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Development of electrochemical biosensors for environmental pollutant and food safety monitoring

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Development of electrochemical biosensors for environmental pollutant and food safety monitoring

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Introduction

The increasing number of potentially harmful pollutants in the environment (such as, endocrine disruptors compounds, volatile organic compounds, explosives) calls for fast and cost-effective analytical techniques to be used in extensive monitoring programs. The requirements, both in terms of time and costs, of most traditional analytical methods (e.g. chromatographic methods) often constitute an important impediment for their application on regular basis. In this context, biosensors appear as suitable alternative or complementary analytical tools.

Biosensors are analytical devices incorporating a biological sensing element. They associate the exquisite sensitivity and specificity of biological receptors in conjunction with physicochemical transducers to deliver complex bioanalytical measurements with simple, easy-to-use formats¹. Potential uses embrace virtually every conceivable analytical task, ranging from medical diagnostics through drug discovery, food safety, process control and environmental monitoring, to defense and security applications. Among the different types of biosensors, the electrochemical ones are particularly interesting due to their high sensitivity inherent to the electrochemical detection and the possibility to miniaturize the required instrumentation providing compact and portable analysis devices²⁻⁴.

In this work, we have developed electrochemical biosensors prepared by immobilizing affinity elements (antibody, bioreceptor) on the surface of miniaturized electrodes for amperometric or impedimetric detection of environmental target pollutants, of food quality and of safety. To optimize biosensors performance, different key steps of their elaboration have been investigated, including the size and geometry of the electrodes and the mode of immobilization of the biological component at the transducer surface. The ideal matrix of immobilization will maintain the functionality of the biomaterial, while providing accessibility towards the target analyte and the proximity between the biomaterial and the transducer. The main methods in reports

are cross-linking, entrapment within polymeric networks (e.g. sol-gel silica, organic polymers), or attachment through chemical binding. Addition of advanced nanomaterials such as gold nanoparticles or carbon nanotubes will be also investigated to enhance biosensor sensitivity⁵⁻⁶.

This thesis is based on three related projects, to develop electrochemical biosensors for monitoring environmental pollutants, food safety and security . After introduction for presentation of the work, chapter I introduces the basis of biosensors, transducers, immobilization strategies, detection methods (bibliographic review). Chapter II, describes an electrochemical estrogen alpha receptor based biosensor for label-free detection of estradiol. Chapter III presents a novel platform for odorant detection based on immobilized olfactory receptors. Chapter IV explains an imprinted Au-nanoparticle composites-based electrochemical sensor for detection of an explosive, TNT. Then a general conclusion and perspectives are presented.

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Chapter I:

Bibliographic Review

Chapter I: Bibliographic Review

1.1 Biosensor history

A biosensor is a device composed of two intimately associated elements, bioreceptor and transducer, Fig.1 presents this scheme¹. Biosensors can be classified into four different basic groups on the basis of signal transduction: electrochemical, optical, mass sensitive and thermal sensors. And biosensors also are classified into immunochemical, enzymatic, non-enzymatic receptor, whole-cell and nucleic acid biosensors, according to the biological recognition elements.

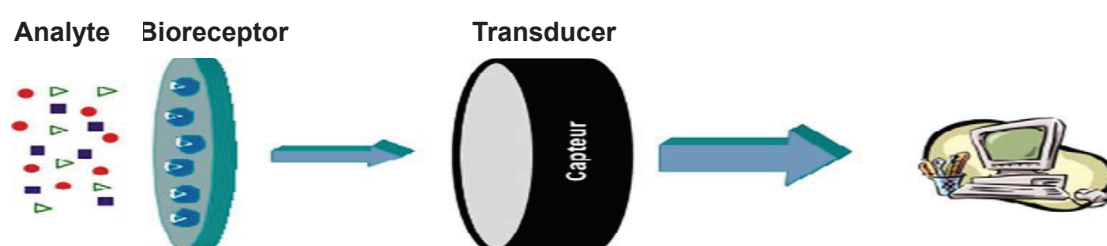


Fig. 1: Scheme of a biosensor

The basic concept of the biosensor was first elucidated by L. C. Clark in 1962, in his seminal description of an "enzyme electrode". Building on his earlier invention of the Clark oxygen electrode, he reasoned that electrochemical detection of oxygen or hydrogen peroxide could be used as the basis for broad range of bioanalytical instruments, by the incorporation of appropriate immobilized enzymes. Two decades later, optical transducers were harnessed in conjunction with antibodies to create realtime bioaffinity monitors. These immunosensors laid the foundation for the second major evolutionary line of biosensing instrumentation. Both the enzyme electrode and the bioaffinity sensors originally found utility in laboratory instruments, but advances in manufacturing coupled with mediated electrochemistry, launched the enzyme-based systems along a new and highly successful trajectory of home use, electrochemistry has come to dominate distributed diagnostics, while optical techniques have found their niche principally in R&D. To complete the picture concerning transduction strategies, advances in acoustic resonance devices are certainly worthy of note, but

both thermometric and magnetic transduction have failed to have any serious practical impact to date².

1.2 Types of bioreceptors in biosensors

1.2.1. Antibody based biosensors

Immunosensors are based on the principles of solid-phase immunoassays, where the immunoreagent, either the antibody (Ab) or the antigen (Ag), is immobilised on a solid support, so that the interaction takes place on the solid–liquid interface. Apart from the sensitivity and selectivity, additional benefits derive from the possibility of conveniently tailoring their affinity and selectivity (e.g. by the design of a suitable hapten and more recently by the design of new recombinant antibodies). Recombinant production and display methods can generate such binding molecules³ allowing faster production of antibodies with new binding properties. Rahman, Md. Aminur⁴ achieved label-free detection of BPA (bisphenol A) with an impedimetric immunosensor. The immunosensor was fabricated by the covalent bond formation between a polyclonal antibody and a carboxylic acid group functionalized onto a nano-particle comprised conducting polymer. By using a commercial reagent 4,4-bis(4-hydroxyphenyl) valeric acid (BHPVA), they have prepared the antigen through the conjugation of BHPVA with bovine serum albumin (BSA) and then produced a specific polyclonal antibody. The immobilization of antibody and the interaction between antibody and antigen were studied using quartz crystal microbalance (QCM) and electrochemical impedance spectroscopic (EIS) techniques. The immunosensor showed specific recognition of BPA with less interference than 4.5% from other common phenolic compounds. The linear dynamic range of BPA detection was between 1 and 100 ng/mL. The detection limit of bisphenol A was determined to be 0.3 +/- 0.07 ng/mL.

1.2.2. MIP based biosensors

Molecularly imprinted polymer(MIP)-based sensors mimic the biological activity of antibodies, receptor molecules, etc.⁵ MIPs combine highly selective molecular

recognition, comparable to biological systems, with typical properties of polymers such as high thermal, chemical and stress tolerance, and an extremely long shelf life without any need for special storage conditions. MIP optical sensors have been developed for detection of pesticides⁶ and for polycyclic aromatic hydrocarbons⁷. Systems still under development are thin MIP membranes on an electrode or optode. The selective transport through the polymeric barriers has to be considered.

1.2.3. Protein receptor based biosensors

Non-catalytic proteins of non-immune origin constitute the recognition element in the so-called protein receptor-based biosensors. These proteins, which span cell membranes, allow the binding signal to be transduced through the membrane by producing the activation of an enzyme (metabotropic receptors) or by opening an ion channel of the membrane (ionotropic receptors). New biorecognition molecules provided by genetic engineering constitute a step further in the development of these biosensors. Hock et al.⁸, for example, compared the binding of estrogens to human estrogen receptors (ERs) α and to recombinant estrogen receptors using the optical sensor BIAcore 2000. In contrast to simple chemical information, by using living intact microorganisms rather than isolated biological components, it is possible to obtain functional information (i.e. information about the effect of a stimulus on a living system) to determine if a substance is toxic to cells.

1.2.4. Whole cell based biosensors

In addition, microorganisms themselves can be used specifically for sensing the bioavailability of a particular pollutant, are usually more tolerant to assay conditions than enzymes or protein, and can be prepared in almost unlimited quantities. The diffusion of substrate and products through the cell wall also results in a slow response relative to enzyme-based sensors. Whole cell biosensors can monitor the metabolism of cells by the measurement of pH, O₂ consumption, CO₂ production, redox potential, electric potential on nervous system cells or bioluminescence in bacteria. In some bioluminescence bacteria, for example, the increase in light is

induced by the presence of a certain contaminant. Genetically engineered microorganism-based biosensors have also been developed for the monitoring of specific contamination. In these ones, genes that code for luciferase are placed under the control of a promoter that recognises the analyte of interest. In the presence of organic pollutants, the genetic control mechanism turns on the synthesis of luciferase, which produces a measurable light emission. Recently, methods that use mammalian cells have been investigated, since they can better resemble the toxicity observed in people than bacteria. Nerve cells growing on array structures, for example, have been implemented in the development of chips and sensors⁹. Tissue-based biosensors such as potato slices for the determination of mono and polyphenols, and cultured human hepatoblastoma Hep G2 cells for the on-site evaluation of environmental waters¹⁰ are of current interest.

1.2.5. DNA based biosensors

Two main strategies have been developed in the area of DNA biosensors. One is the hybridization of nucleic acid sequences from infectious microorganisms detection in which a single stranded DNA molecule immobilized in a sensor is able to seek out, or hybridize to its complementary strand in a sample. However, for the monitoring of endocrine disruptors a second type of DNA biosensors is applied. In this approach, biosensors monitor the interaction of small pollutants with affinities for DNA with the immobilized DNA layer. These biosensors may therefore be used as a general indicator of pollution, integrated in a panel of tests, since they can give rapid and easy to evaluate information on the presence of such compounds.

1.2.6. Enzyme based biosensors

The catalytic biosensors rely on the enzyme-catalysed conversion of a non-detectable substrate into an optically or electrochemically detectable product or vice versa. In general, enzymatic biosensors are based on the selective inhibition of specific enzymes by different classes of compounds. Several enzyme-catalysed reactions involve the production or consumption of a detectable product or low

molecular weight species, such as O₂, CO₂, and ions. A common way of measuring these species is through the use of suitable transducers, usually electrochemical, coupled to the immobilized enzyme. The most important enzymes from an analytical point of view are the oxidoreductases and hydrolases. The use of acetylcholinesterases (AChEs) in biosensor technology has gained enormous attention, in particular with respect to insecticide detection¹¹.

1.3 Types of transducers in biosensors

1.3.1. Optical biosensors

Optical biosensors are the most common, after amperometric and potentiometric biosensors. The various types of optical transducers exploit properties such as simple light absorption, fluorescence/phosphorescence, bio/chemiluminescence, reflectance, Raman scattering and refractive index. Apart from speed, sensitivity and robustness, other attractive features of optical sensors include their suitability to component miniaturisation, remote sensing and their multi-analyte sensing capabilities. In addition, the increasing market of telecommunications has supported development in new optical materials research. Examples of optical techniques usually utilised in biosensors are fibre optic, optical waveguide structures and surface plasmon resonance (SPR). The main advantage of SPR over most optical biosensors is that the analyte presence can be determined directly without the use of labelled molecules.

1.3.2. Mass-sensitive biosensors

Within mass-sensitive biosensors, acoustic wave biosensors operate on the basis of an oscillating crystal that resonates at a fundamental frequency. The crystal element is coated with a layer containing the biorecognition element designed to interact selectively with the target analyte. A measurable change in the resonance frequency occurs after the binding of the analyte on the sensing surface according to the mass change of the crystal. Most of these biosensors utilize piezoelectric materials as the signal transducers¹². Piezoelectric sensors provide real-time data of

binding events but, as the sensitivity is lower than SPR, this method has been used less frequently. An emerging group of mass-sensitive biosensors are the so-called cantilever biosensors, which are based on the bending of microfabricated silicon cantilevers^{12,13}. The mass change, originating from the adsorption of target molecules on the microcantilever surface (where receptor molecules are immobilised), causes a differential surface stress change, and therefore a bending or deflection of the cantilever¹³. The deflection of a few nanometres can be detected by electric or optical methods. Microcantilever biosensors offer various advantages due to their microscopic dimensions (in the range of 10^{-3} mm²): only small quantities of receptor and analyte are necessary and limits of detection achieved are often lower than those obtained by classical methods¹⁴. The feasibility of mass production, real-time monitoring and operation in air, vacuum or liquid environments, are other advantages of these biosensors¹⁵.

1.3.3. Thermometric biosensors

Thermometric biosensors, finally, exploit the absorption or evolution of heat in biological reactions. This is reflected as a change in the temperature within the reaction medium and is transduced by a change in the resistance of a thermistor, which acts as a temperature transducer. As reviewed by Ramanathan and Danielsson¹⁶, thermistor-based calorimetric biosensors have mainly been applied to clinical and industrial process monitoring. Up to now, only several pesticides have been measured by thermometric biosensors.

1.3.4. Electrochemical biosensors

Based on the developing label-free electrochemical biosensors, there various methods that can be used in electrochemistry. For us, among all techniques we chose square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS). They are more rapid and effective for control via many reported papers.

Voltammetry has been practiced for a long time and has revolutionized analytical chemistry. Many electroanalytical techniques have inherent advantages and disadvantages with variable purposes, and therefore may be utilized in a multitude of

different fields of study, encompassing enzyme catalysis¹⁷, free radical generation¹⁸, solar energy conversion¹⁹ and myriad of others. The main advantages of using voltammetric methods over optical spectroscopy or chromatography include their high sensitivity, precision, accuracy and cost effectiveness.

Aicheng Chen and Badal Shah²⁰ review the highlight the differences between three primary electrochemical techniques (mainly for CV,DPV,SWV) and have shown that SWV reigns as the most sensitive of the conventional electrochemical techniques. The sensitivity of SWV coupled with its high frequency was demonstrated as an ideal technique for the development of rapid response and reliable sensors. The usefulness of this technique was elucidated for applications in diagnostics, environmental analysis, the food industry and the measurement of enzymatic activity. The development of novel and improved electrode surfaces and nanomaterials introduces the possibility of sensors that will exhibit even higher sensitivity, with SWV serving as an ideal methodology for its optimization. Concurrent with the greater knowledge of electrochemistry and a better understanding of electrode synthesis and modification, sensors will improve considerably in terms of both sensitivity and detection limits. Micro- and nano-sensors will likely constitute the next disruptive paradigm in the field of sensing and biosensing, and SWV will surely play a critical role in their development.

SWV can be applied for the analysis of reversible and irreversible reactions, reaction with slow electron transfer as well as for catalytic reactions. And SWV is often utilized due to its ability to be operated at high frequency²¹. This means that square wave experiments may be accomplished quickly and can conserve electroactive species with respect to other pulse techniques²². Since there is a decrease in the use of electroactive species, electrode surfaces tend to be less hindered with non-electroactive products. Another advantage of SWV is that the exclusion of oxygen from the analyte solution may not be necessary due to the reduction of oxygen being included in the background current²³. This, of course, is dependent on whether the applied potential is more cathodic than that for the reduction of oxygen. If the potential is indeed more cathodic, the magnitude of the forward and reverse

currents will both include the current from the reduction of oxygen and will be subtracted from the background. The square wave frequency is a parameter that arises from the use of the square wave on the staircase potential and is the frequency at which the analyte is sampled. Each pulse results in a small increment of potential and hence, should the frequency of the pulses increase then an increase in the rate at which the potential is scanned occurs. Similar to cyclic voltammetry, the increase in the sweep rate will also correspond to an increase in the peak current. However, this also involves an increase in the peak potential, and unlike in a CV, this shift in peak potential will be proportional to the logarithm of the square wave frequency.

