DEVELOPMENT OF AN ELECTROCHEMICAL SENSOR FOR COELIAC DISEASE SEROLOGICAL MARKERS

Luis Carlos Rosales Rivera

Dipòsit Legal: T. 1467-2012

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Departament d'Enginyeria Química

UNIVERSITAT ROVIRA I VIRGILI

Tarragona

2012

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CERTIFY THAT:

The Doctoral Thesis entitled: "**Development of an electrochemical sensor for coeliac disease serological markers**", presented by **LUIS CARLOS ROSALES RIVERA** in order to obtain the degree of Doctor by the Universitat Rovira i Virgili, has been carried out under our supervision, at the Department of Chemical Engineering.

Tarragona, 12 November 2012

Dr. Ioanis Katakis

Dr. Pablo Lozano

Acknowledgements

First of all, I would like to express my most sincere gratitude to my supervisors Dr. Ioanis Katakis and Dr. Pablo Lozano for their help, time, patience and continuous guidance along the three years of this PhD.

I would like to thank also Dr. Xavier Rius for the challenging opportunity to continue my studies in Tarragona, for his valuable classes and his internationalization efforts of the URV. My special thanks to Dr. Ciara O'Sullivan for her guidance and uninterrupted help through all my work, without her it would be impossible to finish this *Thesis*.

I would like to thank additionally to all the INTERFIBIO members, to Mary Luz and Jos for their invaluable time and comments, to Diego Bejarano for his excellent comments and support, to MariCarmen for her great patience, knowledge and tireless work, to the present doctorate students Alemayehu, Viji, Mabel, Samuel, Jonathan, Gael, Ewelina, Joan, Ahmed. To all the Irish and other countries partners that came to work in the labs Sarah, Elaine L., Elaine M., Sharon, Ann, Laura, Niamh, Luke, Elaine O.R., Christian and Elif.

Special thanks to former members Dr. Hamdi Joda, Dr. Laia Civit, Dr. Marketa Svobodova, Dr. Pedro Nadal, Dr. Alessandro Pinto. To the senior members of the group Dr. Olivier Henry, Dr. Mayreli Ortiz, Dr. Alex Fragoso, Dr. Teresa Mairal and Dr. Ángel González. To the administrative staff Barbara Vastenavond, Núria Juanpere, Sira Durán, Laura Cortés and Núria Golobardes.

To the Master's in nanoscience classmates and the good moments we shared together: Tilahun, Emre, Luz Mary, Sibel, Xavi, Hany, Surya, Gisaw, Carlos, Josep and Daniele.

Un agradecimiento especial al Dr. Bruno Teixera por su gran apoyo en la parte final de la estructura de esta Tesis, sin su enorme paciencia y tiempo, no hubiera sido posible terminar a tiempo la escritura.

También quiero agradecer al Dr. Valerio Beni por su ayuda a lo largo de este doctorado, por proporcionarme los electrodos de oro necesarios para terminar y por las excelentes charlas que mantuvimos siempre con mucha seriedad.

Quiero agradecer a todos mis amigos de Tarragona, Rafa, Stefy, Alba, Marta, Roser Merlos, Roser Vidal, Belén, Lucie, Felix, Jaumet, Alex (CR7), Juanra, Delfin, Mary por su invaluable apoyo por los momentos inolvidables que vivimos juntos. También a todos los jugadores profesionales de voleibol playa, por esos grandes veranos deportivos.

A mis compañeros de piso, porque gracias a ellos pude integrarme completamente y por enseñarme que el valor de Catalunya está en su gente, Jordi gracias por aceptarme y por enseñarme a cocinar la mejor tortilla de patatas de Tarragona, Rico ha sido un honor haber compartido piso, Antonio espero que termines tu doctorado a tiempo y recupérate pronto, han sido los mejores compañeros de piso que alguien puede pedir. Sin olvidar a las ultimas personas con las que he vivido Alex F. y María. así como por los fichajes estrella que pasaron por el piso.

Un gran abrazo a Martha y Gustavo, los defeños en Tarragona, por su apoyo incondicional y los grandes momentos que compartimos, les deseo lo mejor y que encuentren al fin un lugar donde puedan estar juntos de nuevo. A brindar siempre por los Pumas, aunque sepamos que el Atlas es mucho mejor.

A mi familia, que nunca han dejado de apoyarme por completo, Liz, Mario, Mirna son los mejores hermanos que alguien puede pedir y a mis padres, gracias por estar ahí siempre, espero estar con ustedes muy pronto.

Pero mi mayor agradecimiento es para ti Myrna, sin ti no hubiera sido posible continuar, me has dado el apoyo que necesitaba para terminar.

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This Thesis has been possible thanks to the support of the following research grants:

Doctoral fellowship of the FI-DGR program. Funded by the Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya and the European Social Fund.

Commission of the European Communities, RTD programme "Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CDMEDICS [FP7-2007- ICT-1 IST-2007-2-216031.

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Summary

Coeliac disease (CD), a gluten-sensitive enteropathy, is an autoimmune disorder of the upper small intestine triggered from the gluten ingestion (cereal protein that can be found in wheat, rye and barley) and affects 1% of the population around the globe. The ingestion of gluten, triggers the production of a series of autoantibodies against gliadin (AGA) and tissue transglutaminase (tTG) that are used for the serological detection of the disease. Serological methods are non-invasive, fast and reliable techniques that have become an alternative for diagnostics of several diseases, including the autoimmune (Ciclitira 2001; McGough and Cummings 2005).

Immunosensors are used for the serological detection of proteins and antibodies in complex samples, e.g. serum or blood. They are affinity biosensors based on the specific interaction between antigens and antibodies, this interaction could be monitored by several techniques including electrochemistry. Amperometric detection is one of the most used techniques in immunosensors research and development. This technique represent several advantages compared to other methods, it's very simple to use and also possess the capacity of miniaturization, portability, fast analysis time and low cost of production (Holford et al. 2012; Luppa et al. 2001).

In this *Thesis* it's demonstrated the amperometric detection of serological markers against CD. Using two strategies for the antigen immobilisation on gold electrodes: i) bipodal alkanethiol bounded to the surface by a carboxylic acid terminal for antigen immobilization, ii) thiolation of the protein by conjugation through three different moieties of the antigens: (amine, carboxylic and hydroxyl groups).

Results obtained by amperometric detection, were compared with other available technologies, such as surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS). The usage of these technologies as a complement to the amperometric studies, not only increased the quality of the research, but also helped in the

construction and improvement of assembled immunosensors. They weren't only very reliable, but also depicted excellent properties to be used in miniaturized chip arrays or in flow through devices. This *Thesis* is organised in six chapters and a brief explanation for each of them is shown below:

In *Chapter 1*, a brief introduction about biosensors, its properties and their principal parts, is elucidated. Biosensors classification and definitions, especially immunosensors is reviewed; amperometric based and their specific applications in particular fields. CD description, associated symptoms and latest serological detection advances are also described. Finally, immunosensors involved for the detection of CD autoantibodies using different transduction methods are compared and analysed.

In *Chapter 2*, the general objective of this *Thesis*, and the specific objectives of each chapter are defined.

In *Chapter 3*, the proof-of-principle for the detection of anti-gliadin antibodies, is demonstrated not only in buffer solutions, but also in complex matrices, such as serum samples. Self assembled monolayers of dithiol molecules, with polyethylene glycol groups in order to reduce the non specific adsorption, are studied. Assembly of immunosensors is followed using surface plasmon resonance (SPR) and electrochemical impedance spectroscopy studies (EIS). Finally, amperometric detection of serum samples from ELISA commercial kits are investigated and compared (Rosales-Rivera et al. 2011).

In *Chapter 4*, a similar dithiol-SAM approach investigated in *Chapter 3*, is used to detect and quantify the presence of human antibodies (IgA isotype) in buffer solutions and serum samples from CD patients. Cross reactivity experiments, for antibodies of the IgG isotype, are performed for the assembled immunosensor. Foetal bovine serum solutions, spiked with IgA concentrations are used to analyze the performance of the electrodes in complex protein mixtures. Finally, the quantification of antibodies is done for IgA deficient and non-deficient

patients, and the obtained results compared with ELISA tests using the same patients (Rosales-Rivera et al. 2012).

In *Chapter 5*, the direct adsorption of thiolated proteins (gliadin and tTG) on gold surfaces are studied. Introduction of disulphide groups through different moieties present in the proteins: amine, carboxylic and hydroxyls groups. ELISA tests are done to prove the antigenicity of the chemically modified proteins. Amperometric and SPR detection studies of AGA and anti-tTG antibodies are performed in buffer solutions and using reference serum samples, from commercial ELISA kits. Finally, detection of AGA and anti-tTG antibodies from patients positive to CD are performed, using two serological markers, AGA IgG and anti-tTG IgA, results are compared with ELISA tests.

In *Chapter 6*, the general conclusions about this *Thesis* are presented and future work is discussed.

Keywords: Electrochemical immunosensors; Self assembled monolayer (SAM); Antigliadin antibodies; Human IgA antibodies; Anti-tissue transglutaminase; IgA deficiency; Coeliac disease.

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Abbreviations

Ab/Abs	Antibody/Antibodies
AFM	Atomic force microscopy
Ag/AgCl	Silver/Silver chloride reference electrode
Ag/Ags	Antigen/Antigens
AGA	Anti-gliadin antibodies
Anti-tTG	Anti-tissue transglutaminase antibodies
ARA	Anti-reticulin antibodies
AU	Arbitrary units
BSA	Bovine serum albumin
CD	Celiac disease
CPE	Constant phase element
CRP	C-reactive protein
CV	Coefficient of variation
DBI	Dot-blot immunoassay
DNA	Deoxyribonucleic acid
DT2	22-(3,5-bis((6 mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid
EC50	Half maximal effective concentration
EDC	N-(3-dimethylaminopropyl) -N'-ethylcarbodiimide
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay

EMA	Anti-endomysial antibodies
FBS	Foetal bovine serum
FET	Field effect transistor
Hb	Haemoglobin
HCPS	Hantavirus cardiopulmonary syndrome
HF	Human fibrinogen
HQ	Hydroquinone
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IgA	Immunoglobulin A
IgG	Immunoglobulin G
ISE	Ion selective electrode
LOD	Limit of detection
LSV	Linear sweep voltammetry
mAb	Monoclonal antibody
MES	2-(N-morpholino) ethanesulfonic acid
MUA	11-mercaptoundecanoic acid
NAs	Nucleic acids
NHS	N-hydroxysuccinimide
NSA	Non specific adsorption
ODS	o-dianisidine

pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with tween 20
PEG	Polyethylene glycol
ppb	Parts per billion
ppt	Parts per trillion
PSA	Prostate specific antigen
Rct	Resistance to charge transfer
RIA	Radioimmunoassay
Rs	Solution resistance
RSD	Relative standard deviation
SAM	Self assembled monolayer
SD	Standard deviation
SPCE	Screen printed carbon electrode
SPGE	Screen printed graphite electrode
SPR	Surface plasmon resonance
TMB	3,3,5,5- Tetramethylbenzidine
tTG	Tissue transglutaminase
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
UV-Vis	Ultraviolet-Visible spectrophotometer
θ	Fractional coverage

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CHAPTER 1

Introduction

1.1 Chapter introduction

In this *chapter*, the hypothesis is defined and the principal characteristics of biosensors are explained, including definitions, and their classification according to their biological recognition element and transducer. It focuses however in immunosensors, especially electrochemically operated and some of their applications in the last 10 years. A description of coeliac disease, an autoimmune disease that affects the 1% of most populations, is provided with emphasis on the major advances in the serological detection of this disease and the improvements that have been achieved using amperometric immunosensors.

1.1.1 Motivation for biosensors research

The continuous development, improvement and innovation of biosensors, since its first appearance in 1962, is due to their excellent properties and growth potential. Biosensors are a less expensive alternative to other technologies already in use, e.g. classical immunoassays (ELISA). Its integration with other technologies, such as lab-on-chip devices or able to perform point-of-care testing, have demonstrated the great opportunities that lies within biosensors, when they are efficiently integrated and correctly implemented.

There are many challenges for biosensors, in order to be cost-effective devices, they need to produce rapid results, operate with little sophistication and have a low-cost production. Successful biosensor applications, are already used in medical applications; however, many challenges need to be overcome for other fields such as food analysis, environmental monitoring of pollutants and pharmaceutical development. Commercial potential of biosensors and their impact on the quality of life can be important, if the principal limitations related to cost and some key technical barriers are overcome (Luong et al. 2008).

1.1.2 Hypothesis

The construction of a sensitive, rapid and cost-efficient real-sample-oriented immunosensor can be achieved using thiol SAM formed on gold surfaces. The design of this immunosensor can reduce the time and effort associated with a real case study.

1.2 Biosensor definition

The IUPAC defines a biosensor as a self-contained, integrated receptor-transducer device, which is capable to provide selective quantitative or semi-quantitative analytical information, using a biological recognition element (bio-receptor) and a transducer in direct spatial contact (Thévenot et al. 2001).

Another definition can be found in the journal *Biosensor & Bioelectronics* "biosensors are defined as analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.), a biologically derived material (e.g. recombinant antibodies, aptamers, etc.) or a biomimic (e.g. synthetic receptors, combinatorial ligands, imprinted polymers, etc.) intimately associated with/or integrated within a physicochemical transducer, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical" (Turner et al. 1987), principal parts of biosensors are depicted in Figure 1.1.

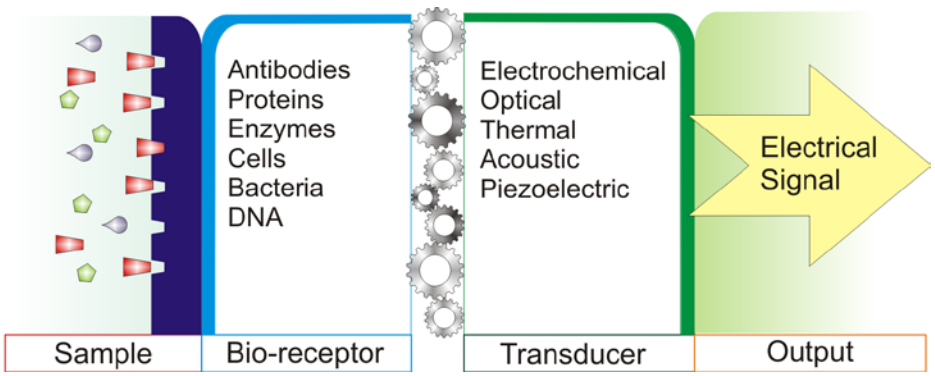

Figure 1.1. Principal parts of biosensors.

1.2.1 Biological recognition element

The biological recognition element confers the ability to recognize an analyte with high specificity. The interaction between the biological recognition element and a specific analyte produces a change that can be detected. For example in the reaction of the analyte, that can be converted by a transducer into a measurable signal. Biosensors can be classified as catalytic, when the biological recognition element reacts with the analyte, or as affinity, when the biological recognition element forms a complex with the analyte (Patel 2002).

1.2.1.1 Catalytic biosensors

Although other biological catalysts exist, enzymatic biosensors comprise the majority of devices based on catalytic biological elements. Enzymatic biosensors use an enzyme for the specific recognition of a substrate, and the catalytic formation of the product, detected later on the transduction element (directly or indirectly). Glucose oxidase (Rubio Retama et al. 2004), urease (Palomera et al. 2011) and laccase (Li et al. 2012) are some examples of enzymes that have been used. In some cases enzymes inside cells can be used, as for example, bacterial cells in the detection of atrazine (Das and Reardon 2012).

1.2.1.2 Affinity biosensors

Affinity biosensors exploit the recognition complex formed between the biological element and its target. Three major groups are found in the literature: receptor sensors, DNA sensors (genosensors) and immunosensors (Cosnier 2005).

Receptor sensors use cellular or membrane proteins as recognition elements, with high affinity and specificity due to their evolutionary process. Receptors are natural targets for a number of toxin and drugs that can be used for clinical and environmental studies. For example for the detection of the neuronal Ca^{+2}-sensor protein VILIP-1 using a nAChR subtype receptor with high-affinity for nicotine in the brain (Zhao et al. 2009), or to characterize the difference in the

receptor-binding specificities of human and avian influenza viruses with glycan chains (Gopinath et al.).

DNA sensors are devices that integrate nucleic acids (NAs), natural and synthetics forms, as biological recognition elements. Some examples can be found using denatured DNA (Dai et al. 2010), synthetic oligonucleotide probes (Zhang et al. 2010), RNA aptamers (Qureshi et al. 2010), DNA aptamers (Cheng et al. 2007), peptide nucleic acids (PNA) (Brandt and Hoheisel 2004).

Immunosensors or antibody based biosensors uses the immobilisation of antigens (Ags) or antibodies (Abs) as biological recognition elements. Because of the inherent high affinity and specificity of Abs, the target (analyte) doesn't need to be purified in order to be detected. Immunosensors are the most used in the majority of rapid detection systems, excluding glucose biosensors (Chambers et al. 2008).

The applications for which immunosensors can be used have increased over the years, not only in the clinical diagnostics field, but also in the environmental field, pharmaceutical and food industries. Novel applications can be found, like for the direct detection of adenovirus (Caygill et al. 2012) or toxic gliadin in foodstuff (Nassef et al. 2008a).

1.2.2 Immunosensor formats

Immunosensors assembly, analogous to immunoassays, can be divided in two main formats: direct or capture (sandwich) assay. Depending on the sample to be detected, either the antigen or the antibody can be immobilised on the surface of the electrode. Competitive, non-competitive and displacement assays, are testing procedures that can be adapted to the direct or capture format, depending on the target to be analysed and can be seen in Figure 1.2.

Non-competitive format (see Figure 1.2(a)), is the most sensitive format. The antibody binding sites are in excess over the target to be detected and the signal increases with the amount of analyte present in the sample. **Competitive format** (see Figure 1.2(b)), is done using a defined concentration of analyte introduced at same time with the sample to be analysed (with unknown concentration); both compete to bound a limited amount of antibody or antigen, present in the sample or at the surface. In this case, the obtained signal decrease as the analyte concentration increases. **Displacement format assay** (see Figure 1.2(c)), is based on displacement of a bound labelled antibody or antigen, using an analyte that posses a higher affinity for the same antigen or antibody, already immobilised on the surface. This displacement produces a decrease on the signal as the amount of analyte in the sample increase (Quinn et al. 2002).

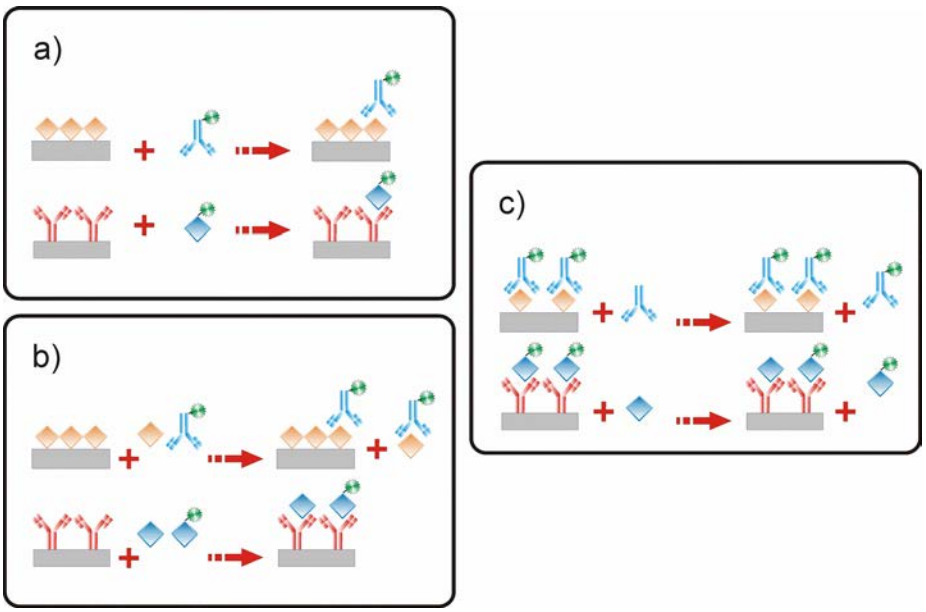

Figure 1.2. Immunosensor formats a) Non-competitive; b) Competitive; c) Displacement.

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Indirect format, an alternative to increase the sensitivity, can be adapted to the non-competitive and competitive procedures. This format is achieved using a secondary labelled antibody, in order to detect a target already bound to a first antibody/antigen depending on the assay type.

Immunosensors normally use labelled antibodies or antigens, in combination with some reagents, to perform the step detection. However, some transducers (e.g. surface plasmon resonance (SPR), electrochemical impedance spectroscopy (EIS), etc.), may operate under a reagentless or a label-free detection mode, or both. Those detection modes can be adapted to the formats explained before, avoiding the use of labels or chemical reagents, that sometimes compromise the sensitivity of the immunosensors (Zhang et al. 2012).

1.2.3 Transducer element

The transducer (or detector), converts the recognition event, done by the bio-receptor, into a measurable signal. Transducers can be classified as: optical, piezoelectric, magnetic, thermometric, acoustic and electrochemical (McGrath et al. 2012). Table 1, presents some examples of immunosensors, classified according to their transduction method.

