Development and optimization of Electrochemical Affinity Biosensors for the control of food safety

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DEVELOPMENT AND OPTIMIZATION OF ELECTROCHEMICAL AFFINITY BIOSENSORS FOR THE CONTROL OF FOOD SAFETY

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…to my mother
…to my father
…to my husband

…to myself

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Malvano, F., Albanese, D., Pilloton, R., Di Matteo, M. **2017**. A new label-free impedimetric aptasensor for gluten detection. *Food control*, 79, 200-206.

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Abstract

The guarantee of food safety requires a fast and accurate control for all chemicals and bacteria, which are harmful for human health. The need to ensure food safety and quality are of great concern to both food industry and consumers. In the food industry, the safety of a product is evaluated through periodic chemical and microbiological analysis; these procedures conventionally use techniques as, chromatography, spectrophotometry, and electrophoresis that are time consuming, require highly trained personnel, are expensive and require steps of extraction or sample pretreatment, increasing the time of analysis. Therefore, among food and drink industries the demand for developing simple, rapid, accurate, low – cost and portable analytical instruments is growing in food sector for the monitoring and detection of chemical and microbiological contaminants (toxins, mycotoxins, pathogenic microorganisms, pesticides and allergens) that endanger the food safety.

Biosensors, analytical devices composed of a biological recognition element (such as enzyme, antibody, receptor or microorganisms) coupled to a chemical or physical transducer (electrochemical, mass, optical and thermal), offer a possible alternative to common approaches, by allowing rapid on site analysis, and providing real – time information during the food production process. Biosensor literature in the field of food safety is focused mainly on affinity biosensors, which are considered as a further subset of biosensors that use an antibody, sequence of DNA or protein interfaced to a signal transducer to measure a binding event; most of them are based on the very high-affinity interaction between antigen and specific antibodies, but novel specific ligands (e.g. aptamers) are emerging. The main characteristic of the immunosensors is linked to their ability to detect very low amount of the specific analyte: this characteristic depends on the effectiveness of the antibody immobilization procedure coupled with transducer system able to detect and amplify the signal coming from the immune-complex formation.

However, the affinity biosensors described in literature belong to the "labelled affinity biosensors" which require the use of labels (commonly enzymes), linked to the target biomolecule, able to detect the immune-complex thanks to the production of substances easily detected by electrochemical or optical transducer systems. The drawback of immunosensors labelled is due to extra costs and time for labelling step and the impossibility of real time detection.

For these reasons, my interest has been focused on the development and optimization of label-free affinity biosensors, based on Electrochemical Impedance Spectroscopy transduction system, able to detect the immune-complex formation. In fact, recent studies have shown that Electrochemical

Impedance Spectroscopy (EIS) is particularly well – suited to the detection of binding events on the transducer surface. The application of EIS as a detection analytical technique, based on the direct monitoring of the interaction between the bioreceptor and its target, enables the production of label – free affinity biosensors for food analysis with significant advantages over labelled ones. Thanks to EIS transduction technique, the detection of analyte of interest is performed in real – time by studying the change in electrical properties of the electrode surface, which depends only on the binding interaction between the analyte and its receptor.

On the basis of the above considerations, my research project has been focused on the development of high sensitive impedimetric biosensors for the quantitative determination of chemical and microbiological food pollutants such as Ochratoxin A (OTA), Gluten, Pesticides and *Escherichia Coli* O157:H7.

In particular, two different immunosensors have been developed for the detection of Ochratoxin A. The first have been prepared on gold electrodes and the influence of oriented and not oriented immobilization techniques on the analytical performances of the developed biosensors have been studied. The comparison between the two immobilization procedures has highlighted the advantages of the oriented immobilization, which showed a more uniform and homogenous antibody layer that favours higher antigen – binding capacity, and sensitivity of the immunosensor. A lower limit of detection (5 pg/ml) for the not oriented immobilization has been reached in contrast to 50 pg/ml registered for the oriented one.

The second biosensor has been prepared on screen printed carbon electrodes. The surface of carbon electrode was modified with electrochemical gold deposition, which has demonstrated a very cheap way to obtain gold-like behaving electrodes using a very small quantity of the metal. For this impedimetric immunosensor capacitance was chosen as the best parameter that describes the electrical changes of the electrode surface due to the immunoreaction between anti-OTA and OTA.

Both impedimetric biosensors for OTA detection showed very low detection limit and high sensitivity, and the registered results regarding the analysis of food samples are suitable for fast OTA measurement in food matrices.

With the aim to guarantee food safety for celiac patients, a label-free impedimetric aptasensor for gluten detection, based on the immobilization of aptamer (Gli1) on the gold electrode modified with PAMAM, has been developed. PAMAM has been proven to increase the sensitivity of the aptasensor with a high binding affinity to gliadin, reaching a very low detection limit of 5 ppm. It is worth noting that this aptasensor is the first one based on the immobilization of aptamer on the modified electrode and all the others, reported in literature, were based on competitive assays: this condition makes the analysis of gliadin very fast and easier than aptamer

competitive assays that required addition of an enzyme labelled aptamer to the food sample and then the enzymatic substrate.

As regards the detection of pesticides in foodstuff, a very innovative impedimetric enzyme inhibition-based biosensor for carbamate and organophosphate compounds has been developed. The high affinity interaction between pesticides and Acetylcholinesterase active site was monitored by EIS, and the impedimetric changes obtained at different pesticide concentrations allow to go up very fast to the presence of the toxic compounds in different food matrices.

Finally, as regard pathogenic bacteria, different anti-E.Coli immobilization procedures were tested, in order to develop a selective label – free impedimetric immunosensor for *Escherichia Coli* O157:H7 detection in food products. The comparison between the immobilization procedures analysed underlined the advantage of the oriented procedure and the use of PAMAM dendrimer, which allow to immobilize an higher number of antibodies, reaching a very high sensitivity, but also the use of activated ferrocene as electron-transferring mediators, which improve the electrical properties of the system, resulting in a better impedimetric response. The lowest limit of detection of 3 cfu/mL was reached in the immunosensor developed on cysteamine/ferrocene-modified electrode, where AFM results confirmed the correct antibody immobilization. Moreover a good agreement between ELISA official methods and this immunosensor was obtained in *E.Coli* cells analysed in food samples.

Introduction

1.1 Food safety

Illnesses resulting from foodborne disease, defined as "*a disease of an infectious or toxic nature caused by or thought to be caused by consumption of food or water*" has become one of the most widespread public health problems in the world today. Internationally, foodborne diseases associated with microbial pathogens, toxins and chemical contaminants in food present a serious threat to the health of millions of individuals (Redmond *et al*., 2003).

Extensive surveillance has also been carried out by epidemiologist to estimate the extent of foodborne disease and food related illness in industrialized countries; it has been estimated that 130 million Europeans (WHO, 2000), 2.1 million to 3.5 million Great Britons from England and Wales, 76 million Americans, and 4.7 million Australians are affected by episodes of foodborne disease and food-related illnesses annually. Direct comparisons of incidence data are not possible because of differences in national surveillance systems; however, it has been suggested that Australia, the United Kingdom, and the United States appear to have similar incidences of foodborne disease. It has also been suggested that individuals from England, Wales, the United States, and Australia may suffer from foodborne disease at least once every 4 to 5 years (Redmond *et al*., 2003).

While these statistics appear high, it is questionable whether they accurately represent the number of foodrelated illness: firstly, food – related illness hospitalization, and death, is under – reported because diagnostic testing could not be done, or test results not forwarded for tabulation; secondly pathogens causing food – related illness may also be transmitted from one person to anther or through other vehicles such as water and thirdly, some food – borne illnesses may be caused by currently unrecognised pathogens, and hence not be attributed to food at all (Mead *et al.*, 1999).

Henson and Traill defined food safety as the invers of food risk: the probability of not suffering some hazard from consuming a specific food (Henson *et al*., 1993). Potential undesirable residues in foods span a broad

range, from natural (e.g. mycotoxins) and environmental contaminants (e.g. dioxins) to agro – chemicals (e.g. nitrates and pesticides), veteran ary drugs, growth promoters, packaging components, and many more. Microbiological considerations are even greater challenge to safety of food because potentially harmful microorganisms have the ability either to grow rapidly from very low numbers in food or to proliferate in the human body once ingested (Wilcock *et al.*, 2004).

The fear of contraction Bovin Spongiform Encephalopathy (BSE), the extensive illegal use of growth hormones, the outbreaks of *Escherichia Coli* O157:H7, the extensive use of artificial chemicals in food manufacturing and many other concerns have severely undermined the confidence of European consumers in the food industry. Similar, although less marked, trends are observed in North America and Japan, with particular concern about both the new food borne pathogens the have resulted in major food poisoning outbreaks and the presence of pesticide residue in foods (Tent, 1999).

The Government of the United Stets first introduced the concept of food safety to North American consumers. The Federal Food, Drug and Cosmetic Act of 1908 prohibited additions of any toxic or deleterious substance to food, and the Food Additive amendment of 1938 required that all additives be proven "safe" before addition to food. In 1976, The Food Safety Council was born in the US; its task was to develop new criteria for evaluating the safety of the food supply, whether it was a food ingredient, a food additive, or a basic foodstuff. The Council also developed documents and regulations in response to scientific research related to food safety (Michaelidou *et al.*, 2007).

The rigorous enforcement of these regulations by the Food Drug Administration (FDA) and the Food Safety Council, and the constant reassurance of the safety of the food supply, has reinforced the concept of absolute food safety to consumers. However, absolute safety is just not possible.

Important actions have been taken in various countries to improve the safety of food supplied to consumers during the years. In Britain, the 1990 Food Safety Act and the 1995 General Hygiene Act have significantly affected the food safety risk management practices in the food sector, shifting the focus from fraud prevention to a proactive scientific – based food safety approach (Sockett, 1991).

In April 1997, the United Kingdom commission approved a communication on "Consumer Health and Food Safety: one of the highlights of this communication was the increased role to be played by independent scientific researchers in the evaluation of potential hazards for the preparation of community legislation. The new Food Standards Agency was created in the UK in April 2000: its mandate includes the surveillance of food in the retail stores and the coordination of research activities in the food

safety area. In May 1997, US President Clinton launched a National Food Safety initiative to enhance surveillance, improve risk assessment, inspection and compliance, educate the consumer and conduct important new research (Tent, 1999).

Today, more than 200 known disease are transmitted through food by a variety of agents that include bacteria, fungi, viruses and parasites; the risk of foodborne illness has increased markedly over the last 20 years, with nearly a quarter of the population at higher risk for illness today.

Even though today food products seem to be safer than ever before, for a technical point of view and due to many safety control programs, consumers in industrialized countries demand food products of high and consistent safety in broad assortments through the year and for competitive prices.

Today's consumer has become increasingly concerned about the safety of food and the negative effects of bio – industrial production. These developments have changed the production, trade and distribution of food products beyond recognition. Governments, both national and international, are responding to this by imposing new legislation and regulations to ensure safe and animal-friendly production, restricted pollution and to economize on the use of resources. Examples are the Codex Alimentarius standards (FAO/ WHO) and The General Food Law (European Union (EU) 2002/178). For food businesses this implies placing more emphasis on safety control, on traceability of food products and on environmental issues and, at the same time, shifting from bulk production towards production of specialities with high added value. Furthermore, because of their embeddedness in the global economy, collaboration with other parties becomes important for all businesses to achieve safe and high-quality food products for the consumer. This means that business strategies must now move their focus from traditional economical and technological interests to topical issues such as the safety and healthfulness of food products, animal friendliness, the environment, etc. These processes are affecting the entire food chain from producer through to retailer.

1.1.1 Mycotoxins

Mycotoxins are a large and varied group of mold – secondary metabolites, with common features they are all produced by fungi and have toxic effect against vertebrates and other organisms. Filamentous fungi produce thousand of toxic compounds, but the more important mycotoxins belong to species *Aspergillus*, Fusarium, and Penicillium. Mycotoxins affect a broad range of agricultural products including cereals, cereal – based foods, dried fruits, wine, milk, coffee beans, cocoa bakery or meat products, which are the basis of many developing countries (Shepard *et al.*, 2012)

Food and Agricultural Organization (FAO) of the Unites Nations estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins. Nowadays, the importance to control mycotoxins levels in foodstuffs is present in all domains.

Among mycotoxins, Ochratoxin A (OTA) remains the single most potent member (Reddy et al., 2010).

OTA is a secondary metabolite produced by *Aspergillus* and *Penicillium* species. It has a polyketide – derived dihydroisocoumarin mojety linked via a 7 – carboxy group to 1 – b – phenylalanine by an amide bond, as shown in Figure 1.1.
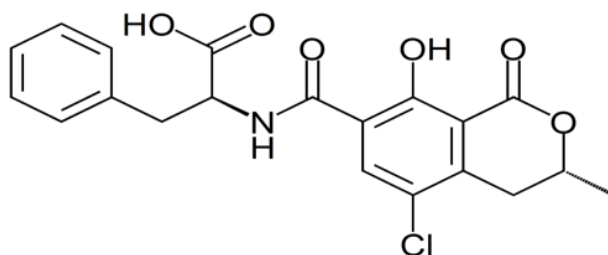
Figure 1.1: *General chemical structure of OTA*

OTA is present in food and food products due to the thermostability of its derivates; it has been widely detected in various agricultural commodities such as cereals, roasted coffee beans, cocoa, milk, beer, wine, grape, juice, spices and dried fruits.

The critical factors that can affect fungal growth during farming, harvesting and storage of commodities susceptible to OTA are temperature, moisture content and exposure time. Additional factors that support fungal growth are the presence of spores, the physical location (the internal part of a vegetables is more vulnerable to fungal invasion than the external area), insects (due to their metabolisms, they increase the moisture and temperature of the plant and break its protective shield), storm and rain damage, moisture stress, the availability of mineral nutrient, pH (in general, mold tolerates acid media and is able to acidify the media themselves), oxygen and carbon dioxide levels, chemical and physical treatments and, for some commodities, the product drying and re – wetting speed. Moreover, poor harvesting practices, improper storage and less than optimal conditions during transportation, marketing and processing can also contriute to fungal growth and increase the risk of mycotoxins production (Kaushik et al., 2013).

OTA is the most detected mycotoxins in human blood globally due to its binding with plasma protein, long elimination half – life (35 days in serum), its enterohepatic circulation and its reabsorption from urine. Human exposure to OTA has been clearly demonstrated through its detection in

human blood, urine and food. In animal species, OTA has shown many adverse effects such as teratogenicity, immunotoxicity, genotoxicity and mutagenicity. The most important toxic effect of this mycotoxin is its nephrotoxicity leading to conditions such as "Balkan Endemic Naphropathy.

In 1993, the National Agency for Research on Cancer (IARC) classified OTA in group "B, a possible human carcinogen due to induction of oxidative DNA.

Keeping these adverse effects in mind, various health organizations have set OTA consumption limits: in the European Union, the acceptable limits established for OTA in various foodstuffs are listed in Commission Regulation (EC) N° 1881/2006 and ranged from 10 μg/kg for instant coffee and dried fruits to 0.5 μg/kg for dietary foods intended specifically for infants.

OTA is a heat-stable molecule within the range of conventional food processing temperatures and no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization.

Official methods for determination of mycotoxins are usually performed in accredited laboratories with sophisticated instrumentation like high – performance liquid chromatography (HPLC) with fluorescence (FLD) or mass (MS) detectors, methods that require expensive equipment as well as complicated and time – consuming solvent clean-up steps. Immune assays can be used as cheaper and quicker alternatives to chromatographic methods for mycotoxin detection: competitive enzyme – linked immune sorbent assay (ELISA) is the most common commercial immune assay used for OTA detection in the food industry based on spectrophotometric reading.

Therefore, the clearly undesirable presence of trace amounts of OTA in foodstuffs requires suitable sampling procedures and highly sensitive techniques to detect and control OTA levels at ppb level; the ability of OTA to persist through the food chain and the observation of both acute and chronic effects in animals and humans have made evident the need to develop reliable, easy, cost – effective and fast analytical methods for its monitoring and control.

1.1.2 Allergens

Each year, millions of people have allergic reactions to food. Food allergens are typically naturally – occurring proteins in foods or derivatives of them that cause abnormal immune responses. Prevalence of food allergies around the world is believed to be increasing, with more than 8% of children and 2% of adults in countries like Australia and New Zealand having allergy to one or more foods. The most common allergens for young children are milk and egg but, fortunately, many children outgrow these allergies by the time they have reached 5-7 years of age. On the other hand, allergies such as those to seafood, peanut and tree nut may develop later and are lifelong

conditions. Practically all foods have the capacity to cause an allergic reaction in a person who has become sensitised to proteins in it.

Allergic reactions to foods vary greatly from mild gastrointestinal discomfort, to skin rashes and potentially life threatening asthma and anaphylaxis.

In Europe, food allergens are monitored and assessed by clinical and scientific expert through the European Food Safety Authority (EFSA): they advise on which food need to be labelled on pre – packed foods; these foods are: cereals containing gluten (namely wheat, rye, barley, oats), crustaceans (e.g. prawns, crabs, lobster, crayfish), eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame, sulphur dioxide/sulphites (where added and at a level above 10 mg/kg in the finished product), lupin, molluscs (e.g. clams, mussels, whelks, oysters, snails and squid).

Among these, gluten is the most common food allergen; it is a complex mixture of proteins comprising the gliadins and glutenins. Gliadin in wheat is an alcohol – soluble compound that contains approximately 50% of the wheat gluten protein and it is also the major allergen responsible for gluten intolerance, bakers' asthma and wheat – dependent exercise – induced anaphylaxis.

Celiac disease (CD) is a genetically determined autoimmune disease of the digestive system, which results in chronic inflammation of the gastrointestinal tract; CD flattens the small intestinal mucosa, hindering nutrient absorption. Once it was considered to be a rare childhood condition, but now it is recognized to be a very common pathology: it affects around 1% of worldwide population and it occurs throughout the entire lifespan (Ludvgsson et al., 2013).

In genetically susceptible individuals, gliadin fragments in the intestinal lumen induce a signaling cascade, causing the autoimmune system activation and it results in a chronic inflammation of the small intestine mucosa with villous atrophy and consequently malabsorption of nutrients.

Due to its unspecific symptoms CD may incur in an incorrect initial diagnosis for irritable bowel syndrome (IBS), colitis, Crohn disease, gastroenteritis of infective nature.

A strict lifelong gluten – free diet is currently the only known effective therapy for celiac disease. Recovery of the small intestinal mucosa is achieved in the vast majority of patients if the daily intake of gluten is less than 20 mg. Maintaining a true gluten – free diet is challenging, because gluten occurs not only in obvious food sources such as bread, pasta and beer. The so – called hidden sources are problematic, because gluten is used extensively also in modified forms as a flavour enhancer, thickener, emulsifier, filler, and fortification ingredients, not only in food products, but also in nutritional supplements, drugs and cosmetics. Additionally, inherently gluten – free grains, such as maize, buckwheat, quinoa and

amaranth, may become contaminated with gluten during crop rotation, milling, transportation or processing.

The European Regulation (EC) N°41/2009) concerning composition and labelling of foodstuffs suitable for people intolerant to gluten indicates that food products can be defined "*gluten-free*" if the gluten content does not exceed 20 ppm in total, while a food with a gluten level between 20 and 100 ppm can be defined as "*very low gluten*".

The method for determination of the gluten content in gluten-free foods accepted by the Codex Alimentarius Commission is the Enzyme-linked Immunoassay (ELISA) Mendez Method (sandwich ELISA based on the R5 and G12 antibody, combined with the cocktail-extraction solution). Conventional methodologies for food analysis provide high reliability and very low limits of detection. Among them chromatography, spectrophotometry, electrophoresis, immunoassays, polymerase chain reaction (PCR) assays and ATP detection methods promise results within 24 hours, but they are expensive and time consuming; moreover, they need samples pretreatments and most of them require the use of highly trained personnel.

1.1.3 Pesticides

In the last decades, organophosphorous and carbamic pesticides have come into widespread use and are progressively replacing organochloride insecticides, fungicides and herbicides (e.g. DDT, aldrin and lindane) due to their lower environmental persistence (Cremisini *et al.*, 1995).They are used extensively to combat agricultural pests. Due to systematic use of these pesticides, their residues accumulate in the food chain and ultimately find their way to human consumers though water, food and soil. The presence of pesticide residues and metabolites in food, water and soil currently represents one of the major issues for the environmental chemistry.

Carbamates (CBs) are organic compounds derived from carbamic acid (NH_2COOH). The structure of biologically active carbamates is displayed in Figure 1.2, where X can be oxygen or sulphur (thiocarbamate), R_1 or R_2 may also be hydrogen, and R_3 is mostly an organic substituent or sometimes a metal.
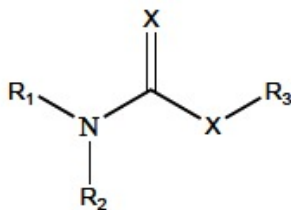
Figure 1.2*: General chemical structure of carbamates*

In addition to their use as therapeutic drugs in human medicine (Alzheimer's desease, myasthenia gravis, glaucoma, Parkinson's disease), these compounds have been applied as pesticides and then as parasiticides in veteran medicine.

Organophosphates (OPs) are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid (Figure 1.3):
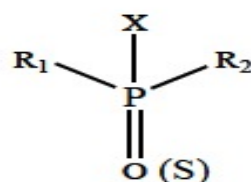
Figure 1.3: *General chemical structure of organophosphates*

R_1 and R_2 are aryl or alky groups that are bonded to the phosphorus atom either directly or through an oxygen or sulphur atom. In some cases R_1 is directly bonded to the phosphorous atom, and R_2 is bonded to an oxygen or sulphur atom. In phosphoramidates, at least one of these groups is $-NH_2$ and the atom double-bonded with phosphorus is either oxygen or sulphur. The $-X$ group, also binding to the phosphorus atom through oxygen or sulphur atom, may belong to a wide range of halogen, aliphatic, aromatic or heterocyclic groups. This "leaving group" is released from the phosphorus atom when OP is hydrolysed by phosphotriesterases or upon interaction with protein targets. In medicine and agriculture, the world "organophophates" refers to a group of insecticides.

CBs and OPs toxicity is mainly due to their inhibitory effect on Acetylcholinesterase (AChE), a key enzyme leads to muscle weakness, miosis, respiratory failure, unconsciousness, convulsion and, eventually, death (Storm et al., 2000).

The principal difference between OP and CB inhibitory action is in the stability of the AChE-OP/CB complex: actually OPs exert their toxicological effects through non – reversible phosphorylation of esterase in the central nervous system, while the CBs acute toxic effects are related to reversible inactivation of AChE (Colovic *et al.,* 2013).

Many methods are available for pesticide detection: chromatographic methods such as high performance liquid chro- matography (HPLC) and gas chromatography (GC) are used as reference methods but present strong drawbacks such as complex and time-consuming treatments of the samples, i.e. extraction of pesticides, extract cleaning and solvent substitutiont. Moreover, the analysis usually has to be performed in a specialised laboratory by skilled personnel and is not suitable for in situ application. However, these issues turn out to be a major problem when rapid and

sensitive measurements are needed in order to take the necessary corrective actions in a timely fashion (Arduini *et al.*, 2006).

1.1.4 Pathogenic bacteria

In recent years, foodborne pathogenic bacteria have emerged as major concerns of public and increasing threaten the health of people; the detection of these bacteria is an important issue for ensuring food safety and public health. In 2005, the World Health Organization estimated that about 2.8 million people worldwide died from diarrhoeal diseases, often caused by the consumption of microbiologically contaminated food or drinking water.

The common foodborne pathogens which are responsible for most of the foodborne disease outbreaks are *Listeria monocytogenes*, *Escherichia Coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica*, *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*. The increasing amount of street foods and the increasing demand for minimally processed ready – to – eat products have begun to concern public health agencies on food safety assurance. Foodborne pathogens are present in various foods such as fruits, vegetables and ready – to – eat products which are consumed without any further treatment; also, food borne diseases are often associated with the consumption of raw or undercooked foods such as seafood, meat and poultry (Chung *et al.*, 2010).

The conventional methods for detecting the foodborne bacterial pathogens present in food are based on culturing the microorganisms on agar plates followed by standard biochemical identifications (Mandal *et al.*, 2011). Conventional methods are usually inexpensive and simple but these methods can be time consuming as they depend on the ability of the microorganisms to grow in different culture media such as pre-enrichment media, selective enrichment media and selective plating media. Usually conventional methods require 2 to 3 days for preliminary identification and more than a week for confirmation of the species of the pathogens. Conventional methods are laborious and they require the preparation of culture media, inoculation of plates and colony counting (Mandal *et al.,* 2011). Furthermore, conventional methods may be limited by their low sensitivity (Lee et al., 2014). False negative results may occur due to viable but non-culturable (VBNC) pathogens. The failure to detect foodborne pathogens would increase the transmission risk of pathogens.

Among the pathogens associated with life – threatening symptoms such as haemolytic uraemic syndrome (HUS), the enterohemorrhagic Escherichia Coli bacteria strains are found to be responsible for serious foodborne outbreaks (Alocilja et al., 2003; Abadias et al., 2008).

Escherichia Coli O157:H7 is so – named because it expresses the 157th somatic (O) antigen identified and the 7th flagellar (H) antigen. It has emerged in the past 20 years as a pathogen of public health importance.

Although most *E.Coli* are normal flora in the colons of humans and other warm – blooded animals, this strain are capable of causing disease in humans. It is one of the deadliest foodborne pathogens because of its combination of virulence and pathogenicity. Illness caused by this organism range from bloody diarrhea to life – threatening conditions, such as hemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopenic purura. The Centre for Disease Control and Prevention estimates that there are 20000 illnesses a year due to *E.Coli* O157:H7 infection (Buchanan et al., 1997; Muhammad – Tahir et al., 2003).

This pathogenic bacteria has been traced to a number of food products like meat, apple juice, milk, unpasteurized fruit juice, lettuce, cheese, curd.

1.2 Biosensors: definition and fundamental aspects

International Union of Pure and Applied Chemistry (IUPAC) recently proposed a very stringent definition of a biosensor: "*A biosensor is a self-confident integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element. A biosensor should be clearly distinguished from a bioanalytical system which requires additional steps, such as reagents addition*" (Thevenot et al., 2001).

Essentially, a biosensor is a reagentless analytical device that incorporates a biological recognition element in close proximity or integrated with a signal transducer to provide a sensing system specific for the target analyte. It transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal (Wang, 2000).

The purpose of the biosensor is to provide rapid, real – time, and reliable information about the biochemical composition of its surrounding environment; ideally, it is a device that is capable of responding continuously, reversibly and does not perturb the sample.

A biosensor consists of three main components: a biological detection system, a transducer and an output system. Figure 1.4 shows a schematic diagram of the typical components in a biosensor.
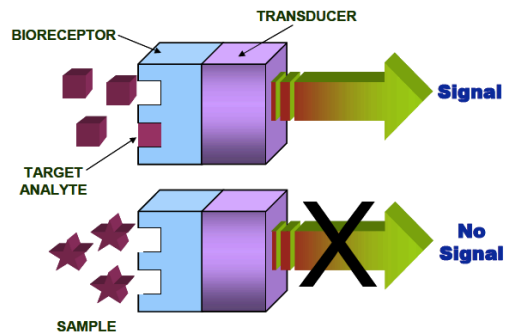
Figure 1.4 *Schematic diagram of a typical biosensor (Parkinson, 2005)*

It is obvious that there are many ways to design biosensor architecture.

The choice of biosensor architecture depends on to a major extent on the biochemical processes involved in the biorecognition process: the processes in close proximity to the electrode surface that are involved in a typical biosensor reaction are rather complex.

It is important to have an overview about the variables that affect the performance of a biosensor and which of these parameters may have a major impact on the signal response (Figure 1.5): this knowledge is fundamental for developing and optimizing biosensors. The choice of the transduction process and transduction material is dependent on this knowledge as well as the chemical approach to construct the sensing layer on the transducer surface.

ELECTRODE VARIABLES	MASS TRANSFER VARIABLES	ELECTRICAL VARIABLES
Materials Surface area Geometry Surface condition	Convection Diffusion Surface Concentration Adsorption	Potential Current Charge Impedance

ELECTROLYTE VARIABLES	EXTERNAL VARIABLES	REACTION VARIABLES
Bulk concentration of electroactive species pH Solvent Trace impurities	Temperature Pressure Time Composition of sample matrix Occurrence of interferences and/or electrode fouling processes	Kinetic and thermodynamic parameters of ET and reaction(s) Rate - determining step of the reaction Stability of reaction partners/ products

POTENTIAL NOISE VARIABLES

Electrochemical noise
Thermal noise
Environmental noise

Figure 1.5: *Variables affecting the electroanalytical performances of a biosensor (Borgmann et al., 2011)*

The choice of the biological recognition element is, however, the crucial decision that is taken when a novel biosensor design has to be developed.

Knowing the most influential parameters of specific biosensor architecture is the basis to understand and fine tune the performances of these devices in a rational manner. Figure 1.6 summarizes the key features of typical biosensors and lists several that are of additional importance for commercial devices. Among these, selectivity, sensitivity, linearity of response, response and recovery time as well as operating lifetime, stability and reproducibility are some of the most important key factors.

KEY PARAMETERS OF BIOSENSOR PERFORMANCE	ADDITIONAL PARAMETERS OF BIOSENSOR PERFORMANCE, RELEVANT FOR COMMERCIALIZATION
Analyte	Cost per measurement
Employed biocomponent for recognition of analyte	Duration of measurement
Type and composition of sample matrices	Turnover of measurements (time resolution)
Selectivity	Warm-up time
Sensitivity	Sample volume
Linear range	Measuring temperature
Detection limit	Size, weight and price of the device
Response Time	Costs, size and weight of required instrumentation
Reproducibility	Delivery time
Precision	Development status of the device/ procedure (commerical product, standard procedure, basic research)
Stability (in use, long-term, storage)	Time between maintenance checks
Calibration aprroach (requirements, intervals, drift)	Potential to be coupled with other analytical techniques
Duration to reach baseline	Data storage and processing

Figure 1.6*: List of key characteristics of a biosensor (Borgmann et al., 2011)*

In fact, while developing a biosensor system, following requirements are extremely essential and considered for successful and commercially viable project:

a) *Selectivity*. The biosensors device should be highly selective for the target analyte and show minimum or no cross reactivity with moieties having similar chemical structure.

b)   *Sensitivity*. The biosensor device should be able to measure in the range of interest for a given target analyte with minimum additional steps such as pre cleaning and pre concentration of the samples.

c)   *Linearity of response*. The linear response of the system should cover the concentration range over which the target analyte is to be measure.

d)   *Reproducibility of signal response*. When sample having same concentrations are analysed several times, they should give same response.

e)   *Quick response time and recovery time*. The biosensor device response should be quick enough so that real time monitoring of the target analyte can be done efficiently. The recovery time should be small for reusability of the biosensor system.

f)   *Stability and operating life*. As such most of the biological compounds are unstable in different biochemical and environmental conditions. The biological element used should be interfaced such that the activity is retained for a long time so as to make the device marketable and practically useful in the filed.

1.3 Historical developments

Leland Charles Clark Jr is considered as the pioneer of the biosensor research. In 1956 he published his first paper on the electrode to measure oxygen concentration in blood (Clark, 1956). In 1962 while addressing at New York Academy of Science symposium he described "how to create electrochemical sensors (pH, polarographic, potentiometric or conductimetric) more smart" by adding "enzyme transducers as membrane enclosed sandwiches". The model was exemplified by an experiment in which glucose oxidase was entrapped at a Clark oxygen electrode using dialysis membrane. The decrease in measured oxygen concentration was proportional to glucose concentration (Clark and Lyon, 1962).

In 1967, Updike and Hicks further extended the work of Clark and demonstrated the first functional enzyme electrode based on glucose oxidase immobilized onto an oxygen sensor. Glucose concentration was measured in biological solutions and tissue in vitro (Updike and Hicks, 1967). In the year 1970, Guilbault and Montalvo (1970) described the first potentiometric enzyme electrode. It was a urea – sensor based on enzyme urease immobilized at an ammonium selective liquid membrane electrode. In the year 1973, Guilbault and Lubrano described glucose and a lactate enzyme sensor based on hydrogen peroxide detection at a platinum electrode. In 1974, Klaus Mosbach developed a heat – sensitive enzyme sensor termed "thermistor" (Mosbach and Danielsson, 1974).

Clark's ideas turned out to be commercial reality in 1975 with the fruitful re – launch (first launch 1973) of the Yellow Springs Instrument Company's

glucose analyser based on the amperometric detection of hydrogen peroxide. The biosensor took a further fresh evolutionary route in 1975, when Divis put forward that bacteria could be harnessed as the biological element in microbial electrodes for the measurement of alcohol (Divis, 1975). Lubbers and Opitz coined the term *optode* in 1975 to describe a fiber – optic sensor with immobilized indicator to measure carbon dioxide or oxygen. They extended the concept to make an optical biosensor for alcohol by immobilizing alcohol oxidase on the end of a fiber – optic oxygen sensor.

In 1976, Clements et al. integrated an electrochemical glucose biosensor in a bedside artificial pancreas and this was later marketed by Miles as the Bio – stator. Although the Bio – stator was commercially unavailable, VIA Medical introduced a novel semi – continuous catheter – based blood glucose analyser. Later in 1976, La Roche (Switzerland) introduced the Lactate Analyzer LA 640 in which the soluble mediator, hexacyanoferrate, was used to shuttle electrons from lactate dehydrogenase to an electrode (Geyssant et al., 1985).

Third generation enzyme sensors bear a resemblance to second generation enzyme sensor based on the use of electron mediators. However, they have advanced into the implementation of co – immobilised enzymes and mediators onto the same electrode instead of freely diffusing mediators in the electrolyte. Direct interaction between the enzymes redox centre and the electrode was possible so either mediator or enzyme was not required. Thus, recurrent measurements were possible that abates the sensor design costs (Cass et al. 1984).

1.4 Classifications

Biosensors can be classified in agreement to the type of involved active biological component in the mechanism or the mode of signal transduction.

Figure 1.7 shows some analytes (substrates) possible to be analysed immobilizing the biological components, separately, in several transducers (Mello et al., 2002).

15
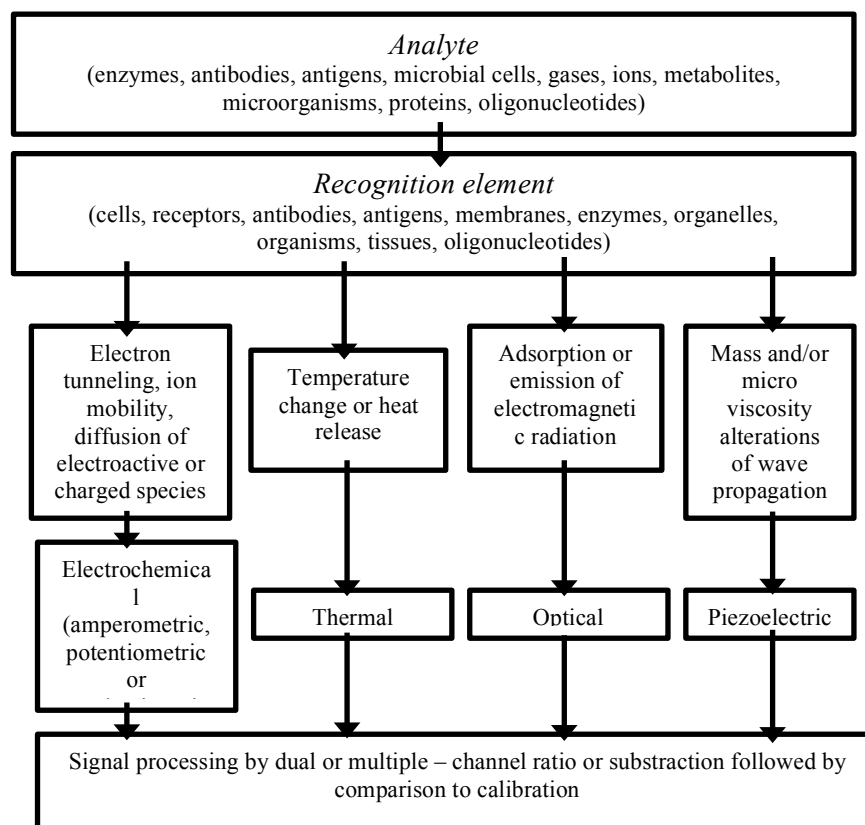
Figure 1.7*: Biocomponent and transducers employed in construction of biosensors (Serna – Cock, 2011)*

The choice of the biological material and the adjusted transducer depend on the properties of each sample on interest and the type of physical magnitude to be measured.

1.4.1 Receptor: biological recognition element

The type of the biocomponent determines the degree of selectivity or specificity of the biosensor. Thus, the biological recognizers are divided in two groups: biocatalytic and bioaffinity receptors.

1.4.1.1 Biocatalytic recognition element

In this case, the biosensor is based on a reaction catalysed by macromolecules which are present in their original environment, have been isolated previously or have been manufactured. Thus, a continuous

consumption of substrate(s) is achieved by the immobilized biocatalyst incorporated into the sensor: transient or steady – state responses are monitored by the integrated detector. Three types of biocatalyst are commonly used:

1. Enzyme (mono – or multi – enzyme), the most common and well developed recognition system;
2. Whole cells (micro – organisms, such as bacteria, fungi, eukaryotic cells or yeast) or cell organelles or particles (mitochondria, cell walls);
3. Tissue (plant or animal tissue slice).