There have so far been many researchers relying on electrochemical impedance spectroscopy (EIS) as detection technique to develop biosensors. For EIS, the electron transfer reaction of a probe at a biosensor is studied. When molecular capture happens at the biofunctionalized electrode surface, there will be changes in the interfacial charge, capacitance, resistance, mass and thickness at the biosensor surface, which in turn have a direct effect on the electron transfer reaction of the probe at the biosensor–electrolyte solution interface. In EIS, a low-amplitude (usually 5–10mV peak-to-peak) sinusoidal potentials is superimposed on a fixed DC potential that promotes the redox reaction of the probe. The sinusoidal current obtained is then used to calculate the impedance over a wide frequency range. A Nyquist plot (imaginary impedance ($-Z_{Im}$) versus real impedance (Z_{Re}) as a function of decreasing frequency) often reveals different features depending on the nature of the electrode reaction. If electron transfer reaction of the probe is the rate-determining electrode reaction step, the Nyquist plot will show a semicircle, and if diffusion of the probe is rate determining, a linear Nyquist plot is obtained. In the former, the high frequency region of the semicircle is dominated by double layer capacitance (C_{dl}) and this region often shows an offset on the Z_{Re} -axis by a value corresponding to the magnitude of the electrolyte solution resistance (R_s). Meanwhile, the intercept of the low-frequency region of the semicircle on the Z_{Re} -axis represents the sum of R_s and the electron transfer resistance (R_{et}). In other words, R_{et} is conveniently estimated from the diameter of the semicircle on the Z_{Re} -axis. Typically, a MHz to mHz

frequency range is used in EIS experiments. Thus, a considerable period may be required to complete a full frequency range in EIS. In addition, noise in the low-frequency region can severely affect the quality of data, making it difficult to extrapolate the intercept on the ZRe-axis for use in estimating Ret. Depending on the nature of a reaction, an ill-defined semicircle may be obtained in some cases after scanning the full spectrum of frequency. In such work, computer simulations based on an equivalent circuit (consisting of Rs, Ret and Cdl) that represents the biosensor–electrolyte solution interface are then performed to obtain fitted Nyquist plots that match with experimental Nyquist plots. This then aids in estimating Ret from the low-frequency intercept on the ZRe-axis. In addition to Ret, EIS is also capable of providing data for capacitive and structural characteristics of the biosensor surface by considering the phase angle calculated from $-Z_{Im}$ and ZRe. However, this is largely ignored in quantitative analysis. As a result, a great deal of time and effort is required to obtain a single parameter, ZRe, which is the sole analytically useful variable in most work reported to-date.

In SWV, it is critical to set a sufficiently long current sampling period at the end of each pulse in order to measure a substantially enhanced Faradaic component of the current. This is equivalent to using low frequencies in EIS to estimate Ret. However, a linear potential scan in SWV only requires several minutes to complete, whereas a significantly longer period is needed in EIS. In addition, SWV provides a more familiar peak-shaped signal for easy interpretation of analytical data.

Liu et al.²⁴, reported square wave voltammetry (SWV) compare to electrochemical impedance spectroscopy (EIS) in developing a label-free electrochemical immunosensor for the estradiol hormone. In this paper, for comparison, significant analysis time and effort are generally required to conduct impedimetric experiments over a frequency range to obtain Ret for constructing a calibration plot. And a high degree of uncertainty is associated with Ret when an ill-defined semicircle is obtained in a Nyquist plot, in which case further analysis time and effort are needed to conduct computer simulations. Moreover, additional EIS data involving phase angle are ignored, as they do not contribute any useful information to quantitative analysis. In

contrast, SWV is a much more rapid detection technique because a voltammetric scan is completed within seconds. A familiar peak-shaped current response is produced and this can be directly used to establish calibration. An appropriate frequency can be quickly determined by multiple pulse chronoamperometry. SWV is very useful for analysis of electrochemically reversible reaction of numerous probes.

In conclusion, for all our experiments, we thought and chose SWV technique as main methods for monitoring analyte concentration. In addition, EIS was used for characterization of the biofunctionalized electrode/electrolyte interface.

1.4 Immobilization strategies

Many immobilization strategies have been reported. Main protocols can be envisioned: adsorption, covalence, entrapment, cross-linking or affinity. In many cases, the immobilization methods are based on combination of several immobilization methods. For these five basic methods, a comparison is presented as following.

According to binding nature, adsorption involves weak bonds; covalent coupling is chemical binding between functional groups of biomolecules and those on the support; entrapment is incorporation of biomolecules within a gel or a polymer; cross-linking is bond between biomolecules, cross linker and insert molecule; affinity is bonds between a functional group on the support and affinity tag on a protein sequence.

For the advantages, adsorption is simple, easy and there is limited loss of biomolecules activity; covalent coupling is no diffusion barrier method, more stable and short response time are obtained; entrapment presents no chemical reaction between the monomer and the biomolecules that could affect the activity and several types of biomolecules can be immobilized within the same polymer; cross-linking is simple; affinity presents a controlled and oriented immobilization.

For the drawbacks, in adsorption there are desorption and non-specific adsorption; covalent coupling leads to not regenerable matrix, coupling involving toxic product leads to high biomolecules activity loss; entrapment leads to diffusion barrier,

biomolecules leakage and high concentrations of monomer and biomolecules; cross-linking leads to high biomolecules activity loss; affinity needs the presence of specific groups on biomolecules (eg.His).

The immobilization protocols appears as a key factor to develop efficient biosensors with appropriate performances such as good operational and storage stability, high sensitivity, high selectivity, short response time and high reproducibility. Immobilized biomolecules have to maintain their structure, their function, to retain their biological activity after immobilization, to remain tightly bound to the surface and not to be desorbed during the use of the biosensor. Moreover, an ideal biosensor has to be stable for long-term application. The type of immobilization method affects activity and stability of biosensors. Factors such as accuracy of measurements, the sensor-to-sensor reproducibility and operational lifetimes are drastically influenced by biomolecule stability. Since the analytical performances of a biosensor are strongly affected by the immobilization process, intensive efforts have been done to develop successful immobilization strategies in order to assure greater sensitivity and stability of biosensors. The choice of the most appropriate and judicious technique also depends on the biomolecule nature, the transducer and the associated detection mode. The best method of biomolecule immobilization varies if the biosensor application requires maximum sensitivity or rather focuses on stability. Reproducibility, cost and difficulty of the immobilization process also need to be considered.

All along my thesis work, the main idea is to develop bioaffinity-sensors and we chose affinity methods for bioreceptor immobilization. Efforts have been achieved in order to develop biosensors based on oriented and site-specific immobilization of bioreceptors. A strategy is to create (bio)affinity bonds between an activated support (e.g. with lectin, avidin, metal chelates) and a specific group (a tag) of the protein sequence (e.g. carbohydrate residue, biotin, histidine). Several affinity methods have been described to immobilize biomolecules through (strept)avidin-biotin, lectin-carbohydrate and metal cation–chelator interactions. We took metal cation-chelator interaction method (in estradiol and odorant detection parts) for fabrication of biosensors.

The strong affinity link between a metal cation and a chelator such as nitrilotriacetic acid (NTA), imidodiacetic acid (IDA) or tag poly(histidine) can also be used to develop biosensors. This method is based on the principle of Immobilized Metal Affinity Chromatography (IMAC), currently used for protein purification and separation. Immobilization of the metal ion on a chromatographic resin by chelation allows the separation of histidine-tagged proteins from untagged proteins. The bound molecule can be eluted from the resin by reducing the pH and increasing the ionic strength of the buffer or using EDTA or imidazole. Based on this principle, biomolecules with His residues in their structure can be easily attached to a support containing a metal chelate. However, few His residues are present on protein surface and are accessible for binding to a chelate-modified surface. To solve this problem, genetic engineering methods allow the production of tagged biomolecules by attaching His at a specific position of the protein. And we also obtained covalent immobilization by pretreatment of electrode surface, yielding to metal ion chelator. The binding of the biomolecules to the solid support is generally carried out by initial activation of the surface using multifunctional reagents (e.g. glutaraldehyde or carbodiimide), followed by biomolecules coupling to the activated support, then the removal of excess and unbound biomolecules. The carrier support can either be an inorganic material (e.g. controlled pore glass), a natural (e.g. cellulose) or synthetic polymer (e.g. nylon). Membranes (e.g. Immunodyne, Ultrabind) that are already pre-activated have also been used. Covalent immobilization can be performed directly onto the transducer surface or onto a thin membrane fixed onto the transducer.

We firstly activated carboxylic groups. Carbodiimides allow the binding between the carboxyl groups of a support and the amino function of a biomolecules. N-hydroxysuccinimide (NHS) can be associated to carbodiimide in order to improve immobilization efficiency. This procedure is widely used to develop biosensors. Carbodiimides also allow the binding between the amino groups of a support and the carboxyl function of biomolecules. It can be also described as biomolecules immobilized onto pre-formed self-assembled monolayers, SAM via covalent binding. After all above steps, analytes were incubated for detection.

In another part of my work, we used nanomaterials, such as gold nanoparticles, for the design of biosensing devices, constituting an exciting and recent approach to improve the stability of detection platforms. The extremely promising prospects of nanomaterials are due to their unique properties. Nanoparticles of different compositions and sizes have also been used in last years as versatile and sensitive tracers for electronic, optical or microgravimetric transduction of different biomolecular recognition events ²⁵. Excellent conductivity of metal nanoparticles allows them to enhance the electron transfer between redox center in proteins and electrode surface ²⁶. Nanoparticles are interesting immobilization surfaces presenting a large surface area ²⁷.

Concerning use of nanoparticles, we chose the entrapment method, especially the use of electropolymerization. Electrochemical polymerization (or electropolymerization) is a simple and attractive approach for the controlled immobilization of biomolecules on electrode surfaces. In this work, entrapment was applied to the analyte, in order to *in situ* form specific molecularly imprinted cavities. This one-step method consists in applying an appropriate potential or current to the transducer soaked in an aqueous solution containing both analyte and monomer molecules. Monomer oxidation gives rise to a radical cation which can either react with a second radical cation or with a neutral monomer in order to obtain a dimer that is then oxidized. Finally, a polymer is formed at the electrode surface. Enzyme molecules that are present in the immediate vicinity of the electrode surface are physically incorporated within the growing polymer network. Most of electropolymerized films used for biomolecule immobilization are conducting polymers such as polyaniline, polypyrrole or polythiophene. Owing to their conductivity, the thickness of the polymer films can be easily controlled and is not restricted to thin films, contrary to non-conducting polymers.

In our work we used polyaniline, it has attracted much attention due to various remarkable characteristics such as controllable conductivity, charge transfer capability and environmental stability. This polymer was described as an interesting material for fabrication of biosensors because it can act as an effective mediator for

electron transfer in redox reactions. Polyaniline transport properties, electrical conductivity or rate of energy migration, provide an enhanced sensitivity²⁸.

Thioaniline functionalized gold nanoparticles were used for the electropolymerization of molecularly imprinted hybrid materials that was called by Willner as Au NPs sponges²⁹.

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Chapter II:
Electrochemical estrogen
receptor alpha based
biosensor for label-free
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2.1 Introduction

A large number of natural and synthetic chemicals have been identified as disruptors of the normal functioning of the endocrine system and produce untoward effects in hormone-responsive target tissues and organs in both humans and animals. These chemicals are referred to as endocrine disrupting chemicals (EDCs), and include alkylphenols, bisphenol A, and a number of pesticides and some chlorinated compounds, the contraceptive 17- α -ethinylestradiol, as well as naturally occurring phytoestrogens (e.g. some coumarin derivatives, flavonoids and isoflavones) and mycoestrogens (e.g. F-2 mycotoxin) and other substances. There is considerable concern about the environmental occurrence of these EDCs. In addition, early life exposure to EDCs may alter gene expression and consequently transmit these effects to future generations¹⁻⁵.

So, public interest in possible health threats posed by chemicals in general and EDCs in particular, is on the rise. To limit environmental threats to human and environmental health, the EU has taken action and made progress in various fields during the last few years, such as the implementation of the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, EC 1907/2006), the Water Framework Directive (WFD), and the agreement on the Industrial Emissions Directive. The public-spirited conscience of citizens, combined with more visible responsibility of public authorities, stimulates demand for more stringent water monitoring. Indeed the WFD Daughter Directive lists two estrogens—17 β -estradiol (17E) and 17- α -ethinylestradiol (EE2) – and several EDCs as priority pollutants (e.g., nonylphenols) and some other candidate substances are also EDCs as polybrominated (PBB) and polychlorinated (PCB) biphenyls. European countries might will require by 2021 to limit EE2 in water bodies to an annual average of no more than 0.035 ng/L⁶. As recently as 2003, the International Programme on Chemical Safety (IPCS) of the United Nations sponsored a joint workshop on “Endocrine

Disruptors: Research Needs and Future Directions,” in Tokyo, and the workshop identified as a research need the development of highly sensitive and selective chemical and biological analytical methods for EDCs and their metabolites, including novel technologies such as biosensors and nanotechnology. At present, only OECD (Organization for Economic Cooperation and Development) tests on the embryonic stages of aquatic organisms are recognized. However, this procedure is extremely long and very time consuming. Also, there is an urgent need for tools for rapid screening of endocrine disruptors, not only in environmental and food samples, but also in order to select substitutes to known EDCs such as bisphenol A, phthalates (DBP, DEHP, BBP) or nonylphenol and to designate any potential EDC. Analytical techniques such as GC/MS and LC/ MS/MS have been used to determine the amounts of EDCs in effluents before and after water treatment plants⁷ and in surface waters and sediments⁸.

EDC determinations require the development of integrated chemistry/toxicity analytical evaluation. Biosensors based on estrogen receptors (ERs) provide significant and useful information about the estrogenic potency of samples. Many endocrine disruptors are believed to bind to estrogen receptors either as agonists or as antagonists. Thus, the binding ability of the chemicals toward the ER is used to test their potential environmental toxicity. The advantage of receptor assays is that they are quite simple to perform and allow the identification of all endocrine disruptors that act through the estrogen receptor. Estrogens act on target tissues by binding to parts of cells called estrogen receptors (ERs). An estrogen receptor is a protein molecule found inside that cell's nucleus, which is a target for estrogen action. These ERs belong to a superfamily of nuclear receptors (NRs). They exhibit a high degree of ligand specificity in that they bind only to estrogens (or closely related molecules). The target tissues affected by estrogen molecules all contain estrogen receptors; other organs and tissues in the body do not. Therefore, when estrogen molecules circulate in the bloodstream and move throughout the body, they exert effects only on cells that contain estrogen receptors. The estrogen receptor undergoes several changes during its proposed cycle in the nucleus, beginning with the association of inactive,

unliganded receptor with heat shock protein (HSP) complexes. After ligand binding and HSP dissociation, structural rearrangement and dimerization of liganded receptors occur. This increases the affinity of produced and purified receptor dimers to recognize specific estrogen response elements^{9,10} (ERE). After it has become attached to estrogen response elements in DNA, this estrogen–receptor complex binds to coactivator proteins and more nearby genes become active. The active genes produce molecules of mRNA, which guide the synthesis of specific proteins. These proteins can then influence cell behavior in different ways, depending on the cell type involved. In all biosensor based assay one of the components has to be immobilized on the solid surface (ligand) and the molecule to be detected (analyte) is present in the solution. The binding between the analyte and the receptor (ligand) occurs on the solid surface, and leads to a change in the refractive index (in case of SPR instruments) or by changes in the fluorescence intensity, ultraviolet light intensity, etc. which can then be detected and directly correlated to the extent of binding on the surface. By its very design the biosensor surface is heterogeneous. This heterogeneity could be due to the nature of the surface or the nature of the immobilized ligand or the immobilization chemistry involved. Since the analyte has to diffuse through the solution to react at the solid surface, some diffusional limitations take place. There is continuous flow of the buffer in the flow cells of the biosensor chip. This may also lead to some convective effects, which may have an influence on the binding assay. Also, there may be depletion of the analyte along the flow channel as the reaction proceeds. This affects the kinetics, and leads to time dependent reaction parameters. Diffusion is presumably the dominant mode of transport of the analyte towards the chip surface. The binding kinetics needs to take into account the coupled effect of the heterogeneity and mass transfer limitations.

Based on this huge societal demand, several electrochemical sensors have already been reported for the detection of 17 β -estradiol (17E) as a model of EDC molecules. Electrochemical sensors are good candidates for detection in real water samples without any preconcentration or any separation steps, and moreover they are associated with light and low-cost instrumentation. Some proposed biosensors are

based on receptor molecules such as a specific aptamer or antibody or estrogen receptor α (ER- α) and the irreversible interaction of 17 β -estradiol with the receptor molecule influences the electron charge transfer with a redox probe present in the measuring medium. Based on this principle and using electrochemical impedance spectroscopy, detection limits in the order of picomolar were obtained¹¹⁻¹⁶.(Tab.1)

Table 1. Relevant electrochemical biosensors for detection of 17 β -estradiol.