Despite the large number of transducers that can be found in the literature, electrochemical transducers have emerged as the most used, easy to handle and fast response immunosensors (Chaubey and Malhotra 2002).

Table 1. Examples of immunosensors classified according to their transducer element.

Transducer	Analyte	Bio-receptor immobilisation	Reference
Optical			
-Fluorescence	Trinitrobenzene(TNT)	Optical waveguide	(Yan et al. 2012)
-Luminescence	Human IgG	Magnetic beads	(Fan et al. 2005)
-Refractive index	Human IgG	Optical fiber	(DeLisa et al. 2000)
-Reflectance	Anti-morphine/morphine competitive assay	BSA modified surface	(Bonanno and DeLouise 2009)
-SPR	Cardiac Troponin T (cTnT)	Carboxymethyl-dextran hydrogel	(Dutra and Kubota 2007)
Electrochemical			
-Amperometric	Staphylococcal enterotoxin A (SEA)	Protein A surface	(Pimenta-Martins et al. 2012)
-Potentiometric	C-reactive protein (CRP)	ZnO nanotubes	(Ibupoto et al. 2012b)
-Conductimetric	Murine double minute-2 (MDM2)	Thiol modified gold surface	(Elshafey et al.)
Electro-chemiluminescence	Prostate specific antigen (PSA) and interleukin-6 (IL-6)	Hydrophobic well	(Sardesai et al. 2011)
STM*	β-amyloid	Au surface	(Kang et al. 2009)
Mass/Acoustic			
-Piezoelectric	Protein 85 (OMP85) of bacterial meningitis	Protein-A thin film	(Reddy et al. 2012)
-Cantilever	Human interleukin-1 (HIL1-β)	Thiol functionalised gold covered microcantilever	(Dutta et al. 2005)
Magnetic	C-reactive protein (CRP)	Magnetic beads	(Meyer et al. 2007)
Thermal	Bovine serum albumin(BSA) and human Fibrinogen(HF)	In solution	(Yuyan and Tadigadapa 2005)

*STM Scanning tunnelling microscopy

1.3 Immobilisation methods

Immunosensor characteristics such as sensitivity, specificity, selectivity, stability, reproducibility and response time, are dependant to a great extent, of the Abs/Ags used for the immobilisation. Maintenance of the native protein structure, biological activity and a good orientation are some key parameters that need to be optimised in order to obtain efficient immunosensors with good performances. The immobilisation method not only affects the stability of biomolecules, but also affects the immunorecognition event of antibodies towards antigens, making the immobilisation process a crucial step for the immunosensors construction (Sassolas et al. 2012).

Assembly of immunosensors are classified in physical or chemical methods. (i) **physical methods**: adsorption and entrapment; (ii) **chemical methods**: cross-linking, affinity and covalent attachment. However, it's always possible to use a combination of them, or to include nanomaterials, such as nanoparticles, to improve the general properties of the immunosensors (Choi 2004).

1.3.1 Physical immobilisation

Physical methods are usually performed with biocompatible materials and solutions, in order to avoid damage of Abs/Ags. Principal problems of these methods are related with the stability of biomolecules, blocking the immunorecognition active sites of Abs/Ags and non-specific adsorption.

Direct adsorption of Abs/Ags on solid supports remains the easiest method to construct functional immunosensors. The adsorption phenomena is controlled by electrostatic, hydrophobic interactions and van der Waal's forces, however, adsorption is commonly done using a fixed amount of Abs/Ags in contact with solid surfaces, washing away unadsorbed molecules. Associated problems could occur, because these biomolecules may desorb with physical or chemical changes, and are easily affected by the non-specific adsorption of other proteins.

Some examples are found for the detection of toxins (Bonel et al. 2010) and drugs (Luangaram et al. 2002).

Entrapment of biomolecules represents a more stable alternative than simpler adsorption in the immunosensors construction. Physical entrapment of Abs/Ags inside micro-pores of polymers, three dimensional matrices or carbon pastes could be used, similar to some enzymatic biosensors. However, the active sites of Abs/Ags need to be available to perform the immunorecognition event. This methodology can diminish the number of available sites or completely block them. Some strategies have overcome those problems and are used for the detection of enterotoxins (Susmel et al. 2005) and pesticides (Sun et al. 2011).

1.3.2 Chemical immobilisation

Chemical immobilisation methods involve the use of chemical reactions to attach Abs/Ags on solid supports, or the chemical modification of these biomolecules in solution and their posterior immobilisation. Chemical methods present inherent risks to Abs/Ags, due to a possible and irreversible cross-linking between them, modifying completely their properties, or suffering a complete denaturation due to the presence of harsh chemicals and/or conditions. Despite these associated problems, they represent an excellent alternative to physical methods, because they offer more control, stability and sometimes the ability to orient the immobilisation through specific groups present in the biomolecules.

Cross-linking is a straightforward method that entails the formation of covalent bonds, either by cross-linking of Abs/Ags or with the addition of inert proteins (e.g. BSA). This method is achieved incorporating bifunctional agents such as glutaraldehyde or gloxal, either directly on the surface or after the adsorption of the Ags/Abs; an alternative is to perform this method in solution, depositing the cross-linked biomolecules after. Some examples can be found using modified screen printed electrodes with chitosan and glutaraldehyde for the

detection of antibodies of a parasitic disease (Zeng et al. 2012), or the cross-linking of a monoclonal antibody for the detection of cardiac markers (Kwon et al. 2011).

Affinity immobilisation exploit the same interaction process explained before for affinity biosensors. This oriented and site specific method uses immobilised active molecules e.g. avidin, lectin, etc., attached to the surface by means of any explained method before. Site orientation is achieved using Abs/Ags with active groups, such as biotin, carbohydrates, etc., that specifically bind the active molecules. Detection of a toxin using a biotin-BSA-gold nanoparticles approach have been studied for the detection of a mycotoxin (Vidal et al. 2011), another example used biotinylated antibodies for the detection of the sex hormone estradiol (Ojeda et al. 2012).

Covalent attachment is a prominent and frequently used immobilisation method, in where surfaces are activated firstly using reagents and afterwards Abs/Ags are joint covalently through a specific chemical group existent in them. For this method, it's possible to use also pre-activated surfaces. Several approaches can be used in order to assembly an immunosensor, for example immobilisation on functionalized controlled pore glass beads (Fernández-Baldo et al. 2010), or using an iridium oxide conducting matrix and immobilisation through the generated aldehyde groups (Wilson 2005).

Self assembled monolayers (SAMs) are of exceptional importance because their inherent properties for the formation of immunosensors, not only by the formation of well ordered and closely packed structures on metal surfaces, but also because of the presence of functional groups that can be linked easily to Abs/Ags through a covalent linkage. SAMs of alkanethiols are described in Figure 1.3. Diluted solution of thiols are used for the formation of well ordered SAMs, sulfur groups are chemisorbed forming a very strong bond of 40 kcal/mol. Sulfur-Au bonds are achieved not only by alkyl sulfides but also from similar structures such as disulfides or related organosulfur compounds (Dubois and Nuzzo 1992; Ulman 1996). Monolayers of

alkanethiols on gold, are probably the most studied approach for the construction of immunosensors and has been described recently in several applications for the detection of benzo[a]pyrene in water samples (Ahmad and Moore 2012), of alpha-fetoprotein in serum samples (Tyan et al. 2011), and for *Staphylococcus aureus* bacteria (Braiek et al. 2012).

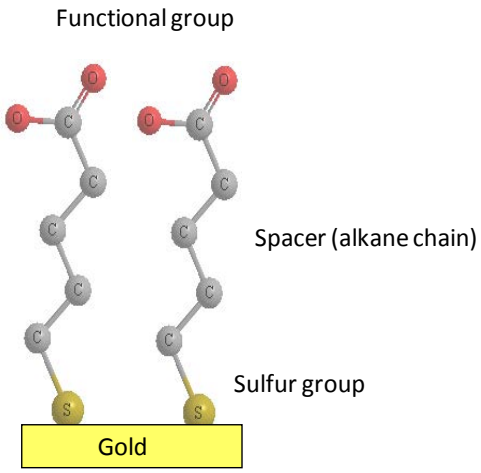

Figure 1.3. Alkanethiols SAMs principal parts.

1.4 Electrochemical immunosensors

Electrochemical biosensors are the most commonly used class for biosensors and immunosensors. During the bio-recognition process, electrochemical species are consumed or generated, and can be easily converted into an electrochemical signal and detected. They can be classified as potentiometric, amperometric and conductimetric (Ybarra 2012). This kind of immunosensors are inexpensive, robust, relatively simple to operate (Ding et al. 2008), have the possibility to work in a reagentless mode and/or in complex matrices, like serum, urine, blood and milk (Caygill et al. 2012; Conzuelo et al. 2012).

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1.4.1 Potentiometric

Potentiometric immunosensors, measure the difference of potential between a reference and a working electrode that is generated by the recognition event. Ion selective electrodes (ISEs) are potentiometric immunosensors. Their principle of operation consists in detecting the variation of concentration for a specific ion. This change can be correlated with the specific analyte concentration present in a sample. The potential change, according to the Nernst equation, is logarithmically proportional to the specific ion activity, with working conditions always near zero of current flow (Luppa et al. 2001).

When the reference and working electrodes are integrated by semiconductor technology field effect transistors (FETs) are produced, comprising a 'source' and a 'drain'. Electrical current flows along a semiconductor path (the channel), connected to two electrodes (the source and drain), that is in direct contact with the sample solution. In combination with a selective ion membrane FETs are known as ISFETs (Ion-Sensitive Field-Effect Transistor) (Chung et al. 2006). Some uses of FETs include immobilised antibodies, that have been used in the detection of herbicides (Mosiello et al. 2003), proteins (Ibupoto et al. 2012a) and bacteria (Dill et al. 1999).

1.4.2 Conductimetric or impedimetric

Conductimetric immunosensors, measure the alterations of electrical conductivity in a solution at constant voltage. However, only few practical clinical applications have been demonstrated using this transduction method. Resistance measurements under AC voltage excitation (impedance) are commonly used and have attracted more attention as a rapid technique in the characterisation of biosensors, and also as, reagentless immunosensor transduction alternative (Farace et al. 2002; Holford et al. 2012). Electrochemical impedance spectroscopy (EIS) measures impedance over a range of frequencies. EIS permits the evaluation of the dielectric properties at the surface in a sensitive, non-destructive and label-free manner that attracts every day more

attention in the biosensors field (Cooreman et al. 2005; Liu et al. 2011; Tsekenis et al. 2008).

Two EIS modes can be used, faradaic or non-faradaic measurements. In the first, the redox species needs to be oxidised and reduced at the surface of the electrode, in the non-faradaic mode, no additional reagent is need. (Daniels and Pourmand 2007).

Some examples can be found in the detection of furazolidone antibiotic residues in food samples, achieved with the detection of its residue 3-amino-2-oxazolidinone (AOZ) in the linear range of 20 to $1x10^4$ ng/mL (Yang et al. 2011). Using disposable screen printed carbon electrodes (SCPEs), it was possible to detect sensitively less than 2.3 pg/mL of human chorionic gonadotropin (HCG) (Truong et al. 2011).

1.4.3 Amperometric

Amperometric immunosensors are based on the direct detection of current changes, in response to electrochemical reactions at a fixed potential. In the majority of cases, the use of electroactive species is needed in the form of redox analytes or redox labels. Changes that occur in the current are directly proportional to the analyte concentration present in the sample (Chaubey and Malhotra 2002). Amperometric immunosensors have high sensitivity, a direct relation between concentration and signal and provide the possibility to select different working potentials (Bojorge Ramírez et al. 2009).

The amperometric enzyme biosensor was the first biosensor reported. Immobilising an enzyme, glucose oxidase, over the surface of an oxygen electrode it was able to quantify the amount of glucose present in a sample (Clark and Lyons 1962). A breakthrough in the field was researched by the Aisawa group, naming the biosensor as "amperometric enzyme immunosensor" the concept was the immobilisation of an antibody through a membrane. In a competitive study they were able to detect from $2 x10^{-2}$ to 10^2 IU/mL of HCG (Aizawa et al. 1979). Using a similar concept, an application for the detection of

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hepatitis B surface antigens, was developed immobilising antibodies through a gelatine membrane. The specific detection was performed using glucose oxidase labelled antibodies in standard glucose solutions (Boitieux et al. 1984).

Immunosensors applications can be encountered in many fields, from clinical diagnostics to environmental detection of pollutants. In the following part, a summary of some amperometric immunosensors, presented in the last decade will be described:

1.4.3.1 Pathogens

Whole bacteria detection can be done, using antibodies raised against the cell lysate of the bacteria. Detection range normally is reported as 'CFU/mL' (colony forming units per mL) or number of 'cells/mL'. Table 2 represent some examples of pathogen detection.

Table 2. Immunosensors for pathogen detection.

Bacteria detected	Immobilisation	Dynamic range (cells/mL)	LOD (cells/mL)	Reference
V.cholerae	Using disposable SPCEs	10^4 to 10^8	10^5 , 55 min	(Rao et al. 2006)
E. coli O157:H7	Adsorbed on a nitrocellulose membrane	0 to 5000	50 , 20 min	(Theegala et al. 2008)
S. aureus	Gold electrodes and covalent attachement to a heterobifunctional cross-linker	$3x10^4$ to $3.7x10^5$	$1.4x10^4$, 30 min	(Escamilla-Gómez et al. 2008)
S. typhimutium	Au/SPEs and a carboxy-methyldextran surface	10^1 CFU/mL to 10^7 CFU/mL	20 CFU/mL, 120 min	(Salam and Tothill 2009)
S. pneumoniae	Au/SPEs and magnetic beads	$4.3×10^4$ to $1x10^7$ CFU/mL	$1.5×10^4$ CFU/mL (serotype 37), 60 min	(Campuzano et al. 2010)

1.4.3.2 Infectious diseases

Serological tests are highly sensitive, specific, and often extremely rapid. One category of such tests includes those designed to detect the presence of antibodies against a known infection. They use similar steps as classical immunoassays (ELISA), in a faster and usually inexpensive way. Table 3 shows some immunosensors applied for diagnosis of some infectious diseases.

Table 3. Immunosensors for diagnosis of infectious diseases.

Infectious disease	Immobilisation	Characteristics	LOD, assay time	Reference
Typhoid disease	Adsorption on SPCEs	100% correlation with the standard test	Not reported, 75 min	(Rao et al. 2005)
Peptic ulcers and chronic gastritis	3-aminopropyl-modified controlled pore glass	Linear range 0–100 U/mL	0.6 U/mL, 30 min	(Messina et al. 2005)
Chagas' disease	Glutaraldehyde on Au/SPEs	8 Positive samples	Not reported, 70 min	(Ferreira et al. 2005)
Peptic ulcers and chronic gastritis	Adsorption on SPCEs	Linear range 0–100 U/mL 20 U/mL CV - 2.7%	0.5 U/mL, 25 min	(Messina et al. 2007)
West Nile virus	Using photo-activable copolymers on glassy carbon electrodes	Antibody dilutions from 1:10 to $1:10^6$	Not reported, 65min	(Ionescu et al. 2007)
Hydatidosis disease	3-aminopropyl-modified controlled pore glass	Linear range 0.5 to 115 ng/mL,	0.091 ng/mL, 26 min	(Pereira et al. 2011)

1.4.3.3 Drugs and hormones

The presence of drugs and hormones can be achieved using specific antibodies, raised against these chemical molecules. They can be purified to detect down to 'ng/mL' of drugs in real samples. Usually, the chemical molecule is attached to a big protein (for example BSA), in order to immobilise it, some examples are presented in Table 4.

Table 4. Immunosensors for the detection of drugs and hormones.

Molecule	Immobilisation	Characteristics	LOD (pg/mL)	Reference
Ecstasy and its analogues	Adsorption on SPCEs	Linear range, ng/mL MDA 0.60–400 MDMA 0.13–400 MDEA 0.026–400	LOD in urine MDA 42 MDMA 190 MDEA 130	(Butler et al. 2006)
17-β estradiol	Adsorption on SPCEs	Linear range 2.44–2500 pg/mL EC50 61.6 pg/mL	0.25 in buffer	(Butler and Guilbault 2006)
Boldenone and methylboldenone	Adsorption on SPCEs	Boldenone EC50 1.0 ± 0.3 ng/mL Methylboldenone EC50 1.5 ± 0.3 ng/mL	Boldenone 30.9 ± 4.3 Methylboldenone 120.2 ± 8.2 Both in urine	(Lu et al. 2006)
Testosterone	Adsorption on SPCEs	Linear ranges 0.03 to 40 ng/mL in buffer 0.03 to 1.6 ng/mL in urine	26 in buffer 1.8 in urine	(Conneely et al. 2007)
Sulfonamide antibiotics	Covalent bonding on magnetic beads	Linear ranges 1.0 to 43 ng/mL in milk 1.2 to 89 ng/mL in buffer	360 in milk 440 in buffer	(Zacco et al. 2007a)

1.4.3.4 Toxins

Detection of toxins in food samples has been always a priority in the food industry. Toxins detection, generated by living organisms like fungus, algae and bacteria, needs to be done in a selective, sensitive and cost effective manner. Table 5 summarize some immunosensors for toxins detection.

Table 5. Immunosensors for toxins detection.

Toxin	Immobilisation	Characteristics	LOD (ng/mL)	Reference
Aflatoxin M1	Adsorption on protein G agarose beads	Linear range 20 to 500 pg/mL	.011 in milk	(Badea et al. 2004)
Staphylococcal enterotoxin B	Thiolated antibodies on gold electrodes	Linear range 1–20 ng/mL	1 in buffer	(Chatrathi et al. 2007)
Okadaic acid	Adsorption on protein-G magnetic beads	Linear range 0.78 to 500 ng/mL	0.5 in buffer	(Hayat et al. 2012)
Staphylococcal enterotoxin A	Covalent binding on a thiol SAM	Linear range 16 to 150 μg/mL	33.9 in buffer	(Pimenta-Martins et al. 2012)

A novel multichannel electrochemical detection (MED) unit, formed by 96-well screen printed patented microplates, was used to detect the presence of aflatoxin B1 (AFB1). Detection of 30 pg/mL with a dynamic range of 0.05 to 2 ng/mL, was achieved using AFB1 spiked corn samples, with very good recoveries (103±8%) (Piermarini et al. 2007).

1.4.3.5 Pesticides and environmental pollutants

Normally used to control the growth of unwanted pests, pesticides can be a mixture of chemical substances or biological agents. Most of pesticides are toxic to humans and animals, and need to be detected from environmental and food samples, using rapid and cost-effective assays so that measures can be taken to limit exposure. Detection of several pesticides and environmental pollutants are summarised in Table 6.

Table 6. Immunosensors for pesticides and environmental pollutants detection.

Pesticide	Immobilisation	Characteristics	LOD	Reference
Atrazine	Adsorption on SPCEs covered with polyaniline	Working range 0.1 to 1.0 μg/mL	0.1 ng/mL	(Grennan et al. 2003)
Polycyclic aromatic hydrocarbons	Adsorption on SPCEs	Indirect co-exposure IC50 7.1 ng/mL Indirect competition IC50 15 ng/mL Indirect displacement IC50 11 ng/mL	Indirect co-exposure 0.8 ng/mL Indirect competition 2 ng/mL Indirect displacement 2 ng/mL	(Fähnrich et al. 2003)
Chlorsulfuron	Adsorption on SPCEs	Linear range 0.01 to 1 ng/mL IC50 0.03 ng/mL	0.01 ng/mL in buffer	(Dzantiev et al. 2004)
Atrazine	Protein A (2%) graphite–epoxy biocomposite electrode	Linear ranges 0.15 to 2.9 ng/mL, buffer 0.017 to 1.3 ng/mL, juice	37 pg/mL in buffer 6 pg/mL in orange juice	(Zacco et al. 2007b)
2,4-dichlorophenoxy-acetic acid	Bacterial cells immobilised on SPGEs and oxygen Clark probes	Linear range 2 to 13 μg/mL SPGEs 4 to 17 μg/mL Clark probe	2 μg/mL for SPGEs 4 μg/mL for Clark probe	(Odaci et al. 2008)

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1.4.3.6 Medical biomarkers

The alteration in the concentration of some proteins or metabolites in the human body can be associated with the presence of a medical condition. Since such variations can be present before the onset of macroscopic findings or symptoms, and since the identification of the metabolites in question can guide medical decisions, early detection and identification are valuable tools for diagnostics and theranostics, some examples are shown in Table 7.

Table 7. Immunosensors for medical biomarkers detection.

