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MSc in Biology

Development of electrochemical immunosensors for celiac disease clinical diagnosis and gluten-free food control

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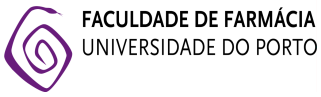

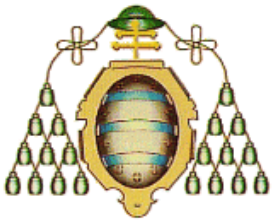

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in Alice's Adventures in Wonderland

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RESUMO

A doença celíaca (DC) é uma enteropatia autoimune que ocorre em indivíduos geneticamente suscetíveis como resultado da ingestão de glúten. Para o diagnóstico clínico desta doença, a metodologia mais usada é o ensaio de imunoabsorção enzimática (ELISA), mas nos últimos anos muitos esforços tem sido feitos para melhorar os testes serológicos para a DC. Contudo, não existe um acordo geral no método imunoquímico a utilizar.

Hoje em dia, os métodos baseados no uso de sensores eletroquímicos estão desempenhando um papel cada vez maior em diversos campos em que sistemas de medida precisos, rápidos e que permitam uma medida em tempo real são necessários. Relativamente à qualidade e custo, os sensores eletroquímicos podem ser uma melhor opção do que os métodos analíticos convencionais. Especialmente, os imunossensores eletroquímicos (IEs), que ao combinar a elevada especificidade dos métodos imunoquímicos tradicionais com o elevado limite de deteção das técnicas eletroquímicas, são poderosas ferramentas analíticas para o desenvolvimento de dispositivos mais rápidos, mais simples, mais sensíveis e de baixo custo que podem ser competitivos o suficiente e aplicados diariamente com êxito no diagnóstico clínico da DC.

O principal objetivo desta tese de doutoramento foi o desenvolvimento, produção e otimização da melhor superfície transdutora de modo a obter IEs sensíveis, reprodutíveis e robustos para análise rotineira do diagnóstico clínico da DC.

A primeira etapa deste trabalho consistiu na busca da superfície apropriada para a imobilização da quantidade adequada de material proteico na superfície transdutora dos sensores eletroquímicos, incluindo também a exploração das potencialidades dos nanomateriais. Para este propósito, elétrodos serigrafados de carbono foram nanoestruturados com nanopartículas de ouro, nanotubos de carbono, nanofibras de carbono, e, com sistemas híbridos de materiais de carbono e metal. A biofuncionalidade dos diferentes transdutores foi avaliada através da sua capacidade para adsorverem e reterem material proteico nas suas superfícies, e empregando, como modelo analito, o complexo altamente estável estreptravidina/biotina. As superfícies transductoras foram também caraterizadas por microscopia eletrónica de varrimento. Os elétrodos serigrafados de carbono modificados com os híbridos de nanotubos de carbono e nanopartículas de ouro revelaram ser os que propocionavam a superfície transductoras mais apropriada.

Depois da seleção do transductor mais adequado, a fase sensora dos IEs foi construída com base na interação antigénio-anticorpo. IEs para a determinação de imunoglobulinas (IgG) classe A (IgA) e classe G (IgG) produzidas contra a gliadina (AGA) e contra a transglutaminase tecidual (anti-tTG) (i.e. os marcadores serológicos específicos para a DC) foram desenvolvidos e otimizados. Um IE de multi-deteção para a determinação simultânea de dois biomarcadores, AGA e anti-tTG, foram também desenvolvidos. Além

disso, a determinação de anticorpos humanos gerados contra a gliadina desamidada foi também efetuada. Os sensores desenvolvidos foram testados usando amostras de soro de pacientes de DC e indivíduos saudáveis. Finalmente, os métodos desenvolvidos foram validados por comparação com os métodos espetrofotométricos convencionalmente utilizados no diagnóstico serológico da DC.

Os imunossensores construídos e optimizados nesta tese de doutoramento tem como vantagem o fato de serem ferramentas analíticas simples de usar e descartáveis, o que facilitará uma futura transferência destes sensores para aplicações *point-of-care*.

Palavras-chave: doença celíaca, transglutaminase tecidual, gliadina, elétrodos serigrafados nanoestruturados, imunossensores eletroquímicos

ABSTRACT

Celiac disease (CD) is an autoimmune enteropathy that occurs in genetically susceptible individuals as a result of gluten ingestion. For CD diagnostic purposes the most used methodology is the enzyme-linked immunosorbent assay (ELISA), and in the past few years many attempts have been made to improve CD serological tests. However, there is no general agreement on the immuno-based analytical method for CD screening.

Nowadays, electrochemical sensing techniques are playing a growing role in various fields in which accurate, fast and online measuring systems are required. With regard to quality and cost, electrochemical sensors can be a better option than standard analytical methods. Especially electrochemical immunosensors (EIs), which combine the high specificity of traditional immunochemical methods with the low detection limits of modern electrochemical techniques, are powerful analytical tools to develop faster, simpler, more sensitive and low-cost devices that can be competitive enough to be successfully applied on a daily basis for CD clinical diagnosis.

The main goal of this PhD thesis was the development, manufacturing and optimization of the best transducer surface to obtain sensitive, reproducible and robust EIs for routine analyses of CD clinical serological diagnosis.

The first stage of this work consisted of the search for a suitable layer for immobilizing an adequate amount of protein material on the transducer's surface of electrochemical sensors, including the exploitation of the potentialities of nanomaterials. For this purpose, screen-printed carbon electrodes were nanostructured with gold nanoparticles (NPAus), carbon nanotubes, and carbon nanofibers, and, with hybrid systems of the carbon/metal materials. The biofunctionality of the different transducers was evaluated by their ability to adsorb and retain protein material on their surface, employing the highly stable and efficient streptavidin/biotin complex as model analyte. The transducers' surfaces were also characterized by scanning electron microscopy. The screen-printed electrodes modified with the hybrid carbon nanotubes/gold nanoparticles revealed to provide the most suitable transducer surface.

After the adequate electrode surface selection, the sensing phase of the EIs was constructed relying on antigen-antibody interactions. EIs for the determination of immunoglobulins (IgG) class A (IgA) and class G (IgG) against gliadin (AGA) and tissue transglutaminase (anti-tTG) (i.e. specific CD serological markers) were developed and optimized. A multiplexed EI for the simultaneous assessment of AGA and anti-tTG CD biomarkers was also developed. Moreover, the determination of human antibodies produced against deamidated gliadin was also assayed. The developed sensors were tested using serum samples from CD patients and healthy individuals.

Finally, the developed methodologies were validated by comparison with the established spectrophotometric immunoassays for CD serological diagnosis.

The immunosensors, that were constructed and optimized within this doctoral thesis, have the advantage of being easy-to-use and disposable analytical tools, which facilitates a future transfer to point-of-care applications.

Keywords: celiac disease, tissue transglutaminase, gliadin, nanostructured screen-printed electrodes, electrochemical immunosensors

RÉSUME

La maladie cœliaque (MC) est une entéropathie auto-immune qui apparait chez des personnes prédisposées génétiquement, due à une ingestion de gluten. La méthode la plus utilisée dans les diagnostiques cliniques est le test du dosage d'immunoabsorption par enzyme liée (ELISA), et ces dernières années, des études ont été réalisées dans le but d'améliorer les tests sérologiques de la MC. Cependant il n'y a pas de consensus au niveau des méthodes analytiques basées sur des réactions immunologiques pour la détection de la MC.

De nos jours, les techniques de mesures électrochimiques jouent un rôle de plus en plus important dans divers domaines dans lesquels sont nécessaires des systèmes de mesures précis, rapides, et en temps réels. Au regard de la qualité et du coût, les capteurs électrochimiques peuvent être une meilleure option que les méthodes de référence actuelles. Plus particulièrement les immunocapteurs électrochimiques (IEs), qui combinent la grande spécificité des méthodes immunochimiques avec les limites de détection basses des techniques électrochimiques modernes, sont des outils analytiques très utiles pour développer des dispositifs plus rapides, plus simple, et à moindre coût, de manière à être suffisamment compétitifs pour être appliqué avec succès dans les diagnostiques cliniques quotidiens de la maladie cœliaque.

L'objectif principal de cette thèse de doctoract fut le développement, la fabrication et l'optimisation du meilleur transducteur pour obtenir des IEs sensibles, reproductibles et robustes pour le diagnostique routinier des marqueurs sérologiques de la maladie cœliaque.

La première de ce travail fut la recherche d'une couche appropriée pour l'immobilisation d'une quantité adéquate de matériel protéique sur la superficie transductrice des capteurs électrochimiques, et dans le même temps l'exploration des propriétés des nanomatériaux. Pour cela des électrodes sérigraphiées de carbone furent nanostructurées avec des nanoparticules d'or, des nanotubes de carbones, des nano fibres de carbone et avec des systèmes hybrides fait de carbones et de métal. La bio fonctionnalité des différents transducteurs furent évaluées pour leurs capacité d'adsorption et rétention de matériels protéiques à sa surface, en utilisant comme modèle analytique, le complexe hautement stable et efficace, streptavidine/biotine. Les superficies transductrices furent caractérisées par microscopie électronique à balayage. Les électrodes sérigraphiées de carbone modifiées avec l'hybride de nanotubes de carbone et nanoparticules d'or se révéla être la superficie transductrice la plus adéquate.

Après avoir déterminée quelle était la meilleure superficie électrodique, la partie de détection des IEs fut construite en se basant sur les interactions antigènes-anticorps. Les immunocapteurs électrochimiques pour la détermination d'immunoglobuline (IgG) de

classe A (IgA) et de classe G (IgG) générée contre gliadin (AGA) et transglutaminase tissulaire (anti-tTG) (i.e. marqueurs sérologiques spécifiques de la maladie cœliaque) furent développés et optimisés. Un IEs multiplexed, pour la détection simultanée des bio marqueurs de la maladie cœliaque AGA et anti tTG a également été développé. De plus, la détermination des anticorps humains produits contre la gliadine desamidée fut aussi testée. Les capteurs développés furent testés en utilisant des échantillons de sérum de patients atteints de la MC et de patients sains. Finalement, les méthodes développées furent validées en comparant les résultats obtenus avec ceux obtenus grâce aux immunotests spectrophotomètriques de référence pour les diagnostiques sérologiques de la MC.

Les capteurs proposés, qui ont été construits et optimisés dans cette thèse, ont l'avantage d'être des outils analytiques faciles à utiliser et jetables, ce qui facilite un futur transfert aux applications point-of-care.

Mots-clés: la maladie cœliaque, transglutaminase tissulaire, la gliadine, électrodes sérigraphiées nanostructurées, immunocapteurs électrochimique

RESUMEN

La enfermedad celíaca (EC) es una enteropatía autoinmune que sufren individuos genéticamente susceptibles como resultado de la ingestión de gluten. Para el diagnóstico de esta enfermedad, los métodos más utilizados están basados en ensayos por inmunoabsorción ligado a enzimas, llamados ensayo de inmunoabsorción enzimática (ELISA), pero en los últimos años la comunidad científica está haciendo esfuerzos para mejorar los tests serológicos de diagnóstico de la EC. Sin embargo, no existe un acuerdo sobre el tipo de método inmunoquímico a utilizar para el diagnóstico de la EC.

Actualmente, los métodos basados en el uso de sensores electroquímicos están jugando un papel muy importante en varios campos en los que es necesario implantar métodos rápidos, fiables, y que puedan realizar medidas en línea. Teniendo en cuenta la calidad y el coste, los sensores electroquímicos pueden convertirse en una buena alternativa a los métodos estándar de análisis. Entre los diferentes sensores electroquímicos, cabe destacar los inmunosensores electroquímicos (IEs), ya que combinan la alta especificidad de las reacciones inmunológicas con los bajos límites de detección las técnicas electroquímicas, lo que los convierten en poderosas herramientas analíticas en el desarrollo de métodos más rápidos, más sencillos, más sensibles and de menor coste que los utilizados actualmente en el diagnóstico de la EC.

El principal objetivo planteado en esta tesis doctoral fue el desarrollo, fabricación y optimización de la superficie del transductor para obtener inmunosensores electroquímicos para el diagnóstico serológico de la EC más sensibles, robustos y reproducibles y con una estabilidad aceptable que permita su comercialización.

La primera parte de este trabajo consistió en la búsqueda de una inmovilización adecuada del material proteico en la superficie del transductor del sensor electroquímico, explotando la potenciabilidad de los nanomateriales. Para ello, electrodos serigrafiados de carbono fueron nanoestructurados con nanopartículas de oro, nanotubos de carbono y nanofibras de carbono, además del uso de materiales nanohíbridos de carbono/metal. La biofuncionalidad de los diferentes transductores ha sido evaluada a través de su habilidad para absorber y retener material proteico en sus superficies, empleando para ello la reacción estreptavidina/biotina. Las superficies de los diferentes transductores también han sido caracterizadas utilizando microscopía de barrido electrónico. Las mejores superficies transductoras han resultado ser los electrodos serigrafiados de carbono modificados con híbridos nanotubos de carbon/nanopartículas de oro.

Después de seleccionar el transductor más adecuado, la interfase sensora de los EIs fue construida utilizando la reacción antígeno-anticuerpo. Así, en el caso que nos ocupa, los EIs desarrollados y puestos a punto en este trabajo determinan inmunoglobulinas A (IgA) y G (IgG) anti-gliadina (AGA) y anti-transglutaminasa tisular (anti-tTG),

marcadores serológicos específicos de la EC. Además, un EI bianalito para la detección simultánea de los marcadores de la EC, AGA y anti-tTG, ha sido también desarrollado. Por último, se ha ensayado un inmunosensor electroquímico para la determinación de anticuerpos contra gliadina deaminada.

Los diferentes sensores desarrollados han sido testados utilizando muestras de suero humano de pacientes celíacos y personas sanas. Finalmente los inmunosensores electroquímicos desarrollados han sido validados con otros inmunoensayos espectrofotométricos ya establecidos para el diagnóstico serológico de la EC.

Los inmunosensores diseñados y optimizados en esta tesis doctoral tienen la ventaja de ser de fácil manejo y de un único uso, lo que facilitaría una futura implantación de estos sensores como dispositivos *point-of-care*.

Palabras clave: enfermedad celíaca, la transglutaminasa tisular, la gliadina, electrodos serigrafiados nanoestructurados, inmunosensores electroquímicos

PUBLICATIONS (PAPERS AND COMMUNICATIONS) RESULTING FROM THE DOCTORAL PROJECT

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M.M.P.S. Neves, M. B. González-García, H.P.A. Nouws, C. Delerue-Matos, A. Santos-Silva, A. Costa-García, Multiplexed Electrochemical Immunosensor for Detection of Celiac Disease Serological Markers, Proceedings of the IMCS 2012 - The 14th International Meeting on Chemical Sensors, Nuremberg, Germany, May 20-23, **2012**
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XIV Iberic Meeting of Electrochemistry & XVII Meeting of the Portuguese Electrochemical Society
Funchal, Portugal, April, **2012**

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Multiplexed Electrochemical Immunosensor for Detection of Celiac Disease Serological Markers
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INDEX

ABBREVIATIONS

AChE	Acetylcholinesterase
AGA	Anti-gliadin antibodies
AgNPs	Silver nanoparticles
Anti-H-IgA-AP	Anti-human IgA antibodies conjugated with alkaline phosphatase
Anti-H-IgG-AP	Anti-human IgG antibodies conjugated with alkaline phosphatase
AP	Alkaline phosphatase
ASV	Anodic stripping voltammetry
BRAC1	Breast cancer 1
BSA	Bovine serum albumin
CA	Cancer antigen
CD	Celiac disease
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
CH	Antibody constant domains
ChOx	Cholesterol oxidase
ChE	Cholesterol esterase
CNF	Carbon nanofiber
CNP	Carbon nanoparticle
CnT I	Cardiac troponin I
CNT	Carbon nanotube
CV	Cyclic voltammetry

DGP	Deamidated gliadin peptide
DMF	Dimethylformamide
DPV	Differential pulse voltammetry
ECM	Extracellular matrix
EB	Electrochemical biosensors
EIs	Electrochemical immunosensors
EIS	Impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial antibodies
ESA	Electrostatic self-assembled
ESPGAN	European society for paediatric gastroenterology and nutrition
F(ab´)2	Antigen binding fragment
Fc	Non-antigen binding fragment
Fe_3O_4 NPs	Iron oxide
FCM	Flow cytometry
GEC	Graphite–epoxy composite
GRF	Graphene
GOx	Glucose oxidase
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
HMW	High molecular weight
H-tTG	Human recombinant tissue transglutaminase
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin

IL-6	Interleukin-6
IL-15	Interleukin-15
IP	Indoxyl phosphate
i_p	Peak current intensity
LMW	Low molecular weight
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MHC	Major histocompatibility complex
MWCNTs	Multiwalled carbon nanotubes
MWCNT-COOHs	Carboxylated multiwalled carbon nanotubes
NASPGHAN	North american society for pediatric gastroenterology, hepatology and nutrition
NIH	National Institutes of health
NPAus	Gold nanoparticles
NSB	Non-specific binding
PCR	Polymerase chain reaction
PfHRP-2	*Plasmodium falciparum* histidine-rich protein 2
POC	Point-of-care
PSA	Prostate specific antigen
QDs	Quantum dots
RIgG	Rabitt imunoglobulin G
ROC	Receiver operating characteristics
RSD	Relative standard deviation
RT	Reverse transcriptase

RT-PCR	Real-time PCR
RP- HPLC	Reversed phase high-performance liquid chromatography
SAMs	Self-assembled monolayers
SARS	Severe acute respiratory syndrome
SEM	Scanning electron microscopy
Si	Silica nanoparticles
SPCEs	Screen-printed carbon electrodes
SPEs	Screen-printed electrodes
SWCNTs	Single-walled carbon nanotubes
SWV	Square wave voltammetry
tTG	Tissue transglutaminase
U	Arbritary units
VH	Antibody heavy chain variable domain
VL	Antibody light chain variable domain

I. STATE OF THE ART

ORGANIZATION AND STRUCTURE OF THE DISSERTATION

The present work includes all scientific articles (5 published and 1 submitted to international peer-reviewed scientific journals) which resulted from this doctoral thesis.

All articles are written in English and the original manuscript content, formatted in accordance with the specific rules of each journal, was maintained. Therefore, there are variations in the structure of the various articles presented along the chapters as well as in the references format. All the literature that is not integrated in the publications is presented according to the Vancouver norm as advised by the "rules of formatting dissertations and doctoral thesis" of the Faculty of Pharmacy, University of Porto.

The texts that appear in Portuguese were prepared in accordance with the new spelling agreement.

The thesis is divided in two main parts: **Part I** corresponds to the **State of the Art** and **Part II** called **Research and Development**, includes all the developed experimental research.

Thus, **in Part I**, with a more theoretical approach, a general introduction to the subject of this dissertation is given in order to contextualize the work, addressing issues such as the problem of celiac disease, namely regarding the diagnostic. An overall view about electrochemical immunosensors principles as well as new analytical alternatives, such as new nanomaterials as transducer surfaces for sensing applications, are also presented. This brief introduction is followed by a description of the main goals of the work. In **Part I** of this thesis are also presented two chapters, **Chapter 1** and **Chapter 2**, that correspond to reviews of the main areas included in the present work.

In **Chapter 1**, a review that was written at the beginning of this project with the aim of gathering information about new methods for CD diagnosis and for quality control of gluten-free foods, is presented. The second review article, presented in **Chapter 2**, describes the state of the art of new analytical trends using screen-printed electrodes nanostructured with nanomaterials as electrochemical sensing transducers for clinical applications.

Part II includes **Chapter 3** and **Chapter 4**. The former chapter consists of an article dealing with the study of nano-modified screen-printed electrodes for general use as electrochemical biosensor transducers. **Chapter 4** includes four articles about the development and application of electrochemical immunosensors for the detection of the selected CD serological biomarkers, using the best transducer surface obtained in the studies carried out in **Chapter 3**. The first article presents the development of an electrochemical immunosensor (EI) for the detection of anti-transglutaminase (anti-tTG) autoantibodies; the second article presents the study of an EI for the detection anti-gliadin

antibodies (AGA). In the third paper, a nanostructured dual-screen-printed electrode was employed for the multiplexed determination of both anti-tTG and AGA antibodies. Finally, the last presented work (corresponding to a manuscript still in preparation) describes an EI for the detection of deamidated gliadin peptides (DGP).

At the end of the dissertation the **Final considerations** are presented. This designation was chosen since partial conclusions have been presented in the different chapters. This final chapter gives an overview of the entire work, highlighting successes and limitations as well as a future outlook.

GENERAL INTRODUCTION

Celiac disease

Celiac disease (CD) is a life-long inflammatory autoimmune condition of the gastrointestinal tract affecting genetically susceptible individuals (Shaoul et al., 2007), and the only treatment available is a strict gluten-free diet (Hill et al., 2006). It is estimated that celiac disease affects celiac disease affects primarily the European ancestry, being an important public health issue. However, CD is still greatly underdiagnosed.

The humoral response is triggered by the ingestion of gluten proteins found in gluten-containing foods such as wheat, rye and barley (Briani et al., 2008). The gluten proteins can be classified according to their solubility: the alcohol-insoluble fractions, the glutenins; and the alcohol-soluble fractions, designed as prolamins due to their high content of the amino acids proline and glutamine. Prolamins are called gliadins in wheat, hordeins in barley, secalins in rye and avenins in oats (Hill et al., 2006). Gliadins are the main gluten proteins involved in the pathophysiology of CD, triggering the immune system for the production of antibodies.

The pathogenesis of celiac disease involves a complex interplay between environmental, genetic, and immunologic factors, involving both adaptive and innate immune system. After the ingestion of gluten, gliadin (or similar peptides), resistant to digestive enzymes, cross the epithelial barrier reaching the cytosol where are deamidated by transglutaminase enzymes, creating epitopes with increased immunostimulatory potential. Then, deamidated gliadin peptides (DGP) are presented, in association with the human leucocyte antigens (HLA) DQ2 or DQ8 of antigen presenting cells, to CD4+ T cell. The expression of pro-inflammatory cytokines by activated T cells promotes the release of matrix metalloproteinases that are responsible for epithelial cell damage and tissue remodeling. The response to gluten also involves the innate immune system, because epithelial cells secrete interleukin (IL)-15 and express nonclassic major histocompatibility complex (MHC) class I molecules in response to gluten exposure. This in turn activates CD8+ cytotoxic T cells expressing the natural killer receptors, which can target and destroy epithelial cells that carry the stress-induced molecule (Briani et al., 2008; Holtmeier et al., 2006).

A 33 amino acid gliadin peptide, a highly specific substrate for deamidation by tissue transglutaminase and capable of stimulating all gliadin specific T cell lines in a very vigorous manner, as well as being resistant to the breakdown of endogenous proteases and

peptidases, has been identified by the group of Shan et al. (2002). In Figure 1 an illustration of the mechanism underlying CD pathogenesis is presented.

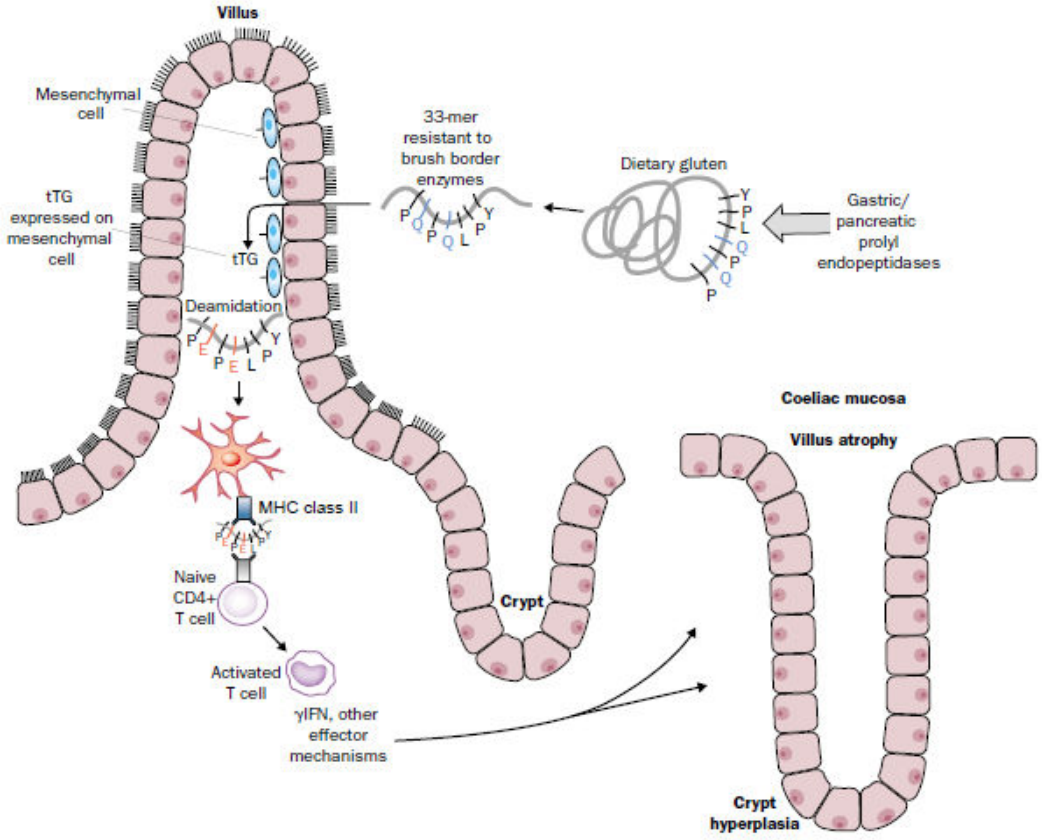

Figure. 1. Hypothetical scheme for digestion of dietary gluten in celiac disease patients. (adapted from Mowat 2003).