Type of probe	Type of electrode	Electrochemical technique	Detection limit	Dynamic range	Reference
Aptamer	Gold chip	SWV with ferri/ferrocyanide probe	1 pM	10 pM 1 nM	[11]
Aptamer	Gold electrode	Impedance with ferri/ferrocyanide probe increase of R_{CT}	2 pM	10 pM 10 nM	[16]
Estrogen receptor α	Gold electrode	Impedance with ferri/ferrocyanide probe increase of R_{CT}	0.1 pM	0.1 pM 1 nM	[15]
antibody	Au NPs/gold electrode	Impedance and SWV with ferri/ferrocyanide probe decrease of R_{CT} , increase of current	SWV: 66 pM EIS: 95 pM	SWV: 0 4.4 nM EIS: 0 3.6 nM	[13]
ER/Lipid bilayer	Au NPs	Impedance with ferri/ferrocyanide probe decrease of R_{CT}	3.7 pM	18 pM 0.55 nM	[12]
MIP	PtNPs/GCE	Impedance and DPV with ferri/ferrocyanide probe	16 nM	30 nM 50 μ M	[14]
No	Nano- Al_2O_3 /GCE + CTAB 2 min accumulation	Voltammetry oxidation peak at 0.45 V	80 nM	0.4 μ M 40 μ M	[18]
No	Pt nanoclusters/MWCNTs/GCE	Voltammetry oxidation peak at 0.45 V	0.5 μ M	0.5 μ M 15 μ M	[19]
No	CNTs/Ni cyclam/GCE	Voltammetry oxidation peak at 0.85 V	60 nM	0.5 μ M 40 μ M	[20]
No	Poly(L-serine) film/GCE 2 min accumulation	Voltammetry oxidation peak at 0.6 V	20 nM	0.1 μ M 30 μ M	[21]
No	LbL (MWCNTs/AuNP)/graphite electrode 200 s accumulation	Voltammetry oxidation peak at 0.9 V	10 nM	70 nM 42 μ M	[22]
ER- α	Au/thiol SAM/NTA-Cu/6His-ER- α	SWV oxidation peak at 0.5 V	1 fM	1 fM 1 nM	This work

Likewise, it is well known that 17 β -estradiol presents an oxidation peak due to oxidation of the phenol function into phenoxy radical and then into a phenoxonium ion, this reaction being followed by the formation of ketone after loss of one proton¹⁷. The direct detection of 17 β estradiol through its oxidation peak after accumulation has been exploited and a detection limit as low as few tens of nM has been achieved¹⁸⁻²²(Table 1).In this work, a new approach is proposed for the conception of a biosensor for the detection of the natural estrogen 17 β -estradiol, as a model of EDC, based on the specific interaction of 17 β -estradiol with the ER- α -receptor, as shown in Reference²³ that reported the first use of the NTA-Cu²⁺-histidine-tagged protein system for the design of an electrochemical biosensor and its electrochemical characterization. The His tag- ER- α -receptor is immobilized on a gold electrode using the 6His-copper interaction. Monitoring of the intensity of the Cu(I)/Cu(II) peak depends on ER- α -receptor conformation, as already shown in the SWV immunosensor²⁴.Through intensity monitoring, the immobilization of the ER α -receptor can be checked (decrease of Cu(I)/Cu(II) peak) as well as the specific

interaction with the ER- α -receptor (increase of Cu(I)/Cu(II) peak). Sensitive and selective detection of 17 β -estradiol is obtained through SWV, monitoring the Cu(I)/Cu(II) peak and 17E oxidation peak.

2.2 Experimental

2.2.1. Reagents and Materials for Estradiol Electrochemical Detection

β -Estradiol ($\geq 98\%$), Estrone ($\geq 99\%$), Estriol ($\geq 97\%$) were purchased from Sigma. Bisphenol A (BPA) (97%), 6-mercaptohexanoic acid ($\geq 90\%$, C6), Na,N-bis(carboxymethyl)-L-lysine hydrate ($\geq 97\%$, NTA), N-hydroxysuccinimide (98%, NHS), sodium acetate, copper acetate were purchased from Aldrich. Acetic acid, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride ($\geq 99\%$, EDC), ethanolamine were purchased from Fluka. Carbon tetrachloride and heptane were purchased from Sigma-Aldrich. Copper acetate solution was obtained by diluting 10 mM copper(II) acetate in 10 mM acetate buffer solution (pH 4.6). The buffer solution used for all the experiments was 10 mM phosphate buffer saline (PBS) at pH 7. All solutions were made in ultrapure water produced by a Millipore Milli-Q system.

2.2.2. ER- α Production and Purification²⁵

His6-ER α (302–552) construction was inserted into the NdeI-BamHI sites of pET15b recombinant plasmid (Novagen) using PCR, leading to fusion proteins with a His tag. Escherichia coli BL21 (DE3) cells were electroporated with 20 mg pET15b recombinant plasmid. The transformed cells were grown in a Lysogeny broth medium supplemented with estradiol and sucrose. Introduction was carried out for 4 hrs at 25 $^{\circ}$ C. The cell disruption buffer contained 2 M of nondetergent sulfobetaines (NDSB). The cells were lysed by sonification, then the extracts were centrifuged and the expression of soluble protein was estimated on SDS-PAGE after passing through a Zn²⁺ affinity chromatography column.

2.2.3. Biofunctionalization of Gold Electrodes

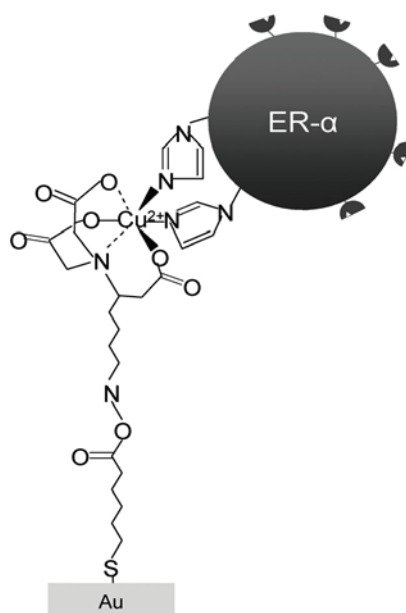
2.2.3.1. Pretreatment

Gold substrates (Pic.1) were provided by the Laboratoire d'Analyse et d'Architecture des Systemes (LAAS, CNRS Toulouse). They were fabricated using standard silicon technologies. (100)-oriented, P-type (3–5 Wcm) silicon wafers were thermally oxidized to grow a 800 nm-thick field oxide. Then, a 30 nm-thick titanium layer followed by a 300 nm thick gold top layer were deposited by evaporation under vacuum. Gold electrodes were first sonicated for 15 min at room temperature in acetone using a Branson 5210 ultrasonic bath (Branson Ultrasonics Corporation, Danbury, USA), rinsed with ultrapure water and dried under nitrogen flow. Then the gold electrodes were cleaned for 5 min with freshly prepared "piranha" mixture (piranha: $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 3 :7, v/v), then rinsed with ultrapure water, ethanol and dried under nitrogen flow.

2.2.3.2. Biofunctionalisation Steps

Tagged ER- α was anchored on the gold electrode surface as presented in Scheme 1. The pretreated electrodes were immersed overnight at -4°C in 1 mM 6-mercaptohexanoic acid solution in ethanol to give alkanethiol SAM bearing carboxylic terminal groups. The modified electrodes were then rinsed with ethanol. An activation step was performed by reaction with a 150 mL drop of 10 mM EDC/10 mM NHS mixture in 10 mM PBS solution for 1 h. Carboxyl groups of gold electrodes were converted into active carbodiimide esters which can then react with NTA terminal amine groups to form covalent bonds (7 mM NTA in 10 mM PBS solution for 1 h). Then the unreacted carbodiimide esters were passivated by rinsing with ethanolamine. After this, the gold electrodes were immediately rinsed with sodium acetate/acetic acid buffer solution and immersed in 10 mM copper(II) acetate in 10 mM acetate buffer solution at pH 4.6 for 45 min, in order to coordinate the Cu^{2+} ions with grafted NTA groups and form a Cu^{2+} metal complex. Finally, 150 mL of cell extract containing 6-His tagged ER- α were dropped onto the surface for 1 h and then thoroughly rinsed with PBS to remove excess ER- α and other non-specifically adsorbed species, before

measurements were made.



Scheme 1: Schematic representation of the 6-His tagged ER α anchored on gold surface

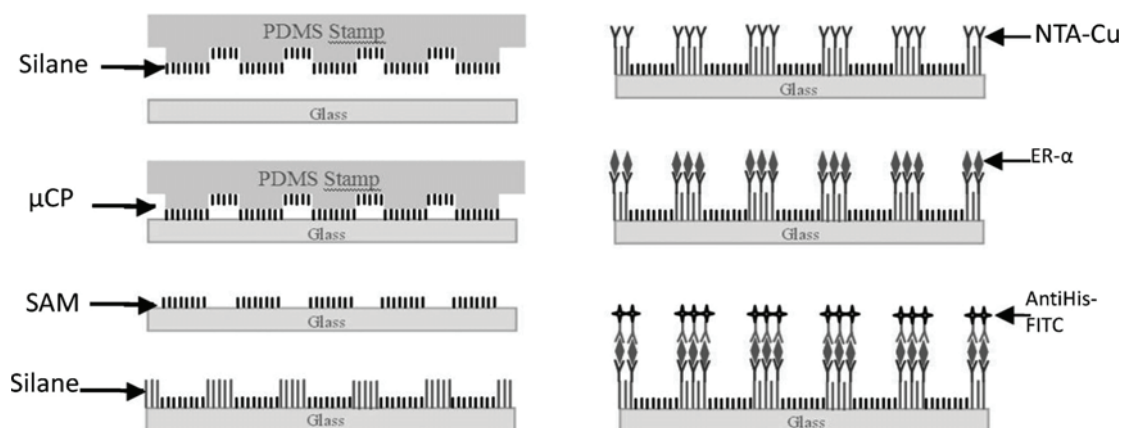
2.2.4. Microcontact Printing for Fluorescence Microscopy

A glass slide electrode was used for fluorescence microscopy. The PDMS stamp (1 cm²) with holes of 50 μ m diameter was used for contact printing of octadecyltrichlorosilane on a glass slide electrode (cf. Scheme 2). First, the PDMS stamps were cleaned with ethanol and were ultrasonicated for 30 min. Then the glass slide was cleaned for 30 min in piranha mixture, rinsed with ultrapure water and then dried with nitrogen. The PDMS stamp was then immersed in octadecyltrichlorosilane (OTS) (5 mM OTS and 0.4 mM of carbon tetrachloride in heptane solution) for 30 seconds and dried under nitrogen flow. Next, the glass was stamped with the inked PDMS for a few seconds. The glass substrates needed to be left in the oven at 100°C for one hour in order to enhance the chemical bonding of OTS to the glass substrate.

The glass substrates were then immersed in 10-(carbomethoxy) decyl-dimethylchlorosilane (CMDCS) solution (10 mM CMDCS and 0.4 mM of carbon tetrachloride in heptane solution) at 4°C for 4 hours. Silanized glass slides were

thoroughly and slowly rinsed with heptane, then water, and dried under a nitrogen flow. The glass substrates needed to be left one more time in the oven at 100 °C for one hour in order to enhance the chemical bonding of CMDCS to the glass substrate. In the end, the substrates were immersed in 1N HCl (37%) overnight at ambient temperature, then rinsed with plenty of water, and finally dried under nitrogen flow. After these treatments, ER- α was immobilized by following the steps described before (see §2.2.3.2.).

After the ER- α immobilization step, 2 mL of FITC conjugated anti-His antibody was maintained in contact with the biofunctionalized glass slide. Then the modified glass slide was observed by fluorescence microscopy (Zeiss Axioplan 2 Imaging apparatus, Carl Zeiss MicroImaging GmbH, Jena, Germany).



Scheme 2: Microcontact Printing and fluorescence detection of ER- α .

2.2.5. Electrochemical Measurements

Square wave voltammetry (SWV) was performed using a Voltalab 80 (Hach Lange, France). A three-electrode cell was used, including a saturated calomel electrode (SCE) as reference electrode, a platinum plate as counter electrode (0.54 cm²) and a modified gold working electrode (0.19 cm²). The procedure of electrochemical detection were carried out at 4 °C using a Minichiller thermostated bath (Huber, company) and used a PBS solution at pH 7.0. The square wave voltammetry measurements were carried out in the potential range from -400 mV to +600 mV after maintaining potential at -400 mV for 5 min for the total reduction of Cu(II) to Cu(I). The

scan rate, duration, amplitude were 100 mV/s, 0.05 s, 5 mV, respectively²⁴.

The impedance spectroscopy measurements were carried out in the frequency range from 0.05 kHz to 100 kHz at different dc potentials at 0 mV vs.SCE electrode, using a modulation voltage of 10 mV.

2.2.6. Incubation of Estradiol

The 17 β -estradiol should first be dissolved in organic solvents such as methanol. A 10⁻³ M mother solution of 17E in methanol was prepared and then diluted in PBS containing 1% of methanol for each concentration. The concentration range from 10⁻¹⁵ M to 10⁻⁹ M of 17E was chosen for analysis. The ER- α functionalized gold electrodes were exposed to increasing concentrations of 17E at 4 °C. For each concentration, the incubation time was 1 hour at 4 °C.

2.3 Results

2.3.1. Control of Copper Complexation by Voltammetry

In order to check that NTA/Cu²⁺ complex has successfully been formed on the gold electrode, square wave voltammetry was performed. After maintenance at -400 mV for 5 min, the oxidation peak Cu(I) to Cu(II) appears at +33 mV (Fig.1). This result demonstrates that copper ions are well immobilized as NTA complex at the gold electrode surface and its peak surface area allows the determination of a copper surface coverage of 1x10⁹ mol/cm², with an RSD of 20%, showing that a dense monolayer of chelated copper ion is available on grafted electrode surface.

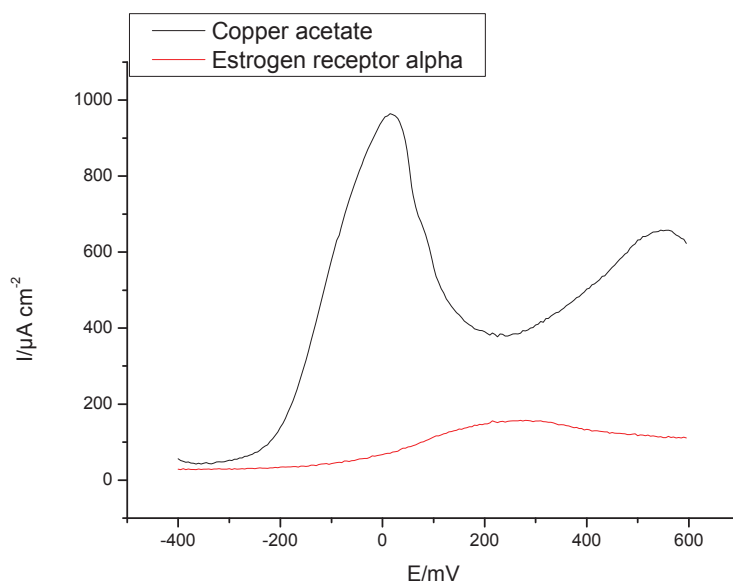


Fig.1. The square wave voltammetry response of copper complexation and ER- α immobilization in pH 7.0 PBS solution at the amplitude, scan rate and potential range at 5mV, 100mV/S and -0.4 to +0.6V, respectively.

2.3.2. Control of ER- α Immobilization by Voltammetry and Fluorescence Microscopy

2.3.2.1. Square Wave Voltammetry

As shown in Figure 2 (black curve), after ER- α is immobilized, the Cu(II)/Cu(I) peak (Cu peak) shifted to 285 mV, showing that a different Cu(II) complex is formed. Its peak maximum decreased by a factor of 10, showing that the electron transfer rate greatly decreased after ER- α immobilization. This phenomenon has already been observed in the case of 6 His-tagged reduced antibody immobilization²⁴. The Cu(II)/Cu(I) peak maximum is correlated to the surface density of immobilized ER- α .