The biocatalytic – based biosensors are the best known and studied and have been the most frequently applied to biological matrices since the pioneering work of Clark. One or more analytes, usually named substrates S and S', react in the presence of enzyme(s), whole cells or tissue culture and yield one or several products, P and P', according to the general reaction scheme:

$$S + S' \rightarrow P + P'$$
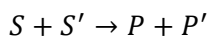
There are four strategies that use adjacent transducers for monitoring the analyte S consumption by this biocatalysed reaction:
- detection of the co – substrate S' consumption, e.g. oxygen depleted by oxidase, bacteria or yeast reacting layers, and the corresponding signal decrease from its initial value;
- recycling of P, one of the reaction products, e.g. hydrogen peroxide, H^+, CO_2, NH_3, etc. production by oxidoreductase, hydrolase, lyase, etc., and corresponding signal increase;
- detection of the state of biocatalyst redox active centre, cofactor, prosthetic group evolution in the presence of substrate S, using an immobilized mediator which reacts sufficiently rapidly with the biocatalyst and is easily detected by the transducer;
- direct electron transfer between the active site of a redox enzyme and the electrochemical transducer.

The third strategy attempts to eliminate sensor response dependence on the co – substrate, S', concentration and to decrease the influence of possible interfering species. The first goal is only reached when reaction rates are much higher for immobilized mediator with biocatalyst than those for co – substrate with biocatalyst.

Biosensors that use microorganisms, plant or animal tissue, as biocomponents, have the advantage of unnecessary tedious procedures of extraction and purification; microbial sensors are less sensitive to the inhibition for other compounds present in the sample, are more tolerant to

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the pH variations, temperature and generally have a longer lifetime. These devices are based on the contact of electrodes and immobilized living cells, and they are easily regenerated, for immersion in a solution of nutrients. On the other hand, biosensors with these characteristics presents a slow response and low selectivity of the with isolated enzymes, due to a variety of metabolic processes occurring in a living cell (Phadke et al.,1992). These sensors are generally based on the detection of organic compounds assimilated by the microorganisms or monitoring changes occurring in respiration activity during metabolism. Some microbial biosensors have been used for food analysis, but principally, in biological oxygen demand (BOD) measurements.

Problems like selectivity and the slow response characteristics of microbial sensors can be overcome by the use of enzymes that, not surprisingly, represent the most commonly used sensing agents due to their selectivity (Davis et al., 1995). Among the enzymes commercially available, the oxidases are the most often used; this type of enzyme offers the advantages of being stable and in some situations does not require coenzymes or cofactors.

1.4.1.2 Bioaffinity recognition element

The biosensor operation is based on interaction of the analyte with macromolecules or organized molecular assemblies that have either been isolated from their original biological environment or engineered. Thus, equilibrium is usually reached and there is no further net consumption of the analyte by the immobilized biocomplexing agent. These equilibrium responses are monitored by the integrated detector.

Affinity – based biosensor provide selective interactions with a given ligand to form a thermodynamically stable complex.

The most developed examples of biosensors using biocomplexing receptors are immunosensors, based on immunochemical reactions, i.e. binding of an antigen (Ag) with a specific antibody (Ab). Formation of such Ab – Ag complexes has to be detected under conditions where non – specific interactions are minimized. Each Ag determination requires the production of a particular Ab, its isolation and, usually, its purification.

From their mode of production, there are three kinds of antibodies: polyclonal (pAb), monoclonal (mAb) and recombinant (rAb). Polyclonal are purified from the blood of immunized animals having the benefits of low cost and easy development; but it is very important careful purifying of the conjugate (to gain selectivity) and sometimes appears cross-reactivity to the protein used for this conjugate (e.g. BSA). MAbs are produced from positive hybridomas by fusing murine myeloma cells and spleen cells from immunized mice; uniformity with same analytical properties and unlimited production are their benefits. The third generation in Ab technology are

recombinant antibodies (rAbs), which does not require animals. The functional gene of some Ab can be cloned and transmitted into prokaryotic or eukaryotic organisms (genetically modified organisms) from positive hybridoma or spleen cells with or without immunization (Li et al., 2009).

In order to increase the sensitivity of immunosensors, enzyme labels are frequently coupled to Ab or Ag, thus requiring additional chemical synthesis steps. Even in the case of enzyme – labelled Ab, these biosensors will essentially operate at equilibrium, the enzymatic activity being there only to quantify the amount of complex produced. As the binding or affinity constant is usually very large, such systems are either irreversible (single – use biosensor) or placed within an FIA environment where Ab may be regenerated by dissociation of complexes by chaotropic agents, such as glycine – HCl buffer at pH 2.5.

Even if most of affinity – based biosensors are based on the high affinity interactions between antigen and specific antibodies, but novel specific ligands (e.g. aptamers) are emerging. Aptamer are short sequences (20 – 90 oligonucleotides) of single stranded nucleic acids (DNA, RNA) that can bind with high affinity and specificity to a wide range of targets, ranging from large proteins to small molecules like amino acids or drugs. They are obtained by an in vitro selection process named SELEX (systematic evolution of the ligand by the exponential enrichment process), which was first reported in 1990. Aptamers have the ability in certain physicochemical conditions to fold into defined three – dimensional conformations, which facilitate specific interactions with target molecules having high affinity constants (Hayat et al., 2012). While antibodies have been the standard for molecular recognition of mycotoxins for several decades, aptamers have emerged owing to inherent advantages with respect to antibodies: immunization of animals is not required, more chemical and thermal stability, less variability compared to pAbs, or inexpensive in – vitro synthesis. Particularly, aptamers are not susceptible to denaturation in the presence of solvent commonly used in the extraction of mycotoxins (Vidal et al., 2013)

A developing field in electrochemical affinity biosensors is the use of chips and electrochemical methods to detect binding of oligonucleotides (gene probes). There are two approaches currently developed: the firs one intercalates into the oligonucleotide duplex, during the formation of a double stranded DNA on the probe surface, a molecule that is electroactive; the second approach directly detects guanine that is electroactive.

Affinity – based biosensors have been used for the detection of pathogens, antibodies, toxins, biomarkers, among other analytes. The wide spectrum of application of affinity biosensors ascertains a great future for this type of biosensors.

1.4.2 Detection or measurement mode

The activity of the biological component with a substrate can be monitored by the oxygen consumption, hydrogen peroxide formation, fluorescence, adsorption, pH change, conductivity, temperature or mass. Thus, the biosensor can be classified in several types according to the transducer: electrochemical, optical, thermal and acoustic.

1.4.2.1 Electrochemical transducers

An electrochemical biosensor uses a transducer where electrochemical signals are generated during biochemical reactions and are monitored using suitable potentiometric, amperometric or conductimetric systems of analyses (Thakur et al., 2013).

Biosensors based on electrochemical transducer have the advantage of being economic and present fast response; the possibility of automation allows application in a wide number of samples. The electrochemical biosensors can be classified in amperometric, potentiometric, conductimetric and impedimetric ones (Mello et al. 2002).

Amperometric biosensors systems are commonly used devices and are available in the market today. The firs ever commercial biosensor designed by Leyland and Clark for monitoring glucose was an amperometric electrode.

Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species. It is usually performed by maintaining a constant potential at Pt, Au or C based working electrode or on array of electrodes with respect to the reference electrode, which may also function as the auxiliary electrode, if current are low (10^{-9} to 10^{-6} A). The resulting current is directly interrelated to the bulk concentration of the electro active species or its production or consumption rate within the adjacent biocatalytic layer. As biocatalytic reaction rates are frequently preferred to be first order dependent on the bulk analyte concentration (Thevenot et al., 2001). Analytes like sugars, alcohols, phenols, oligonucleotides and O_2 can be determined using amperometric biosensors.

On food analysis, the majority of the electrochemical biosensors are based on the amperometric transducers in combination with oxidases. Among the amperometric, transducers that are based on the monitoring of hydrogen peroxide present a higher sensitivity than those with detection of the oxygen consumption. However, these are more suitable when the biological components are microbial cells, vegetables or animal tissue.

Potentiometric measurements involve the determination of the potential difference between both an indicator and a reference electrode, or two reference electrodes separated by a permselective membrane, when there is

no significant current flowing between them. The transducer may be an ion – selective electrode (ISE) which is an electrochemical sensor based on thin films or selective membranes as recognition elements (Buck et al., 1994). The most common potentiometric devices are pH electrodes; several ion (F^-, I^-, CN^-, Na^+, K^+, Ca^{2+}, NH_4^+) or gas (CO_2, NH_3) selective electrodes are available. The potential differences between these indicator and reference electrodes are proportional to the logarithm of the ion activity or gas fugacity (or concentration). The response of potentiometric biocatalytic sensor is, as for amperometric biosensor, steady – state or transient, but it is never an equilibrium response. The situation is more complex for enzyme – labelled immunosensors: although the antibody – antigen complex is expected the reach an equilibrium and reactions to be either reversible or irreversible, the labelled enzyme activity is measured under steady – state analyte consumption conditions. Another important feature of the ISE based biosensors, such as pH electrodes, is the large dependence of their response on the buffer capacity of the sample and on its ionic strength (Thevenot et al., 2001).

Conductimetric biosensors are based on the principle of change of conductivity of the medium when microorganisms metabolize uncharged substrates, such as carbohydrates, to intermediates, such as lactic acid. The amount of charged metabolites is directly proportional to the growth rate of the organism and is easily quantifiable. Conductimetric biosensors are usually non-specific and have a poor signal/noise ratio, and therefore have been little used (Mello et al. 2002).

Impedimetric biosensors use the impedance principle as transduction technique, most indicated to monitor quality and detect food pathogens, detection of bacteria and sanitation microbiology. Impedance appears to be an excellent technique for the investigation of both bulk and interfacial electrical properties of electrode systems, which can be used to determine quantitative parameters of electrochemical processes. If biorecognition events, such as reactions catalysed by enzymes, biomolecular recognition events of specific binding proteins, lectins, receptors, nucleic acid, whole cell, antibodies or antibody – related substances, occur at the modified electrode surface, the interfacial properties change (Maalouf et al., 2007). One of the advantages of impedance technique is the small amplitude perturbation from steady state, which makes it a non – destructive technique. The impedance can be measured in the presence or absence of a redox couple, which is referred to faradic and non – faradic impedance measurement, respectively. The faradic biosensors detect biorecognition events which occur at the modified electrode by measuring the change in the faradaic current (interfacial electron transfer rate) owing to steric hindrance caused by the biomolecular interaction and/or by the electrostatic repulsion between the free charges of the target molecules and the electroactive species in the supporting electrolyte. Redox probe selection depends on

various parameters such as the charge, hydrophobicity/hydrophilicity, size of the redox couple and the chemical and physical properties of the modified electrodes (Elshafey et al., 2013).

1.4.2.2 Optical transducers

Optical biosensors are also known as "optodes" because of their resemblance with electrodes; they are receiving considerable attention nowadays, with advances in optical fibres and laser technology. These sensors had extended the limits of application of the spectrophotometric methods in analytical chemistry, specially, for miniaturized systems. Optical biosensors integrate optical technique with a biological element to identify chemical or biological species. Many optical biosensors were developed based on Surface Plasmon Resonance, spectroscopy and evanescent waves.

In particular, the optical biosensors are based on methods such as UV – Vis adsorption, bio/chemiluminiscence, fluorescence/phosphorescence, reflectance, scattering and refractive index, caused by the interaction of the biocatalyst with the target analyte. Optodes are constructed with an immobilized selective biocomponent at one end of an optical fiber, with both the excitation and detection components located at the other end. The change in the intensity of adsorbed or emitted light from an indicator dye that can in turn interact with the selective biocomponent is the principle the pH, pO_2 and pCO_2 fiber – optic probes that achieve transduction via the indicator dye alone. This change is directly proportional to the amount of analyte present in the sample. The principle of these fiber – optic probes is the total internal reflection (TIR) phenomenon in a light guide using evanescent waves, an electromagnetic wave that exists at the surface of many forms of optical waveguides, to measure changes in refractive index at the sensor surface. TIR – based biosensors make use of the evanescent wave penetrating only a fraction of a wavelength into the optically rarer medium when light coming from an adjacent denser medium is incident on the interface at an angle above the critical angle. Changes in the surface refractive index or absorptivity reduce the transmission of light through the guide. Systems of $NAD(P)^+/NAD(P)H$ dependent dehydrogenase enzymes are indicated for use in optical devices as NAD(P)H adsorbs light strongly at 340 nm (ultraviolet) and emits fluorescent light in the blue range /at 460 nm). These coenzymes have been used for analysis of acetaldehyde, alanine, malate, glucose, glycerol, ethanol, galactose, but show restriction because a high instability and high cost (Mehrvar et al., 2000; Dremel et al., 1989; Wangsa et al., 1988).

Another optical TIR – based biosensor that internal reflection in a light guide is SPR (Surface plasmon resonance). SPR devices combine an evanescent wave detector with a biocomponent, generally, an antibody.

Maybe SPR is a further important sensing technique that allows non – labelled immunoassay. The SPR method is a charge – density oscillation that may exist at the interface of two media with dielectric constant of opposite sign, for instance, a metal and a dielectric. An SPR optical sensor, generally, comprises an optical system, a transducing medium which interrelates the optical and bio – chemical domains, and a electronic system supporting the optoelectronic components of the sensor and allowing data processing. SPR is a quantum electro – optical phenomenon; energy carried by photons of light can be coupled or transferred to electrons in a metal. This coupling results in the creation of a plasmon, a group of excited electrons on the surface of the metal. The intensity of the plasmon is influenced by the type of metal and the environment of the metal surface. Changes in chemical properties within the range of the plasmon field (such as the protein interaction in antibody – antigen binding) cause changes in plasmon resonance. These changes can be measured as a change in the angle of incidence or shift in the wavelength of light adsorbed and can be measured as a change in the SPR signal. Most of SPR instruments measure changes in the angle of incidence (Homola et al., 1999). SPR biosensor are potentially useful for environmental and food safety analysis because they are relatively easy to use, do not require labelling of either molecule in the reaction, and can assay crude samples without purification.

1.4.2.3 Thermal and Acoustic transducers

Although the electrochemical and the optical biosensors dominate, other forms of transducer such as thermal and acoustics are used, which can be sufficiently effective in analytical applications. Despite the lack of selectivity, which is a characteristic problem of these transducers, they present the advantage of miniaturization and the possibility of construction of arrays of sensors for simultaneous determination of several compounds (Mello et al., 2002).

Biosensors with thermal transducers are based on the monitoring of energy changed, under the heat form, over time, which occurs in a chemical reaction catalysed by enzymes or microorganisms. However, the heat cannot be perfectly confined in an adiabatic system and always presents a loss of information since the produced heat is partly wasted by irradiation, conduction or convection. The use of thermal biosensors in food analysis is still limited, probably due to tradition and relative complex instrumentation involved. Despite this, several important compounds for the quality control of food have been determined using thermal transducers, including ascorbic acid, glucose, lactate, galactose, ethanol, sucrose, penicillin G, cephalosporin and oxalic acid (Ramanathan et al., 2001; Ramanathan et al., 1999; Mosbach et al., 1995; Bataillard et al., 1993).

The piezoelectric transducers (surface acoustic wave) are more applied in immunosensors. In these devices, an antigen or antibody is immobilized in the surface of crystal. The interaction of these elements with the analyte highly specific can be monitored through the oscillation of the immersed crystal in liquid, which will produce a modification of mass in the crystal, perceptible by means of its frequency of oscillation. The immunosensors with wave acoustics principles, among others types, can be used for detection of pathogenic microorganisms, gases, aromas, pesticides, hormones and others (Babacan et al., 2000; Bizet et al., 1999; Ivnitski et al., 1999; Abad et al., 1998; Horacek et al., 1998).

1.5 Biomolecules immobilization strategies

In the progressing field of biosensors, most prominent footstep is to immobilize bioreceptors onto the transducer. Immobilization step in the development of biosensors is engaged with an extremely crucial role. The rewards provided by an effective immobilization method are extended utilization of the biosensor and predicted lengthened storage stability, maintaining free diffusion of substrates and products into and out of the immobilization layer.

Moreover, the choice of right immobilization technique gives rise to an efficient, simple and cheap biosensor, which can be commercialized certainly.

Various methods have been described for enzyme or protein immobilization (Table 1.1).

Table 1.1: *Immobilization procedure for biomolecules (Scouten et al., 1995)*

Method	Advantages	Disadvantages
Adsorption on insoluble matrices (e.g. by van der Waals forces, ionic binding or hydrophobic forces)	Simple, mild condition, less disruptive to enzyme/protein	Enzyme/Protein linkages are highly dependent on pH, solvent and temperature;
Entrapment in a gel (eventually behind a semipermeable membrane)	Universal for any enzyme, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a result of free radicals
Crosslinking by a multifunctional reagent (such as glutaraldehyde)	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilizing physically adsorbed enzymes or proteins that are covalently bound onto a support	Difficult to control the reaction, requires a large amount of enzyme, the protein layer has a gelatinous nature (lack of rigidity), relatively low enzyme activity
Covalent bonding onto a membrane, insoluble supports	Stable enzyme – support complex, leakage of the biomolecule is very unlikely, ideal for mass production and commercialization	Complicated and time – consuming: possibility of activity losses due ti the reaction involving groups essential for the biological activity (can be minimized by immobilization in the presence of the substrate or inhibitor of the enzyme

The *entrapment*, or retainment, of the biomolecule behind a membrane is often a straightforward process, particularly for the immobilization of living cells or crosslinked enzymes or proteins. Even so, the choice of semipermeable membrane may have a significant effect on the sensitivity and background of the resulting signal. A good common semipermeable membrane is the highly negatively charged prefluorinated sulfonate polymer

Nafion: this membrane suppresses interference by ascorbic acid, uric acid and acetaminophen in the redox system; due to the charge repulsion between these compounds and the membrane, they are hindered in their ability to reach the electrode and, therefore, are not able to cause interference.

Non-covalent methods of protein immobilization are widely employed and involve either passive adsorption onto hydrophobic surfaces or electrostatic interactions with charged surfaces. Simplest manner of explaining adsorption is, the act of sticking of atoms, ions, biomolecules or molecules of gas, liquid or dissolved solids to a surface; this isaccomplished by non-covalent interactions like electrostatic, van der Waals, hydrophobic between sensing molecule and surface (Wong *et al*., 2009).

Here, the use of nitrocellulose membranes or polystyrene microtiter plates for hydrophobic adsorption and polylysine coated slides for electrostatic binding are perhaps the most widely familiar. The major advantage of immobilization in this manner is that neither additional coupling reagents nor modification to the protein of interest is required. However, non-covalent immobilization typically involves relatively weak and reversible interactions. As a result, proteins can leach out from the support, which in turn results in loss of activity over time and contamination of the surrounding media. It is also well – known that adsorption of protein onto surfaces often results in conformational changes and denaturation of proteins that can result in massive losses in protein activity. Furthermore, since there is no control over the packing density of the immobilized proteins, their activity may be further reduced by steric congestion (Zhang *et al*., 2010).

For more stable attachment, the formation of *covalent bonds* is required, and these are generally formed through reaction with functional groups present on the protein surface. In common with non-covalent adsorption, these methods can be used on unmodified proteins since they rely only on naturally present functional groups; functional groups available for this method mainly originate from amino acid side chains, such as the amino groups of lysine, carboxyl groups from aspartame and glutamate, sulfhydryl groups from cysteine, and phenolic hydroxyl groups from tyrosine (Sullivan et al., 2003).

For example, the exposed amine groups of lysine residues readily react with supports bearing active esters, with the most common being N – hydroxysuccinimide (NHS) esters, to form stable amide bonds. However, one disadvantage of using NHS esters is that they are unstable in aqueous conditions, and thus, the attachment of proteins in aqueous buffers will compete with ester hydrolysis, resulting in only modest immobilization yields. As an alternative, aldehyde groups can be coupled with exposed amines on proteins to produce an imine that can be reduced using sodium cyanoborohydride or other reagent to form a stable secondary amine linkage (Hahn *et al*., 2007).

The nucleophilicity of the amine group also allows reaction with epoxide-functionalized materials; epoxides have the advantage of being relatively stable to hydrolysis at neutral pH, which allows easy handling of the materials.

For the acidic aspartate and glutamate residues, the generic method in which they can be used for immobilization is by conversion to their corresponding active esters in situ with a carbodiimide coupling agent and an auxiliary nucleophile. The most commonly used example of the former is 1-ethyl- 3-(3-dimethylaminopropyl) carbodiimide (EDC), while NHS is widely used as the auxiliary to generate the NHS ester on the protein. This active ester can then react with amine-bearing supports. The advantage of this combination of reagents is that both are water-soluble and may be used in aqueous media, although the instability of carbodiimides and the subsequently generated active esters under these conditions means that the reaction yields are rather low. There is also the risk that the NHS esters formed on the protein molecule may then couple to other protein molecules to give poorly defined polymers (MacBeat *et al*., 2000).

Probably the most important development in immobilization technology for miniaturized biosensors using microelectrodes is the use of *electrochemically created polymer films*, Certain monomers such as pyrrole, N-methylpyrrole, aniline and tyramine will polymerize at a sufficiently positive charged electrode surface; such conditions also bring most proteins to the surface. The best known example of electrochemical polymerization is that of pyrrole, which produces a polypirrole (PPY) membrane. Although a precise understanding of this electropolimerization process is still unclear, its general scheme involves the oxidation of the pyrrole monomer, to give the radical cation that can either react with a second radical cation or a neutral monomer molecule, to give the dimeric species. Further oxidation and coupling reactions of this type lead to oligomers, and eventually to the deposition of a polycationic polymer at the electrode. The morphology of the film is governed by the nature of the underlying electrode, the speed of deposition, the ionic and polyionic species present, and the solution pH. Enzymes, whole cells and protein fragments can be entrapped in this membrane, or modified pyrroles, such as 3- and N-derivatized carboxylate pyrroles, or N-amino-substituted pyrroles can be used so that enzymes can be bound covalently to the monomer before electropolymerization (Yam *et al*., 2006, Subramanian *et al*., 2006).

In working toward even greater levels of miniaturization and high-specification sensors, researcher are now exploiting "bottom – up" nanotechnology – derived platform such as *Self – Assembled Monolayer*, which promise a greater degree of sensitivity and miniaturization.

Self – assembly involves spontaneous arrangements of atoms and molecules in an ordered functional entity.

SAMs are organic assemblies formed when molecules in solution or the gas phase adsorb and spontaneously organize into a single layer on a surface.

The molecules constituting SAMs have chemical functionality with a specific affinity for a substrate. There are a number of chemical functionalities for binding to specific metals, metal oxides, semiconductors and glass. The most widely applied class of SAMs derives from the adsorption of an alkanethiol on gold; in fact, it forms good (well-packed) SAMs and it is historically the most studied. Moreover, gold is a reasonably inert metal, easy to prepare and pattern by a combination of lithographic tools and chemical etchants.

Gold binds thiols with a high affinity and it does not undergo any unusual reaction with them; In particular, Au(III) yields SAMs having both the highest density and the highest degree of regularity. Nonetheless, this thiol–gold bond leads to facile exchange of the adsorbates.

SAMs of alkanethiols on gold provide suitable systems for studying biological and biochemical processes: the stability, the uniform surface structure and the relative ease of varying their functionalities make SAMs an appealing choice for immobilizing sensing molecules on a transducer surface. Non-covalent protein immobilization methods are widely employed and involve either passive adsorption onto hydrophobic surfaces or electrostatic interactions with charged surfaces. Moreover, a number of different classes of organic reactions have been explored for modifying the surface of SAMs, including nucleophilic substitutions, esterification, acylation and nucleophilic addition. Under appropriate experimental conditions, the terminal groups exposed on the surface of a SAM immersed in a solution of ligands can react directly with the molecules present in solution (Frasconi *et al.*, 2010).

Finally, in the last few years, functionalization of surfaces with highly branched dendrimer molecules has gained attractiveness for various applications because the number of functional groups exceeds those of surfaces functionalized with self-assembled monolayers. So far, little is known about the physicochemical properties of dendrimer functionalized surfaces, especially if the flexibility of dendrimer structure remains after covalent immobilization.

Dendrimers, a family of special synthetic polymer molecules, efficiently present multivalent motifs since they possess a high amount of uniform chemical functionalities arranged along multiple, flexible, branches which are in direct proximity to each other due to their fixation to a central core molecule (Boas *et al.*, 2004). Their steric order leads to an extraordinarily high density of surface functional groups on the exterior of the dendrimer and gives rise to the 'dendritic effect', which is generally referred to as a synergistic process enhancement achieved by replacing a monomeric by a multimeric structure. Since size and shape of dendrimer molecules resemble

that of proteins, their interaction potential with a variety of biomolecules is a matter of actual research (Jasmine *et al.*, 2010; Shi *et al.*, 2010).

Physicochemical properties of dendrimers in solution have been studied experimentally as well as by molecular dynamics revealing that pH, ionic strength and polarity of the solvent as well as the type of dendrimer internal and terminal functions have a strong influence on the resulting structure of the molecule. This kind of variation in three-dimensional architecture offers the possibility to control structure–biocompatibility relationships between dendrimers and bioactive molecules (Porcar *et al.*, 2010; Prabal *et al.*, 2005).

Many biotechnological applications make use of dendrimer molecules immobilized to solid surfaces: advantages over self-assembled monolayer surface coatings are that biomolecule activity, bio-availability and sensor sensitivity are increased (Mori *et al.*, 2009, Lee *et al.*, 2009).

Physicochemical properties of dendrimer-modified surfaces clearly differ from those of self-assembled monolayers. In contrast to SAMs, not only the terminal functional groups but also the presence of internal polar and chargeable functions and the flexibility of the spacer branches contribute to the wetting behaviour and charge generation of dendrimer-modified surfaces (Katzur *et al.*, 2012).

Therefore, it can also be assumed that beside SAMs, which have gained a lot of attention in studying interface phenomena such as protein adsorption, bio-mineralization, bacteria and cell adhesion, also dendrimer surface coatings provide a valuable platform to examine multivalent biological processes. Furthermore, dendrimer coatings might be useful for the selective modification of biomaterial surfaces providing specific properties such as non-fouling or bactericide.

1.6 Label and label – free detection techniques

The importance of biosensors relies on their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex samples with a minimum sample pre – treatment.

Because some biomolecules or molecules cannot be transformed or cannot give electroactive signal for measurement, another electroactive molecule must be used for electroactive signal; so an extra preparation process (the labelling process) is necessary to detect the analyte.

Consequently, most affinity – based biosensor systems requires secondary molecules for amperometric or voltammetric experiments, which are attached to analyte or biorecognition receptor molecule to obtain an electroactive signal for measurement: during readout the amount of label is detected and assumed to correspond to the number of bound targets. Labels can be fluorophores, magnetic beads, active enzymes with an easily

detectable product, or anything else allowing facile target conjugation and convenient detection.

Labelled immunosensors normally operate using either a direct or indirect sandwich procedure or a competitive format as depicted in Figure 1.8.
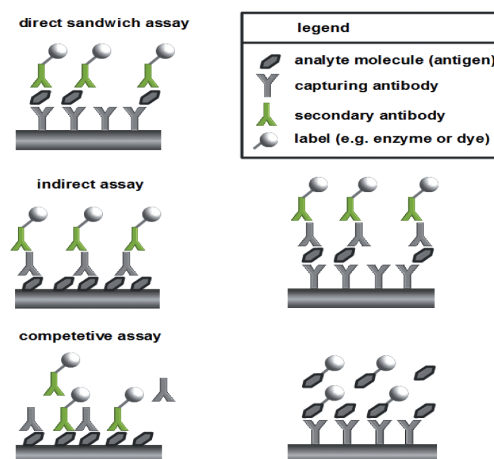
Figure 1.8*: Analytical task of immunosensors (Santos et al., 2014)*

Sandwich assays rely on secondary antibodies binding to the target antigen or antibody after the primary biorecognition reaction. In a competitive assay format, labelled and non labelled antibodies (or antigens) compete for the binding sites of the biorecognition layer. In direct assay formats basically all binding events of the target analyte can be addressed by the secondary antibody leading to typically higher sensor signals than in competitive assays. Competitive assays, however, require less working steps leading to potentially more cost and time – effective sensors.

Even if labelling can augment selectivity (e.g. using the sandwich approach with second probe) and enhance sensitivity (e.g. using a label that greatly changes the impedance), the labelling process can drastically change the binding properties of the biomolecules, and the yield of the target – label coupling reaction is highly variable.

Besides the time and expense benefits of omitting the labelling step, label – free operation enables detection of target – probe binding in real time, which is generally not possible with label – based systems.

Detection of biological recognition reactions between antibodies and antigens omitting labels typically relies on sophisticated instrumental. Among others, mass spectrometry and chromatographic approaches can be used to detect immunoreactions. Among optical methods, surface plasmon resonance (SPR) spectroscopy is an interesting way to detect antibody –

antigen binding events. Mass – sensitive biosensors based on vibrating cantilevers or the quartz crystal microbalance offer a straightforward way to detect antibody – antigen binding. Instrumental electroanalysis offers some alternatives for the detection of biological recognition events in immunosensors such as capacitive immunosensors and sensors based on electrochemical impedance spectroscopy. Both techniques are universal platform for the detection of immunoreactions and hence bacteria, viruses, tumor markers and more have been detected with these methods. Capacitive biosensors detect changes in the capacitance of an electrode upon the occurrence of binding event. The capacitive structure comprises a series of components such as the electrochemical double layer including the diffuse layer from ions in solution, the grafting layer and the biorecognition layer. Since the contribution of the biorecognition layer to overall capacitance is typically large compared to that of the other components, changes in the biorecognition layer upon the binding of antibodies or antigens can be probed by measuring the changes in the capacitance of the biosensor. This is often accomplished by potential – step experiments that require relatively cost – effective electrochemical equipment.

Electrochemical impedance spectroscopy (EIS) measures the complex resistance of an electrochemical system; the components of an electrochemical system including a biorecognition layer are altered upon a biorecognition event and can thus be probed by EIS. The occurrence of an immune recognition reaction typically results in a change of the impedance of the electrochemical system. Consequently, EIS detection schemes have been employed in biosensors and immunosensors for the quantification of different analytes.

In conclusion, instrumental electroanalysis, namely capacitance measurements and EIS allow for label – free detection of immunoreactions.

1.7 Fundamentals and Applications of Electrochemical Impedance

Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) is a powerful interfacial analytical tool and has been applied extensively to corrosion studies (Wang *et al.*, 2014), the characterization of charge transport across membrane and battery development (Noak *et al.*, 2014); its utility in detection interfacial binding events has been known for some two decades (Daniels *et al.*, 2007; K'Owino *et al.*, 2005; Berggren *et al.*, 2001; Lisdat *et al.*, 2008).

As explained above, when a target biomolecule interacts with a probe – functionalized surface, changes in the electrical properties of the surface (e.g. dielectric constant, resistance) can result solely from the presence of the target molecule; these changes can be detected by the impedimetric technique.

This approach has been used to study a variety of electrochemical phenomena over a wide frequency range: it is a powerful method of analysing the complex electrical resistance of a system and it is sensitive to surface phenomena and changes of bulk properties (Daniels et al., 2007).

In the field of biosensors, it is particularly well – suited to the detection of binding events on the transducer surface; in particular, if the impedance of the electrode – solution interface changes when the target analyte is captured by the probe, EIS can be used to detect that impedance change.

Impedance measurement does not require special reagents and is amenable to label – free operation: this is arguably the major motivation for studying impedance biosensor. In other words, in order to detect molecules without electrochemical reaction, EIS can be employed as a measurement technique "to see electrode surface modifications just by looking impedance curves"; it can be used for biomolecular recognition, biomolecular bindings and biomolecular interactions between molecules such as DNA – DNA, DNA – protein, receptor – ligand, protein – ligand, antibody – antigen and ion channels – ligands. As a consequence of this affinity provides label – free detection without chemical transformation and this binding property can be monitored by EIS expeditiously.

The impedance Z of a system is generally determined by applying a voltage perturbation with small amplitude and by detecting the current response. From this definition, the impedance Z is the quotient of the voltage – time function V(t) and the resulting current – time function I(t):

$$Z(t) = \frac{V(t)}{I(t)} = \frac{V_0 \sin(2\pi f t)}{I_0 \sin(2\pi f t + \varphi)} = \frac{1}{Y} \qquad (1.1)$$

Where V_0 and I_0 are the maximum voltage and current signals, f is the frequency, t the time, φ the phase shift between the voltage – time and current – time functions, and Y is the complex conductance or admittance.

The impedance is a complex value, since the current can differ not only in terms of the amplitude but it can also show a phase shift φ compared to the voltage – time function. Thus, the value can be described either by the modulus |Z| and the phase shift φ or alternatively by the real part (Z') and the imaginary part (Z'') of the impedance (Figure 1.9).

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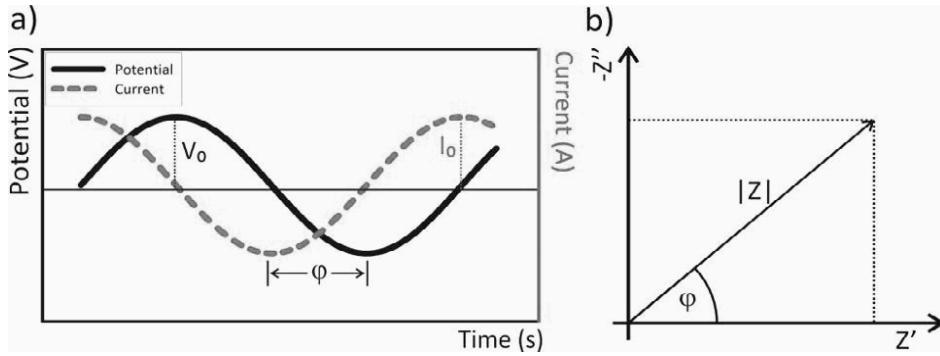
Figure 1.9: *(a) Time domain measurements of an impedance analysis: a sinusoidal voltage perturbation (amplitude V_0) and the sinusoidal current response (amplitude I_0), with a phase angle φ. (b) The impedance value can be expressed as the modulus |Z| and the phase angle φ, or it can be specified by the real (Z') and the imaginary (Z'') parts.*

Therefore the results of an impedance measurement can be illustrated in two different ways: using a Bode Plot (log |Z| and φ as a function of log f), or using a Nyquist Plot, which plots Z' and Z'' (Figure 1.10).
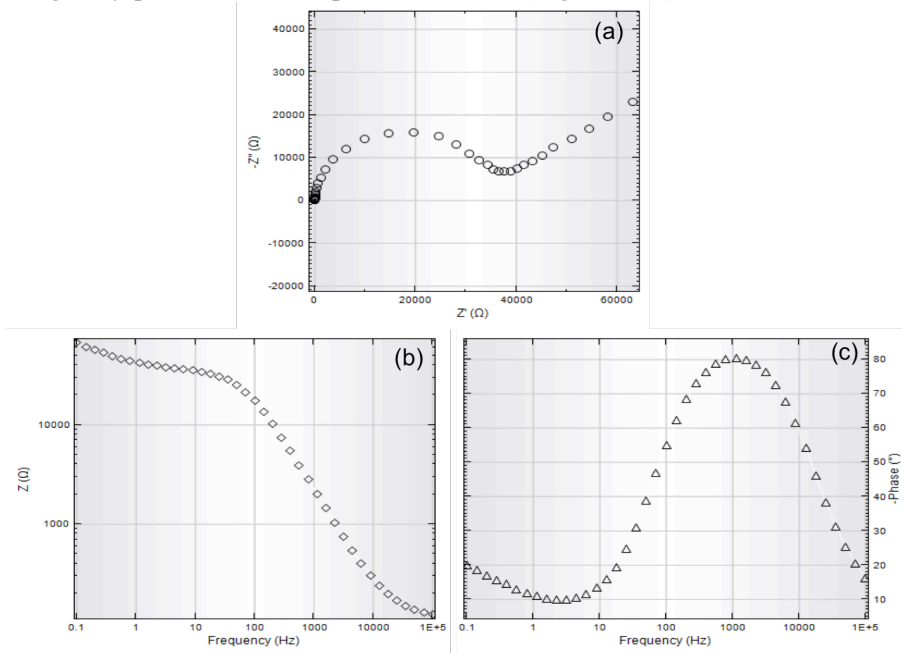
Figure 1.10: *(a) Nyquist plot. (b) Bode Plot: frequency vs Z. (c) Bode Plot: frequency vs phase.*

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The name impedance "spectroscopy" is derived from the fact that the impedance is generally determined at different frequencies rather than just one. Thus, an impedance spectrum is obtained that allows the characterization of surfaces, layers or membranes as well as exchange and diffusion processes. To achieve this, the impedance spectrum is often analysed using an equivalent circuit. This circuit, which commonly consists of resistances and capacitances, represents the different physicochemical properties of the system under investigation.

However, it is only possible to describe a system of interest, but the technique can also be used for analytical purposes. In this case, the change of one impedance element (a resistance or a capacitance) as a function of the solution composition is evaluated. In some cases it is also possible to correlate the overall impedance to a change in concentration. This can simplify measurements, since it is often sufficient to determine the impedance at just one selected frequency or within a limited frequency window (where the relative changes are largest) in such cases.

In all form of impedance, as noted above, the data acquirement is that of current as a function of time as the voltage waveform is applied. The relationship between applied oscillating voltage and observed oscillating current is analysed by considering the experimental configuration (that is, the surface and its associated solution) to be composed of circuitry elements (an equivalent circuit of resistors and capacitors). Four elements are used to understand the relationship between current and voltage: the current impedimetric in an AC potential. These are solution resistance (R_s), double layer capacitance (C_{dl}), electron transfer resistance (R_{ct}) and Warburg impedance (Z_w). The solution resistance is related to the conductivity/mobility of the redox probe in solution and is not affected by biorecognition events at the electrode surface. The double layer capacitance is an intrinsic feature of polarized electrodes immersed in electrolyte solution. Depending on the potential applied to the electrode, ions of opposite charge will approach the surface, forming a separated double layer. The capacitive element is usually replaced by a constant phase element (CPE) to reflect the non – ideality of the surface capacitance. The electron transfer resistance quantifies the electrostatic and/or steric barrier presented to the redox probe at the surface (Figure 1.11). The Warburg impedance, which is not used for analytical purposes, represents the unperturbed diffusion of the redox probe in bulk solution towards the electrode, down a diffusion gradient.