Currently, the diagnosis of celiac disease requires a small intestinal (jejunal) biopsy. In case of a positive result, clinical improvement is based on a gluten-free diet. Valuable immunological assays have been developed for the non-invasive diagnosis of CD, which are useful to prevent the underdiagnosis of CD and avoiding jejunal biopsies (Bahia et al., 2001). The most widely used methodology for CD clinical diagnosis is an enzyme-linked immunosorbent assay (ELISA). Serological tests, based on highly specific antibody-antigen interactions, are fundamental to identify gluten intolerance and have also been employed in monitoring the response to a gluten-free diet (Kaukinen et al., 2007).

Measurement of serum antigliadin antibodies (AGA) was first used as a diagnostic tool and, then, as a means of assessing continued inflammation of the small intestine, allowing the monitoring of the patients' compliance with a gluten-free diet, which is the only treatment available so far. However, in recent years, there is a tendency to no longer recommend AGA tests for routine diagnosis because the immunoglobulin A (IgA) isotype antibodies toward tissue transglutaminase (anti-tTG), identified as the CD autoantigen, are generally acknowledged as the first choice test (Kaukinen et al., 2007, Dahlbom et al., 2005). According to Rostom et al. (2006), anti-tTG IgA antibody tests have sensitivities higher than 90% and specificities higher than 95%. In contrast, AGA IgA tests have a sensitivity of about 80% and a specificity ranging from 80 to 90% (Shuppan 2000; Hill et al., *2005*) However, some patients are IgA deficient, which jeopardizes the pathology's detection by serological tests. In these cases, the determination of AGA IgG and anti-tTG IgG has been suggested as an alternative (Dahlbom et al., 2005).

In 2004, Schwertz et al. (2004) showed that the detection of antibodies against DGP could be valuable for the diagnosis of celiac disease. Particulary, IgG anti-DGP antibodies are more sensitive and more specific for CD than AGA IgG (Volta et al., 2008) and their performance is at least as good as that of IgA AGA (Sugai et al., 2006, Niveloni et al., 2007, Volta et al., 2008*)*. The new test displayed a higher diagnostic accuracy than AGA antibody tests and, although less sensitive than tTG antibodies, showed a significantly higher specificity than tTG antibody tests (Volta et al., 2008). The combined use of tTG and DGP antibodies seems to be a very useful tool for celiac disease diagnosis. Moreover, antibodies toward DGP can be helpful in disease follow-up (Volta et al., 2008).

It is important to note that despite the poor sensitivity and specificity of native gliadin antibody tests in comparison with the tests for the deamidated peptide or even tTG antibodies; commercial ELISA kits for AGA antibody detection are still commercialized by several companies (e.g. Phadia, Biosystems, Zedira, etc…). There is no discussion about tTG being the main autoantigen in CD, however, due to the lack of higher standardization of the reference methods for the different immunoassays, the development of a tool that allows a more complete serological screen of CD still was not yet accomplished and is of the utmost importance.

Due to the many recent development in the electrochemical biosensors field, this decentralized analytical tool can be an excellent alternative option to perform CD clinical diagnosis.

In Chapter 1 of this dissertation a Review about the state of the art regarding the conventional and recent alternative methods for celiac disease diagnosis, as well as gluten-free food control, is presented.

Electrochemical biosensors

Nowadays, the (bio)analytical field assists to a continuing demand for faster and simpler analytical methods for the determination of relevant analytes in the fields of clinical, environmental and food analysis. Therefore, biosensors have become interesting analytical devices that offer new possibilities within (bio)analysis.

A biosensor (Figure 2) can be defined as a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information (using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction system (Thévenot et al., 2001, Farré et al., 2009). Once the interaction of the biochemical receptor with the analyte is converted into a signal detectable by the transduction system, the signal is send to a readout or display by appropriate electrical equipment (Farré et al., 2009, Thévenot et al., 2001, Justino et al., 2010). Biosensors can be classified according to their biorecognition principle used for sensing (e.g. enzymes, nucleic acids, antibody or whole cells) and according to the transduction element employed (e.g., electrochemical, optical, piezoelectrical or thermal). Most of the biosensors described in the literature are electrochemical (Farré et al., 2009; Justino et al., 2010).

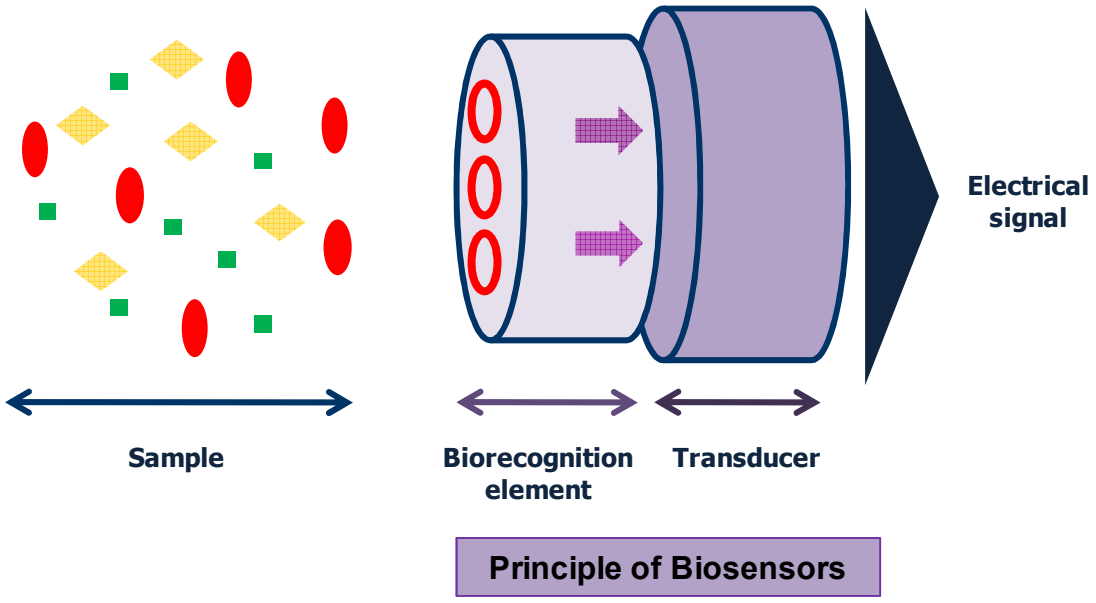

Figure 2. Squeme of a biosensor design.

Electrochemical Immunosensors

The development of immune-based assays for the detection and quantification of antibody-antigen interactions continues to be a subject of intensive research and development efforts. Therefore, within the electrochemical biosensors, electrochemical immunosensors (EIs) are in many cases the best option for *point-of-care* (POC) diagnostics because they provide specific and sensitive detection. EIs are affinity ligand-based solid-state biosensor devices in which the immunochemical reaction is coupled to an electrochemical transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex, which is similar to the immunoassay methodology (Luppa et al., 2001, Farré et al., 2009, Tudorache and Bala 2007).

Immuno-based assays that rely on antibody–antigen interactions provide a promising means of analysis owing to their specificity[1] and sensitivity[1] (Zhang et al., 2008). High specificity is achieved by the molecular recognition of target analytes (usually the antigens) by antibodies to form a stable complex on the surface of an immunosensor (Killard et al., 1995). On the other hand, the sensitivity depends on several factors including the use of high affinity analyte-specific antibody(ies), their orientation after immobilization on the immunosensor surface, and the detection system used to measure the analytical signal (Killard et al., 1995).

Antibodies are a family of glycoproteins known as immunoglobulins (Ig). There are five distinct classes of glycoproteins (IgA, IgG, IgM, IgD, and IgE) with IgG being the most abundant class (approximately 70%) and the most often used in immunoanalytical techniques. Antibodies are usually represented as a Y-shaped molecule (Figure 2) based upon two distinct types of polypeptide chains that differ according to their molecular weight. There is a smaller chain, which is the “light” chain, with a mass of approximately 25 kDa and a larger chain with a mass of approximately 50 kDa, known as the “heavy” chain. In each Ig molecule, there are two light and two heavy chains which are held together by disulfide bonds. Both heavy and light chains are divided into constant (C) and variable (V) domains based on their amino acid sequence variability. The light chains have

[1] In the field of biosensors, the concept of specificity is in straight correlation with the selectivity, which is the ability of the sensor device to detect, in an unequivocal way, the analyte of interest, and sensitivity appears in terms of detection limits. In terms of qualitative diagnosis in medical sciences, sensitivity and selectivity are based on the fact that the results of tests performed with a diagnostic tool, can be positives (a: trues; b: false positives) or negatives (c: false negatives; d: trues). In this context, sensitivity is defined as the ratio between the number of true positive tests (a) and their sum, and the number of false negative tests (c), as: (a / (a + c)). And selectivity is defined as the ratio between the number of true negative tests (d) and their sum, and the number of false positive tests (b), as: (d/ (d+b)) (Aguilera-Herrador et al., 2010).

a single variable domain (VL) and a single constant domain (CL). In comparison, a heavy chain consists of a single variable domain (VH) and three constant domains (CH1, CH2, CH3). In general, the antibody molecule may be divided into two main fragments, the non-antigen binding fragment (designate as Fc) and the antigen-binding fragment (F(ab´)2), as can be observed in Figure 3. The variable domains in both chain types are the most important regions with regard to the antibody–antigen binding interaction. The specificity of an antibody towards the binding site (or epitope) of its antigen is a function of its amino acid sequence. Within the VL and VH domains, there are three distinct subregions of high sequence variability, known as hypervariable regions. There are three on each light chain and three on each heavy chain, forming six hypervariable loops known as complementarity determining regions, which constitute the antigen binding site. It is the diversity in this region that allows the production of high-affinity antibodies against almost any antigen (Cass 1990).

Antibodies can be divided in polyclonal antibodies and monoclonal antibodies. A polyclonal antiserum will contain many different antibody molecules with varying affinities and specificities. In contrary, monoclonal antibodies are produced by a single clone of antibody-producing cells and therefore all molecules from the clone have the same specificity and affinity (Cass 1990).The intermolecular forces which contribute to the stabilization of the antibody-antigen complex are the same as those involved in the stabilization of the configuration of proteins and other macromolecules (Cass 1990). It is estimated that 108 antibody specificities can be produced from this one basic molecular structure, and an individual antibody will usually recognize only one antigen, although there are possible cross-reactivities (Zhang et al., *2008*).

Therefore, EIs present high selectivity and sensitivity which allows early detection of many diseases. Furthermore, EIs are also cost-effective. In the EI sensing strategy, most antibodies and antigens are intrinsically unable to act as redox partners, therefore an appropriate label is often conjugated to a particular component of the immunocomplex to promote an electrochemical reaction. The produced electrochemical signal is then quantitatively related to the amount of analyte present in a sample solution and is recorded with the use of bench or portable instruments which are usually capable of applying different electrochemical techniques such as voltammetry, amperometry, potentiometry, impedance spectroscopy, and conductimetry (Farré et al., 2009, Justino et al., 2010, Ricci et al., 2012).

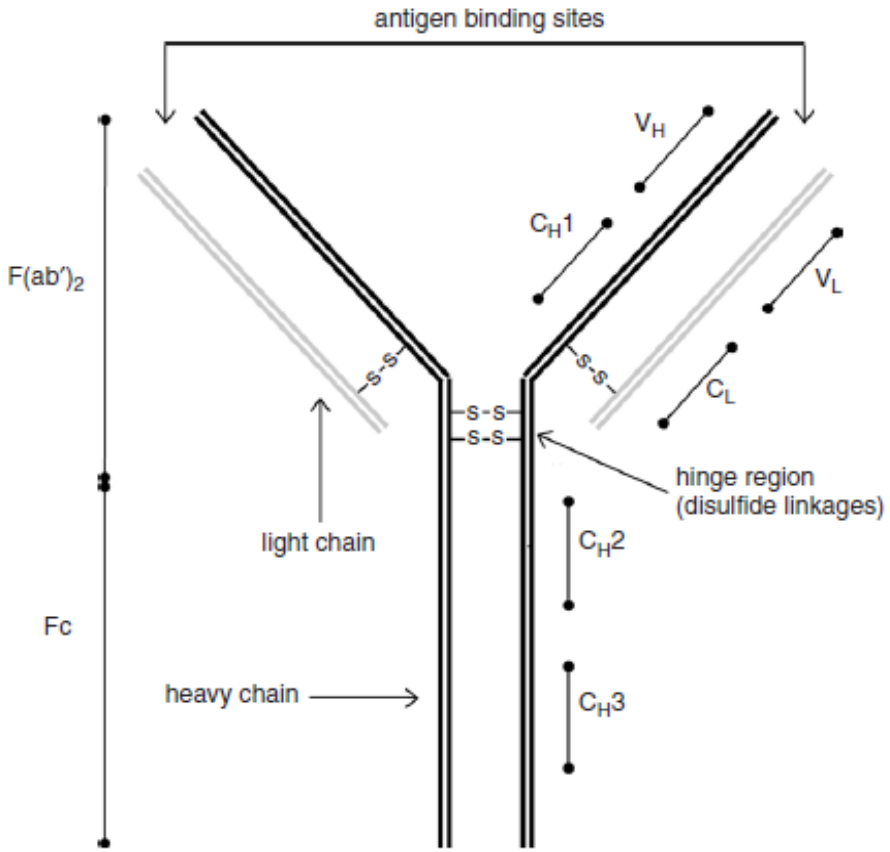

Figure 3. Schematic representation of the "Y"-shaped structure of an antibody (Zhang *et al.* 2008).

The enzyme linked immunosorbent assay (ELISA) is the accepted method for the detection of any molecule suitable for antibody targeting. The use of electrochemical transducers for the development of immunosensors has important advantages compared with ELISA, which make them interesting alternatives to the conventional immunoassays (Ricci et al., 2012). The employment of small sample volumes, the possibility of real-time analysis due to the direct translation of the electrical signal into the readout signal, and the achievement of low detection limits are some of the advantageous features of EIs. Moreover, there is a continuous effort on the miniaturization of the instrumentation. All these characteristics make EIs attractive tools for immunosensing development.

Immobilization procedures in solid phase bioassays

Electrochemical immunosensors are usually obtained through the immobilization of a recognition element (i.e. antigen or antibody) on the electrode surface (Ricci *et al.* 2012). The manner in which the recognition element is immobilized on the solid phase is a critical aspect in the immunosensor's architecture and requires special consideration. Immobilization of the biological materials onto the transducer surface is the key step for the successful fabrication of biosensors. Biomolecules have been immobilized by adsorption, covalent attachment, entrapment, cross-linking, and affinity binding (Albareda-Sirvent et al., 2000; Tudorache and Bala 2007; Zhang et al., *2008;* Sassolas et al., *2012*). The chosen immobilization method should provide an orientation of the recognition element with minimal steric hindrance in order to guarantee a favourable interaction between the analyte and the binding sites (i.e. the paratopes or epitopes, for an antibody or an antigen, respectively).

Adsorption

Adsorption is a rapid and simple procedure for the immobilization of antigens and antibodies onto a solid phase, especially for disposable biosensors. Physical adsorption based on *van der Waals* attractions and electrostatic and hydrophobic interactions between biomolecule and solid surface, is the most often used method because of its simplicity. For this reason, physical adsorption is frequently used as a preliminary test before undertaking further improvement or designing more complex biosensors. The most important drawback of this technique is that the forces between biomolecule and support are weak and cannot be controlled easily. As a consequence, the biological component can be leached during the assay, depending on experimental conditions such as pH, ionic strength, temperature, and solvent (Albareda-Sirvent et al., 2000).

Covalent attachment

Covalent immobilization of the biological recognition component on the solid electrode surface can be achieved through direct attachment or by use of, for example, the self-assembled monolayer (SAM) procedure. In the direct attachment on gold surfaces, thiol groups of biomolecules can form covalent bonds with the transducer's surface. The activation of solid carbon surfaces has also been described for the covalent immobilization of biomolecules. In this case, the covalent coupling of biomolecules can be achieved between the carboxyl groups of a support and the amino function of the biomolecule, or reversely, employing a support modified with amino groups for the binding of carboxyl-

terminated molecules (Sassolas *et al. 2012*). In the SAM process the electrode surface should be made of gold or should be coated with a gold layer. Then, by use of sulfur-containing compounds (thiols, sulfides, and disulfides) a monomolecular layer can be formed on the gold surface by simple immersion of the electrode in the sulfur-containing solution. Active groups (–COOH, $–NH_2$, –OH) on the surface of the monolayer are then reacted with the biomolecule of interest, leading to covalent bonding. SAMs haves important characteristics which are necessary for the immobilization procedure, such as stability, reproducibility, and uniformity of the monolayer (Tudorache and Bala 2007).

Entrapment

The immobilization of the biocomponent by entrapment within a suitable matrix, which is then deposited on the electrode support, can improve its stability. Immobilization in matrices such as gels, polymers, pastes, or inks can also be as simple as physical adsorption. The biological material is usually mixed and well homogenized with the supporting material and then applied on the electrode's surface as an additional membrane that must be dried or polymerized. The matrices used include gelatins, polyurethanes, poly(vinyl alcohol), carbon paste, and carbon ink. The principal advantage of this technique is its compatibility with mass fabrication techniques. *Electropolymerisation* is a special example of the entrapment technique in which the biological molecule is homogenized in a monomer matrix and is then deposited on the transducer surface as a polymeric film produced by electropolymerisation (Tudorache and Bala 2007).

Cross linking

Immobilization by intra- or intermolecular cross-linking has also been used to coat electrode surfaces with specific biomolecules. The method is based on the formation of three-dimensional links between the biological material and bi- or multifunctional reagents. The resulting modified biological material is completely insoluble in water and can be adsorbed on a solid surface (Tudorache and Bala 2007, Albareda-Sirvent et al., *2000*).

Affinity

Specific affinity interactions for biomolecule immobilization have been widely used in immunoassay systems in recent years. The (strept)avidin–biotin interaction is the most employed (Weber et al., 1989). Usually it involves biotinylating the capture element and coating a solid phase with either avidin or streptavidin. The dissociation constants of biotin–avidin and biotin–streptavidin interactions are of the order of 10^{-15} mol L^{-1} and are

some of the largest free energies of association observed for non-covalent interactions (Gitlin et al., 1987*)*. The complexes also withstand high temperatures, pH variations, and are resistant to dissociation when exposed to chemicals such as detergents and protein denaturants (Jones and Kurzban 1985). Another used affinity-based immobilization technique for the immobilisation of antibodies in immunoassay systems involves a bacterial antibody-binding protein. The two most common of which are Protein A and Protein G. These proteins bind specifically to antibodies through their non-antigenic (Fc) regions, which allow the antigen binding sites of the immobilized antibody to be oriented away from the solid phase and be available to bind the target analyte. As these proteins interact directly with the Fc region of antibodies, there is no need for antibody biotinylation.

Immunosensor formats

Most of the developed immunosensors are based either on a competitive or non-competitive assay, when applied to the detection of low and high molecular weight molecules, respectively (Ricci et al., 2007*;* Zhang et al., 2008). In a *competitive immunoassay*, sample analyte and labelled analyte compete for a limited number of antibody-binding sites. In electrochemical immunoassays, an enzyme label or an electroactive label is commonly used. The signal produced by the bound labelled analyte is usually inversely proportional to the amount of sample analyte. When immobilised antibodies react with the free antigens in competition with labelled antigens (Figure 4A) or, reversely, when immobilised antigens compete with free antigens for labelled free antibodies (Figure 4B), the immunoassay format is a defined as *direct competitive*. But when a secondary antibody is used as label after binding with the Fc region of the primary antibody, the format is denoted as *indirect competitive* immunoassay (Figure 4C) (Ricci et al., 2007; Ricci et al., 2012).

In a *non-competitive immunoassay* (also known as a *two-site "sandwich" immunoassay*), the sample analyte is captured by an excess of a capture antibody, separating it from the bulk sample (Figure 4D). After the interaction between immobilised antibodies and free antigens, an excess of secondary labelled antibodies, directed toward a second binding site of the antigen, is added. These labeled antibodies will only bind to the existing capture antibody–analyte complex. In an ideal non-competitive immunoassay, no signal would be produced in the absence of analyte because there are no appropriate binding sites available for the secondary labelled antibody. However, in practice, this is not the case due to non-specific interactions between the secondary antibody and other

components of the immunoassay. Therefore, it is always desirable to use a blocking agent to reduce these non-specific interactions. (Ricci et al., 2007; Zhang et al., 2008).

The ideal format to perform an immunoassay should be as simple as possible. Thus, a label-free direct competitive format, as shown in figure 4A, would be the ideal. However, besides simplicity other aspects, such as selectivity and sensitivity, have to be considered. Therefore, in some cases it is important to use a non-competitive assay with two antibodies, one of them monoclonal (usually the capture element), that react with the same antigen to ensure the selectivity of the assay.

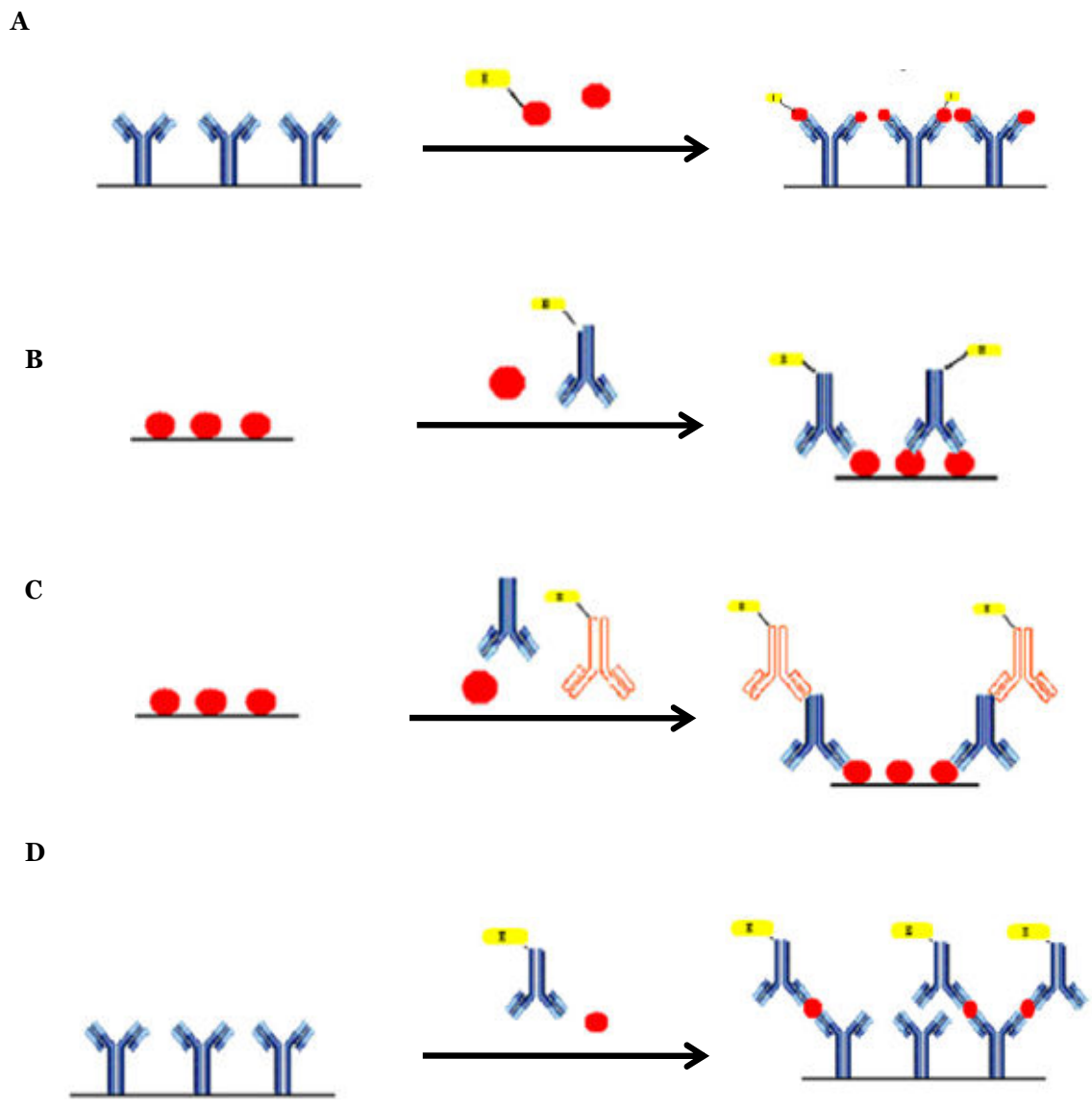

Figure 4. Schematic representation of different immunoassays formats. (adapted from Ricci *et al.* 2007).