Biomarker	Immobilisation	Linear range	LOD, assay time	Reference
Human heart fatty acid binding protein	Adsorption on SPCEs	4 to 250 ng/mL CV below 9 %	4 ng/mL, 50 min using whole blood	(O'Regan et al. 2002)
Complement 3	Adsorption on carbon paraffin electrodes	0.06–10 µg/mL	0.06 µg/mL, 30 min	(Zhou et al. 2003)
Histamine	Graphite electrodes, no immobilisation	200–2000 ng/mL	200 ng/mL, 30 min in whole blood	(Lim et al. 2003)
Amylase	Covalent attachment on a cystamine monolayer	0.01–1.0 µg/mL	80.9 ng/mL, 5 min in human saliva	(Aluoch et al. 2005)
Pepsinogens 1 & 2	Adsorption on a polystyrene surface	0 to 50 ng/mL for both analytes	0.6 ng/mL for both analytes, in 60 min	(Ogasawara et al. 2006)
HCG	Adsorption on nano-Au and methylene blue layers	0.1 to 10 ng/mL (1.0–100.0 mIU/mL)	0.03 ng/mL (0.3 mIU/mL), in 40 min	(Chai et al. 2008)

1.4.3.7 Cancer proteins

The elevated presence of some proteins can establish the presence or recurrence of cancer in a person. Although only a few are very specific, routine clinical diagnosis of tumour markers has been studied extensively and can be associated directly as predictive factors. An example is the carcinoembryonic antigen (CEA), a glycoprotein used as a part of annual medical checkups in many countries, detection of this protein and some others are presented in Table 8.

Table 8. Immunosensors for different cancer proteins detection.

Tumour marker	Immobilisation	Linear range	LOD, assay time	Reference
AFP	Entrapped in a chitosan matrix	0 to 20 ng/mL and 20 to 150 ng/mL	0.74 ng/mL in 35 min	(Yu et al. 2004)
CEA	Adsorption on a porous organometallic material	0.25 to 160.0 ng/mL	0.18 ng/mL	(Zhuo et al. 2008)
CEA & AFP	Using microchip capillary electrophoresis and amperometric detection	0.5 to 66.0 ng/mL for CEA 0.5 to 80.0 ng/mL for AFP	CEA 0.25 ng/mL AFP 0.13 ng/mL In 15 min	(Zhang et al. 2009)
AFP	Adsorption on SPCEs modified with gold nanoparticles and a sulfhyrdryl viologen	1.25 to 200 ng/mL	0.23 ng/mL	(Liang et al. 2009)
CEA	Covalent attachment to a SAM of a dithiol	0 to 200 ng/mL	0.2 ng/mL, in 90 min	(Laboria et al. 2010)
PSA & IL-6	Adsorption on SPCEs modified with gold nanoparticles	0.23 to 5 pg/mL for PSA 0.30 to 20 pg/mL for IL-6	0.23 pg/mL for PSA 0.30 pg/mL for IL-6 40 min for both	(Chikkaveeraiah et al. 2011)

1.5 Coeliac disease (CD)

1.5.1 Autoimmune diseases

Autoimmune diseases result from the activation of the immune system to the body's own cells, or an excessive response for non-threatening substances present in the environment. This aberrant autoimmune response triggers the presence of large amounts of Abs in the system (autoantibodies). The produced antibodies can be deposited on tissues and organs, sometimes, generating acute and chronic inflammation, increasing the risk of other diseases (Pihoker et al. 2005).

Clinical diagnostics are done routinely to detect and quantify Abs related to certain autoimmune diseases, in those cases, specific Ags are known (autoantigens) (Robinson et al. 2003). Detection is normally done from serum or whole blood samples. Presence and quantification of Abs/Ags in clinical diagnostics, should not be considered as a marker more with high limitations, and should be used only to confirm or exclude patients of a suspected disease (Bogdanos 2012).

1.5.2 CD description

CD or gluten-sensitive enteropathy, is an autoimmune disease that entails a permanent intolerance to gluten proteins present in wheat, barley and rye and in some cases to oats (van Heel and West 2006). Prevalence of CD has been always attributed to people from Europe, including countries where Europeans emigrated like North America and Australia, but in fact, CD affects the 1% of most populations. Despite the use of mass screening technologies, recognition of the disease remains mainly unrecognized (Dube et al. 2005; Rubio-Tapia and Murray 2010).

Coeliac disease is present in genetically susceptible individuals that have one of two types of the human leukocyte antigen (HLA) proteins. Approximately 95% of the patients express isoform HLA-DQ2, and the other 5% express isoform HLA-DQ8. The presence of those proteins is necessary for the disease development, however, that's not the exclusive factor; because 30-40% of healthy individuals present the same HLA proteins (Mäki et al. 2003).

Actually, the only known and effective treatment for CD is a gluten free diet (GFD), that represents the complete elimination of gluten from consumption. However, it has been demonstrated, that in practice is very difficult to achieve. Complete exclusion of any cereal that contains wheat, gluten or prolamins; is difficult, expensive and sometimes can provoke dietary deficiencies. Alternatives to a GFD are available using pharmacotherapy, but still underdeveloped. Some novel approaches are being investigated e.g. consumption of a detoxified dietary gluten, gene therapy and vaccines that contain specific peptides, but most of them are in early studies(McAllister and Kagnoff 2012).

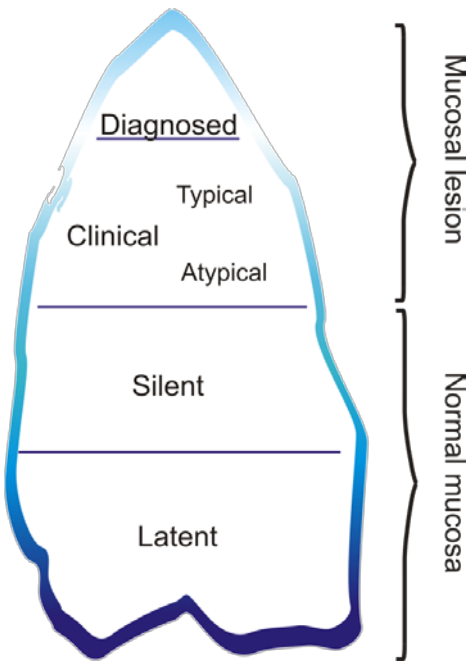

Figure 1.4. Coeliac disease Iceberg, after (Ferguson et al. 1993).

Occurrence for CD can be described from the coeliac iceberg (see Figure 1.4). Four cases are normally found: (i) **typical**, the easiest to detect, because patients present normal gastrointestinal symptoms; (ii) **atypical**, gastrointestinal signs are minimal but other conditions can be present; (iii) **silent**, no associated symptoms are present, but the disease can be detected using serology; (iv) **latent**, persons are positive to serology and present genetic compatibility, but not always present symptoms (Setty et al. 2008). It can be seen from the Figure 1.4, that the latent cases represent the vast majority of undiagnosed persons, and diagnosed cases represent only the tip of the iceberg (Fraser and Ciclitira 2001).

1.5.2.1 Symptoms

CD diagnosis are established by the identification of some key indicators, normally present in patients, and summarised in Table 9 Silent version of CD can also present some symptoms, in where Fe-deficiency anaemia is the most common condition (Hin et al. 1999; Tursi et al. 2001).

Table 9. Principal symptoms associated with CD, adapted from (McGough and Cummings 2005).

Symptoms	Adults	Children
Common	Diarrhoea, altered bowel habit Abdominal pain, bloating Aphthous ulcers Anaemia , weight loss Dermatitis herpetiformis Malabsorption, oedema Osteoporosis, low impact fracture	Loss of appetite, failure to thrive Malabsorption, diarrhoea Abdominal distension Small stature, muscle wasting
Uncommon	Tiredness, depression, muscle weakness, amenorrhea, arthritis , infertility, dental enamel defects	

1.5.3 Serological detection

The use of serological studies have become a standard in diagnostics for some autoimmune diseases, they represent a non-invasive alternative to detect them (Konstantinov et al. 2009). Anti-reticulin antibodies (ARA) were used as a serological marker for CD detection, but became obsolete. Anti-gliadin antibodies (AGA) detection, since its introduction in 1958, became the principal CD biomarker and at some stage was used at the same time than ARA (Unsworth et al. 1988). Due to a lack of sensitivity and poor specificity, along with the measure of new and more efficient biomarkers, AGA detection is unadvised. Special cases e.g. children below 18 months, IgA-AGA and more recently IgA antibodies against deamidated gliadin (DGP) can be used with good results (Lagerqvist et al. 2008; Prause et al. 2009). The discovery of anti-endomysial antibodies (EMA), and its high specificity against CD (almost 100%), replaced completely the AGA tests. However, they also have some problems related with the interpretation of results and are expensive to perform.

Tissue transglutaminase (tTG) has been recognized as the principal autoantigen for CD detection. Its discovery, lead to generation of recombinant tTG and production of more specific antibodies, making serological tests more efficient with higher specificity and sensitivity. Due to its accuracy, low cost and reliability, detection of IgA antibodies against tTG (Anti-tTG) is actually the only recommended test for CD. When the presence of total IgA antibodies is low, measurement of IgG anti-tTG can be used as an alternative. The confirmation of CD diagnosis for all cases requires a duodenal biopsy, that shows mucosal villous atrophy in patients that consume food with gluten (Armstrong et al. 2011; Hill et al. 2005).

New approaches for CD serological detection have promoted simpler and cost effective screening methods. Despite the fact that anti-tTG detection, IgA isotype, is the current and only recommended test for CD screening, other alternatives as EMA and anti-DGP can be used as well, but the use of experienced personal is recommended. AGA tests

have been discarded and only few applications have been found, e.g. as a complementary test for CD detection in young children (Harris et al. 2012). Available tests with their principal characteristics using immunoassays, are summarised in Table 10.

Table 10. Principal characteristics of CD serological tests, adapted from (Leffler and Schuppan 2010).

Test	Sensitivity (%)	Specificity (%)
AGA-IgA	85	90
AGA-IgG	85	80
Anti-DGP-IgA	88	95
Anti-DGP-IgG	80	98
Anti-tTG-IgA	98	98
Anti-tTG-IgG	70	95
EMA	95	99

1.5.4 State of the art of immunosensors for CD detection

1.5.4.1 AGA detection

Detection of CD serological markers using immunosensors are an excellent and reliable alternative to ELISA tests, which normally are more expensive, require associated equipment, and more time to be completed. The use of electrochemical immunosensors for detection of CD related antibodies has been reported a few times in the literature, normally comparing the obtained results with ELISA or another immunoassay.

AGA detection, has been reported using an impedimetric immunosensor, in where gliadin was immobilised on gold electrodes modified with a polyelectrolyte layer of poly(4-styrenesulfonic acid sodium salt). Despite the good sensitivity reported, the linear range from 1.5 to 150 μg/mL is difficult to understand, because there's no difference for low concentration signals, less than 15 μg/mL. The serum sample analysis was short on samples and dilutions, making difficult to prove it was able to distinguish positive and negative patients to CD (Balkenhohl and Lisdat 2007a). Another study used an automated microfluidic system, for the IgG-AGA detection. Immobilisation of gliadin was done on controlled pore glass particles, modified with 3-aminopropyl. Amperometric detection of standard serum samples was compared with ELISA experiments from a commercial kit, obtaining an excellent correlation. Measurement of the response signal, despite being a microfluidic device, was measured using cyclic voltammetry which needs more time and no flow to be used (Pereira et al. 2010).

Amperometric detection of AGA from CD serum samples was achieved using a cyclodextrin-based supramolecular approach. Carboxymethylcellulose polymers modified with adamantane and gliadin were immobilised on cyclodextrin SAMs. Detection was done for two different serum samples and compared with ELISA, achieving similar responses in both cases (Ortiz et al. 2011b). The same approach was used for the detection of follow-up samples from CD patients, after their adherence to a GFD. A comparison between ELISA and the constructed immunosensor was done using reference serum calibrators, obtaining a very good correlation. Two patients were studied, obtaining highly reproducible signals and excellent correlation with ELISA (Ortiz et al. 2011a). More recently, voltammetric detection of AGA was done using modified screen printed carbon electrodes, with multiwall carbon nanotubes (MWCNTs) and gold nanoparticles. Response for the achieved immunosensor was compared with a commercial ELISA kit, obtained responses by ELISA and the immunosensor couldn't be completely compared, because the same dilution was used for all the patients (Neves et al. 2012b).

1.5.4.2 Anti-tTG detection

Detection of anti-tTG antibodies, the only recommended CD serological test, has been reported in more recent publications. Using an impedimetric biosensor, similar to the used for AGA detection, was used to detect anti-tTG antibodies in human serum samples. A logarithmic linear range, showing the values in terms of dilution, was obtained. Quantification of antibodies from human serum samples showed no correlation, probably due to saturation of the used immunosensor (Balkenhohl and Lisdat 2007b). A reusable graphite-epoxy electrode was used for the amperometric detection of A-tTG antibodies, IgG isotype. Experiments focused on the NSA reduction of other antibodies present in serum samples, normally found to be of the IgG isotype. Only 50% of the positive patients were demonstrated, because the rest were very near the cut-off line of the immunosensor (Pividori et al. 2009).

Another approach used gold interdigitated array electrodes for the label-free impedimetric detection of anti-tTG antibodies. It was possible to detect antibodies in buffer samples and compare the obtained signals with ELISA based tests. Further use of gold nanoparticles, as a label, showed a sensitivity enhancement, but only for high concentrations of anti-tTG. However, the proposed work didn't quantify the amount of anti-tTG (Singh et al. 2011). Screen printed carbon electrodes, covered with an overoxidized polypyrrole film, were used for the impedimetric detection of anti-tTG antibodies in buffer samples. Impedimetric results showed it was possible to follow the construction of the immunosensor, however, specific response of anti-tTG antibodies was only detected at high concentrations (West et al. 2011). A similar approach, using carbon nanotubes and gold nanoparticles, used before for the AGA detection, was used for the voltammetric detection of anti-tTG antibodies. Only qualitative results could be obtained for positive CD patients because few patients matched with the ELISA response (Neves et al. 2012a).

A novel immunomagnetic sensor was proposed for the detection of anti-tTG antibodies. Capture of anti-tTG antibodies, with magnetic particles functionalised with the antigen, was done in solution. Using a magnet below the surface of the electrodes, the particles were collected at the working electrode. Electrochemical signal was recorded using differential pulse voltammetry (DPV). It was possible to analyse different human serum samples and compare them with ELISA. The immunosensor was able to distinguish all positive samples to CD (46 patients), from 61 healthy patients, all but one, was discriminated (Adornetto et al. 2012). Multiplexed detection of CD AGA and anti-tTG antibodies, was performed using dual screen printed carbon electrodes. Using a similar strategy, it was possible to achieve a semi-quantification of studied antibodies in serum samples (Neves et al.).

Thiol-based immunosensors have been studied for the specific detection of CD related antibodies. The use of dithiol molecules for the covalent attachment of proteins, has been demonstrated before: for the label-free detection of PSA using EIS in buffer solutions, in where the dithiol SAMs characteristics were analysed, showing that it's possible to achieve a complete monolayer in 3 hours of exposure (Fragoso et al. 2008). Detection of gluten from extracted food samples was showed using the same dithiol, achieving a very low LOD of 5.5 ng/mL for the analysed protein (Nassef et al. 2008b). More recently, CEA detection was performed using the same dithiol approach on gold chip arrays; able to detect 0.2 ng/mL of the protein. Studies were performed using serum samples and compared with ELISA based tests, achieving an excellent correlation (Laboria et al. 2010).

In this *Thesis* it was possible not only to achieve the successfully detection of CD related antibodies in buffer samples, but also in serum samples from CD patients with an excellent correlation with ELISA studies. In this *Thesis*, the use of the same dithiol and a faster immobilisation approach using thiolated antigens, have been successfully demonstrated for the detection of CD antibodies in real patients' sera.

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CHAPTER 2

Objectives

The construction of an electrochemical amperometric immunosensor, using thiolated molecules on gold surfaces in a cost effective, rapid and real-sample oriented. Demonstration of this objective is achieved through the accomplishment of specific objectives for each chapter. This demonstration involves, not only the construction of a functional immunosensor to detect serological markers in real serum samples from CD patients', but also other important characteristics such as reliability, specificity and sensitivity.

The construction of immunosensors using dithiol-self assembled monolayers is described in *Chapter 3* and *Chapter 4*. The proof-of-concept for the detection of antibodies in buffer solutions, is studied in *Chapter 3*. In *chapter 4* the objective is the detection and quantification of total human IgA antibodies. Detection of anti-gliadin and anti-tTG antibodies, using self assembled thiolated antigens on gold surfaces, is studied in Chapter 5.

In *Chapter 3* surface characterisation and immobilisation steps are followed using SPR and EIS, antibodies detection is investigated using amperometry. The specific detection of human antibodies using amperometry is described in *Chapter 4*. In *Chapter 5*, studies concerning the surface coverage of thiolated antigens on gold samples are done by SPR and amperometric experiments, as wells as, investigations concerning the detection of antibodies using those modified surfaces.

Non-specific adsorption (NSA) of proteins, which can interfere with the detection of specific targets, is a crucial study for all immunosensors. Studies using a dithiol, that possess PEG groups for reduction of the NSA are done in *Chapter 3* through amperometric experiments. Fouling of the immunosensor is analysed in *Chapter 4*, using foetal bovine serum solutions as dilution buffer, along with the analysis of cross reactivity for other antibodies that can be encountered in real samples.

In *Chapter 5* the use of a poly-(ethylene glycol) on gold surfaces is investigated in order to reduce the NSA. PEG-thiol immobilisation is studied using SPR and amperometry, in order to prove it, as a potential backfiller for the constructed immunosensors.

The correlation of electrochemical experiments with ELISA standardized tests is done in *Chapter 3*, *Chapter 4*, and *Chapter 5*. In the case of *Chapter 3*, the correlation is done using reference serum calibrators from commercial ELISA kits and using CD real patient's serum samples. In *Chapter 4*, quantification of total IgA antibodies is investigated, compared using amperometric and ELISA based tests for CD patient's sera (deficient and non-deficient). In *Chapter 5,* the antigenicity of thiolated proteins is compared using SPR, ELISA, and amperometric studies. Immunosensors Investigations are done using clinical reference serum solutions and CD real patients' sera using the obtained immunosensors.

CHAPTER 3

"Electrochemical immunosensor detection of antigliadin antibodies from real human serum"

3.1 Chapter introduction

In this *chapter* the use of a carboxyl-terminated bipodal alkanethiol, that contains poly-ethyleneglycol (PEGs) groups, is explored using the properties of thiols to form self assembled monolayers (SAMs), as a base to construct functional immunosensors. SAMs properties in the reduction of non specific adsorption (NSA) and diffusion of electroactive species, is demonstrated. Information obtained from the development of a functional immunosensor, served as basis for the construction of the biosensors in *Chapter 4* and *Chapter 5*. Electrochemical, chemical and biological parameters such as covalent linkage, immobilisation conditions, different mediators and substrates are investigated. Alternative immunosensors technologies, like surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS) are used in the characterization of the surface and for a better understanding about the complete construction of immunosensors. The proof of principle of the immunosensors involved in the detection of anti-gliadin antibodies is demonstrated, with the achievement of a very low limit of detection, due to the an almost zero NSA. Detection of anti-gliadin antibodies in complex matrices reached an excellent correlation with ELISA.

3.2 Article "Electrochemical immunosensor detection of antigliadin antibodies from real human serum"

3.2.1 Abstract

The determination of antigliadin antibodies from human serum samples is of vital importance for the diagnosis of an autoimmune disease such as coeliac disease. An electrochemical immunosensor that mimics traditional ELISA type architecture has been constructed for the detection of antigliadin antibodies with control over the orientation and packing of gliadin antigen molecules on the surface of gold electrodes. The orientation of the antigen on the surface has been achieved using a carboxylic-ended bipodal alkanethiol that is covalently linked with amino groups of the antigen protein. The bipodal thiol presents a long

poly(ethyleneglycol) modified chain that acts as an excellent non-specific adsorption barrier. The bipodal nature of the thiol ensured a good spacing and hence good diffusion properties of electroactive species through the self assembled monolayer, which is vital for the efficiency of the constructed electrochemical immunosensor.

The electrochemical immunosensor was characterized using surface plasmon resonance as well as electrochemical impedance spectroscopy. Amperometric evaluation of the sensor with polyclonal antigliadin antibodies showed stable and reproducible low limit of detection (46 ng/mL; % RSD = 8.2, n = 5). The behaviour and performance of the electrochemical immunosensor with more complex matrixes such as reference serum solutions and real patient samples was evaluated and compared with commercial ELISA kits demonstrating an excellent degree of correlation; the electrochemical immunosensor not only delivers a positive or negative result, it allows the estimation of semi quantitative antibody contents based on the comparison against clinical reference solutions.

3.2.2 Introduction

Celiac disease (CD) is a gluten-sensitive enteropathy that affects 1% of the population around the globe (Briani et al. 2008). It is an autoimmune disorder of the upper small intestine that is triggered from the ingestion of gluten, a protein that consists of a mixture of gliadins and glutenins and that can be found in cereals like wheat, rye oats or barley. The ingestion of gluten triggers the production of a series of autoantibodies against gliadin and tissue transglutaminase, which has been identified as the autoantigen triggering CD (Dieterich et al. 1997).Anti-gliadin antibodies, isotypes IgA and IgG, can be detected in peripheral blood and in small intestinal mucosa of CD patients (Lycke et al. 1989). ELISA tests for the monitoring of anti-gliadin antibodies are performed for persons with CD and results show sensitivities of up to 96% for IgA and IgG-AGA and specificities of up to 92% (David 1996).