This approach, which involves the addition to solution of a diffusing redox probe, is known *"faradaic"*.
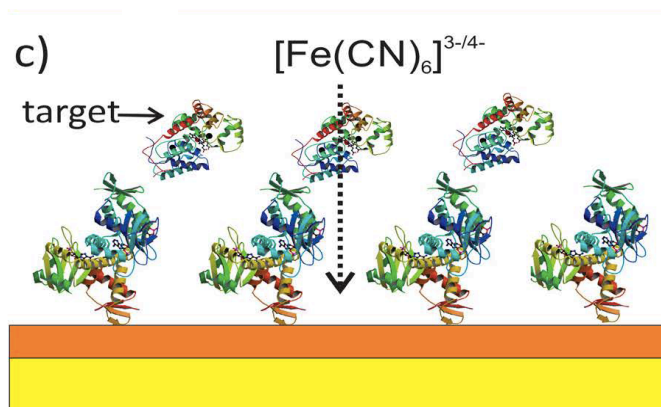
Figure 1.11*: Schematic representation of an faradaic impedance measurement (Santos et al., 2014)*

In "*non faradaic*", transduction occurs not through changes in the impediment presented to a solution phase probe but to surface dielectric, charge distribution or local conductance, most commonly assessed through capacitance. The capacitance arises when an electrode is immersed in an electrolyte solution and a certain potential is applied. In this condition, charged species and dipoles will be oriented on the interface electrode/solution, generating the previously mentioned electrical double layer capacitance, a physically measurable quantity potentially very sensitive to interfacial change. This change may be induced when a protein target binds to the receptor, previously attached on the electrode, displacing water and ions from the surface, or due a changing in protein conformation.

The capacitance of a biorecognition surface in non – faradaic approach can be described as a combination of two capacitances (Figure 1.12); the first capacitance consists of an insulating layer, comprised by SAM on gold and double layer (C_m); the second capacitance (C_{rec}) is related to the formation of a receptor biofilm (biorecognition layer). The binding event (target – receptor biorecognition) generates a third layer, (C_a).
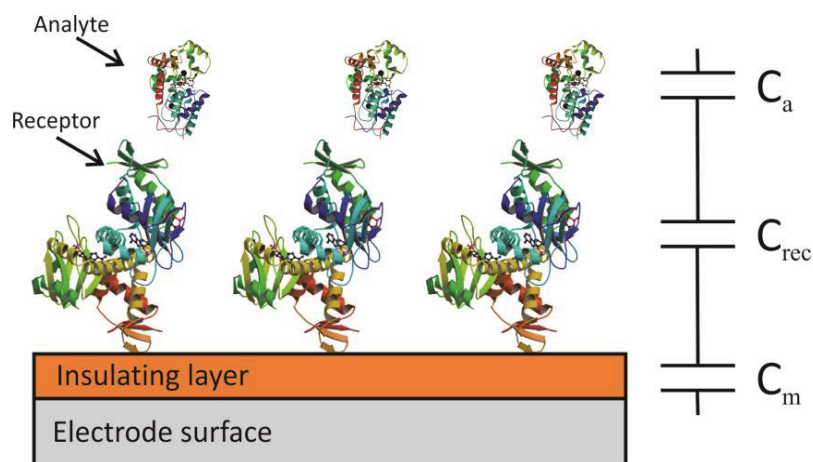
Figure 1.12*: Schematic representation of a capacitive biosensor (Santos et al., 2014).*

Therefore, when taking measurements with such surface – modified sensing electrodes, redox – active compounds are commonly added to the solution, resulting in a well – defined charge transfer resistance R_{ct}; if the redox – active compound is omitted or a blocking layer is applied to the electrode, rather capacitive impedance behaviour will be observed (since R_{ct} will become extremely large).

Thus, a binding event at the electrode can be detected by following the change in R_{ct} in the first case or the change in the capacitance in the second case.

Anyway, equivalent circuits are used in order to approximate the experimental impedance data with ideal or distributed impedance elements arranged in series and/or in parallel. Many electrochemical systems have been analysed according to this procedure. For the situation of an electrode in contact with an electrolyte, the so – called Randle's Circuit is used (Figure 1.13), comprising the solution resistance R_s, the charge transfer resistance R_{ct}, the double layer capacitance C_{dl} and the Warburg impedance W.
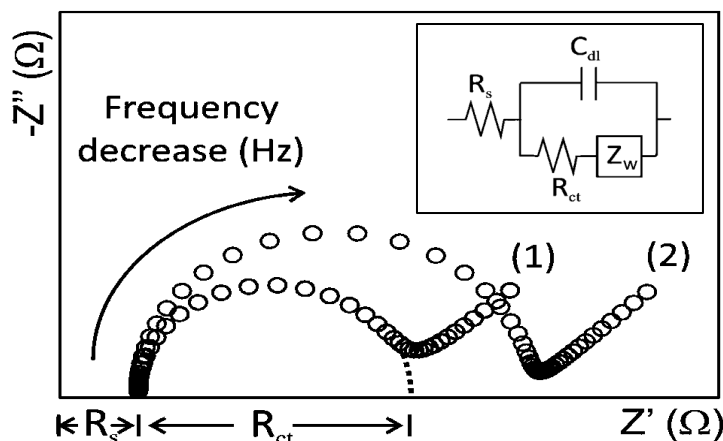
Figure 1.13: *Nyquist Plots for electrodes in contact with an electrolyte (a). Inset: Randles' equivalent circuit.*

In the Nyquist plot shown in Figure 1.13, the values for R_s and R_{ct} can be easily determined. The double layer capacitance can be calculated from the frequency at the maximum of the semicircle ($\omega = 2\pi f = 1/R_{ct}C_{dl}$). The product of R_{ct} and C_{dl} is often termed the time constant τ of the electrochemical process. The 45° line indicating Warburg – limited behaviour can be extrapolated to real axis. The intercept is equal to $R_s + R_{ct} - 2\sigma C_{dl}$, from which σ and subsequently diffusion coefficients can be calculated.

For analytical applications, however, the equivalent circuit is often simplified by neglecting the Warburg impedance. This can be done by choosing a frequency range where no 45° line is observed in the Nyquist plot and the interfacial or bulk impedance is dominant.

When performing electrochemical impedance spectroscopy, it is often necessary to control the situation at the working electrode. In some cases the impedance can be measured at the "open circuit" potential (OCP). This means a situation that has equilibrated at the electrode in dependence on the solution composition. However, this situation must be stable during the measurements. Here, a simple two – electrode arrangement can often be used. Alternatively a DC potential must be fixed at the working electrode in order to define a situation at the electrode or to force a process to occur (e.g. an oxidation process). This is a typical potentiostatic mode, where a three – electrode arrangement is necessary. Another approach is to define a current to flow over the working electrode. This can be achieved in galvanostatic mode, again with a three – electrode arrangement.

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Modern impedance analyser also contain data evaluation software and fitting programmes which allow the choice of an equivalent circuit from the implemented software as a starting point for the description of an individual system. In order to verify the validity of the chosen circuit, the quality of the fit to the experimental curve must be evaluated, and experimental parameters such as the electrolyte or analyte concentrations, or a layer thickness or the electrode area must be varied. The frequency range needed to determine the parameter of interest can also deduced from such an analysis of the impedance data.

For analytical applications, it is also possible to not only reduce the frequency range but also to limit the measurements to a few or even just one selected frequency. For example, this approach can be used when measuring chemically sensitive semiconductor structures that show highly capacitive behaviour. The frequency range that must be analysed varies with the system under investigation, but generally ranges from several tens of kilohertz to 10^{-1} or 10^{-3} Hz. Commercial instruments easily cover the range from 10^{-5} or 10^{-6} Hz. The impedance parameter of interest (e.g. the resistance or capacitance as a function of the concentration to be determined) can be obtained from a fit of the measured impedance data with a verified equivalent circuit, or the overall impedance can be directly correlated to the concentration (Lisdat *et al.*, 2008).

1.8 Affinity biosensors application in agri – food industry

Safety and quality are key issues of today's food industry. Food contamination can originate from a number of different sources, such as the improper use of veterinary drugs and pesticides, the formation of toxins and mycotoxins, bacterial contamination and the creation of chemicals during processing techniques. Analysis of food contaminants is not only vital for our protection but it also aids the global trade process by helping to identify and limit the trade in contaminated produce. Contaminants such as pathogens, toxins, pesticides and veterinary drug residues all have the potential to have serious health implications in terms of causing illness or by limiting the efficacy of medicines through developed drug resistance.

Therefore, the analysis of foods to assess the presence of both biological (pathogen bacteria) and chemical contaminants is a practice of crucial importance for ensuring food safety and quality.

The majority of chemical contaminants are commonly using separative techniques coupled to various detectors such as GC-FID, GC-ECD, GC-MS, HPLC-UV, HPLC-FL, HPLC-MS, while conventional bacterial testing methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in food. These last traditional methods are very sensitive and inexpensive, but require several days to generate results

because they rely in the ability of microorganisms to multiply and to produce visible colonies.

Since the food chain is becoming more and more complex, powerful analytical methods are required to verify the performance of food safety and quality system with high sensitivity, selectivity, ability for rapid implementation and capability of automatic screening.

Electroanalytical chemistry has, for decades, played a relevant role in food safety and quality assessment, taking more and more significance over time in the solution of analytical problems. At present, the implementation of electrochemical methods in the food is evident. This is in a large part due to the relevant results obtained by combining the attractive advantages of electrochemical transduction strategies (in terms of relatively simple hardware, versatility, interface with automatic logging and feasibility of application outside the laboratory environment) with those from biosensors technology.

The development of portable, rapid and sensitive biosensor technology is crucial for this purpose. The importance of biosensors relies on their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex samples with minimum sample pre-treatment. Biosensor methods can be used as a rapid screening tool, greatly reducing the cost and time of analysis.

The food industry is benefitting from major advances in the development of biosensors with different transduction system that can be applied in the areas of food safety, quality and process control; studies are focused mainly on affinity biosensors, where antibodies or antibody fragments are used as molecular recognition element for specific analytes (antigens) to form a stable complex, with a label – free approach, which guarantees a direct quantification of the specific analyte.

Important examples of enzyme electrochemical biosensors are those dedicated to the determination of glucose, alcohol or cholesterol are important examples. In addition, other types of different electrochemical biosensing approaches have emerged strongly in the last years. Among these, the strategies involving affinity interactions have been shown to possess a large number of applications. Therefore, electrochemical affinity based biosensor have been widely used to determine major and minor components in foodstuffs, providing sufficient data to evaluate food freshness, the quality of raw materials, or the origin of samples, as well as to determine a variety of compounds at trace levels related to food safety such as mycotoxins, allergens, drugs residues or pathogen microorganisms.

1.9 Ochratoxin A affinity biosensors: state of art

The need to develop high – performing methods for OTA analysis able to improve the traditional ones is evident: owing to their high sensitivity, good

specificity and less dependence on sample clean-up, electrochemical sensors based on immunological procedures seem most promising, thanks to their low cost, compatibility with miniaturization and portability (Muchindu *et al.*, 2010). Therefore, in the last years, immunosensors have aroused a very great interest with expectations of providing fast and highly sensitive detection of proteins peptides, toxins, viruses and bacteria or part of these, finding widespread applications in clinical diagnostics, food safety and environmental monitoring.

Tsai *et al.* (2007) fabricated a piezoelectric immunosensor based on self – assembled monolayer of 16 – mercaptohexadecanoic acid to detect OTA using quartz crystal microbalance; the fabricated immunosensor exhibited detection range from 50 to 1000 ng/mL and detection limit of 16.1 ng/mL with negligible effect of interference.

Adànyi *et al.* (2007) reported the development of a rapid and highly sensitive competitive immunoassay for the detection and quantification of OTA; OTA was quantified by measuring the formation of the fluorescent immunocomplex on the waveguide surface. The limit of detection for OTA in several cereals has been reported in a range of 3.8 – 100 ng/g, while in coffee and wine, detection limits were 7 and 38 ng/g respectively. In this study the authors used the optical waveguide light mode spectroscopy (OWLS) techniques for the detection of aflatoxin and OTA on both competitive and direct immunoassay wherein a microchip utilized a flow – injection analyser (FIA) system.

Prieto-Simon *et al.* (2008) reported OTA detection through immobilization of polyclonal and monoclonal antibodies. The immunosensors, both HRP and alkaline phosphatise (ALP) labelled immunosensors showed detection limit of 0.7 and 0.3 ng/mL, respectively.

Bonel *et al.* (2010) and Liu et al. (2013) studied indirect competitive enzyme-linked immunosorbent assays (ELISA) strategies, developing labelled immunosensors for wheat and corn samples analysis respectively. All measurements were conducted by differential pulse voltammetry technique.

In all these cases, the immunosensors required a label attached to the target: during readout the amount of label is detected and assumed to correspond to the number of bound targets. However, labelling a biomolecule can drastically change its binding properties and the yield of the target-label coupling reaction is highly variable (Daniels *et al.*, 2007).

Moreover, the use of labels is also a source of higher costs and analysis times (Ricci *et al.*, 2007).

Moreover, among all of the possible transducers used for immunosensors development (electrochemical, optical, microgravimetric) the electrochemical ones rank highly owing to their sensitivity, low cost, simplicity and, in some cases, miniaturization, portability, and integration in automated devices (Farrè *et al.*, 2009).

For all these reasons, in the last years, the potential use of Electrochemical Impedance Spectroscopy (EIS) technique has been examined in the immunosensor development; it is, in fact, a powerful, nondestructive and informative technique, which can be used to study the electrical properties of the sensing device interface and to trace the reactions (Ciania *et al*., 2012).

The application of EIS on modified electrodes on which antibodies have been immobilized let to develop label free immunosensors based on the impedimetric change that occurs when the immunocomplex occurred on the electrodes surface.

Radi *et al*., (2009) prepared a novel, label – free, impedimetric immunosensor to detect OTA that exhibited linearity in the range 1 – 20 ng/mL, with a detection limit of 0.5 ng/mL. Muchindu *et al*. (2010) reported the development of impedimetric immunosensors for the detection of OTA in a linear range of 2–10 ng/mL.

Solanki *et al.,* (2010) have immobilized anti – OTA antibody onto self – assembled monolayer of 11 – Amino – 1 – undecanethanol for the selective detection of OTA in coffee sample using EIS technique; the results of the studies revealed linearity from 0.5 ng/dL – 6.0 ng/dL and detection limit of 0.08 ng/dL.

Recently, studies on the development of electrochemical aptasensor for detection of OTA have been published (Mishra *et al*., 2015). Even if aptamers offer many advantage in contrast to antibodies, i.e. they are easier and more economical to produce, the analysis of food samples require clean up procedures increasing time and cost analysis and thus reducing the advantages of the biosensors.

DNA aptamers based electrochemical impedance biosensor was utilized for the detection of OTA which exhibited detection range of 0.1 – 100 nM with the detection limit of 0.44 nM (Castillo *et al*., 2011). Prabhakar *et al*., (2011) utilized a Langmuir – Blodgett film of polyaniline (PANI) – stearic acid (SA) to fabricate an aptamer based impedimetric immunosensors to detect OTA: the aptasensor exhibited linear range of 0.1 – 10 ng/mL with detection limit of 0.1 ng/mL in 15 min.

Therefore, different impedimetric label – free OTA affinity biosensors have been described previously by immobilization of polyclonal or monoclonal antibodies and aptamers on the surface of gold or platinum electrodes. However, in recent years, the applications of disposable screen – printed carbon electrodes (SPCEs), characterized by low – cost fabrication and mass production, have attracted an increasing interest for the development of labelled immunosensors (especially enzyme immunosensors) but it is noteworthy that few studies on electrochemical label – free immunosensors integrated onto SPCEs have been developed (Lin *et al*., 2016; Lien *et al*., 2015).

In this PhD thesis two different novel label – free impedimetric immunosensors have been developed for the detection of Ochratoxin A, characterized by a very low detection limit and high sensitivty. The first one has been developed on gold electrodes and the influence of oriented and not oriented monoclonal antibody immobilization techniques on the biosensors analytical performances of the has been investigated (Malvano et al., 2016a).

With the aim to reduce the cost of the sensor device, the other biosensor has been developed on screen-printed carbon electrodes, properly modified with electrochemical gold deposition, which has demonstrated a very cheap way to obtain gold-like behaving electrodes using a very small quantity of the metal (Malvano et al., 2016b).

1.10 Gluten affinity biosensors: state of art

To ensure the safety of products for celiac disease patients, foods bearing gluten – free label must not exceed the level of 20 mg gluten per kg product (Codex Standard 118-1979, 2008). This requirement sets the standard for analytical methods for gluten detection, because these must be capable of detecting gluten traces well below the threshold of 20 mg gluten/kg. Availability of fast, cheap but sensitive methods for gluten detection are necessary for an effective gluten – free products labelling and thus protecting celiac people from the unaware content of gluten in food higher than official limit (20 ppm) set by the European regulation.

Currently, enzyme-linked immunosorbent assays (ELISA) based on the R5 and G12 antibodies are recommended by legislation (Codex Committee of Methods of Analysis and Sampling, 2015). ELISAs are most commonly used for gluten analysis, not only due to their specificity, sensitivity, and suitability for routine analysis, but also for lack of an independent reference method. Alternatives to ELISA based on immunosensors, proteomics, mass spectrometry, genomics and novel approaches such as aptamers, microarrays, and multianalyte profiling are being developed.

Electrochemical immunosensors for gluten detection in food products have been developed in the last years exploiting the capability of specific monoclonal antibodies to detect gliadin antigens: a sandwich immunosensor that needs labelling steps followed by enzymatic reaction before measurement, and an amperometric competitive immunosensor based on gliadin immobilization on disposable carbon – nano gold screen – printed electrodes (Manfredi *et al.*, 2016).

Recently (Chiriacò *et al.*, 2015) proposed a lab on chip platform based on impedimetric immunosensors to detect gluten at 1 ppm.

Nucleic acid aptamers, obtained by the in vivo selection process SELEX represent a new kind of receptors for gliadin detection. The use of aptamers as the biomolecular recognition element for developing gluten sensors is justified by their low cost synthesis and high reproducibility; high affinities

comparable to those of monoclonal antibodies but with higher stability due to their nucleic – acid chemical nature and additionally they can be easily combined with different chemical labels/groups that provide flexibility for adaptation to different platforms (Miranda – Castro *et al*., 2016).

In the last years aptamers against the 33-mer peptide (amino acid sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) recognizing the hydrophobic immunodominant fragment of α2 – gliadin, have been studied pointing out their capability to bind gliadin from several gluten source (Pinto *et al*., 2014, Amaya-Gonzalez *et al*., 2014, Amaya-Gonzalez *et al*., 2015). Aptamers against the 33-mer peptide , termed Gli1 and Gli4, have been applied in an electrochemical competitive enzyme – linked assay on magnetic particles. The assay based on Gli4 – aptamer was able to achieve a gluten detection of 0.5 ppm but it failed in detecting gluten in heat treated and hydrolysed food samples contrary to Gli1 who was kinetically favoured. Recently, Lopez-Lopez *et al*. (2017) developed a competitive electrochemical enzyme labelled aptasensor for the analysis of gluten in food samples.

In this PhD thesis the first impedimetric label – free aptasensor based on the immobilization of Gli1 aptamer for gliadin detection on the modified gold electrode was developed: this condition makes the analysis of gliadin very fast and easier than the common aptamer competitive assays that require the addition of an enzyme labelled aptamer to the food sample and then the enzymatic substrate (Malvano et al., 2017a).

1.11 Carbamate and Organophosphate biosensors: state of art

During the last two decades, the enzymatic biosensors have gained considerable analytical interest for the determination of pesticides due to advantages of portability, routine detection and fast processing time (Mulchandani *et al*., 2001); in this context, the use of cholinesterase enzymes has shown great promise to assemble enzyme sensors for screening analysis.

The most common biosensors developed for pesticides detection are based on AChE inhibition: CBs and OPs, in fact, are able to form a covalent link with the serine residue present in the catalytic site of the enzyme, blocking the hydrolysis of the acetylcholine in choline acetic acid. The principle of this type of biosensors is based on the quantification of the inhibitor, measuring the enzymatic activity in absence and in presence of the inhibitor by different transduction techniques such as amperometric, piezoelectric and optical transducers (Arduini *et al*., 2013; Albanese *et al*., 2012; Valdes – Ramirez *et al*., 2008a; Caetano *et al*., 2008; Andreescu *et al*., 2006; Vakurov *et al*., 2004).

Because the AChE inhibition occurs for both pesticide groups AChE – based biosensors are not specific: they give information about the toxicity

level of a sample but they can be used only as an allarm system for this class of toxic compounds, followed, in the case of positive response, by the HPLC or GC-MS analyses to exactly detect the type and the amount of pesticide in the sample (Moscone *et al*., 2016).

Thanks to their sensitivity, selectivity, rapid response and low – cost manufacturing, various electrochemical biosensors, have been proposed for the detection of OPs and CBs based on amperometric analysis: Arduini et al. (2006) studied inhibition effect on AChE of different pesticides, observing a higher sensitivity towards alicarb (50% inhibition with 50 ppb) and carbaryl (50% inhibition with 85 ppb); real samples were also tested in order to evaluate the matrix effect and recovery values comprised between 79 and 123% were obtained.

Afterwards, Arduini *et al*. (2013) have proposed a mono-enzymatic AChE amperometric biosensor developed on gold screen – printed electrode able to detect very low paraoxon concentrations, until 2 ppb.

During last ten years, a lot of pesticides have been tested with amperometric biosensors, exploiting the inhibition mechanism of pesticides versus AChE, such as carbaryl (Caetano *et al*. 2008; Valdes – Ramirez *et al*., 2008a), dichlorvos (Wu *et al*; 2013; Valdes - Ramirez *et al*., 2008b), malathion and chlorpyrifos (Chauchan *et al*., 2011).

More recently, an immunosensor for highly sensitive detection of parathion was developed, using electrochemical impedance spectroscopy as transduction technique (Mehta *et al*., 2016): the immunosensor detect parathion in a broad linear range (0.1 – 1000 ng/L) with a very low limit od detection (52 pg/L), showing an high selectivity towards parathion in the presence of malathion, paraoxon and fenitrothion. The viability of this biosensor was demonstrated by detecting parathion in real sample such as tomato and carrot.

In this PhD thesis a very innovative impedimetric enzyme inhibition-based biosensor for carbamate and organophosphate compounds has been developed: the high affinity interaction between pesticides and Acetylcholinesterase active site was monitored by impedimetric transduction technique, and the impedimetric changes obtained at different pesticide concentrations allow to go up very fast to the presence of the toxic compounds in different food matrices (Malvano et al., 2017b).

1.12 *Escherichia Coli* O157:H7 affinity biosensors: state of art

Nowadays, biosensors play a significant role in the determination of pathogens: in particular most of immunosensors use labeled antibodies to monitor the formation of antigen – antibody complex (Leng *et al*., 2017; Yang *et al*., 2012; Heyduk *et al*., 2012).

However, label – free immunosensors, in which the immune interaction is directly monitored, exhibit some important advantages in terms of speed and simplicity of operation.

Several approaches concerning the developed of label – free immunosensors for the detection of the pathogenic strain *E.Coli* O157.H7 have been reported in literature: these include different transduction techniques, such as Surface Plasmon Resonance (SOR) (Subramanian *et al.*, 2006), Quartz Crystal Microbalance (QCM) (Li *et al.*, 2011), Surface Acoustic Wave (SAW) (Berkenpas *et al.*, 2006).

Among all the possible immunosensors (optical, microgravimetric, electrochemical), the electrochemical ones, rank highly owing to their sensitivity, low cost and simplicity. Among them, different impedimetric label – free immunosensors for the detection of *E.Coli* bacteria have already been developed (Escamilla – Gomez *et al.*, 2009; Geng *et al.*, 2008; Yang *et al.*, 2016), while few studies have been conducted on the specific detection of the pathogenic strain *E.Coli* O157:H7.

Chowdhury *et al.* (2012) immobilized polyclonal anti – E.Coli O157:H7 on a conductive polyaniline film, and electrochemical impedance spectroscopy was used to test the sensitivity and effectiveness of the sensor electrode by measuring the change in impedance values of electrodes before and after incubation with different concentrations of bacteria. As small concentration as 10^2 cfu/mL of *E.Coli* O157:H7 were successfully detected on the immunosensor with the upper detection limit of 10^7 cfu/mL. Moreover, the specificity of the sensor has been tested by comparing with two other strains of similar bacteria, *Salmonella typhi* and *E.Coli* BL21, and found to be satisfactory.

However, the lowest limit of detection (2 cfu/mL) was reported by Barreiros dos Santos *et al.* (2013), where anti - E.Coli polyclonal antibodies have been immobilized onto gold electrodes via self – assembled monolayer of mercaptohexadecanoic acid: electrochemical impedance spectroscopy was used to monitor the immune – complex. A large linear rate ($3*10 – 3*10^4$ cfu/mL) was achieved and no significant adsorption of *Salmonella typhimurium* was observed; any tests on real food matrices were performed.

Whit the aim to develop a very high sensitivity and selectivity label-free immunosensor for *Escherichia Coli* O157:H7 detection, in this PhD thesis different immobilization procedures of monoclonal anti-E.Coli were tested (Manuscript for submission*, Biosensor journal,* Invitation for the Special Issue: "Label-free Biosensing").

1.13 Aim of the work

The aim of this work has been the development of label-free affinity biosensors for the quantitative determination of some chemical and microbiological food pollutants and allergens, exploiting specific

biomolecules as recognition element coupled with the Electrochemical Impedance Spectroscopy as transduction technique.

In particular, the project has been focused on the development and optimization of four different label – free affinity biosensors:

- Affinity biosensors for Ochratoxin A detection
- Aptasensor for Gluten detection
- Enzymatic Biosensor for Pesticides detection
- Affinity biosensor for *Escherichia Coli* O157:H7 detection

The research activities have focused on the identification of the right technique for the immobilization of biomolecules on the electrode; appropriate materials have been chosen for the increase of sensitivity and stability of the biosensors highlighting the advantages obtained with label-free working principle, which guarantees a direct reading of the target molecule, avoiding the use of additional elements.

The developed biosensors have been characterized in all the preparation steps with the aim to obtain extremely selective, sensitive and reproducible biosensors able to reach limits of detection, regarding the analyte of interest, at least equal to the concentration limits imposed by the food legislation.

1.14 References

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A highly sensitive impedimetric label free immunosensor for Ochratoxin A measurement in cocoa beans

A high sensitive impedimetric label free immunosensor for Ochratoxin measurement in cocoa beans

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2.1 Abstract

In this work the development and optimization of an impedimetric label free immunosensor for the detection of Ochratoxin A (OTA) is reported.

Two antibody immobilization methods (oriented and not oriented) were compared highlighting a lower limit of detection (5 pg/ml) for the not oriented immobilization but a closer linear range in contrast to oriented anti – OTA immunosensors which showed linearity in the range of 0.01- 5 ng/mL OTA. The analysis of the Atomic Force Microscopy (AFM) images showed two different nanostructures indicating that the use of oriented immobilization created a more ordered and highly dense antibody surface. Finally the oriented immunosensor was used to quantify OTA in spiked cocoa bean samples and the results were compared with those registered with competitive ELISA kit. The immunosensor was sensitive to OTA lower than 2 μg/kg that represents the lower acceptable limit of OTA established by European legislation for the common food products.

2.2 Introduction

Mycotoxins are toxic secondary metabolites responsible for the contamination of approximately 25% of the world's crops, causing spoilage of agricultural products. In pollutants risk assessment, experts consider these contaminants as the most important chronic dietary risk factor (Prieto-Simon, Campas, Marty & Noguer, 2008).

Ochratoxin A (OTA) is a secondary metabolite produced by several species of Aspergillus and Penicillium fungi. The toxin, which is a nephrotoxic and nephrocarcinogenic compound, has mainly been found in cereals but significant levels of contamination may also occur in coffee, cocoa beans, wine, dried fruits, beer and grape juice spices. OTA is a proven carcinogen in animals and is classified as a class 2B, possible human carcinogen by the International Agency for Research on Cancer (Reddy & Bhoola, 2010). The National Toxicology Program (NTP) has designated OTA as "reasonably anticipated to be a human carcinogen" based on sufficient evidence of carcinogenicity in experimental animals (Clark & Snedeker, 2006). Regulations relating to mycotoxins have been established in many countries to protect the consumer from the harmful effects of these compounds. In several countries, these contaminants are subject to legislation that is based on the establishment of an Acceptable Daily Intake (ADI) or Tolerable daily intake (TDI). In the European Union the acceptable limits established for OTA in various foodstuffs are listed in Commission Regulation (EC) No 1881/2006 and ranged from 10 μg/kg for instant coffee and dried vine fruits to 0.5 μg/kg for dietary foods intended specifically for

infants. OTA is a heat-stable molecule within the range of conventional food processing temperatures and no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization. Thus the accurate knowledge of OTA contamination level in food products represents a key factor in the food safety at worldwide level. Research studies have been conducted to develop appropriate methods for the detection of OTA in food and feed samples (Kaushik, Arya, Vasudev & Bhansali, 2013). Traditional methods include gas chromatography, thin layer chromatography, capillary electrophoresis and high-performance liquid chromatography. However, these techniques require expensive equipment as well as complicated and time-consuming solvent cleanup steps. Owing to their high sensitivity, good specificity, and less dependence on sample cleanup, electrochemical sensors based on immunological procedures seem most promising, thanks to their low cost, compatibility with miniaturization and portability (Muchindu et al., 2010). Therefore, immunosensors have aroused a very great interest with expectations of providing fast and highly sensitive detection of proteins, peptides, toxins, viruses and bacteria or part of these, finding widespread applications in clinical diagnostics, food safety and environmental monitoring.

In the field of food safety some studies have been focused on the development of electrochemical immunosensor for OTA determination; Bonel, Vidal, Duato and Castillo (2010), Liu, Yang, Zhang and Yub (2013) and Prieto – Simon et al. (2008) studied indirect competitive enzyme-linked immunosorbent assays (ELISA) strategies, developing labelled immonusensors for wine, wheat and corn samples analysis respectively. All measurements were conducted by differential pulse voltammetry technique. In these cases, the immunosensors required a label attached to the target: during readout the amount of label is detected and assumed to correspond to the number of bound targets. However, labelling a biomolecule can drastically change its binding properties, and the yield of the target-label coupling reaction is highly variable (Daniels & Pourmanda, 2007). Moreover, the use of labels is also a source of higher costs and analysis times (Ricci, Volpe, Micheli & Palleschi, 2007).

For these reasons, in the last years, the potential use of Electrochemical Impedance Spectroscopy (EIS) technique has been examined in the immunosensor development; it is, in fact, a powerful, nondestructive and informative technique, which can be used to study the electrical properties of the sensing device interface and to trace the reactions (Ciania et al., 2012). The application of EIS on modified electrodes on which antibodies have been immobilized let to develop label free immunosensors based on the impedimetric change that occurs when the immunocomplex occurred on the electrodes surface. Muchindu et al., (2010) and Radi, Munoz – Berbel. Latesc & Martyc (2009) reported the development of impedimetric immunosensors for the detection of OTA in a linear range of 2-10 and 1–20

ng/mL respectively. Studies on the development of electrochemical aptasensor for detection of OTA have been recently published (Mishra, Hayat, Catanante, Istamboulie & Marty 2015). Even if aptamers offer many advantages in contrast to antibodies, i.e they are easier and more economical to produce, the analysis of food samples require clean up procedures increasing time and cost analysis and thus reducing the advantages of the biosensors.

An important aspect that has to be considered during the fabrication of an immunosensor is the orientation of the sensing molecules on solid phase for improving sensitivity, specificity, and analyte-binding capacity.

From the above the aim of this work is the development of a highly sensitive label-free impedimetric immunosensor for the detection of OTA in food samples. A Self Assembled Monolayer (SAM) procedure coupled with the oriented immobilization of the monoclonal anti-OTA was used for the construction of the immunosensor and the EIS with Cyclic Voltammetry were used to characterize the immobilization steps and the performance of the immunosensors. The sensitivity and the topography by Atomic Force Microscopy (AFM) of oriented and not oriented immunosensors was also investigated. Finally the immunosensor was used to quantify OTA in spiked cocoa bean samples and the results compared with competitive ELISA kit.

2.3 Materials and Methods

2.3.1 Reagents

4-mercaptobenzoic acid (MBA, 99%), 2-(N-morpholino)ethanesulfonic acid (MES >99.5% purity), N-Hydroxysucciminide (NHS, 99%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, >99%), Sulfuric acid (H_2SO_4, 99.9%), Ethanolamine ($NH_2CH_2CH_2OH$, >99.5%), Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), Tween 20, Ethanol (>99.8%) and Ochratoxin A were purchased from Sigma-Aldrich (Milano, Italy). Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$) was obtained from Carlo Erba reagent (Milano, Italy). Anti-Ochratoxin A antibody (anti-OTA)(1 mg/mL) was purchased from Abcam (Cambridge, United Kingdom), while Protein A/G (5 mg/mL, 59.7 kDa, >98%) was obtained from BioVision Inc. (San Francisco, USA). NaH_2PO_4, Na_2HPO_4, NaCl and KCl used in the preparation of phosphate buffered saline (PBS: 0.1 M KCl, pH 7.4) were received from Sigma Aldrich (Milano, Italy) .

2.3.2 Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat and Nova software. Au thin-film single-electrodes were obtained from Micrux Technologies (Oviedo, Spain). The electrodes incorporate a conventional three – electrode configuration, with an Au working (diameter 1 mm), reference and counter electrodes.

2.3.3 Immunosensor manufacturing

Before modification, gold electrodes were cleaned by applying 13 potential cycles between -1.0 and +1.3 V with 100 mV/s scan rate in 0.05 M sulfuric acid. SAM was carried out on the surface of the electrode using an ethanol solution containing MBA 30 mM under a constant potential of 1.2 V for 20 min.

The terminal carboxylic groups on gold electrode surface were activated by dropping on the Au modified electrode a solution of 75 mM EDC and 15 mM NHS in 100 mM MES buffer (pH 7.4) for 2 hours.

Thereafter, the immobilization of anti-OTA was carried out in oriented and not oriented way. In the oriented immobilization method 20 μL of Protein A/G 5 mg/mL were dropped on the modified electrode and left to react for 1hour. After incubation, 100 μL of 1 M ethanolamine (pH 8.5) solution was dropped onto the modified surface and incubated for 15min to block unreacted active sites. After thorough rinsing with PBS buffer, the modified electrode was covered with 10 μL of anti-OTA solution at four different concentrations (0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL) for 30 min at room temperature. Finally the electrode was rinsed in PBS to remove unbound antibodies. In the not oriented construction of the immunosensor, the anti-OTA solution was added after the activation of carboxylic groups with EDC/NHS. Then the electrode was rinsed in PBS to remove unbound antibodies and finally the unreacted active sites were blocked with 1 M ethanolamine.

The schematic diagram of the immunosensors fabrication is presented in Figure 2.1.
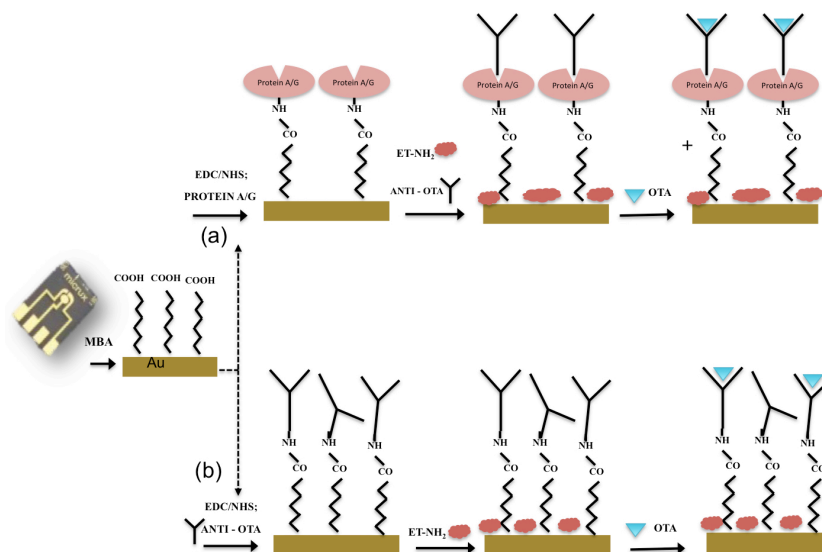
Figure 2.1:*Schematic diagram of the oriented (a) and not oriented (b)
immunosensors fabrication.*

2.3.4 Experimental measurements

EIS measures the response of an electrochemical system to an applied
oscillating potential as a function of the frequency resulting in an impedance
spectra (Nyquist plot) where the complex impedance is displayed as the sum
of the real and imaginary components (Z^{I} and Z^{II} respectively).

For electrochemical impedance studies, a sinusoidal AC potential (10
mV) in the frequency range from 0.1 to 10^{5} Hz was super imposed to a 0.00
mV (vs. reference electrode) DC potential. The measurements were
performed in a solution of 1 mM ferri/ferrocyanide redox couple
($[Fe(CN)_6]^{4-/3-}$, 1:1) in PB, pH 6.8, as background electrolyte at room
temperature.

The CV was also used to characterize each step of electrode modification
and anti-OTA immobilization. The measurements were performed from -0.6
to 0.6 V vs. reference electrode with a scan rate of 0.05 V/s; the redox
couple used for the CV was the same as that used for impedance
measurements, 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$,1:1) in
PB, pH 6.8.