Cyclic voltammetry as a tool for development of electrochemical immunosensors

In electrochemical analysis involving voltammetry, a three-electrode cell configuration is used and is constituted of a working electrode (i.e. the electrode at which the reaction of interest occurs); a reference electrode (to maintain/ control the potential of the working electrode), and a counter or auxiliary electrode to carry the cell current (Cass 1990; Wang 2000). When the potential of the working electrode is maintained or shifted to a value where the oxidation or the reduction of species in solution occurs, a current flows between the solution and the electrode. With an appropriate control of the experimental conditions, the intensity of this current can be related to the analyte concentration and the applied potential can provide information about the identity of the species. Since the intensity of the measured current results from the oxidation (anodic current) or reduction (cathodic current) of chemical species in solution, voltammetric techniques can only be applied to electroactive species or non-electroactive species that are previously converted into electroactive species or coupled to faradaic processes.

The current resulting from an electrochemical reaction is called faradaic current, because it obeys Faraday's law (i.e. the reaction of one mole of electroactive substance involves a charge change of 96,487 coulombs). In non-faradaic processes no charge transfer occurs across the interface between the solution and the electrode. However, there are changes in the structure of this interface that may cause a transient flow of external currents. These two processes occur simultaneously, so the total current is the sum of the faradaic current and the non-faradaic (background) current. Thus, the most recent voltammetric techniques aim to minimize the contribution of non-faradaic processes in order to improve the signal to noise ratio (Wang 2000).

Cyclic voltammetry (CV) is a voltammetric technique widely used to generate the signal in EIs devices. CV involves sweeping the potential between two limits, E1 and E2, at a fixed scan rate. Once potential E_2 is reached the scan direction is reversed whilst maintaining the same scan rate (Figure 5 and 6). CV is a useful technique for the study of electrochemical characteristics and also for examining more complex electrochemical systems such as enzyme-coupled reactions. Bearing in mind that enzymes are common labels for immunoassays (e.g., alkaline phosphastase has been used extensively as label in immunoassays, including EIs) the employment of CV as detection technique is a good option to understand the mechanism of electron transfer at the electrode surface.

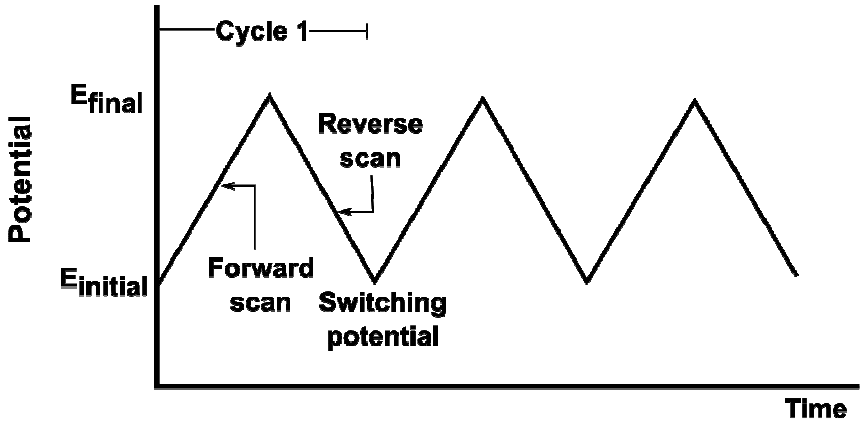

Figure 5. Potential-time excitation signal in a cyclic voltammetric experiment (Wang 2000).

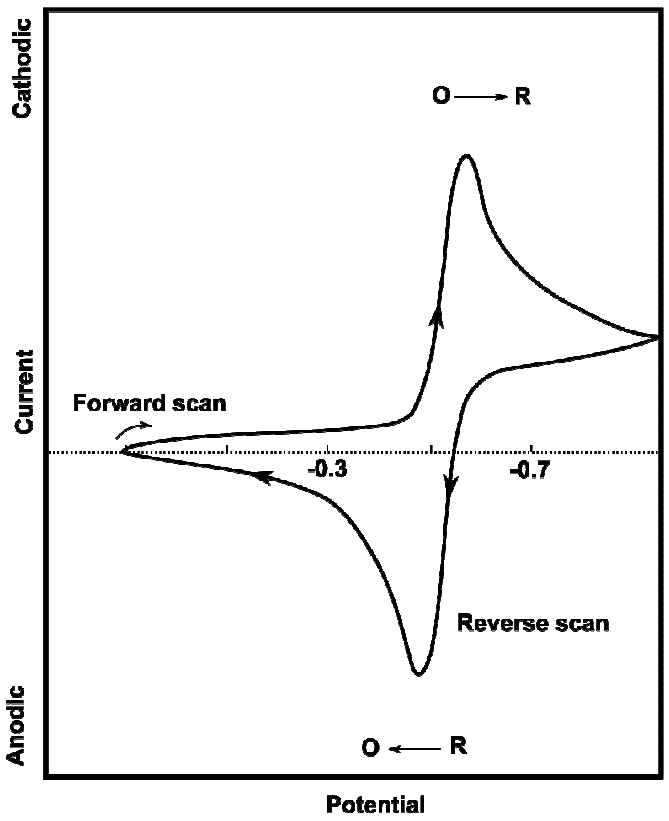

Figure 6. Typical cyclic voltammogram for a reversible $O + ne^- \leftrightarrow R$ redox process (Wang 2000).

Alkaline phosphatase as electrochemical enzymatic label

Alkaline phosphatase (AP) (*ortho*phosphoric monoester phosphohydrolase) is widely used as enzyme label in immunoassays. AP has a molecular weight in the range of 80 kDa and exibiths optimum activity at alkaline pH values. Magnesium is also considered essential for maximum AP enzymatic activity. AP's role as lable in immunoassays is largely exploited because AP is easily conjugated to haptens, antibodies, and other proteins, and is the most active of all alkaline phosphatases; moreover it presents a high turnover number and broad substrate specificity (Fernández-Sánchez et al., 1998, Preechaworapun et al., 2008).

In EIs, AP is used to generate electroactive organic products, most of which can be detected and quantified. Electrochemical detectors are generally sensitive and rapid for the analysis of the product of the enzyme hydrolysis of an AP substrate. Several AP substrates, useful for electroanalysis, have been studied for the use in immunoassays, such as: catechol monophosphate (Szydlowska 2006), 3-indoyl phosphate (IP) (Martinez-Montequin et al., 2000; Diaz-Gonzalez et al., 2002; Fanjul-Bolado et al., 2004; Diaz-Gonzalez et al,. 2005), hydroquinone diphosphate (HQDP) (Wilson and Rauh 2004), 4-nitrophenol phosphate (NPP) (Rosen and Rishpon 1989; Kim and Kwak 2005; Fanjul-Bolado et al., 2006), *p*-aminophenyl phosphate (APP) (Rosen and Rishpon 1989; Hart et al., 1997; Pemberton et al., 1999; Gyurcsanvi et al., 2002; Moore et al., 2003; Dong et al., 2006; Kwon et al., 2006), 1-naphthyl phosphate (NTP) (Pemberton et al., 1999; Chikae et al., 2007), phenyl phosphate (PheP) (Rosen and Rishpon 1989; Hart et al., 1997; Wilson and Rauh 2004; Sun et al., 2006), and 2-phospho-l-ascorbic acid (AAP) (Kokado et al., 2000; Gyurcsanyi et al,. 2002; Moore et al., *2003*). During the enzymatic process, these substrates are converted to the electroactive species catechol, indigo carmine (IC), hydroquinone (HQ), 4-nitrophenol (NP), 4-aminophenol (AP), 1-naphthol (NT), phenol (Phe), and l-ascorbic acid (AA), respectively.

A new method in which AP catalyzes the deposition of metallic silver was developed by Fanjul-Bolado et al., (2007). In this method 3-indoxyl phosphate/silver (3-IP/Ag+) is used as the AP electrochemical substrate. 3-IP is basically constituted by an indolic ring substituted in position 3 by a phosphate group. In the presence of AP this substrate is hydrolyzed in position 3, giving an indoxyl intermediate. This indoxyl intermediate will oxidize, producing indigo blue, and reduce silver ions present in solution into a metallic deposit. Therefore, metallic silver is co-deposited with indigo blue. The deposited silver is electrochemically stripped into solution and the resulting current intensity is measured by anodic stripping voltammetry. The described mechanism is illustrated in Figure 7. Metallic silver will be deposited where the enzymatic AP label is attached, which is

especially important to avoid cross-contamination and to ensure that the measured signal correspondes to the immune-labelled reaction.

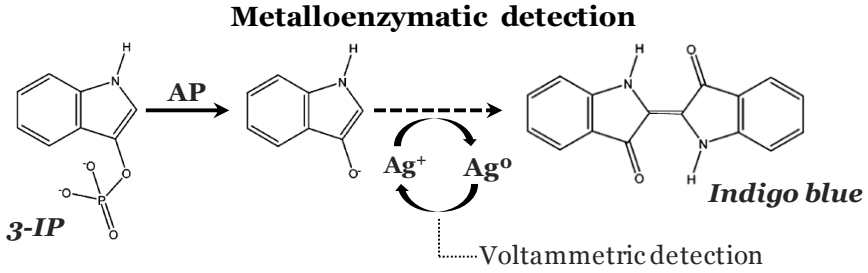

Figure 7. Mechanism of alkaline phosphatase-catalyzed silver deposition for electrochemical detection (adapted from Fanjul-Bolado et al., 2007).

Screen-printed electrodes

A wide variety of electrodes have been used as support to fabricate biosensor devices, including carbon paste electrodes (Fernández-Sánchez et al., 2000*)*, glassy carbon electrodes (Dai et al., 2003*),* and gold electrodes (Ouerghi et al., 2001). Recently, several biosensor devices have been developed on screen-printed electrodes (Hernández-Santos et al., 2004; Díaz-González et al., 2005; Singh et al., 2005*)*. The screen-printing microfabrication technology is nowadays well established for the production of thick-film electrochemical transducers (Tudorache and Bala 2007). This technology allows the mass production of reproducible, inexpensive, customized and mechanically robust solid-strip electrodes. Moreover, typical disadvantages of conventional electrodes such as memory-effects due to difficult, time-consuming and sometimes inefficient cleaning steps can be surpassed with these disposable SPEs. Another important feature that these electrodes exhibit is related to the miniaturisation of the corresponding device, along with their ease of handling and manipulation. Several SPE configurations based on different materials can be constructed. The process of production and design of SPEs consists of sequential layer-by-layer deposition of an ink onto an insulating support or substrate, which will define the geometry of the sensors (Bilitewski et al., 1992; Albareda-Sirvent et al., 2000; Mooring et al., *2005*, Tudorache and Bala 2007). The substrates are commonly made of

alumina, ceramics, PVC, gold, iron, etc. and the conducting path of the electrode consists of carbon ink/paste, or platinum, gold, or other metal pastes. SPEs can also consist of two (working and reference) or three electrodes (working, reference and auxiliary), designated first-generation or secondary generation, respectively (Bilitewski et al., 1992; Albareda-Sirvent et al., 2000; Mooring et al., 2005; Tudorache and Bala 2007).

The sensing element is the analyte-specific part of biosensors. The capture element can be immobilized onto the surface of the working electrode, employing the previously referred immobilization strategies. (Timur et al., 2004; Domínguez-Renedo et al., 2007; Tudorache and Bala 2007; Alonso-Lomillo et al., 2009; Sassolas et al., 2012) Therefore, SPE-based biosensors seem well suited to complement, or replace, standard analytical methods in fields such as clinical diagnostics, environmental analysis, and food quality control. Indeed, easy-to-use self-testing glucose strips, based on screen-printed enzyme electrodes, coupled to pocket-size amperometric meters, have dominated the $5 billion/year diabetes monitoring market over the past two decades (Newman and Turner 2005). The current research in the field of biosensors has revealed that SPEs are perfect candidates to construct more sensitive, reproducible and low–cost biosensors for routine analysis (Alonso-Lomillo et al., 2010*)*. Figure 8 shows the design of a typical SPE and the corresponding dimensions.

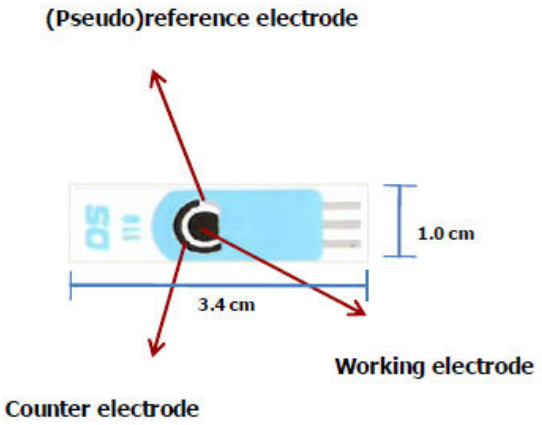

Figure 8. Example of screen-printed electrodes.

Nanomaterial-based electrochemical transducers

The SPE's surface, besides playing an important role as transducer, also works as the sensing phase. Therefore, new strategies are being explored to better amplify the

transduction of biological events in a sensitive way. The nanostructuration of electrode surfaces with carbon-based nanomaterials and gold nanoparticles provides outstanding properties for the solid sensing platform.

Since their discovery in the early 1990s, attributed to Iijima (Iijima 1991), the application of carbon nanotubes (CNTs) has been growing in different areas of research. CNTs are formed by one (single-walled carbon nanotubes, SWCNTs) or multiple cylindrical layers of graphene sheets (multi-walled carbon nanotubes, MWCNTs) (Fernández-Abedul and Costa-García 2008; Agüí et al., 2008). These CNTs have diameters ranging from fractions of nm to several centimeters cm, with both their ends normally capped by fullerene-like structures. (Trojanowicz 2006). CNTs present large length-to-diameter aspect ratios which provide high surface-to-volume ratios and the ability to promote electron transfer in electrochemical reactions, making them excellent nanomaterials for sensing applications (Agüí et al., 2008). One important aspect to consider when working with CNTs is their insolubility both in polar and non polar solvents as a consequence of their hydrophobic walls. Therefore, to obtain stable dispersions it is necessary to modify their surface with, for example, surfactants or biopolymers or to functionalize their structure in acidic or basic media. For example, the oxidation of CNTs in acidic medium is a simple and widely employed method to generate functional groups and, at the same time, to eliminate impurities. Obtaining a homogeneous CNT dispersion is very important for the subsequent nanostructuration of electrode surfaces.

Another carbon-based material that started to capture researcher's attention at the same time were carbon nanofibers (CNFs) (Rodriguez 1993). CNFs are cylindrical nanostructures with graphene layers arranged as stacked cones, cups or plates (Rodriguez 1993). They have lengths in the order of μm, while their diameter varies between some tens to several hundred nm (Wang and Lin 2008). The use of CNFs for biosensor development has grown in recent years because of their adequate physical and chemical properties (e.g., conductivity, surface area, inherent and induced chemical functionalities, and biocompatibility) (Wang and Lin 2008).

Despite the fabrication method, the initially obtained CNFs had the same disadvantages of low solubility and impurities as CNTs. The methods used to overcome these problems are the same as described for the CNTs, i.e. the generation of functional groups on their surfaces using acidic media. However, it is noted that the CNFs are more susceptible to undergo structural changes due to thermal treatments (Kuvshinov et al., 2009) and the oxidation of their surfaces. Therefore CNFs present higher solubilities than CNTs, which facilitates their dispersion in aqueous media.

CNTs and CNFs share the same basic composition (graphene) and appearance with diameters of 3-100 nm and lenghts of up to mm (Jong 2000). Moreover, both structures

can be obtained through chemical vapor deposition. The process consists essentially of thermal decomposition of a carbonaceous gas (such as acetylene, ethylene, methane) due to the reaction at high temperatures with other gases (ammonia, nitrogen, hydrogen) in the presence of a metal catalyst. The nanomaterials are deposited on the metal substrate in a random or, in certain conditions, an orderly manner. The major drawback of this method is the great number of structural defects of the resulting carbon nanomaterials. Since they have similar formation and growth mechanisms, the type of nanostructure obtained depends therefore on the experimental conditions employed (Jong 2000; Chuang et al,. 2008; Kuvshinov et al., 2009).

As was referred before, chemical, electrochemical, and thermal treatments are applied to generate functional groups on the CNT/CNF surfaces which can allow covalent binding of biomolecules. Moreover, covalent binding of biomolecules employing cross-linking agents or affinity binding through the biotin-(avidin/streptavidin) interaction can also be employed (Wang and Lin 2008) to augment the CNTs and the CNFs ability to adsorb protein material. Physical adsorpion of biomolecules onto the carbon nanomaterial's surface or direct entrapment in carbon ink or paste are also options for biomolecule immobilisation. Figure 9 shows typical representative schemes of the described carbon nanomaterials.

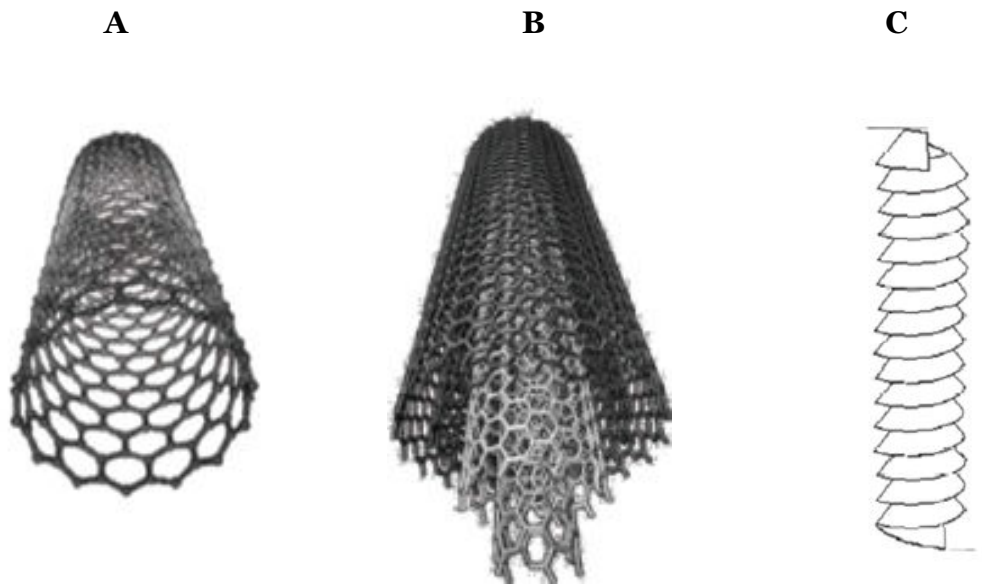

Figure 9. 3D schemes of (A) SWCNT, (B) MWCNT, and (C) CNFs .

In addition to carbon nanomaterials, metal nanoparticles have become extremely suitable for creating new sensing assays. Gold nanoparticles (NPAus) are of great promise for the construction of bioelectroanalytical devices because of their unique properties, which allows the improvement of the biocompatibility of the solid sensing phase (Yáñez-Sedeño et al., 2005; Suprun et al,. 2010). Moreover, gold nanoparticles present a high surface-to-volume ratio, a high surface energy, the ability to decrease the distance between proteins and the transducer surface and the ability to act as an electron-conducting pathway between prosthetic groups and the electrode surface, which may facilitate electron transfer between redox proteins and the electrode surface (Yáñez-Sedeño et al., 2005; Liu et al., 2003).

Among the conventional methods for the preparation of gold nanoparticles based on the reduction of Au(III) compounds, the most popular has been the reduction of tetrachloroauric acid (HAuCl4) with citrates in water. This method was introduced in 1951 by Turkevitch et al. (1951), and gives rise to gold nanoparticles of approximately 20 nm in diameter. The method was subsequently improved by other researchers in order to control the diameter of the nanoparticles (Frens 1973). Other methods are based on the reduction of the gold species (HAuCl4) with sodium borohydride (NaBH4) in sodium citrate (Raj et al., 2003) or by applying microwave-radiation (Xu 2008). The use of the photochemical reduction of the tetrachloroaurate anion ($AuCl_4^-$) makes a controlled particle size possible (Dong and Zhou 2007).

Gold nanoparticle-modified electrode surfaces can be prepared by covalent binding of gold nanoparticles with functional groups of self-assembled monolayers (SAMs); thiols or dithiols in case of gold electrodes, or with amino groups; by entrapment, or electropolymerization, of colloidal gold into the electrode by mixing the gold with the other components in the composite electrode matrix or by direct deposition of nanoparticles onto the electrode surface (Yáñez-Sedeño et al., 2005). Immobilization through affinity using the (strept)avidin–biotin interaction is also a possibility, for example by modifying the electrode surface with biotin and the NPAus with avidin or streptavidin. Electrochemical deposition of NPAus has recently been reported as the method of choice for simple, low-cost nanostructuring and nanopatterning of electrode surfaces (Figure 10). Electrochemical deposition methods allow a fast, simple, efficient and reproducible modification of the electrode surface with gold nanoparticles. Furthermore, these methods are applicable to any type of electrode.

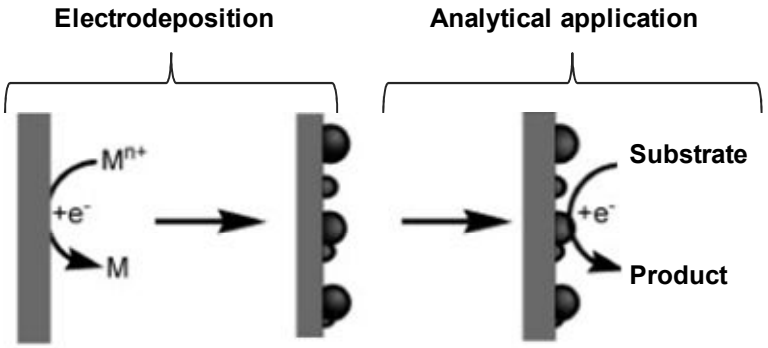

Figure 10. Schematic representation of the electrodeposition process of nanoparticles on the electrode surface and their use for analytical purposes.

One of the new trends is the use of hybrid materials, formed between carbon and gold nanomaterials, as last generation transducers for the development of biosensors. Carbon and metal-based nanomaterials have exceptional properties that once conjugated in hybrid surfaces lead to a synergic effect on each material property, presenting collective characteristics that are drastically different from the individual components (Gu et al. 2009).

The use of nanomaterials relies on the principle that some materials whose structural dimensions are in the nanometer scale are capable of presenting very different properties than the same material with larger dimensions.

The role of nanomaterials, as interface between biological recognition events and electronic signal transduction, can lead to exiting opportunities in the construction of novel biosensor devices. The ease of modification is one of the features that make SPE excellent candidates to be applied as nanostructured electrodic transducer surfaces.