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Immunosensors are a type of biosensor based on the specific recognition between antigens and antibodies (Ekins 1999) and the way in which this phenomenon is monitored depends on the principle used to detect the immune interaction, optical, electrochemical or micro gravimetric methods are common transduction methods (Luppa et al. 2001). The use of a solid support to immobilise either the antigen or the antibody is used in classical techniques like enzyme linked immunosorbent assays (ELISA) (Dahlbom et al. 2008), radioimmunoassays (RIA) (Oftedal et al. 2008) or fluorescence immunoassays (Agiamarnioti et al. 2006), where the interaction is measured with the help of labelled antibodies.

The use of electrochemical immunosensor is supported by their selectivity, sensitivity, versatility and simplicity and short analysis time (Bange et al. 2005); they also are renowned for their potential for miniaturization, portability and low cost. They have been successfully used in the detection of proteins, antibodies, bacteria and whole cells (Ghindilis et al. 1998; Khaled et al. 2008; Kim et al. 2006; Konstantinov et al. 2009; Laczka et al. 2009; Mata et al. 2010; Melanson 2007).

Many different classical strategies are employed to try and mimic the architecture of classical immunoassays on electrode surfaces; the assembly of the immunosensor with the attachment of antibodies or antigens on the surface can be carried out by several methods such as polymer or sol–gel entrapment (Ramanavicius et al. 2010) covalent attachment (Hays et al. 2006) or physical adsorption (Diaz-Gonzalez et al. 2005). Other approaches exploiting the use of nanoparticles and carbon nanotubes have recently been explored (Cid et al. 2008; Li and Gao 2008; Rusling et al. 2009; Takeda et al. 2005). A widely reported procedure for biomolecule immobilisation is the use of self-assembled monolayers (SAM). The utilization of thiol-ended molecules with different moieties provides extensive and stable bonds with electrode surfaces while allowing strong covalent bonds with different biomolecules via simple conjugation chemistry. The nature of the interaction with the biomolecules provides good control over their

orientation, adding to the selectivity and specificity of the electrochemical immunosensors. The use of self-assembled monolayers based on bipodal alkanethiols with polyethylene groups incorporated has been shown great potential and the excellent performance of electrochemical biosensors based on this surface chemistry has been demonstrated and applied to the detection of different biomolecules for food quality control and clinical diagnostics (Fragoso et al. 2008; Laboria et al. 2010; Nassef et al. 2009; Nassef et al. 2008).

The determination of autoantibodies from human serum using alternative methods to classical ELISA has been reported. Methods where the antigen is immobilised onto a metallic, predominantly gold, surface include the utilization of quartz crystal resonance techniques coupled with peptide-modified carbon nanotubes for the determination of autoantibodies related to rheumatoid arthritis in a label-free format that displayed higher sensitivity than classical ELISA (Drouvalakis et al. 2008). Surface plasmon resonance techniques have been used to detect the interaction of autoantibodies used for the diagnosis of antiphospholipid syndrome or systemic lupus erythematosus with their corresponding antigens covalently immobilised on a gold microchip (Thaler et al. 2009). This method has proved useful with highly reproducible signals and provided information to enable the identification of disease-relevant autoantibodies linked to disease progress. An electrochemistry-based determination of autoantibodies for the rapid detection of antichromatin antibodies in human sera, typical in systemic lupus erythematosus has also been developed (Konstantinov et al. 2009). A sandwich-type assay is built with the antigen immobilised on a membrane; the generated electroactive species are detected on a nearby screen-printed electrode in a total assay time of ca. 20 min.

An amperometric DNA biosensor for the determination of Aleutian mink disease related autoantibodies using the interaction of Pt(II) complex with the immobilised DNA or autoantibodies has also been reported (Babkina et al. 2004). The generation of ferrocenyl

glycopeptides as electrochemical probes to detect autoantibodies in multiple sclerosis patients' sera has been investigated by exploring the shift in potential recorded in voltammetric techniques upon interaction of the modified peptide with the target autoantibodies (Real-Ferndndez et al. 2008).

Celiac disease related antibodies, anti gliadin and anti-tissue transglutaminase, have been detected and reported using electrochemical immunosensors. Using a polyelectrolyte layer of poly (sodium-4-styrensulfonic acid) and BSA as blocking agent or 3-mercaptopropionic acid surface modification methods the detection of anti-tTG and anti-gliadin by impedance measurements has been reported (Balkenhohl and Lisdat 2007a, b). Serial dilutions of standard solutions were explored and some serum samples were also tested to estimate higher or lower contents of antibodies. These studies showed that a high charge transfer resistance values in impedance spectroscopy measurements can be related to increasing amounts of antibodies. However no real proportionality of the actual values was established and the comparison with some ELISA tests showed some false positives.

Antigliadin antibodies have been detected using a low-volume microsystem where the immunorecognition using a labelled antihuman IgG took place in a gliadin-coated controlled pore glass bed and the modified electrochemical reporter molecule is detected by a bare gold electrode in a total assay time of ca. 30 min and results were comparable to those obtained from commercial ELISA kits (Pereira et al. 2010). The detection of anti-tTG by direct adsorption of tTG onto graphite electrodes and immunorecognition by labelled antihuman IgA within a total assay time of just over 1 h has also been reported. The results of analyzing serum samples showed high specificity and about 70% sensitivity when compared with commercial ELISA methods (Pividori et al. 2009).

The electrochemical immunosensor presented in this work used a surface chemistry based on carboxyl-terminated an alkane chain bipodal thiol SAM containing polyethyleneglycol groups. Conjugation of

the coating antigen gliadin was achieved through its amine groups using carbodiimide chemistry (Grabarek and Gergely 1990; Staros et al. 1986), to the bipodal SAM. The presence of the polyethyleneglycol groups have been demonstrated to be an efficient barrier for non-specific adsorption phenomena while the bipodal nature of the alkane thiol preserved a good diffusion of electroactive species towards the electrode and also offers enhanced stability of the monolayer (Fragoso et al. 2008). Amperometric detection of polyclonal anti-gliadin antibodies and analysis of reference standard solutions showed reproducible responses. Real patient samples were analyzed and compared with commercial clinical diagnosis ELISA kits, showing an excellent correlation between the two techniques. A depicted diagram of the assay architecture and electrochemical detection format is shown in Figure 3.1.

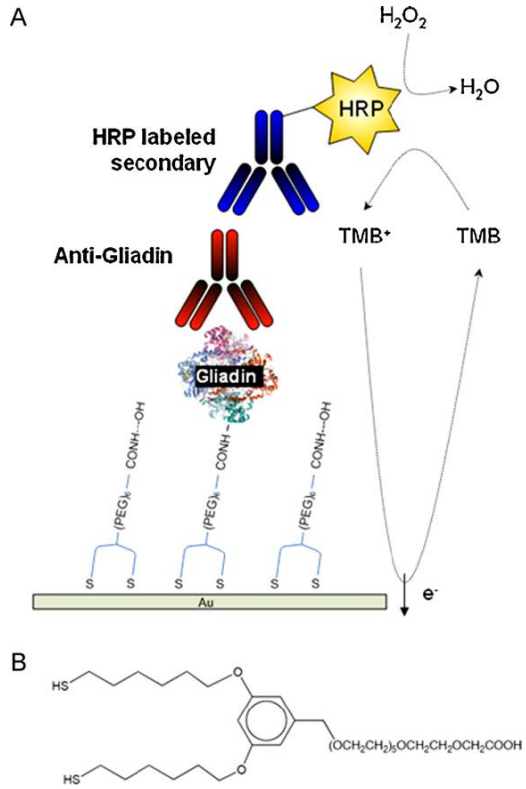


Figure 3.1. A) Schematic of the electrochemical immunosensor assay architecture. B) (22-(3,5-bis((6 mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid dithiol PEG-6 carboxylate (DT2).

3.2.3 Experimental section

3.2.3.1 Reagents and materials

Gliadin, polyclonal anti-gliadin antibodies developed in rabbit, anti-rabbit labelled with HRP, potassium ferrocyanide (III) and potassium ferrocyanide (II), sulfuric acid, strontium nitrate, phosphate-buffered saline (PBS) with 0.05% (v/v) Tween 20, carbonate–bicarbonate capsules for preparation of carbonate buffer (0.05 M, pH 9.6), and 3,3,5,5-tetramethylbenzidine (TMB) were purchased from Sigma–Aldrich Co. Hydrogen peroxide 30% (v/v), ethanolamine, glycine, N-(3-dimethylaminopropyl)-Nethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), sodium acetate, and acetic acid were purchased from Scharlau (Spain); (22-(3,5-bis((6 mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid (DT2) (see Fig. 1) was purchased from SensoPath Technologies (USA). Anti-human IgA and anti-human IgG labelled with HRP antibodies used for electrochemical measurements were those provided in Gliatest ELISA kits, Eurospital SpA (Italy). Amine coupling kit and Biacore SPR gold sensor chips were purchased from Pharmacia Biosensor AB (Sweden). Aqueous solutions were prepared with Milli-Q water (Millipore) and all reagents were used as received. Nunc MaxiSorp flat-bottom well plates were purchased from VWR International Eurolab (Barcelona, Spain).

3.2.3.2 Instrumentation

Electrochemical impedance measurements were carried out with a CH Instruments Electrochemical Analyzer 660A (CH Instruments Inc., US) and electrochemical studies using an Autolab PGSTAT 10 potentiostat, with measurements performed using a conventional three-electrode cell. Gold disk electrodes (2 mm-diameter) (CHI 101, CH Instruments Inc., US) were used as working electrode and a standard silver/silver chloride (sat. KCl) was used as a reference electrode (CHI 111 CH Instruments); platinum gauze was the counter electrode. Surface plasmon resonance analyses were performed on a Biacore 3000 GE Healthcare (USA). Experiments were performed using the Biacore

implemented temperature controller module. (For detailed surface plasmon resonance experiments conditions see Supplementary Information provided.) Measurements of pH were carried out using a pH Basic 20, Crison (Spain). Absorbance values were measured with a SPECTRAmax 340PC plate reader from bioNOVA científica (Barcelona, Spain).

3.2.3.3 Electrode modification and electrochemical detection

Gold electrodes were first polished with aqueous alumina slurries of 25 and 1 m (Buehler, US) and then rinsed with Milli-Q water, sonicated for 1 min and dried with argon; the electrodes were then treated with cold Piranha's solution (1:3, v/v, H2O2 and H2SO4) for 30 s and subsequently washed with Milli-Q water and argon dried. (Warning: Piranha's solution is highly corrosive and violently reacts with organic materials; this solution is potentially explosive and must be used with extreme caution.) A preliminary electrochemical cleaning in 0.1 M potassium hydroxide was performed by linear sweep voltammetry between −0.2 and −1.8 V; then cyclic voltammetry was performed for 30 cycles or until a stable voltammogram was obtained, at 0.2 V/s in 2 M sulfuric acid (Carvalhal et al. 2005). Electrodes were immersed in an ethanolic solution of 1 mM DT2 for 3 h, then rinsed with ethanol and dried with argon. To activate the carboxylic acids of the DT2 the electrodes were then immersed in a mixture 50% (v/v) EDC (0.4 mM) and NHS (0.1 mM) for 30 min and then rinsed with Milli-Q water. Electrodes were subsequently incubated with gliadin (100 g/mL) dissolved in acetate buffer, pH 4.5, for 30 min and any activated unreacted carboxylic acid groups remaining were blocked by immersion of the electrodes for 15 min in 2 M ethanolamine, pH 8.5, followed by a final rinse with Milli-Q water. The electrodes were incubated for 15 min in different antibody concentrations, calibrator reference solutions or patients' real serum followed by another 15 min the corresponding anti-rabbit or antihuman horseradish peroxidase-labelled antibody solution.

Amperometric measurement was carried out at 0.15 V in a 5 mL electrochemical cell containing PBS buffer and TMB (7.5:1, v/v, final ratio), and the TMB oxidized by the peroxidase enzyme, is reduced back to its original form generating a cathodic current at the electrode (Parker et al. 2009). All the electrochemical measurements were performed at room temperature and under the same stirring conditions. Impedance measurements were performed in 0.1 M PBS buffer solution, pH 7.4, containing 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide at a bias potential of 0.25 V in a frequency range from 0.1 Hz to 100 kHz with an amplitude of 5 mV. The impedance data was represented in the form of Nyquist plots and the parameters were obtained with the aid of the simulation software Z-view®.

3.2.4 Results and discussion

3.2.4.1 Surface plasmon resonance studies

The conjugation of gliadin to the thiol-modified gold surface and each step of the sensor assembly was monitored using surface plasmon resonance (see supplementary information for experimental details). The results indicated the successful binding of the gliadin to the surface as well as the subsequent recognition of a range of anti-gliadin antibodies concentrations (see Figure 3.2).

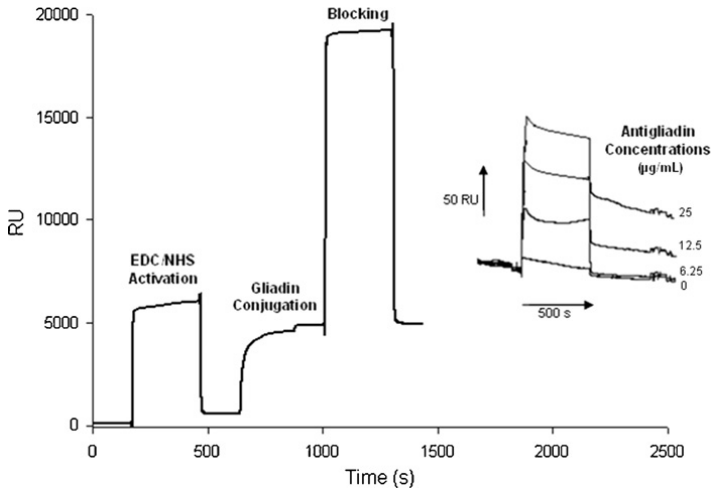


Figure 3.2. Surface plasmon resonance monitoring of the electrochemical sensor assembly. The inset shows the response of the assay to a range of polyclonal anti-gliadin antibody concentrations.

3.2.4.2 Impedance characterization of the immunoelectrode architecture

The fractional coverage (θ) of the dithiol self-assembled monolayer was evaluated

using the equation:

$$(1-\theta) = \frac{{R_{ct}}^0}{R_{ct}}$$

with Rct^0, being the resistance to charge transfer of the bare electrode, and Rct after the dithiol self-assembled monolayer. The θ values estimated were 0.994, which is indicative of a nearly complete monolayer and agrees with the reported literature values of similar dithiol self-assembled monolayers (Fragoso et al. 2008). Electrochemical impedance spectroscopy was utilized to explore each step of the electrochemical immunosensor construction. (See supplementary information). Electrodes were also tested by directly adsorbing gliadin on the surface followed by the antigliadin antibodies. The impedance

response was not only lower but opposite to the expected, indicative of the low level of efficiency of direct chemisorption as compared with bipodal thiol carbodiimide-based covalent attachment.

3.1.4.3 Amperometry-based antibody determination

Rabbit-produced IgG type human anti-gliadin antibodies were used to evaluate the performance of the electrochemical immunosensor in solutions of known concentration. The gliadin-modified electrodes were incubated for fifteen minutes with the solutions containing anti-gliadin antibodies prior to fifteen minutes incubation with horseradish peroxidase-labelled antirabbit IgG antibodies to complete the electrochemical immunosensor architecture. The measured amperometric response shown in Figure 3.3, demonstrated the efficiency and reproducibility of the immunosensor yielding a limit of detection of 46 ng/mL with an average RSD of 8.2 %, n = 5. The signal of the zero concentration value plus three times its standard deviation was used to estimate the limit of detection.

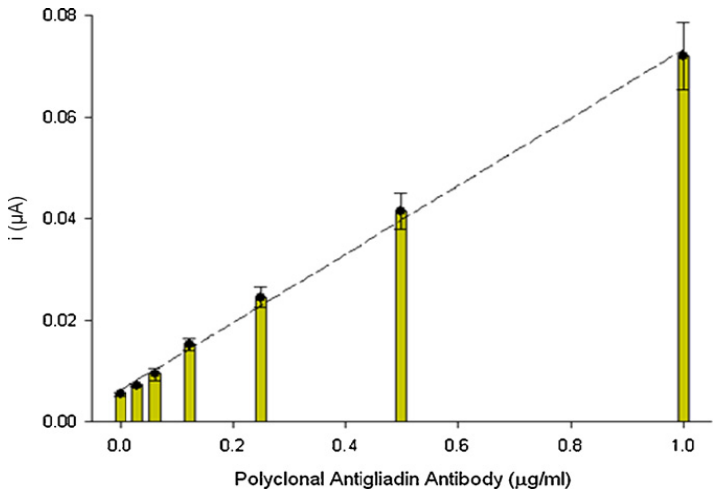


Figure 3.3. Amperometric calibration plot of the electrochemical immunosensor against different antibody concentrations. Each data point represents the average of three measurements on three separate sensors.

Calibrator serum solutions from ELISA commercial kits, normally used as clinical reference solutions to estimate anti-gliadin contents, were used to evaluate the performance of the electrochemical immunosensor in real complex matrixes. In order to explore the electrochemical immunosensor response compared with ELISA kits, both electrochemical and ELISA based evaluation of the calibrator solutions were carried out using the horseradish peroxidase labelled antibody solution provided in the commercial ELISA kits. The results clearly demonstrated the ability of the electrochemical immunosensor to discriminate clinically relevant anti-gliadin concentrations from sera matrices. Calibrator serum solutions labelled as zero are examples of sera with non significant levels of anti-gliadin antibodies. The response of the electrochemical sensor to those sera was comparable to that found in ELISA methods, (see Figure 3.4), which is indicative of a good non-specific adsorption control. Furthermore electrochemical and ELISA responses demonstrated an excellent correlation to the expected antigliadin contents in the sera tested.

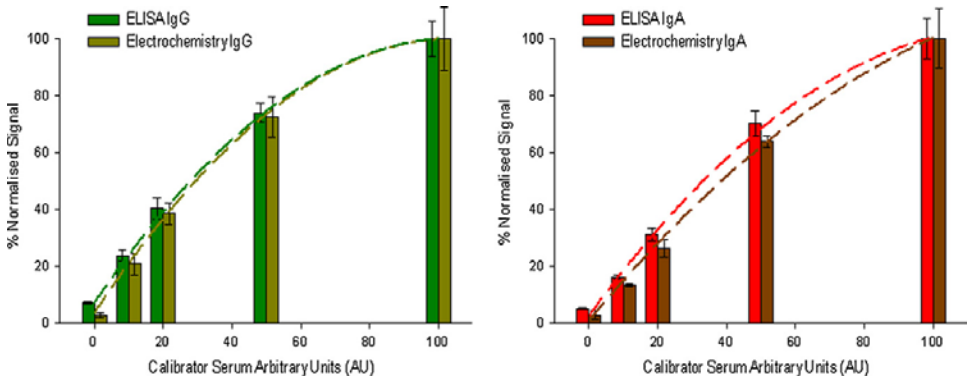


Figure 3.4. Comparison of electrochemical and ELISA responses to different calibrator serum solutions. Each data point represents the average of three measurements on three separate sensors.

The electrochemical sensor and equivalent ELISA assay were compared (see Figure 3.5) analyzing real celiac disease patients' serum. The dynamic range of both assays was explored by serial dilutions of

patients' serum to ensure the best sensitivity in the determination of anti-gliadin antibodies.

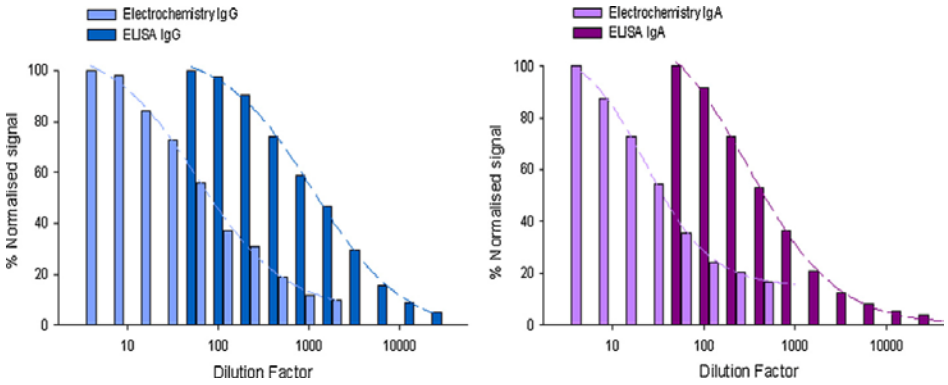


Figure 3.5. Electrochemical and ELISA assay performance for antigliadin determination in real patients' serum

The response of the electrochemical sensor showed a typical immunoassay behaviour, where saturation and non-sensitive zones were clearly identified. The lower dilution levels shown observed using the electrochemical platform is attributed to the oriented immobilisation of antigen in comparison with the random adsorption of antigen protein in the equivalent ELISA assay, as the random orientation may restrict the antigenicity of the protein, hence resulting in a proportionally lower antibody binding efficiency. The performance of the electrochemical immunosensor was evaluated against a commercial ELISA kit by analysis of real patient sera. The electrochemical immunosensor currents were extrapolated to the calibrator solution plot to obtain equivalent arbitrary units to those found when analyzing the same samples by commercial ELISA kits. The comparison of the results between the electrochemical immunosensor and commercial kits displayed (see Figure 3.6) a linear relationship with slope value of ca. 1.00 between the results obtained, indicative of an excellent degree of correlation.