For the OTA measurement, 10 µL of OTA at different concentrations in
PBS were dropped onto the working area of the immunosensor and
incubated for 20 min.   Before the impedance measurements the

immunosensor was rinsed thoroughly with copious amount of bi-distilled water.

2.3.5 AFM studies

AFM studies were performed by using Veeco Dimension 3100 AFM with Nanoscope III controller.

Measurements were carried out by using a silicon cantilever with a nominal tip radius of 20 nm. Topographic images were taken in tapping mode with a scan size of 1x1 μm.

2.3.6 Preparation of cocoa beans samples

The preparation of the cocoa bean samples was according to the procedure described in I'screen Ochra Elisa kit (Tecna, Italy). 2.5 g of finely grinded cocoa beans was added to 5 mL of 0.1 M phosphoric acid and 50 mL of chloroform; the solution was shaken in a low – speed shaker (400 rpm) for 15 min. 25 mL of filtrate were collected and added to 5 mL of the 0.13 M sodium bicarbonate solution; the solution was shacked for 30 seconds. The upper aqueous phase was recovered and centrifuged to remove solvent traces. Finally 4mL of sodium bicarbonate solution was added to 1 mL of surfactant.

This procedure was applied for four different amounts of OTA spiked to grinded cocoa bean samples in order to obtain 1, 1.5, 2.5 and 5μg/kg. The total samples analyzed were 12, three for each OTA concentration. The results obtained by the immunosensors were compared with those measured with the competitive Elisa kit (I'screen Ochra Elisa kit -Tecna, Italy) for OTA detection.

2.4 Results and discussions

2.4.1 Assembling of immunosensors

The design of the immobilization layout is a very important factor in the performance of immunosensors, as it greatly affects both the sensitivity and the specificity of the biointerface.

The surface modification of the Au electrodes for the preparation of OTA oriented immunosensor was monitored using EIS and CV.

The voltammograms of the Au electrode at the different immobilization steps display well defined anodic and cathodic peaks due to the reversible interconversion of $[Fe(CN)_6]^{4-/3-}$ redox solution (Figure 2.2A). The formation of the consecutive layers used for the biosensor construction, causes the

decrease of both peaks due to hindering effects of the layers on the electron transfer rate.
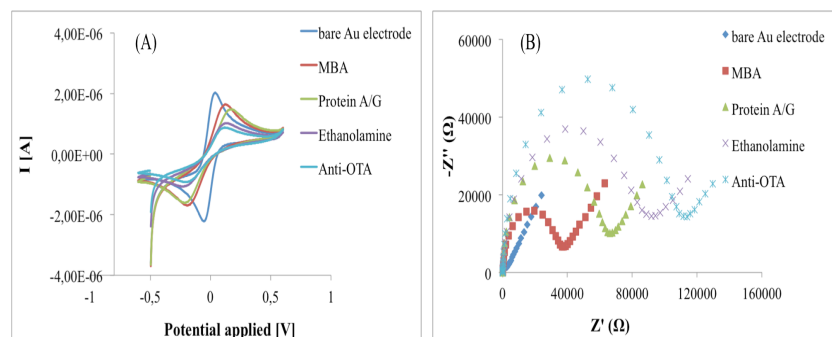
Figure 2.2: *(A) Cyclic voltammograms in 1 mM [Fe(CN)$_6$]$^{3-/4-}$ after each step of immunosensor construction; (B) EIS response to all immunosensor fabrication steps.*

The impedance data belonging to the all fabrication steps of the immunosensor, plotted in the form of Nyquist Plots, were shown in Figure 2.2 (B). The linear part of the plots, which correspond to lower frequencies, is related to a diffusion process while the semicircle diameter, at higher frequencies, corresponds to an electron transfer resistance. Because this last property depends on the dielectric and insulating features at the electrode/electrolyte interface, it can be used to describe the interface properties of the electrode (Prabhakar, Martharu & Malthotra, 2010).

As expected, when chemical species get covalently immobilized on the Au electrode surface, the impedance of the electrochemical system increases (Figure 2.2B); this could be ascribed to the blocking layer coating on electrode surface, which became thicker with the assembly procedure. In particular, as result of the immobilization on the electrode surface of MBA, protein A/G and the final addition of ethanolamine, the diffusion of the redox probe close to the Au modified surface was dramatically reduced. In fact, the permeability of [Fe(CN)$_6$]$^{4-/3-}$ through the immobilization layers was strongly reduced with a significant increase of the electron transfer resistance. A further increase of the impedance was observed when the anti – OTA is immobilized. The EIS and CV results revealed that the chemical and biomolecular layers acted as effective barriers to the charge transfers (Anandan, Gangadharan & Zhang 2009; Radi et al., 2009).

2.4.2 Optimization of anti – OTA concentration

Because the sensitivity of immunosensors depends on the immunochemical reactions of antigen with the antibody, the optimization of

the amount of anti – OTA on electrode surface is a crucial factor for the performances of the immunosensor in the same way as dilution of antibodies plays an important role in the preparation of ELISA plates. For this purpose, immunosensors were produced immobilizing four different amounts of anti – OTA on the modified electrodes. Figure IV.3 shows the Nyquist plots of oriented OTA immunosensors at different concentrations of anti – OTA (0.5 µg/mL (Figure 2.3A), 1 µg/mL (Figure 2.3B), 5 µg/mL (Figure 2.3C) and 10 µg/mL (Figure 2.3D) for OTA increasing amount.
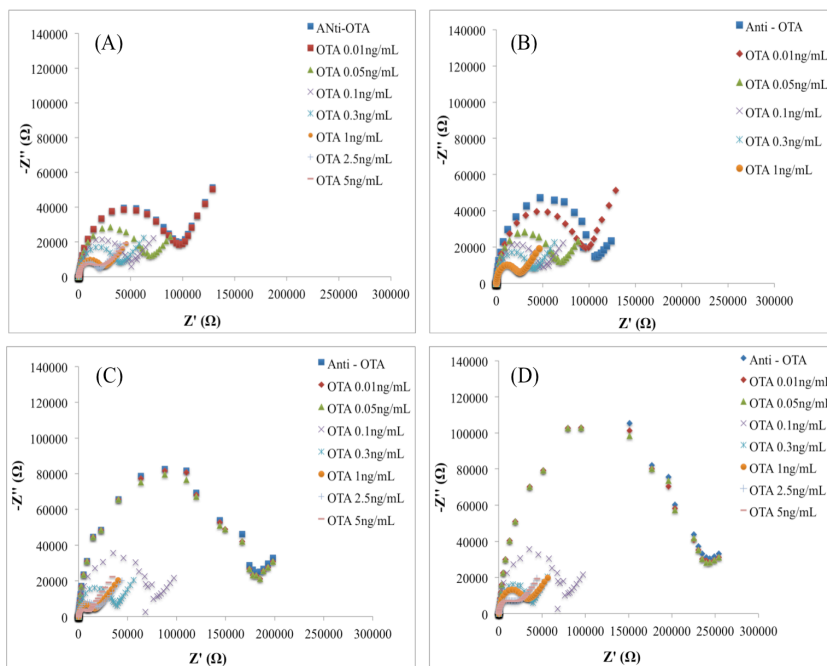
Figure 2.3: *Nyquist plots of immunosensors with anti – OTA concentration 0.5 µg/mL (A), 1 µg/mL (B), 5 µg/mL (C), 10 µg/mL (D). The Nyquist plots for each anti – OTA concentrations are the average of three different immunosensors.*

The Nyquist plots were fitted using Nova software by the equivalent circuit shown in Figure 2.4.
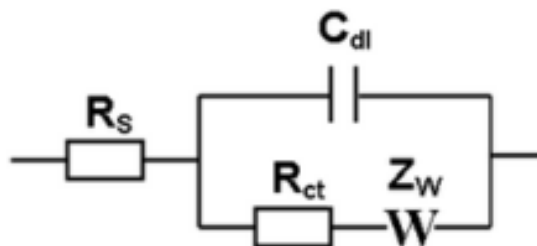
Figure 2.4: *Equivalent circuit used to fit impedance spectra.*

This equivalent circuit model, commonly applied for the impedimetric immunosensor characterization (Daniels & Pourmanda, 2007) consists of resistive and capacitive elements, as well as a Warburg element. In particular, R_s represents the resistance of the working solution while CPE (Constant Phase Element) is connected with the capacitance of the complex bioactive layer; R_{ct} is related to the electron transfer resistance through the electrode surface and the Warburg impedance describes the normal diffusion to the electron surface through the complex layer. In the given frequency range, the most significant changes were observed in the R_{ct} values, as shown in Table 2.1; in particular, it can be seen the R_{ct} value decrease by increasing OTA concentration for all the four different tested immunosensors.

Table 2.1: *Values of the equivalent circuit parameters for the OTA-binding of immunosensors fabricated at different anti-OTA concentrations. Data are the mean values of three different immunosensors.*

		R_s [Ω]	R_{ct} [Ω]	Q [μF/n]	W [Ω]
Anti-OTA 0.5 μg/mL	Anti – OTA	145±12	106±10.4	160.1±20.4	27.4±5.7
	OTA 0.01ng/mL	140±12.3	100±9.5	151.6±18.4	30.5±6.8
	OTA 0.05ng/mL	142±10.6	65±11.2	150.9±15.7	24.5±9.6
	OTA 0.1ng/mL	143±8.6	50±10.2	150.2±18.19	26.4±4.6
	OTA 0.3ng/mL	139±8.2	45±6.3	147.1±16.2	28±3.5
	OTA 1ng/mL	140±7.9	26±8.6	145.2±118.9	27.5±6.4
	OTA 2.5ng/mL	141±6.2	25.5±2.5	141.3±12.6	27.3±5.7
	OTA 5ng/mL	143±6.8	24±5.8	140.3±10.6	27.9±4.6
Anti-OTA 1 μg/mL	Anti – OTA	143±4.6	110.0±19.5	147.1±19.3	41.1±5.7
	OTA 0.01ng/mL	146±5.7	71.6±16.7	140.1±12.4	43.4±9.6
	OTA 0.05ng/mL	145±9.3	54.9±18.4	137.8±13.6	32.9±3.6
	OTA 0.1ng/mL	138±2.6	50±10.5	131.4±17.4	33.4±5.9
	OTA 0.3ng/mL	139±4.2	40.6±15.6	126.9±12.9	36.9±4.1
	OTA 1ng/mL	141±7.4	35.1±11.4	122.8±15.7	36.7±4.9
	OTA 2.5ng/mL	143±6.3	23.9±18.9	120.5±17.2	36.3±6.2
	OTA 5ng/mL	145±8.5	20.8±8.8	120.2±13.6	36.0±8.1
Anti-OTA 5 μg/mL	Anti – OTA	146±5.8	207±25.7	177.6±13.5	26±7.4
	OTA 0.01ng/mL	140±6.7	205±21.7	175.4±12.7	18.5±3.6
	OTA 0.05ng/mL	139±2.5	200±19.6	170.3±14.0	19.9±4.9
	OTA 0.1ng/mL	141±4.5	71.5±16.4	166.3±17.3	20.7±2.5
	OTA 0.3ng/mL	140±6.3	34.3±9.4	160.3±15.8	19.9±4.6
	OTA 1ng/mL	145±8.4	19.9±5.6	154.1±12.6	20.8±8.4
	OTA 2.5ng/mL	146±7.4	14.4±2.5	149.4±18.3	21±7.2
	OTA 5ng/mL	142±6.2	11.7±2.1	146.8±16.2	20.9±6.1
Anti-OTA 10 μg/mL	Anti – OTA	143±9.1	222±21.4	172.8±12.5	41.8±2.5
	OTA 0.01ng/mL	145±5.6	215±20.5	167.9±15.7	36.7±5.7
	OTA 0.05ng/mL	142±6.7	212±23.1	159.1±15.9	36.5±6.9
	OTA 0.1ng/mL	145±4.6	154±15.7	151.1±15.2	35.8±7.8
	OTA 0.3ng/mL	142±5.7	94.5±11.9	144.9±16.9	39.8±4.7
	OTA 1ng/mL	141±2.5	71±10.3	143.6±12.5	42.6±5.8
	OTA 2.5ng/mL	140±4.9	52.8±12.4	141.8±17.3	41.7±2.5
	OTA 5ng/mL	137±8.1	47.3±9.5	141.5±18.5	40.5±5.1

These results were in agreement with Muchindu et al., (2010) who developed an OTA immunosensor on Pt disk electrode modified with doped Polyaniline film. The decreasing of R_{ct} could be due to the formation of immunocomplex that causes differences in the dielectric or conductivity properties of the electrode surface (Darain, Park, Park & Shim, 2004).

As regards the differences among the impedance spectra (Figure 2.3) for the four immunosensors developed, it is possible to see that the immunosensor with 1 μg/mL anti – OTA showed remarkable decrease of the semicircle diameter and in R_{ct} for all the OTA amounts. For the

immunosensor at 0.5 μg/mL anti – OTA, OTA concentrations lower than 0.05 ng/mL did not bind sufficiently and thus the electron transfer resistance did not show the expected decrease.

Finally, immunosensor with 5 μg/mL and 10 μg/mL anti – OTA concentrations, because of high density of anti – OTA, showed higher impedances even if an evident decrease of R_{ct} was observed only when OTA was dropped at concentrations higher than 0.1 ng/mL. in this case the electrode surface is so dense that the binds between anti – OTA and OTA are insufficient to cause any charge transfer resistance decrease. Consequently the denser bioactive layer probably resulted in an activity loss in the immunosensor performance. Hence 1 μg/mL anti – OTA was chosen as the optimal concentration for the further characterization of the immunosensor.

2.4.3 Analytical characteristics of immunosensor

As discussed above the value of the equivalent circuit that showed the most remarkable differences at different OTA amount was the electron transfer resistance (R_{ct}). This parameter was then used to characterize the immunosensor fabricated with 1 μg/mL anti – OTA. The calibration curve was obtained by plotting the logarithmic value of OTA concentrations versus ΔR_{ct}. ΔR_{ct} was calculated by the following equation:

$$\Delta R_{ct} = R_{ct(anti-OTA)} - R_{ct(OTA)} \qquad (2.1)$$

where $R_{ct(anti-OTA)}$ is the value of the electron transfer resistance when anti – OTA is immobilized on the electrode surface and $R_{ct(OTA)}$ is the value of the electron transfer resistance after the bind between anti – OTA and OTA.

The calibration curve showed a linear correlation in the range from 10 pg/mL to 5 ng/mL, for values higher than 5 ng/mL, no linear ΔR_{ct} were observed probably due the saturation of the specific binding sites (Figure 2.5).
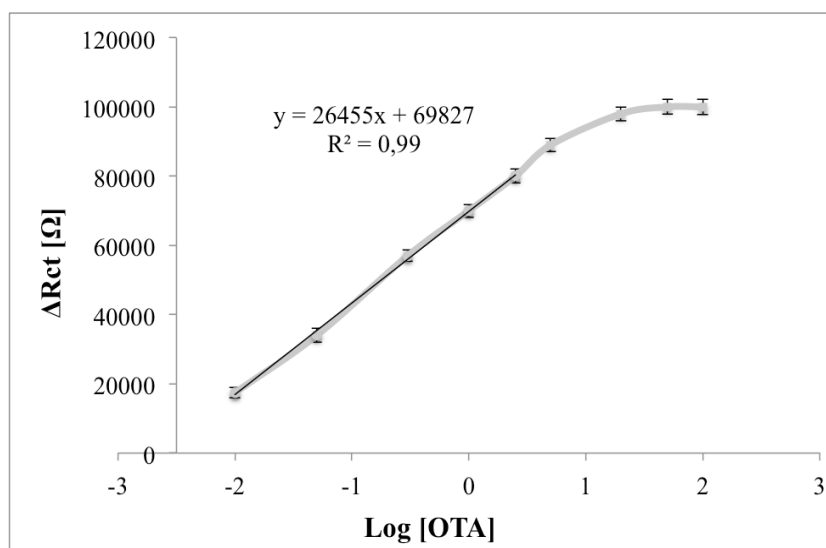
Figure 2.5: *Calibration curve of OTA immunosensor. The data represent
the average values of five immunosensors.*

The limit of detection (LOD), based on the sum of average blank solution
and three times the standard deviation, was estimated to be 0.01 ng/mL with
a response time of 30 min including the incubation time.

The reproducibility calculated on five different OTA immunosensors at
OTA 0.3 ng/mL showed a Relative Standard Deviation (RSD) of 5.6%.

The possible re – use of OTA immunosensors was also investigated. For
this purpose after the immunocomplex formation between anti – OTA and
OTA the electrode was dipped in 10:10:80 methanol:acetonitrile:water
solution for 40 min and the immunoassay, using the same OTA
concentration (0.05 ng/mL), was performed. The impedance spectra (Figure
IV.6) obtained after the binding anti – OTA and OTA for the immunosensor
pre-treated for OTA detachment showed a lower impedance highlighting a
decrease of anti – OTA immobilized on the electrode surface and thus a
change on the analytical performance.
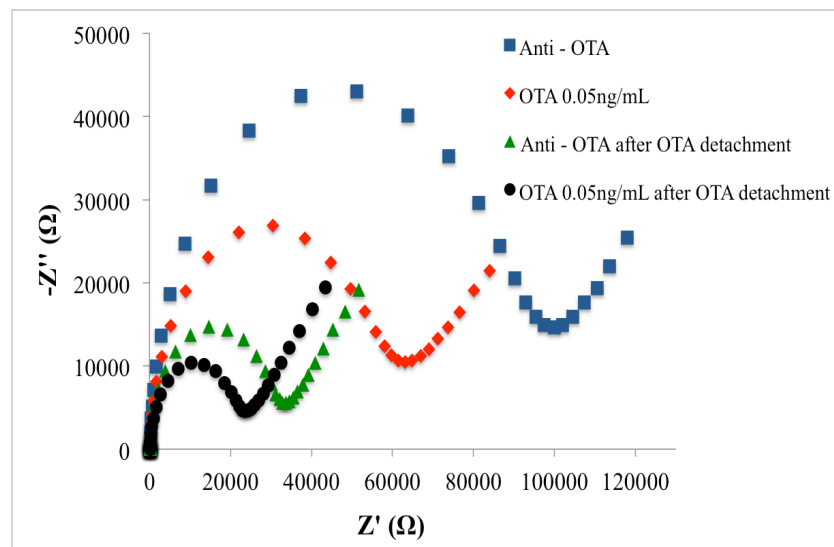
Figure 2.6*: EIS responses of immunosensor after and before the treatment in detachment solution.*

Finally the storage stability was also determined. For this purpose different immunosensors were stored for 21 days at 4 °C without chemical preservatives and characterized at regular interval times. After the investigated storage period the immunosensors showed a negligible loss of activity.

2.4.4 Oriented and not – oriented anti – OTA immobilization

The method of biomolecules immobilization on conductive surfaces plays a crucial role in the performance of an immunosensor. The method for the immobilization of antibodies used for immunoaffinity assays should orient the interacting sites (Fab) of antibodies towards the test solution with the antigen molecules. Protein A/G is an immunoglobulin (Ig)-bonding protein that shows specificity for the heavy chains on the Fc region of antibodies, thus it effectively orients the immobilized antibodies with antigen – binding sites facing outward. When Protein A/G is not used in the immobilization procedure the covalent bond between the activated SAM and the NH_2 groups of anti – OTA occurs in non – specific position. In this condition the probability of interaction between OTA and anti – OTA decreases (Fig.2.7A). In this regard, OTA immunosensors with 1 μg/mL anti – OTA were fabricated using the two immobilization methods and their analytical performances were compared (Fig.2.7B).
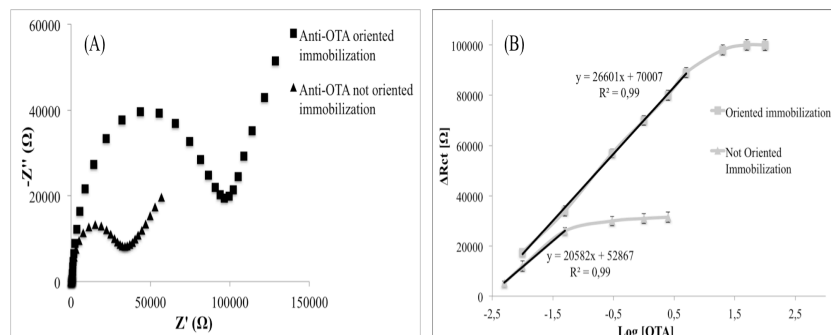
Figure 2.7: *Impedance spectra for oriented and not oriented anti-OTA immobilization (A); Calibration plots of oriented and not oriented OTA immunosensor (B).*

When the antibody immobilization occurred random, a lower charge transfer resistance was registered probably due to a lower number of antibodies immobilized on the surface of the electrode. The analytical characterization of the two immunosensors versus different concentration of OTA (Figure 2.7B) showed a closer linear range but a lower detection limit (5 pg/mL) for the not oriented immunosensor. This means that in case of not oriented antibodies a number of molecules, lower than in the oriented one, is effectively exposed to antigen interaction and that the receptor based biosensors decrease their LOD when the effectively immobilized receptor molecules are optimally minimized in order to still obtain a signal from the transducer (Cremisini, Di Sario, Mela, Pilloton & Palleschi, 1995). On the contrary, in the oriented immunosensor a wider linear range and a higher sensitivity were observed and thus a higher antigen – binding capacity (Kausaite – Minkstimiene, Ramanaviciene, Kirlyte & Ramanavicius, 2010).

The comparison of the analytical performance of the label free immunosensors developed in this study with the other impedimetric bioaffinity sensors (immuno and aptasensors) in the literature is reported in Table 2.2. The OTA oriented and not oriented immunosensors reported in this study showed a linear range and the lowest LOD when compared with the other immunosensors.

Table 2.2: *Comparison among impedimetric OTA label free biosensors*

Schematic biosensor assembly	Linear Range [ng/mL]	LOD [ng/mL]	Sensitivity [KΩ mL/ng]	References
SPCE/4-CP/OTA-Apt	$2 - 10$	2.00	1.12	Mishra et al. (2015)
SPCE/PTH/IrO$_2$NP$_s$-OTA-Apts	$4*10^3 - 4*10^7$	$5.6*10^3$	$2.48*10^6$	Rivas et al. (2015)
Au/4-CP/Ab	$2 - 10$	2.00	10.12	Radi et al. (2009)
Pt/PANI-PV-SO^{3-}/Ab	$1*10^{-2} - 5$	$1*10^{-2}$	0.56	Muchindu et al. (2010)
Au/MBA/Ab	$5*10^{-3} - 5*10^{-2}$	$5*10^{-3}$	20.33	Malvano et al. 2016
Au/MBA/protA-G/Ab	$1*10^{-2} - 5$	$1*10^{-2}$	26.45	Malvano et al. 2016

SPCE: screen printed carbon electrode; PTH: Polythionine; Apt:Aptamer;
PANI-PV-SO^{3-}: Polyaniline polyvinylsulfonate; 4-CP: 4-carboxyphenyl;
MBA: 4-mercaptobenzoic acid

2.4.5 AFM results

AFM is a very useful technique able to obtain information about the morphological characteristics of the resulting surfaces of the immunosensors.

AFM 3D - images of an oriented and a not oriented immunosensor surface are reported in Figure 2.8.
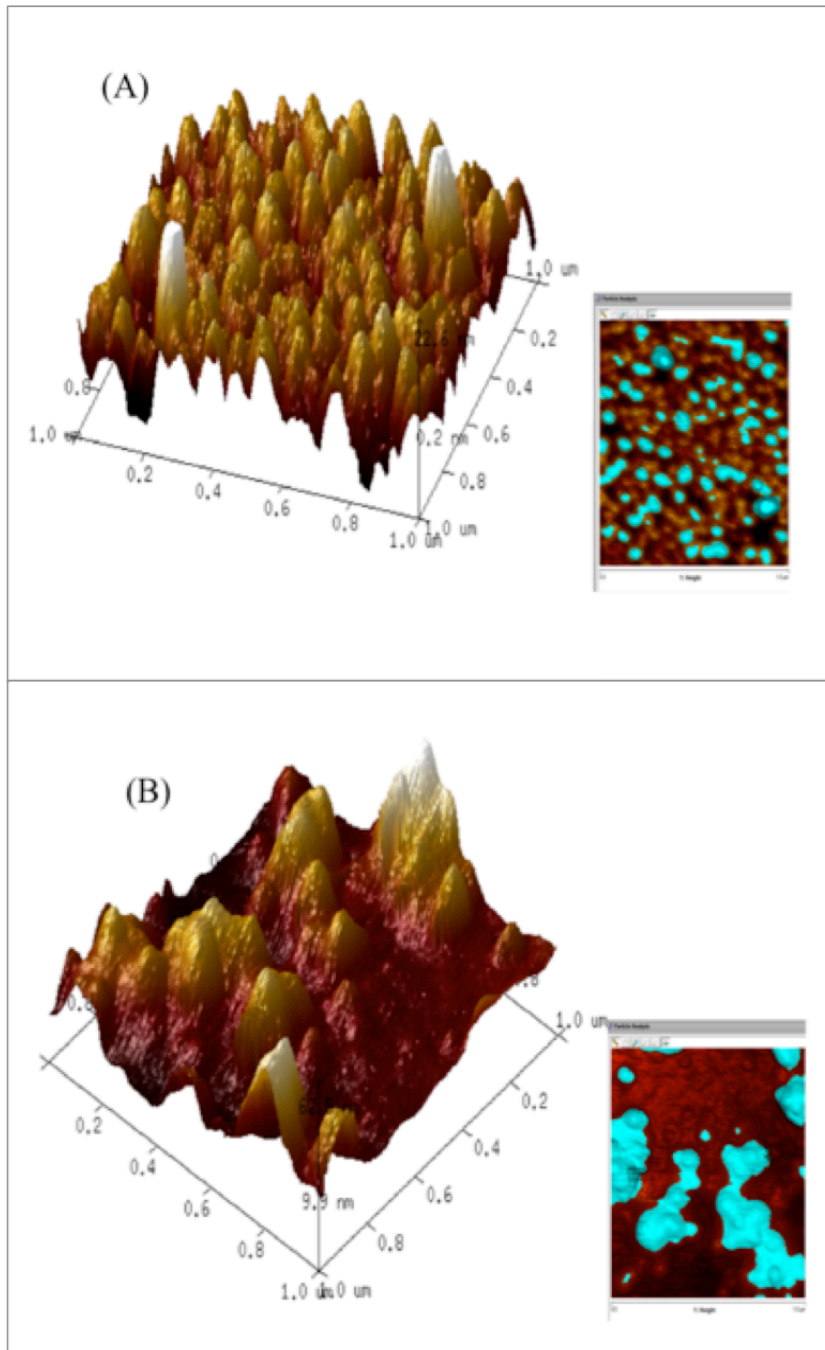
Figure 2.8: *AFM 3D – images of an oriented (A) and a not oriented (B)
immunosensor surface*

The surface of the oriented immunosensor (Figure 2.8A) shows a number of homogeneous spheres – like structures, which were expected from a densely packed protein monolayer. The surface of not oriented immunosensor (Figure 2.8B), instead, shows not well-distributed structures, with different sizes and heights.

AFM "roughness analysis" parameters were chosen as key descriptors of surfaces morphology: average roughness (R_a), which is the average deviation of the measured z – values from the mean plane, root – mean – square roughness (R_q), which effectively describes the standard deviation of an entire distribution of z – values and maximum roughness (R_{max}) which indicates the difference between the largest positive and negative z – values. The results of the roughness analysis (Table 2.3) showed all roughness value lower for oriented surface, pointing out that, in the case of oriented antibody, a more homogeneous surface is obtained.

Table 2.3: *Roughness parameters for oriented and not oriented immunosensor surface*

	Oriented	Not oriented
R_a [nm]	3.61	12.1
R_q [nm]	5.36	15.2
R_{max} [nm]	59.2	89.5

Moreover, by using the Particle Analysis Method the mean size (diameter) and the density of the nanostructures observed in a scanning size 1 μm^2 were calculated. The mean size of the oriented structures is found to be 57.22 nm, against the 121.64 nm of the not oriented structures. In AFM analysis, due to the tip broaden effect, the observed single spheres have larger size than those of a real protein molecule.

The real diameter of the structures (protein layer) can be calculated, according to Zhengjian et al. 2010, by the following formula:

$$D = 2\sqrt{2Rr + r^2} \tag{2.2}$$

where R is the tip radii, r is the real width of protein molecule and D is the apparent width of protein molecule, respectively. For a tip of 20 nm, the real size of protein structure in the oriented immunosensor was very close to the theoretical estimation of Ig equal to 14.2 nm (Zhengjian *et al.*, 2010). A higher real size (44nm) was calculated for not oriented immobilization probably due to the random covalent bond between activated SAM and the anti – OTA. Moreover the higher density value (66 particles/μm^2) observed for oriented immobilization in contrast to that calculated for not oriented one

(13 particles/μm^2) confirm the well known capability of Protein A/G to create an ordered a highly dense antibody surface.

2.4.6 Measurement of OTA in cocoa beans

The feasibility of applying the proposed immunosensor for the detection of OTA in food matrices was studied. We chose cocoa beans due to their complex composition that could interfere during the analysis and their very low level (maximum 2 $\mu g/Kg$) permitted by European standard.

Cocoa beans samples were spiked with four different concentrations of OTA and analysed by the developed immunosensors and competitive ELISA Kit for OTA detection. The sample preparation used was the same for both the analytical methods. The results are shown in Table 4 where good recovery percentage exhibited by the immunosensor is reported, calculating as follow:

$$Recovery \: [\%] = 100 - \left[\left(\frac{c_{spiked} - c_{found}}{c_{spiked}}\right) * 100\right] \qquad (2.3)$$

Taking into consideration the LOD of the anti – OTA immunosensor and the dilution factors used for the OTA detection in food matrices that can range from 5 to 50, the impedimetric immunosensors developed in this study show a detection limit of 0.05 and 0.5 $\mu g/kg$ respectively.

Table 2.4*: OTA results in spiked cocoa beans samples obtained by impedimetric immunosensors and ELISA kit.*

Spiked concentration [μg/kg]	Immunosensor		ELISA	
	Found concentration [μg/kg]	Recovery [%]	Found concentration [μg/kg]	Recovery [%]
1	1.04±0.04	104.00±5.03	1.13±0.05	113.50±4.95
1.5	1.044±0.05	96.00±0.03	1.57±0.05	104.69±7.00
2.5	2.41±0.03	96.40±4.51	2.69±0.09	107.60±5.66
5	5.46±0.30	109.30±6.10	5.13±0.12	102.68±2.40

According to Table 2.4, the sensitivity of the label free impedimetric immunosensor developed in this work is enough to allow the detection of OTA levels in cocoa beans established by EU that recommends a concentration up to 2 $\mu g/kg$.

2.5 Conclusions

In this study, a label free impedimetric immunosensor for sensitive detection of Ochratoxin A in food matrices (in chocolate samples) was developed and the electrochemical impedance spectroscopy was used to investigate it. The comparison between the two immobilization procedures (oriented and not oriented antibody) used for the fabrication of immunosensors underlines the advantages of the oriented immobilization, which showed a more uniform and homogenous antibody layer that favors higher antigen – binding capacity, and sensitivity of the immunosensor.

The results obtained with AFM analysis were in good agreement with those obtained from impedance characterization studies, underlining that in case of oriented antibody a more ordered surface guarantees a higher number of molecules effectively exposed to antigen interaction. Finally the linear range, the very low detection limit and high sensitivity showed the potential of the proposed immunosensor as a highly capable analytical device for a fast OTA measurement in food matrices.

2.6 Acknowledgments

2.7 References

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Impedimetric Label-Free Immunosensor on Disposable Modified Screen-Printed Electrodes for Ochratoxin A

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3.1 Abstract

An impedimetric label-free immunosensor on disposable screen-printed carbon electrodes (SPCE) for quantitative determination of Ochratoxin A (OTA) has been developed. After modification of the SPCE surface with gold nanoparticles (AuNPs), the anti-OTA was immobilized on the working electrode through a cysteamine layer. After each coating step, the modified surfaces were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The capacitance was chosen as the best parameter that describes the reproducible change in electrical properties of the electrode surface at different OTA concentrations and it was used to investigate the analytical parameters of the developed immunosensor. Under optimied conditions, the immunosensor showed a linear relationship between 0.3 and 20 ng/mL with a low detection limit of 0.25 ng/mL, making it suitable to control OTA content in many common food products. Lastly, the immunosensor was used to measure OTA in red wine samples and the results were compared with those registered with a competitive ELISA kit. The immunosensor was sensitive to OTA lower than 2 µg/kg, which represents the lower acceptable limit of OTA established by European legislation for common food products

3.2 Introduction

Mycotoxins are a large and varied group of mold-secondary metabolites with common features because they are all produced by fungi and have toxic effects against vertebrates and other organisms. Mycotoxins affect a broad range of agricultural products, including cereals, cereal-based foods, dried fruits, wine, milk, coffee beans, cocoa, or meat products, which are the basis of the economies of many developing countries [1]. Moreover, mycotoxins are presently considered as the most important chronic dietary risk factor, higher than food additives or pesticide residues [2].

Ochratoxin A (OTA) is one of the most abundant mycotoxins that contaminates food products; it is found in tissue and organs of animals, including human blood and breast milk, and is known to produce nephrotoxic, teratogenic, carcinogenic, and immune toxic activity in several animal species [3]. OTA affects humans mainly through consumption of improperly stored food products, causing cancer.

The International Agency of Research on Cancer (IARC) has classified OTA as a possible carcinogenic compound for humans since it causes immune suppression and immune toxicity [4].

From the mid-2000s, approximately 100 countries (covering 85% of the world's inhabitants) had specific regulations or detailed guidelines for the

occurrence of mycotoxins in food [2]. The European Union has established with the Regulation (EC) No 1881/2006, stating the acceptable limits for OTA in many products at high risk of contamination: OTA is allowed in very small concentrations (about 0.5–10 μg/Kg) depending on the kind of food [5]. These regulatory limits force all Member States to monitor and control mycotoxin levels in foodstuffs in order to reduce the intake of this toxic compound.

The methods most frequently used for OTA determination are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), coupled to fluorescence or mass spectrometry detectors [6]. The chromatographic techniques are highly sensitive and specific, but require well-equipped laboratory facilities, time-consuming pretreatment steps, and highly-trained personnel that strongly limit the routine implementation of chromatography-based approaches [7]. Immune assays can be used as cheaper and quicker alternatives to chromatographic methods for mycotoxin detection. Competitive enzyme-linked immune sorbent assay (ELISA) is the most common commercial immune assay used for OTA detection in the food sector based on spectrophotometric reading, however, it suffers the drawbacks of time-consuming (for example, 50 min for I'screen Ochra Elisa kit (Tecna-Trieste,Italy); 90 min for OTA ELISA, (Abraxis LLC-Warminster, PA, USA), and the narrow dynamic range up to 16 ng/mL.

In recent years there has been a strong interest in the development of immunosensors based on the antigen-antibody interaction, but novel specific ligands (e.g., aptamers) are emerging.

Among all of the possible immunosensors (electrochemical, optical, microgravimetric) the electrochemical ones rank highly owing to their sensitivity, low cost, simplicity and, in some cases, miniaturization, portability, and integration in automated devices [8].

In the literature are reported two different types of electrochemical OTA immunosensors: label- and label-free-based. For the labelled immunosensors, the interaction between OTA and anti-OTA, based on direct and/or indirect competitive immunoassay, is detected by the use of enzymes exploiting the classical electrochemical techniques, such as amperometric, potentiometric, and conductimetric methods [9-13].

This immunosensor exhibits a detection limit ranging from 0.008 [12] to 0.12 [10] ng/mL with a dynamic range up to 250 ng·mL [13].

The application of electrochemical impedance spectroscopy (EIS), as a transduction technology, enables the label-free detection and quantification of the immune complex and, thus, for the development of biosensors for food hazards [14,15]. EIS is a powerful informative and non-destructive technique due to the small voltage excitation used during detection, which can be used to study the electrical properties of the sensing device interface and tracing the reactions occurring on it [16,17].

Different impedimetric label-free OTA affinity biosensors have been described previously by immobilization of monoclonal antibody and aptamers on the surface of gold or platinum electrodes [18-21]. This impedimetric immunosensor exhibits detection limit ranging from 0.01 [19] to 2 [20] ng/mL, with and a dynamic range up to 25 ng/mL [21].

In recent years the applications of disposable screen-printed carbon electrodes (SPCEs), characterized by low-cost fabrication and mass production, have attracted an increasing interest for the development of labelled immunosensors (especially enzyme immunosensors) but it is noteworthy that few studies [22,23] on electrochemical label-free immunosensors integrated onto SPCEs have been developed.

Exploiting the advantages of gold nanoparticles (AuNPs), which have been extensively used as matrices for the immobilization of macromolecules, such as proteins, enzymes, and antibodies, in addition to providing a microenvironment similar to what obtained under physiological conditions [24], it is possible to design a new electrochemical sensor by the modification of a working electrode surface. Moreover, AuNPs have attracted considerable attention in electroanalysis because of their excellent physical and chemical properties, such as high surface to volume ratio, good electrical properties, strong adsorption ability, and good surface properties. Electrodeposition of metallic nanoparticles on an electrode surface is a better process than deposition from solution, as the former is comparatively easier, faster, and generates a more stable surface. In addition, electrochemical deposition allows Au(III) to be addressed only on a polarised working electrode as Au(0) not on auxiliary or reference electrodes. Compared to gold electrodes, the electrodeposition of metallic nanoparticles or aggregated clusters (simply abbreviated as nanoparticles in this paper) on SPEs is more advantageous because of the unique properties offered by the metallic nanoparticles as described earlier [25]. Moreover the gold deposition on the electrode avoids poisoning and cross-contamination, which interferes with the analysis. These problems are absent in disposable SPCEs. Consequently, the combination of screen-printing and electrodeposition of metallic nanoparticles is a very promising technique for the mass production of electrochemical sensors with enhanced sensitivity [25].