In Chapter 2, a revision on the state of the art of the use of electrochemical biosensors based on nanostructured screen-printed electrodes for clinical applications can be found.

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Main goals

Attending to the specificity of immunological reactions with the advantages of electrochemical techniques, the main goal this dissertation was the development, manufacturing and optimization of the best transducer surface to obtain sensitive, reproducible, robust and low-cost electrochemical immunosensors for routine analyses of CD clinical serological diagnosis.

In order to achieve the proposed immunosensing strategy several development and integration steps were addressed:

- ✓ Development, characterization, optimization, and reproducible production of nanostructured SPCEs in order to provide an adequate transducer surface for the sensor devices. The nanostructuration of the SPCEs with multiwalled carbon nanotubes (MWCNTs), CNFs, NPAus, and hybrids of MWCNTs/NPAus and CNFs/NPAus was explored.

- ✓ Evaluation of the biofunctionality of the developed transducers through their ability to adsorb protein material on their surface using direct adsorption of the streptavidin/biotin system. The biofunctionality was evaluated and optimized using alkaline phosphatase and 3-indoxyl phosphate/silver (3-IP/Ag^{+}) to produce a product (metallic silver) which is detectable by anodic stripping voltammetry.

- ✓ Development and optimization of individual electrochemical immunosensors for the determination of CD serological markers (AGA and anti-tTG autoantibodies, and DGP).

- ✓ Construction of a multiplexed EI for the simultaneous determination of AGA and anti-tTG autoantibodies, integrating the individually optimized sensors in the design of the multiplexed sensor.

- ✓ Validation of the developed methods by comparing the obtained results with the results obtained by the established methods for CD serological diagnosis (i.e. ELISA).

- ✓ Application of the developed and optimized immunosensors for quantification of the serological CD markers in human serum samples.

Chapter 1

Celiac disease: from gluten to diagnosis

Celiac disease diagnosis and gluten-free food analytical control

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Celiac disease diagnosis and gluten-free food analytical control

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Abstract

Celiac disease (CD) is an autoimmune enteropathy, characterized by an inappropriate T-cell-mediated immune response to the ingestion of certain dietary cereal proteins in genetically susceptible individuals. This disorder presents environmental, genetic, and immunological components. CD presents a prevalence of up to 1% in populations of European ancestry, yet a high percentage of cases remain underdiagnosed. The diagnosis and treatment should be made early since untreated disease causes growth retardation and atypical symptoms, like infertility or neurological disorders. The diagnostic criteria for CD, which requires endoscopy with small bowel biopsy, have been changing over the last few decades, especially due to the advent of serological tests with higher sensitivity and specificity. The use of serological markers can be very useful to rule out clinical suspicious cases and also to help monitor the patients, after adherence to a gluten-free diet. Since the current treatment consists of a life-long glutenfree diet, which leads to significant clinical and histological improvement, the standardization of an assay to assess in an unequivocal way gluten in gluten-free foodstuff is of major importance.

Keywords: Celiac disease. Autoimmune. Transglutaminase. Gliadin.

Abbreviations

AGA anti-gliadin antibodies
CD celiac disease
EIs electrochemical immunosensores
EMA endomysial antibodies
ELISA enzyme-linked immunosorbent assay
Ig immunoglobulin
ROC receiver operating characteristics
tTG tissue transglutaminase

Introduction

Celiac disease (CD) is a disorder of the small intestine caused by an inappropriate immune response to wheat gluten and similar proteins of barley and rye in genetically susceptible individuals. CD can be also referred as celiac sprue, nontropical sprue, gluten-sensitive enteropathy or idiopathic steatorrhea [1]. The classic presentation, with malabsorption, was first described by Samuel Gee in 1888 [2], but the relation between the disease and wheat was not reported until the late 1940s by Willem Karel Dicke, who observed that the ingestion of certain cereal grains was harmful to children with celiac disease [2, 3]; later, John W Pauley described the associated histologic changes in the intestine [4].

CD is found mainly in Caucasians, occurring in 1 per 130-300 individuals in the western European population [5-7]. Although CD is one of the most common immune-mediated disorders, still remains a considerable prevalence of undetected cases [8].

It is more appropriate to consider CD as a multisystem disorder, rather than mainly gastrointestinal. A considerable number of conditions are associated to CD namely osteoporosis, malignancy and infertility. It is also associated with other autoimmune disorders, such as dermatitis herpetiforms, type 1 diabetes or autoimmune thyroiditis, and also with neurological and genetic disorders [4, 9-12].

Clinical presentation

Celiac disease can be diagnosed at any age; however, it presents most commonly in early childhood (between 9 and 24 months) or in the third or fourth decade of life [13-16]. As occurs in other autoimmune disorders, CD is more common in females than in males at a ratio of 3 to 1 [17].

Although, it is a disorder that primarily affects the small bowel, the symptoms can range from classical gastrointestinal symptoms, such as diarrhea and abdominal distension, which are more common in infants and young children, to non-specific gastrointestinal symptoms and extra-intestinal manifestations, typical of older age groups; moreover, some patients are asymptomatic [8, 18]. The concept of the “celiac iceberg” has been used to emphasize the clinical variability of celiac disease and that many cases are still undiagnosed [10].

The clinical presentation of CD is, therefore, very heterogeneous, ranging from an asymptomatic or silent stage to a clinically overt or symptomatic form [19]. The asymptomatic forms are characterized by extraintestinal symptoms, but with typical histological changes and positive serology. The classical or symptomatic form presents typical gastrointestinal symptoms, histological changes and positive serology.

The term “latent" CD characterizes the subjects with genetic predisposition to develop CD. They do not have a flat mucosa, despite a gluten-containing diet, but probably will develop clinically overt CD later in life [20-22].This patients usually present increased intraepithelial lymphocytes (IELs) and positive serology for endomysial antibodies (EMA) and tissue transglutaminase (tTG) antibodies with HLA-DQ2 or DQ8 predisposing genotype [23-25].

Finally, the term “refractory” CD refers to patients who do not respond to a gluten-free diet or who previously responded but later became non-responsive, presenting severe villous atrophy despite maintenance of a strict gluten-free diet [26].

The severity of symptoms is not necessarily proportional to the severity of the mucosal lesions. In fact, currently, there are more subjects with asymptomatic or mild celiac disease than with the classical symptoms of severe malabsorption [3, 21].

Genetics and pathogenesis

The disease is genetically determined, affecting 10% of first degree relatives and 75% of the monozygotic twins being both affected. The human leucocyte antigen HLA-DQ2 is presented in 90-95% of CD patients and the remaining 5-10% of patients is HLA-DQ8 positive [18]. Although the possession of the HLA proteins is necessary, it is not sufficient for CD development, since about 30% of the healthy population possesses them [27-29].

Gluten is a complex mixture of wheat storage proteins that can be alcohol-soluble, fractions, the gliadins, and alcohol-insoluble fractions, the glutenins [28]. Based on their differential N-terminal sequence, size and electrophoresis mobility [30] gliadins are subdivided into α, β, γ, and ω-gliadins. Glutenins consist of low molecular weight (LMW) and high molecular weight (HMW) glutenins [31].

Gliadins are also called prolamins due to their high content in the aminoacids proline and glutamine. It is generally accepted that prolamins are the major triggering factors in CD [32]. Gliadins have analogous proteins that are present in barley (hordeins), rye (secalins) and oats (avenins). Recent studies failed to identify the toxic aminoacid sequence in oats [33] being considered toxic in only a minority of patients with CD [18].

The mechanism underlying CD pathogenis can be explained by the ingestion of the alcohol-soluble protein components of wheat, barley, and rye. These gluten peptides are resistant to digestion by gastric and pancreatic enzymes due to their high content in proline, reaching the epitelial cell membrane and passing into the cytosol [34]. The deamidation of these proline-rich gluten peptides is mediated by tTG enzyme [35] creating epitopes with increased immunostimulatory potential. The deamidated epitope are then presented, in association with the human leucocyte antigens DQ2 and DQ8 of antigen presenting cells, to CD4+ T cells expressing α/β T-cell receptor [36]. These T cells become activated and express proinflamatory cytokines [37] that, in turn, promote the release of matrix metalloproteinases which cause epithelial cell damage leading to the development of the flat mucosa, typical of CD [37]. The resulting tissue injury leads to further release of tTG [29].

The in-vitro study by Lu Shan and colleagues [38] reported a highly stable 33-mer peptide, rich in proline and glutamine, which has been isolated from gliadin and is thought to contain the toxic sequence. This 33 amino acid peptide has been reported to have immunodominant characteristics, being resistant to degradation by all gastric, pancreatic and intestinal brush border membrane proteases in the human intestine, and is readily available for T-cell recognition and activation.

Diagnostic and management

The diagnosis of CD is complicated by the diversity of clinical manifestations that are related to the age at onset and symptomatology.

Biopsy

Diagnostic criteria for CD in both children and adults are still based on the guidelines proposed in 1990 by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) [39]. Small bowel biopsy has been the standard diagnostic test for CD during the last 30 years. The biopsy is performed during an upper endoscopy and should only take place during a normal gluten-containing diet, when villous atrophy and crypt hyperplasia can be detected. Multiple samples are taken from the second or third part of the duodenum. Inadequate sampling and patchy villous atrophy can lead to an incorrect

diagnosis. Moreover, it should also be considered that isolated marked villous flattening and IELs can be found in other diseases [40].

Recently, there has been an increase in atypical forms of CD, including cases without significant gastrointestinal symptoms, and cases presenting symptoms and complications of CD before the development of villous atrophy [41-42].

Biological markers

Recent guidelines from the ESPGAN [39], and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) [43] have suggested an algorithm for diagnosis (Fig. 1), in which the serological tests appear as the first test to clear clinical suspicion of celiac disease in patients presenting characteristic symptoms or in those who belong to a risk group. These patients at risk are those with celiac disease associated disorders, as endocrine, neurological, liver, genetic and autoimmune diseases; first- and second-degree relatives of celiac patients are also at risk.

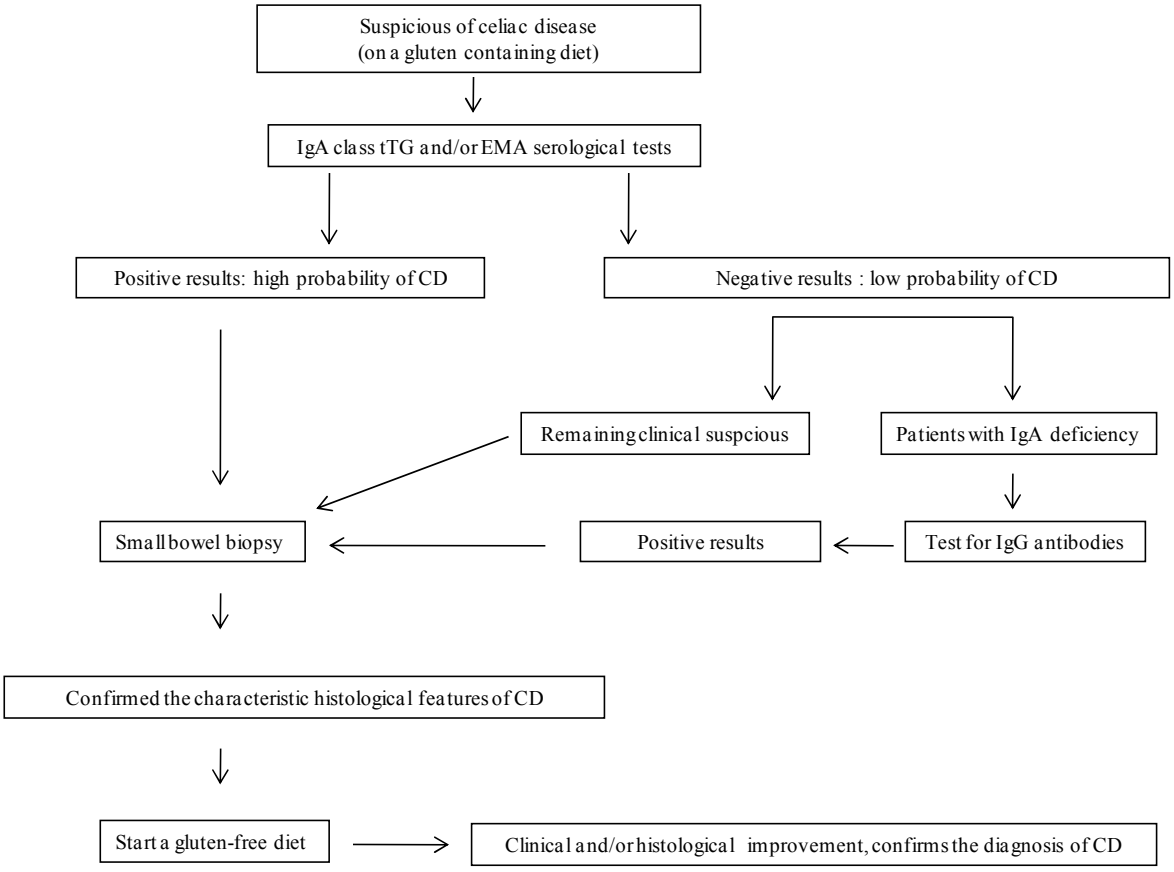

Fig. 1 Proposed approach for the evaluation of patients with suspected celiac disease [39, 43].

Antibody tests cannot replace histological studies of bowel biopsies; however they are very important as a screening tool, for early detection of CD cases [1]. Serological tests can also be employed in the follow-up and management of CD. Indeed, according to the ESPGAN criteria, when the diagnosis of CD is established by clinical, analytical and histologic studies, repeated endoscopy with duodenal biopsy is not necessary if the patient's condition improves after introducing a gluten-free diet; the results of repeated endoscopy could be rather confusing, since normalization of the histology may take up to 8 years [44].

The serologic tests use highly specific antibody-antigen interactions and are fundamental to identify the gluten intolerance and to monitor the response of the CD patients to a gluten-free diet [45]. There are two types of serologic tests. One detects the antibodies against the antigen gliadin (i.e. anti-gliadin antibodies (AGA)), in which the immunoglobulin A (IgA) isotype is considered to be the most specific [46], and the other, the autoantibody test, which detects antibodies to IgA tissue transglutaminase (IgA-tTG antibodies) and IgA endomysial antibodies (IgA-EMA) [18], that are usually present in serum during the active phase of CD [47]. However, some patients are IgA deficient, and, therefore, in that case, the detection of the pathology by the serological tests is jeopardized. Selective IgA deficiency affects about 2-5% of patients diagnosed with CD [48]. In these cases, the determination of the IgG class of antibodies against gliadin (IgG-AGA), endomysium (IgG-EMA) and tTG (IgG-tTG antibodies) has been suggested as an alternative [47].

Since their description in 1958, AGA have been used as the serological markers for CD [28]. Both IgA and IgG AGA are present in the sera of patients with CD, although they lack of specificity, as gliadin may cross the normal gut mucosa, being present in 5-10% of healthy population.

Endomysium is a connective tissue protein found in the collagenous matrix of human and monkey tissue [3]. EMA association with CD was first described in 1984 [49] and its detection rapidly became the serological test of choice, due to its specificity of almost 100%. The use of human umbilical cord as subtract has been proposed as a valid alternative to the monkey esophagus [50-51].

Tissue transglutaminase (tTG) is a calcium-dependent enzyme expressed both intra- and extracellulary, and is implicated in physiologic processes like extracellular matrix (ECM) formation, cell adhesion and apoptosis [53]. tTG serves as a cross-linker of different ECM proteins, resulting in the formation of an ε-(γ-glutamyl)-lysin bond. Gliadin is the preferred substrate for tTG [3]. It was suggested that it has the ability to crosslink itself to gluten leading to antibody formation [54]. In 1997 tTG was identified as the auto-antigen recognized by the EMA [55] and was identified as the main autoantigen in CD.

Sensitivity and specificity of serological markers

The most sensitive and specific serologic markers of celiac disease are the EMA and the tTG IgA antibodies [48]. While two different types of tests are used for detect these autoantibodies, they detect antibodies to the same antigen, the tTG [56]. IgA anti-tTG antibody and IgA anti-EMA tests have sensitivities higher than 90% and specificities higher than 95%, and, therefore, the serological tests to detect those antibodies are recommended for initial screening [57]. In contrast, IgA AGA has a sensitivity of about 80% and a specificity ranging from 80-90%. For routine diagnosis, the determination of gliadin antibodies in serum is no longer recommended, since they are less sensitive and specific than EmA and tTG antibody tests [43, 58].

IgG anti-EMA and anti-tTG antibodies, in spite of having a specificity rounding 95%, present poor sensitivities (around 40%) and for this reason these antibodies have been used less frequently as serological markers [57]. However, because IgA deficiency has an increased prevalence among celiac patients [29], care should be taken in interpreting the results of IgA antibody tests. In the case of IgA deficiency, measurement of IgG anti-EMA/tTG and IgG anti-gliadin antibodies should be performed. Positive results for IgA anti-EMA/tTG antibodies, or IgG anti-EMA/tTG antibodies and AGA in the case of IgA deficiency should be followed by intestinal biopsy. A biopsy might also be recommended in cases of negative serology, when there is a high clinical suspicion.

In positive serological cases, followed by a negative biopsy, it is important to consider HLA typing, since the absence of both HLA-DQ2 and/or HLA-DQ8 alleles has a very high negative predictive value, helping to rule out the disease in cases of equivocal biopsy results [3, 4, 56].

It is established that the most widely used methodology for CD clinical serological diagnostic purposes is an enzyme-linked immunosorbent assay (ELISA). Several high quality commercial kits are available to detect IgA antibodies to tTG antibodies, which appear to have equivalent diagnostic sensitivity and specificity to EMA IgA. The advantages of tTG testing is that the ELISA test, eliminates the disadvantages associated with the use of EMA, namely the higher cost, time-consuming protocol which is unsuitable for testing large numbers of samples, the use of monkey oesophagus (or human umbilical cord) and the subjective interpretation of the immunofluorescence analysis [15]. Several studies have compared the analytical and clinical utility of commercially anti-transglutaminase ELISAs assays [59-73], and found that the use of tTG as antigen for CD diagnosis presents an adequate sensitivity and specificity. Therefore, major efforts have been concentrated on developing a tTG-based ELISA, using either the commercially available guinea pig tTG or human recombinant tTG.

The first generation assays for tTG antibodies detection used guinea pig liver tTG as the antigen. Second generation kits using purified human tTG or human recombinant tTG (H-tTG) were developed and introduced in routine practice. Several studies compared the first and second generation kits and concluded that the human antigens improve assay sensitivity and selectivity [63, 74-76]**.** A third generation of kits using tTG-gliadin peptide complexes as the antigen, has also been proposed, however it seems to have no advantage over human recombinant antigen kits, specially regarding specificity [77, 78].

Several second-generation assays are commercially available, and were introduced in routine practice of clinical laboratories. These assays use either recombinant human tTG or purified H-tTG as antigen.

Van Meensel [79] and colleagues evaluated 10 different commercially available second-generation IgA anti-tTG ELISA kits, and showed that most of these assays presented excellent performance, with good linear ranges. According to the optimal receiver operating characteristics (ROC) curve cut-off employed, the sensitivity values ranged from 91% to 97% and the specificity ranged between 96% and 100%. Since the areas under the ROC curve did not differ significantly, the results of the kits could be compared; however, there is some variability between these immunosorbent assays which needs to be solved in order to reach higher homogeneity.

Gluten-free food control

Two guidelines concerning the management of CD were recently published: "Recommendations of NASPGHAN" [43] and "National Institutes of Health (NIH) Consensus Development Conference Statement on Celiac Disease" [80].

After a positive diagnosis for CD, the only treatment available, so far, is a lifelong strict adherence to a gluten free diet, which will permit the recovery of the intestinal mucosa [18]. However, a diet completely free of gluten would be difficult, if not impossible, to maintain. Gluten is a common component in the human diet, and its exclusion presents a big challenge for celiac disease patients. Gluten plays a key role in determining the unique baking quality of wheat by conferring water absorption capacity, cohesivity, viscosity and elasticity to dough [81]. After sugar, it is perhaps the second most widespread food component in Western civilization [82]. Since about 10% of gluten seems to be made up of potentially toxic gliadin peptides [83] it is extremely important to evaluate the purity of gluten-free products, in order to ensure a safe diet for celiac patients. To certify gluten-free products, the use of highly sensitive assays is mandatory. The European Union, World Health Organization and Codex Alimentarius require reliable measurement of the wheat prolamins, gliadins, rather than all wheat-derived proteins [84]. There is still no general agreement on the analytical method to measure gluten in ingredients and food products

[85], although the official limits described in the Codex Draft Revise Standard (2000) are 20 ppm for foodstuffs naturally gluten-free and 200 ppm for foodstuffs rendered gluten-free [86]. Nevertheless, to measure gluten traces in food, immunochemical methods are usually chosen to determine gliadins [87-92]. Besides the quality control of gluten-containing products, it is important to assess gluten in foodstuff that can be contaminated with native or heated proteins from wheat, barley and rye.

In the recent years several analytical possibilities for the detection of the wheat protein component gliadin in food products have been exploited, such as the polymerase chain reaction (PCR) technology, mass spectrometry or high-performance liquid chromatography (HPLC). PCR allows the amplification of a specific DNA fragment, flanked by two oligonucleotides that act as primers in the amplification reaction carried out by DNA polymerase. The amplified product is visualized by staining with a fluorescent dye or by Southern blotting after a gel electrophoresis. The amplification of gluten-specific DNA fragments by PCR has been reported [93, 94]. Normally, PCR results are only qualitative, however, by incorporating internal standards, the results provide semiquantitative measurements. Employing real-time PCR (rt-PCR) highly accurate quantitative results can be obtained. A quantitative competitive PCR system has been constructed, evaluated and compared to ELISA, obtaining a good correlation of the results between the two methods [95]. In this study a wheat-, barley- and rye-specific WBR11/WBR13 primer pair was used. These primers were also used in a quantitative competitive PCR system to detect gluten traces in flours and “gluten-free” bakery products [96]. Piknova and colleagues achieved detection limits of 200 mg/kg of wheat in flour using real-time PCR [97]. Henterich and colleagues performed a rt-iPCR (real-time immuno-polymerase chain reaction (iPCR)) for the detection of the cereal protein gluten, gliadin. Using iPCR a detection limit of 16 mg gliadin/100 g food was achieved [98].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry allows the detection of large proteins from highly complex protein mixtures such as those present in gluten prolamins [99]. The high resolution and sensitivity of this technique allows the elucidation of protonated molecular masses of most of the gliadin, hordein, secalin and avenin components. Méndez and colleagues [100] concluded that the analysis of gliadin-containing foods by this technique allows the immediate identification of the characteristic gliadin mass pattern, consequently permitting easy identification of gliadins in such samples with a detection sensitivity of 50-100 ng total protein loaded. In another study the first non-immunological alternative to quantify gluten gliadins in food samples was presented [101]. The procedure allowed the microquantification of gluten in processed and unprocessed gluten-containing food samples below toxic levels for CD patients, with a linear response in the 0.4 - 10 mg per 100 g range and a detection

sensitivity similar to that of ELISA systems. A new protocol for determining small amounts of gliadins in foods that contain relatively large amounts of other prolamin proteins from maize and/or rice was also described [102]. This strategy combines a two-step procedure of extraction (60% aqueous ethanol followed by 1 M acetic acid) with subsequent MALDI-TOF analysis to corroborate the presence of these ethanol-soluble wheat prolamin fraction. HPLC allows the separation and qualitative and quantitative determination of compounds with analytical interest. A widely used HPLC technique is the reversed phase high performance liquid chromatography (RP-HPLC). In reversed phase system, stationary phase is slightly polar or non-polar, while mobile phase has stronger polarity. A RP-HPLC system has already been described for the separation and quantitative determination of wheat prolamins in food [103,104]. Also a flow cytometry (FCM) method for to the quantitative determination of picogram levels of gliadin was developed [105]. FCM is a high-throughput technique that is able to analyse a large numbers of cells individually using light-scattering and fluorescence measurements [106]. In the work of Capparrelli and colleagues, rat antibodies against a 16-residue peptide of gliadin, common to the α, β, γ, and ω-gliadins, were used. A detection limit under 10 pg/ml was achieved.