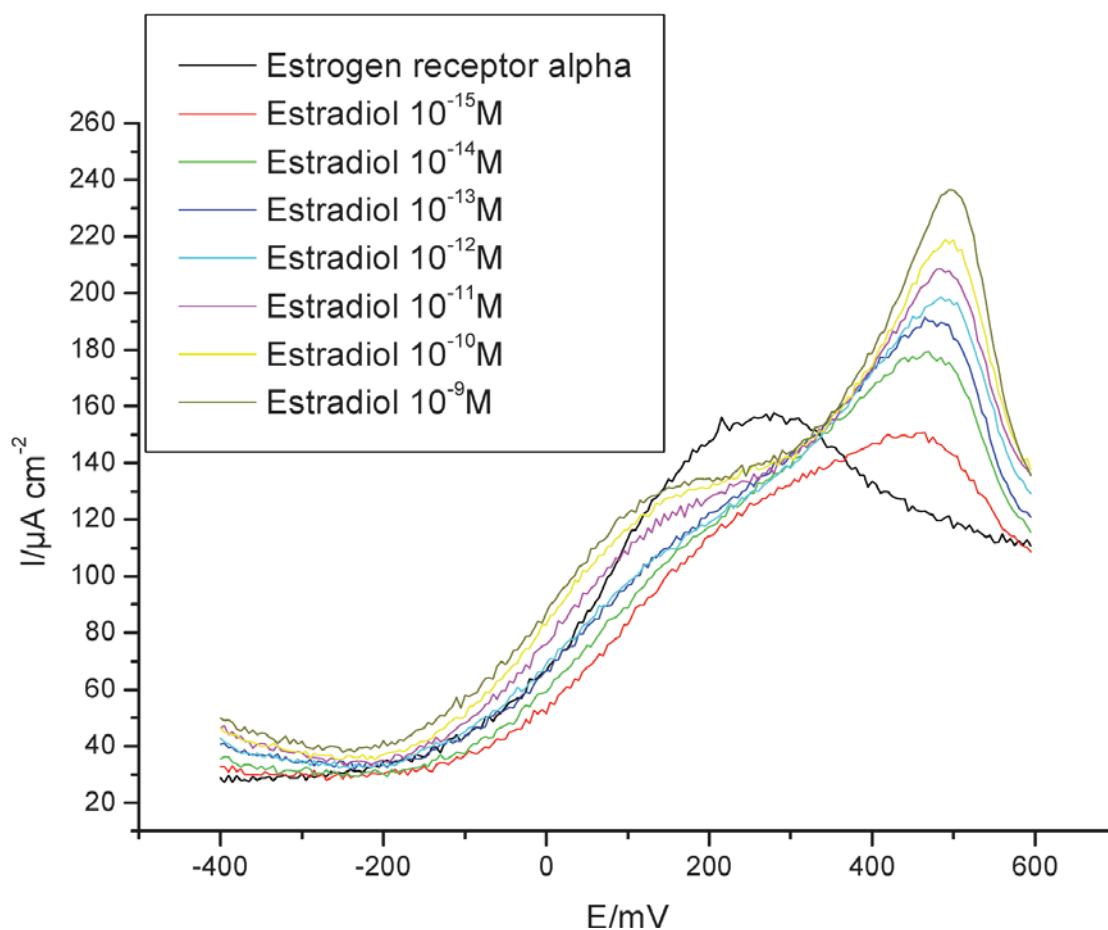


Fig. 2. SWV of ER- α biolayer before and after interaction with 17 β -estradiol.

2.3.2.2. Fluorescence Control of ER- α Immobilization

Due to the use of FITC conjugate anti His-antibody, it is possible to reveal the position of the immobilization of ER- α . As shown in Figure 3, where OTS was stamped, there is no fluorescence (black part). Green spots of 50 mm diameter appear, due to the presence of 6-His tagged ER- α immobilized on Cu-NTA. This result demonstrates that ER- α was successfully immobilized on surface Cu-NTA, and so it can be used for further target detection.

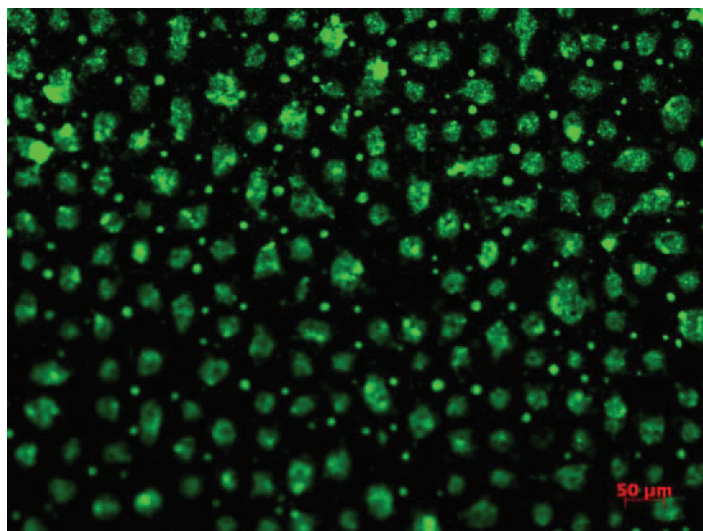


Fig. 3. Fluorescence image of FITC conjugate anti His-antibody on the ER- α grafted on microcontact printed glass substrate.

2.3.3. Electrochemical Detection of Estradiol

2.3.3.1. Impedance Control

After 17E was incubated for 1 h with ER- α functionalized gold electrode, a decrease of the impedance of the electrode/electrolyte interface is observed (cf. Figure 4), reflecting a decrease of the charge transfer resistance of the bilayer after ER- α /17E interaction. This observation is due to a change of conformation of ER- α after its interaction with 17E. This phenomenon has already been observed during olfactory binding protein/odorant interaction²⁶.

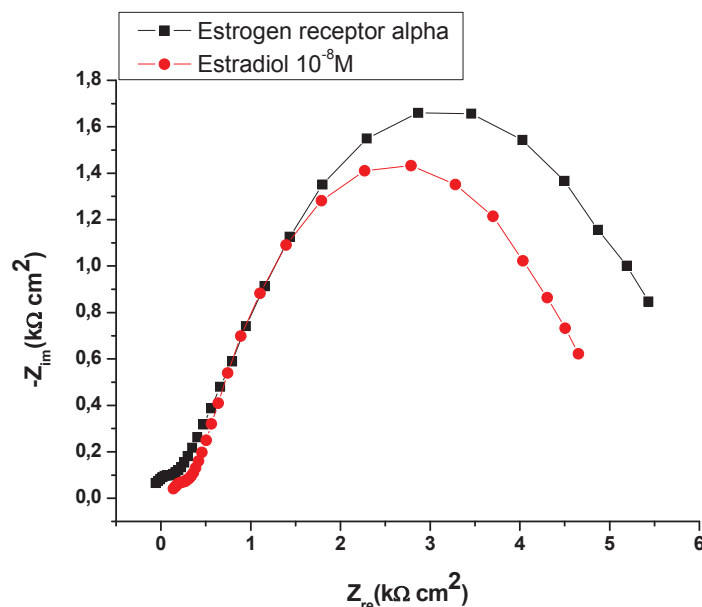


Fig. 4. Nyquist diagram of the ER- α functionalized gold electrode/ electrolyte interface before and after 17 β -estradiol interaction.

2.3.3.2. SWV Measurements

After incubation of E17, typical SWV curves obtained are presented in Figure 2. A typical E17 oxidation peak appears at 500 mV. The position of this peak has already been observed in the range of 450 mV–600 mV^{18,19,21}. The E17 peak maximum increases with successive increases of E17 concentration increases. This observation is in agreement with the decrease of the charge transfer resistance of the bilayer after ER- α /E17 interaction.

When incubated E17 concentration increases, the Cu peak maximum is shifted to lower potential (from 285 mV to 125 mV when concentration varies from 10⁻¹⁵M to 10⁻⁹ M) and it increases in intensity. This phenomenon must be probably due to the change of conformation of Cu surrounding when ER α -E17 complex is formed.

As a calibration curve, the E17 peak/Cu peak ratio was plotted as a function of -logarithm of concentration of 17 β -estradiol, as presented in Figure 5. Through this representation, a standard deviation of 10% was obtained with three different electrodes.

The selectivity of the ER- α biosensor was evaluated by incubating solutions of different concentrations of 17 β -estradiol (10⁻¹⁵M to 10⁻⁹ M) containing a mixture of

interfering substances (EE2, estrone, estradiol, BPA) at a concentration of 10^{-9} M. The measured points are presented in Figure 5. For concentrations of E17, up to 10^{-13} M, no interfering effect is observed. When E17 concentration is higher than 10^{-13} M, in the presence of interfering substances, the E17 oxidation peak does not increase, due to a competition effect on ER- α .

The performances of the ER- α biosensor developed in this work are compared to those of the relevant electrochemical biosensors for detection of 17β -estradiol, in Table 1. The lowest detection limit was obtained with our ER- α biosensor. The obtained limited dynamic range is quite compatible with the observed concentrations in real waters.

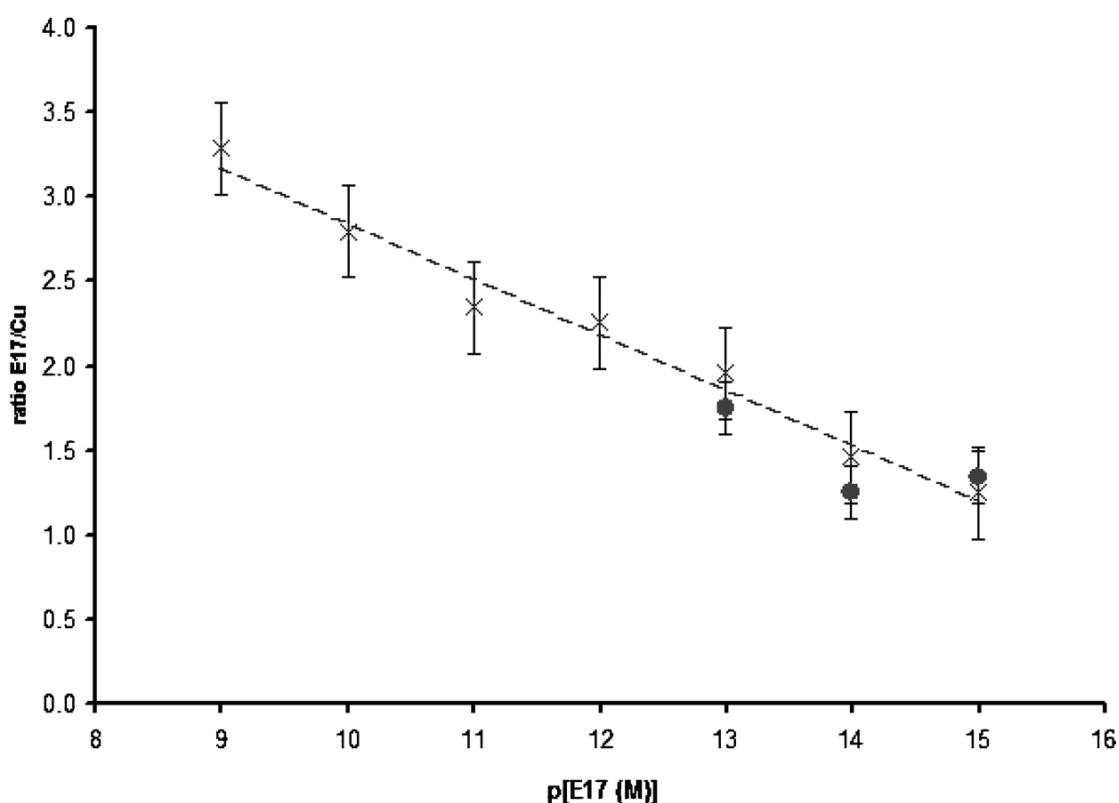


Fig. 5. Ratio of E17 peak/Cu peak as a function of -logarithm of concentration of 17β -estradiol. (x) E17 in 10 mM PBS solution ,
(●) E17+ 10^{-9} M of interfering substances (estrone, estriol, BPA).

2.3.3.3. Detection of 17β -Estradiol in Hospital Effluents

The method of standard additions was applied to real water samples, using the ER- α biosensor. Hospital effluents before and after the Bellecombe water treatment

plant were sampled from the SIPIBEL pilot site (<http://www.graie.org/graie/sipibelpublic/a-sipibel.htm>). The results are presented in Figure 6. It is noticeable that the E17/Cu ratio is lower for input effluents, compared to output effluents. This is due to the fact that the Cu peak maximum is higher for input effluents, the concentration of EDC substances being higher. The measurement of the Cu peak could be used in an early warning system for the assessment of a global endocrine disrupting effect of an effluent.

The determined concentration of 17β -estradiol in the input effluent was 262 ± 30 fM and 14 ± 0.2 fM in the output effluent. These samples were analysed using the LC-MS/MS technique, whose detection limit is 0.4 pM for 17β -estradiol. No estradiol was detected.

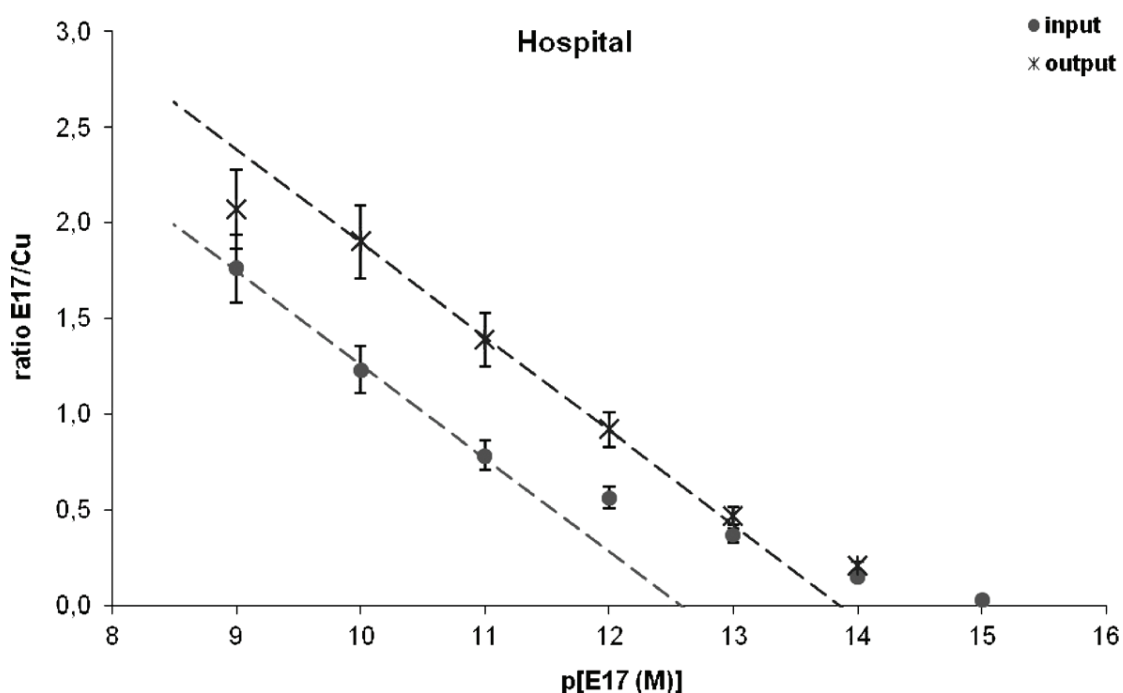


Fig. 6. E17/Cu ratio versus $-\log$ of E17 concentration obtained with the method of standard additions applied to input and output hospital effluents from SIPIBEL pilot site.

2.4 Conclusions

In summary, a sensitive, selective, rapid and simple biosensor for the detection of

17 β -estradiol has been developed using ER- α immobilized on gold electrodes. The detection was based on SWV of the E17 oxidation peak and the Cu peak, the calibration curve being presented as E17/Cu ratio versus $-\log[E17]$. This device has been demonstrated to be able to detect E17 in the linear range from 1 fM to 1 nM, with a detection limit of 1 fM. Good selectivity was obtained for interfering substances at nanomolar level, for concentration of E17 up to 0.1 μ M. The E17 was detected in hospital effluents. It is shown that the measurement of the Cu peak could be used in an early warning system for the assessment of a global endocrine disrupting effect of an effluent.