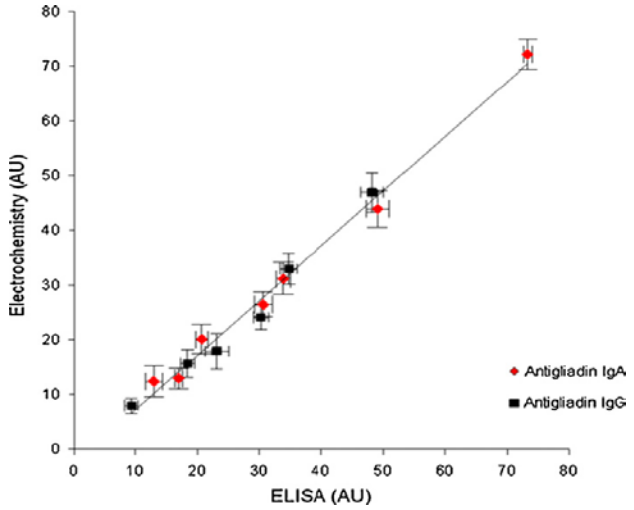


Figure 3.6. Comparison of the electrochemical immunosensor and commercial ELISA for the determination of antigliadin antibodies from real patients' sera.

3.2.5 Conclusions

A simple but robust and reliable electrochemical immunosensor for the determination of anti-gliadin antibodies has been built and characterized. The performance of the developed immunosensor has yielded low limits of detection (46 ng/mL) with a high degree of reproducibility (% RSD = 8.2, n = 5). Complex matrixes such as calibrator serum or real patient blood serum did not affect the performance of the sensor. The high sensitivity inherent of electrochemical detection and the optimal packing of molecules on the surface provided by the dithiol chemistry employed facilitated the electrochemical immunosensor to match the behaviour and sensitivity achieved by standard ELISA procedures in shorter time operation times (30 min total assay time against the 90–135 min of commercial kits), allowing a semi-quantitative estimation of antibody contents and facilitating an excellent prospective for portability and in situ measurements.

3.2.6 Supplementary information

3.2.6.1 ELISA dilution curve of real samples

Typically, 50 μl of a 10 μg/ml of gliadin from wheat were immobilised on a NUNC MaxiSorp 98 well plate for 60 min at 37 ºC using 50 mM carbonate-bicarbonate pH 9.6 as coupling buffer. After blocking the well surface with 200 μL of 10 mM PBS-Tween for 60 min at 37 ºC, 50 μL of various dilutions of the real patients' samples prepared in EUROSPITAL's sample diluent were added into the wells for 60 min at 37 ºC, 50 μL of anti-human IgA modified with HRP enzyme (308 ng/mL in 10 mM PBS-Tween) was added for 60 min at 37 ºC to detect human anti-gliadin IgA antibodies. In the same manner, anti-human IgG labelled with HRP (74 ng/mL) was added to detect human anti-gliadin IgG antibodies. Finally, the presence of the HRP label was detected using 50 μL of TMB liquid substrate, and the enzymatic reaction was stopped with 50 μL of a 1 M H_2SO_4 solution after 15 min, and measured at 450 nm using the SPECTRAmax 340PC plate reader. In between all the mentioned steps a three-cycle washing procedure using PBS-Tween (200 μL) was adopted.

3.2.6.2 Surface plasmon resonance chip preparation and measurements

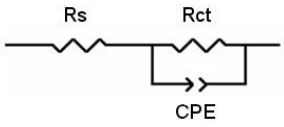
J1 gold chips were cleaned in cold Piranha's solution for 30 seconds and then thoroughly rinsed with distilled water followed by an ethanol rinsing and subsequently dried with argon. The chips were then immersed for 3 hours in an ethanolic solution of 1mM DT2, followed by rinsing them in ethanol and again drying with argon. The functionalized chips were then introduced into the BIACORE 3000 instrument, and the chip allowed to condition by flowing running buffer (PBS-Tween, 10 mM, 7.4 pH), at a flow of 20 μL/min for 30 min or until a stable baseline was achieved. For immobilization of the gliadin, a flow rate of 5 μL/min was used, and a mixture of 10 μL 50 % v/v EDC (0.4 M) and 50 % v/v NHS (0.1 M) was injected into each channel to be modified. Gliadin (30 μg/mL) in acetate buffer 0.1 M at a pH 4.5 was flowed for 7 min. Inactivation of

the remaining activated carboxylic groups to hydroxyl groups was achieved by injection for 7 min of ethanolamine 1 M pH 8.5.

The incubation steps of anti-gliadin antibodies was carried out at a flow rate of 20 µL/min. Anti-gliadin antibodies were incubated for 7 min for association and 7 min for dissociation. The regeneration of the channels was done using glycine 10 mM pH 1.8, using 2 pulses of 1 min, separated by 1 min of PBS-Tween.

3.2.6.3 Impedance characterization of the immunoelectrode architecture

The impedance data was represented in the form of Nyquist plots and the parameters were obtained with the aid of the simulation software Z-view® according to the equivalent depicted circuit.



Rs represents the solution resistance, *Rct* charge transfer resistance and *CPE* is a constant phase element that represents the capacitive effect on the electrode surface.

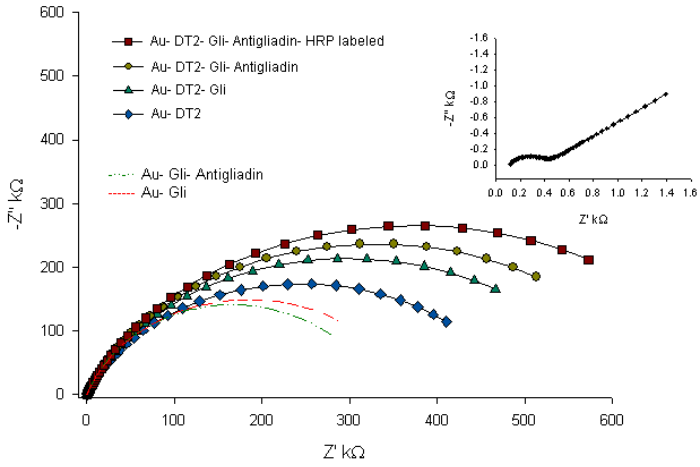


Figure 3.7. Typical Nyquist plots obtained from the different stages of the construction of the electrochemical immunosensor for the detection of 1 μg/mL of polyclonal antigliadin antibodies. The inset shows the impedance plot recorded for a bare electrode.

3.2.7 Acknowledgements

This work has been carried out with financial support from the Commission of the European Communities, RTD programme "Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CD MEDICS [FP7-2007-ICT-1 IST-2007-2-216031]. The authors would like to acknowledge Dr Julia Ellis from King's College London for the kind supply of real patients' samples; Eurospital SpA (Italy) for the kind supply of Gliatest IgA and IgG ELISA kits and reagents; Mr. Rosales- Rivera acknowledges Universitat Rovira i Virgili for the financial support within the MSc Nanoscience and Nanotechnology programme and Generalitat Catalunya for the FI predoctoral grant.

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CHAPTER 4

"Amperometric immunosensor for the determination of IgA deficiency in human serum samples"

4.1 Chapter introduction

In *Chapter 3* the proof-of-concept for the construction of a functional immunosensor was fully demonstrated, using a bipodal alkanethiol. The platform is used with complex matrices and with ability to detect different reference serum solutions used for the detection of CD anti-gliadin human antibodies. In *Chapter 4*, using a similar and optimized approach, it will be demonstrated the detection and quantification of human anti-IgA antibodies present in serum samples from CD patients. Using amperometry as electrochemical detection technique, key experiments will be studied. Firstly in buffer solutions, in order to obtain a calibration curve. Cross reactivity studies are done in order to assure the low interference of other antibodies, in this case IgG isotype antibodies. Specificity of the immunosensor and its use in complex matrices, such as foetal bovine serum (FBS) samples, is also explored. The reliability of the constructed immunosensor has been proved, as well as its versatility, concerning the immobilisation and detection of IgA antibodies. Finally, total IgA concentrations are quantified for deficient and non-deficient CD patients' sera.

4.2 Article "Amperometric immunosensor for the determination of IgA deficiency in human serum samples"

4.2.1 Abstract

An electrochemical immunosensor for the detection of human IgA deficiency in real human blood serum has been developed. The performance of the immunosensor presents a large but sensitive dynamic range that allows the determination of non-deficient IgA levels (>70 μg/mL) as well as of severe IgA deficiencies (0.5–5.0 μg/mL). The assay architecture involves the immobilisation of a coating antibody on an electrode surface using carboxylic-ended bipodal alkane-thiol self-assembled monolayers (SAMs). The long chain bipodal SAM presents intercalated poly(ethylenglycol) groups that confer the immunosensor the ability to retain its optimum performance in very complex matrices and serum with negligible non-specific adsorption phenomena.

Amperometric optimisation of the assay resulted in limits of detection of 142 ng/mL in just 30 min total assay time. Real patients' serum samples were analysed using the developed electrochemical immunosensor demonstrating an excellent correlation in terms of sensitivity and reproducibility compared with standard enzyme linked immunosorbent assays (ELISA).

4.2.2 Introduction

Immunoglobulin A (IgA) deficiency is the most common immunodeficiency among healthy individuals, with incidence levels of up to 1:163 (Clark et al. 1983; Hunt et al. 1985). Values of IgA from ca. 1 mg/mL and above are considered as a normal concentration (Sinclair et al. 2006), with IgA levels below 50–70 μg/mL considered to be indicative of a global immunodeficiency (Thibault et al. 2006; Yuan and Goldfinger 2008). The value of 70 μg/mL as the deficiency levels for IgA was partially chosen because lower concentrations than these cannot be easily detected by nephelometry, the most widely available method for determination of IgA levels, but at the same time a relatively inaccurate optical method (Goldblum et al. 1986). The determination of more acute deficiencies require the utilisation of more sensitive assays such as passive haemagglutination inhibition (Mohabir and Rees 1995), solidphase RBC adherence assay (Schulenburg et al. 1991), particle-gel immunoassay format (Salama et al. 2001) (gel card technology), or enzyme-linked immunosorbent assay (Sanz et al. 1999). However the distinction between a deficiency and a severe deficiency (between 0.5 and 5.0 μg/mL) (Yuan and Goldfinger 2008) has clinical and diagnostic implications. Pathologies that benefit from a close control of IgA levels and monitoring are those caused by several drugs or viral infections (Latiff and Kerr 2007) type 1 diabetes (Nicoloff 2007) or coeliac disease (Sinclair et al. 2006).

For example, approximately 2–3% of coeliac patients are immunoglobulin-A (IgA) deficient, which are known to produce false negative IgA tTG results (Cataldo et al. 2000). For this reason, it is recommended that serum IgA levels be measured in those individuals at a higher risk of the disease, i.e. those with symptoms or with a family history, as the selective IgA deficiency is 10–20 times more common in coeliac disease patients than in the rest of the population (Collin et al. 1992; Sinclair et al. 2006). Enzyme-linked immunosorbent assays are time-intensive and require skilled operators and necessarily require to be carried out in centralised laboratories. Electrochemical immunosensors offer many advantages such as ease-of-use, rapid response, reduction of reagent consumption, high throughput analysis as well as great potential for miniaturisation and production of portable devices (Warsinke et al. 2000). Electrochemical immunosensors are normally constructed by immobilisation of a biomolecule onto the electrode surface, using techniques such as non-covalent binding, polymer entrapment (Cosnier 1999; Cosnier and Gondran 1999), hydrophobic adsorption (Grant et al. 2003) or polyelectrolyte adsorption techniques (Beissenhirtz et al. 2004; Ladam et al. 2002), and covalent binding via the formation of self-assembled monolayers, exploiting carboxyl or amino terminated thiol compounds that can then be activated for covalent coupling, thus controlling the orientation, distribution or spacing of the immobilised molecules (Gooding and Hibbert 1999). The chemisorption of thiols on gold surfaces provides a stable and conductive surface that has been largely exploited for electrochemical immunosensors (Arya et al. 2009; Azzaroni et al. 2003; Duarte et al. 2009; Vaughan et al. 1999). The work presented here takes advantage of the properties of a carboxyl-terminated bipodal alkanethiol, which incorporates a long polyethyleneglycol chain, facilitating electron transfer whilst acting as an efficient barrier against non-specific interactions of matrix components (Fragoso et al. 2008; Laboria et al. 2010; Nassef et al. 2009; Nassef et al. 2008) .This bipodal alkanethiol-based assay architecture has recently been applied in alternative electrochemical immunosensors with good sensitivity and

control over non-specific binding phenomenon in serum samples (Dulay et al. 2011; Rosales-Rivera et al. 2011).

Electrochemical determination of immunoglobulin's is a topic that has been reported and when searching for low concentration detections most of the work is referred to the predominant immunoglobulin isotype G. Relevant work carried out by (Wilson 2005) reported reasonable limits of detection (5 ng/mL) with 2 h total incubation time but with showed limited detection range (up to 55 ng/mL).

Recently some works have reported extended dynamic ranges (up to 1 μg/mL) with enhanced detection limits down to 0.1 ng/mL; however very strict experimental conditions are required, as well as some pre-concentration of the electroactive species prior to voltammetric reading (Kang et al. 2009). Determination of low levels of immunoglobulin A has been recently investigated using various surface chemistries and electrochemical techniques. A capacitive immunosensor based on a CuS ultra thin film generated on the electrode surface has been reported, where the film is generated by a sol–gel preparation technique that provides a CuS film to which amino and carboxyl moieties present in biomolecules can bind through chelate bonding. Capacitance measurements are then recorded following a potentiostatic step procedure, achieving low limits of detection (1.81 ng/mL) using spiked buffered solutions but with a very limited linear range (0–94 ng/mL) (Wu et al. 2010). Other reports have detailed the use of carbon spheres fabricated by microwave-hydrothermal technique to form arrays on the surface of indium–tin oxide electrodes, where the carbon spheres have aldehyde and carboxyl groups on the surface that are capable of forming amide bonds with antibodies.

Using impedimetric transduction, the carbon sphere-modified electrode was exploited for the detection of IgA antibodies, with a very low limit of detection (0.1 ng/mL) but again a limited linear range (0–200 ng/mL) (Chen et al. 2010).

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Other analytical methods, such as chemiluminiscent ones (Zhou et al. 2006), have also been applied to achieve extended IgA detection range whilst having low detection limits (1.2 ng/mL), however the level of complexity of the operational procedures (magnetic beads and polymer conjugated based pre-concentration from sample matrices along with several temperature controlled centrifugation step) highlights the need for a fast, sensitive and simple method for the determination of low levels of IgA.

In this work an electrochemical immunosensor for the detection of human IgA has been developed and tested in patient sera. The sensor architecture involves the immobilisation of a coating antibody on the electrode surface, incubation with samples containing IgA and finally incubation with a horseradish peroxidase labelled antihuman IgA antibody, as depicted in Figure 4.1. The resulting electrochemical immunosensor, based on a SAM architecture of a bipodal alkanethiol, was reliable enough to allow the use of amperometric techniques with low detection limits and extended detection ranges in buffered solutions, foetal bovine serum and finally in real patients' serum detecting in the severe deficiency values (ng/mL) matching standard ELISA results.

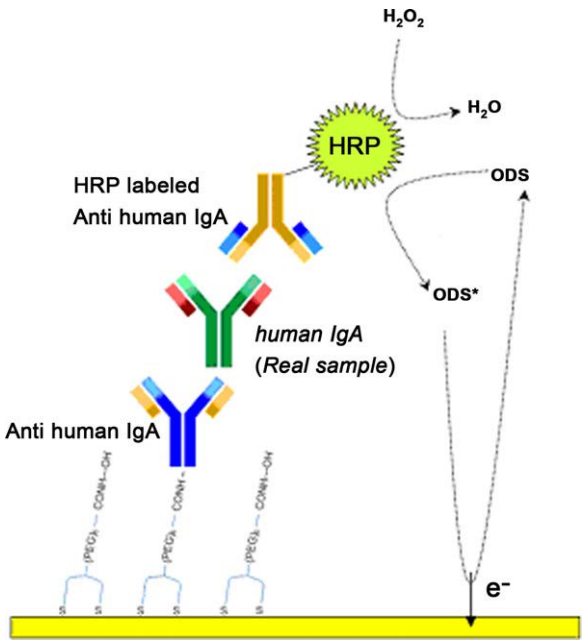

Figure 4.1. Electrochemical immunosensor architecture.

4.2.3 Experimental section

4.2.3.1 Reagents and materials

Bipodal thiol 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid (DT2) was purchased from Sensopath Technologies (Bozeman, MT, USA) (see Fig. 4.1). Stock solutions of 1 mM in ethanol were prepared, purged with nitrogen and stored at −20 ◦C when not in use. Horseradish peroxidase (HRP), anti-human IgA (α- chain specific) antibody produced in goat, IgA from human colostrum and human IgG, anti-human IgA and IgG peroxidase antibody produced in goat, foetal bovine serum, o-dianisidine (ODS), N-hydroxy-succinimide (NHS), potassium hexacyanoferrate (II) trihydrate, and N-(3-dimethylaminopropyl)- N'-ethyl-carbodiimide (EDC) were obtained from Sigma–Aldrich Chemical Co. (Spain) and were used as received. MES monohydrate was obtained from Acros organics. Hydrogen peroxide 30%, sulfuric acid 97%, ethanolamine, ethanol, potassium chloride were obtained from Scharlau S.L. (Spain).

4.2.3.2 Electrochemical Instrumentation

All electrochemical measurements were performed in a standard three-electrode system in a 4 mL glass conical electrochemical cell under constant stirring with a platinum mesh counter electrode and Ag/AgCl reference electrode (Bioanalytical System, IN, USA). Standard gold disk electrodes 1.6 mm diameter (BAS, IN, USA) were used as working electrodes. Amperometry and cyclic voltammetry were carried out using a CHI 660A electrochemical workstation (CHInstruments, USA). Standard disk electrodes were prepared by polishing with aqueous slurries of 25, 0.6 and 0.3 mm alumina on a polishing cloth (Buehler, USA). The electrodes were then sonicated for 5 min and rinsed with Milli-Q water, followed by cycling from −0.2 V to 1.8 V vs. Ag/AgCl in 2 M sulphuric acid at 0.2 V/s. The electrodes were then rinsed with Milli-Q water and ethanol and Argon-dried prior to further use.

4.2.3.3 Electrode surface self-assembled monolayer formation

Electrodes were immersed in 1 mM ethanolic solutions of the bipodal alkanethiol (DT2) for 3 h. The electrodes were then rinsed with ethanol and Milli-Q before being argon dried. The thiol modified electrodes were then immersed in a solution of 50 mM NHS/200 mM EDC in 0.1 M MES buffer pH 5 for 30 min to activate the terminal carboxylic groups of the immobilised thiol molecules. Electrodes were immersed for 20 min in 100 μg/mL of antihuman IgA in pH 7.4 PBS, followed by immersion in 1 M ethanolamine for 15 min to block any remaining activated carboxyl groups. Coating antibody concentration is shown in Supplementary information part.

4.2.3.4 Electrochemical detection of IgA antibodies

The electrodes were incubated for 15 min at room temperature with different concentrations of (i) commercial human IgA antibodies, (ii) different percentages of foetal bovine serum solution mixed with pH 7 phosphate buffer and spiked with 1 μg/mL of commercial human IgA antibodies or (iii) real patient serum samples followed by a final rinse

with PBS, followed by incubation for another 15 min with 6 μg/mL of HRP-labelled anti-human IgA antibodies. In all cases the amperometric response was recorded for 60 s at 0.2 V vs. Ag/AgCl in 0.2 M acetate buffer, pH 4.7, containing 0.15 mM of o-dianisidine as mediator and 1 mM of H_2O_2 as peroxidase substrate.

4.2.4 Results and discussion

4.2.4.1 Characterisation of the electrochemical immunosensor

The coverage θ (between 0 and 1) values estimated for the formation of the bipodal SAM were 0.992, which is indicative of a nearly complete monolayer and agrees with reported literature values of similar dithiol self-assembled monolayers (Fragoso et al. 2008). The different stages of electrochemical immunosensor fabrication, such as dithiol self-assemble monolayer formation, coating anti-human IgA antibody immobilisation and target human IgA antibody binding, were characterised using impedimetric methods (see Supplementary information).

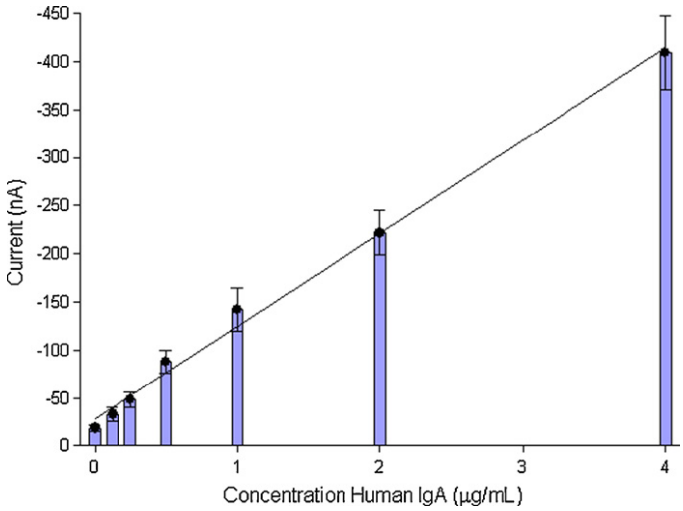

Figure 4.2. Calibration plot for the detection of human IgA antibodies using amperometric methods.