For this reason, the aim of this work is the development of a label-free impedimetric immunosensor for OTA detection, realized on an AuNP-modified SPCE. EIS and CV were used to characterize each step of electrode modification and the analytical performances of the immunosensors developed. Finally the possibility to use a fast and cheap disposable biosensor like that proposed in this study could represent a key factor for the monitoring of mycotoxins in food products.

3.3 Materials and methods

3.3.1 Chemicals

Glutaraldehyde solution ($C_5H_8O_2$, 50 wt % in H20), cysteamine (C_2H_7NS, ≤98%), gold (III) chloride hydrate ($HAuCl_4$, 99.9%), sulfuric Acid (H_2SO_4, 99.9%), ethanolamine ($NH_2CH_2CH_2OH$, >99.5%), potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), and Ochratoxin A were purchased from Sigma-Aldrich (Milano, Italy). Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$) was obtained from Carlo Erba reagent (Milano, Italy). Anti-Ochratoxin A antibody (Anti OTA, 1 mg/mL) was purchased from Abcam (Cambridge, UK), NaH_2PO_4, Na_2HPO_4, NaCl, and KCl used in the preparation of phosphate-buffered saline (PBS: 0.1 M KCl, pH 7.4) were also obtained from Sigma Aldrich (Milano, Italy). I'screen Ochra ELISA kit for the detection of Ochratoxin A was purchased from Tecna (Italy).

3.3.2 Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat (Metrohm), equipped with an impedance module (FRA32M) and the experimental data were analysed with Nova software (Metrohm). Screen-printed carbon electrodes (SPCEs), based on a three-electrode layout (working/auxiliary/reference, Figure 3.1), were produced in three screen-printing steps as described in Albanese et al [26]. Specifically, a first layer of a carbon/graphite ink (G-Went, Pontypool-UK) was deposed to define the conducting tracks, the working and auxiliary electrodes. The second was a silver/silver chloride ink (Acheson Colloiden B.V., Scheemda, NL), used as a pseudo-reference electrode. The third layer consisted in an insulating ink (G-Went, Pontypool-UK). Between the first and the second screen-printing steps, the strips were cured at 80 °C for 25 min to dry off residual solvents and cure the patterned pastes after every step of screen-printing. The diameter of the working electrode was 2.8 mm. Scanning electron microscopy (SEM) images were obtained on a Raith Turnkey 150 SEM.

3.3.3 Immunosensor Manufacturing

3.3.3.1 Preparation of Gold – Modified SPCEs

The gold deposition on the homemade SPCEs was conducted after an electrochemical treatment at 1.7 V vs. Ag/AgCl as a reference electrode for 360 s in PBS.

The electrochemical AuNP deposition was carried out using a solution of 1 M $HAuCl_4$ in 0.5 M H_2SO_4 under a constant potential of −0.4 V vs. Ag/AgCl in a range of 50–400 s.

3.3.3.2 Electrochemical Deposed Multilayer (EDM)

EDM was employed to create thiol layers attached onto AuNPs. Cysteamine 20 mM was dropped onto the AuNPs modified working electrode and a constant potential of 1.2 V vs. Ag/AgCl for 20 min was applied. After the electrode was thoroughly rinsed with water, to remove physically-adsorbed cysteamine, 100 μL of glutaraldehyde solution 12% (v/v) were dropped onto the modified working electrode for 1 h and then, again, rinsed with water.

3.3.3.3 Antibody immobilization

Different concentrations of anti-OTA solution (1 μg/mL, 5 μg/mL, 10 μg/mL) were dropped onto the modified electrode for 30 min at room temperature, then the electrode was rinsed in PBS to remove unbound antibodies. After the immobilization step, ethanolamine 1 M (pH 8.5) for 15 min was used to block unreacted active sites. The schematic diagram of immunosensor fabrication is shown in Figure 3.1B.
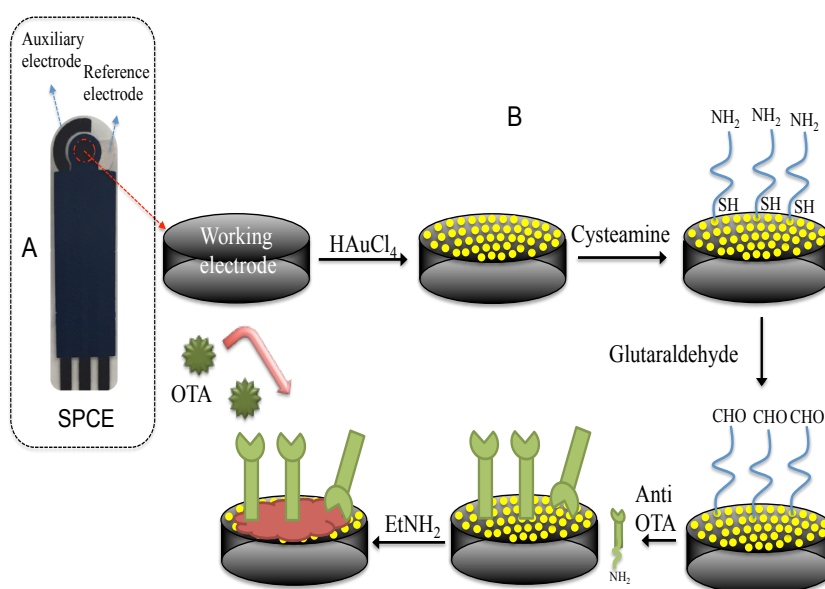
Figure 3.1. (*A) Screen-printed carbon electrode layout; and (B) steps used for the fabrication of the immunosensor.*

3.3.4 Experimental Measurement

EIS and CV measurements were used to characterize each step of the electrode modification.

For the impedance measurements, a sinusoidal AC potential (10 mV) in the frequency range from 0.1 to 10^4 Hz was superimposed to 0.00 mV (vs. reference electrode) DC potential. The impedance spectra were plotted in the form of Nyquist plots, where the complex impedance is displayed as the sum of the real and imaginary components (Z' and Z'' respectively), and in the form of Bode diagram where the total impedance of the system (Z) is plotted versus frequency. All measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-}/^{3-}$, 1:1) in PBS, pH 7.5, as background electrolyte at room temperature.

The voltammetric measurements were performed from −0.6 to 0.6 V vs. Ag/AgCl with a scan rate of 0.05 V/s; the redox couple used for the CV was the same as that used for impedance measurements.

For the OTA analysis, 20 μL of OTA at different concentrations in PBS were dropped onto the working area of the immunosensor and incubated for 20 min. Before the impedance measurements, the immunosensor was rinsed thoroughly with copious amounts of PBS.

3.3.5 Preparation of Wine Samples for OTA Measurement

The preparation of red wine samples was conducted according to the procedure described in the I'screen Ochra ELISA kit. 5 mL of wine was added to 5 mL of 1 M HCl and 10 mL of dichloromethane; the solution was shaken in a low-speed shaker (400 rpm) for 15 min and centrifuged at 2200 rpm for 15 min. Five millilitres of solvent phase was added to 2.5 mL of the 0.13 M sodium bicarbonate solution; the solution was shaken for 30 s. The upper aqueous phase was recovered and centrifuged to remove solvent traces and finally diluted two times with sodium bicarbonate solution.

This procedure was replicated for the three different OTA (1.5 ng/mL, 5 ng/mL, and 10 ng/mL) spiked wine samples. The OTA results obtained by the immunosensors were compared with those measured with the competitive ELISA kit for OTA.

3.4 Results

3.4.1 Characterization of the Electrode Modifying Process

The electrodeposition of gold nanoparticles (AuNPs) on the carbon electrode surface was strongly affected by the electrodeposition times, which have been optimized to obtain the best analytical performances in our device. As Figure 3.2 shows, an increase of the electrodeposition time from 50 to 300 s leads to significant current increase, while an effect that plateaued at a longer deposition time is observed (inset Figure 3.2). Therefore, an electrodeposition time of 300 s was selected as optimum.
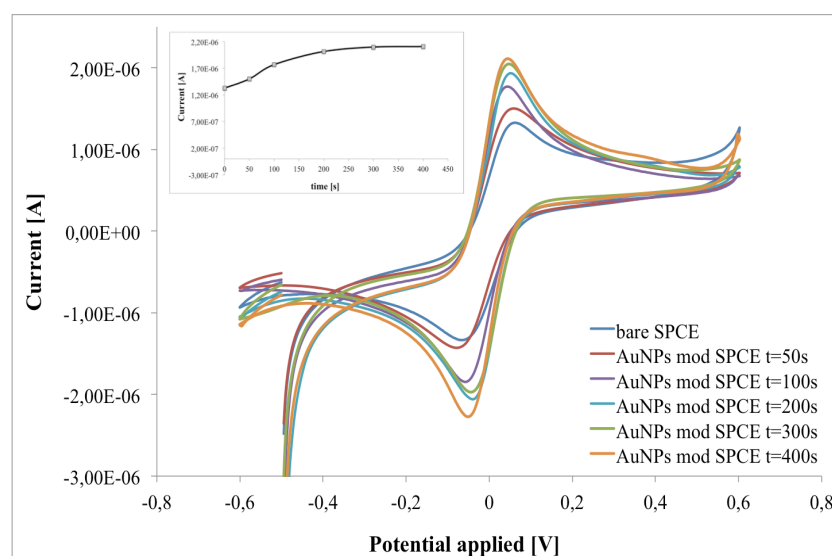
Figure 3.2*: CV of bare and AuNP-modified SPCE at different deposition time. Inset: current intensity of the anodic peak at different deposition times, in 1 mM ferri/ferrocyanide redox couple [Fe(CN)$_6$]$^{4-/3-}$, in PB, pH 6.8.*

As expected, the working electrode modified with AuNPs exhibited the characteristic increase of the anodic and cathodic peaks, thus confirming the successful modification process. Moreover increasing the electrodeposition time for 50 to 300 s, larger peak current and a smaller peak – to – peak potential separation (ΔE) were observed. In particular, the optimized AuNPs deposition time increased the electrochemical performance if the electrode with anodic current of 2.1 μA and ΔE=91 mV for AuNPs/SPCE, in contrast to 1.3 μA and ΔE=135 mV for SPCE (Figure 3.2). This behaviour was attributed to the enhanced electrochemical activity of the AuNPs, which

allowed the increase of the electrode active area and shifted the peak potential near to 0 V giving rise to a smaller peak – to – peak separation.

This fact suggests a slight improvement in the electrocatalytic properties of the electrode produced by the addition of the AuNPs, which facilitated the electron – transfer process [27].

SEM images (Figure 3.3) of the carbon-working electrode before and after the gold electrodeposition had been carried out, in order to verify the presence of the AuNPs.
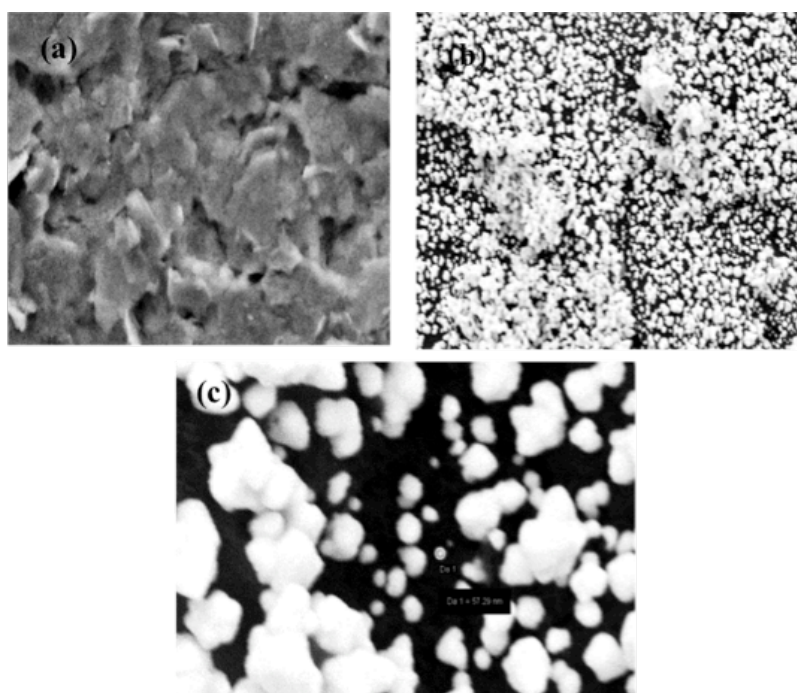
Figure 3.3*: SEM surface images of (a) a bare carbon electrode; and (b,c) a carbon electrode after gold Electrodeposition*

As expected, the surface of the bare carbon electrode shows the typical flake – shaped graphite particles along with large cavities. When the electrode is biased to deposit (reduce) the gold, the tips, the reliefs and depression of the SPCE surface suffer from a heterogeneous distribution of electric charge that promotes the formation of a heterogeneous dimension of Au particles, besides considering that a high tima of deposition favours the interfusion of NPs in larger gold ones so the nano – structure was destroyed. Cluster agglomeration of AuNPs and single NPs are evident in Figure 3.3. Moreover the quantity of deposed gold on carbon printed surface (122 pg per electrode) has been calculated from the deposition current (0.6 μA) and time (300 s), considering the Faraday constant = 96485.34 C/mol, the number of electrons (3) involved in the reduction of Au(III) to Au(0), and the atomic

weight of Au (196.96 D). AuNPs deposed on SPCE resulted in a cheap gold surface since it was obtained with a smaller quantity of gold than the expensive commercial solid gold electrodes. Experimental complex plane impedance spectra for the bare SPCE and SPCE – AuNPs are shown in Figure V.4. The almost linear and close – to – vertical spectra, observed for both electrodes, is caused by a faster mass – transfer limited process due to electron flow from the electrode surface in the bulk solution. This behaviour indicates a purely capacitive response of the electrode properties [28]. When the AuNPs are deposed, a decrease of curve slope was observed.
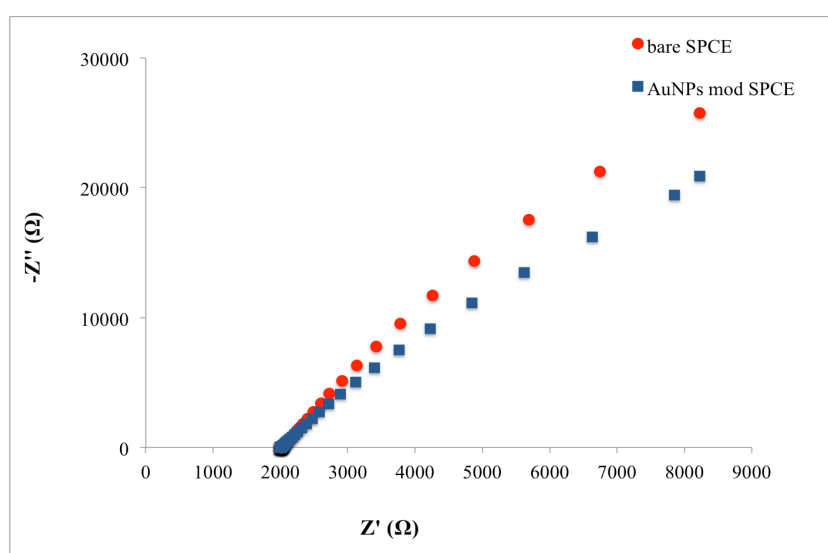
Figure 3.4*: Nyquist plots in impedance measurements of bare and AuNP-modified SPCEs*

In order to characterize the behaviour of the sensing layers, EIS and CV measurements have been carried out during each step of the immunosensor construction (Figure 3.5 and Figure 3.6).

The cysteamine layers, as well as the consecutive immobilization of anti – OTA and ethanolamine caused a significant decrease of the electron transfer rate, measured by CV, with a lowering of both anodic and cathodic peaks due to hindering effects of the layers [29,30].
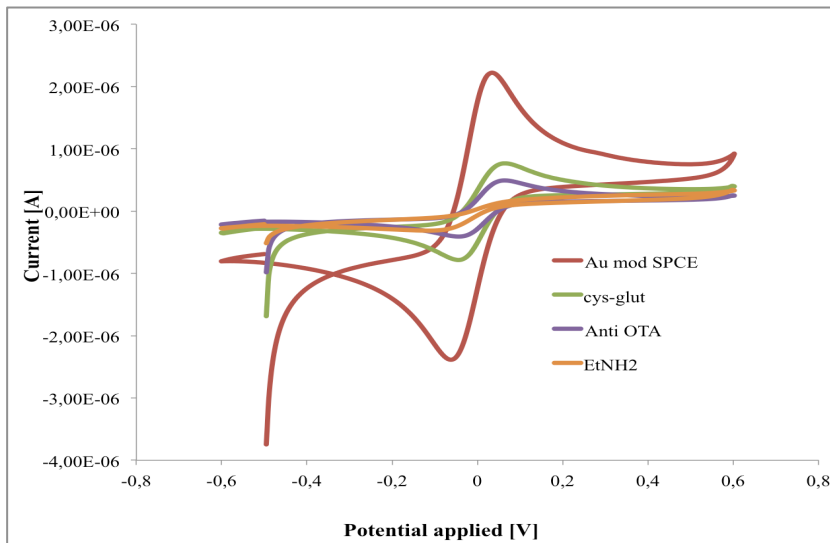
Figure 3.5*: Cyclic voltammograms recorded in 1 mM [Fe(CN)6]4-/3-, in
PB pH 6.8, during the fabrication of the immunosensor.*
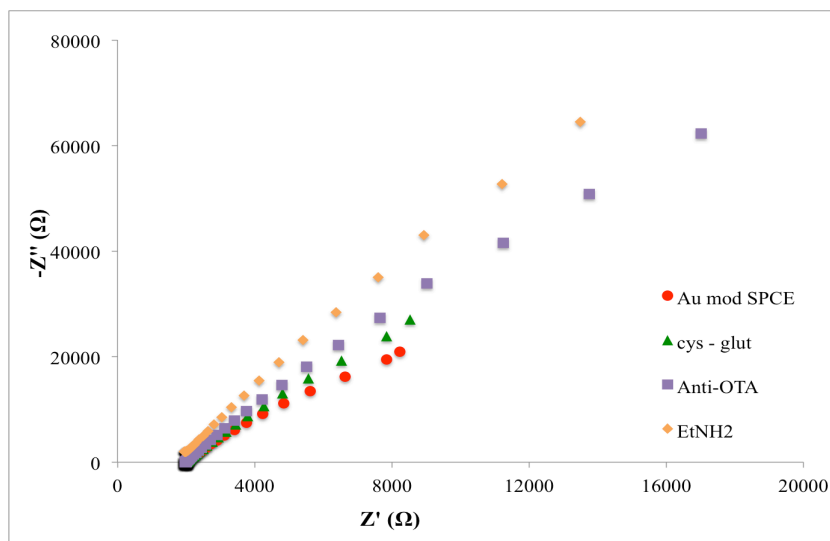
Figure 3.6*: Nyquist plots in impedance measurements during the fabrication
of the immunosensor*

Impedance spectra, during the immunosensor fabrication, represented as
the change of the impedance components (Z' resistive component, Z''
capacitive component), as a function of frequency, were shown in Figure
3.6. The increase of both Z components is due to a change in the electron

transfer resistance caused by the bio composite layer on the surface of the electrode that also induces a capacitance decrease because of the increased distance in the plate separation between the surface of the electrode and the electrolyte solution. Moreover it is evident that a significant change of impedance components occurs only at low frequencies. For a better description of the change caused by the immobilization steps on the impedance properties of the immunosensor, the Bode plot (total impedance in function of frequency) have been reported (Figure 3.7). While no differences were shown at the higher frequency region (inset Figure 3.7) significant total impedance changes were shown from 0.1 to 1 Hz.
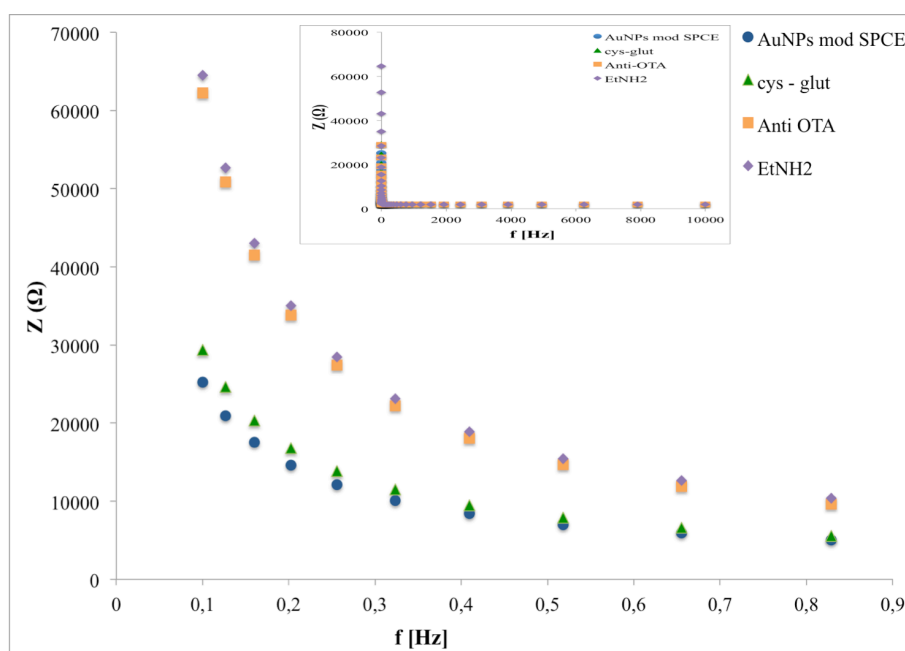
Figure 3.7*: Bode plots in impedance measurements after all immunosensor fabrication steps in the frequency range 0.1 – 1 Hz. The inset shows Bode plots in the frequency range 0.1 – 10000 Hz*

In this range no significant differences were observed when cysteamine layer was attached to AuNPs, in contrast to the immobilization of anti – OTA molecules, which gives rise to a substantial total impedance increase. No changes in impedance value were observed after the blocking of active sites with $EtNH_2$.

The Nyquist plots of the developed immunosensors after the incubation with three different OTA concentrations are reports in Figure 3.14. In the given frequency range, the binding of OTA with anti – OTA affect the sensor impedance signal; in particular, we observe a decrease in the capacitive component (-Z'') of total impedance at low frequencies.

According to other studies [31-34] on impedimetric immunosensors, the making of the immunocomplex induces a capacitance decrease, which can be directly related to the amount of analyte to be quantified.
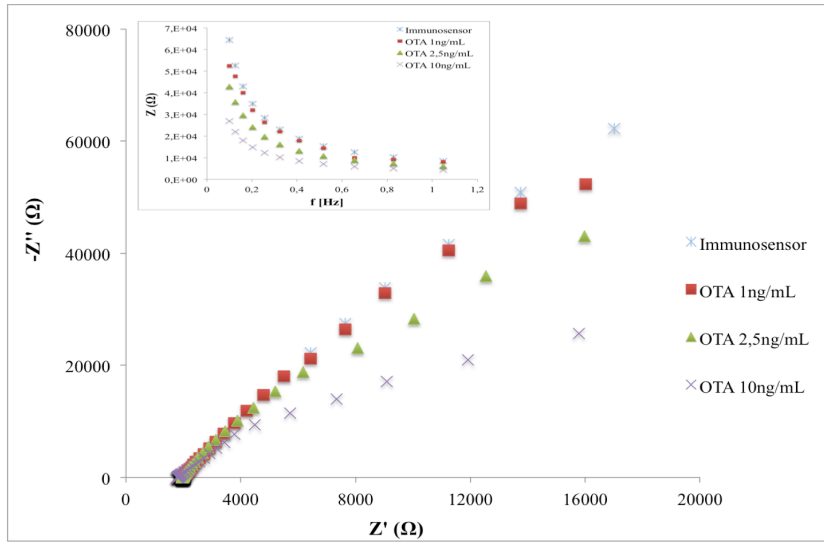
Figure 3.8: *Nyquist plot in impedance measurements of the immunosensor before and after the interaction with different OTA concentrations.*

As shown in the inset of Figure 3.8, the maximum differences among the Bode plots corresponding to different OTA amounts were observed at 0.1 Hz. The latter was chosen as the operating frequency for all impedance measurements during the analytical performances of the immunosensor.

3.4.2 Optimization of Anti – OTA Concentration

The influence of the antibody concentration on the immunosensor analytical performance was investigated. For this reason, immunosensors were developed by the immobilization of three different amounts of anti – OTA (1 μg/mL, 5 μg/mL, 10 μg/mL) and the capacitance (C) was measured for OTA from 0.3 ng/mL to 40 ng/mL, after an incubation time of 20 min. the capacitance of the system was calculated according to Yang et al. [33], using the following equation:

$$C = -\frac{1}{2\pi f Z''} \tag{3.1}$$

where f is the operating frequency (Hz), and Z'' is the capacitive component of the total impedance.

Throughout the whole study, the change in capacitance (denoted as ΔC),
taken as a measure before and after the immunoreaction, is calculated by the
following equation:

$$\Delta C = C_{antiOTA-OTA} - C_{antiOTA} \tag{3.2}$$

where $C_{antiOTA-OTA}$ he value of the capacitance after OTA coupling to
the anti – OTA and C_{OTA} represents the value of the capacitance of the native
immunosensor.

For the immunosensor with 1 μg/mL anti – OTA no significant changes
in capacitance was measured before and after the immunocomplex in the
range of OTA investigated. The calibration curves of the OTA
immunosensors with 5 μg/mL and 10 μg/mL, obtained by plotting the
logarithmic value of OTA concentrations versus ΔC, are shown in Figure
3.9.

As reported in our previous study [29], lower antibodies amounts allow
detecting lower OTA concentrations; in particular, for 5 μg/mL anti – OTA,
the immunosensor shows a significant ΔC in the range from -0.52 to 1.30 log
OTA (0.3 to 20 ng/mL), while ΔC changes only for values higher than o.69
log OTA (5 ng/mL) with 10 μg/mL anti-OTA. Moreover, higher antibody
amounts allow obtaining higher sensitivity and a higher capacitance signal
due to the higher antigen – binding capacity.
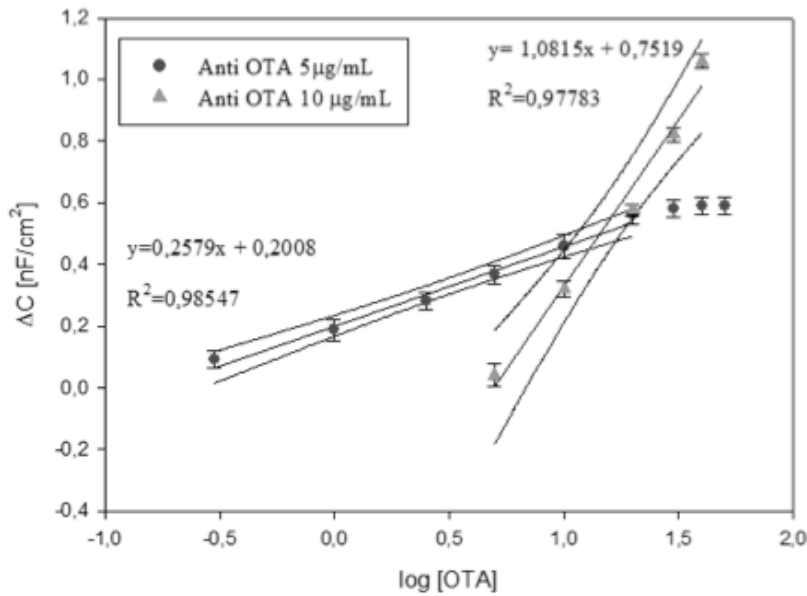
Figure 3.9*: Calibration curves of OTA immunosensors at 5 μg/mL and
10 μg/mL anti – OTA. Data represent the average values of five
immunosensors with error bars and 95% confidence curves. The inset shows
the measured capacitance in the range of OTA investigated for the
immunosensors with 5 μg/mL and 10 μg/mL anti – OTA.*

The detection limit (LOD), calculated using the sum of average blank
solution and three times the standard deviation, was 0.37 ng/mL and 5.42
ng/mL for immunosensor with 5 μg/mL and 10 μg/mL, respectively.

The comparison of the analytical performance of the label – free
immunosensors on screen – printed AuNP – modified carbon electrodes
developed on this study with the other impedimetric immunosensor reported
in the literature is reported in Table 3.1.

Since no previous studies have been published on the label – free
immunosensors on screen – printed carbon electrodes which shows
capacitive behaviour, we have compared the results with resistive
immunosensors developed on Au and Pt eelctrodes. In Table 3.1 the
sensitivity of our immunosensor has been calculated using change in total
impedance as a function of different OTA amounts.

Table 3.1: *Comparison among impedimetric OTA label – free biosensors*

Schematic Immunosensor Assembly	Sensitivity [kΩmL/ng]	Linear Range [ng/mL]	LOD [ng/mL]	Sensitivity x LOD	References
Pt/PANI-PV-SO^{3-}/Ab	0.56	2-10	2.00	1.12	[20]
Au/TA/GA/BSA/Ab-MNP	6.50	0.05-1	0.01	0.06	[19]
Au/4-CP/Ab	20.25	1 -20	0.50	10.12	[18]
Au/MBA/ProtA-G/Ab	14.03	0.01-5	0.01	0.14	[29]
Au/MBA/Ab	377.78	0.005 -0.05	0.005	1.89	[29]
SPCE/AuNPs/Cys.Glut/ Ab(5μg/mL)	2.56	0.3-20	0.37	0.64	This work
SPCE/AuNPs/Cys.Glut/ Ab(10μg/mL)	3.09	5-40	5.42	7.72	This work

PANI-PV-SO3_: Polyaniline–polyvinylsulfonate; TA: Thiolamine SAM; GA: Glutaraldehyde; MNP: Magnetic
Nanoparticles. 4-CP: 4-Carboxyphenyl; MPA: 4 Mercaptobenzoic acid; Ab: OTA Monoclonal Antibody

The analytical parameters obtained by out label – free impedimetric immunosensors are suitable for the analytical determination of OTA in the range of interest for food matrices. In addition, considering that the electrodes used for the current fabrication of impedimetric immunosensors are pure gold electrodes (e.g., thin – film gold electrodes require 2270 pg of gold) our impedimetric immunosensors are cheaper, because of the use of disposable SPCE modified with a very small quantity of gold (calculated to be 122 pg of gold per electrode).

The reproducibility calculated on five different immunosensors showed a good relative standard deviation (RSD) for both immunosensors: 5.18% for 5 μg/mL anti – OTA and 2.5 ng/mL OTA, 5.69% for 10 μg/mL anti – OTA and 20 ng/mL OTA.

The feasibility of applying the proposed immunosensor for the detection of OTA in wine was studied. Since the maximum OTA concentration permitted by European Commission (EC) No 1881/2006 is 2μg/kg we have tasted the immunosensot at 5 μg/mL anti – OTA. Finally, the storage stability was also determined. For this purpose, different immunosensots were stored for three weeks at 4 °C without chemical preservatives and characterized at regular interval times. After the investigated storage period, the immunosensors showed a negligible loss of activity.

Red wine samples were spiked with three different concentrations of OTA, and analysed by the developed immunosensors and a competitive ELISA kit for OTA detection. The sample preparation used was the same for both the analytical methods. The result, reported in Table 3.2, show a comparable performance for both methods and, thus, the capability of the immunosensor is a fast analytical technique for the control of OTA in food samples.

Table 3.2*: OTA results in red wine samples obtained by ELISA and the developed impedimetric immunosensor*

	Spiked Concentration [μg/mL]	Found Concentration [μg/mL]	Recovery [%]
ELISA	1.50	1.49	99.77
	5.00	6.12	122.45
	10.00	9.90	98.96
Impedimetric Immunosensor	1.50	1.36	94.56
	5.00	4.99	99.79
	10.00	10.29	102.91

3.5 Conclusions

The first immunosensor for OTA detection based on EIS with a modified SPCE is reported. The surface of ca arbon electrode was modified with electrochemical gold deposition, which has demonstred a very cheap way to obtain gold-like behaving electrodes using a very small quantity of the metal (122 pg per electrode). Thus, a label-free impedimetric immunosensor for Ochratoxin A detection was developed on Au modified SPCE and EIS was used to analyze the analytical immunosensor performance. Capacitance was chosen as the best parameter that describes the electrical changes of the electrode surface due to the immunoreaction between anti-OTA and OTA at different concentrations. The developed immunosensor, with its very low detection limit and high sensitivity, exploits the advantages of cheapness, simplicity, and versatility of the SPCE and its results are suitable for fast OTA measurement in food matrices.

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A new label – free impedimetric aptasensor for gluten detection

A new label-free impedimetric aptasensor for gluten detection

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4.1 Abstract

Celiac disease is a serious autoimmune disorder caused by the ingestion of gluten. Gliadin is the gluten fraction responsible for the triggering of disease. The only cure for the celiac patients, known until now, is a diet with gluten-free foods, classified by the European regulation doesn't exceed 20 ppm in gluten. With the aim to guarantee the food safety for celiac patients in this study the developing and optimization of a fast and reliable label free impedimetric aptasensor for gliadin detection is reported. The aptamer (Gli1) at 0.5 mmol and poly (amidoamine) dendrimer of fourth generation (PAMAM G4) at 2mg/ml were chosen during the developing steps of the sensing platform because the best ones for the detection of low gluten concentrations with the highest sensitivity. The aptasensor showed linearity in the range of 5-50 mg/l and 50-1000mg/l in gliadin, a limit of detection of 5 mg/l corresponding to 5 ppm of gluten, a reproducibility lower than 5% and a storage stability at 4°C of two months. Finally the aptasensor was used to measure gluten, in gluten and gluten-free food products, showing a good agreement with the results obtained with official R5 ELISA method.

4.2 Introduction

Gluten is a mixture of gliadin, an allergenic protein family responsible for the autoimmune enteropathy generated by Celiac disease, one of the most common chronic digestive disorders defined as a 'small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals' (Ludvigsson et al., 2013). After the diagnosis the only effective safe therapy for celiac disease is based on a rigorous and permanent diet that excludes sources of gluten (wheat, rye, barley) and any foods made with these grains.

Maintaining a true gluten-free diet isn't very simple, because gluten can occurs from cross- contamination by hypothetic gluten free raw material or flavour enhancer, thickener, emulsifier, filler and fortification ingredient (Hüttner & Arendt, 2010) posing potential risks for the most sensitive celiac patients.

The main methods for the detection of gluten in foods are based on directly targeting the gliadin (allergenic proteins in the gluten) or its peptide fragments. The detection can occur by isoelectric focusing (IEF), A-PAGE, SDS-PAGE, reversed-phase (RP)-HPLC, size-exclusion HPLC (SE-HPLC), high-performance resolution capillary electrophoresis (HPCE), the combination of HPLC with electrospray ionization (ESI), tandem mass spectrometry detection (LC–MS/MS) and enzyme-linked immunosorbent assay (ELISA). This latter is the currently accepted method for gluten

determination in native and processed foods. However, these gliadin analysis methods are time intensive, expensive and require trained operators

Availability of fast, cheap but sensitive methods for gluten detection are necessary for an effective gluten-free products labelling and thus protecting celiac people from the unaware content of gluten in food higher than the official limit (20 ppm) set by the European regulation.

Electrochemical immunosensors for gluten detection in food products have been developed in the last years exploiting the capability of specific monoclonal antibodies to detect gliadin antigens: a sandwich immunosensor that needs labeling steps followed by enzymatic reaction before measurement, and an amperometric competitive immunosensor based on gliadin immobilization on disposable carbon-nano gold screen-printed electrodes (Manfredi et al., 2016). Recently Chiriacò, De Feo, Primiceri, Monteduro, De Benedetto (2015) proposed a lab on chip platform based on impedimetric immunosensors to detect gluten at 1 ppm.

Nucleic acid aptamers, obtained by the in vitro selection process SELEX represent a new kind of receptors for gliadin detection. The use of aptamers as the biomolecular recognition element for developing gluten sensors is justified by their low cost synthesis and high reproducibility; high affinities comparable to those of monoclonal antibodies but with higher stability due to their nucleic-acid chemical nature and additionally they can be easily combined with different chemical labels/groups that provide flexibility for adaptation to different platforms (Miranda-Castro, de-los-Santos-Alvarez, Miranda-Ordieres & Lobo-Castanon, 2016).

In the last years aptamers against the 33-mer peptide (amino acid sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) recognizing the hydrophobic immunodominant fragment of α2-gliadin, have been studied pointing out their capability to bind gliadin from several gluten sources (Amaya-González, De-Los-Santos_Alvarez, Miranda-Ordieres & Lobo-Castanon, 2014, 2015; Pinto et al., 2014) Aptamers against the 33-mer peptide, termed Gli1 and Gli4, have been applied in an electrochemical competitive enzyme-linked assay on magnetic particles. The assay based on Gli 4-aptamer was able to achieve a gluten detection of 0.5 ppm but it failed in detecting gluten in heat treated and hydrolyzed food samples contrary to Gli1 who was kinetically favored. Recently Lopez-Lopez, Miranda-Castro, De-Los-Santos-Alvarez, Miranda-Ordieres and Lobo-Castanon (2017) developed a competitive electrochemical enzyme labeled aptasensor for the analysis of gluten in food samples.