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Chapter III:
A novel platform based on
immobilized olfactory
receptors for detection odorant
molecules characteristic of
boar taint

Chapter III: A novel platform based on immobilized olfactory receptors, for detection odorant molecules characteristic of boar taint**3.1 Introduction and Current state of the art in detection of odorants**

Odorant molecules can form a signature of metabolic states or diseases, participate in aromas in food or liquid and allow detection of drugs, explosives or domestic and environmental pollutants. Hence, there is an increasing interest in emerging technologies allowing rapid and noninvasive assessment of volatile odorant compounds¹. Previously, electronic nose devices were based on physical or chemical sensing elements; however they are not adequate to mimic the performances of the natural olfactory system. In recent years, biomimetic olfactory-based biosensors have become a promising research field and great effort has been put into the development of novel highly sensitive olfactory biosensors using various biological olfactory components as sensing elements.

The mammalian olfactory system has an excellent capacity in recognizing and discriminating tens of thousands of odorants. The olfactory receptors (ORs) expressed at the cell membranes of olfactory sensory neurons, are the natural sensing elements for odorants, and are thus crucial for odorant detection. These receptors are members of the class A rhodopsin-like family of G protein-coupled receptors². They are made up of seven transmembrane helices, joined together by three extracellular and three intracellular loops. Activated olfactory receptors are the initial partners in a signal transduction cascade which ultimately produces a nerve impulse which is transmitted to the brain. The receptors activate the intracellular G protein, which causes effectors to produce a secondary signal inside the cell, causing the cell to react to the original external chemical signal³. These transduction proteins can thus receive chemical signals outside of the cell and transmit them into the interior of the cell.

This work is focused on the detection of boar taint. Boar taint spoils taste and odor of pork meat. This is due to some volatile compounds, including androstenone (cf. Scheme 3). They are especially concentrated in the fat, but are also found in several

parts of pig body, such as submaxillary salivary glands and testes^{4a,4b,5}. The olfactory receptor OR7D4 from chimpanzee was identified as being specific to androstenone, with an improved sensitivity relative to OR7D4 from human or other species⁶.

Recent discussions focused on the fabrication of OR-based biosensors and detection methods. At present the research is still in early stage and needs to be extended.

To produce functional ORs, some groups have used various OR expression systems, either mammalian cells^{7-13,14}, *Saccharomyces cerevisiae*-based¹⁵⁻¹⁸ or *E.coli*^{19,20} also regarded as most promising systems. Here, ORs are produced in *Saccharomyces cerevisiae*, which constitutes an efficient heterologous expression system. The rationale is that yeast cells withstand growing at low temperature (15°C), which probably improves ORs folding and trafficking into the plasma membrane. The temperature downshift may also induce cold shock proteins, some of which able to serve as molecular chaperones.

As for detection, the main transducing techniques used in olfactory receptor-based biosensors are presented in a recent review²¹. Electrochemical, resonant and optical sensors are most widely used. Among electrochemical transduction techniques, microelectrodes for recording extracellular potentials of olfactory sensory neurons, light addressable potentiometric sensors, field effect transistors and electrochemical impedance spectroscopy have already been used. In the present study, we used the square wave voltammetry to detect odorant-dependent signal change from ORs carried by nanosomes²² grafted through the N-terminal 6-His tag of the ORs onto a gold electrode previously modified with NTA-copper complex²³. SWV was determined to be a more rapid, efficient, and low cost detection technique than Electrochemical Impedance Spectroscopy²⁴, and it has not yet been employed for odorant detection. Moreover, the instrumentation used for SWV measurements is much more simple, and miniaturizable, which is an advantage for use in biosensors arrays.

The related OR7D4 olfactory receptors immobilization is visualized through fluorescence microscopy. The relative variation of current Cu(I)-Cu(II) peak increases linearly versus log (concentration of androstenone) from 10^{-14} M to 10^{-4} M, in buffer

solution. Negative tests were performed, using an unrelated odorant, helional, itself a ligand of OR 1740. Cross-selectivity was also tested after immobilization of OR 1740.

This new bio-electronic nose platform can overcome some drawbacks of metal oxide semiconductors or conducting polymers and also can help in increasing sensitivity and selectivity²⁵.

3.2 Experimental section

3.2.1. Materials and reagent

Saccharomyces cerevisiae yeast strain MC18 (MATa *gpa1 :: lacZ [LEU2] ade2-1 his3-11, 15 leu2-3,112 trp1-1 ura3-1 can1-100*), kindly provided by Prof. I. Connerton (University of Nottingham, UK) was transformed in order to express the relevant OR carrying the 6HIS tag at its N-terminus. Yeast colonies were grown in 0.67% yeast nitrogen base (Difco), synthetic drop-out CSM media without HIS, LEU, TRP, URA (Bio101, Inc., CA, USA), 40 mg/ml adenine, 80 mg/ml tryptophane, complemented with 2 % glucose.

ORs expression was obtained by induction of the transformed yeasts with 2 % galactose at 15°C for a duration of 108h, optimized from the 60 hours described previously²⁶. Preserving the integrity and functionality of expressed receptors remains a challenge. This can only be achieved by maintaining at all times the presence of a lipidic environment around the receptors. ORs being located within the yeast membranes, the yeast membrane fraction was prepared in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenyl methyl sulfonyl fluoride and the Complete protease inhibitor cocktail (Roche, Meylan, France). The protein concentration of the membrane fraction preparation was determined using the BCA reagent (Pierce, Perbio Science, Brebieres, France) with bovine serum albumin as a standard. In addition, for all experiments the membrane fraction was sonicated prior to use, for 10 minutes at 2x160 W, 35 kHz (Sonoclean S2600 sonicator, Labo-moderne, Paris, France) in ice-cold water. The average diameter of nanosomes is between 89±23 nm, as evaluated by Dynamic Light Scattering²⁷. Nanosomes were filtered prior to preparing the working solutions at 80µg/ml^{16,28}.

6-Mercaptohexanoic acid ($\geq 90\%$, C6), Na, Na-bis(carboxymethyl)-L-lysine hydrate ($\geq 97\%$, NTA), N-Hydroxysuccinimide (98%, NHS), Sodium acetate, Copper acetate were purchased from Aldrich. Acetic acid, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride ($\geq 99\%$, EDC), Ethanolamine were purchased from Fluka. DMSO was purchased from Sigma.

The buffer solution used for all experiments was phosphate buffer saline (PBS, tablet): One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C. All solutions were made in ultrapure water produced by a Millipore Milli-Q system.

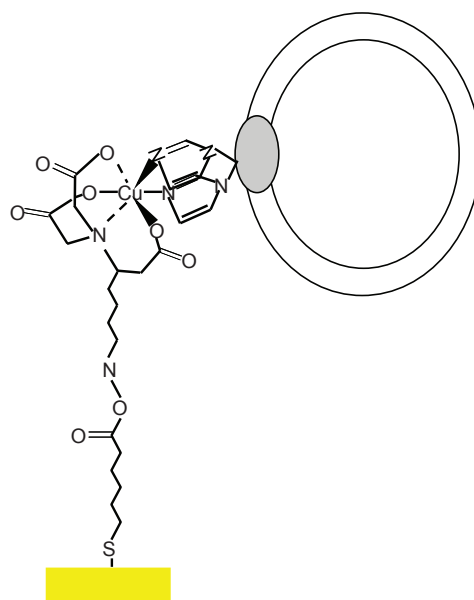
The odorant androstenone was purchased from Sigma-Aldrich and helional was a gift from Givaudan-Roure, courtesy of B. Schilling, Dübendorf, Switzerland).

3.2.2. Pretreatment and biofunctionalization of electrodes

The gold electrodes were fabricated by evaporation of a gold layer of 300 nm thickness on silicon substrate using a titanium layer as adhesion layer of 30 nm thickness. These gold electrodes were provided by the RENATECH platform in LAAS, CNRS Toulouse. Gold electrodes were first sonicated for 15 mins at room temperature in acetone using Branson 5210 ultrasonic bath (Branson Ultrasonics Corporation, Danbury, USA) and rinsed with ultrapure water and dried under nitrogen flow. Then gold electrodes were cleaned for 5 mins with freshly prepared "piranha" mixture (piranha: $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 3:7, v/v), then rinsed with ultrapure water, ethanol and dried under nitrogen flow²⁹.

6-His tagged ORs (carried by nanosomes) were anchored on the gold electrode surface as presented in Scheme 1. The pretreated electrodes were immersed over night at -4°C in 1 mM 6-mercaptohexanoic acid solution in ethanol to give an alkanethiol SAM bearing carboxylic terminal groups. The modified electrodes were then rinsed with ethanol. Activation step was performed by reaction with a 150 μl drop of 10mM EDC/10mM NHS mixture solution for 1h. Carboxyl groups of gold electrodes were converted into active carbodiimide esters which can then react with NTA

terminal amine group to form covalent bonds (7mM NTA solution on gold surface for 1h). Then rinsing with ethanolamine (1:1000 diluted in PBS) was performed to passivate unreacted carbodiimide esters. After this, the gold electrodes were immediately rinsed with sodium acetate/acetic acid buffer solution and immersed in 0.01M copper (II) acetate in 0.01M acetate buffer solution at pH 4.6 for 45 min for the coordination of Cu^{2+} ions with grafted NTA groups to form a Cu^{2+} metal complex. Finally, 150 μl of a solution of nanosomes carrying 6-His tagged ORs, was dropped onto the surface and incubated for 1h in room temperature, prior to rinsing with PBS and use for detection of odorants.



Scheme 1: Schematic representation of 6-His tagged OR in nanosome anchored on gold electrode

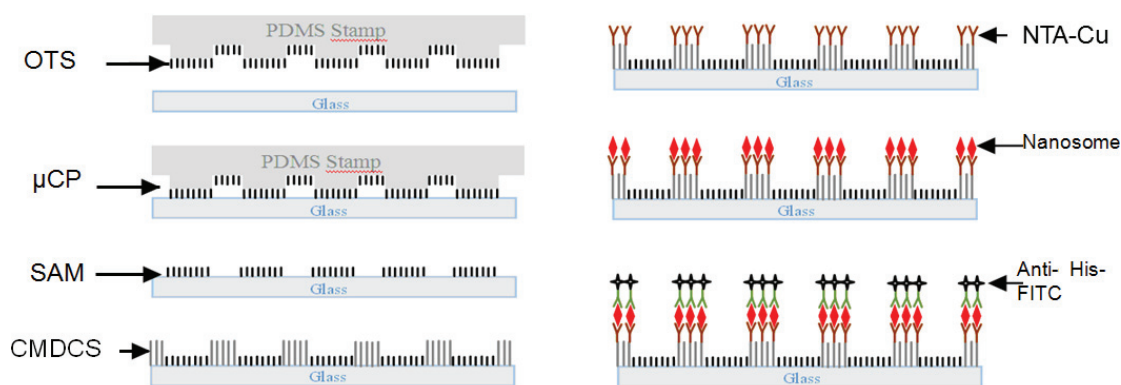
3.2.3. Microcontact printing for fluorescence microscopy

A glass slide electrode was used for fluorescence microscopy. The PDMS stamp (1 cm^2) with holes of 50 μm of diameter, was used for contact printing of octadecyltrichlorosilane on glass slide electrode (cf. Scheme 2). First, PDMS stamps were cleaned with ethanol and they were then ultra-sonicated for 30 mins. The glass slide was cleaned for 30 mins in piranha mixture, then rinsed with ultrapure water and dried with nitrogen. Then, PDMS stamp was immersed in octadecyltrichlorosilane (OTS) (5 μM OTS and 0.4 mM of carbon tetrachloride in heptane solution) for 30

seconds and dried under nitrogen flow. Next, stamping of the glass with the inked PDMS was performed for a few seconds. The glass substrates must be left in the oven at 100°C for one hour in order to enhance the chemical bonding of OTS to the glass substrate.

The glass substrates were then immersed in 10-(carbomethoxy)decyl-dimethylchlorosilane (CMDCS) solution (10 mM CMDCS and 0.4 mM of carbon tetrachloride in heptane solution at 4°C for 4 hours). Silanized glass slides were thoroughly rinsed slowly with heptane, water and dried under a nitrogen flow. The glass substrates must be left one more time in the oven at 100°C for one hour in order to enhance the chemical bonding of CMDCS to the glass substrate. In the end, substrates were immersed in 1N HCl (37%) overnight at ambient temperature, then rinsed copiously with water, and finally dried under nitrogen flow. After these treatments, the immobilization of 6-His tagged OR was performed according to biofunctionalization steps (cf. §2.2.). After 6-His tagged ORs immobilization step, 2ml of FITC conjugated anti-His antibody was maintained in contact with biofunctionalized glass slide.

Then the prepared glass slide was observed by fluorescence microscopy (Zeiss Axioplan 2 Imaging apparatus, Carl Zeiss MicroImaging GmbH, Jena, Germany).



Scheme 2: Microcontact printing and fluorescence detection of 6-His tagged OR

3.2.4. Preparation of odorant solutions

Stock solutions of odorants (androstenone 10^{-2} M and helional 10^{-1} M) were prepared freshly on the day of the experiment in DMSO, and then 10^{-4} M dilutions were performed in PBS directly from the stock solution. Further dilutions were prepared by successive 1:10 dilutions in PBS from 10^{-4} M to 10^{-14} M.

With OR7D4 functionalized electrodes, androstenone was used as the specific odorant and helional as a negative control. In order to test the specificity of functional response, OR1740 functionalized electrodes were used, on which helional was used as the specific odorant and androstenone as the negative control.

3.2.5. Electrochemical measurements

Square wave voltammetry (SWV) was performed using a Voltalab 80. A three-electrode cell was used, including a saturated calomel electrode (SCE) as reference electrode, a platinum plate as counter electrode (0.54 cm^2) and a modified gold working electrode (0.19 cm^2)³⁰. The square wave voltammetry measurements were carried out in the potential range from -400 mV to $+600 \text{ mV}$ after maintaining

potential at -400mV for 5mins for the total reduction of Cu(II) to Cu(I).The scan rate, duration, amplitude were 100mV/S, 0.05s, 5mV, respectively³¹.

The impedance spectroscopy measurements were carried out in the frequency range from 0.05 kHz to 100 kHz at different dc potentials at 0mV vs.SCE electrode, using a modulation voltage of 10 mV. After each measurement, the solution was removed and the cell rinsed with PBS.

A voltammetric scan is completed within seconds. A familiar peak-shaped current response is produced and this can be directly used to establish calibration. An appropriate frequency can be quickly determined by multiple pulse chronoamperometry. In contrast, a significantly 10 times longer analysis time and substantial effort is required to complete the EIS determinations relative to SWV.

3.3 Results

3.3.1. Control of 6 His tagged chimpanzee OR7D4 immobilization by fluorescence microscopy

Due to the use of FITC conjugate anti His-antibody, it is possible to visualize the immobilization of 6-His OR7D4 nanosomes. As shown in Figure 1, where OTS was stamped there is no fluorescence (black part). Green spots of 5 μm diameter appear, due to the presence of 6-His tagged OR7D4 nanosomes immobilized on Cu-NTA. This result demonstrates that OR7D4 nanosomes were successfully immobilized on Cu-NTA surface, and so it can be used further for target detection.

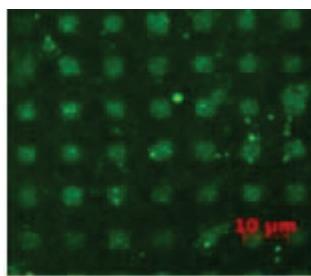


Fig. 1. Fluorescence image of FITC conjugate anti His-antibody with the chimpanzee 6xHis OR7D4 carried by nanosomes grafted on microcontact printed glass substrate.

3.3.2. Control of copper complexation by voltammetry

In addition, in order to check electrically that NTA/Cu²⁺ complex has successfully been formed on the gold electrode, square wave voltammetry was performed. After maintaining at -400 mV for 5 min, the oxidation peak Cu(I) to Cu(II) appears at +33 mV (data not shown). This result demonstrates that copper ions are well immobilized as NTA complex at the gold electrode surface and its peak surface area allows the determination of a copper surface coverage of 1×10^{-9} mol cm², with an RSD of 20%, showing that a dense monolayer of chelated copper ion is available on grafted electrode surface.