4.2.4.2 Electrochemical detection of human IgA antibodies

Using the optimised immunosensor assembly conditions the anti-human IgA coated electrodes were exposed to different concentrations of human IgA (see Figure 4.2). The signal of the zero concentration value plus three times its standard deviation was used to estimate a limit of detection of 142 ng/mL, falling into the clinically relevant range for the determination of severe IgA deficiencies. The specificity of the immunosensor towards IgA was checked in presence of IgG as a typical example of other common immunoglobulin found in high concentration in human serum samples. The specificity obtained by the immunosensor was checked presenting the anti human IgA-coated gold platform to either IgA or IgG as target antibodies and to HRP-labelled anti human IgA or IgG secondary antibodies (see Figure 4.3). The response of the immunosensor recorded negligible cross-reactivity with other relevant immunoglobulin antibodies. Prior to testing the immunosensor performance against real serum the assay was evaluated for matrix effects by subjecting it to amperometric measurements in different concentrations of foetal bovine serum solutions spiked with human IgA antibodies.

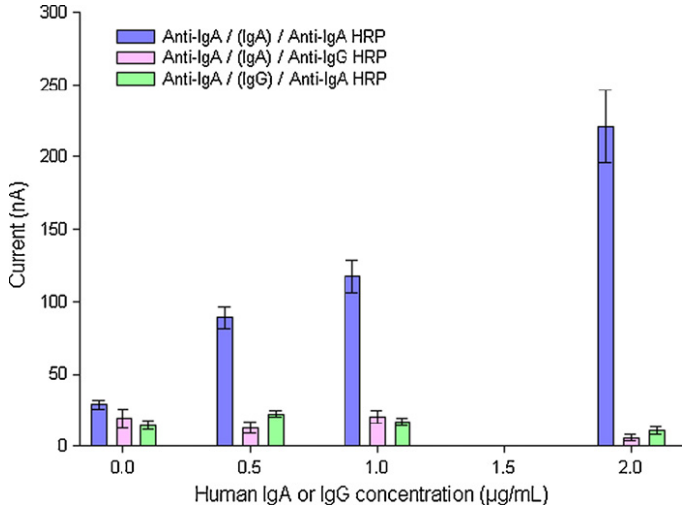

Figure 4.3. Amperometric response of the immunosensor to different assay architectures (n = 3).

The current measured were normalised being 100% the current recorded in absence of foetal bovine serum. The immunosensor was demonstrated to maintain signal intensity even in pure serum, with observed of blank non-specific binding response of ca.14% (see Figure 4.4). Undesired non-specific binding events, recorded as high blank currents, could be recorded in the presence of matrices, such as foetal bovine serum, with very high contents of various biological substances. Instead of this the electrochemical immunosensor registered little interference from non-specific binding, proving the suitability of the PEG-bipodal alkanethiol to act as efficient nonspecific binding barrier. A perfect discrimination between specific antibody signals and blank controls (performed in 100% foetal bovine serum signal) was observed; unlike some other examples reported in the literature (Arya et al. 2009) the bipodal alkanethiol SAM based assay did not require the implementation of additional non-specific adsorption barrier mechanism to prevent unwanted binding phenomena. Poly(ethylenglycol) groups intercalated in the long chain of the alkanethiol proved efficient to allow the utilisation of the immunosensor in very complex matrices with no need for backfilling SAMs or antifouling proteins (Ladam et al. 2002).

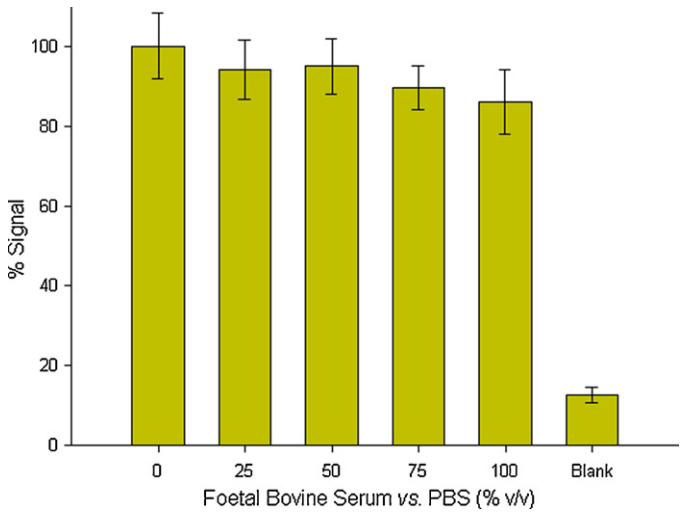

Figure 4.4. Amperometric response of the immunosensor in different foetal bovine serum solutions spiked with 1 μg/mL of human IgA (n = 3).

Real patient serum samples were studied for the detection of human IgA levels and different dilutions were analysed to establish the optimum dilution value for the analysis of different real samples and were analysed both by amperometric and ELISA methods. Both electrochemical and spectrophotometrical dilution curves showed the expected behaviour, where signal saturation zone, dynamic detection range and non-sensitive region can be clearly determined, as shown in Figure 4.5. The wide dynamic range observed confers the electrochemical sensor very profitable detection flexibility, facilitating analysis of samples with a range of concentrations of two orders of magnitude difference.

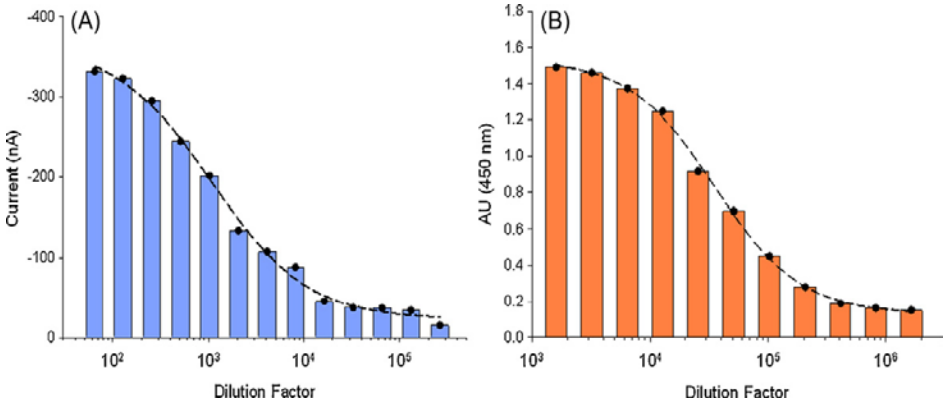

Figure 4.5. Amperometric (A) and ELISA (B) responses for the detection of human IgA antibodies for different dilutions of an IgA non-deficient real patient's serum sample.

Several human real samples with clinically suspected different levels of IgA (deficient and non-deficient) were analysed and the values of current obtained from each sample linear range of the dilution curve extrapolated to the calibration plots to obtain quantitative data (see Figure 4.6).

The results from the various samples with different levels of IgA showed an excellent degree of correlation between the electrochemical immunosensor and ELISA, highlighting the potential of the electrochemical immunosensor developed for the in situ measurement of human IgA levels in sera samples. The electrochemical immunosensor was able to reliably quantify normal IgA levels in serum samples and was also able to determine IgA deficiencies of severe cases ([IgA] between 0.5 and 5.0 μg/mL). These results highlight the advantageous performance of the electrochemical immunosensor i.e. wide operation range, ability to work in real serum samples and rapid assay time. The performance of the immunosensor clearly improves that of other simple and rapid electrochemical immunosensors – limit of detection of 0.77 μg/mL (Sanchez Ordonez and Fabregas 2007) – or classical immunosorbent assays – limit of detection of 1.0 μg/mL (Sato et al. 2000). The excellent performance specifications of the sensor combined with the prospective for integration in multielectrode array platforms and potential for miniaturisation and portability of the instrumentation associated positions the reported immunosensor as a perfect candidate for analysis at the point-of-care.

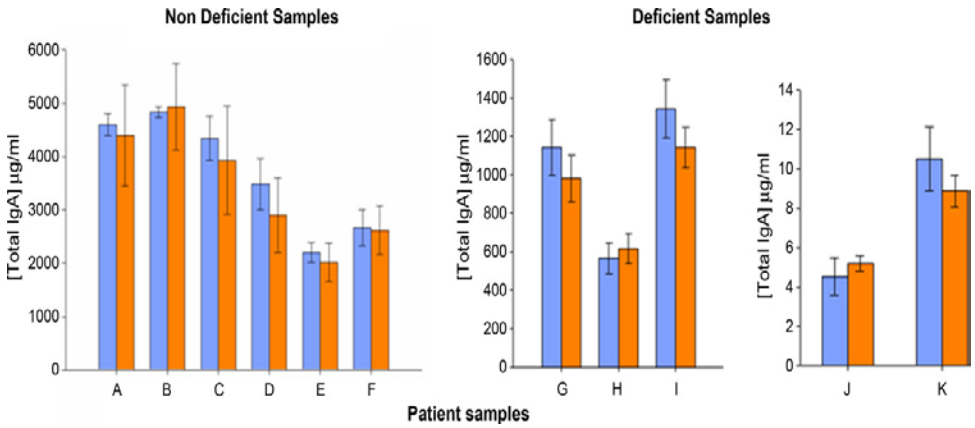

Figure 4.6. Analysis of IgA antibody contents in real patients' sera by electrochemical immunosensor and ELISA methods.

4.2.5 Conclusions

The dithiol SAM-based electrochemical amperometric immunosensor described in this work has been investigated and optimised to yield reliably low limits of detection, indicative of the robustness and sensitivity of the achieved assay. The amperometric immunosensor presented low levels of non-specific adsorption, which allowed an optimal performance in complex matrices such as foetal bovine serum or directly human blood serum.

The behaviour of the electrochemical immunosensor correlated excellently with classical ELISA methods not only in the capability to quantitatively determine IgA contents from real serum samples, but also allowing the testing of different concentration range samples, accompanied by the advantages of a rapid assay time (30 min), low-cost apparatus, low time-consuming and the possibility for in situ measurements. The potential for miniaturisation of the electrochemical immunosensor and its implementation within a multi-electrode array for detection of multiple markers confers the reported electrochemical immunosensor a great future for integration in automated electrochemical-detection platforms as a more convenient alternative to classical optical immunoassays.

4.2.6 Supplementary information

4.2.6.1 Coating antibody concentration

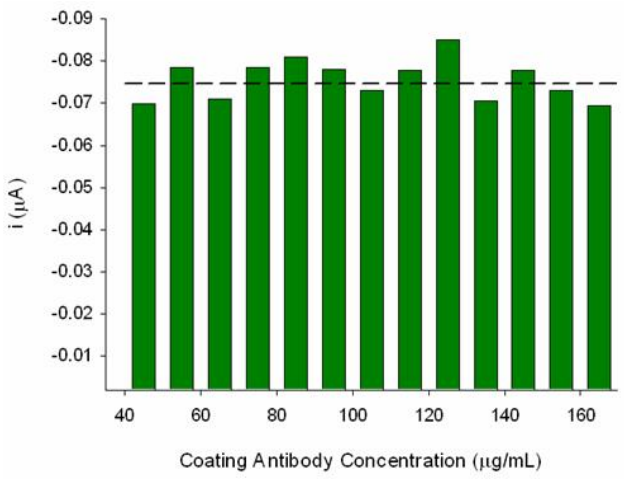

Figure 4.7. Effect of the different coating antibody concentration on the electrochemical immunosensor performance.

4.2.6.2 Fabrication of the electrochemical immunosensor using EIS

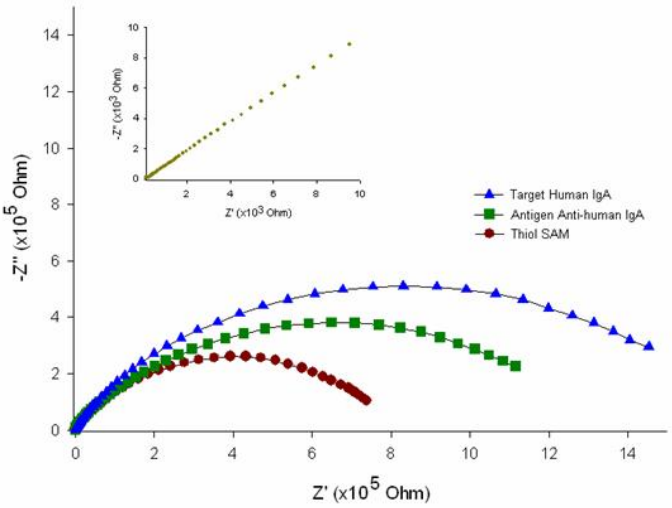

Figure 4.8. Typical Nyquist plots reflecting increasing impedance due to immunosensor construction and target binding. The inset shows the impedance plot recorded for a bare electrode.

4.2.6.3 Nyquist plot for increasing concentrations

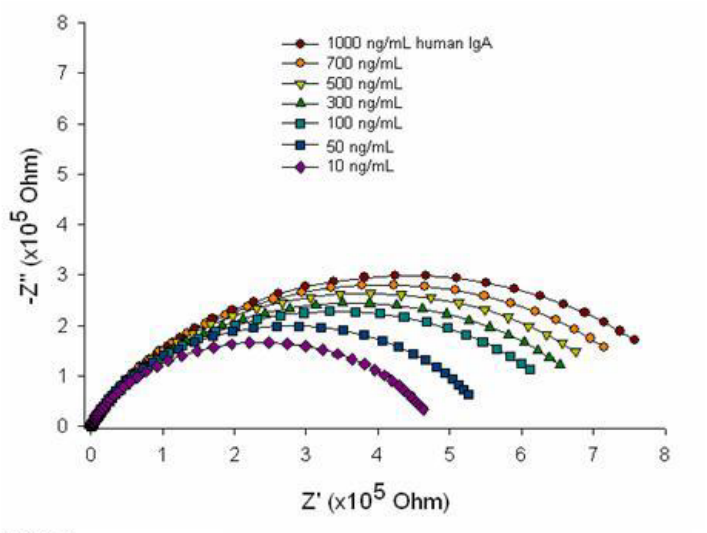

Figure 4.9. Typical Nyquist plots reflecting increasing impedance for the determination of human IgA antibodies.

4.2.7 Acknowledgements

This work has been carried out with financial support from the Commission of the European Communities, RTD programme “Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System”, CD MEDICS [FP7-2007- ICT-1 IST-2007-2-216031]. The authors would like to acknowledge Dr. J. Ellis from King’s College London and Dr. Federico Biagi from San Mateo Hospital, Pavia, Italy for the supply of real patients’ samples. Mr. Rosales-Rivera acknowledges the Generalitat de Catalunya for financial support.

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CHAPTER 5

"Modified proteins as self-assembled antigens monolayer for amperometric and SPR detection of antibodies"

5.1 Chapter introduction

In this chapter, proteins are self-assembled over gold surfaces, after its thiolation through different moieties (amine, carboxylic and hydroxyl). Introduction of disulphide bonds is reached using standardized chemistry pathways. The antigenic properties of the modified proteins is tested using standard enzyme linked immunosorbent assays (ELISA), surface plasmon resonance (SPR), and amperometric studies. Immunosensor assembly protocol is very similar to the used at *Chapter 3*, and even some parts of the immobilisation protocols are equal, but instead of using a dithiol, the antigen is attached directly over the surface, and the possible remaining sites are blocked with thiol molecules that posses PEG groups. Detection of anti-gliadin (AGA) and anti-tissue transglutaminase (tTG) antibodies, in buffer samples, are compared for the different obtained surfaces using amperometry and SPR studies. Reference serum calibrators from commercial ELISA kits are tested and compared in all obtained surfaces, through amperometric and SPR detection. Finally, serum samples from coeliac disease (CD) patients' sera, IgG-AGA and IgA-tTG antibodies, are detected and compared using amperometric and ELISA based tests.

5.2 Article "Modified proteins as self-assembled antigens monolayer for amperometric and SPR detection of antibodies" In preparation

5.2.1 Abstract

A simple and fast immunosensor for the determination of coeliac disease (CD) related antibodies, anti-gliadin and anti-tissue transglutaminase (anti-tTG), was investigated. Antigenic proteins, gliadin and tTG were chemically modified introducing disulphide groups through different moieties of the molecules (amine, carboxylic and hydroxyl). Modified antigens were self-assembled over gold surfaces and used for the monitoring of the immune-recognition of the related specific antibodies using surface plasmon resonance (SPR) and amperometric measurements. ELISA studies showed no changes in the antigenicity of all different modified gliadin proteins, whilst only

introduction of thiol groups in the tTG through amine moieties preserved its antigenic properties. Amperometric detection was performed using a secondary horseradish peroxidase labelled antibody and the non specific adsorption phenomena was controlled using a poly-(ethylene glycol) alkanethiol as backfiller. For gliadin modified surfaces, SPR best response yielded a limit of detection (LOD) of 0.63 µg/mL, for amperometric measurements, the LOD improved down to 85 ng/mL. In the case of tTG modified surfaces, SPR yielded a LOD of 6.1 µg/mL and 260 ng/mL when evaluated by amperometry. Immunosensor performance in presence of complex matrices, including clinically relevant serum reference solutions and coeliac disease patient's sera, was evaluated using SPR and amperometric studies. The thiolation of the antigenic proteins enabled a simple and convenient two-step surface modification procedure involving spontaneous gold-thiol covalent binding and backfilling of the remaining sites. Complete assay time was 30 min for amperometry and 12 min in the case of SPR.

5.2.2 Introduction

Immunosensors are devices able to monitor or sense affinity binding events between immunological molecules, antigens and antibodies. When both molecules are in close proximity to a transducing surface, occurrence of the recognition event can be detected by measuring changes in the physical-chemical properties of that surface. These immunochemical reactions have been widely recorded and reported using a number of analytical techniques. In order to provide enough sensitivity for the detection at least one of the immuonreactants must be kept in close contact or immobilised onto the transducing surface. Different procedures have been used to ensure the immobilisation of immunoreactants to solid supports, in order to form an immunoaffinity layer, *i.e.*, physical adsorption (Corry et al. 2003); polymer entrapment (Barton et al. 2009), sol-gel entrapment (Shalev and Miriam 2011), covalent attachment (Albarghouthi et al. 2000), Langmuir–Blodgett deposition (Singhal et al. 2002) or self assembled monolayers (SAM) (Chapman et al. 2000). One of the main requirements

for immobilisation techniques is the maintenance of the biological properties for the immobilised molecule, which in the case of immunoreactants is mainly related to the maintenance of their functionality and protein conformation to ensure the occurrence of antigen-antibody recognition events (Yoshimoto et al. 2010).

A widely reported immobilisation method of biomolecules onto metallic solid supports for biosensor application is the chemisorption of a monolayer of molecular thickness; also know as self assembled monolayer (SAM), they can be mainly formed directly by adsorbing a molecule that contains a ligand with affinity towards the surface or by attaching the molecule to an already SAM-modified surface. The direct adsorption of thiolated biomolecules to metallic surfaces, in most cases gold, is normally used for optical and electrochemical immunosensors. This approach not only creates controlled SAMs, but also prevents the loss of immunogenic activity and ensures a stable immobilisation through a strong sulphur-gold bond (Love et al. 2005). Modified proteins with thiol/disulphide groups can form ordered SAMs on gold surfaces (Baltus et al. 2007; Bonroy et al. 2006; Brogan et al. 2003; Choi et al. 2005; Lee et al. 2003), sometimes with less preparation and time of immobilisation than thiols (Fowler et al. 2006). Functionalisation of whole molecules or just fragments with thiol/disulphide groups, and later attachment to gold surfaces has been successfully implemented in biosensor devices (Nassef et al. 2009; Viana et al. 2002).

Immunosensors are an ever growing research topic in the area of immunochemistry and an alternative to classical methods like enzyme-linked immunosorbent assays (ELISA) and radio immunological assays (RIA) in point-of-care and portable testing scenarios (Willmott and Arrowsmith). Immunosensor development using electrochemical and optical detection is one of the most promising research areas. Characteristics including portability, low cost, miniaturization potential and high sensitivity, allows them to be used in a wide range of applications: environmental controls, food analysis, bacteria detection, cancer and tumour biomarkers (Adányi et al. 2006; Lin and Ju 2005; Ricci

et al. 2007; Rusling et al. 2009; Tokarskyy and Marshall 2008). Integration with other technologies allows continuous operation and multi-analysis procedures (Mastichiadis et al. 2008). Examples of immunosensors applied to clinical diagnosis have been reported using both electrochemical (Dulay et al. 2011; Fragoso et al. 2010; Hao et al. 2007; Laboria et al. 2010; Rosales-Rivera et al. 2011b) and optical transduction (Bonroy et al. 2006; Choi et al. 2005).