Electrochemical impedance spectroscopy (EIS) is a powerful informative and non-destructive technique due to the small alternating voltage excitation used during detection, which can be used to study the electrical properties of the sensing device interface and tracing the reactions occurring on it. The application of EIS as a detection analytical technique, based on the direct monitoring of the interaction between the bioreceptor and its target, enables

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the production of label-free biosensors for food analysis with significant advantages over labeled ones. By avoiding the laborious and expensive labeling steps, which can cause loss of affinity between the labeled receptor and its target, and decrease reproducibility, sensitivity and selectivity of the biosensor, the use of the label-free monitoring reduces biosensor costs and allows analysis in short time (Rhouati, Catanante, Nunes,Hayat & Marty, 2016). Thanks to EIS transduction technique, food biosensor analysis are performed in real-time by studying the change in electrical properties of the electrode surface which depends only on the binding interaction between the analyte and its receptor (Malvano, Albanese, Pilloton & Di Matteo, 2016a).

In response to industrial demand that requires simple and fast analytical methods for routine and in situ control of gluten in food processing we propose the first label-free electrochemical aptasensor for the gluten analysis in food products.

Poly (amidoamine) dendrimers of fourth generation (PAMAM G4) was used for the biofunctionalization of the gold electrode in order to increase the sensitivity and at the same time reach low detection limit. EIS and cyclic voltammetry (CV) were used to characterize each step of electrode modification and the analytical performances of the aptasensors developed. Finally the aptasensor was used to quantify gluten in raw and processed food samples and the results compared with the official ELISA method based on R5 monoclonal antibody.

4.3 Materials and Methods

4.3.1 Chemicals

Sulfuric Acid (H2SO4, 99.9%), Cysteamine (95%), Glutaraldehyde solution (50% in H2O), Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), Polyamidoamine (PAMAM) dendrimer generation 4 (ethylenediamine core), were purchased from Sigma-Aldrich (Milano, Italy).

Gli1 aptamer, 5'-tagged with 6-carboxyfluorescein (6FAM), was obtained from Sigma-Aldrich (Milano, Italy) according to the following sequences:
5'(6FAM)CTAGGCGAAATATAGCTACAACTGTCTGAAGGCACCCA AT .

Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$), was obtained from Carlo Erba reagent (Milano, Italy).

Sodium phosphate monobasic (NaH2PO4), Sodium phosphate dibasic anhydrous (Na2HPO4), and Potassium Chloride (KCl) were obtained from Sigma Aldrich (Milano, Italy). The Gliadin standard of Prolamin Working Group (PWG Gliadin) was purchased from R-Biopharm Italia Srl (Melegnano, Italy).

4.3.2 Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat (Metrohm), equipped with an Impedance module (FRA32M); the experimental data were analyzed with Nova software (Metrohm). Au thin-film single-electrodes, based on a three-electrode layout (working/auxiliary/reference) were purchased from Micrux Technologies (Oviedo, Spain). The diameter of Au working electrode was 1mm.

4.3.3 Aptasensor manufacturing

Before modification, gold electrodes were cleaned by applying 13 potential cycles between -1.0 and +1.3 V with 100 mV/s scan rate in 0.05 M sulfuric acid. Cysteamine water solution 20 mM was dropped on the surface of the electrode and a constant potential of 1.2 V vs. Ag/AgCl for 20 min was applied. After the electrode was thoroughly rinsed with water, to remove physically – adsorbed cysteamine; then 100 μL of glutaraldehyde solution 5% (v/v) were dropped onto the modified working electrode for 1 h and, again, the electrode was rinsed with water.

Before the immobilization, the terminal carboxylic group of Gli1 aptamer was activated in a solution of 75 mM EDC and 15 mM NHS in 100 mM MES buffer (pH 7.4) for 2 h and then the activated aptamer was dropped on Au modified electrode. Afterwards, the immobilization of Gli1 aptamer was carried out in presence and in absence of PAMAM dendrimer. For the immobilization without PAMAM, cysteamine modified electrode was covered with 10 μL of aptamer solution at three different concentrations (0.5 μM, 1 μM 1.5 μM) for 1h at room temperature. The functionalization with PAMAM was carried out by glutaraldehyde deposition on the cysteamine layer, then three different concentrations of PAMAM solution (1 mg/mL, 1.5 mg/mL, 2 mg/mL) were dropped on it and left to react for 3 hours. After that, activated aptamer was incubated on electrode surface for 1 h.

Finally, the electrode was rinsed in PBS (pH 7.5) to remove unbound aptamers. The schematic diagram of gliadin aptasensor fabrication is presented in Figure 4.1
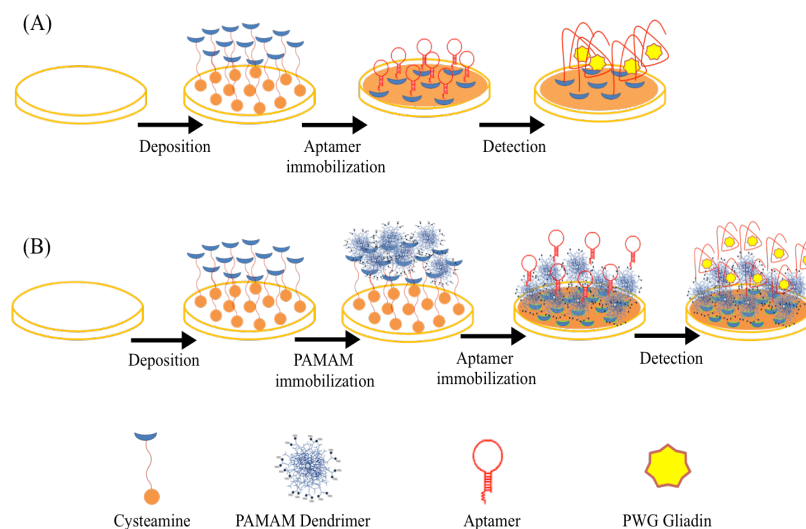
Figure 4.1: *Schematic diagram of the aptasensor with and without PAMAM*

4.3.4 Experimental Measurements

Electrochemical Impedance Spectroscopy was used to detect the immobilization processes and the interaction Gli1-gliadin.

For the impedance measurements, a sinusoidal AC potential (10 mV) in the frequency range from 0.1 to 10^4 Hz was superimposed to 0.00 mV (vs. reference electrode) DC potential. The impedance data were plotted in the form of Nyquist plots, where the complex impedance is displayed as the sum of the real and imaginary components (Z^I and Z^{II} respectively) and in the form of Bode diagram, where the total impedance of the system (Z) is plotted versus frequency. All measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4/3}$, 1:1) in PBS, pH 7.5, as background electrolyte at room temperature.

Cyclic Voltammetry measurements (CV) were also used to characterize each step of the electrode modification. The measurements were performed from -0.6 to 0.6 V vs. reference electrode with a scan rate of 0.05 V/s; the redox couple used for the CV was the same as that used for impedance measurements.

PWG gliadin standard solutions and food samples extracts were dropped onto the working area of the aptasensors and incubated for 45 min. Before the impedance measurements, the sensor surface was rinsed thoroughly with copious amounts of PBS.

4.3.5 Preparation of food samples for gluten measurement detection

The preparation food samples was carried out according to Mendez, Vela, Immer & Janssen (2005).

Five commercial samples were chosen for our tests: beer and gluten-free beer, gluten-free toasted bread, rice and corn flour. 5 mg for grinded solid or 5 ml for liquid samples were mixed with 2.5 mL of "cocktail solution" and incubated for 40 min at 50°C. Cocktail solution contains denaturing and reducing agents, ensuring a very good recovery of gluten proteins also from heat-treated food. After the cooling of the sample at room temperature, 7.5 mL of 80% ethanol were added. After shaking for 2 h at room temperature, the extract was centrifuged at 8000 rpm for 10 min and the supernatant was recovered and diluted with PBS obtaining a final dilution factor of 500.

The results obtained with the aptasensor were compared with those measured with R5 ELISA KIT (R-Biopharm Italy) for gliadin detection.

4.4 Results and Discussion

4.4.1 Aptasensor development and optimization of experimental conditions

The amount of the bioreceptor is an important factor in the performance of a biosensor as it greatly affects the capability of the biointerface to detect the target in a range of interest.

With the aim to develop an aptasensor able to detect gluten in a wide range of raw and processed foods Gli1 was selected, among the aptamers against 33-mer (Amaya-Gonzalez et al., 2014), because of its high affinity not only to intact gliadin but also to its peptide fraction caused by enzyme reaction in fermentation process. (e.g. the beer). Three different amount of Gli1 aptamers were directly immobilized on the surface of cysteamine modified gold electrodes.

The immobilization steps were monitored by EIS and CV (Figure 4.2).

The voltammogramm of the Au electrode display well defined anodic and cathodic peaks due to the reversible interconversion of $Fe(CN)_6^{3-/4-}$ (Figure 4.2a).
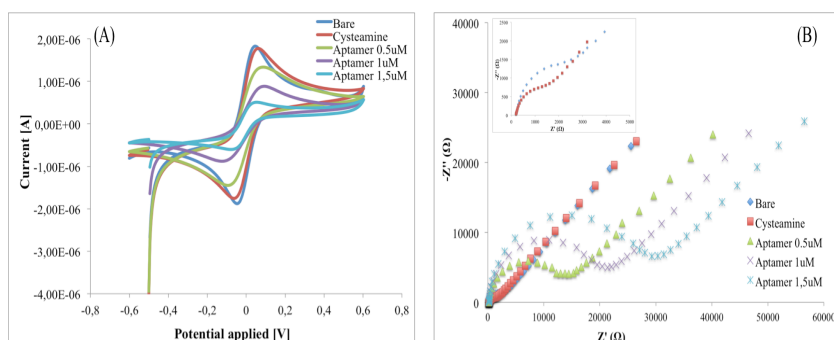
Figure 4.2: (*A) Cyclic voltammograms in 1 mM [Fe(CN)6]4-/3- and (B) EIS response after each step of aptasensor construction with different amount of Gli1. The inset correspond to the impedance spectra for bare and cysteamine modified electrodes.*

The formation of the cysteamine layers and the aptamer binding, causes the decrease of both peaks due to hindering effects of the layers on the electron transfer rate. Nyquist plots showed an increase of impedance during the immobilization steps due to the blocking layer coating on electrode surface, which became thicker with the assembly procedure (Figure 4.2B). Moreover, impedance increases with the increasing of aptamer concentration pointing out a greater immobilization of aptamer on the surface of the electrode.

When the aptasensor reacts with increasing concentration of PWG gliadin standard an increase of semicircle diameters of Nyquist plots was observed (Figure 4.3) corresponding to the charge transfer resistance R_{ct} of Randle circuit used for data fitting.
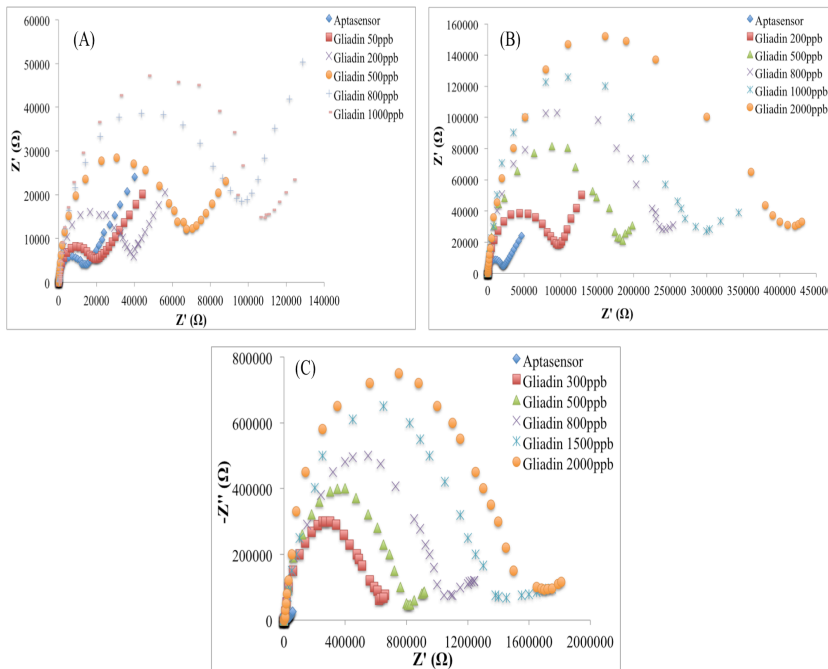
Figure 4.3: *Nyquist plots of aptasensor with aptamer concentration 0.5 μM (A), 1.5 μM (B), 2 μM (C). The Nyquist plots for each anti – OTA concentrations are the average of three different immunosensors.*

This parameter versus the concentration of PWG gliadin was then used to calibrate the three aptasensor fabricated (Figure 4.4).
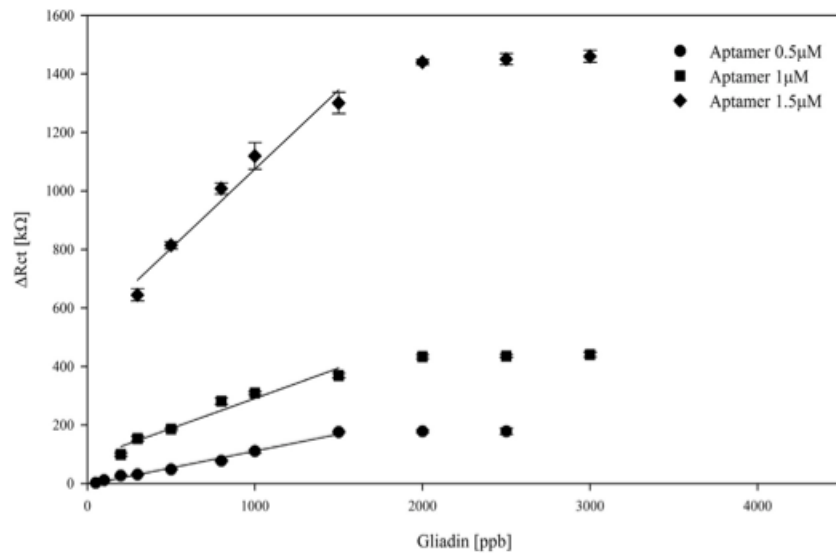
Figure 4.4: *Calibration curves for PWG gliadin with different aptamer Gli1 loading*

Even if the amount of Gli1 result in higher signals, the aptamer at 0.5 μmol showed the lowest limit of detection (LOD) equal to 50 μg/L of gliadin, calculates using the sum of average blank solution and three times the standard deviation.

The process related to gliadin coupling with Gli1 aptamer was monitored by single frequency impedance (SFI) that is able to monitor total impedance in a single frequency versus time. In this study SFI tests were carried out at 0.1 Hz chosen on the basis of the maximum differences among the Bode plots corresponding to different gliadin concentration (Figure 4.5).
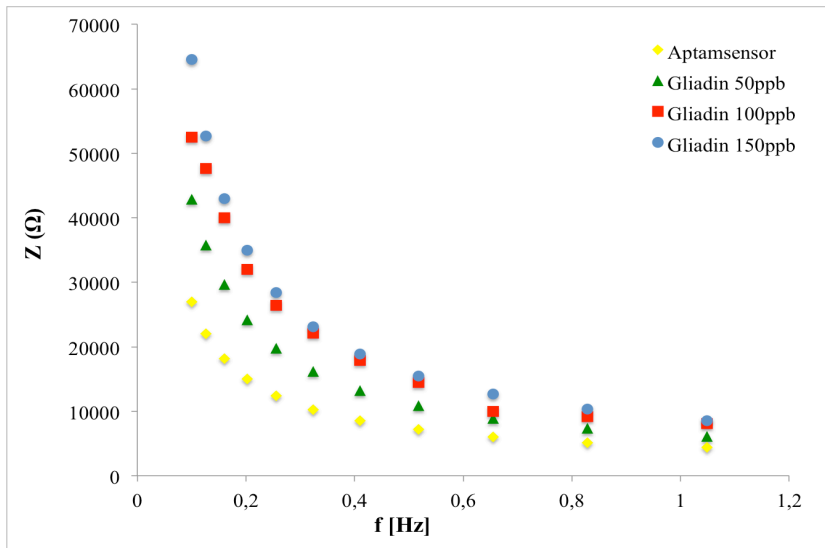
Figure 4.5:*Bode plots the frequency range 0.1 – 1 Hz before and after the interaction with different PWG concentrations*

A significant change in impedance was observed for an incubation time of 40 min, then no change was registered (Figure 4.6).
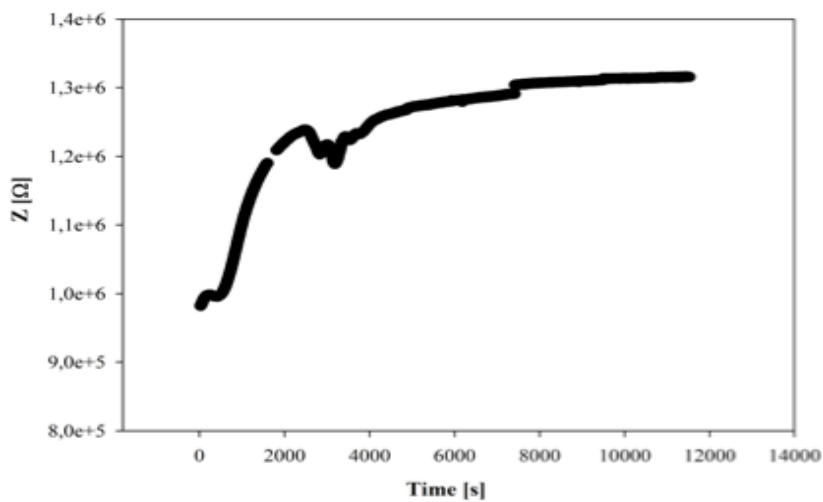
Figure 4.6: *Incubation Time*

These results justified the incubation time of 45 min used in this study for Gli1 PWG gliadin coupling.

Taking into consideration that gluten is a 50/50 blend of gliadin and glutenin and in a typical analysis of gluten in food matrix a dilution 1:500 is

required, the LOD of the aptasensor was calculated equal to 50 ppm of gluten. With the aim to improve the analytical performances of the aptasensor to detect the official limit in gluten (20 ppm) imposed by the European Regulation for the labelling of gluten – free foods, a PAMAM dendrimer was used as linker for Gli1 aptamer.

Three different concentrations of PAMAM (1, 1.5 and 2 mg/mL) were used for the immobilization of Gli1 at 0.5 µmol. Also in this case the immobilization of different layers was investigated by EIS showing the increase of total impedance in each immobilization step used during the fabrication of the aptasensors (Figure 4.7).
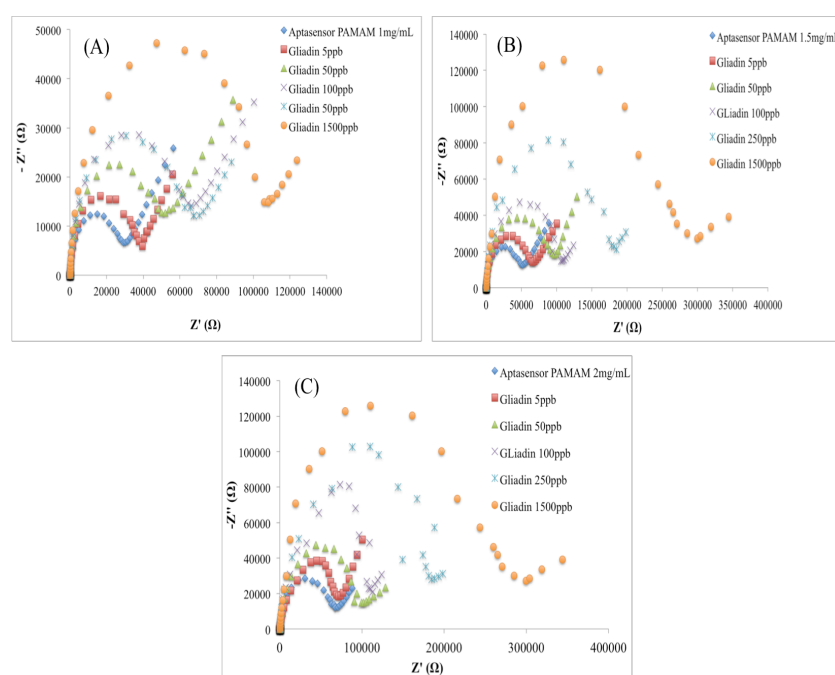
Figure 4.7: *EIS response to aptasensors developed with three different PAMAM amount: 1 mg/mL (A), 1.5 mg/mL (B), 2 mg/mL (C)*

When PAMAM was loaded on cysteamine modified electrode, R_{ct} progressively increased with PAMA concentration (42 KΩ for 1 mg/mL; 53 KΩ for 1.5 mg/mL, 70 KΩ for 2 mg/mL) suggesting that higher amount of dendrimer were immobilized on the electrode surface.

The three aptasensors constructed with PAMAM were able to detect lower PWG gliadin amount (equal to 5 mg/mL) than those registered without the dendrimer. This data is consistent with Lee et al. (2009) and Mori et al. (2009) who verified the advantages of PAMAM dendrime, over self – assembled monolayer surface coatings (SAMs), to increase the bio –

availability and sensor sensitivity. Moreover PAMAM maintains flexibility of the branches after fixation to the solid surface exposing bioactive moieties in a more effective way than in monolayer linkers (Katzur et al. 2012).

For all three aptasensors the linearity was in separated concentration ranges of 5 – 50 mg/L and 50 – 1000 mg/mL in gliadin with a LOD of 5 mg/L corresponding, as stated above, to 5 ppm of gluten in food products (Figure 4.8). This result was consistent with Amaya-Gonzalez et al. (2015) who verified the ability of Gli1 aptamer in gluten detection by a competitive electrochemical aptamer based assay.

The highest sensitivity was found for the aptasensor fabricated with 2 mg/mL of PAMAM.
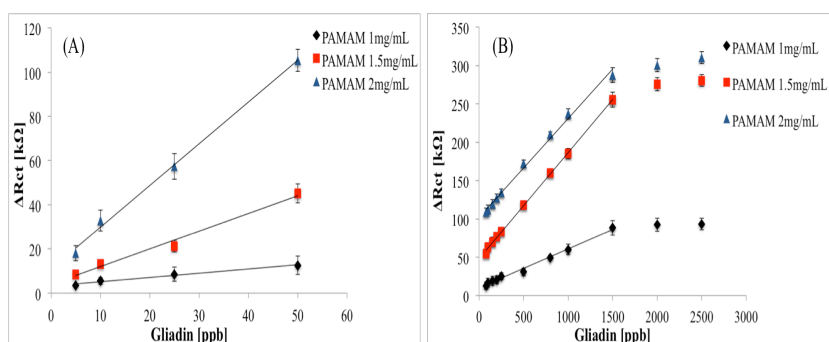
Figure 4.8: *Calibration curves for PWG gliadin for aptasensor with 0.5μmol Gli1 and PAMAM at 1, 1.5 and 2 ng/mL (A). Linear range PWG gliadin 5 – 50 μg/L. (B) Linear range PWG gliadin 50 – 1000 μg/L.*

This result could be due to the spherical shape of the dendrimers and the high density of reactive groups on their surface that raise the specific surface areas of the probe, thereby increasing the amount of immobilized aptamer (Benters, Niemeyer & Wohrle, 2001; Degenhart, Dordi, Schonherr & Vancso, 2004; Ajikumar et al., 2007).

This latter was confirmed by the analysis of EI spectra registered during the construction of the sensor with dendrimer who highlighted the increase in ΔR_{ct} (56 KΩ, 58 KΩ and 95 KΩ for 1, 1.5 and 2 mg/mL of PAMAM respectively) calculated before and after the binding of the aptamer at 0.5 μmol.

Moreover as previous reported (Malvano, Albanese, Crescitelli, Pilloton & Esposito, 2016b), higher level of biomolecule immobilization raised the sensitivity of the sensor increasing the coupling capacity between bioreceptor and target.

4.4.2 Analytical performance of aptasensor

The reproducibility calculated on five different aptasensors, at 100 μg/l PWG standard, showed a good relative standard deviation (RSD) for all aptasensors developed with PAMAM: 4.56%, 5.12% and 4.25% for 1, 1.5 and 2 mg/ml PAMAM respectively.

The storage stability of aptasensors fabricated with 2 mg/ml PAMAM was also determined. For this purpose different aptasensors were stored for 2 months at 4°C without chemical preservatives and characterized at regular interval times. After the investigated storage period the aptasensors showed a negligible loss of activity.

In Table 4.1 a performance comparison of aptamer – based electrochemical assays, previously developed, for gliadin detection is reported. Gli 4 showed highest affinity for gliadin even if it fails in detection the peptide fraction in solution (Amaya – Gonzalez et al., 2015).

Table 4.1: *Comparison among electrochemical aptasensor based assays for gluten.*

	Assay type	Trasduction technique	LOD [gluten ppm]	References
33-mer onto magnetic beads+biotin-Gli1+gliadin+streptavidin-peroxidase	competitive	amperometry	4.9	Amaya Gonzalez *et al.*, 2015
33-mer onto magnetic beads+biotin-Gli1+gliadin+streptavidin-peroxidase	competitive	Amperometry	0.5	Amaya Gonzalez *et al.*, 2015
SPCE-33mer . biotin-Gli4.gliadin . streptavidin-peroxidase	competitive	Amperometry	0.11	Lopez-Lopez *et al.*, 2017
GE-Cys-PAMAM-Gli1	direct	impedance	5.0	This work

Anyway a good agreement with literature was observed for Gli1. It is worth noting that our aptasensor is the first one based on the immobilization of aptamer on the modified electrode and all others were based on competitive assay. This condition makes the analysis of gliadin very fast and easier than aptamer competitive assay that required addition of an enzyme labelled to the food sample and then the enzymatic labelled aptamer to the food sample and the enzymatic substrate.

The analysis of the impedimetric data registered during the interaction tests between the aptasensors, with and without dendrimer, and increasing PWG gliadin concentration, also provides an estimation of dissociation

constant (K_d) for aptamer PWG gliadin interaction. The dissociation reaction for PWG gliadin and Gli1 complex could be expressed for the dissociation constant, K_d as following:

$$AG \rightarrow A+G \qquad (4.1)$$

in which A is the aptamer, G is the target and AG is the aptamer – target complex.

The equilibrium can be descry bed using dissociation constant K_d:

$$K_d = [A][G]/[AG] \qquad (4.2)$$

Assuming the surface coverage of PWG gliadin – aptamer complex is α, the surface coverage of unbound aptamer will be 1- α:

$$K_d = \left(\frac{1-\alpha}{\alpha}\right)[G] \qquad (4.3)$$

K_d can be obtained experimentally by measure of fraction occupied sites by:

$$f = \frac{R_{cteq} - R_{ct0}}{R_{ct0}} \qquad (4.4)$$

where R_{cteq} is R_{ct} at equilibrium and R_{ct0} is the initial R_{ct} (Fan, Zhao, Shi, Liu & Li *, 2013; Jing & Bowser, 2011).

According to Langmuir adsorption isotherm, this fraction f can be directly related to the surface coverage of the gliadin – aptamer complex:

$$f = \alpha * f_{sat} \qquad (4.5)$$

where with $\alpha = 1$, the standard signal reaches the maximum value f_{sat}.

The use of dimensionless f data allows transformation of the adsorption isotherm in to the Hanes – Wool form, where overweighting of the low concentration results is avoided (Schuler and Kargi *, 2002).

$$f = \frac{f_{sat} * [G]}{K_d + [G]} \qquad (4.6)$$

A plot of [G]/f versus [G] for the aptasensors with and without PAMAM was shown in Figure 4.9.
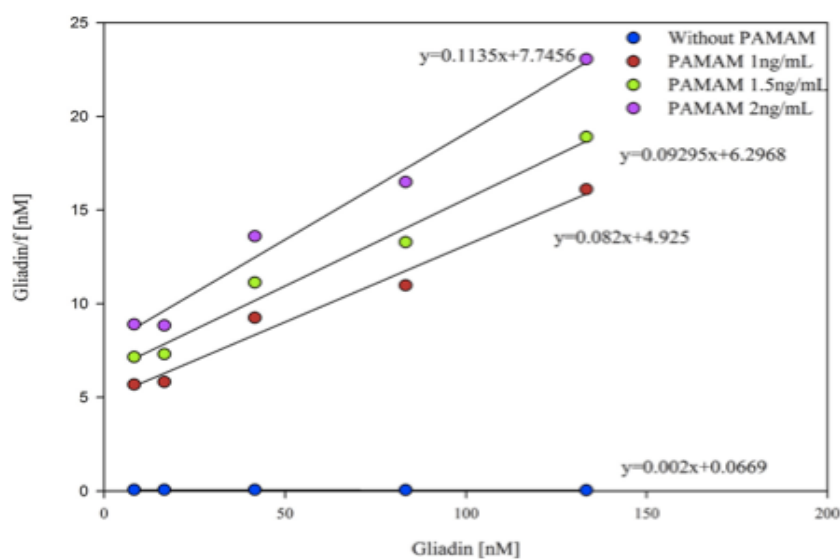
Figure 4.9: *Hanes – Woolf plots for determing the dissociation constant*

The slope of linear regressions and the y – intercept for each aptasensors let the calculation of K_d as reported in Table 4.2.

Table 4.2: *Dissociation constant (K_d) of the aptamer Gli1 immobilized on sensor surface with and without PAMAM.*

Aptamer Gli1	without PAMAM	PAMAM 1 mg/mL	PAMAM 1.5 mg/mL	PAMAM 2mg/mL
K_d/nM	73.63	6.66	9.03	9.86

Highest affinity was calculated when PAMAM was used for the aptamer anchorage on the electrode confirming the advantages of dendrimer to preserve the native biomolecule conformation.

For all aptasensor developed Gli1 showed higher affinity to PWG gliadin and the value estimated were consistent with other authors (Amaya-Gonzalez et al. 2015) who calculated a K_d value of 58 nM for Gli1 – PWG gliadin interaction. It is worth noting that K_d value is influenced by the analytical technique used for the calculation, the experimental procedure (aptamer immobilized or free in solution), if it is native or 5 – tagged (for its binding on solid surface), and by the type of moiety used for the tagging (Jing & Bowser, 2011).

4.4.3 Analysis of gluten in food samples

In order to highlight the gluten detection capability of the aptasensor (with PAMAM at 2 mg/ml) processed and unprocessed food labelled as "gluten – free" were chosen as samples. The aptasensor was also tested with corn flour and rice to evaluate the reactivity of Gli1 versus other prolamines non celiac disease triggering. The extraction of gluten was carried about by cocktail solution (Mendez et al., 2005) according to extraction protocol of official method based on R5 immunoassay. R5 ELISA was used as comparison method, the amount of gluten detected in food samples by both methods was reported in Table 4.3.

Table 4.3: *Gluten results obtained by impedimetric aptasensor and R5 ELISA kit.*

Sample	Aptasensor [ppm]	R5 ELISA kit [ppm]
lager beer	34.29±0.20	21.67±6.13
gluten free beer	6.03±0.04	6.59±0.27
gluten free bread	7.97±0.40	8.08±0.29
rice	nd	nd
corn flour	21.02±0.65	18.19±0.88

Both methods gave concordant results for beer and bread "gluten – free" labelled confirming their safety in celiac patients. Contrasting gluten concentrations were measured by aptasensor and ELISA kit. The capability of Gli1 aptamer to recognize also hydrolysed gliadin in contrast to R5 sandwich ELISA could be an explanation of different gluten amount detected in larger beer samples. finally corn flour contains gluten near the threshold established by European regulation. R5 recognizes only the amino acid sequence QQPFP and similar sequences present in prolamins from wheat (gliadin), rye (secalin), and barley (hordein) (Valdes, Garcia, Lorente & Mendez, 2003), thus the amount of about 20 ppm measured by ELISA kit and aptasensors could be due to a cross – contamination during the manufacturing process of corn flour.

4.5 Conclusion

A new label-free impedimetric aptasensor for gluten detection, based on the immobilization of aptamer (Gli1) on the gold electrode modified with PAMAM, was reported. PAMAM has been proven to increase the sensitivity of the aptasensor with a high binding affinity to PWG gliadin. The aptasensors developed were very sensitive to gluten with a detection limit of 5 ppm. Moreover, a good agreement between R5 ELISA official method and

aptasensor was obtained in gluten content analyzed in food samples. The analysis of gluten does not require the use of other reagents but only the gliadin extract, thus it makes the impedimetric aptasensor

a fast and simple method for the control of food safety in food products addressed to celiac patients diet.

4.6 References

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A new label free impedimetric affinity sensor based on cholinesterases for detection of organophosphorous and carbamic pesticides in food samples: impedimetric vs amperometric detection

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A new label-free impedimetric affinity sensor based on cholinesterases for detection
of organophosphorous and carbamic pesticides in food samples:
impedimetric vs amperometric detection

5.1 Abstract

Due to their increasing use in agriculture, the presence of pesticides
residues in food and water currently represents one of the major issues for
the food safety. Among the pesticides, organophosphate and carbamate
species are the most used and their toxicity is mainly due to their inhibitory
effect on Acetylcholinesterase (AChE), a key enzyme for the nerve
transmission; the inhibition of this enzyme leads to respiratory failure,
unconsciousness, convulsion and, eventually, death. For this reason, a mono-
enzymatic acetylcholinesterase impedimetric biosensor was developed
immobilizing the AChE enzyme on cysteamine modified gold electrode, in
order to sensitively detect carbamate and organophosphate compounds with
a very fast response.

In fact, the common amperometric evaluation of AChE inhibition degree
was correlated to the impedimetric changes of the electrode surface, due to
the formation of enzyme complex with carbamate or organophosphate
compounds. The results showed a proportionality between pesticide's
concentration and charge transfer resistance decrease for all pesticides
analysed, highlighting that a very fast impedimetric analysis would reveal
the pesticide inhibiting capacity, and, therefore, its toxicity.

The influence of lead and mercury on biosensor response was examined
and finally and it was used to measure Carbaryl and Dichlorvos in tap water
and lettuce samples.

5.2 Introduction

Pesticide is a term associated to organic toxic compounds used to protect
crops and seeds by destroying insects, bacteria, weeds, rodents and other
pests (Sassolas *et al*., 2012).

The presence of pesticides residues in food, water and soil currently
represents one of the major issues for the environmental chemistry; they are,
in fact, among the most abundant environmental pollutants due to their
increasing use in agriculture. Among the pesticides, organophosphate and
carbamate species are the most used because of their high insecticidal
activity and relatively low persistence with respect to organochlorine
pesticides as well as aldrin or lindane (Arduini *et al*., 2006).

Carbamates (CBs) and organophosphates (OPs) toxicity is mainly due to
their inhibitory effect on Acetylcholinesterase (AChE), a key enzyme for the
nerve transmission: the inhibition of this enzyme leads to muscle weakness,
miosis, respiratory failure, unconsciousness, convulsion and, eventually,
death (Storm *et al*., 2000).

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A new label-free impedimetric affinity sensor based on cholinesterases for detection
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For these reasons, there is a general concern on pesticide food contamination and, consequently, the development of simple and sensitive strategies for detecting toxic compounds is critically important in order to carry out the measurement "in situ" using miniaturized, cost-effective and easy to use analytical system.

In particular, the biosensors based on AChE inhibition are reliable tools for the detection of OP and CB compounds (Andreescu and Marty, 2006; Vakurov *et al.*, 2004). These pesticides are able to form a covalent link (irreversible for OPs and pseudo irreversible for CBs) with the serine residue present in the catalytic site of the enzyme, blocking the hydrolysis of the acetylcholine in choline and acetic acid.

The most common biosensors developed for pesticides detection are based on Ache inhibition. In particular, the enzymatic activity of AChE is measured before and after the exposure to pesticide samples by different transduction techniques such as amperometric, piezoelectric and optical transducers (Arduini *et al.*, 2013, Albanese *et al.*, 2012, Valdes – Ramirez *et al.*, 2008, Caetano and Machado 2008)

Because the AChE inhibition occurs for both pesticide groups is not specific, AChE based biosensors give information about the toxicity level of a sample, thus they can be used as a warning system for this class of toxic compounds, followed, in the case of positive response, by the HPLC or GC-MS analyses to exactly detect the type and the amount of pesticide in the sample (Moscone *et al.*, 2016).

The electrochemical impedance spectroscopy (EIS) is an interesting transduction technology which enables the direct analyte detection by studying the electrical properties of the sensing device interface: the EIS measurements involve the analysis of impedimetric changes, at the electrode interface when a biological or chemical element interacts with the electrode surface functionalized with a bioreceptor. In particular, EIS combines the analysis of both resistive and capacitive properties of the electrode surface, based on the small amplitude perturbation of a system from steady, which makes it a non – destructive technique (Guan *et al.*, 2004; Bahadir and Sezginturk 2016)

This type of transduction allows the development of affinity-binding biosensors with noteworthy advantages due to their direct and label-free detection of analyte of interest.

Exploiting the inhibition mechanism of pesticides versus AChE, and the capability of EIS to measure interaction between the analyte and the bioreceptor, we considered to apply the EIS as transduction method for the of AChE based biosensors for the direct measurement of pesticides inhibition compounds in real matrix.

For this study AChE was immobilized via glutaraldehyde on an electrochemical deposed multilayer of cysteamine on printed gold electrode.

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EIS and Cyclic Voltammetry (CV) were used to characterize each step of electrode modification and the analytical performance of the biosensor versus CB and OP compounds. Comparison between the impedimetric characterization and the amperometric ones was also reported. The influence of lead and mercury on biosensor response was examined and finally and it was used to measure Carbaryl and Dichlorvos spiked in tap water and lettuce samples.