3.3.3. Functional response of 6 His tagged chimpanzee OR7D4 by Electrochemical detection

3.3.3.1. Detection of odorant by impedance measurements

After androstenone was incubated for 1 hr with OR7D4 nanosomes functionalized gold electrode, a decrease of the impedance of the electrode/electrolyte interface was observed (cf. Fig. 2), reflecting a decrease of the charge transfer resistance of the bilayer after OR7 D4/androstenone interaction. This observation may arise from a change of conformation of OR7D4 after its interaction with androstenone. This phenomenon has already been observed during olfactory binding protein/odorant interaction³².

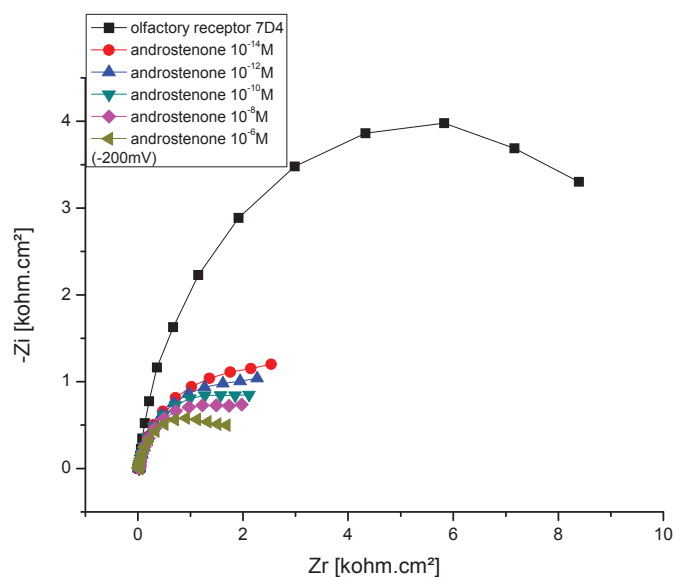


Fig. 2. Nyquist diagram of the OR7D4 nanosomes functionalized gold electrode/electrolyte interface before and after interaction with androstenone at various concentrations.

3.3.3.2. Square wave voltammetry measurements

As shown in Figure 3 (black curve), after 6 His tagged chimpanzee OR7D4 nanosomes were immobilized, the Cu(II)/Cu(I) peak (Cu peak) shifted to 190 mV, showing that a different Cu(II) complex is formed. Its peak maximum decreased by a factor of 10, showing that the electron transfer rate greatly decreased after OR 7D4 immobilization. This phenomenon has already been observed in the case of 6 His-tagged reduced antibody immobilization³¹. The Cu(II)/Cu(I) peak maximum is correlated to the surface density of immobilized OR7D4 nanosomes.

After incubation of androstenone, typical SWV curves obtained are presented in Fig 3. Cu(II)/Cu(I) peak maximum increases when the odorant concentration increases from 10^{-14} M to 10^{-4} M. This phenomenon may be due to the change of conformation of Cu surrounding OR7 D4 when it is activated in presence of androstenone.

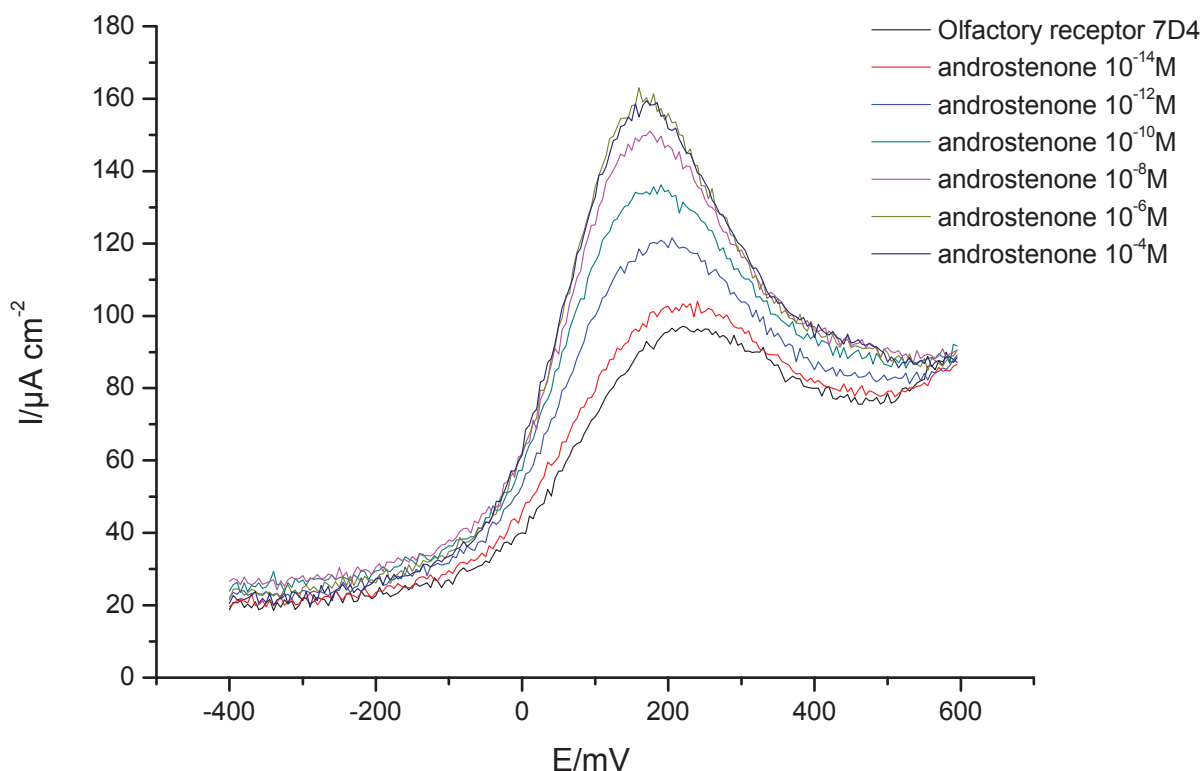
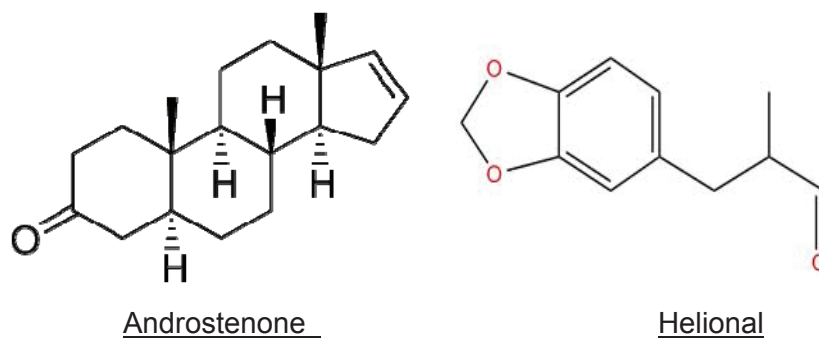


Fig. 3. The square wave voltammetry of OR7D4 nanosomes biolayer before and after incubation with androstenone, in PBS. The amplitude, scan rate and potential range are 5 mV, 100 mV/s, -0.4 V to +0.6 V, respectively.

3.3.3.3. Study of cross selectivity of ORs

As a calibration curve, the relative variation of Cu(II)/Cu(I) peak maximum was plotted as a function of logarithm of concentration of androstenone, as presented in Figure 4. Through this representation, a standard deviation of 10% was obtained with three different electrodes.

We also used helional, which has a completely different chemical structure compared to androstenone (cf. Scheme 3), as an unrelated odorant. It was incubated with OR7D4 nanosomes functionalized electrode. Relative variation of Cu(II)/Cu(I) peak maximum as function of logarithm of concentration of helional is very low, compared to its variation with androstenone (cf. Figure 4).



Scheme 3: Chemical formula of odorant

A cross selectivity test was performed with OR 1740 nanosomes functionalized electrode, as presented in Figure 5. Relative variation of Cu(II)/Cu(I) peak maximum as function of logarithm of concentration of helional was found higher than that obtained with androstenone. The observed sensitivities, measured using SWV are in good agreement with the known odorant /OR affinities⁶.

Within electrochemical methods, and compared with the references reported, our detection limit (10^{-14} M) seems lower than others, such as presented by Sankaran and references herein (1 ppm)²⁵.

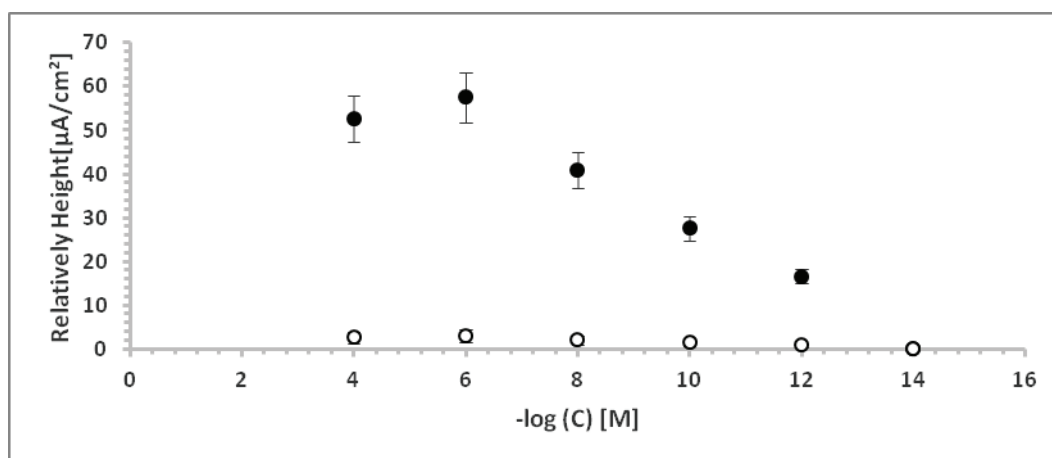


Fig. 4. Relative variation of Cu(II)/Cu(I) peak maximum obtained with OR7D4 nanosomes functionalized electrode as a function of concentration of odorant.

(● androstenone and ○ helional)

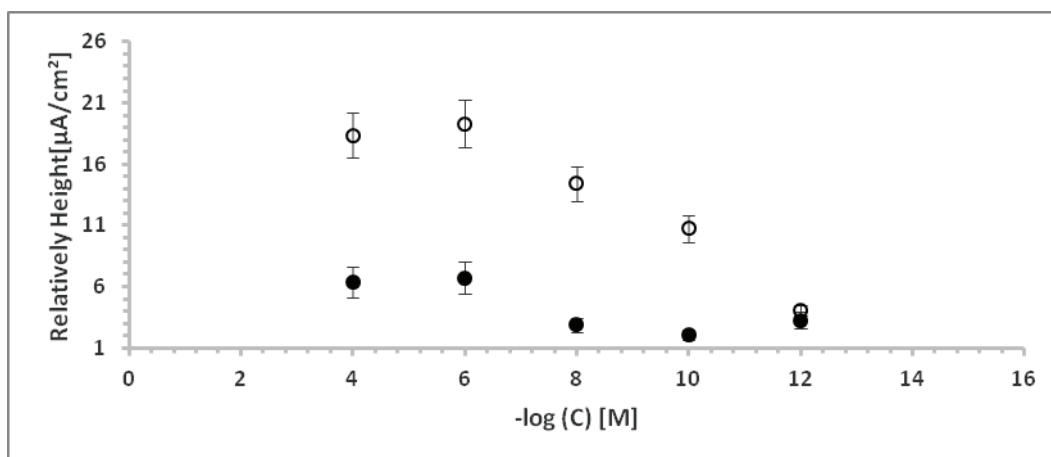


Fig. 5. Relative variation of Cu(II)/Cu(I) peak maximum obtained with OR 1740 nanosomes functionalized electrode as a function of concentration of odorant.

(○ helional and ● androstenone)

3.4 Conclusion

In this study, we presented an effective tool for monitoring odorant in solution based on relatively specific olfactory receptors (OR7D4 and OR 1740) carried by nanosomes immobilized on gold electrodes. This platform can detect odorant binding onto the receptor with label-free SWV measurements, indicating that functionality is preserved, and that it is sensitive and selective. In the future, multisensor assay, based on arrays of ORs for the detection in parallel of multiple volatile organic compounds will be performed.

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**Chapter IV:
TNT recognition by biomimetic
sensor based on
electropolymerization of
oligothioaniline-functionalized
gold nanoparticles**

Chapter IV: TNT recognition by biomimetic sensor based on electropolymerization of oligothioaniline-functionalized gold nanoparticles**4.1 Introduction**

Increasing efforts have been focused on developing devices for detection of explosives with significant impact on environmental protection and homeland security. Nitro-aromatic explosives, such as trinitrotoluene (TNT), are used in landmines and for military purposes, leading to contamination of the environment, infiltrating in soil and groundwaters in war zones and near military facilities ¹. These compounds are highly toxic and carcinogen to living organisms, causing health problems, such as aplastic anemia, toxic hepatitis, hepatomegaly, cataract and skin irritation ². Various sensors have been reported in the recent years for the detection of TNT, based on fluorescence of different materials quenched by TNT, such as creatinine-functionalized quantum dots ³, graphene quantum dots ⁴ or functional polymers ⁵, on surface-enhanced Raman spectroscopy substrates based upon highly ordered gold nanoparticles (AuNPs) ⁶ or silver nanoparticles/graphene nanosheets composites ⁷, on surface plasmon resonance ^{8,9} or on the redox activity of the nitro groups of TNT in electrochemical sensors ^{10,11}.

The molecular imprinting technique is a versatile method for the development of biomimetic sensors. The method is based on polymerization and crosslinking of functional monomers in the presence of a template molecule. The subsequent removal of the template from the resulting polymer network leads to the formation of cavities that are complementary in geometry and chemical functionality with the guest molecule. With the advantages of high affinity and specificity, good mechanical strength, thermal and chemical stability, low cost and ease of preparation, molecularly imprinted polymers (MIPs) have found potential applications in many fields such as chromatography ¹², drug analysis ^{13,14}, immunoassays ¹⁵ or biomimetic sensors ^{16,17}. The non-covalent approach for imprinting materials, based on the formation of template-monomer adduct by non-covalent interactions and pioneered by Wulff and co-workers, has been proven to be more versatile than the alternative covalent

approach, due to the less complicated preparation process and the broad selection of functional monomers and possible target molecules¹⁸. The binding of the target molecule to the recognition sites in MIPs can be assessed by means of electrochemical^{19,20}, optical²¹ or piezoelectric²² methods. Electrochemical methods have been widely applied, due to their rapidity, simplicity and low cost. The preparation of MIPs by direct electropolymerization on the sensing surface has gained increased attention lately, offering the advantage of a better control of the film thickness at the electrode surface. Moreover the use of conducting polymers leads to an increased sensitivity. Different types of polymers have been investigated for the preparation of MIPs such as polypyrrole, aniline or poly(*o*-phenylenediamine). Few MIP-based sensors for the detection of TNT have been reported. Among them different polymer synthesis approaches coupled with electrochemical^{23, 24}, piezoelectric²⁵ and optical^{26,27} detection techniques.

A significant research interest has been given for the developing of nanomaterials for the construction of sensing devices. Among them, gold nanoparticles (AuNPs) arised great attention and have been extensively studied, owing their unique physicochemical attributes²⁸. The sensitivity of AuNPs properties, such as color, surface plasmon resonance, electrical conductivity and binding affinity is significantly enhanced when they are subjected to functionalization with an appropriate metal, organic or biomolecular functional groups²⁹. The integration of nanoparticles in MIP materials has the benefits of enhancing the number of accessible complementary cavities and the catalytic activity of the surface and of fast equilibration with the analyte³⁰. Microporous metal-organic frameworks have attracted interest lately and proven to be a highly sensitive and selective platform for the detection of explosives, due to the unique electronic properties of both microporous metal-organic frameworks and nitroaromatics and the nature of their interactions³¹. Willner et al used AuNPs and molecular imprinting technique for the development of sensitive and selective matrixes for the detection of picric acid, *N,N'*-dimethyl-4,4'-bipyridinium, and *N,N'* dimethylbipyridinium-4,4'-ethylene dichloride³² and for the analysis of various antibiotics³³.