Despite the efforts in developing new analytical strategies for gluten control in foodstuffs, the most used method of measurement of gliadin still relies on the ELISA-like techniques. Thus, two commercial immunoassays are currently available to assess gluten content of gluten-free foods. The association of Analytical Communities endorses the method originally developed by Skerritt and Hill [93], the sandwich ω-gliadin ELISA, which uses a monoclonal antibody to the heat-stable ω-gliadin fraction. Since the ω-gliadin fraction is not denatured when heated for cooking or processing, this assay can be used to assess gluten content of foods containing both native and heated protein. One major drawback of this assay is that measurements of this subfraction with the extrapolation to total gliadin have theoretical errors of -44 to +80% [94]. Moreover it is unable to accurately detect and quantify barley prolamins and cannot accurately quantify hydrolyzed gluten [109].

The other test is the R5 ELISA [110] that has been proposed as the standard method for gluten analysis in gluten-free foods by the Codex Committee on Methods of Analysis and Sampling [111], promoted by the Codex Committee on Nutrition and Foods for special dietary uses [112]. This test uses the R5 monoclonal antibody directed to the potentially celiac toxic epitope QQPFP (glutamine-glutamine-proline-phenylalaline-proline) present in wheat, rye and barley prolamins. This immunoassay is able to quantify native and heated gluten although it seems to over estimate barley hordein [113] and to be unable to accurately quantify hydrolyzed gluten [109].

New strategies for celiac disease diagnosis and gluten-free food analytical control

Currently, biosensor development is widespread in many fields and a considerable effort is being focused on the development of even more rapid, sensitive, high sample throughput and, specially, on-site analytical strategies that can be applied in point-of-care analysis. Two kinds of sensors are found in the bibliography: optical and electrochemical.

Optical biosensors

De Stefano and colleagues [114] proposed the development of a porous silicon-based (PSi) optical biosensor for the detection of trace amounts of gliadin using a recombinant glutamine-binding protein (GlnBP) from *Escherichia coli* as a molecular probe. The solutions containing the molecular probe and the analyte, peptic-tryptic (PT)-gliadin, were directly spotted on the sensor surface. GlnBP was covalently linked to the surface of the PSi surface via a functionalization process.The proposed optical protein microsensor with a PSi-based transducer sensor allows a sensitive, fast and easy readable optical response; moreover it is able to work under reducing conditions, which solves some problems related to prolamin extraction. The results showed that about 45% of the spotted proteins have selectively bound the respective peptide. A fiber-optic biosensor for the detection of anti-gliadin antibodies was also developed [115].The biosensor has been developed by coating a tapered optical fiber by immobilization of gliadin using the electrostatic self-assembled (ESA) method which allows the construction of nanometric scale recognition surfaces on the fiber optic, allowing a real time monitorization of the sensor behavior. Gliadin antigens were successfully immobilized onto the surfaces of tapered optical fibers using the ESA method which has been proved to be an efficient immobilization strategy. The biosensors were tested using antibodies conjugated with and without peroxidase. A high sensitivity sensor was obtained, with fast response times as compared with standard ELISA tests.

Electrochemical immunosensors

Yet, the most commonly used biosensor strategy relies on electrochemical sensors. New electrochemical immunosensors (EIs), which employ cost-effective, user-friendly and highly sensitive analytical transduction devices, have appeared as new exciting alternatives to the conventional immunochemical tests which are based on indirect detection compromising real-time analysis. EIs are a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information

using an immobilized immunological recognition element (for detecting a target analyte by structural complementarity) and an electrochemical based-transducer which converts the biological interaction into a measurable signal [116].

Recently, two EIs for the detection of celiac disease toxic gliadin in foodstuffs were reported. Nassef and colleagues [117] developed an electrochemical immunosensing strategy for the detection of toxic gliadin using an antibody, coined CDC5, which was raised against the putative immunodominant celiac disease toxic epitope of α-gliadin, 56-75. For anchoring the captured antibody, two different surfaces, based on a gold electrode modified with acidic self-assembled monolayers (SAMs), were proposed. A good performance regarding sensitivity, specificity and reproducibility was obtained. When applied to real sample analysis an excellent performance correlation was achieved when compared with ELISA as well as considerable decrease in the time to perform the assay. In order to improve sensitivity, the use of antibody fragments instead of whole antibodies was also exploited. A new electrochemical immunosensor based on the spontaneous adsorption of antigliadin Fab fragments (CDC5-Fab) on gold surfaces was developed [118]. CDC5-Fab forms a stable monolayer on gold after 15 min and presents a 2 months long-term stability, when stored at 4ºC with more than 90% of antigen recognition ability. By using amperometry to evaluate the ability of Fab modified electrodes to detect gliadin a limit of detection of 3.29 ng/mL was achieved. This Fab immunosensor has shown to be highly sensitive, rapid, simple and to have a short assay time.

Regarding clinical diagnosis of CD using EIs, some advances are also being achieved. Balkenhohl and Lisdat developed impedimetric immunosensors for the detection of antibodies directed against gliadin [119] and for the detection of autoantibodies against transglutaminase [120] in human serum. The immunosensors were based on the immobilization of gliadin and transglutaminase onto disposable screen-printed gold electrodes which were covered with a polyelectrolyte layer of poly (sodium-4-styrensulfonic acid). Although the results suggest a lower precision, as compared to ELISAs, an acceptable sensitivity was achieved, which makes the developed sensors reliable and promising methodologies for the analysis of anti-transglutaminase and anti-gliadins antibodies in human serum. On the other hand, Pividori and colleagues proposed an amperometric electrochemical immunosensor based on the physical adsorption of tTG from guinea pig liver on graphite–epoxy composite (GEC) electrodes [121]. For 10 positive and 10 negative processed serum samples a sensitivity of 70% and a specificity of 100% were achieved, as compared with the commercial ELISA method. The developed sensor appears as a promising alternative to the conventional ELISA assays, as it is a simple, low cost and point-of-care analytical method. The authors also proposed the evaluation of the benefits of transfer the developed methodology to disposable screen-printed electrodes.

Although the immunosensor technology seems promising, some limitations still remain, such as long-term stability, surface effects, and interferences resulting from complex sample matrices.

Conclusions

The knowledge of celiac disease has grown in the last two decades and there has been a sharp increase in the number of newly diagnosed individuals. The different clinical presentations of CD can complicate the diagnosis and, therefore, delay the treatment of the disease. The advances in the efficacy of serological antibody testing potentiate the possibility of future accurate screening programmes in the community, working as a first-line method to clarify clinically suspicious cases in an underdiagnosed stage and also to manage the follow-up of this multifactorial disease.

Patients with a low to moderate probability of presenting the disease should be submitted to blood studies rather than to small bowel biopsy. Several commercially available enzyme-linked immunoasorbent assay kits can be employed in CD screening. Among them, those using the serological markers IgA anti-tTG antibodies present the highest diagnostic accuracy. Although the overall diagnostic performance of the tests is good and similar for the different assays, greater standardization is required.

After a positive diagnosis, the implementation of a gluten-free diet is the only treatment available for CD. Two immunological methods for gluten food analysis are commercially available, and both use monoclonal antibodies toward gluten proteins. Further studies are necessary to develop an analytical method that can discriminate and quantify the celiac-toxic polypeptides in food ingredients and processed foods.

At the moment, ELISA assays are the recommended approach to the diagnosis of CD. Regarding gluten assessment in food products, ELISA R5 was provisionally endorsed by Codex Alimentarius, although there is not a standard methodology that receives universal agreement. There are several problems regarding the pretreatment of the food products which starts immediately with the extraction process.

The need for a standardized methodology to perform an unequivocal clinical diagnosis of CD as well as to determine quantitatively the gluten content in food products with the gluten-free label still remains.

Future perspectives

Possible areas of future study may be directed toward new immunosensing strategies that combine the high specificity of traditional immunochemical methods with miniaturized systems that allow development of a point-of-care test for CD clinical

diagnosis and gluten-free food quality control. Nevertheless, the reference methods need to present better international agreements, in order to achieve a higher standardization for the different immunoassays.

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Chapter 2

New trends: nano, disposable and electrochemical

Nanostructured screen-printed electrochemical biosensors for clinical applications

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Nanostructured screen-printed electrochemical biosensors for clinical applications

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Abstract

Point-of-care testing is used to rapidly provide reliable results in an easy manner, and is therefore one of the current tendencies in research and development in analytical chemistry. Biosensors are excellent alternatives to perform decentralized analytical operations. Among the different transducers of these sensors, electrochemical transducers stand out due to their attractive features such as high sensitivity and selectivity, rapid response and low cost. The development of disposable screen-printed electrodes was an important contribution to the electrochemical biosensor (EB) field due to their electrical properties and small size, which allows the reduction of the electrochemical instrumentation down to small pocket-size devices, making them applicable for both personal and professional use. In recent years, the integration of nanomaterials on the electrode surface has been gaining relevance since it improves the transducer's sensing platform, promoting aspects such as the increase of the electroactive area, the improval of the electron transfer, the enhancement of biocompatibility, and the maintenance of the biological element's activity. This review presents an overview of the progress in the field of EBs that are based on nanostructured screen-printed electrode transducer surfaces and their application in clinical analysis.

Keywords:

Electrochemical Biosensors. Screen-printed electrodes. Nanomaterials. Point-of-care testing.

Abbreviations

AChE	Acetylcholinesterase
AgNPs	Silver nanoparticles
ASV	Anodic stripping voltammetry
AuNPs	Gold nanoparticles
BRAC1	Breast cancer 1
BSA	Bovine serum albumin
CA	Cancer antigen
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
ChOx	Cholesterol oxidase
ChE	Cholesterol esterase
CNF	Carbon nanofiber
CNP	Carbon nanoparticle
CnT I	Cardiac troponin I
CNT	Carbon nanotube
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
EB	Electrochemical biosensors
EIS	Impedance spectroscopy
Fe_3O_4 NPs	Iron oxide
GOx	Glucose oxidase
GRF	Graphene
hCG	Human chorionic gonadotropin
IL-6	Interleukin-6
MWCNTS	Multiwalled carbon nanotubes
PfHRP-2	Plasmodium falciparum histidine-rich protein 2
POC	Point-of-care
PSA	Prostate specific antigen
QDs	Quantum dots
RIgG	Rabitt imunoglobulin G
SAMs	Self-assembled monolayers
Si	Silica nanoparticles
SPE	Screen-printed electrodes
SWCNTs	Single-walled carbon nanotubes
SWV	Square wave voltammetry
tTG	Tissue transglutaminase

1. Introduction

Nowadays, an evolution of analytical (bio)chemistry towards simpler, faster and *in situ* analytical performances is observed. Since the 1990s, patient self-testing has grown in popularity and there are commercially available test kits for pregnancy, blood pressure, drugs of abuse, blood glucose, cholesterol, among others [1]. The need to save time and money, gaining, simultaneously, in efficacy, implies the decentralization of analytical operations to a point-of-care (POC) system platforms [2, 3]. Warsinke [1] proposed an algorithm to simplify the process of clinical testing through POC tests (Fig. 1).

Moreover, POC testing can also be very useful in resource-limited settings [4]. For this purpose, electrochemical biosensors (EBs) are becoming exiting alternatives to the centralized clinical setting diagnostic techniques. EBs present advantages such as short analysis times, low assay costs and real-time measurements. With regard to quality and cost, EBs can be a better option than standard analytical methods. For the design of an EB, before the immobilisation of the capture element onto the electrode surface, the choice of the base electrode is a crucial step [5]. A wide variety of electrode materials have been used as transducers to fabricate EBs, including platinum [6], gold [7, 8, 9], and distinct forms of carbon [10], such as carbon paste [11], glassy carbon [12, 13], carbon fiber [14], epoxy graphite [15], and graphene [16]. Recently, several EBs have been developed on disposable and small-size screen-printed electrodes (SPEs) [17,18]. Bearing in mind that the achievement of high sensitivity requires innovative approaches that couple different amplification platforms [19], the modification of transducer surfaces with carbon, metal, and gold nanoparticles is being subject of intense investigation. One of the new trends is the use of hybrid nanomaterials as last generation transducers for the development of electrochemical biosensors. This article intends to give a wide perspective of advances in the last years regarding the employment of nanostructured screen-printed electrodes as transducer surfaces in the construction of electrochemical biosensors for clinical diagnosis.

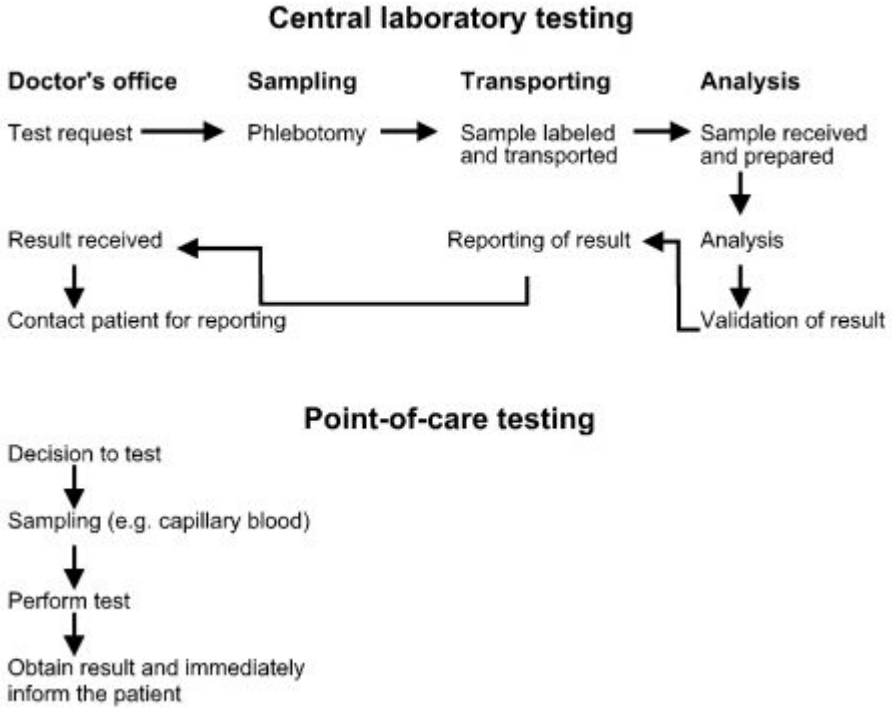

Figure 1. Proposed algorithm of the process of clinical testing using central laboratory versus decentralized POC testing (from [1]).

2. Screen-printing technology

The main disadvantage of EBs based on conventional electrodes, such as carbon paste and glassy carbon, among others, is the regeneration of their surfaces. To overcome this problem, in recent years the application of disposable SPEs has been gaining special attention for use as electrochemical transducers [20]. The screen-printing technology, adapted from the microelectronics industry, offers high-volume production of extremely inexpensive, and yet, highly reproducible and reliable single-use sensors; a technique which holds great promise for on-site monitoring [17]. There are many commercial sources of SPEs in different configurations (e.g. Pine Research Instrumentation, http://www.pineinst.com/echem; PalmSens Electrochemical Sensor Interface, http://www.palmsens.com; BioSens Technology, http://www.rusens.ru; DropSens, www.dropsens.com) [18]. These disposable robust solid strip electrodes have successfully been employed in the development of analytical methodologies that respond to the

growing need to perform rapid *in situ* analyses [17]. Other important features of these electrodes are related to the miniaturisation of the measuring device, their ease of handling, and their manipulation in a disposable manner. The construction of SPEs includes a series of basic stages, namely: selection of the screen, selection and preparation of the inks, selection of the substrate, and the printing, drying and curing steps [17]. Like this, several SPE configurations based on different materials can be made. In summary, the design and production process of SPEs consists in sequential layer-by-layer deposition of an ink onto an insulating support or substrate, which will define the geometry of the sensors [21, 22, 23]. The substrate is commonly composed of alumina, ceramics, PVC, gold, iron, etc and the conducting path of the electrode are made of carbon ink/paste, or platinum, gold or other metal pastes. SPEs can also contain two (working and reference) or three electrodes (working, reference and auxiliary), designated first-generation and secondary generation SPEs, respectively. The sensing element is the analyte-specific part of the biosensors. Enzymes, microorganisms, antibodies, nucleic acids and receptors have commonly been employed in the construction of screen-printed biosensors. They can be immobilized onto the surface of the working electrode, employing a variety of immobilization strategies [18, 24, 25, 26]. The electrochemical instrumentation used with SPE biosensors has been reduced to small pocket-size devices which make them applicable for both personal and professional use. Thus, SPE-based biosensors seem well suited to complement, or replace, standard analytical methods in fields such as environmental analysis, food quality control and clinical diagnostics. In fact, SPEs have progressively been incorporated in routine analysis in the clinical and environmental field. The possibility of working with customized SPEs that can be produced because of their versatility of design is another interesting characteristic since it allows multiplex detection. The electrode mask can have multiple working electrodes, and therefore different analytes of interest can be determined. This is especially important for multiple analyses, saving time and money. In critical clinical situations this type of analysis has an even higher importance because it can discard different pathologies and conduct the patient to the correct treatment.

3. Nanomaterial-based electrochemical transducers

The emergence of nanotechnology is opening new horizons for the application of nanomaterials in biosensors and bioassays. These nanomaterials are of considerable interest due to their unique physical and chemical properties, which offer excellent prospects for chemical and biological sensing. Nanotechnology has therefore been applied to improve the performance of biosensors and in recent years great attention has been

given to nanostructured materials of different chemical composition, produced as nanoparticles, nanowires or nanotubes [27]. Bearing in mind that the electrode surface simultaneously works as the support for the immobilization of the recognition element and as the sensing surface, SPEs are being modified with different nanomaterials in order to improve their electrochemical behavior [28, 29, 30]. The modification of the electrode surface with nanomaterials provides an additional sensing platform that minimizes problems which may occur during the biosensor's construction, such as: the poisoning of the electrode surface due to non-specific adsorption, irreproducibility due the washing steps, and incorrect orientation of the capture element's/analyte's free-binding sites.

Different strategies were developed for efficient deposition of nanomaterials and nanoparticles onto solid phase transducers as well as for the immobilization of the biological elements on their surfaces. The manner in which the nanomaterials or the recognition element are immobilized on the solid phase is a critical aspect in the biosensor's architecture and requires special consideration. The most widely used strategies are: physical adsorption, covalent binding, entrapment (including electropolymerisation), cross-linking and affinity binding [18, 22, 26, 31]. Electrochemical deposition approaches are often reported as methods of choice for the simple low-cost nanostructuring and nanopatterning of electrode surfaces.

The chosen method should provide an excellent patterning and immobilization of the chosen nanomaterial on the electrode surface as well as a good orientation of the recognition element with minimal steric hindrance to interact favorably with the analyte.

4. Clinical applications of screen-printed-based transducers surfaces modified with nanomaterials

In clinical analysis, SPEs present added advantages. Among them, the employment of small sample volumes and the possibility of developing disposable biosensors due to their low cost can be cited. Besides avoiding the tedious and time-consuming electrode surface cleaning steps, SPEs enable the use of low reaction volumes because of their reduced dimensions, which reduces reagent consumption. Moreover, the decrease in the diffusion distances for the analytes to reach their surface-bound receptor partners allows shorter incubation periods and, thus, faster assays [32]. Enzymatic sensors, genosensors and immunosensors can be manufactured by modifying screen-printed electrode surfaces with nano-scale materials.

4.1. Carbon nanomaterial-based biosensing

Carbon materials such as graphene (GRF), carbon nanotubes (CNTs), and carbon nanofibers (CNFs) [10, 27, 33, 34] are widely exploited for the construction of electrochemical transducers. GRF is a flat monolayer of carbon atoms tightly packed into a two-dimensional (2D) honeycomb lattice, and is a basic building block for graphite materials of all other dimensions. It can be wrapped up into 0D fullerenes, rolled into 1D nanotubes or stacked into 3D graphite [35]. GRF fascinated many researchers as it provides a large detection area, biocompatibility, and unique electronic properties such as ultra-high mobility and an ambipolar field-effect with much lower synthesis costs [35].

CNTs consist of a graphite sheet rolled up into a nanoscale-tube, with a diameter of 1–2 nm in case of single-wall carbon nanotubes (SWCNTs), or can be concentric, closed graphite tubules with diameters of 2–50 nm and an interlayer distance approximately 0.34 nm, which are multi-walled CNTs (MWCNTs) [10, 27, 34].

CNFs are cylindrical nanostructures with graphene layers arranged as stacked cones, cups or plates [36]. They have lengths in the order of μm, while their diameter varies between some tens to several hundred nm [34].

Fullerene (C_{60}) is another carbon-based material that is characterized by having multiple redox states in a wide range of potentials and being stable in many redox forms [37], therefore it has been reported that it plays an important role in electron transfer. Fullerene consists of a spherical molecule that has a truncated icosahedron shape that resembles a cage-like fused-ring structure.

These carbon nanomaterials present excellent electrochemical properties and their application as sensing phases contributes to the increase of the electroactive area and the electron transfer rate. However, carbon-based materials lack bioreactivity and therefore some methods have been employed to solve this limitation. For instance, chemical, electrochemical, and thermal treatments are applied to generate functional groups on the material's surface which allows covalent binding of biomolecules. Moreover, covalent binding of biomolecules employing cross-linking agents or affinity binding through the biotin-avidin/streptavidin interaction can also be employed [34]. Fisioadsorption of biomolecules onto the carbon nanomaterial's surface or direct entrapment in carbon ink are also options.

Several studies regarding the use of SPEs modified with carbon-based materials, either CNTs or graphene, have been reported. Recently, a novel screen-printed electrode, based on low-cost vegetable parchment technology, was developed and employed graphene nanosheets as the sensing phase for the determination of prostate specific antigen (PSA) in human serum samples [38]. In this study, amine-modified graphene oxide (GRF-NH_2)

was physically adsorbed on the electrode surface providing an excellent coating platform for the immobilization of the capture antibodies through glutaraldehyde cross-linking. Using linear sweep voltammetry the proposed "sandwich-type" immunoassay showed a wide linear concentration range (over 6 orders of magnitude) with a minimum value of 2 pg mL^{-1}. In Fig. 2 a squeme of the described immunosensor is presented. A genosensor for the impedimetric detection of DNA polymorphism correlated to cystic fibrosis was proposed by Bonanni et al. [39]. In this case, screen-printed carbon electrodes (SPCE) containing carboxyl functionalized MWCNTs were used for the covalent immobilization of an amino-modified oligonucleotide probe, complementary to the cystic fibrosis (CF) mutant gene. A non-complementary DNA sequence and a three-mismatch sequence corresponding to the wild DNA gene (present in healthy people) were used as negative controls. The developed protocol allowed the very sensitive detection of the triple base deletion in a label-free CF-related DNA sequence, achieving a limit of detection (LOD) of around 100 pM.

In table 1 a summary of other carbon nanomaterial-based SPE transducers is presented. Sensors for the detection of PSA [38], IgG immunoglobulins [40, 44], human chronic gonadotropin [41], anti-carcinoembryonic antigen (CEA) [42, 43], thrombin [47, 49] and to assay DNA hybridization [45, 46, 48, 50] were developed. Several enzymatic sensors were also reported [51, 52, 53].

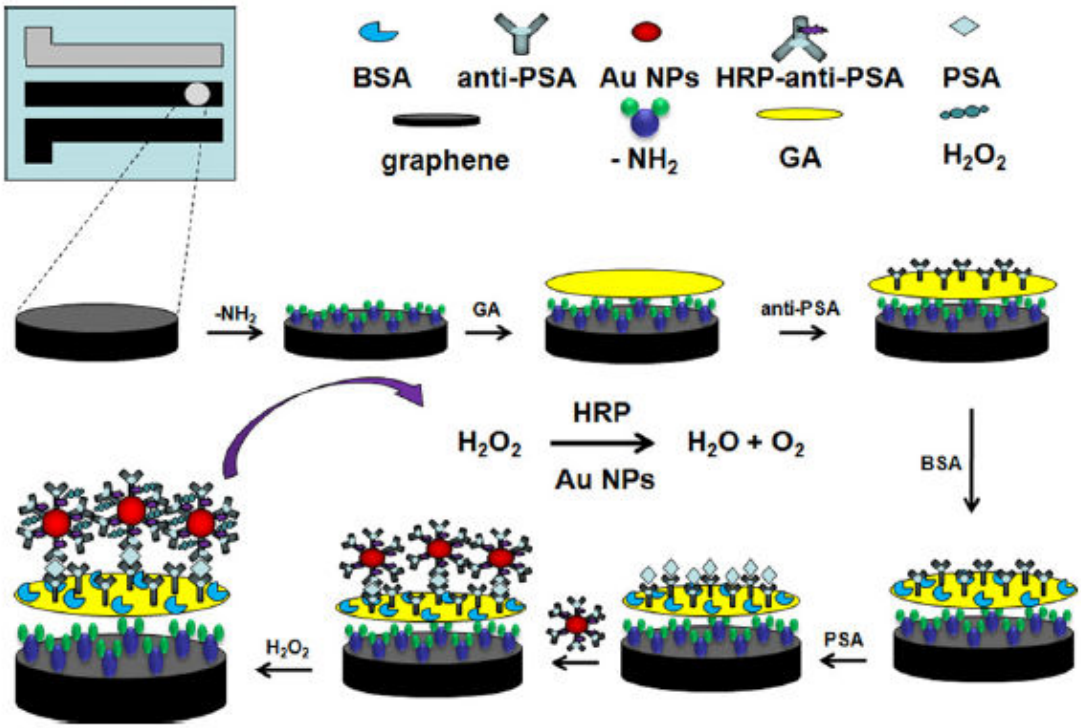

Figure 2. Schematic representation of the immunosensing strategy followed by Yan et al. to dectect PSA using a disposable graphene nanosheets-modified SPE (from [38]).