5.3 Materials and Methods

5.3.1 Reagents

Acetylcholinesterase (AChE 2 KU, 149 U/mg$_{solid}$), Cysteamine (95%), Glutaraldehyde solution (50% in H_2O), Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), Hexane ($CH_3(CH_2)_4CH_3$, 99%), Carbaryl ($C_{10}H_7OCONHCH_3$), Paraquat dichloride hydrate ($C_{12}H_{14}C_{l2}N_2 \cdot H_2O$), Dichlorvos ($C_4H_7C_{l2}O_4P$), Chlorpyrifos - methyl ($C_9H_{11}C_{l3}NO_3PS$), Phosmet ($C_{11}H_{12}NO_4PS_2$), Kresoxim-methyl ($C_{18}H_{19}NO_4$) were purchased from Sigma-Aldrich (Milano, Italy). Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$), Lead Acetate Trihydrate (99.9%) and Mercuric (II) chloride was obtained from Carlo Erba reagent (Milano, Italy). Acetylthiocholine Chloride was purchased from Molekula (Rimini, Italia). Sodium phosphate monobasic (NaH_2PO_4), Sodium phosphate dibasic anhydrous ($Na_2HPO_{4)}$, and Potassium Chloride (KCl) used in the preparation of phosphate buffered (PB: 0.1 M KCl, pH 7) were received from Sigma Aldrich (Milano, Italy).

5.3.2 Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat and NOVA software. Au thin-film single-electrodes and the all-in-one electrochemical cell were obtained from Micrux Technologies (Oviedo, Spain). The electrodes incorporate a conventional three-electrode configuration, with an Au working (diameter 1mm), reference and counter electrodes.

5.3.3 Acetylcholinesterase immobilization procedure

Before enzyme immobilization, gold electrode was cleaned by applying 10 potential cycles between -1.0 and +1.3 V vs reference electrode with 100 mV/s scan rate in 0.05 M sulfuric acid. Then 20 mM Cysteamine water solution was electrodeposited on the electrode surface under a constant

potential of 1.2 V vs reference electrode for 10 minutes. After that, the electrode was covered with an aqueous solution of glutaraldehyde 2.5% (v/v) for 30 minutes; then it was thoroughly washed with bi-distilled water to remove the unreacted glutaraldehyde. Finally the resulting modified electrode surface was incubated with 2.5 U of AChE over night at 4°C.

5.3.4 Electrochemical Measurements

5.3.4.1 Impedimetric measurement

The Electrochemical Impedance Spectroscopy (EIS) measurements were carried out over imposing an excitation voltage of 10.00 mV to 0.00 mV (vs. reference electrode) DC potential, in the frequency range from 0.1 to 10^5 Hz. The measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$, 1:1) in PB, 0.1 M pH 7, as background electrolyte, at room temperature.

Each step of the biosensor build-up was interrogated also by cyclic voltammetry (CV): the measurements were performed from -0.6 to 0.6 V vs reference electrode with a scan rate of 0.05 V/s; the redox solution used for the CV measurements was the same one used for EIS measurements.

For pesticide measurements, 20 μL of CB and OP compounds respectively at different concentrations were dropped onto the electrode working area and incubated for 30 minutes; before the impedance

measurement, the biosensor was rinsed with copious amount of bi-distilled water. Single frequency impedance (SFI), at 0.1 Hz was used for kinetic analysis to calculate the kinetic dissociation constants of OP and CB with AChE.

5.3.4.2 Amperometric measurement

The degree of pesticides inhibition was calculated using the following equation:

$$I\% = \frac{i_0 - i_i}{i_0} * 100 \tag{5.1}$$

where (i_0) and (i_i) represent the amperometric biosensor response, before and after the incubation time respectively, at 2 mM substrate (acetylthiocholine) concentration (Albanese et al., 2012). Acetylthiocholine measurements were performed using amperometric analysis in a Flow Injection Analysis (FIA) apparatus, under a constant potential of 0.4 V. Carrier solution (0.1 M phosphate buffer in 0.1 M KCl, pH 7) from a reservoir was pumped with a peristaltic pump (Miniplus 3, Gilson, France) at

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flow rate of 0.5 mL/min to the injection valve (Sample injection valve, Omnifit) equipped with a 100mL sample loop.

5.3.4.3 Kinetic analysis

Kinetics studies of AChE enzymatic reaction were carried out in order to analyse mechanism of enzyme inhibition. Tests were monitored by single frequency impedance (SFI), at 0.1 Hz, obtaining a plot of total impedance of the system versus time.

The inhibition reaction of AChE by a CB or OP compound takes place in two-step process: the first step involves the formation of the enzyme-inhibitor complex with subsequent carbamylation (with CBs) or phosphorylation (with OPs) of the serine hydroxyl resulting in inhibition of the enzyme.

The reaction scheme is the following one (Fukuto, 1990):

$$E-OH + PX \overset{K_I}{\leftrightarrow} E*PX \overset{k_3}{\rightarrow} EP + X \qquad (5.2)$$
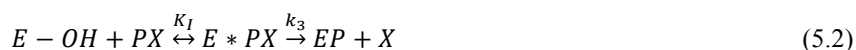
where $E-OH$ represents AChE in which the serine hydroxyl moiety (-OH) is emphasized, PX is the pesticide inhibitor, E*PX is the complex enzyme-inhibitor, EP is the carbamylated/phosphorylated enzyme, X is the leaving group, K_I is the dissociation constant for enzyme-inhibitor complex and k_3 is the first order rate constant for the conversion of the enzyme-inhibitor complex to carbamylated/phosphorylated enzyme.

For the analysis, three different CBs (Carbaryl, Paraquat, Kresomix-methyl) and three different OPs (Dichlorvos, Chlorpyrifos-methyl Pestanal, Phosmet) were used at five different concentrations (5ppb, 10ppb, 32 ppb, 48ppb, 80ppb) and inhibition parameters were evaluated.

5.3.4.4 Analysis of food samples and interference study

AChE affinity biosensor was used for the determination of Carbaryl and Dichlorvos in tap water and lettuce samples.

In order to verify the accuracy of the developed biosensor, three water samples were spiked with three different concentrations of Carbaryl (25ppb, 50ppb, 100ppb) and three different concentrations of Dichlorvos (25ppb, 50ppb, 100ppb).

The preparation of vegetables samples was carried out according to Xavier et al. 2000: 2.5 g of the homogenate lettuce sample were spiked with known amounts of Carbaryl (25ppb, 50ppb, 100ppb) and Dichlorvos (25ppb, 50ppb, 100ppb). The samples were mixed with 5 ml of pure hexane and sonicated for 15 min. The mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was collected; blank samples were prepared following the previous procedure without pesticide spiking.

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Additionally, the influence of two interfering substances, such as lead and mercury, was examined. Two different concentrations of Pb(II) (20ppb and 100ppb) and Hg(II) (500ppb – 1000ppb) was put in contact with AChE enzyme and interference effects were analysed.

5.4 Results and Discussion

5.4.1 Electrochemical Characterization of electrode modifying process

Cyclic voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) were widely used as a convenient tools to monitor the various steps of the biosensor buildup.
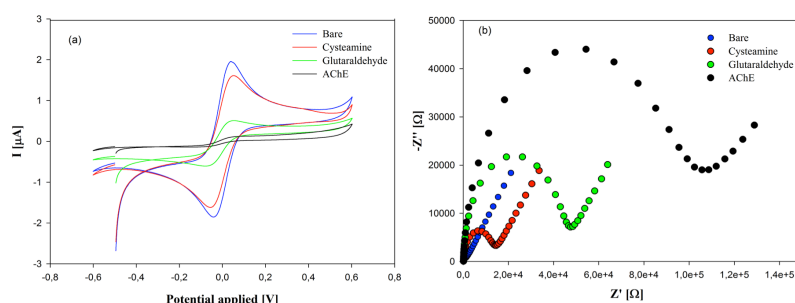
Figure 5.1: *Cyclic voltammograms in 1 mM [Fe(CN)$_6$]$^{4-/3-}$ after each step of biosensor construction (a). EIS response to all biosensor fabrication steps (b)*

Figure 5.1(a) and Figure 5.1(b) show the typical cyclic voltammograms and Nyquist plots respectively obtained after each step of electrode modifying process and enzyme immobilization.

The voltammograms of the Au electrode displayed a well defined anodic and cathodic peaks due to the reversible interconversion of $[Fe(CN)_6]^{3-/4-}$. As result of the immobilization on the electrode surface of cysteamine, glutaraldehyde and the final addition of AChE enzyme, the diffusion of the redox probe close to the electrode surface was dramatically reduced, causing a significant decrease of both peaks.

Nyquist plots, showed an increase of total impedance of the system during the immobilization steps due to the layer coating on the electrode surface, which became thicker with the assembly procedure (Figure 5.1b). As expected, the permeability of $[Fe(CN)_6]^{3-/4-}$ through the immobilization layers was strongly reduced with an increase of the electron transfer resistance.

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The CV and EIS results pointed out that chemical and biomolecular layers act as effective barriers to the charge transfers (Malvano et al. 2016a).

5.4.2 Determination of OP and CB Pesticides by Amperometric detection

Amperometric inhibition biosensor require an initial calibration using the enzyme's substrate; the monoenzymatic AChE biosensor uses acetylthiocholine as substrate, according to the following equation:

$$\text{Acetylthiocholine} + H_2O \rightarrow \text{Thiocholine} + \text{Acetate Acid} \qquad (5.3)$$

The substrate was enzymatically hydrolysed to thiocholine, which was then subjected to electrocatalytic oxidative dimerization at 0.4 V t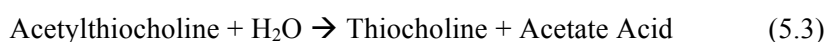o give the disulphite compound. This response, at the working electrode, was correlated to the activity of AChE (Chauhan and Pundir 2011).

The optimization of biosensors based on AChE inhibition is closely linked to amperometric signal read by electrochemical transducer. Although a high enzyme activity is requires for reproducibility and long – term measurement with suitable electrode response on acetylthiocholine, sensitive inhibitor determinations are favoured at low enzyme loading. From preliminary tests and according to previous studies (Albanese et al., 2012) 2.5 U of AChE was chosen for the construction of AChE biosensor. The calibration curve of the amperometric AChE biosensor (Figure 5.2), obtained for different substrate concentrations, showed an high sensitivity 477.34 nA/mM in the range from 0.1 to 2 mM, a limit of detection of 0.1 mM, defined as the acetylthiocholine concentration that yields a signal – to – noise (S/N) ratio equal to 3, and a good repeatability of the current response with relative standard deviation (RSD) of 3.78% (calculated on five different injection at 0.5 mM).

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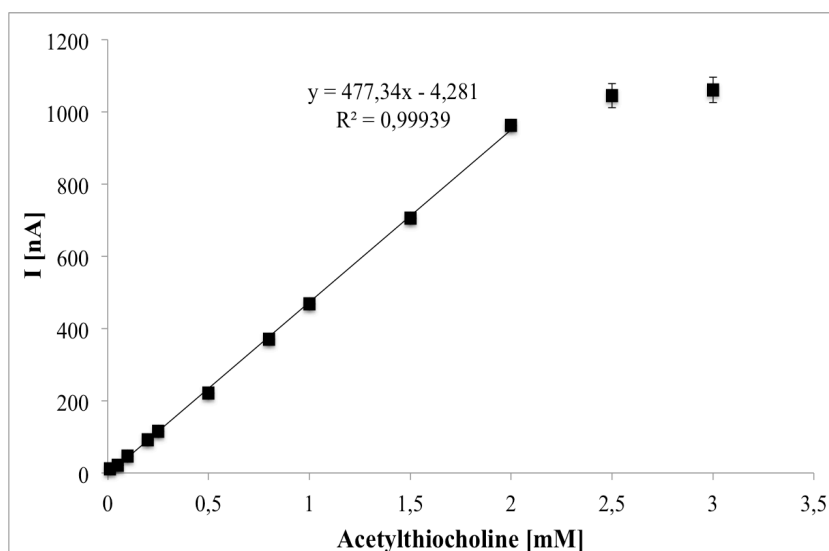
Figure 5.2: *Calibration curve of AChE biosensors with 2.5 U of AChE
enzyme. Data represent the average of three biosensors. Applied potential
400 mV. 0.1 M phosphate buffer 0.1 M KCl, pH 7.*

For inhibition measurement, with the aim to ensure that the biosensor is
working under kinetic - controlled conditions, the concentration of the
substrate used must be near the upper limit of the system and within the
linear range of response (Liu and Lin 2006).
According to above results, 2 mM acetylthiocholine was used to carry out
the inhibition test and the degree of pesticides inhibition was calculated
using the following equation

$$I\% = \frac{i_0 - i_i}{i_0} * 100 \qquad\qquad (5.4)$$

As reported in the official methods for pesticides detection (EAPA), the
extraction of pesticides is usually carried out using organic solvent; because
pesticides are often extracted with pure hexane (Xavier et al., 2000), the
effect of this solvent on the AChE activity was evaluated.

The influence of this organic solvent, which is completely insoluble in
aqueous phase, gave satisfactory results. In fact, according to previous
studies (Albanese et al. 2012; Andreescu et al. 2002), after 45 min of
biosensor incubation in pure hexane, only a marginal reduction of biosensor
response (less than 5%) was detected compared with initial value (data not
shown)

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Therefore, this solvent was used for the preparation of pesticides standard solutions without effect on enzyme activity and the calibration curves of the AChE biosensor tested with three different CBs (Carbaryl, Paraquat, Kresomix-methyl) and three different OPs (Dichlorvos, Clorpyrifos – Methyl Pestanal, Phosmet) are showed in Figure 5.3a,b, respectively.
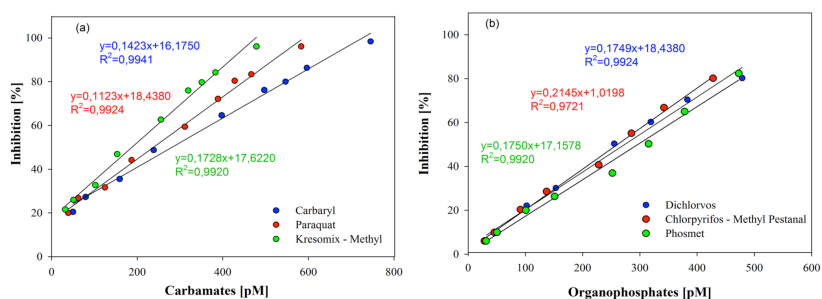
Figure 5.3: *Inhibition curves for Carbaryl, Paraquat, Kresomix – methyl (a) and for Clorpyrifos – methyl Pestanal, Phosmet and Dichlorvos (b).*

The AChE biosensor showed a similar sensitivity for CBs and OPs and the same linear range for all analysed compounds: in fact, considering molecular weight of each pesticide, it was able to detect both of compounds in a range of 10 – 150 ppb.

The detection limits, calculated as the pesticide concentration that yield the inhibition signal three times higher than noise, were 8 and 10 ppb for CBs and OPs compounds respectively. These results are competitive with previous studies (Arduini et al., 2006, Arduini et al., 2013, Caetano and Machado 2008, Chauhan and Pundir 2011) who used printed gold electrodes and AChE as monoenzymatic system.

Finally, the biosensor was characterized by a reproducibility (RSD%) interelectrode, calculated on five different AChE biosensors, of 3.1 and 4.8% for OP and CB, respectively.

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5.4.3 Impedimetric detection of carbamates and organophosphate compounds

The capability of EIS to detect the binding between OP and CB compounds, responsible of enzymatic activity inhibition of AChE, is showed in Figure VII.4. The AChE biosensor was put in contact with increasing concentrations of Carbaryl (Figure 5.4a) and Dichlorvos (Figure 5.4b) chosen as CB and OP compounds, respectively, and EIS spectra were analysed.
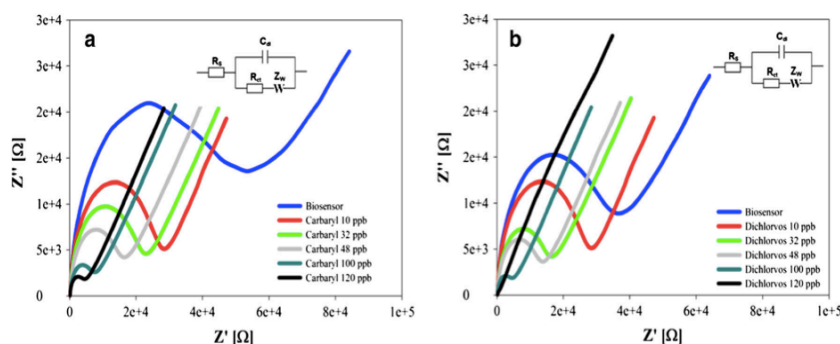
Figure 5.4: *Nyquist plots of biosensor after incubation of different Carbaryl (a) and Dichlorvos concentrations (b). The inset corresponds to the equivalent circuit used to fit impedance spectra*

In particular, experimental data were fitted using Nova software by the common Randle's circuit (inset Figure 5.4). In the given frequency range, for both pesticides classes, a decrease of semicircle diameter of Nyquist plots, was observed by increasing pesticides concentration, which correspond to the decrease of R_{ct} values. The ΔR_{ct} value, expressed as the difference between the R_{ct} value before and after the contact enzyme inhibitor, at different pesticide concentrations, was used to characterize the biosensor.

The same behaviour was observed after contact with the other CB and OP pesticides analysed in this work (Figure 5.5).

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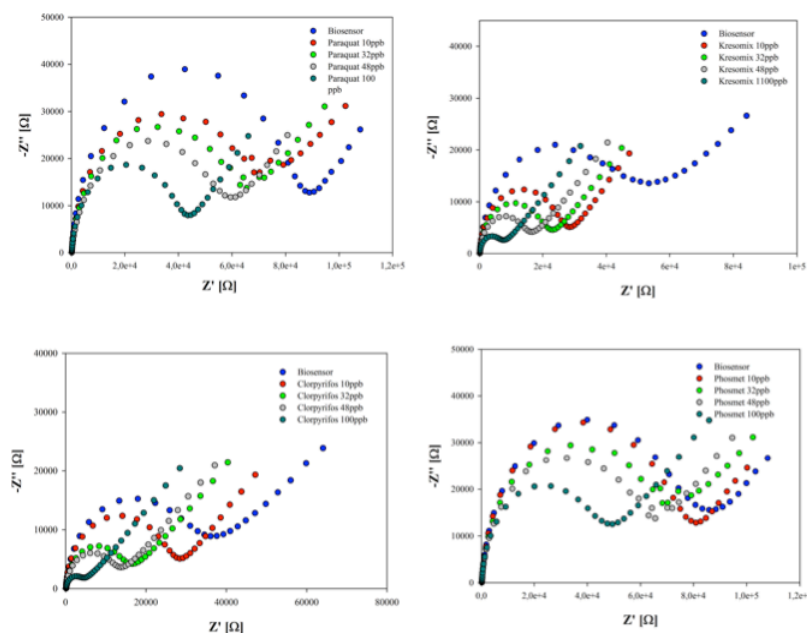
Figure 5.5: *Nyquist plots of biosensor after incubation of different Paraquat (a), Kresomix (b), Clorpyrifos (c), Phosmet (d) concentrations.*

The calibration curves obtained for all tested pesticides (Figure 5.6 a,b) showed a linear correlation in the range 5 – 170 ppb for CBs and 2.5 – 170 ppb for OPs, with a LOD, based on the sum of average blank solution and three times the standard deviation, equal to 5 ppb for Carbaryl, Paraquat and Kresomix – methyl, and 2.5 ppb for Dichlorvos, Clorpyrifos Methyl Pestanal and Phosmet. AChE biosensor showed a reproducibility (RSD%) interelectrode, calculated on five different AChE biosensors, of 3.1 and 4.8% for OP and CB, respectively.
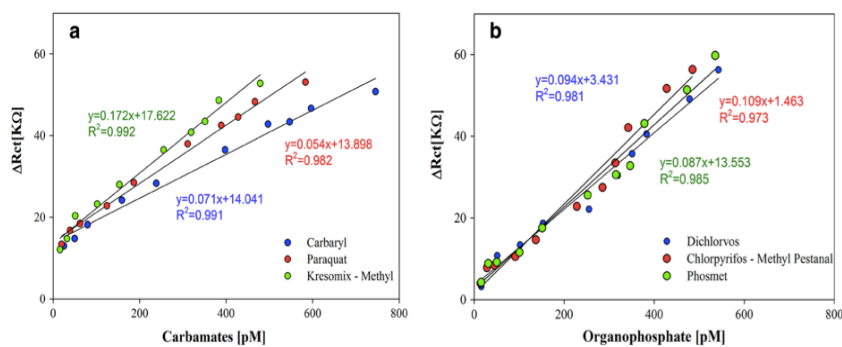
Figure 5.6: *Calibration curves by impedimetric analysis for all CBs (a) and OPs compounds (b).*

Finally, the storage stability of biosensor was also determined by impedimetric analysis: for this purpose, different AChE biosensors were stored for 3 months at 4°C without chemical preservatives and characterized at regular interval times. After the investigative storage period, the biosensor showed a negligible loss of activity.

5.4.4 Incubation time of AChE biosensor

The incubation time used for the characterization of amperometric and impedimetric AChE inhibition – based biosensors was 40 min. this latter was estimated by single frequency impedance (SFI) that is able to monitor total impedance in a single frequency versus time, in our study, SFI tests were carried out a 0.1 Hz chosen on the basis of the maximum differences among the Bode plots corresponding to different Carbaryl and Dichlorvos concentrations (Figure 5.7a,b). A significant change in impedance, for both pesticides, was observed for an incubation time of 40 min, and then no change was registered.

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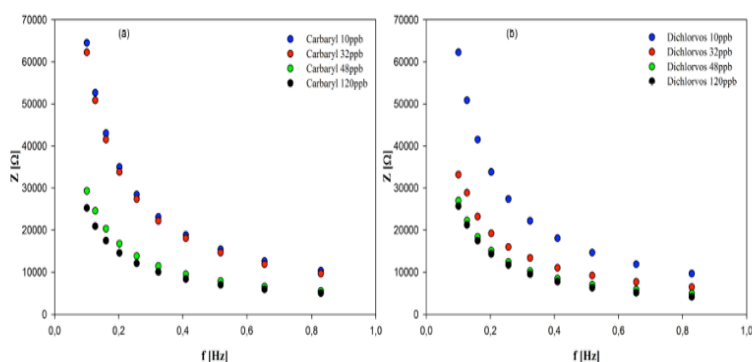
Figure 5.7*: Bode Plots in impedance measurements for biosensor after
incubation with different Carbaryl (a) and Dichlorvos (b) different
concentrations in the frequency range 0.1–1 Hz.*

5.4.5 Comparison between amperometric and impedimetric AChE
biosensor

The comparison of the results obtained with the impedimetric and
amperometric transduction shows that the firs one is able to detect a wider
linear range and lower LODs for all pesticides tested (Table 5.1).

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Table 5.1: *Comparison between Acetylcholinesterase inhibition-based biosensors developed by amperometric and impedimetric transduction.*

		Transduction method			
		Amperometry		Electrochemical Impedance Spectroscopy	
		Linear Range (ppb)	Limit of Detection (ppb)	Linear Range (ppb)	Limit of Detection (ppb)
Organophosphates	Dichlorvos	10-150	10	2.5-170	2.5
	Chlorpyrifos methyl	10-150	10	2.5-170	2.5
	Phosmet	10-150	10	2.5-170	2.5
Carbamates	Paraquat	10-150	8	5-170	5
	Carbaryl	10-150	8	5-170	5
	Kresoxim-methyl	10-150	8	5-170	5

High correlation was found between enzymatic inhibition and change in electrical resistance for all CBs and OPs investigates (Figure 5.8) confirming that the binding AChE pesticide observed by impedance transduction is responsible of the decrease of enzymatic activity.

Figure 5.8: *Linear correlations between AChE inhibition degree and ΔRct for Carbaryl, Paraquat, Kresomix-methyl (a) and for Dichlorvos, Clorpyrifos methyl Pestanal and Phosmet (b)*

It is worth to note that as well as the amperometric detection, the impedimetric technique is not able to discriminate the type of pesticide

linked to the enzyme but only the toxicity level given the high relationships between I% and ΔR_{ct} for both carbamate and organophosphate compounds.

Moreover, the EIS transduction method results to be extremely faster than amperometric one. In fact, at difference of amperometric measurements that require the injection of AChE substrate before and after the exposure with pesticide solution with the impedimetric transduction, only the exposure with the pesticide is required.

In this experimental tests, amperometric measurements need of a total time of 20 min versus the 4 min for impedimetric measurement, excluding the pesticide incubation time.

5.4.6 Kinetic analysis

Inhibition mechanism of AChE activity by pesticides is presented in the reaction scheme (5.2), explained above. Time dependent inhibition is commonly described by the following equation:

$$ln\frac{E}{E_0} = -\frac{k_3}{1+\frac{K_I}{I}}t \tag{5.5}$$

where E/E_0 represents the percentage of the remaining enzyme activity in relation to the initial activity (E_0), K_I is the dissociation constant for the enzyme – inhibitor complex, k_3 is the first – order rate constant for the conversion of the enzyme – inhibitor complex to carbamylated/phosphorylated enzyme, I is inhibitor concentration and t is incubation time (Krstic et al. 2008).

If $I >> E_0$ the slope of Eq. (5.5), dependent on pesticide concentration, can be expressed in the form (Colovic et al, 2013):

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_1}{k_3}\frac{1}{(I)} \tag{5.6}$$

Since the high correlation between the enzymatic activity inhibition and the impedimetric changes for all pesticides studied, we modified Eq. (5.5) as follow:

$$ln\frac{R_{ct}}{R_{ct0}} = -\frac{k_3}{1+\frac{K_I}{I}}t \tag{5.7}$$

where R_{ct0} and R_{ct} are the impedimetric signals of the AChE biosensor in absence and after the exposure of pesticide solution, respectively.

The values of k_{app} were obtained, for each pesticide at different concentrations, from the slope of the linear dependence of ln R_{ct}/R_{ct0} versus

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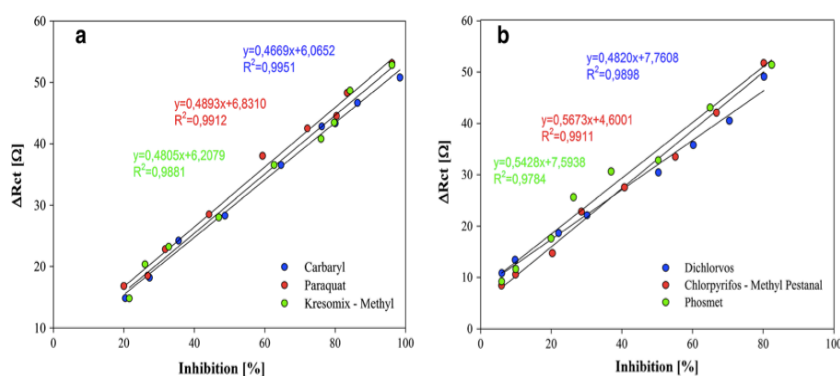impedimetric vs amperometric detection

t. the change of R_{ct} in time was monitored for five different pesticide concentrations by single frequency impedance (SFI): the tests were carried out at 0.1 Hz, as reported above. The change of R_{ct} and ln R_{ct}/R_{ct0} versus t for Carbaryl at five concentrations is shown in Figure 5.9a. The slopes of the plots (Figure 5.9b) give the k_{app} values. R_{ct} change during the time was studied for all CBs and OPs used in this study (Figure 5.10).
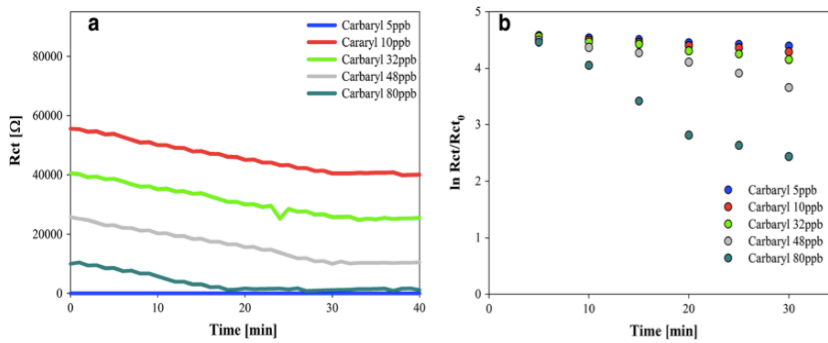
Figure 5.9: *Single frequency impedance data for Carbaryl at different concentrations (a). Progressive Rct decrease produced by reaction of AChE with different concentrations of Carbaryl plotted as semi-logarithmic curve in accordance with Eq. (5.7) (b).*

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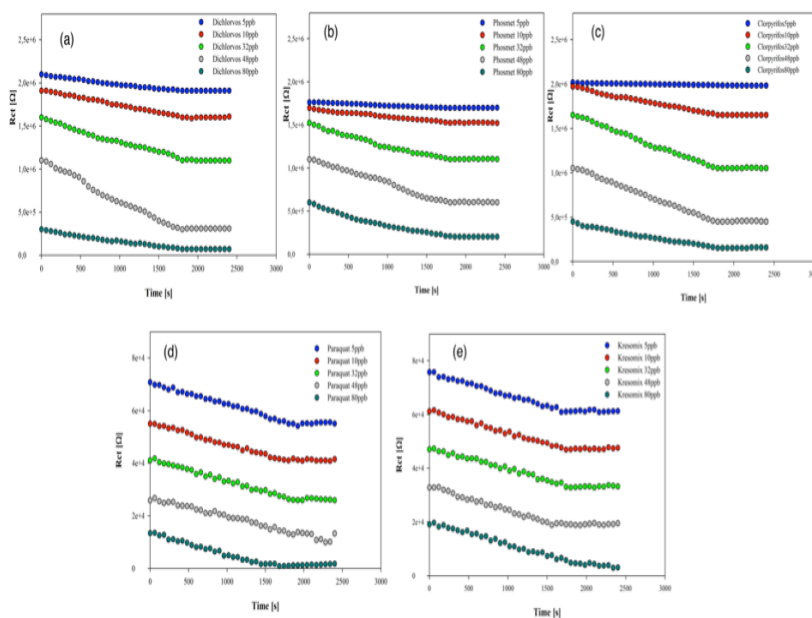
Figure 5.10: *Single Frequency Impedance data for Dichlorvos (a), Phosmet (b), Clorpyrifos (c), Paraquat (d), Kresomix (e) at different concentrations.*

Inhibition process by different pesticide concentrations, which progressed with time in accordance with Eq. (5.6) was analysed also for the all other pesticides.

The value of k_{app}, obtained from the slope of the dependence of ln R_{ct}/R_{ct0} versus t for all CBs and OPs tested were then plotted according to Eq (5.7) and the results are reported in Figure 5.11.
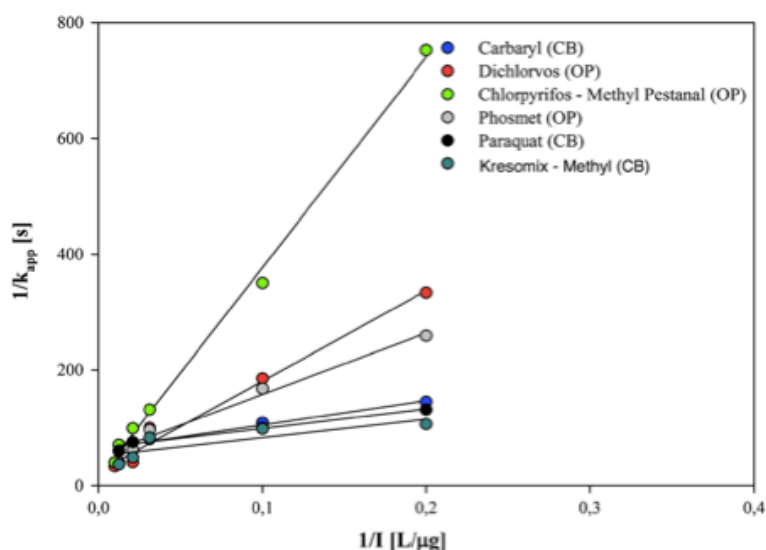
Figure 5.11: *Dependence of K_{app} upon the concentration of Clorpyrifos methyl Pestanal, Dichlorvos, Phosmet, Carbaryl, Paraquat and Kresomix methyl plotted as reciprocal in accordance with reaction.*

The inhibition parameters K_I and k_3 were calculated from the slope and the intercept of Eq. (5.7), for all carbamates and organophosphate compounds and the results are showed in Table 5.2.

Table 5.2: *Inhibition parameters KI and k3 for Carbaryl, Paraquat and Kresomix-methyl (as carbamates) and Clorpyrifos-methyl Pestanal, Dichlorvos and Phosmet (as organophosphates)*

	Pesticides	**K_I [mol/L]**	**k_3 [1/s]**
Carbamates	Carbaryl	3.00×10^{-6}	1.58
	Paraquat	7.92×10^{-6}	1.73
	Kresomix – methyl	7.44×10^{-6}	1.66
Organophosphates	Clorpyrifos-methyl Pestanal	7.56×10^{-7}	0.73
	Dichlorvos	6.91×10^{-7}	1.15
	Phosmet	2.75×10^{-8}	1.68

Dissociation constants (K_I) calculated for the OPs tested were in accordance with Colovic et al. 2013 and Mehta *et al.*, 2016 who evaluated the inhibition of AChE with Diazioxon and Clorpyrifos and parathion, respectively.

No K_I measurements were found in literature about CBs compounds, Since KI provides a measure of the dissociation of the enzyme – pesticide complex, the data obtained for the investigated CBs and OPs confirm the

irreversibility of binding between AChE – OP in contrast to the CBs where the acylated intermediate is slowly (about 1 h) hydrolysed to reactivate the enzyme (Darvesh et al., 2008).

5.4.7 Carbamates and Organophosphate compounds detection in real samples and interference studies

The possibility to apply the developed affinity enzymatic biosensor for the detection of pesticide in real food matrices was studied; Dichlorvos as organophosphate compound and Carbaryl as carbamate were selected. Tap water and lettuce samples were spiked with three different concentrations of Carbaryl and Dichlorvos and analysed by developed biosensor through impedimetric detection.

The results (Table 5.3) highlight that the developed biosensor exhibited a good recovery percentage for both carbamate and organophosphate compounds.

Table 5.3: *Carbaryl and dichlorvos results in spiked tap water sample obtained by developed biosensor*

Sample	Pesticide	Spiked concentration [ppb]	Biosensor result [ppb]	Recovery [%]
Tap water	Carbaryl	25	26.53±1.45	106.12±5.90
		50	49.69±1.86	11.62±1.60
		100	97.50±3.79	102.49±1.29
Tap water	Dichlorvos	25	24.87±3.79	100.50±2.48
		50	52.48±2.78	95.02±5.26
		100	97.92±3.98	102.07±0.95
Lettuce	Carbaryl	25	27.12±2.35	110.00±5.94
		50	52.14±1.49	104.28±3.63
		100	102.10±2.78	102.10±2.44
Lettuce	Dichlorvos	25	25.99±1.45	108.48±4.88
		50	51.08±2.67	102.16±3.75
		100	101.82±0.98	101.82±1.40

Heavy metal ions can inhibit the activity of AChE affinity biosensor. Two different concentrations of PB(II) (20 and 100 ppb) and Hg(II) (500 – 1000 ppb) were evaluated as inhibitors versus AChE. This interference study was carried out in absence of any pesticides. An interfering effect lower than 5% was measured at the tested lead concentrations. However, according to literature (Sanllorente-Mendez *et al.*, 2010), the most important interference was caused by Hg(II), which produces an interfering effect of 28 and 42% at

mercury concentrations equal to 500 and 1000 ppb, respectively. The interference study was carried out\ in absence of any pesticides; no interfering effect was measured at the tested lead concentrations.

5.5 Conclusions

A new impedimetric enzyme inhibition – based biosensor for carbamate and organophosphate compounds was proposed, immobilizing acetylcholinesterase enzyme on cysteamine modified gold electrode. The high - affinity interaction between pesticides and active site of the enzyme was monitored by electrochemical impedance spectroscopy, and the impedimetric changes obtained at different pesticide concentration allows to go up very fast to the inhibition degree of the toxic compounds. The developed affinity AChE biosensor, with its high number of attractive characteristics associated to the use of EIS transduction, namely very fast response and low cost of instrumentation and the ability to be miniaturized, is considered as promising candidate for pesticide detection on-site applications.

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Impedimetric affinity biosensors for *Escherichia Coli* O157:H7 detection

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Manuscript for submission

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6.1 Abstract

In this work the development and optimization of impedimetric label free immunosensors for the detection of *Escherichia Coli* O157:H7 is reported.

Different immobilization techniques of monoclonal anti – E.Coli were tested, in order to reach very low limit of detections.

The comparison between the immobilization procedures analyzed underlines the advantage of the oriented procedure and the use of a dendrimer, which allow to immobilize an higher number of antibodies, reaching a very high sensitivity, but the use of activated ferrocene as electron-transferring mediators, which improve the electrical properties of the system, results in a very low limit of detection equal to 3 cfu/mL.

This immunosensor was used to analyze milk and meat samples obtaining a good agreement with ELISA official methods results.

6.2 Introduction

Foodborne bacterial pathogens are believe to be the most frequently occurring hazard in the nation's food and water supply. *Escherichia Coli* O157:H7 has emerged as one of the deadliest foodborne pathogens because of its combination of virulence and pathogenicity (Buchanan *et al.*, 1997).

Illness caused by this organism range from bloody diarrhea to life – threatening conditions, such as hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. The Centre for Disease Control and Prevention estimates that there are 20000 illnesses a year due to *E.Coli* O157:H7 infection (Muhammad-Tahir & Alocilja, 2003).

Conventional methods for *E.Coli* O157:H7 detection include multiple – tube fermentation (MTF), membrane filter (MF), plate count; although these methods are very sensitive and selective, they are laborious and time – consuming: require hands – on preparation and 24 to 48 h of incubation time before suspected pathogens can be identified, which is not practical considering the short shelf – life and cost of storage of the food products (Deisingh *et al.*, 2002).