CD is an autoimmune disorder that is triggered in persons genetically predisposed by ingestion of gluten and related proteins (Dieterich et al. 1997; Stern et al. 2001); damage is done by the ingestion in the small intestine and it leads to the presence of several symptoms that can go from the malabsorption of nutrients to complicated symptoms like chronic diarrhoea, weight loss, abdominal distension and general malnutrition (Mäki and Collin 1997). The only successful treatment against CD is a gluten free diet (Janatuinen et al. 2002), with recorded improvements in days or weeks after the adherence to the gluten free program (Briani et al. 2008). Current diagnosis and routine clinical tests of the disease include serological tests to measure the levels of antibodies against gliadin, tTG and endomysium (EMA). (Farrell and Kelly 2001) The detection of Anti-tTG (IgA) and Anti-EMA (IgA) are highly sensitive and specific to detect CD in children and adults >95% (Hill 2005), due to poor sensitivity AGA tests have been discarded, further studies have revealed than for children below 18 months the detection of both AGA-IgA and tTG-IgA are an optimal test for the detection of untreated CD patients, making AGA tests still useful for specific cases (Lagerqvist et al. 2008).

In this work the introduction of disulphide groups through three different moieties, carboxylic, hydroxyl and amino of antigens (gliadin and tTG) were used in the detection of CD related antibodies (AGA and anti-tTG); electrochemistry and SPR studies were performed in order to characterise the immunosensor, explore associated non-specific adsorption and monitor specific binding detection of related antibodies. Results proved that the use of direct immobilization of thiolated

antigens on gold surfaces could be used to detect CD related antibodies without losing any antigenicity and showing performances similar to unmodified proteins in different analytical techniques, such as ELISA, SPR or electrochemistry. Analysis of reference sera showed how the range of antibody concentration covered was of clinical relevance. The thiolation of the antigenic proteins enabled a simple and convenient two-step surface modification procedure involving only spontaneous gold-thiol covalent binding, using short assay times.

5.2.3 Experimental section

5.2.3.1 Reagents and materials

Gliadin, polyclonal anti-gliadin antibodies developed in rabbit, anti-rabbit IgG and IgA labelled with HRP, potassium ferrocyanide (III) and potassium ferrocyanide (II), strontium nitrate, cystamine dihydrochloride, N-(3-dimethylaminopropyl) - N - ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), sodium cyanoborohydride, HEPES buffer, phosphate-buffered saline (PBS) with 0.05% v/v Tween 20 (PBS-T), carbonate bicarbonate capsules for preparation of carbonate buffer (0.05 M, pH 9.6), ready to use 3,3,5,5- Tetramethylbenzidine (TMB) liquid substrate and dithiopropionic acid succinimidyl ester (DTSP) were purchased from Sigma-Aldrich (Spain). Ethanol, acetone, dimethyl sulfoxide (DMSO), hydrochloric acid, sodium di-hydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) were obtained from Panreac Quimica (Barcelona, Spain). Hydrogen peroxide 30% v/v, sulphuric acid, sodium acetate, acetic acid, potassium hydroxide, sodium chloride, potassium chloride, ethylene glycol, sodium periodate and sodium hydroxide were purchased from Scharlau (Barcelona, Spain).

2-(2-(2-(11-mercaptoundecyloxy) ethoxy) ethoxy) ethanol (PEG-SH) was obtained from SensoPath Technologies (Bozeman, USA). Anti-tTG rabbit produced polyclonal antibody was purchased from Zedira (Darmstadt, Germany). Anti-human IgA and anti-human IgG labelled with HRP antibodies used for electrochemical measurements were those provided in Gliatest ELISA kits (AGA) and Eu-tTG ELISA kits, tTG was

supplied from EUROSPITAL (Trieste, Italy) and real patient samples were provided by King's College London (UK). Centrifugal filter membranes 0.5 mL (MWCO 30 kDa) were purchased from Whatman GmbH (Dassel, Germany). Biacore bare gold sensor chips (SIA Au Kit) were obtained GE Healthcare Europe (Barcelona, Spain); Nunc MaxiSorp flat-bottom well plates were purchased from VWR International Eurolab (Barcelona, Spain). Aqueous solutions were prepared using de-ionized water from a Milli-Q RG system, Millipore (Madrid, Spain) and all reagents were used as received.

5.2.3.2 Chemical thiolation protocols

Disulphide groups were covalently introduced into the structure of both gliadin and tTG proteins, via amine (Swaim et al. 2004), carboxylic (Lin et al. 1990) and hydroxyl moieties (Geoghegan and Stroh 1992) that exists at different parts of the proteins. (See supplementary information for thiolation protocols). Modification procedures are depicted in Figure 5.1.

5.2.3.3 Electrode modification

Gold disk electrodes CHI 101 (CH Instruments Inc, US) were first polished with aqueous alumina slurries of 25 and 1 µm (Buehler, US) and then rinsed with Milli-Q water, sonicated for 1 min and dried with nitrogen. The electrodes were treated with a mixture of H_2O_2 (30%) and KOH (0.1 M) for 10 min, after that an electrochemical cleaning was done using a CHI Electrochemical Analyzer 660A (CH Instruments Inc, US) in 0.1 M potassium hydroxide, performed using one linear sweep voltammetry between -0.2 and -1.8 V (Heiskanen et al. 2008) using a conventional three electrodes cell, standard silver/silver chloride (sat. KCl) CHI111 (CH Instruments, US) as reference, platinum gauze was the counter electrode. Electrodes were immersed in a solution of 0.1 mg/mL of the modified antigen (gliadin or tTG) dissolved in PBS-Tween 0.01 M pH 7.4 for 1 hour, rinsed with water and dried with nitrogen. To block non specific adsorption (NSA), the electrodes were immersed in an

ethanolic solution of 1 mM PEG-SH for 30 min and then rinsed with ethanol.

5.2.3.4 Surface plasmon resonance analysis

Biacore gold chips (SIA Au kit), were first cleaned using a Piranha's solution (1:3 v/v H2O2 and H2SO4) *(Warning: Piranha's solution is highly corrosive and violently reacts with organic materials; this solution is potentially explosive and must be used with extreme caution)* for 1 min over the surface and then rinsed several times with ethanol and repeated twice for each gold chip.

Real time analysis was performed using a SPR Biacore 3000® (GE Healthcare, US). All SPR experiments were done using 0.01 M PBS-Tween (0.5%), filtered and degassed, as running buffer and unless stated, in all dilutions used. Gold chips were first conditioned using a 20 μL/min flow of running buffer until a stable signal was achieved. Protein binding was performed at a flow rate 5 μL/min one channel at a time, injecting 150 μL of 0.1 mg/mL of modified antigen. Subsequently in order to block non-specific interactions 100 μL of 1 mM PEG-SH solution was flowed in all the channels. In all experiments a control channel was set using only PEG-SH on the gold surface. Antibody determination was performed at a flow rate of 20 μL/min, with 6 min of association and dissociation steps, always injecting buffered solution in the presence and absence of target antibody.

5.2.3.5 Electrochemical analysis

Amperometric measurements were carried out at 0.15 V in a 5 mL electrochemical cell containing PBS buffer and TMB (7.5:1, v/v, final ratio), in where the TMB oxidized by the peroxidase enzyme, is reduced back to its original form generating a cathodic current at the electrode, as in previous work (Rosales-Rivera et al. 2012). All the electrochemical measurements were performed at room temperature and under the same stirring conditions. The electrodes were incubated for 15 min in different antibody concentrations or reference serum solutions followed

by another 15 min of the corresponding anti-rabbit or anti-human horseradish peroxidase-labelled antibody solution. Immobilization and detection schematic is depicted in Figure 5.1.

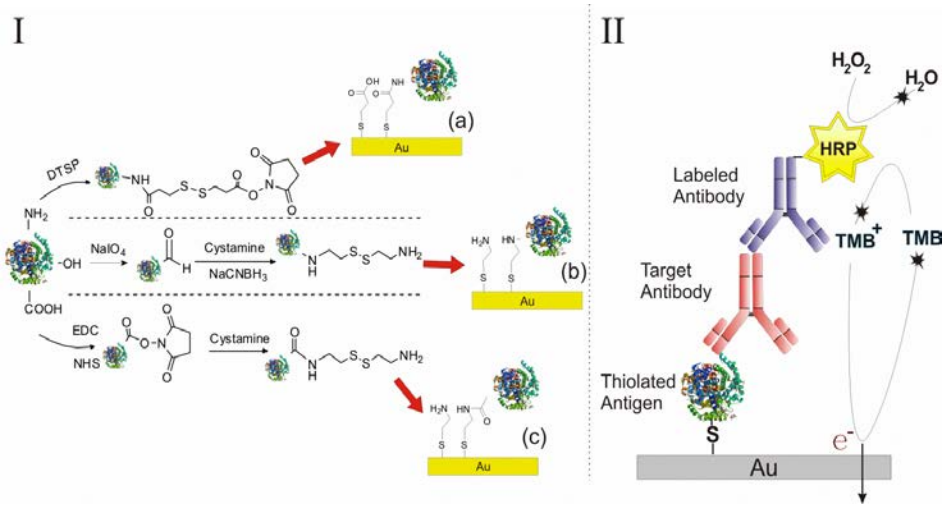

Figure 5.1. I) Thiolation of the antigen protein through the different moieties (a) Amine, (b) Hydroxyl and (c) Carboxyl groups. II) Architecture of the immunoassay including electrochemical detection.

5.2.4 Results and discussion

5.2.4.1 Antigenicity of the modified protein

The antigenicity of the modified proteins was studied by ELISA prior to the adsorption of the proteins onto gold surfaces, the reason being that different procedures and modifications could cause a loss of antigenic potential of the gliadin and tTG proteins, if their structure or epitopes sites had been affected. Therefore the modified proteins were compared in terms of antigenicity against unmodified gliadin and tTG (see supplementary information for ELISA procedure details).

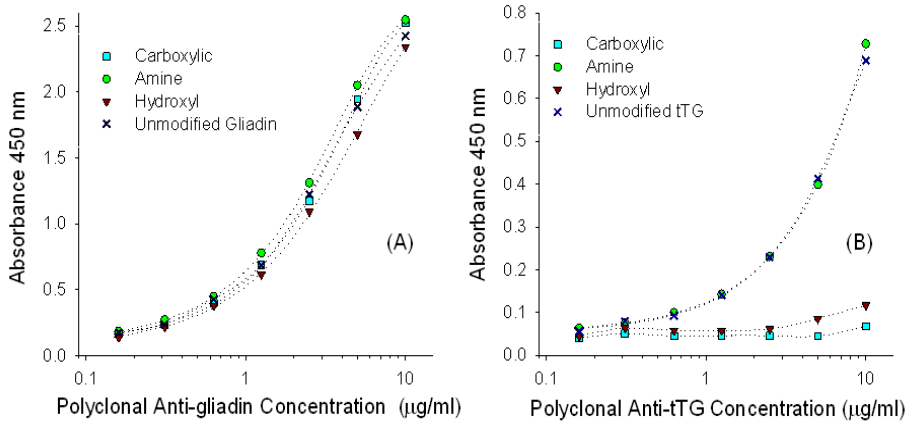

Figure 5.2. ELISA analysis of the antigenicity of the modified (A) gliadin and (B) tTG.

The results showed how thiolated gliadin maintained its antigenicity regardless of the moiety used to introduce the thiol modification. The obtained limits of detection were 28, 50 and 55 ng/mL in the case of amine, hydroxyl and carboxylic based gliadin thiolation. On the other hand it appeared clear that in the case of tTG only the thiolation of the protein through the amine groups maintained the antigenicity of the protein. The obtained LOD was 0.50 µg/mL. Modification of the tTG via carboxylic and hydroxyl groups showed an associated effect of loss of antigenicity of the protein even before the adsorption on gold electrodes (see Figure 5.2).

5.2.4.2 Surface plasmon resonance characterisation

The immobilisation of the thiolated antigens onto gold surfaces was characterized by means of SPR. The amount of thiolated antigen adsorbed was quantified and results are shown in Table 5.1.

Table 5.1 SPR estimated thiolated antigen surface concentration.

	Antigen surface concentration (pmol/cm^2)		
	Amine	**Hydroxyl**	**Carboxylic**
Gliadin	7.79	7.33	5.66
tTG	2.96	1.85	3.15

The results showed a similar successful degree of immobilisation onto the gold surface for the different modified antigens. Slightly higher concentrations were recorded in the case of modified gliadin with respect to tTG. In all cases the level of immobilisation of PEG-SH as barrier for non-specific adsorption was similar yielding in average 5.5 x 10^{-10} moles/cm^2 for modified gliadin and 2.8 x 10^{-10} moles/cm^2 for modified tTG (See supplementary information for SPR protocol). In all cases the values are typical for protein immobilisation and thiol monolayer formation (Fragoso et al. 2008; Nassef et al. 2009; Widrig et al. 1991).

5.2.4.3 Polyclonal antibodies detection

The antigenic performance of the modified proteins was tested when efficiently adsorbed on gold surfaces via the sulphur-gold bond formation. First SPR response was recorded for different concentrations of polyclonal IgG type anti-gliadin and anti-tTG (see Figure 5.3). Bulk responses were subtracted for all the graphs and signals are an average of two complete experiments. In all cases control experiments with only PEG-SH did not show any relevant non specific binding (data not shown).

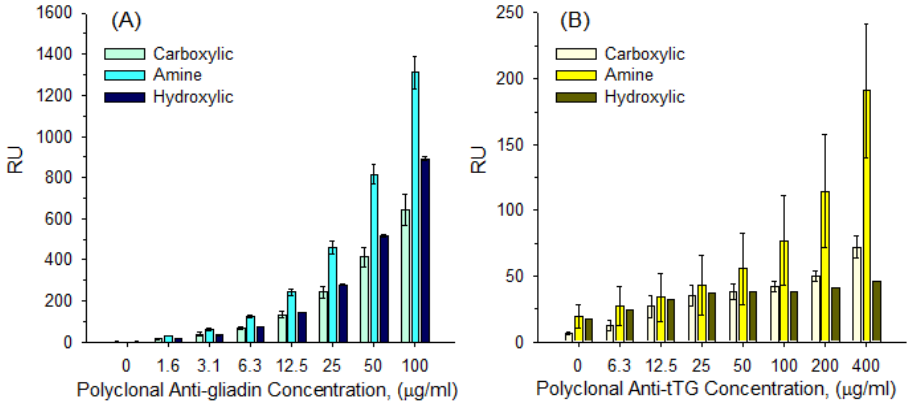

Figure 5.3. SPR analysis of the antigenicity of the adsorbed (A) gliadin and (B) tTG.

The antigenic response recorded showed in the case of thiolated gliadin that the thiolation through the carboxylic and hydroxyl groups implied lower antigenic response than in the case through amine groups. The obtained limits of detection were 0.63, 0.77 and 0.78 µg/mL in the case of amine, hydroxyl and carboxylic based gliadin thiolation, with associated sensitivities of 16.34, 10.36 and 8.23 RU/µg•mL^{-1} respectively. In the case of tTG the results confirmed the findings obtained by ELISA and only the thiolation through amine groups of the tTG showed a trend in its antigenic response. The LOD obtained was 6.11 µg/mL with a sensitivity of 0.54 RU/µg•mL^{-1}. The response of the modified proteins towards the detection of polyclonal antibodies was studied on gold surfaces by means of an electrochemical sensor platform are depicted in Figure 5.2.

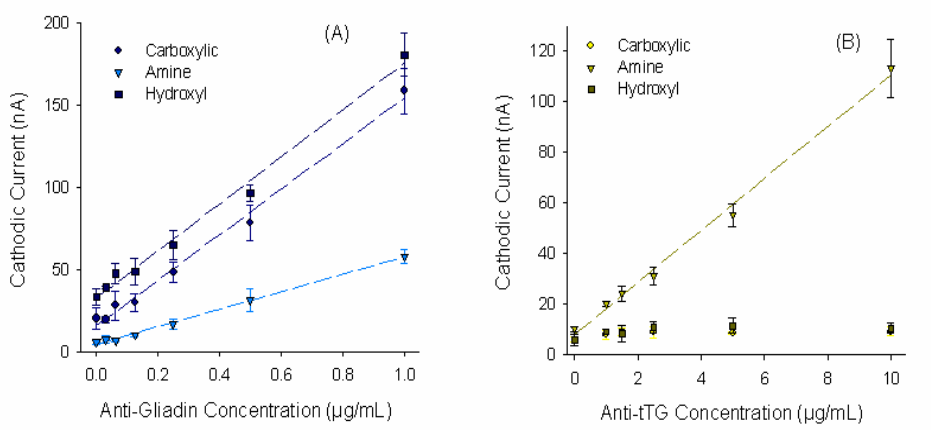

Figure 5.4. Electrochemical detection of polyclonal antibodies over (A) gliadin (B) tTG-modified electrodes.

Electrochemical detection (see Figure 5.4), showed more sensitivity and a lower LOD in both antibodies used, anti-gliadin and anti-tTG. In the case of anti-gliadin the obtained limits of detection were 0.085, 0.117 and 0.178 µg/mL in the case of amine, hydroxyl and carboxylic based gliadin thiolation, with associated sensitivities of 53, 143 and 139 nA/µg•mL^{-1} respectively. Only the thiolation through amine groups of the tTG showed an antigenic response. The LOD obtained was 0.26 µg/mL with a sensitivity of 10 nA/µg•mL^{-1}.

The main difference between the different thiolated gliadin antigens was the lower sensitivity recorded when using amine-based thiolation of the gliadin, this decrease in sensitivity was compensated by a lower level of background signal, allowing to a lower LOD overall. In the case of thiolated tTG, as expected, only the amine-based thiolation showed antigenicity and a reproducible trend for the detection of anti-tTG antibodies.

5.2.4.4 Reference serum solutions analysis

Clinical reference serum solutions from ELISA commercial kits were used in order to evaluate the performance of the modified antigens in the presence of complex matrices; IgG and IgA isotypes for anti-gliadin and anti-tTG were explored and compared using SPR and

electrochemical experiments (see Figures 5.5 & 5.6). Certainly showing the thiolated proteins maintains its antigenicity and can be used in order to discriminate clinically relevant antibody concentrations.

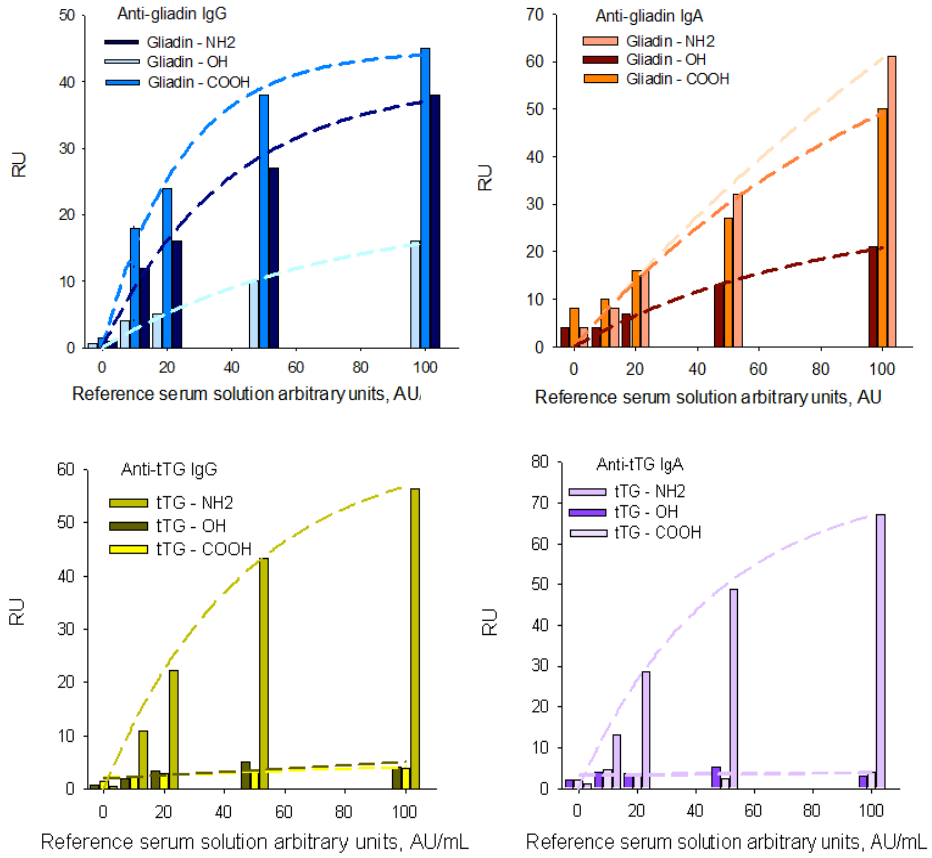

Figure 5.5. SPR analysis of anti-gliadin and anti-tTG antibodies, IgG and IgA isotypes, from reference serum solutions.

The serum-based complex detection matrix in which the antibodies are detected require an efficient non-specific binding mechanism. The use of PEG-SH proved successful upon observation of the low signal generated by the zero antibody concentration from reference solutions. The serum solutions showed how the contents for isotype IgG saturated the signal reaching a maximum after 50 arbitrary units (AU). This type of saturation of the signal is typical of solutions provided in commercial ELISA kits (see supplementary information). For

both isotypes the gliadin modified through hydroxyl group showed the least sensitivity, while modification through amine or carboxyl groups was similar. In the case of anti-tTG antibodies from reference serum solutions only the tTG modified through amine group showed the antigenicity necessary to discriminate the different reference solutions.