Table 1. Analytical characteristics of clinical biosensors based on SPE modified with carbon nanomaterials as transducer surface

Screen-printed electrode	Nanomaterial	Nanomaterial immobilization procedure	Recognition element	Recognition element immobilization technique	Analyte	Analysed sample	Electrochemical technique	Limit of detection	REF*
IMMUNOSENSORS									
Graphite	MWCNTs	Entrapment	Anti-RIgG antibodies	Phase inversion	RIgG	-	Amperometry	1.66 μg ml^{-1}	40
Graphite	MWCNTs	Entrapment	Anti-hCG antibodies	Phase inversion	hCG	-	Amperometry	14.6 mIU mL^{-1}	41
Graphite	CNP	Entrapment	Anti-CEA antibodies	Covalente attachment	CEA	Urine	ASV	32 pg mL^{-1}	40
Graphite	MWCNT	Entrapment	Anti-CEA antibodies	Covalence attachment	CEA	Saliva and serum	SWV	1×10^{-12} g mL^{-1}	43
Carbon	Graphene	Fisioadsorption	Anti-PSA antibody	Cross-linking	PSA	Serum samples	CV	0.46 pg mL^{-1}	38
Graphite	CNTs	Entrapment	Anti- human IgG	Fisioadsorption	Human IgG	Human serum samples	DPV	0.06 pg mL^{-1}	44
GENOSENSORS									
Carbon	Fullerene (C_{60})	Entrapment	*E. coli* 16S rDNA probe	Fisioadsorption	*E. coli* 16S rDNA target	-	DPV	-	45
Graphite	MWCNTs	Fisioadsorption	DNA probe	Fisioadsorption	DNA target	-	EIS	22 fmol	46
Carbon	MWCNTs	Fisioadsorption	Oligonucleotide probe complementary to the CF mutant gene	Covalence attachment	CF mutant gene	-	EIS	100 pM	39
Carbon	MWCNTs	Fisioadsorption	Thrombin aptamer	Covalence attachment	Human alpha thrombin	Human serum spiked with human alpha thrombin	EIS	10^{5} pM	47
Graphite	SWCNTs	Fisioadsorption	(BRCA1) DNA probe	Fisioadsorption	(BRCA1) DNA target	-	DPV	378.52 nM	48
Graphite	Graphene	Fisiadsorption	Thrombin aptamer	Fisiasorption	Thrombin	-	EIS	-	49
Graphite	MWCNTs	Fisiadsorption	DNA aptamer	Covalence attachment	Lysozyme	-	EIS	12.09 lg ml^{-1}	50
ENZYMATIC SENSORS									
Carbon	MWCNTs	Fisioadsorption	Lactate dehydrogenase	Cross-linking	Lactate	Blood samples	Amperometry	7.5×10^{-6} mol L^{-1}	51
Rhodium–graphite	MWCNTs	Fisioadsorption	CytochromeP450scc (CYP11A1)	Fisioadsorption	Cholesterol	-	Amperometry	-	52
Carbon	MWCNTs	Cross-linking	Tyrosinase	Fisioadsorption	Methimazole	Pharmaceuticals	CV	0.056 μM	53

ASV (Anodic stripping voltammetry); BRCA1 (breast cancer 1); CEA (carcinoembryonic antigen); CF (Cystic fibrosis); CNP (carbon nanoparticle); CV (Cycliv voltammetry); Differential pulse voltammetry (DPV); GRF (graphene); hCG (human chorionic gonadotropin); Eis (impedance spectroscopy); MWCNTS (multiwalled carbon nanotubes); PSA (prostate specific antigen); RIgG (rabitt imunoglobulin G); SWCNTS (singlewalled carbon nanotubes); SWV (Square wave voltammetry).
*References.

4.2. Metal nanomaterial-based biosensing

Besides carbon nanomaterials, noble metal nanoparticles (NPs) are receiving a high share of attention since they allow the immobilization of biomolecules, retaining their biological activity, and the formation of an efficient conducting interface with electrocatalytic properties [54]. Chemical and physical methods have been employed to synthesize noble metal NPs in order to obtain a good level of homogeneity and to provide fine control over size, shape and surface properties, to better take advantage of their unique physicochemical properties for biosensing [55]. Metal nanoparticles are also widely used as labels, however this review focuses on their role in transducer surface modification.

Gold is the most commonly employed metal in the construction of electrochemical biosensors. As electrode material, gold is not as good as carbon but its chemical reactivity still remains. An especially interesting reaction occurs between gold and thiols (RSH) and disulphides (RSSR'). In this reaction the homolytic rupture of S-H or S-S bonds implies the formation of self-assembled monolayers (SAMs). Depending on the thiols or disulphides, gold can be modified simply for possessing different functionalities, making it possible to achieve a high degree of surface functionality. This extends the possibilities for making other modifications by means of common chemical reactions. Moreover, cysteine and cystine residues from proteic material allow direct chemisorption to gold surfaces. This is of tremendous interest in the construction of biosensors. Due to the spontaneity of these reactions, the trend in system development is directed towards the increase in the number of interactions between gold and proteic material. Therefore, apart from the chemical reactivity, the gold surface's structure is very important. The use of continuous gold surfaces has the inconvenience of promoting a high number of interactions, which can imply a less selective orientation as well as drastic changes in the structure of the chemisorped proteic material. As a consequence, the biological activity of the recognition element can be altered and, in turn, the efficacy of the immunosensors is low. A solution to this problem is the utilization of gold nanostructures because they possess the same chemical reactivity but are more biocompatible [9, 56]. Therefore, gold NPs (NPAus) provide stable and high surface-to-volume ratio surfaces for the immobilization of biomolecules while retaining their biological activity. Due to their high surface energy, their ability to decrease the distance between proteins and metal particles, and their ability to act as an electron-conducting pathway between prosthetic groups and the electrode surface, may facilitate electron transfer between redox proteins and the electrode surface [9]. Moreover they are cost-effective and easy to prepare. Gold nanoparticle-modified electrode surfaces can be prepared in three ways: (a) binding of NPAus with functional

groups of self-assembled monolayers (SAMs); (b) direct deposition of NPAus on the bulk electrode surface; (c) incorporating colloidal gold into the electrode by mixing the gold with the other components in the composite electrode matrix [9]. Silver NPs (NPAgs) have recently become one of the most popular materials to fabricate sensors for the detection of a variety of compounds as they are capable of facilitating the electron transfer of some biological molecules [54]. Synthesis of metallic NPAgs through reduction of silver ions with different reagents [33], UV or electron beam irradiation [33], or electrochemical methods [35–40] are some of the methods described in the literature. The electrochemical deposition of silver onto solid surfaces is probably the easiest and fastest option.

Wei Wu et al. [57] developed a novel transducer surface based on a nanosilver-doped DNA polyion complex membrane (PIC).This was achieved by the modification of a screen-printed carbon electrode (SPCE) using Nafion-entraped double-stranded DNA molecules. The double-stranded DNA provides a three-dimensional network that adsorbs the silver ions, providing a facile pathway for the formation of NPAgs through the reduction of the silver ions using $NaBH_4$. CEA antibodies bind to the surface of the NPAgs through their amino acids that form complexes with the metal. The developed immunosensor was used to analyze CEA in 50 real samples and the results were in good agreement with the results provided by a commercial enzyme-linked immunosorbent assay (ELISA). The authors concluded that the PIC membrane enhanced the sensitivity of the assay.

Moreno et al. [58] proposed the immobilization of oligonucleotide-functionalized gold nanoparticle probes (affinity modules) by electrodeposition on arrayed screen-printed gold electrodes (Fig. 3). The final hybridization reaction of complementary oligonucleotides and PCR products was carried out with amperometric detection using horseradish peroxidase (HRP) as an enzymatic reaction. The authors pointed out that the proposed hybridization strategy can be applied to many other kinds of molecules such as aptamers, enzymes or antibodies. Chikae et al. [59] exploited saccharide–protein interactions in order to detect Alzheimer's amyloid-beta (Aβ) peptides. An SPE modified with (electrodeposited) NPAus coated with an acetylenyl-terminated SAM was used as the sensing phase. The saccharide immobilized in the SAM monolayer captured Aβ peptides and the oxidation peak current response of the tyrosine (Tyr) residue of Aβ was detected by differential pulse voltammetry.

Although NPAus are the most frequently reported metal nanoparticles for surface enhancement, Fe_3O_4 [69, 72] nanoparticles were also reported as biocompatible metal nanomaterials for modification of SPEs.

In table 2 other biosensors for clinical applications based on gold nanostructured [60, 61, 62, 64, 65, 66, 67, 68] and silver [57, 70, 71] nanoparticles, using SPEs as transducer surfaces are presented.

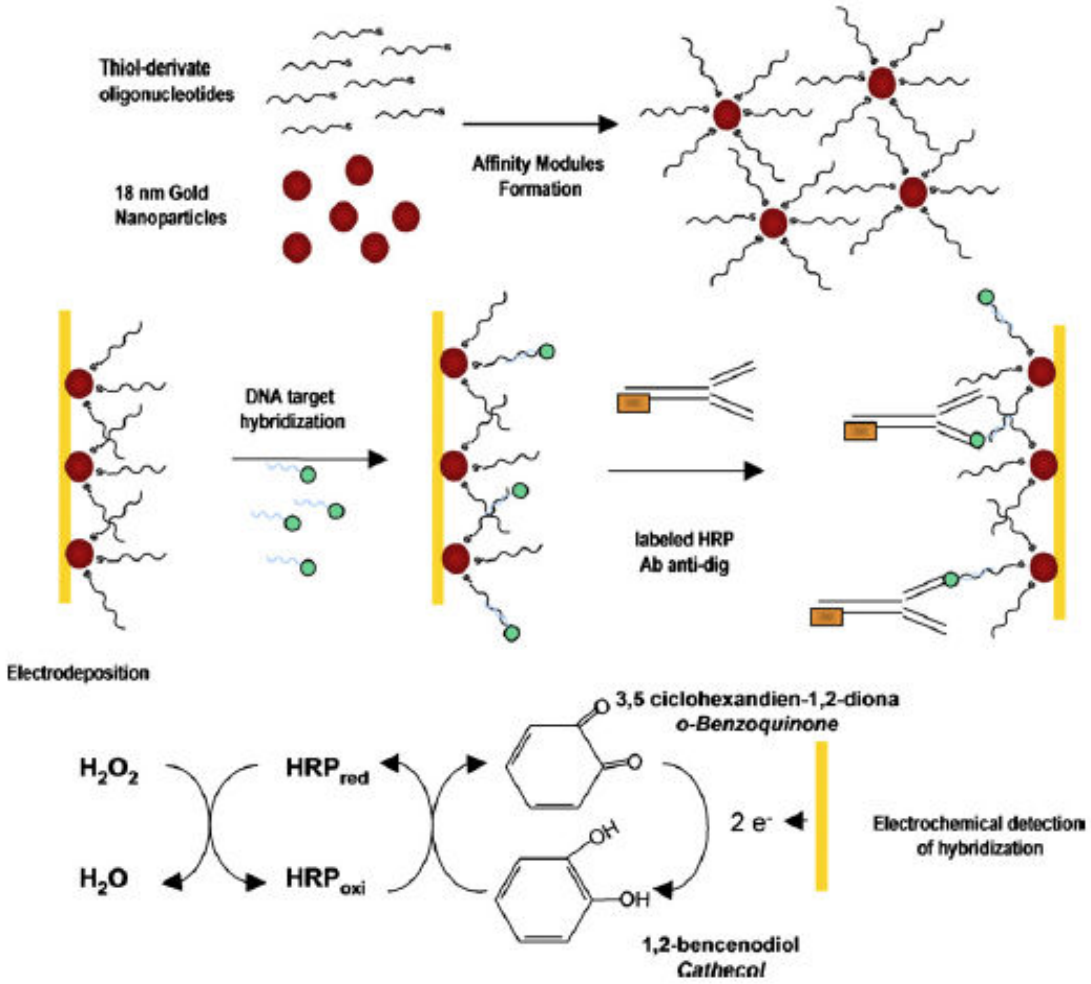

Figure 3. Illustration of a proof of concept for the selective immobilization of affinity modules of olignucelotides functionalized with gold nanoparticles on SPEs, as proposed by [58].

Table 2. Analytical characteristics of clinical biosensors based on SPE modified with metal nanoparticles as transducer surface

Screen-printed electrode	Nanomaterial	Nanomaterial immobilization procedure	Capture element	Capture element immobilization technique	Analyte	Analysed sample	Electrochemical technique	Limit of detection	REF*
IMMUNOSENSORS									
Graphite	Colloidal gold	Entrapment	IL-6 antibody	Entrapment	IL-6	-	DPV/ EIS	1.0 ng L^{-1}	60
Carbon	AuNPs	Electrochemical deposition	PSA 8A6 antibody PSA 5G6 antibody	Fisioadsorption	fPSA and tPSA	Cell cultures of prostate tumor cells	CV	1 ng mL^{-1}	61
Carbon	AuNPs	Entrapment	Anti-myoglobin	Fisioadsorption	Myoglobin	Human plasma	CV	10 ng ml^{-1}	62
Carbon	AgNPs	Chemical reduction	Anti-CEA	Fisioadsorption	CEA	Serum samples	DPV	10 pgmL^{-1}	57
Carbon	Colloidal gold	Electrochemical deposition	Anti-CnT I antibodies	Fisioadsorption	CnT I	-	EIS	0.2 ng mL^{-1}	63
GENOSENSORS									
Carbon	AuNPS	Electrochemical deposition	Sars DNA probe	Streptavidin/Biotin	Sars DNA target	-	CV	2.5 pmol L^{-1}	64
Graphite	AuNPs	Entrapment	Thrombin aptamer	Covalence attachment	Thrombin	-	Cv/ SWV	10-9 M	65
Gold	AuNPs	Electrochemical deposition	DNA probe	Electrodeposition of affinity modules	DNA target	-	Amperometry	-	58
Carbon	AuNPs	Electrochemical deposition	Four *pneumoniae* probes	Affinity (streptavidin–biotin interaction)	Four *pneumoniae* targets	-	CV	3-8 pM	66
Gold	AuNPs	Electrochemical deposition of affinity modules	LiKMP-11 aptamer	Electrodeposition	LiKMP-11	-	Amperometry	0.025 mg mL^{-1}	67
Carbon	NPAus	-	RT-specific peptide	Cross link	HIV-1 RT	Human serum	SWV	0.8 pg mL^{-1}	60
Graphite	Fe_3O_4 NPs	Entrapment	DNA probe	Covalence attachment though SAMs	HIV-1 sequences tare7get	-	EIS/ SWV	50 pM	69
ENZYMATIC SENSORS									
Carbon	AgNPs	Electrochemical deposition	-	-	Lamotrigine	-	DPV	3.72×10^{-7} M	70
Carbon	AuNPs	Electrochemical deposition	Saccharide	Covalence attachment	Amyloid-beta peptide	-	DPV	-	59
Carbon	AgNPs	Entrapment	GOx	Entrapment	Blood glucose	Rabbit serum	Amperometry	-	71
Carbon	Fe_3O_4 NPs	Entrapment	ChOx; ChE	-	Cholesterol	-	Chronoamperometry	-	72

ASV (Anodic stripping voltammetry); AgNPs (silver nanoparticles); AuNPs (gold nanoparticles) CnT I (cardiac troponin I); CEA (carcinoembryonic antigen); CF (Cystic fibrosis); CV (Cyclic Voltammetry); Differential pulse voltammetry (DPV); GOx (Glucose Oxidase); hCG (human chorionic gonadotropin); EIS (Impedance spectroscopy); Interleukin-6 (IL-6); MWCNTS (multiwalled carbon nanotubes); PSA (prostate specific antigen); RT (reverse transcriptase); SARS (Severe Acute Respiratory Syndrome); SAMs (self-assembled monolayers); SWV (Square wave voltammetry).

*References.

4.3. Hybrid nanomaterial-based biosensing

The use of hybrid materials as last generation transducers is generating great expectative for their introduction in the development of electrochemical transducers for biosensors. This hybrid conjugation can consist of carbon and gold nanoparticles or of a bimetallic system. Like was referred before, carbon nanomaterials have exceptional electrochemical characteristics but lack some reactivity. This problem can be surpassed by combining them with metal-based nanomaterials [73, 74]. Hybrid surfaces lead to a synergic effect on each material property presenting collective characteristics that are drastically different from the individual components [75]. Thus, screen-printed electrodes have been modified with different hybrid nanomaterial conjugates. However, despite good characteristics such as sensitivity and reproducibility of these nanohybrid modified screen-printed electrodes, their use is still reduced.

Recently, a disposable amperometric biosensor (Fig. 4) was developed for the detection of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP-2) in the sera of humans with P. falciparum malaria [76]. For this purpose, disposable SPEs were modified with MWCNTs and NPAus. Bare SPEs, MWCNT-modified SPEs, and NPAu- and MWCNT-modified SPEs (Nano-Au/MWCNT/SPEs) for the amperometric detection of PfHRP-2 were compared, and Nano-Au/MWCNT/SPEs yielded the highest immunosensing performance among the tested electrodes, with a detection limit of 8 ng/ml. The sensor was compared with a commercial ELISA kit and showed to be more sensitive and specific. These kind of cost-effective and portable diagnosis methods assume even more importance in regions with severe economic limitations. An electrochemical transducer surface nanostructured with a hybrid conjugation of carbon nanotubes and gold nanoparticles for the simultaneous detection of IgA and IgG anti-transglutaminase (tTG) and anti-gliadin antibodies was recently reported by our team [77]. The respective antigens (i.e. tTG and gliadin) were successfully immobilized on a nanohybrid screen-printed carbon electrode surface and 10 different human sera were assayed. A good correlation between the results and the results from a commercial ELISA kit was obtained, indicating that the dual-electrochemical immunosensor is a trustful analytical screening tool.

Table 3 summarizes the principal analytical characteristics of recently developed biosensors based on hybrid-SPE surfaces.

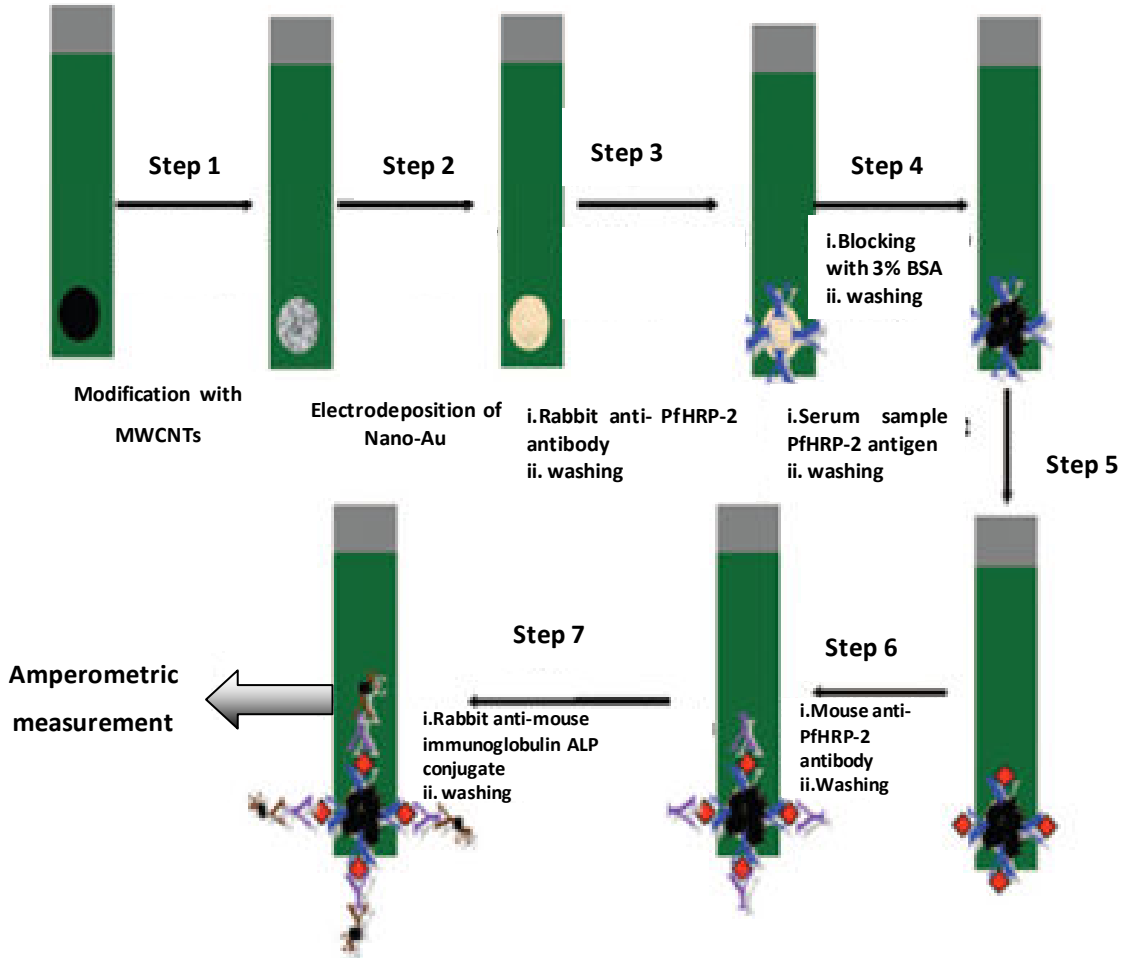

Figure 4. Schematic representation of the fabrication and the assay procedure of the immunosensor based on nano-hybrid modified SPE for PfHRP-2 antigen (red diamonds) detection. BSA, bovine serum albumin (adapted from [76]).

5. Conclusions and Future Prospects

The use of thick-film technology for the production of screen-printed electrodes seems to be one of the most promising methods for the development of electrochemical biosensors. Advantages such as miniaturization, mass production, customization, portability and low cost stand out with these electrodes, placing SPEs as the ultimate and versatile electrodic surface for analytical sensing operations. The advent of interesting nanomaterials allows the modification of SPE surfaces which improves electrochemical and biochemical features of the sensing platform. Disposable, sensitive, specific and fast nanostructured-based SPE surfaces are gaining special attention in the clinical analysis field. The relevance of EBs for clinical diagnosis will increase even more as the necessity to save money raises as well as the growing need for decentralized clinical applications. Moreover, the greatest impact of EBs will be felt at point-of-care testing locations without laboratory support, either regarding electric equipment or concerning sampling procedure. Nonetheless, relevant and not yet totally controlled aspects such as the storage and stability of biological materials immobilized onto the electrode surface has to be improved. The philosophy of rapid *in-situ* tests requires patient-friendly sampling techniques. The employment of small volumes of blood or other matrices (e.g. saliva, sweat, tears, etc.) are good alternatives. The continuous miniaturization of this technology into reliable, robust and easy-to-use instrumentation is a key to the success of the use of EBs in point-of-care testing.