In the present study, an electrochemical sensor for the sensitive and selective detection of TNT is proposed, based on molecularly imprinted technology in a conductive film of polythioaniline deposited on gold electrodes by means of an intermediate monolayer of *p*-aminothiophenol (PATP) and PATP functionalized-AuNPs. Firstly, a self-assembling monolayer of PATP is formed on the gold electrode surface through Au-S bonds. Then, a conductive hybrid film was deposited on the electrode via electropolymerization of the aniline moiety in PATP immobilized on gold electrodes and on AuNPs. In order to obtain MIP films the electropolymerization process was performed in the presence of TNT as template molecule, the subsequent removal of TNT leaving cavities that serve as specific recognition sites. Combining the advantages of molecularly imprinting and electrodeposition with those conferred by AuNPs, the developed sensor exhibited good sensitivity, with a low detection limit (LOD) and high selectivity.

4.2 Materials and methods

4.2.1. Chemicals and instrumentation

Trinitrotoluene (TNT), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), dinitrotoluene (DNT), *p*-aminothiophenol (PATP), tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium borohydride ($NaBH_4$), methanol and phosphate buffered saline (PBS) tablets, were purchased from Sigma-Aldrich with highest available purity. The gold sols were made up and stored in clean glass vials.

All solutions were prepared using Milli-Q water.

Electrochemical measurements were performed with VOLTALAB-80 PGZ/402 potentiostat-galvanostat, in three-electrode electrochemical cell, with, a gold working electrode (0.19 cm^2), a platinum plate (0.54 cm^2) as auxiliary electrode and a saturated calomel electrode (SCE) as reference.

UV-vis absorption spectra of the AuNPs nanoparticles were recorded at room temperature with a Perkin Elmer Lambda 900 UV/Vis/NIR spectrophotometer between 300 and 800 nm.

4.2.2. Fabrication of the molecular imprinting sensor

4.2.2.1. Preparation of the functionalized AuNPs

The AuNPs were synthesized according to the procedure described in the literature³⁴. For a typical preparation of gold nanoparticles, 31.6 mg (8×10^{-5} mol) of tetrachloroauric(III) acid trihydrate were dissolved in 30 mL of methanol in a round flask of 0.1 L equipped with a condenser. A solution of *p*-aminothiophenol (1.6×10^{-4} mol) in 12 mL of a mixture methanol/water (v/v) was added dropwise to the gold salt solution under stirring, which changes its color from yellow to dark brown. After 10 min, 30.4 mg of NaBH₄ (8×10^{-4} mol) dissolved in 2.2 mL of water were added dropwise to the mix under vigorous stirring. After 10 min, stirring was stopped and the solution was kept in darkness for 1 hour. The suspension was then filtered on a polymer membrane and washed successively with water and ether. The resulting black powder was dried and stored either as a solid or dispersed in 0.1 N HCl solution. The UV-vis spectra of gold nanoparticle colloids display a small surface plasmon band centered around 535 nm.

4.2.2.2. Synthesis of biomimetic materials onto the gold electrodes

The protocol used for the fabrication of MIP sensor was similar to the procedure reported before with slight changes³⁵.

Prior to modification, the gold surface of the electrodes was cleaned by exposure to UV/ozone. The gold slides were then immersed in 50 mM PATP ethanolic solution for 24 hours at 4°C. The electrodes were then rinsed with ethanol and double distilled water to remove the physically adsorbed PATP.

Cyclic voltammetry was employed for the deposition of poly-thioaniline AuNPs films onto the PATP-modified electrodes. In order to obtain imprinted films, the electropolymerization was carried out in a solution of 10 mM [Fe(CN)₆]^{-3/-4} in PBS pH 7.2 containing 0.1 mg/mL PATP-functionalized Au NPs and 44 mM TNT, by cycling the potential from -0.35 V to +0.80 V vs SCE, at a scan rate of 100 mV/s, for 10 cycles, followed by applying a fixed potential of +0.80 V for 30 min. The non-imprinted films (NIPs) were prepared in a similar manner, without the addition of TNT to the

polymerization mixture.

The extraction of the template from the film was performed by immersing the modified electrodes in a 1:4 (v/v) mixture of acetone: PBS solution for 30 min at room temperature .

4.2.2.3. *Electrochemical measurements on MIP and NIP-modified electrodes*

Linear sweep voltammetry (LSV) was carried out in 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in PBS pH 7.2 in order to investigate the release and the uptake process for variable concentrations of TNT solution, in the range of potential between +0.80V and -0.35V vs SCE, at a scan rate of 50 mV/s. Prior to LSV measurements the electrodes were left in contact with the solution to be analyzed for 1h at room temperature, for the uptake of the substrates, followed by a washing step with PBS, in order to remove the TNT molecules physically adsorbed on the surface of the polymer, and by applying a fixed potential of +800 mV for 5 min. During this step Fe (II) ions were oxidized to Fe (III) and a more stable signal for LSV was obtained.

The calibration curves for the imprinted and non-imprinted polymer were constructed upon the analysis of various concentrations of TNT ranging from 4.4 fM to 0.44 nM. The limit of detection (LOD, n=3) was also calculated.

4.2.2.4. *Surface characterization by atomic force microscopy (AFM)*

AFM was used to investigate the surface morphology of the electropolymerized films. The AFM measurements were carried out using an Agilent PicoPlus® AFM (Agilent Technologies, Palo Alto, CA,USA). Silicon tips with a nominal spring constant of 3 Nm⁻¹ were used in tapping mode at a frequency of ~70 kHz.

4.2.2.5. *The measurement of TNT in real samples*

The prepared MIPs were used to analyze TNT in tap and river water samples. The water samples did not contain any TNT, thus they had to be spiked with different concentrations of analyte. For this purpose, the MIP-sensor was incubated with the spiked solutions for 1h and the electrochemical measurement were carried out as

described in section 4.2.2.3. The recovery was calculated for each sample.

4.3 Results and discussion

4.3.1. Mechanism of molecular imprinting

Fig. 1. depicts the steps in the construction of the MIP sensor. In the first step, a monolayer of PATP is formed at the surface of gold electrodes via Au-S bonds between gold and thiol groups of PATP. In the next step, the MIP film is deposited on the electrode via electropolymerization in a mixing solution containing PATP-functionalized AuNPs and TNT. The π - π interactions between the electron-poor TNT and the electron-rich PATP promote the embedding of the host molecules in a three-dimensional polymer-AuNPs network. Finally, the template is removed from the complex matrix, forming surface imprinted sites. AuNPs have an important role in the matrix, for the enhancement of the conductivity by increasing the electron transfer, and for the amount of imprinted sites, leading to more homogenous distribution of the recognition sites.

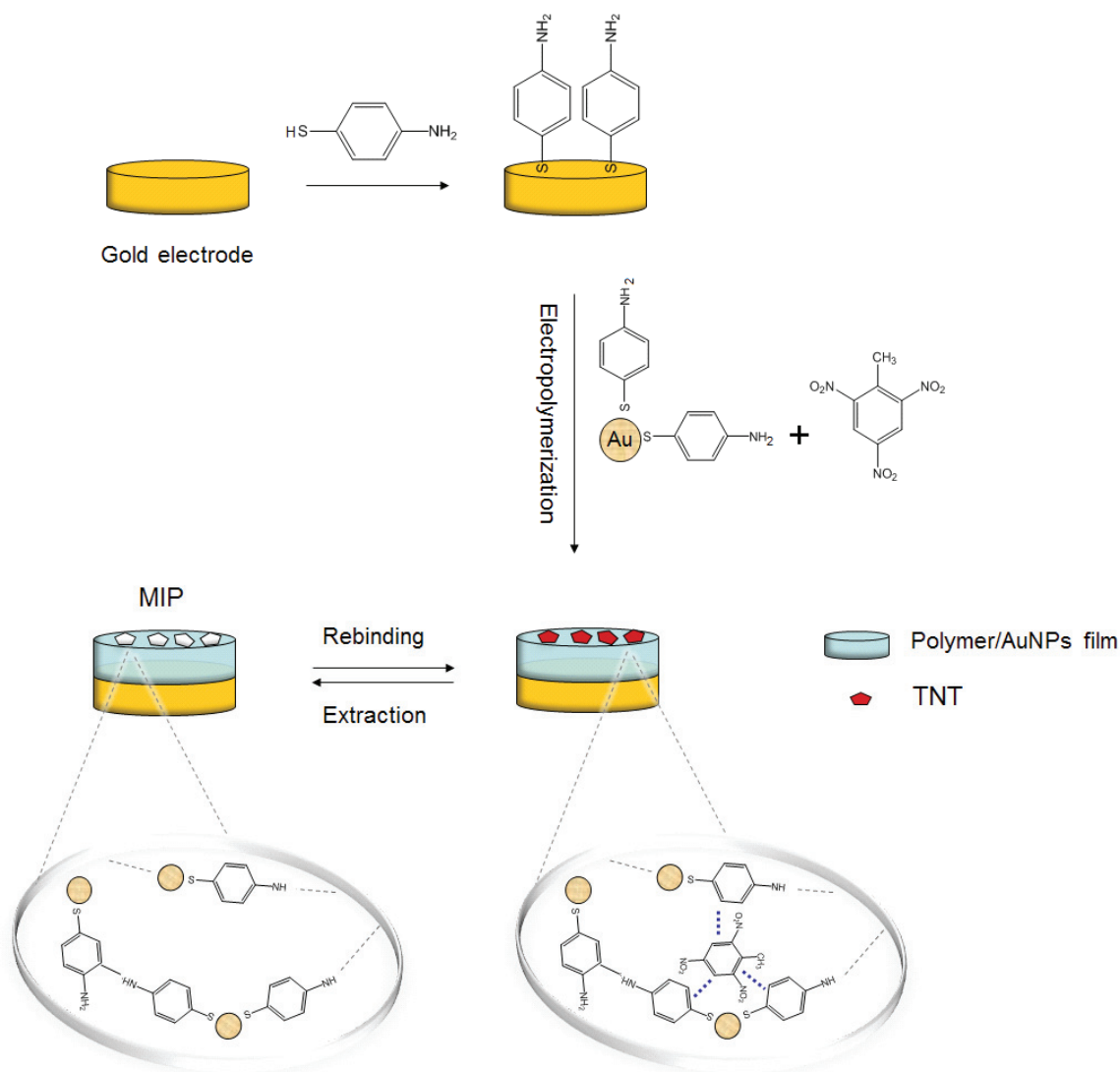


Figure 1. Schematic representation of the procedure for preparing MIP-based sensors for TNT detection

4.3.2. Electrochemical control of the preparation of the MIP and NIP based sensor

Figure 2 shows the electropolymerization process for the deposition of MIP films on the electrode surface in 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in PBS. The current gradually decreases with each scan cycle, due to the continuous formation of polythioaniline/AuNPs/TNT composite films that obstruct the access of the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the surface of the gold electrode, demonstrating that a polymeric film was formed on the surface of the electrode.

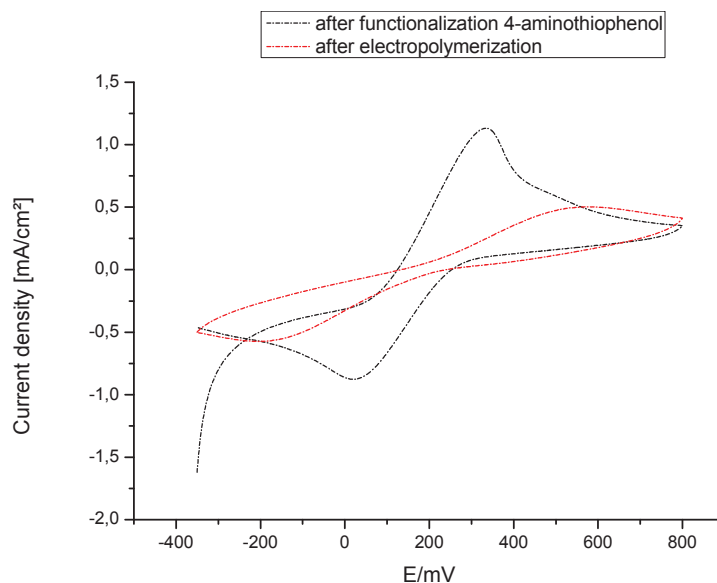


Fig. 2. Cyclic voltammograms after functionalization of the gold surface with PATP (black line) and after electropolymerization of thioaniline functionalized AuNPs on the surface of gold electrodes in the presence of 44 mM TNT solution (red lines). Scan rate 100 mV/s. Potential range: -350 to 800 mV vs SCE.

4.3.3. Electrochemical and Morphological characterization of MIP and NIP films

After the electropolymerization of MIP and NIP films, their electrochemical behaviour is quite different, as shown by voltammograms presented in Figure 3. Charge transfer through NIP film is very low, compared to that through MIP. It shows that their morphology should be quite different and that the presence of template molecule (TNT) could help for charge transfer.

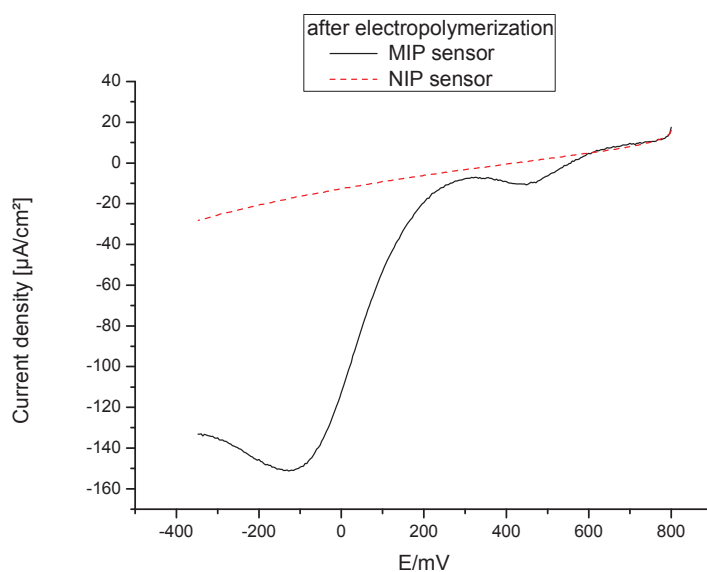


Fig. 3. The MIP and NIP sensors LSV behavior after electropolymerization.

Figure 4 shows the morphology of the prepared films observed by AFM. The obtained images confirm the electrodeposition of the polymer films onto the electrodes and offer important informations about the topography, porosity and surface roughness of the films. The AFM images of the NIP film (c, f) show a good coverage of the surface of the electrodes with a homogeneous, compact film, while for the MIP film (b, d) a polymer-AuNPs network with higher surface roughness and a good dispersion of AuNPs can be observed.

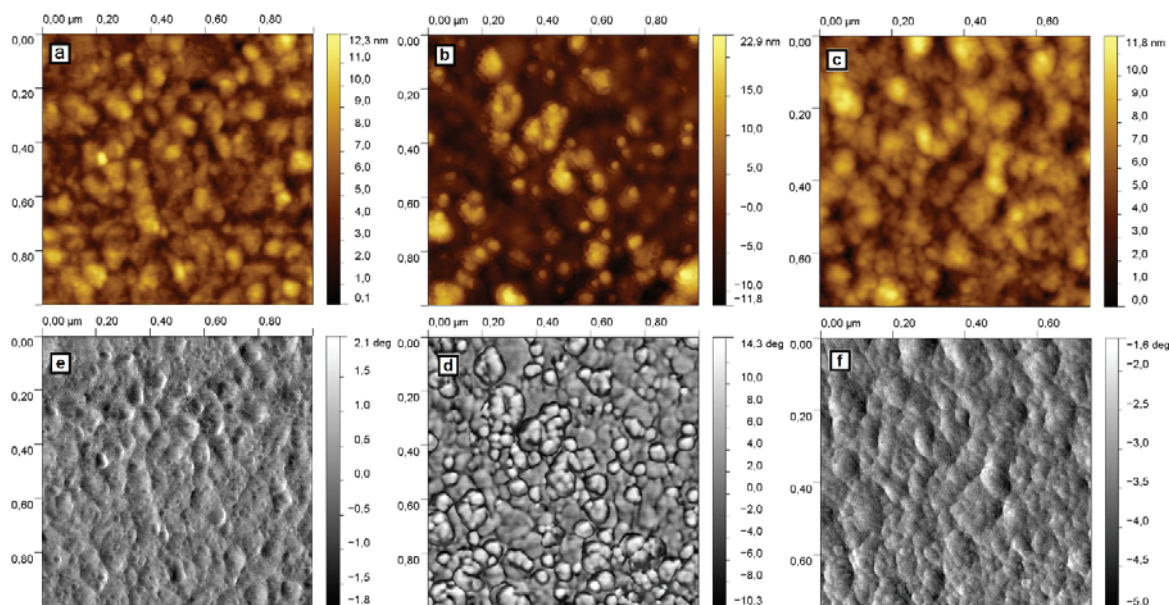


Fig 4. AFM analysis of bare- (a,e), MIP- (b, d) and NIP- (c, f) electrodes: topography (a, b, c) and phase (e, f, g) 2D images. The analysis was made at the end of a complete release-rebinding cycle of five concentrations of TNT (10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M) for both MIP and NIP.