Similarly anti-gliadin and anti-tTG reference serum solutions were evaluated using the modified electrochemical immunosensor.

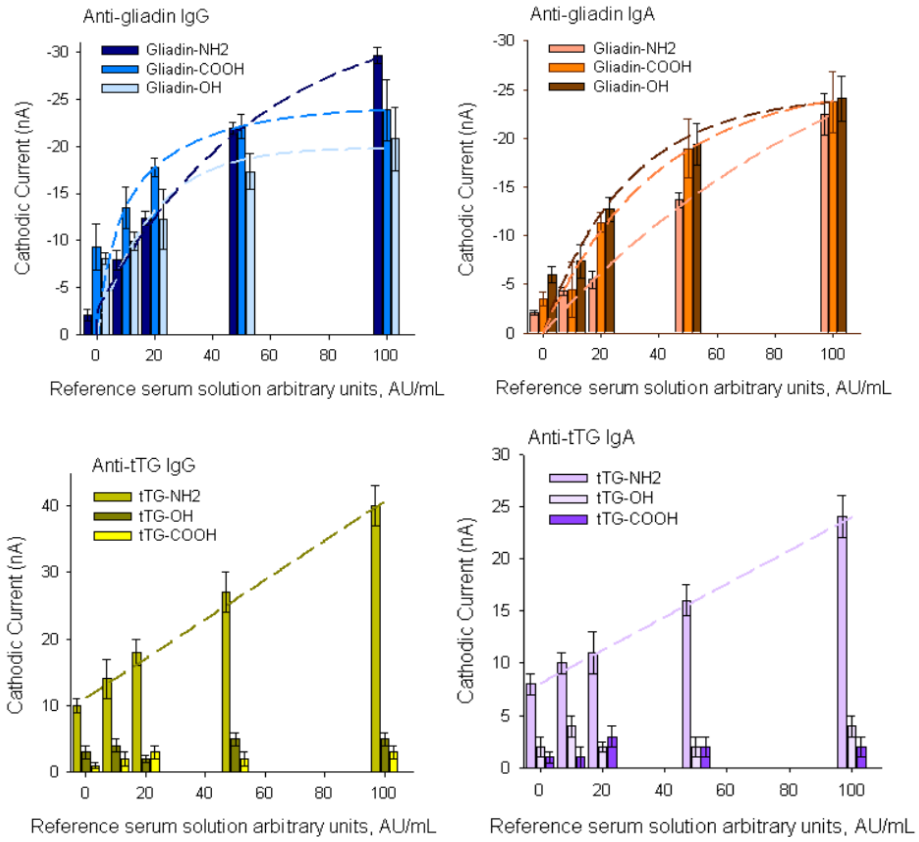

Figure 5.6. Electrochemical analysis of the anti-gliadin and anti-tTG antibodies, IgG and IgA isotypes, from reference serum solutions.

The optimal performance of the electrochemical sensor became clear at view of the results obtained. Results depicted, in the case of anti-gliadin, a previously observed saturation for the signal for reference

serum solutions at above 50 AU/mL, in this case for both isotypes. Despite this saturation it appeared that gliadin thiolated through amine groups was able to show a more sensitive performance with a larger linear range than the other modifications in all cases. The antigenicity of tTG modified through amine groups was again confirmed when using the electrochemical detection. Dynamic range of the sensor allowed the determination of both anti-tTG isotypes, from reference serum solutions with no signal saturation, showing the sensor could be used at higher concentrations. The efficiency of the immobilised PEG-SH as non-specific binding barrier was also confirmed using the electrochemical immunosensor format, showing a very low signal in the case of blank samples.

Electrochemical detection of CD patient's sera was compared with an equivalent ELISA assay (see Figure 5.6). In order to assure the best response from a limited amount of patient sample, the most clinically relevant isotypes, IgG in the case of anti-gliadin and IgA for anti-tTG antibodies were used. Antigens that showed the best response, in both cases thiolated through amine groups, ensured the best sensitivity of the detection. Obtained amperometric currents were extrapolated to the calibrator solutions plot to obtain equivalent arbitrary units and compared to those found when analyzing the same samples using an ELISA standardized test with the same calibrators.

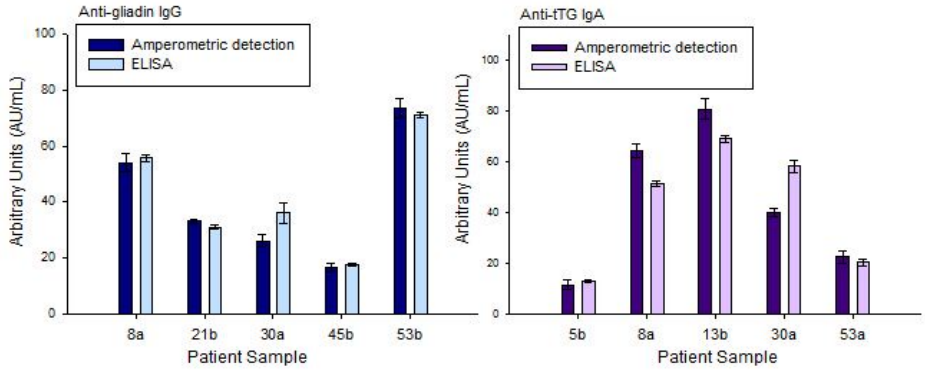

Figure 5.6. Comparison of electrochemical vs. ELISA-based results in the evaluation of CD patient's sera.

5.2.5 Conclusions

Proteins thiolation through different moieties and its further immobilization on gold surfaces was achieved. CD related antibodies detection was done with the aid of SPR and electrochemistry studies. The antigenicity of gliadin was preserved in all studied modifications, while in the case of tTG, only the amine-based modification maintained its antigenic properties. It was demonstrated that the use of PEG-alkanethiols, as backfillers, reduces the NSA of other molecules and improves the characteristics of the immunosensors. Electrochemical immunosensors performance showed better sensitivity and lower limits of detection, in buffer and reference serum solutions, than its SPR counterparts. The results of the work presented confirmed the potential and suitability of proteins modification for its integration at immune-detection platforms. Based on the obtained characteristics, these platforms can be easily oriented for the detection directly from real serum samples using simple, low-cost, portable transduction mechanisms such as electrochemistry-based ones. This advantageous transduction mechanism constitutes an added value to the suitability of the modified protein as straight forward antigenic agent in immunodetection platforms, for its use in multiplex detection platforms, with little sample manipulation.

5.2.6 Supplementary information

5.2.6.1 Surface Plasmon Resonance Immobilization

Biacore gold chip (SIA Au kit) was cleaned as before (see Experimental section). First channel was used always as control and no antigen was immobilised. Concentration of 0.1 mg/mL of the antigen was passed over the surface for 30 min, at a flow rate of 5 μL/min. Values of antigen bounded to the surface were obtained in resonance units (RU) and converted to surface concentration knowing that 1000 RU are approximately 1 ng/mm^2, for proteins. The distinct surface coverage achieved, in pmol/cm^2 (10^{-12}) for all the thiolated antigens is summarized in Table 1. Protein surface coverage can be compared with the immobilization of a molecule 3 times smaller like cytochrome-c (MW 12,000), in where 17 pmol/cm^2 have been reported, using covalent attachment over a thiol self assembled monolayer (Jiang et al. 1997). Comparison can be done with previous work at the group, in where using the same antigens and a carboxylic terminated dithiol, it was possible to obtain values of 9.88 and pmol/cm^2 for gliadin (MW 35,000) and 3.24 pmol/cm^2 for tTG (MW 78,000) (Dulay et al. 2011; Rosales-Rivera et al. 2011a).

In order to reduce the non specific adsorption of other molecules, PEG-SH alkanethiol was used (MW 335.5). A concentration of 1mM was injected for 20 min in all the channels using a flow rate of 5 μL/min. The first channel was used as control for all the experiments, in where no thiolated antigen was immobilised; an average of 2000 RU of PEG-SH were immobilized in this channel for both cases, gliadin and tTG. Immobilisation for the rest of the channels can be seen in Table 2.

Table 2. Poly-ethylene glycol alkanethiol (PEG-SH) immobilisation on the different thiolated antigen covered surfaces.

		Resonance units (RU)		
		Amine	**Hydroxyl**	**Carboxylic**
Modified protein	**Gliadin**	1700	1726	1623
	tTG	812	986	831

5.2.6.2 Thiolated antigens ELISA methodology

Modified antigens (gliadin and tTG) were evaluated colorimetrically by sandwich ELISA and absorbance measured using a SPECTRAmax PC plate reader (bioNova científica, Spain). All ELISA experiments were performed using 0.01 M PBS-Tween pH 7.4 buffer solutions, 60 min of immobilisation time and 37°C of incubation temperature, unless stated. Immobilisation of the thiolated antigens was done using a concentration of 5 μg/ml for both antigens dissolved in 0.05 M Carbonate Buffer at pH 9.6. A volume of 50 μl was immobilized on NUNC MaxiSorp 98 well plates at, in where the surface was blocked using 200 μl of 0.01 M PBS-Tween.

For gliadin tests, dilutions of anti-gliadin rabbit produced antibodies (0.16–10 μg/ml). In the case of tTG, dilutions of anti-tTG rabbit produced antibodies (0.16 – 10 μg/ml); a volume of 50 μl was added to each well in both cases. After that 90 ng/mL of anti-rabbit IgG peroxidase labelled antibody, 50 μl was to each well. The presence of HRP was detected using 50 μl of commercial TMB ELISA liquid substrate and stopping the reaction using 1 M of H_2SO_4 after 20 min. Absorbance was measured at 450 nm.

5.2.6.3 ELISA analysis of the reference serum solutions

Immobilisation of the antigens (gliadin and tTG) was done using a 5 μg/ml concentration dissolved in 0.05 M carbonate Buffer pH 9.6. A volume of 50 μl was immobilised on NUNC MaxiSorp 98 well plates. Surface was blocked using 200 μl of 0.01M PBS-Tween for 60 min at 37°C. Incubation of the calibrators from EUROSPITAL kits (Gliatest and Eu-tTG) was done using 50 μl of each calibrator. Labelled antibody incubation and detection of the signal was done as before (thiolated antigen ELISA methodology). Response curves in the case of the antigens (gliadin and tTG) are depicted in Figure 5.7.

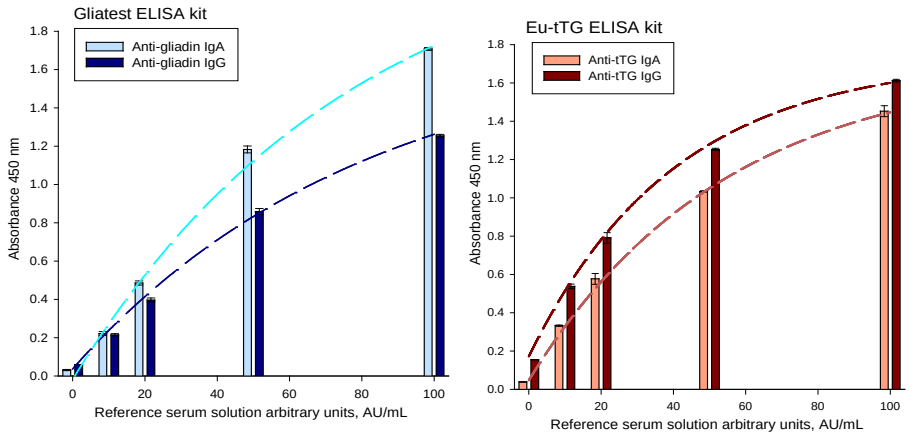

Figure 5.7. Reference serum solutions ELISA analysis for the used antigens (A) gliadin and (B) tTG.

5.2.6.4 Chemical thiolation protocols

a) Thiolation through amine groups $-NH_2$

The introduction of disulphide groups was done using an homobifunctional protein cross linker, DTSP, this chemical contains two N-hydroxysuccinimide esters separated by an average of 8°A (Green et al. 2001); amine groups present in the protein react with this ester and forms stable amide bonds. Introduction of disulfides by this synthetic route was performed by reacting terminal primary amines, lysine, serine and tyrosine residues present in the molecules (Swaim et al.

2004),giving rise to covalent attachment between disulfide groups and the proteins.

Gliadin (0.25 mg) and tTG (0.25 mg) were dissolved, separately, in 0.5 mL of 0.01 M Carbonate buffer pH 4.5 and mixed with 0.05mL DMSO containing 0.07 mg of DTSP. They were mixed in dark conditions for 5 hours at room temperature under vigorous stirring. After that the excess of DTSP was removed by ultrafiltration (30 kDa MWCO membranes). The modified protein was collected in 0.1 M PBS pH 7.4 and the concentration was determined using a Cary 100 UV-Vis spectrophotometer (Varian, Spain) at a wavelength of 280 nm. The modified proteins were labelled as gliadin-NH_2 and tTG-NH_2.

b) Thiolation through carboxylic groups –COOH

Disulphides were covalently attached to the antigen structure through the terminal carboxylic acids of aspartic and glutamic acid residues. At acidic pH, carboxylic acids can react with carbodiimide derivates in the presence of NHS to form an active ester, a primary amine. Cystamine residues can easily attack the electrophile carbon of the ester and form amide bonds (Lin et al. 1990).

Gliadin (0.25 mg) and alternatively tTG (0.25 mg) were dissolved in 0.1mL of 0.01M acetate buffer pH 4.5; 7.8 mg of sulfo-NHS and 6.9 mg of EDC were mixed and allowed to react for 10 min under vigorous stirring at room temperature. Following that 0.9 mL of 0.05 M HEPES buffer pH 8.5 containing 20 mg of cystamine was added to the reaction for 2 hours at room temperature. The modified protein was separated and quantified as described before for the gliadin-NH_2. Modified proteins were labelled as gliadin-COOH and tTG-COOH.

c) Thiolation through hydroxyl groups –OH

Disulphide groups were attached to hydroxyl groups present in the glycosides chains of the proteins. First the hydroxyl groups were partially oxidized with sodium periodate, converting them into aldehydes. After that amine groups present at cystamine molecules

reacted forming unstable Schiff's bases and finally converted into stable bonds using reductive amination with sodium cyanoborohydride (Geoghegan and Stroh 1992).

Gliadin (0.25 mg) and tTG (0.25 mg) were dissolved, in different reactions, in 0.1 mL of 0.01 M Acetate buffer pH 5.0. Sodium periodate was added to form a 5 mM solution, in order to oxidize hydroxyl groups into aldehydes. Present at the carbohydrates derivatives of the proteins. The reaction took place for one hour under dark and stirring conditions. The oxidized antigen solution was slowly added to 0.9 mL of 0.1 M cystamine solution dissolved in 0.05 M carbonate buffer pH 9.5, they were left to react for 3 hours. Final step was the reduction of unstable imines (Schiff's bases) to amine bonds adding 10 mM sodium cyanoborohydride into the solution for 1 hour. The modified protein was separated and quantified as described for the gliadin-NH_2. Modified proteins were labelled as gliadin-OH and tTG-OH.

5.2.7 Acknowledgements

This work has been carried out with financial support from the Commission of the European Communities, RTD programme "Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CDMEDICS [FP7-2007- ICT-1 IST-2007-2-216031]. The authors would like to acknowledge Dr. J. Ellis from King's College London for the kind supply of real patients' samples; EUROSPITAL® for the kind supply of Eu-tTG, Gliatest IgA and IgG ELISA kits and reagents; Mr. Rosales-Rivera acknowledges the support from the Comissionat per a Universitats I Recerca del DIUE de la Generalitat de Catalunya i del Fons Social Europeu for his FI grant.

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CHAPTER 6

Conclusions

6.1 Conclusions

Coeliac disease, an autoimmune disease with intestinal and sometimes extra-intestinal manifestations, entails a lifelong intolerance to gluten. Its detection prevails mainly unrecognized and diagnosis is recommended as early as possible, to reduce the possibility of health complications. Detection using serological tests, represent a faster and non-invasive approach with possibilities of high throughput screening.

Immunosensors are an alternative to classical immunoassays (ELISA) based tests, commercially available for the serological detection of CD. Commercial ELISA kits can be time consuming, expensive and may need associated equipment, however electrochemical immunosensors with characteristics such as portability, potential of miniaturization and high sensitivity may represent a good and cheaper alternative for the detection of related antibodies to CD.

In this *Thesis*, the construction of amperometric immunosensors for the serological detection of CD markers, was explored using two strategies: (i) the covalent attachment of the specific antigens, using a dithiol self assembled monolayer, (ii) the direct immobilisation of the antigens, with the introduction of disulphide bonds through amine, carboxylic and hydroxyl groups of the used proteins. Use of polyethylene glycol molecules for both cases, demonstrated a reduction of non specific adsorption and better immunosensors characteristics. Standard protocols were constructed for both strategies, demonstrating short assay times and potential of portability, multi-electrode array detection and in situ measurements.

The proof-of-concept for antibodies detection, was explored in *Chapter 3*, using a dithiol SAM based immunosensor and using amperometric detection of anti-gliadin antibodies. A robust and reliable immunosensor was built and characterised, using not only amperometry, but also real-time SPR experiments. Protocols were constructed and optimised for concentration levels of antigens and secondary labelled antibodies. Detection of reference serum samples

from commercial ELISA kits, was done using the obtained immunosensor, it was possible to characterize all the calibrators present in the kit and distinguish between positive and negative reference calibrators. Detection of human serum samples, compared with ELISA procedures, showed a similar behaviour and sensitivity, but with the use of shorter immobilisation times. Finally, human anti-gliadin antibodies from both isotypes (IgA and IgG) were compared with ELISA based tests. Very good degree of correlation and semi-quantitative estimation of antibody levels, were reached.

IgA quantification of serum samples from CD patients was performed in *Chapter 4*, using a similar dithiol SAM approach for the immunosensor construction, as in *Chapter 3*. Electrodes were successfully tested in complex matrices, such as human and foetal bovine serum samples. Cross reactivity studies showed specificity only, towards detection of IgA antibodies. Finally, behaviour of the obtained immunosensors presented an excellent correlation with ELISA based tests for the quantitative determination of IgA levels, from deficient and non-deficient CD real serum samples. Despite only three samples were truly IgA deficient, it was demonstrated that the immunosensors could be used to determine clinically relevant IgA levels in a rapid, low cost and low time consuming, with the possibility of in-situ measurements.

In *Chapter 3 and 4*, using impedance measurements, nearly complete monolayers were reached using the bipodal alkanethiols, with ideal packing of molecules on gold surfaces. Carboxylic-acid-terminated-thiols were successfully used the immobilisation of antigens, by a simple and fast bio-conjugation method. The presence of polyethylene glycol groups, reduced the non-specific adsorption, making possible the detection in complex matrices, avoiding a decrease in the specific signal response of constructed immunosensors.

Self-assembly of thiolated proteins on gold surfaces for the amperometric detection of CD related antibodies, was achieved in *Chapter 5*. The introduction of disulphide groups was done using standardized chemical protocols through amine, carboxylic and hydroxyl

groups, present in the antigens (gliadin and tTG). Preservation of antigenicity was proven for all the gliadin modifications; while in the case of tTG, only one modification (amine) retained its antigenic properties, probably due to the damage of the antibody binding site, or denaturation of the tTG protein. Non specific adsorption of other proteins was reduced using as backfiller a PEG-alkanethiol SAM. Assembled electrochemical immunosensors showed better sensitivity and LOD than SPR sensor chips, however, the last ones could be used several times with real-time detection. Obtained platforms were effectively used for the detection of commercial calibrators from ELISA kits and from CD serum samples, proving that they could be used in complex matrices. Finally, detection of serum samples from CD patients was performed using two serological markers, anti-gliadin IgG and anti-tTG IgA. Obtained results were compared with ELISA tests for the same markers, reaching a good correlation for the obtained signals. A simple and fast immobilisation method was successfully proven for the construction of immunosensors, using a rapid immobilisation of thiolated antigens (30 min). Immunosensors proved to be highly sensitive, specific and reliable for its use in complex matrices, like serum and its use in multiplex detection platforms.

6.2 Future work

Multiplex detection of CD related antibodies using multielectrode array chips, in order to detect not only several patients at the same time, but also several serological markers with high precision and sensitivity, e.g. anti-tTG antibodies and IgA antibody at the same time by triplicate in the same chip. Integration of immunosensors into microfluidic platforms using online amperometric measurements or similar electrochemical approaches.

Scientific contributions

- Rosales-Rivera, L.C., Acero-Sanchez, J.L., Lozano-Sanchez, P., Katakis, I., O'Sullivan, C.K., 2011. Electrochemical immunosensor detection of antigliadin antibodies from real human serum. Biosensors & Bioelectronics 26(11), 4471-4476.

- Rosales-Rivera, L.C., Acero-Sánchez, J.L., Lozano-Sánchez, P., Katakis, I., O'Sullivan, C.K., 2012. Amperometric immunosensor for the determination of IgA deficiency in human serum samples. Biosensors and Bioelectronics 33(1), 134-138.

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