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Table 3. Analytical characteristics of clinical biosensors based on SPE modified with hybrid nanomaterials as transducer surface

Screen-printed electrode	Nanomaterial	Nanomaterial immobilization procedure	Capture element	Capture element immobilization technique	Analyte	Analysed sample	Electrochemical technique	Limit of detection	REF*
IMMUNOSENSORS									
Carbon	MWCNTs NPAUs	Fisioadsorption Electrochemical deposition	Anti- PfHRP-2 antibody	Fisioadsorption	PfHRP-2	Human serum	Amperometry	8 ng ml^{-1}	76
Carbon	MWCNTs AuNPs	Entrapment Entrapment	Anti- AChE antibody	SAM	AChE	Human red blood cells	CV SWV	0.05 nM.	78
Carbon	MWCNTs AUNPs	Fisioadsorption Electrochemical deposition	tTG	Fisioadsorption	Anti-tTG	Human serum	CV	n/a	79
Carbon	MWCNTs NPAUs	Fisioadsorption Electrochemical deposition	Gliadin	Fisioadsorption	Anti-gliaidin IgA Anti-gliadin IgG	Human serum	CV	9.1 U mL^{-1} 9.0 U mL^{-1}	80
C arbon	MWCNTs AUNPs	Fisioadsorption Electrochemical deposition	tTG Gliadin	Fisioadsorption	Anti-tTG IgA Anti-tTG IgG Anti-gliadin IgA Anti-gliadin IgG	Human serum	CV	2.45 U mL^{-1} 2.95 U mL^{-1} 3.16 U mL^{-1} 2.82 U mL^{-1}	77
Carbon	Graphene AuNPs	Fisioadsorption Electrochemical deposition	Anti-CA CA 153 Anti-CA 125 Anti-CEA	Fisioadsorption	CA 153 CA125 CEA	Serum samples	ASV	1.5 x 10^{-3} UmL^{-1} 3.5 x 10^{-4} UmL^{-1} 1.2 x 10^{-3} UmL^{-1}	81
Graphite	MWCNTS Colloidal gold,	Entrapment with	Ricin antigen	Fisioadsorption	Ricin antibody	Serum samples	Amperometry	2.1 ng mL_1	82
Carbon	MWCNTs Graphene AuNPs	Entrapment Fisioadsorption Fisioadsorption	RaHIgG	Fisioadsorption	H IgG	Serum samples	DPV	44 pg/mL	83
GENOSENSORS									
Gold	QDs/Si	Fisioadsorption	Thrombin aptamers	Fisioadsorption	Thrombin	Human blood serum	DPV	0.1ngmL_1	84

AChE (Acetylcholinesterase); ASV (Anodic stripping voltammetry); AuNPs (gold nanoparticles); CA (cancer antigen); QDs/Si (quantum dots/ silica nanoparticles); CEA (carcinoembryonic antigen); CV (Cyclic Voltammetry); DPV (Differential pulse voltammetry); NPAus (gold nanoparticles); MWCNTS (multiwalled carbon nanotubes); PfHRP-2 (Plasmodium falciparum histidine-rich protein 2); PSA (prostate specific antigen); SAMs (self-assembled monolayers); SWV (Square wave voltammetry); tTG (tissue transglutaminase)

*References.

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II.

RESEARCH AND DEVELOPMENT

Chapter 3

Hybrid transducer surfaces

Nanohybrid Materials as Transducer Surfaces for Electrochemical Sensing Applications

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Nanohybrid Materials as Transducer Surfaces for Electrochemical Sensing Applications

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Abstract

A nanohybrid electrochemical transducer surface was developed using carbon and gold nanomaterials. The strategy relayed on casting multiwalled carbon nanotubes or carbon nanofibers onto a screen-printed carbon electrode surface, followed by in situ generation of gold nanoparticles by electrochemical deposition of ionic gold, in a reproducible manner. These transducers, so fabricated, were characterized using both electrochemical and microscopic techniques. Biofunctionality was evaluated using the streptavidin-biotin interaction system as the biological reaction model. These platforms allow to achieve low detection limits (in the order of pmoles), are reproducible and stable at least for a month after their preparation, being a perfect candidate to be used as transducer of different sensor devices.

Keywords: Carbon nanofibers, Carbon nanotubes, Electrochemical transducer surfaces, Gold nanoparticles, Hybrid materials

1. Introduction

Amplified transduction of biological events remains a major challenge to electrical bioassays [1]. The development of electrochemical transducers that make use of electrode surfaces modification with nanomaterials became an exciting area of development for modern analytical science. The use of nanomaterials extend the potentialities of those electrodes surfaces improving their original electrochemical properties providing a low-background current, high signal to noise ratio and fast electron transfer [2].

Carbon nanotubes (CNTs) and carbon nanofibers (CNFs) based electrochemical biosensors have been used in the development of new designs of electrochemical biosensors which have found widespread use in several analytical applications [3–10]. Most applications of these materials rely on the modification of the working electrodes [8]. An application involving carboxylated-multiwalled carbon nanotubes (MWCNTs) dissolved in a DMF:water mixture used to modify commercially available screen-printed electrodes (SPEs), with considerable advantages regarding the conventional SPEs, was also reported [11]. Carbon-nanostructures exhibit a unique combination of excellent mechanical, electrical and electrochemical properties, such as large length-to-diameter aspect ratios, good electrical conductivity and a wide potential range. However, these present some lack of reactivity which can eventually lead to a decrease in protein adsorption. To overcome this disadvantage the transducers surface can be simultaneously modified with gold nanoparticles (NPAus) which have high surface energies and are therefore quite reactive [12]. Several studies employing NPAus in electrochemical applications such as bioassays, biosensor, chemical sensor and electrocatalysis have also been reported [13–15]. There is a variety of methods for depositing NPAus onto different substrates [16–18]. For instance, Martínez-Paredes and colleagues [19] proposed an electrochemical method to generate gold nanostructured screen-printed carbon electrodes. The developed electrochemical surface was applied to the detection of lead underpotential deposition and to the construction of geno- [20] and immunosensors [21]. NPAus works as an excellent adsorbent support, solving carbon limitations and enhancing biocompatibility [14].

The achievement of high sensitivity requires innovative approaches that couple different amplifications platforms [22]. Hence, the metal nanoparticle/carbon nanostructure hybrid system allows a synergic effect on each material property since the hybrid system may have collective characteristics that are drastically different from individual component [23]. The physical deposition of gold on bulk carbon substrates is an attractive method for the preparation of nanostructured electrodes and some applications were already published [1, 24-27].

To study the advantages of the transducer surface nanostructuration, the interface between protein and the nanomaterials can be accomplished using the classic streptavidin-biotin system. Streptavidin coated-surfaces have been widely applied to solid-phase assays, mainly immune ones, due to the extraordinary properties of streptavidin-biotin interaction [28]. Streptavidin-biotin technology relies on the extremely tight and specific affinity between these two molecules associated with the exceptional stability of these proteins [29-30].

In this work we studied different methodologies for modify the screen-printed carbon electrodes (SPCEs) in a reproducible manner in order to obtain the best nanohybrid transducer surface. As far as we are concerned, there are no previous reports of SPCEs modification with nanohybrid materials and their application as electrochemical transducer surface.

Several nanostructured-based electrochemical transducers were studied. SPCEs surface was modified with carboxylated multiwalled carbon nanotubes (MWCNTs) or carboxyl-funcionalized carbon nanofibres (CNFs) and, moreover, with an hybrid conjugation of these carbon nanomaterials with gold nanoparticles (NPAus). The results of the modification were evaluated using the biological streptavidin-biotin complex. The transducer performance was optimized by effective blocking of non-specific binding (NSB) of the labels using bovine serum albumin (BSA) and casein. Alkaline phosphatase (AP) worked as the enzymatic label and a mixture of 3-indoxyl phosphate with silver ions (3-IP-Ag^{+}) as the substract. The analytical signal obtained resulted from the anodic redissolution by cyclic voltammetry of metallic silver generated enzymatically. The electrochemical behavior of these modified electrodes was carefully evaluated assessing aspects as detection limits, sensitivity, reproducibility, stability and biocompatibility. Chronocoulometry and scanning electron microscopy (SEM) were also employed in the characterization of the hybrid surface.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric experiments were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266MHz computer system and controlled by Autolab GPES 4.8 (software version for Windows 98). All the measurements were carried out at room temperature.

Single-use screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Oviedo, Spain). The DropSens electrodes incorporate a conventional three-electrode configuration, printed on ceramic substrates (3.4-1.0 cm). Both working (disk-shaped 4 mm diameter) and counter electrodes are made of carbon inks, whereas pseudoreference electrode and electric contacts are made of silver. An insulating layer was printed over the electrode system, leaving uncovered the electric contacts and a working area which constitutes the reservoir of the electrochemical cell, with an actual volume of 50 μL. The SPEs were easily connected to the potentiostat through a specific DropSens connector (Spain, ref. DSC).

A JEOL JSM-6100 scanning electron microscope (20 kV, Japan) was used to characterize the working electrodes. A pHmeter MicropH2001 (Crison Instruments S.A., Spain) and an ultrasonic bath Ultrasons (J.P. Selecta, Spain) were also employed.

2.2. Reagents and solutions

Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate, bovine serum albumin fraction V (BSA), β-Casein from bovine milk (casein), streptavidin (molecular weight, 66 000), biotin conjugated to alkaline phosphatase (B-AP; dimer, four units of B per molecule of AP, molecular weight, 160 000), were purchased from Sigma (Madrid, Spain). Standard gold (III) tetrachloro complex ($AuCl_4^-$), silver nitrate, hydrochloric acid fuming 37% and nitric acid (HNO_3) were obtained from Merck (Germany). Biosynth (Switzerland) supplied 3-indoxyl phosphate disodium salt (3-IP). Ultrapure water obtained with a Milli-RO 3 plus/Milli-Q plus 185 purification system from Millipore Ibérica S.A. (Madrid, Spain) was used throughout this work. Carboxyl modified multiwalled carbon nanotubes (MWCNTs) were purchased from Nanocyl (ref. 3151) and carboxyl modified carbon nanofibers (CNFs) were kindly provided by the company “Grupo Antolín” (Valencia, Spain).

Working solutions of streptavidin were made in 0.1 M Tris-HNO_3 pH 7.2 buffer (buffer 1). Working solutions of B-AP were prepared in 0.1 M Tris-HNO_3 pH 7.2 containing 2 mM $Mg(NO_3)_2$ (buffer 2). A mixture solution of 1.0 mM 3-IP and 0.4 mM silver nitrate were

prepared daily in 0.1 M Tris-HNO_3 pH 9.8 and 20 mM $Mg(NO_3)_2$ (buffer 3) and stored in opaque tubes at 4 °C. BSA and casein lyophilized powder was reconstituted in buffer 1.
The MWCNTs solutions were prepared by dilution with a mixture of DMF:water (1:1) as it was reported in a previous work [11]. CNFs solutions were made in water. The solutions were prepared in water using an ultrasonic bath for 30 minutes. Gold tetrachloroaurate dilutions were prepared in 0.1 M HCl.

All chemicals employed were of analytical reagent grade.

2.3. Analytical Procedure

2.3.1. Screen-printed Carbon Electrodes (SPCEs) Nanostructuration with Carbon Nanomaterials

The modification of the SPCEs was carried out by depositing 4μL or 10 μL of the MWCNTs or CNFs dispersion, respectively, on the working electrode surface. The solution was left to dry at room temperature until its absolute evaporation. Finally, modified SPCEs were carefully washed with water and dry at room temperature.

2.3.2. Generation of the Hybrid Surface between Carbon Nanomaterials and Gold Nanoparticles (NPAus)

Gold nanostructures were generated in situ over SPCE-MWCNTs and SPCE-CNFs (SPCE-MWCNT-NPAus and SPCE-CNF-NPAus, respectively) applying a constant current intensity for a certain period of time in an acidic solution of $AuCl_4^-$. After that, and in the same medium, a potential of + 0.1 V was applied during 2 minutes, in order to desorb hydrogen. Finally, the gold nanostructures were generously rinsed with water.

2.3.3. Evaluation of the Nanostructured SPCEs using the Streptavidin-Biotin Reaction

A drop of 10 μL of streptavidin solution with an adequate concentration was placed on the pretreated surface of the SPCE and left there for a fixed time at adequate temperature. Then, the electrode was washed with buffer 1 to remove the excess of protein. Free surface sites were blocked with an adequate concentration of BSA or casein by placing a drop of 40 μL solution for 30 min. Thus, after another washing step with buffer 2, an aliquot of 40 μL of B-AP ($1x10^{-10}$ M) was dropped on the streptavidin modified electrode for an hour reaction. After a washing step with buffer 3, the enzymatic reaction was carried out dropping an aliquot of 40 μL of a mixture of 1.0 mM 3-IP and 0.4 mM silver nitrate solutions. The redissolution peak of the silver deposited on the electrode surface was recorded when anodic stripping scan was carried out from + 0.03 V to + 0.4 V in the case of the electrodes modified with the carbon nanostructures; and from – 0.002 V to + 0.4 V

in the case of the presence of gold nanoparticles, at scan rate of 50 mV/s using cyclic voltammetry.

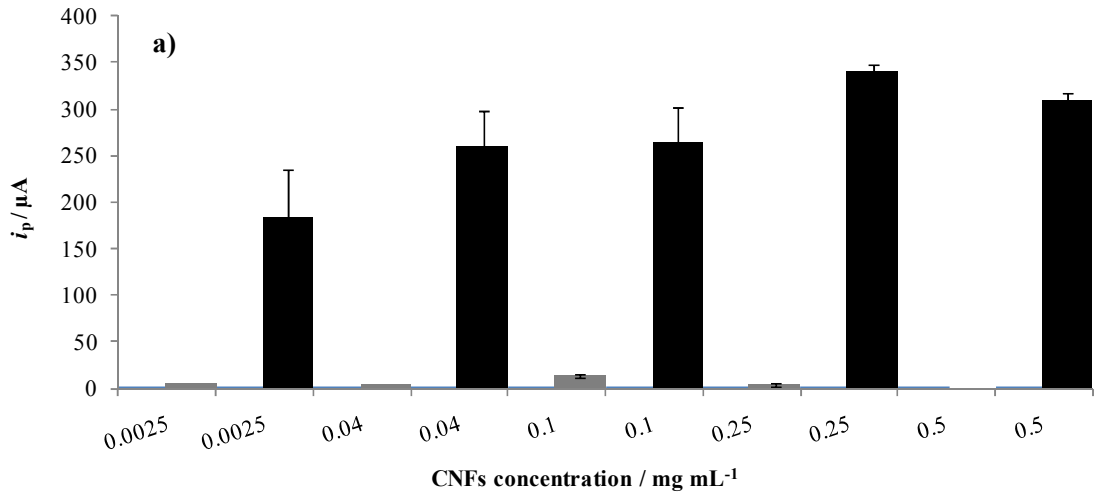
2.3.4. Characterization of the hybrid surface

The hybrid system between the carbon nanomaterials and the gold nanoparticles was characterized by scanning electron miscroscopy (SEM) and chronocoulometry. With this purpose, oxidation of gold were carried out by holding the electrode at a potential of + 0.85 V in a 0.1 M hydrochloric acid medium recording the current vs. time curve. The area under the curve was used to relate the amount of transfer charge with the mass of gold involved in the process using the Faraday equation [19].

3. Results and discussion

3.1. Optimization of the SPCEs coating process with the CNFs and MWCNTs

Five concentrations, between 0.025 mg mL^{-1} and 0.5 mg mL^{-1}, of functionalized CNFs were studied using the procedure explained in section 2.3.1. Electrochemical detection of the redissoluted peak of silver was performed as described in section 2.3.3. Figure 1a shows the results of the dependence of i_p with the different concentration of the CNFs modified SPCEs. It can be observed that as CNFs concentration increases, the analytical signal also increases until the value of 0.25 mg ml^{-1} since there is an increment of the superficial area available for the adsorption of streptavidin. The surface reaches the saturation for a concentration of 0.25 mg ml^{-1}. For 0.5 mg mL^{-1} a slightly decrease seems to take place. The modification of the electrode surface with carbon nanomaterials improves aspects such as the electroactive area and heterogeneous rate constants for the electron transfer. Although, the excessive modification of the electrode surface can produce an increment of this mesoporous film thickness, producing higher capacitances [11].The best relation between the faradaic and capacitive intensities was achieved for 0.25 mg mL^{-1}. No significant background signals were detected for all the studied concentrations. Cyclic voltammograms recorded for the concentration of 0.25 mg ml^{-1}, regarding analytical and background signals, were presented in Figure 1b.



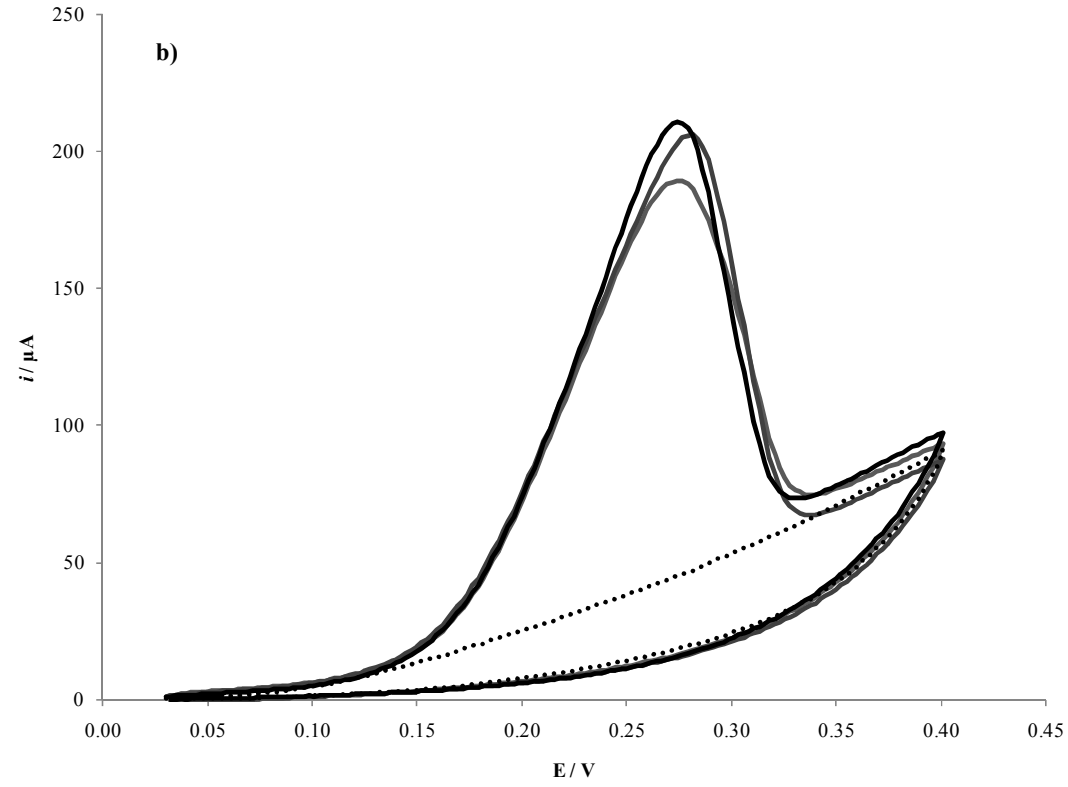


Fig. 1. a) Dependence between i_p and the concentration of CNFs solution in water. Streptavidin concentration, 10^{-7} M; B-AP concentration, 5.0 x 10^{-10} M; 3-IP concentration, 1.0 mM; silver ion concentration, 0.4 mM; BSA 1% ; analytical signal (black bars) and background signal (grey bars). Data are given as average ± *SD* (n = 3). b) Cyclic voltammograms of B-AP 5.0x10^{-10} M at a SPCE-modified with 0.25 mg ml^{-1} solution of CNFs. Analytical signal (solid line) and the background signal (dashed line). Other experimental conditions as in Figure 1a.

SPCEs working electrodes were modified with MWCNTs dispersed in DMF:water (1:1) with a concentration of 0.1 mg mL^{-1} as it was reported in a previous work [11]. These authors showed that the amount of MWCNTs employed not only improves electrodic surface features as the rate constants for the electron transfer and the electroactive area but also have a raising effect the double-layer capacitance. For the optimized value of 0.1 mg mL^{-1} this analytical disadvantage was minimized without compromising the analytical signal.

The optimization of the blocking solution is a key step for the development of any transducer surface. In this way, different blocking agents, casein and BSA at 1%, 2% and 3% of concentrations were studied. Moreover, streptavidin concentration was other optimized parameter. Streptavidin works as primary coating being used to immobilize biotin, which is associated with alkaline phosphatase (B-AP). An alkaline phosphatase concentration of $5.0x10^{-10}$ M was used through the optimization procedure. The response of the electrode surfaces to the two studied parameters was evaluated. The obtained results demonstrate that casein 3% was the most efficient blocking solution for SPCEs (data not show). BSA didn't avoid NSB, being the reproducibility jeopardized (data not show). Regarding blocking solution optimization for the modified electrodes, BSA revealed to be the most efficient in avoiding protein unspecific adsorption. Moreover, the fact of being more ease of handling and less expensive then casein, are also positives aspects of using BSA as the blocking solution. The obtained results suggest that BSA 1% was highly effective in minimizing NSB. Regarding streptavidin concentration, the value of 10^{-7} M showed to be the unanimous choice for the screen-printed surfaces considered, being established as the optimum concentration level ensuring sensitivity. This behavior was due to the full recover of the porous surface at the refereed value, since streptavidin was left on the surfaces to dryness. Figure 2 shows the effect of different concentrations of streptavidin on the analytical response of all the transducer, except for SPCE-NPAus format which streptavidin concentrations was optimized in a previous work [31].

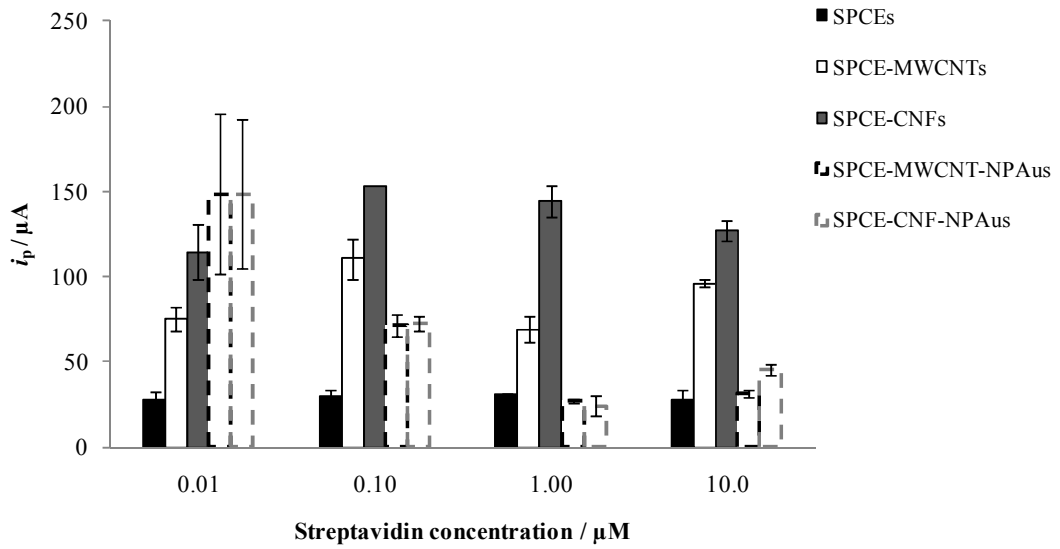


Fig. 2. Effect of the different concentrations of streptavidin (10^{-8} M; 10^{-7} M; 10^{-6} M; 10^{-5} M) on the i_p. B-AP concentration, 5.0 x 10^{-10} M; 3-IP concentration, 1.0 mM; silver ion concentration, 0.4 mM; casein 3% (SPCEs) BSA 1% (SPCEs modified electrodes). Data are given as average ± *SD* (n = 3).

3.2. Electrochemical generation of gold nanostructures on the carbon-modified electrode surface

Gold nanoparticles were electrochemically deposited onto the surface of SPCEs previously modified with carbon nanotubes or carbon nanofibers in optimized conditions. Gold nanoparticles have been formed from $AuCl_4^-$ of different concentrations prepared in 0.1 M HCl by applying a constant current intensity for a period of time. Three gold concentrations (0.1 mM $AuCl_4^-$, 0.5 mM $AuCl_4^-$, 1 mM $AuCl_4^-$), 3 current intensities (-5 µA, -10 µA, -100 µA) and 3 deposition times (60 s, 120 s and 300 s) were studied using the procedure described in section 2.3.2..