4.3.4. Analytical performance of the imprinted and non-imprinted polymer

Nitrobenzene units undergo stepwise reduction³³. The nitro groups are reduced to hydroxylamine in the first step, followed by a reduction of hydroxylamine to amine groups. In order to check the uptake process for variable concentrations of TNT, LSV measurements were employed in a solution of PBS pH 7.2 containing 10 mM $[\text{Fe}(\text{CN})_6]^{-3/-4}$ (Figure 5). After the extraction of the template the prepared MIP was incubated with solutions of different concentrations of TNT. An increase in the current peak and a shift of the peak potentials to a more positive potential can be observed with the increase of TNT concentration from 4.4 fM to 0.44 nM (Fig.5) confirming the binding of the analyte in its specific cavities.

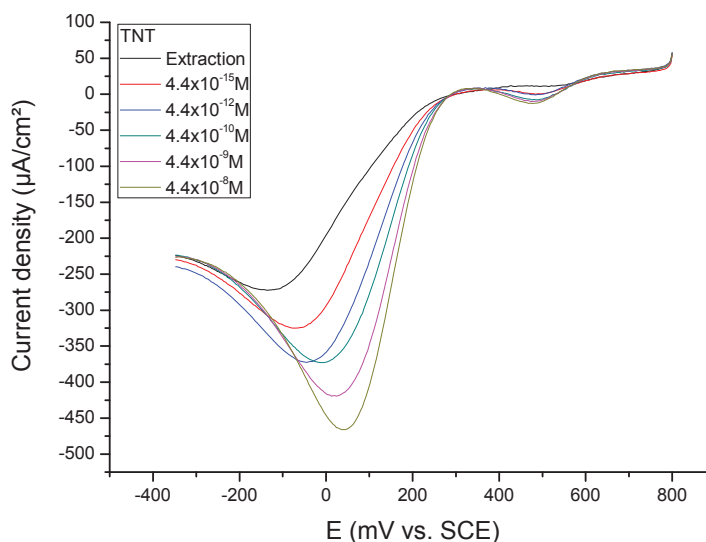


Fig 5. LSVs upon extraction and rebinding of various concentrations of TNT. Potential range: 800 mV to -350 mV vs SCE. Scan rate 50 mV/s.

Rebinding studies were performed in the range of concentration from 4.4 fM to 4.4 μ M, observing an increase in the current peak as the concentration increased for the MIP sensor. Upon the analysis of NIP in the same conditions, the current peaks were significantly reduced, due to the fact that for the polymerization in the absence of TNT no cavities were obtained. The current response in the case of NIP is probably due to the adsorption of the analyte on the surface of the polymer. These results confirm the formation of specific binding cavities in the MIP after the removal of the template. A linear relationship between the signal-blank/signal ratio ($(I-I_0)/I_0$) and the logarithmic concentration of TNT was obtained in the range of 4.4 fM to 0.44 nM. At higher concentrations saturation is achieved (data not shown). The limit of detection was calculated interpolating the mean current obtained for the blank for three replicates plus three times its standard deviation in the linear equation. Blank is the signal obtained after extraction of the template, upon analysis of a solution containing 10 mM $[\text{Fe}(\text{CN})_6]^{-3/-4}$ in PBS pH 7.2 that does not contain TNT. In order to test the reproducibility three determinations on different electrodes were assayed for each concentration. A low limit of detection of 0.04 fM was obtained for the MIP sensor.

The selectivity of the prepared MIP sensor was assessed using 2,4-dinitrotoluene (DNT), another nitro-aromatic compound with similar structure. LSV was employed as previously described of detection DNT alone (10^{-10}M , 10^{-8}M). The response of the MIP sensor towards binding of TNT is illustrated in Figure 6, TNT have a higher ability to form charge-transfer complexes, induce a stronger current signal change than other nitro-substituted aromatic molecules of a less electron withdrawing nature. Which make MIP-sensor more suitable specific detection TNT.

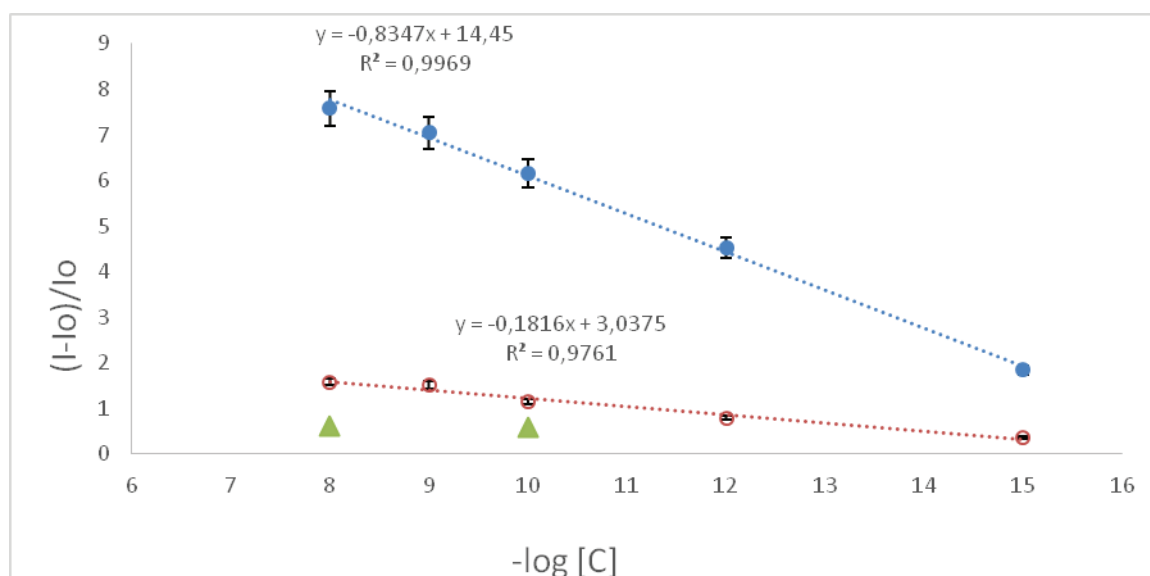


Fig. 6. Calibration curves upon analysis of various concentration of TNT on MIP-modified sensor (●), NIP-modified sensor (○), selectivity of MIP sensor upon analysis of concentrations of DNT(▲) (at 10^{-10}M , 10^{-8}M).

4.3.5. Water samples application

In order to assess the suitability of the developed sensor for the analysis of TNT in complex matrixes, different water samples were analyzed using the protocol previously described. The tap and river water samples did not contain any TNT, thus they were spiked with the analyte at certain concentrations (10^{-15}M , 10^{-12}M , 10^{-9}M). The results are summarized in Table 1. The obtained recoveries ranged between 96.7% and 102.17% , showing that the method is suitable for the analysis of TNT in complex matrixes, with future perspectives on environmental applications. For each

concentration a number of three determinations were performed.

Table I. Determination of TNT in various water samples

Sample	TNT added -log (C)/ M	TNT detected -log (C)/ M	Recovery (%)	RSD % (n=3)
Tap water 1	15	15.19	101.30	1
Tap water 2	12	11.60	96.70	2
Tap water 3	9	9.19	102.17	2
River water 1	15	15.03	100.25	10
River water 2	12	12.03	100.25	3

4.4 Conclusion

The easy fabrication and robustness of MIPs make them ideal candidates for the development of sensing devices. The development of an electrochemical sensor for sensitive detection of TNT based on MIP-based polymer/AuNPs hybrid membrane was reported herein. The MIP was prepared on the surface of PATP functionalized gold electrodes by electropolymerization of PATP-functionalized AuNPs in the presence of TNT as template molecule. The developed sensor showed a wide linear range between 0.44 nM and 4.4 fM with a LOD of 0.04 fM, lower than that reported for other TNT sensors. Compared with the similar sensor reported by Willner group³³ we strikingly improved the sensitivity from 200 pM to 0.04 fM using ferro/ferricyanide as redox probe, lowering also the manufacturing cost by a 10-fold decrease of the concentration of the reagents. With a good sensitivity and reproducibility, the MIP hybrid sensor was shown to have potential to be an effective method for the electrochemical detection of TNT in water samples for environmental protection.

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Chapter V:
General Conclusion and
Perspectives

Chapter V: General Conclusion and Perspectives

In summary, we first developed receptors based biosensors (affinity) for electrochemical detection of estradiol and odorant molecules, respectively. Sensitive, selective, rapid, simple, repetitive, stable and effective biosensors were produced.

For estradiol detection part, our biosensor device has been demonstrated to be able to detect E17 in wide range with a detection limit of 1 fM, which is extremely sensitive in nowadays reported literature. Good selectivity was also obtained at nanomolar level. In addition, E17 was detected in hospital effluents. Moreover, this work indicated that measurements of the Cu peak could be used in an early warning system for the assessment of a global endocrine disrupting effect of an effluent.

For odorant detection part, we detected odorant molecules characteristic of boar taint, a novel platform was successfully completed. We monitored odorant in solution at very low concentration, 10^{-14} M (also lower compared to reported literature) as well as indicating that the chemical/biological functionality was preserved. Selectivity was also tested herein.

And following, another immobilization strategy, non-covalent entrapment of the analyte, integrating nano-materials, was used to produce a biomimetic sensor. An easy fabrication of a robust MIPs/metallic nanoparticles hybrid based electrochemical sensor was obtained for detection of TNT. π donor– π acceptor interactions was applied herein. The sensor performance is quite good: a wide linear range and a low detection limit (0.04 fM). We strikingly improved the sensitivity using ferro/ferricyanide as redox probe. Selectivity and real samples tests were also explored. It constitutes a potential device with significant impact for environmental protection and homeland security.

In future research, there are lots of points to do in order to develop proper microsystems for biomedical and environmental monitoring and for food safety.

Certain significant expectative are presented below:

1. Multi-biosensor assay should be undertaken for effective approach to develop more sophisticated in parallel multi-analyte detection systems.

2. Miniaturized biosensor development for suitable and easy-control, more stable measurements with resource-limited settings in low costs should be addressed.

3. Graphene and quantum dots also could be especially targeted for developing novel biosensors.

The nanomaterials are of high interest due to their unique electrical, chemical and physical properties. And nanomaterial-based biosensors provide an extensive range of applications for analysis of environmental and biological systems, given that they are minimally susceptible to matrix effects and are selective for the compound of interest.

4. The biosensors ought to be validated for analytical performance and alternate-validated with already well-established analytical methods.

5. Appropriate field-assess researches are required to make clear the performance of the biosensors under a broad range of robust conditions and to show how they could survive in the “real world”.

ANNEXE 1: Abbreviations not shown full name above

EDTA: Ethylenediaminetetraacetic acid

SAM: Self-assembled monolayer

DPV: Differential pulse voltammetry

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

PDMS: Polydimethylsiloxane

HCl: Hydrogen chloride

FITC: Fluorescein isothiocyanate

RSD: Relative standard deviation

E.coli: *Escherichia coli*

BCA: Bicinchoninic acid

DMSO: Dimethyl sulfoxide

ANNEXE 2: Detection technological Principle

References:

SWV: Square wave voltammetry

http://en.wikipedia.org/wiki/Squarewave_voltammetry

CV: Cyclic voltammetry

http://en.wikipedia.org/wiki/Cyclic_voltammetry

LSV: Linear sweep voltammetry

http://en.wikipedia.org/wiki/Linear_sweep_voltammetry

EIS: Electrochemical impedance spectroscopy

http://en.wikipedia.org/wiki/Dielectric_spectroscopy

Fluorescence microscope

http://en.wikipedia.org/wiki/Fluorescence_microscope

Atomic force microscopy

http://en.wikipedia.org/wiki/Atomic_force_microscopy

ANNEXE 3: Publications and Conferences

Publications:

1. Electrochemical Estrogen Receptor alpha Based Biosensor for Label-Free Detection of Estradiol.

ELECTROANALYSIS, 25(7)1765-1772, 2013.

Zhenzhong Guo¹ et al.

2. A novel platform based on immobilized olfactory receptors for detection of odorant molecules characteristic of boar taint.

Submitted.

Zhenzhong Guo¹ et al.

3. TNT recognition by biomimetic sensor based on electropolymerization of oligo(4-aminophenyl)thiophene-functionalized gold nanoparticles.

Submitted.

ZhenZhong Guo¹ et al.

Conferences:

1. The 2011 Biomolecules day of Rhône-Alpes. 27, Mai, 2011. Université de Lyon 1, France.

2. 3rd International Conference on Occurrence, Fate, Effects, and Analysis of Emerging Contaminants in the Environment. 23-26, August, 2011. Copenhagen, Denmark.

3. The 2012 Biomolecules day of Rhône-Alpes. 1 June 2012. Université de Grenoble, France.

4. 13th symposium of Groupe français de Bioélectrochimie and 8th Workshop of France-China. 24-28, Septembre, 2012. Lacanau-Océan, France.

5. 6th French-Spain day of CMC2 – IBERNAM. 22-23 Novembre 2012. Marseille (France)

6. Summer school on electrochemistry for environmental and biomedical applications. 17-21, June, 2013. Cluj-Napoca, Romania.

7. The electrochemistry days, .8-11, July, 2013. Université Paris Descartes(7), Paris.

8. Visiting scholar in Seoul National University. 7-12, December, 2014. (In planning)

9. 3rd International Conference and Exhibition on Biosensors & Bioelectronics. 11-13, August, 2014. San Antonio, USA. (Accepted)

DISCIPLINE
ANALYTICAL BIOTECHNOLOGY

Development of electrochemical biosensors for environmental pollutants and food safety monitoring.**Abstract**

Nowadays, electrochemical biosensors are rapid, reliable, cost-effective and label-free tools for in field monitoring of environmental pollutants, for assessing the food safety criterion and for detection of the territory military residues.

We developed biological receptors based affinity biosensors for electrochemical detection of estradiol in environment samples and odorant molecules in pork meat adulteration. The sensitive, selective, rapid, simple, repetitive, stable and effective biosensors were produced.

In addition, another strategy entrapment based on integrated nano-material was used to produce a biomimetic sensor for explosives detection, which has shown good valuable in environmental protection and homeland security.

In the end, certain significant prospective are discussed.

Key words: Environmental pollution; Food safety; Electrochemistry; Biosensor; Immobilization strategies; Nano-materials; Detection methods.

Développement de biocapteurs électrochimiques pour la détection de polluants environnementaux et pour la sécurité alimentaire.**Résumé**

De nos jours, les biocapteurs électrochimiques constituent des outils rapides, fiables, rentables et sans marquage pour la détection sur site des polluants environnementaux et l'évaluation des critères de sécurité alimentaire et pour la détection des résidus militaires sur le terrain.

Nous avons développé des biocapteurs d'affinité basés sur des récepteurs biologiques pour la détection électrochimique de l'estradiol dans des échantillons environnementaux et de molécules odorantes dans l'adultération de viande de porc. Des biocapteurs sensibles, sélectifs, rapides, simples reproductible, stables et efficaces ont été élaborés.

En outre, une autre stratégie basée sur des nanomatériaux intégrés a été employée pour élaborer un capteur biomimétique pour la détection d'explosifs, qui a montré de bonnes qualités dans la protection de l'environnement.

A la fin du manuscrit, certaines perspectives sont discutées.

Mots clés: polluants environnementaux; sécurité alimentaire; électrochimie; Biocapteur; stratégie d'immobilisation; Nano-matériels; méthodes de détections.