As consequence, detection of pathogens such as bacteria by a rapid, sensitive and cost effective method is therefore a subject of great interest in the field of food industry, environmental monitoring, clinical and diagnostic analysis.

Nowadays, biosensors, in particular immunosensors, play a significant role in the determination of pathogens: most of them use labeled antibodies to monitor the formation of the antigen – antibody complex (Leng *et al.*, 2017; Yang *et al.*, 2012; Heyduk *et al.*, 2012).

However, label – free immunosensors, in which the immune interaction between antibody and antigen is directly monitored, exhibit some important advantages in terms of speed and simplicity of operation. Several approaches

concerning the developed of label – free immunosensors for the detection of the pathogenic strain *E. Coli* O157:H7 have been reported in literature: these include different transduction techniques, such as Surface Plasmon Resonance (SPR), (Subramanian *et al.*, 2006), Quartz Crystal Microbalance (QCM) (Li *et al.*, 2011), Surface Acoustic Wave (Berkenpas *et al.*, 2006).

Among all the possible immunosensors (optical, microgravimetric, electrochemical), the electrochemical ones rank highly owing to their sensitivity, low cost and simplicity. In particular, the Electrochemical Impedance Spectroscopy (EIS) becomes promising transduction method for foodborne pathogenic bacteria detection due to its portability, rapidity and sensitivity: it is regarded as an effective way for monitoring the formation of antigen – antibody by probing the interfacial electrical properties of electrode surface. Thanks to EIS transduction technique, food biosensor analysis are performed in real-time by studying the change in electrical properties of the electrode surface which depends only on the binding interaction between the analyte and its receptor (Malvano *et al.*, 2016)

Different impedimetric label – free immunosensors for the detection of *E.Coli* bacteria have already been developed (Escamilla – Gomez *et al.*, 2009; Geng *et al.*, 2008; Yang *et al.*, 2016), while few studies have been conducted on the specific detection of the pathogenic strain *E.Coli* O157:H7.

Chowdhury *et al.* (2012) immobilized polyclonal anti – E.Coli on a conductive polyaniline film, reaching detection limits of 10_2 cfu/mL: a significant decrease in sensitivity of the sensor toward *Salmonella typhi* and *E.Coli* BL21 has been reached.

The lowest limit of detection (2 cfu/mL) was reported by Barreiros dos Santos *et al.* (2013), where anti – E.Coli antibodies have been immobilized onto gold electrodes via self – assembled monolayer of mercaptohexadecanoic acid: a larger linear rate ($3*10 - 3*10^4$ cfu/mL) was achieved but any tests on real food matrices were performed.

In order to detect *E.Coli* O157:H7 pathogen in food products, a highly sensitive and selective label – free impedimetric immunosensor has been developed in this study, immobilizing monoclonal anti – *E.Coli* O157:H7 on gold electrodes functionalized through different immobilization schemes.

The performances of all design as impedimetric immunosensor upon binding of *E.Coli* cells to the electrode was evaluated by measuring the electron – transfer resistance by EIS in presence of $[Fe(CN)_6]^{3/4}$ as redox probe.

Finally, the immunosensor developed with immobilization scheme that has shown the lowest limit of detection was used to analyze milk and meat samples inoculated with *E.Coli* O157:H7 cells.

6.3 Materials and Methods

6.3.1 Chemicals

Ferrocenecarboxylic acid (>97%), Cysteamine (95%), Glutaraldehyde solution (50% in H2O), Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), Polyamidoamine (PAMAM) dendrimer generation 4 (ethylenediamine core), were purchased from Sigma-Aldrich (Milano, Italy). Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$), was obtained from Carlo Erba reagent (Milano, Italy).

4-mercaptobenzoic acid (MBA, 99%), 2-(N-morpholino)ethanesulfonic acid (MES >99.5% purity), N-Hydroxysucciminide (NHS, 99%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, >99%), Sulfuric acid (H_2SO_4, 99.9%), Ethanolamine (EtNH$_2$ - $NH_2CH_2CH_2OH$, >99.5%), Ethanol (>99.8%) were purchased from Sigma-Aldrich (Milano, Italy), while Protein A/G (5 mg/mL, 59.7 kDa, >98%) was obtained from BioVision Inc. (San Francisco, USA).

The E.Coli O157:H7 antibody (1.4mg/ml) was purchased from Fitzgerald, while the E.Coli O157:H7 (heat killed) was received from SeraCare (Gaithersburg, USA).

Sodium phosphate monobasic (NaH2PO4), Sodium phosphate dibasic anhydrous (Na2HPO4), and Potassium Chloride (KCl) used in the preparation of phosphate buffered saline (PBS: 0.1 M KCl, pH 7.4) were obtained from Sigma Aldrich (Milano, Italy).

6.3.2 Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat (Metrohm), equipped with an Impedance module (FRA32M); the experimental data were analyzed with Nova software (Metrohm). Au thin-film single-electrodes, based on a three-electrode layout (working/auxiliary/reference) and the all-in-one electrochemical cell were purchased from Micrux Technologies (Oviedo, Spain). The diameter of Au working electrode was 1mm.

6.3.3 Immunosensors Manufacturing

Before modification, gold electrodes were cleaned by applying 13 potential cycles between -1.0 and +1.3 V with 100 mV/s scan rate in 0.05 M sulfuric acid.

Different immobilization schemes were adopted for the electrode functionalization with anti – *E.Coli* O157:H7:
1. Oriented (b) and not oriented (a) anti – *E.Coli* O157:H7 on MBA self – assembled monolayer;

2. Anti – *E.Coli* O157:H7 on electrochemically deposited cysteamine layers (c);
3. Anti – *E.Coli* O157:H7 on cysteamine and ferrocene layers (d)
4. Anti – *E.Coli* O157:H7 on PAMAM and ferrocene layers (e)

At the end of functionalization steps for all the above schemes, 10 μL of anti – *E.Coli* O157:H7 solution (1.4 ng/mL) was dropped on the surface of modified electrodes for 30 min at room temperature.

The immobilization of anti – *E.Coli* O157:H7 in oriented and not oriented way was carried out according the procedure described by Malvano *et al*. (2016). A constant potential of 1.2 V for 20 min was applied to the gold electrode dropped with 30 mM MBA ethanol solution; then the terminal carboxylic groups on gold electrode surface were activated with a solution of 75 mM EDC and 15 mM NHS in 100 mM MES buffer (pH 7.4) for 2 h.

In the oriented immobilization method, 20 μL of Protein A/G 5 mg/mL were dropped on the modified electrode and left to react for 1 h. After incubation, 100 μL of 1 M ethanolamine (pH 8.5) solution was dropped onto the modified surface and incubated for 15 min to block unreacted active sites. In the not oriented immobilization the anti – *E.Coli* O157:H7 solution was added after the activation of carboxylic groups with EDC/NHS. Then the electrode was rinsed in PBS to remove unbound antibodies and finally the unreacted active sites were blocked with 1 M ethanolamine.

For the immobilization schemes (c), (d), (e), cysteamine water solution 20 mM was dropped on the surface of the electrode and a constant potential of 1.2 V vs Ag/AgCl for 10 min was applied. After the electrode was thoroughly rinsed with water to remove physically – adsorbed cysteamine. After that three different schemes were tested:

1. Glutaraldehyde solution 5% (v/v) was dropped onto the cysteamine modified working electrode for 1 h and again the electrode was rinsed with water. After that, the modified electrode was covered with 10 μL of anti – *E.Coli* solution (1.4 ng/mL) for 30 min at room temperature. Finally the unreacted active sites were blocked with 1 M ethanolamine and the electrode was rinsed in PBS to remove unbound antibodies (scheme c).

2. The terminal carboxylic groups of ferrocene carboxylic acid were activated with a solution of 75 mM EDC and 15 mM NHS in 100 mM MES buffer (pH 7.4) for 2 h; then, the activated solution was dropped on Au cysteamine modified electrode and left to react for 1 h. After that, cysteamine water solution 20 mM was dropped again on the surface of the electrode and incubated overnight. After the treatment with 100 μL of anti – *E.Coli* solution (1.4 ng/mL) for 30 min at room temperature. Finally the unreacted active sites were blocked with 1 M ethanolamine and the electrode was rinsed in PBS to remove unbound antibodies (scheme d).

3. The immobilization of anti – *E.Coli* in presence of PAMAM dendrimer was carried out by glutaraldehyde deposition on the cysteamine layer; then PAMAM 2 mg/mL was dropped on it and left to react for 3 h. After that, activated ferrocene was incubated on electrode surface for 1 h. Finally, the electrode was covered with 10 µL of anti – *E.Coli* solution (1.4 ng/mL) for 30 min at room temperature. Finally the unreacted active sites were blocked with 1 M ethanolamine and the electrode was rinsed in PBS to remove unbound antibodies (scheme e).

6.3.4 Experimental Measurements

Electrochemical Impedance Spectroscopy (EIS) was used to characterize each step of the electrode modification and the interactions antibody - *E.Coli* O157:H7 cells.

For electrochemical impedance studies, a sinusoidal AC potential (10 mV) in the frequency range from 0.1 to 10^5 Hz was superimposed to 0.00 mV (vs. reference electrode) DC potential; the measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$, 1:1) in PB, 0.1 M pH 7, as background electrolyte, at room temperature.

The impedance data were plotted in the form of Nyquist plots, where the complex impedance is displayed as the sum of the real and imaginary components (Z^I and Z^{II} respectively) and in the form of Bode diagram, where the total impedance of the system (Z) is plotted versus frequency. Experimental spectra were fitted with a proper equivalent circuit using the facilities of FRA32M (Nova Software).

Cyclic Voltammetry measurements (CV) were also used to monitor the construction of the immunosensors design: the measurements were performed from -0.6 to 0.6 V vs. reference electrode with a scan rate of 0.05 V/s; the redox couple used for the CV was the same as that used for impedance measurements.

For *E.Coli* measurements, 1 mL of *E.Coli* cells at different concentrations were dropped onto the electrode working area and incubated for 90 minutes; before the impedance measurement, the immunosensors were rinsed with copious amount of PB.

6.3.5 Preparation of food samples for E.Coli detection

25 g of meat sample was stomached for 2 minutes at 120 rpm in 250 mL of PBS. The sample was filtered. This procedure was applied for three different *E.Coli* concentrations spiked to grinded samples in order to obtain 10^3 cfu/mL, $5*10^3$ cfu/mL and 10^4 cfu/mL.

As regards the milk sample, 25 mL of milk properly spiked, was mixed in 250 mL of PBS and then it was spiked with the same three different *E.Coli* concentrations used for meat sample.

The results obtained with the immunosensor were compared with those measured with *E.Coli* O157:H7 Test Kit (Bioo Scientific, United States) for *E.Coli* detection.

6.4 Results

6.4.1 Design and comparison of different immobilization layers: CV and EIS characterization

In the fabrication of a biosensor, the method of antibodies immobilization on conductive surface plays a crucial role in the analytical performances of the immunosensor. Here, five different immobilization schemes were analysed and compared, named as:

a) $MBA + EtNH_2 + anti\text{-}E.Coli$
b) $MBA + ProteinA/G + EtNH_2 + anti\text{-}E.Coli$
c) $Cys + anti\text{-}E.Coli + EtNH_2$
d) $Cys + Ferrocene + Cys + anti\text{-}E\text{-}Coli + EtNH_2$
e) $Cys + Ferrocene + PAMAM + anti\text{-}E.Coli + EtNH_2$

The surface modifications of the Au electrodes for the preparation of E.Coli immunosensor was monitored using Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS), which provide useful information on the electrical changes of the electrodes behaviour after each assembly step.

The voltammograms of the Au electrode display well defined anodic and cathodic peaks due to the reversible interconversion of redox probe $[Fe(CN)_6]^{3/4}$. After the Au electrode was covered with short – chain thiols of MBA (immobilization schemes a and b – Figures 6.1a and 1b) and cysteamine (immobilization schemes c, d and e – Figures 6.1c, 1d, 1e), the permeability of ion through it was so low that the redox probe doesn't penetrate in and therefore the response current decreases (Figure 6.1).
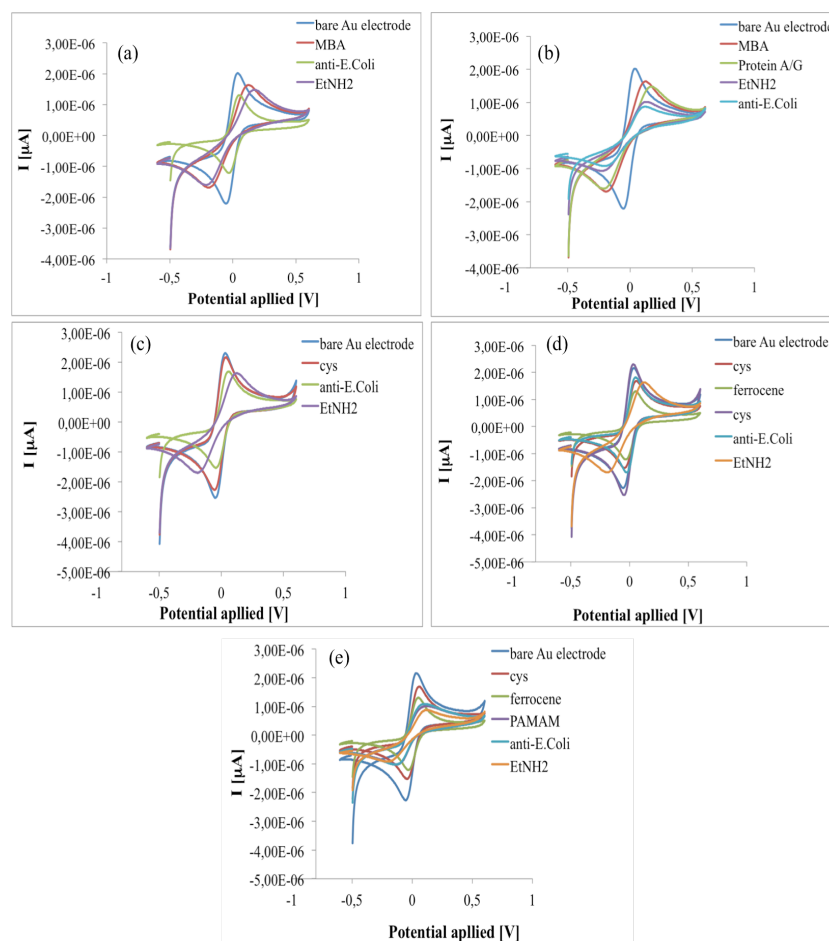
Figure 6.1: *Cyclic Voltammograms in 1mM [Fe(CN)$_6$]$^{3/4}$ after each step of immunosensors construction for all immobilization schemes tested in this work: MBA+anti-E.Coli+EtNH$_2$ (a), MBA+ProteinA/G+EtNH$_2$+anti-E.Coli (b), Cys+anti-E.Coli+EtNH$_2$ (c), Cys+Ferrocene+Cys+anti-E.Coli+EtNH$_2$ (d), Cys+Ferrocene+PAMAM+anti-E.Coli+EtNH$_2$ (e)*

According to these results, Nyquist plots showed an increase of the impedance, most significant for the electrode modified with MBA (Figure 6.2 a and b) instead of cysteamine (Figure 6.2 c, d and e), caused by dramatic reduction of the redox probe diffusion close to the Au modified electrode surface due to the hindering effects of the layers in the electron transfer rate.

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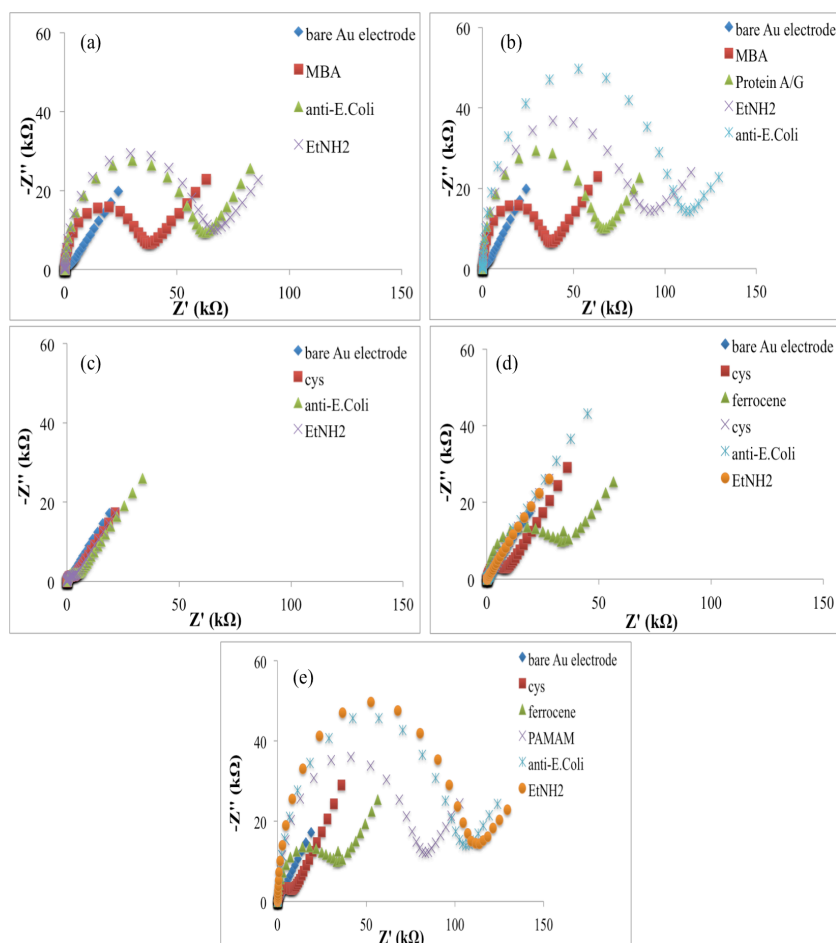
Figure 6.2*: EIS responses to all immunosensor fabrication steps for all immobilization procedures tested in this work: MBA+anti-E.Coli+EtNH$_2$ (a), MBA+ProteinA/G+EtNH$_2$+anti-E.Coli (b), Cys+anti-E.Coli+EtNH$_2$ (c), Cys+Ferrocene+Cys+anti-E.Coli+EtNH$_2$ (d), Cys+Ferrocene+PAMAM+anti-E.Coli+EtNH$_2$ (e)*

As result of the immobilization on the MBA-modified electrode surface of EtNH$_2$ (Figure 6.2a), for a not oriented antibody immobilization (procedure a), and ProteinA/G+EtNH$_2$ (Figure 6.2b), for an oriented antibody immobilization (procedure b), the permeability of $[Fe(CN)_6]^{4-/3-}$ through the immobilization layers was strongly reduced with a significant increase of the electron transfer resistance.

In the immobilization schemes (d) and (e), ferrocene was used to act as an electron-transferring mediators to shuttle electrons between the electroactive probe and electrode surface; thanks to its conductivity, it

improves the electric properties of the system, in order to reach very low detection limits (Kim *et al.,* 2003).

In order to verify the correct immobilization of ferrocene molecules on cysteamine-modified electrodes, the total amount of immobilized ferrocene was calculated according to Radi *et al.,* 2009, through the following equation:

$$\Gamma = \frac{Q}{nFA} \qquad\qquad (6.1)$$

where n is the number of electrons transferred (n=1), F the Faraday constant, A is the effective square area and Q is the Faradaic charge required for the full oxidation of the immobilized layer; this value is reached from the integration of the area under either the anodic or the cathodic peak, corrected from the background current, measured at a slow potential scan rate.

According to Eq. (6.1), the amount of ferrocene molecules immobilized on cysteamine-modified electrodes, for both immobilization procedure d and e, is equal to $3.29*10^{-9}$ mol/cm^2: this amount is enough to restrict slightly the access of the redox probe to the electrode, resulting in an increase of the electron transfer resistance (Figure 6.2 d, e).

Exploiting the electrocatalytic properties of ferrocene, in the last configuration (e), fourth generation PAMAM dendrimer (having 64 surface amines) was used as linker for the antibody in order to increase the sensitivity: when PAMAM was loaded on cysteamine/ferrocene – modified electrode, the current response decreased (Figure 6.2 e) with an increase of the impedance (Figure 6.2 e), suggesting that dendrimer molecules were correctly immobilized on electrode surface.

6.4.2 Immunosensors analytical performances

When the immunosensors react with increasing concentration of *E. Coli* cells, an increase of semicircle diameters of Nyquist plots was observed for each immobilization procedure tested in this work (Figure 6.3), corresponding to the charge transfer resistance R_{ct} of the Randle's Circuit used for data fitting.
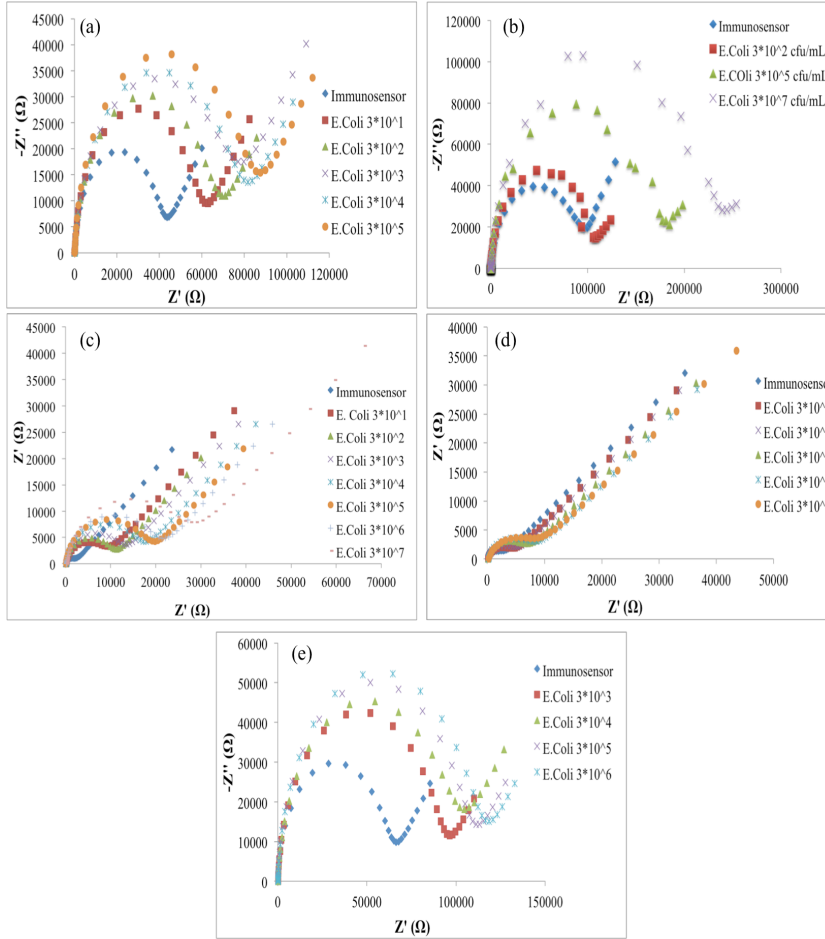
Figure 6.3: *Nyquist plots in impedance measurements before and after the interaction of the immunosensors with different E.Coli concentrations, for all immobilization procedures tested in this work: MBA+anti-E.Coli+EtNH$_2$ (a), MBA+ProteinA/G+EtNH$_2$+anti-E.Coli (b), Cys+anti-E.Coli+EtNH$_2$ (c), Cys+Ferrocene+Cys+anti-E.Coli+EtNH$_2$ (d), Cys+Ferrocene+PAMAM+anti-E.Coli+EtNH$_2$ (e)*

This parameter was used to characterize the immunosensors fabricated; in particular, ΔR_{ct} was used to obtain the calibration curves, expressed as a function of the logarithmic value of the *E. Coli* cells concentration. ΔR_{ct} values were calculated by the following equation:

$$\Delta R_{ct} = R_{ct(E.Coli)} - R_{ct(AntiE.Coli)}$$

(6.2)

where R_{ct} is the value of electron transfer resistance when anti – *E.Coli* is immobilized on the electrode surface and $R_{ct(E.Coli)}$ is the value of electron transfer resistance after the bind between antibody – *E.Coli* cells.

The calibrations curves of all immunosensors developed are showed in Figure 6.4:

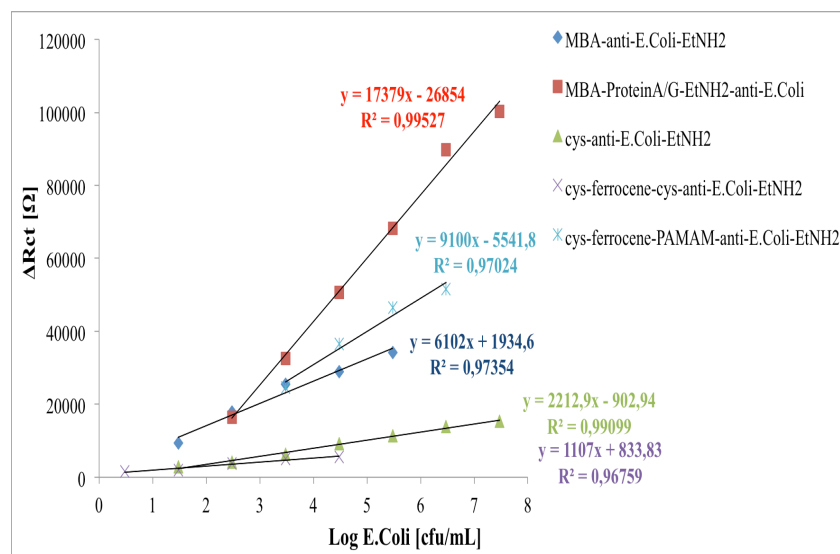**Figure 6.4**: *Calibration curves of immunosensors developed*

The analytical characterization of the immunosensors developed on MBA-modified electrodes versus different concentration of *E.Coli* showed, for the not oriented immunosensor, a closer linear range ($3x10^1$ - $3x10^5$cfu/mL) then oriented one ($3x10^2$ - $3x10^7$cfu/mL), but a lower detection limit (LOD), calculated using the sum of average blank solution and three times the standard deviation ($3x10^1$ cfu/mL for not oriented versus $3x10^2$ cfu/mL for oriented): this means that in case of not oriented antibodies a number of molecules, lower than in the oriented one, is effectively exposed to cells interaction and that the immunosensor decrease its LOD when the effectively immobilized antibodies are optimally minimized in order to still obtain a signal from the transducer.

On the contrary, among the immunosensors developed with anti-E.Coli immobilization by a self – assembled monolayer of cysteamina, the highest sensitivity was found for the immunosensor fabricated with PAMAM dendrimer. As reported in literature (Yoon *et al.*, 2000), the immobilization network containing dendrimers as a building unit represent a number of advantages such as: structural homogeneity and high density of identical functional chain end groups, which enables structurally stabilized constructs, and internal porosity which preserves the permeability of the resulting layer, still maintaining the rigidity of the construct.

Moreover, the alternate deposition of dendrimers and conductive ferrocene molecules has allowed to improve electrical response of the system and to guarantee, thanks to PAMAM functional groups, an higher number of immobilized anti-E.Coli. Moreover, higher level of biomolecule immobilization raised the density of the sensor increasing the coupling capacity between bio receptor and target.

Even if the PAMAM dendrimer was used in order to increase the sensitivity, the lowest detection limit (3 cfu/mL) was reached in the immunosensor developed with cysteamine/ferrocene procedure (procedure d); as explained above, a lower number of antibodies immobilized allow reaching a lower detection limit.

The reproducibility, calculated on five different immunosensors at 3×10^3 cfu/mL *E.Coli*, showed a good relative standard deviation for all immunosensors developed.

The comparison of the analytical performances of developed label free impedimetric immunosensors with other recent impedimetric label – free immunosensor for *E.Coli* O157:H7 detection is reported in Table 6.1.

Table 6.1*: Comparing study between the present work with some recent impedimetric label – free immonosensors for E.Coli O157:H7 detection*

Schematic Immunosensor Assembly	Sensitivity [$\Omega \bullet mL/cfu *cm^2$]	Linear Range [cfu/mL]	LOD [cfu/mL]	References
Au+PANI+Glu+ anti-E.Coli		$1.0 \times 10^2 - 1.0 \times 10^7$	100	Chowdhury *et al*., 2012
Au+MHDA	772050.0	$3.0 \times 10^1 - 3.0 \times 10^8$	2	Barreiros dos Santos *et al*., 2013
MBA+anti-E.Coli +EtNH$_2$	784615.3	$3 \times 10^1 - 3 \times 10^5$	30	This work
MBA+Protein A/G+ EtNH$_2$+anti-E.Coli	2228076.9	$3 \times 10^2 - 3 \times 10^7$	300	This work
Cys+anti-E.Coli +EtNH$_2$	283589.7	$3 \times 10^1 - 3 \times 10^7$	30	This work
Cys+Ferrocene+ Cys+ anti-E.Coli+EtNH$_2$	141923.0	$3 \times 10^0 - 3 \times 10^5$	3	This work
Cys+Ferrocene+P AMAM+anti-E.Coli+EtNH$_2$	1166666.6	$3 \times 10^3 - 3 \times 10^6$	3000	This work

PANI: Polyaniline; Glu: Glutaraldheyde]
MHDA: 16-mercaptohexadecanoic acid

The immunosensor developed with antibodies immobilization on cysteamine/ferrocene (procedure d) showed a wide linear range and the lowest LOD when compared with the other impedimetric label – free immunosensors.

In order to obtain information about the morphological characteristics E.Coli cells, AFM technique was carried out (Figure 6.5).
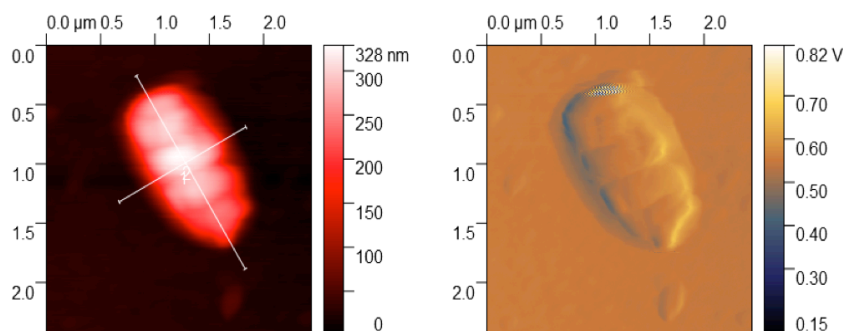

Figure 6.5: *AFM images of a E.Coli cell*

Figure 6.5 shows an AFM picture of a selected working area functionalized with E. coli antibodies that was exposed to 10^6 cfu/ml of E. coli solution. A micro-object of approximately 2μm*0.5μm can clearly be seen in this picture. The dimensions of the mirco-object correspond to the dimensions of an E. coli bacterium that is known to be a cylindrical object, measuring approximately 2μm in length and 0.8 μm in diameter (Duplan *et al.*, 2011). The slightly reduced height of the bacterial cell observed in this experiment could be explained by the possible flattening occurring due to the pressure exercised by the AFM cantilever tip as well as shrinking of bacteria due to the drying and fixing procedure.

6.4.3 Analysis of E.Coli O157:H7 in food samples

In order to highlight the *E.Coli* O157:H7 cells detection capability of the immunosensor developed with activated ferrocene on cysteamine – modified electrode (procedure d) that showed the lowest detection limit, meat and milk samples were spiked with three different *E.Coli* concentrations and analyzed by the immunosensor and the ELISA kit for *E.Coli* detection. The sample preparation used was the same for both analytical methods. The results are shown in Table 6.2.

Table 6.2: *E.Coli results in spiked milk and meat samples obtained by impedimetric immunosensors and Elisa kit.*

Sample	Spiked Concentration [cfu/mL]	Immunosensor		Kit	
		Immunosensor [cfu/mL]	Recovery [%]	Found concentration [cfu/mL]	Recovery [%]
Milk	1.00×10^3	0.72×10^3	72	1.08×10^3	108
	$5.00 * 10^3$	4.90×10^3	98	4.76×10^3	95.2
	1.00×10^4	9.14×10^3	95.5	11.69×10^4	116.9
Meat	1.00×10^3	0.98×10^3	98	0.89×10^3	89
	5.00×10^3	5.24×10^3	104.8	4.16×10^3	83.2
	1.00×10^4	9.55×10^3	95.5	9.62×10^4	96.2

The immunosensor developed with activate ferrocene on cysteamine modified electrode exhibited a good recovery percentage, highlighting the potential of proposed immunosensor as a highly capable analytical device for a fast *E.Coli* O157:H7 measurement in food products.

6.5 Conclusions

Different anti-E.Coli immobilization procedures were tested in this work, in order to develop a new label – free impedimetric immunosensor for the *Escherichia Coli* O157:H7 detection in food products.

The comparison between the immobilization procedures analyzed underlines the advantage of the oriented procedure and the use of PAMAM dendrimer, which allow to immobilize an higher number of antibodies, reaching a very high sensitivity, but also the use of activated ferrocene as electron-transferring mediators, which improve the electrical properties of the system, resulting in a better impedimetric response

However, the lowest limit of detection of only 3 cfu/mL was obtained in the *E.Coli* immunosensors developed on cysteamine/ferrocene – modified electrode: a good agreement between ELISA official methods and this immunosensor was obtained in *E.Coli* cells analyzed in food samples.

6.6 References

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Conclusions

This work has been focused on the development and optimization of label -free affinity biosensors, based on Electrochemical Impedance transduction system. The developed affinity biosensors has been targeted for quali-quantitative detection, in real time, of chemical pollutants, pathogenic bacteria and allergens in food products or during their manufacturing process.

In particular, my interest has been turned to four analites: Ocrhatoxin A (among mycotoxins), carbamates and organophosphate compounds (among pesticides), gluten (among allergens) and, finally, *Escherichia Coli* O157:H7 (among patogenic bacteria)

A label – free impedimetric immunosensor for sensitive detection of Ochratoxin A (OTA) in food matrices was developed: the comparison between the two immobilization procedures (oriented and not oriented monoclonal antibody) used for the fabrication of immunosensors underlines the advantages of the oriented immobilization, which showed a more uniform and homogenous antibody layer that favors higher antigen-binding capacity and sensitivity of the immunosensor. The results obtained with AFM analysis underlining that in case of oriented antibody a more ordered surface guarantees a higher number of molecules effectively exposed to antigen interaction. Finally the linear range, the very low detection limit and high sensitivity showed the potential of the proposed immunosensors as a highly capable analytical devices for a fast OTA measurement in food matrices.

After that, in order to obtain a cheaper device, the first impedimetric immunosensor for OTA detection based screen printed electrode was developed. The surface of carbon electrode was firstly modified with electrochemical gold deposition, which has demonstrated a very cheap way to obtain gold-like behaving electrodes using a very small quantity of the metal (122 pg per electrode). Capacitance was chosen as the best parameter that describes the electrical changes of the electrode surface due to the immunoreaction between anti-OTA and OTA at different concentrations.

The developed immunosensor, with its very low detection limit and high sensitivity, exploits the advantages of cheapness, simplicity, and versatility

of the SPCE and its results are suitable for very fast OTA measurement in food matrices.

With extreme interest towards celiac disease and with the aim to guarantee the food safety for celiac patients a label-free impedimetric aptasensor for *Gluten* detection, based on the immobilization of aptamer (Gli1) on the gold electrode modified with PAMAM, has been developed.

The dendrimer has been proven to increase the sensitivity of the aptasensor with a high binding affinity to PWG gliadin. The aptasensors developed is very sensitive to gluten with a detection limit of 5 ppm. Moreover, a good agreement between R5 ELISA official method and aptasensor has been obtained in gluten content analysed in food samples. The analysis of gluten does not require the use of other reagents but only the gliadin extract, thus it makes the impedimetric aptasensor a fast and simple method for the control of food safety in food products addressed to celiac patients diet.

It is worth noting that this aptasensor is the first one based on the immobilization of aptamer on the modified electrode and all the others were based on competitive assays. This condition makes the analysis of gliadin very fast and easier than aptamer competitive assays that required addition of an enzyme labelled aptamer to the food sample and then the enzymatic substrate

As regards pesticides contamination, a very innovative impedimetric enzyme inhibition-based biosensor for carbamate and organophosphate compounds has been developed, immobilizing Acetylcholinesterase enzyme on cysteamine-modified gold electrode. The high affinity interaction between pesticides and active site of the enzyme was monitored by electrochemical impedance spectroscopy, and the impedimetric changes obtained at different pesticide concentrations allow to go up very fast to the presence of the toxic compounds in tap water or food matrices. The developed affinity AChE biosensor, with its high number of attractive characteristics associated to the use of EIS transduction, can be considered as promising candidate for pesticide detection on-site applications.

Finally, ss regard pathogenic bacteria, different monoclonal anti - E.Coli immobilization procedures were tested: the comparison between the immobilization procedures analyzed underlined the advantage of the oriented procedure and the use of PAMAM dendrimer, which allow to immobilize an higher number of antibodies, reaching a very high sensitivity, but also the use of activated ferrocene as electron-transferring mediators, which improve the electrical properties of the system, resulting in a better impedimetric response. However, the lowest limit of detection of only 3 cfu/mL was obtained in the *E.Coli* immunosensors developed on cysteamine/ferrocene – modified electrode: a good agreement between ELISA official methods and this immunosensor was obtained in *E.Coli* cells analyzed in food samples.

The study of different immobilization techniques and the use of Electrochemical Impedance Spectroscopy as transduction technique allowed the development of label – free affinity biosensors extremely sensitive and selective, able to detect some of the most common food contaminants and allergens; the use of this rapid and cheap innovative analytical method could be of great importance to ensure safety in food sector.

…in quegli occhi
la debolezza
di chi sa di essere
dolcemente forte…