Figure 3 shows the results obtained for the gold concentration of 1 mM with the transducer previously modified with MWCNTs. In all cases it can be observed that the analytical signal obtained is similar for all times assayed. Regarding the three current intensities applied (-5, -10, -100 µA) there is no considerably difference from -5 to -10 µA since the slightly enhancement of the current can be justified with the standard deviation. As was reported in a previous work from Martínez-Paredes and colleagues [19] the formation of gold nanoparticles is influenced by the current intensity that is applied, the

time of gold deposition and gold concentration. Namely, the particle diameter seems to be indirectly proportional with time deposition and reduction intensity. For large deposition times occurs a shift in potential towards more negative potentials (-0.70 V) during gold electrodeposition. In the acid medium the generation of hydrogen occurred at this potential improving the nucleation of gold on the electrode surface in a detriment of growth of nanoparticles. The bar diagrams in Figure 3 shows that current decreases when increases the time for electrodeposition. For a 60 s deposition time the best analytical signal was achieved applying a constant current intensity of -100 μA. However, the results revealed that for these experimental conditions (-100 μA) there was a considerable standard deviation. Instead, a current intensity value of -5 μA was chosen for further studies. These results were also observed for the other gold concentrations in study (data not show). In order to achieve the best relation between sensitivity and reproducibility the concentration of 0.1 mM of $AuCl_4^-$ was selected (Figure 4). For the hybrid transducer surface between CNFs and NPAus was verified a similar behavior (data not show), therefore the selected values for the studied parameters were the same.

So, in both hybrid system, whether had carbon nanotubes or carbon nanofibers, the results indicate that the best compromise between sensitivity and reproducibility was achieved for a 0.1 mM $AuCl_4^-$ electrodeposited at -5 μA during 60 seconds. In Figure 5 SEM images of the hybrid electrodic surfaces were shown. The mean diameter of the gold nanoparticles formed on the working electrodes of carbon nanostructured SPCEs was predicted from these SEM images and 92 ± 15 nm was the value obtained. For this situation, the amount of gold deposited over the SPCE, determined by chronocoulometry, was of 76 ± 2 ng.

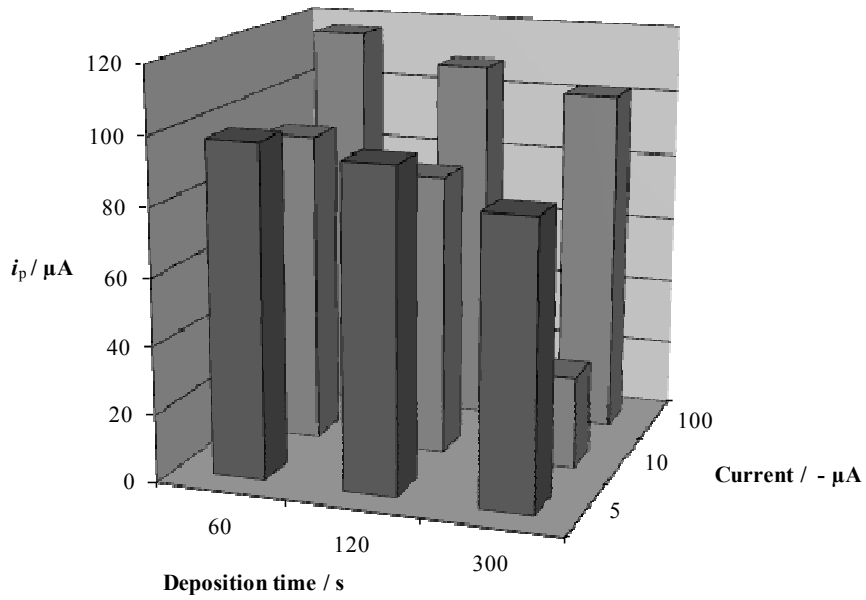


Fig. 3. Effect of the current intensity and deposition time of gold on the analytical signal. B-AP concentration, 1.0 x 10^{-10} M ; 3-IP concentration, 1.0 mM; silver ion concentration, 0.4 mM; BSA 1%. SPCE-MWCNTs (0.1 mg mL^{-1} MWCNTs) and $AuCl_4^-$ (1.0 mM).

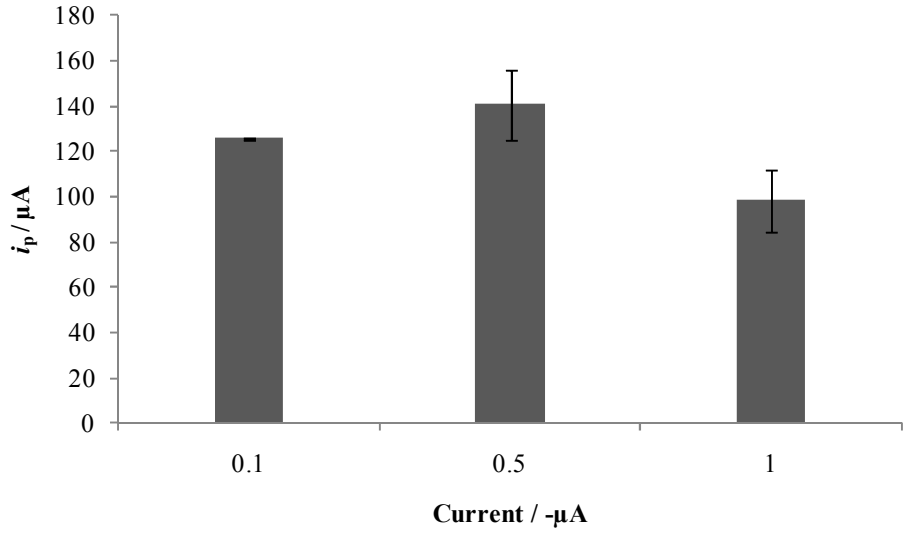


Fig. 4. Comparison of the effect of three gold concentrations on the analytical signal for the same deposition time (60 s) and current intensity (-5 µA). Rest of experimental conditions as in Figure 3. Data are given as average ± *SD* (n = 3).

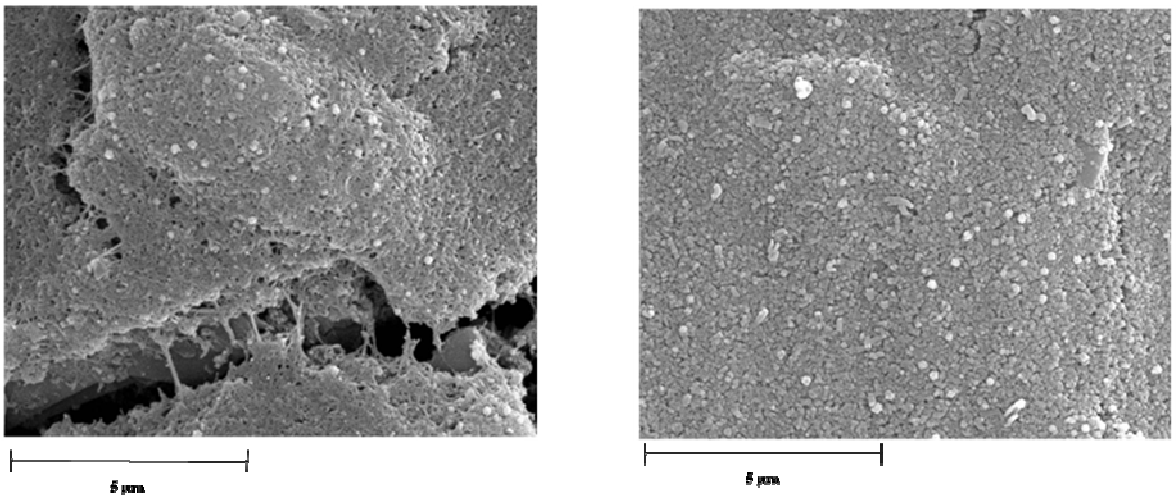


Fig. 5. SEM images of the working electrode of the optimized nanostructured screen-printed carbon electrodes. SPCE-MWCNT-NPAus (A); SPCE-CNF-NPAus (B). Gold electrodeposition at -5 μA for 60 s. Gold concentration: 0.1 mM. (amplification factor: 10 x 10^3).

3.3. Comparison of different transducer platforms

In order to study the effect of the transducers surfaces on the analytical signal six different transducers (SPCEs, SPCE-NPAus, SPCE-CNFs, SPCE-MWCNTs, SPCE-CNF-NPAus, SPCE-MWCNT-NPAus) were evaluated and compared using the streptavidin-biotin reaction as model analyte. Figure 6 gives the results obtained.

The electroactive surface area (*A*) of each transducer was also calculated (Table 1). The *A* of modified SPEs was calculated using the Randle–Sevcik equation for a reversible electrochemical process under diffusive control (T_a =25 °C) (Equation 1):

(1) $$i_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$$

where n is the number of electrons involved in the redox reaction, A is the electroactive area of the electrode (cm^2), D is the diffusion coefficient of the molecule in solution, C is the concentration of the analyte molecule in the solution (mol cm^{-3}) and v is the scan rate (Vs^{-1}). The A was calculated from the cyclic voltammograms for 1.0×10^{-3} M ferricyanide in $HClO_4$ 0.1M solution (D= 7.77×10^{-6} cm^2 s^{-1} [32]). The geometric area of a SPCEs is 12.56 mm^2.

The data present in Figure 6 shows that the nanostructured surfaces present better analytical signals comparing with the nonnanostructured SPCEs. It was also observed that the SPCE-NPAus allows to obtain similar sensitivity as that obtained with SPCE-MWCNTs. However in the case of SPCE-CNFs the analytical signal was lower, probably

due to the fact that CNFs are less functionalized than MWCNTs. The hybrids nanostructuration improved considerably the analytical signal respect to the other surfaces. In the case of the hybrid surfaces, the SPCE-MWCNT-NPAus are better in terms of sensitivity then SPCE-CNF-NPAus. However, the data provided in Figure 6 are not supported by the electroactive areas obtained. When we compare the electrodes modified with CNTs and CNFs, the last ones present a higher *A*. The same situation is observed regarding the hybrids with CNTs and CNFs. Nevertheless, the data obtained in Figure 6 indicates that CNTs are more effective in adsorbing streptavidin. Moreover, the *A* of the transducer SPCE-NPAus is the smallest. Although they are also the best transducers regarding the analytical response, standing out the hybrid surfaces. This is maybe due to the fact that NPAus improves the adsorption processes, besides provide a stable surface for the immobilization of biomolecules retaining their biological activities. The obtained results indicate that the electroactive area available is not directly related with the efficiency for the adsorption of the protein material, instead the nature of the transducer surface is the factor that determines the analytical response. For the same concentration of streptavidin (1.0×10^{-7} M) the hybrid surfaces present high adsorption efficacy. The hybrid structures formed by NPAus and carbon nanomaterials seem to present positive synergic features resulting collective in a more interesting analytical tool than when they appear individually.

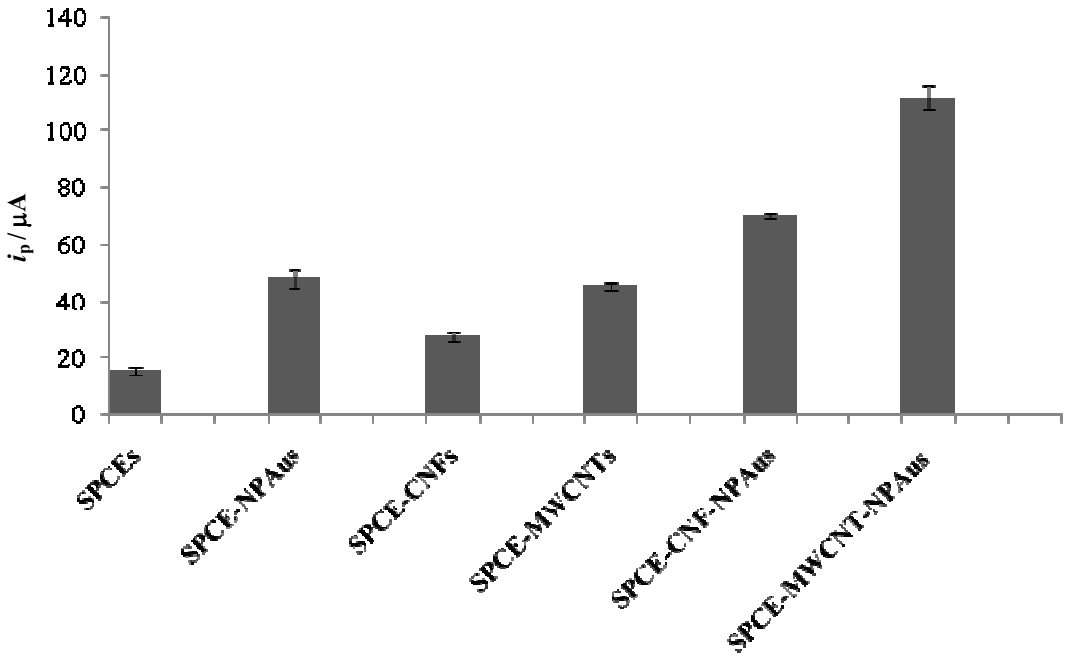


Fig. 6. Effect of the different transducers on the analytical signal for a B-AP concentration of 1.0 x 10^{-10} M. [Streptavidin] = 10^{-7} M. Other experimental conditions as in Figure 2. Data are given as average ± *SD* (n = 3).

Table 1. Electroactive areas (mm^2) data of the studied transducers. Data are given as average ± *SD* (n = 3).

SPCES	SPCE-NPAuS	SPCE-CNTS	SPCE-CNFs	SPCE-CNT-NPAus	SPCE-CNF-NPAus
8.73 ± 0.07	8.28 ± 0.09	9.92 ± 0.14	11.3 ± 0.33	10.10 ± 0.02	12.2 ± 0.16

3.4. Alkaline phosphatase calibration plot

Using the optimum conditions described previously, calibration plots of B-AP were obtained for all transducers. Table 2 lists the characteristics of the calibration plots obtained for each transducer. The reported limit of detection (LOD) and quantification (LOQ) were calculated from the calibration plots using the equations: LOD=3s/m and LOQ=10s/m (where s is the standard deviation of the intercept and m is the slope of the calibration plot) [32]. To evaluate the reproductibility of the electrode response, analysis of B-AP at three concentrations on the calibration plot was studied by three replicate measurements in three different days. The obtained LOD for the SPCEs showed small variations (from 1.1 to 5.1×10^{-11} M) regarding the electrodes nanostructured with the carbon nanostructures and NPAus. The hybrid transducers showed a better overall analytical performance, standing out their lowest LOD in the order of 10^{-12} M. The wider linear ranges (10^{-12} to 10^{-10} M) and lower LOD (6.1×10^{-12} M) were found for the hybrid SPCE-MWCNT-NPAus. This transducer also presented the highest slope.

3.5. Long-term stability

To study the transducer stability four series of several SPCE-MWCNT-NPAus and SPCE-CNF-NPAus were prepared and stored at 4ºC and at room temperature. In order to evaluate the stability of those single-use electrodes the transducers were tested following the procedure described in Section 2.3.3.. The voltammetric measurements were made on the same day of preparation and past 7, 15 and 30 days. The results, present in Figure 7, did not show any appreciable change among the analytical signal.

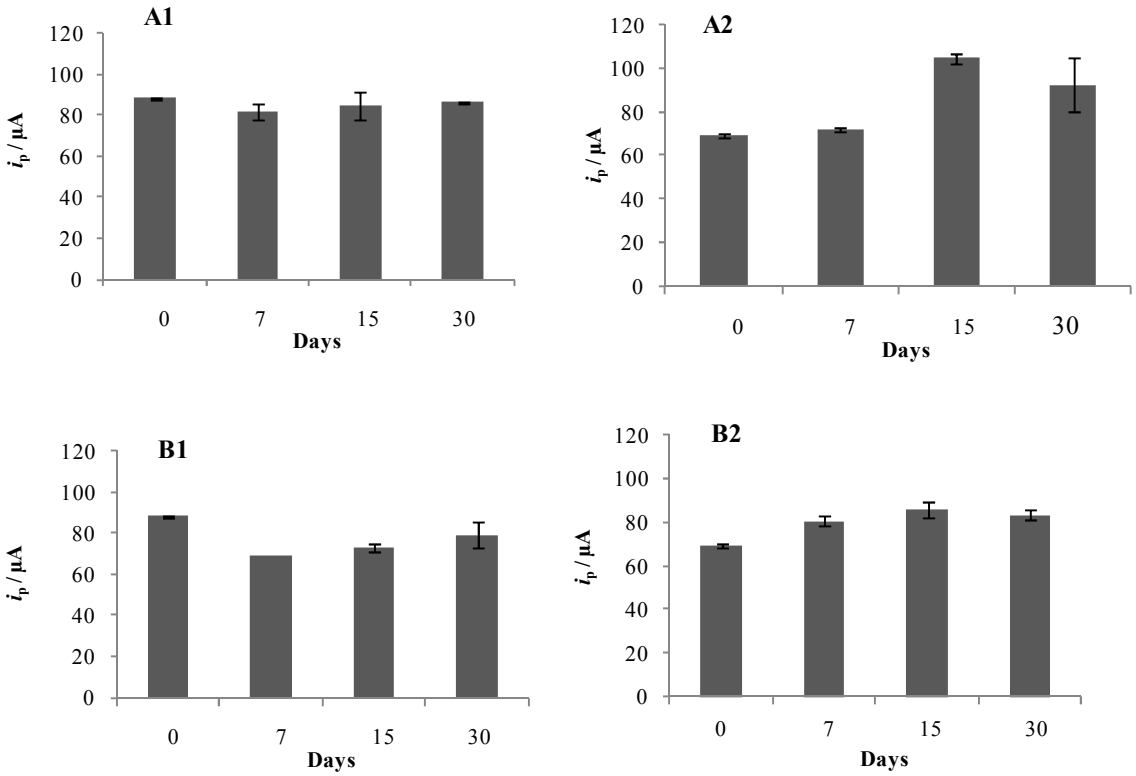


Fig. 7. Stability of the hybrid transducers for a month. (A) SPCE-MWCNT-NPAus and (B) SPCE-CNF-NPAus stored at 4ºC (1) and room temperature (2). Other experimental conditions as in figure 3. Data are given as average ± SD (n = 3).

Table 2. Analytical data from the calibration graphs obtained with different nanostructured surfaces in the determination of alkaline phosphatase.

	Regression equation (ip (A); CB-AP (mol L^{-1})	n*	Linear range (mol L^{-1})	Correlation coeficient	LOD (mol L^{-1})	LOQ (mol L^{-1})	Reproductibility (RSD%)**
SPCEs	$i_p = (14.19 \pm 0.39) \times 10^4 C_{B\text{-}AP} + (1.04 \pm 0.24) \times 10^{-6}$	4	$2.5 \times 10^{-11} - 1.0 \times 10^{-10}$	0.996	1.1×10^{-11}	3.74×10^{-11}	13.4 (0.01) 7.70 (0.05) 25.3 (0.075)
SPCE-NPAus	$i_p = (41.03 \pm 1.38) \times 10^4 C_{B\text{-}AP} + (3.9 \pm 0.79) \times 10^{-6}$	5	$1.0 \times 10^{-11} - 9.0 \times 10^{-11}$	0.993	1.3×10^{-11}	4.3×10^{-11}	0.40 (0.03) 1.40 (0.05) 2.00 (0.07)
SPCEs-CNFs	$i_p = (18.01 \pm 0.27) \times 10^4 C_{B\text{-}AP} + (1.09 \pm 0.14) \times 10^{-5}$	4	$5.0 \times 10^{-11} - 1.0 \times 10^{-9}$	0.999	5.1×10^{-11}	1.7×10^{-10}	1.70 (0.075) 1.20 (0.10) 12.0 (0.50)
SPCEs-MWCNTs	$i_p = (39.98 \pm 0.89) \times 10^4 C_{B\text{-}AP} + (6.64 \pm 1.35) \times 10^{-6}$	5	$1.0 \times 10^{-11} - 2.5 \times 10^{-10}$	0.998	2.3×10^{-11}	7.6×10^{-11}	23.6 (0.05) 13.4 (0.10) 7.20 (0.15)
SPCE-CNF-NPAus	$i_p = (83.08 \pm 9.4) \times 10^4 C_{B\text{-}AP} - (4.17 \pm 0.41) \times 10^{-6}$	5	$5.0 \times 10^{-12} - 7.5 \times 10^{-11}$	0.999	3.3×10^{-12}	1.1×10^{-11}	7.60 (0.01) 6.90 (0.035) 0.90 (0.05)
SPCE-MWCNT-NPAus	$i_p = (92.65 \pm 1.0) \times 10^4 C_{B\text{-}AP} - (3.36 \pm 0.85) \times 10^{-6}$	10	$5.0 \times 10^{-12} - 1.2 \times 10^{-10}$	0.999	6.1×10^{-12}	2.0×10^{-11}	15.8 (0.01) 6.80 (0.05) 1.80 (0.10)

* Number of points;** Between the parentheses are shown the concentrations (nmol L^{-1})

4. Conclusions

Sensitive transducer surfaces were compared in terms of their analytical potential using the high affinity streptavidin-biotin interaction as biological reaction model. The optimized transducers were studied regarding sensitivity, reproducibility, biocompatibility and stability. The best results were achieved with the hybrid conjugation between carbon nanomaterials, especially carbon nanotubes, and gold nanoparticles. The developed hybrid surfaces can work as excellent platforms for a wide range of electrochemical biosensor devices, moreover, once modified with streptavidin offers an at least 30 days stable surface, perfect to use on solid-phase assays with biotinylated molecules.

Acknowledgments

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Chapter 4

Screening for celiac disease serological markers

Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes

M.M.P.S. Neves, M.B. González-García, H.P.A. Nouws, A. Costa-García

Biosensors and Bioelectronics, 2012, 31 (1), 95–100

Voltammetric immunosensor for the diagnosis of celiac disease based on the quantification of anti-gliadin antibodies

M.M.P.S. Neves, M.B. González-García, A. Santos-Silva, A. Costa-García

Sensors and Actuators B, 2012, 163 (1), 253–259

Multiplexed Electrochemical Immunosensor for Detection of Celiac Disease Serological Markers

M.M.P.S. Neves, M.B. González-García, C. Delerue-Matos, A. Costa-García

Sensors and Actuators B, 2012 (*in press*)

Detection of antibodies against deamidated gliadin in human serum samples

Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes

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Abstract

Celiac disease is a gluten-induced autoimmune enteropathy characterized by the presence of tissue tranglutaminase (tTG) autoantibodies. A disposable electrochemical immunosensor (EI) for the detection of IgA and IgG type anti-tTG autoantibodies in real patient's samples is presented. Screen-printed carbon electrodes (SPCE) nanostructurized with carbon nanotubes and gold nanoparticles were used as the transducer surface. This transducer exhibits the excellent characteristics of carbon-metal nanoparticle hybrid conjugation and led to the amplification of the immunological interaction. The immunosensing strategy consisted of the immobilization of tTG on the nanostructured electrode surface followed by the electrochemical detection of the autoantibodies present in the samples using an alkaline phosphatase (AP) labelled anti-Human IgA or IgG antibody. The analytical signal was based on the anodic redissolution of enzymatically generated silver by cyclic voltammetry. The results obtained were corroborated with a commercial ELISA kit indicating that the electrochemical immunosensor is a trustful analytical screening tool.

Keywords: Electrochemical immunosensors. Transglutaminase. Celiac disease. Screen-printed electrodes. Carbon nanotubes. Gold nanoparticles.

1. Introduction

Celiac disease (CD) is a gluten-sensitive enteropathy triggered by dietary gluten in genetically susceptible individuals. CD has a strong association with human leucocyte antigen HLA-DQ2 and HLA-DQ8. Although the presence of these HLA proteins is necessary for developing celiac disease, it is not enough since about 30% of the healthy population possess them (McGough and Cummings, 2005). CD patients normally experience a massive immune reaction that leads to the production of autoantibodies and to the destruction of the small-intestinal mucosal morphology and intestinal dysfunction (Lindfors et al., 2009). Over time there has been a substantial increase in background prevalence of the disease, affecting around 1% of most populations (Lohi et al., 2007; Rubio-Tapia and Murray, 2010).

The diagnostic criteria for CD, which require endoscopy with small bowel biopsy, have been changing over the last few decades, especially due to the advent of serological tests with higher sensitivity and specificity for the measurement of these antibodies (Neves et al., 2010). Serologic testing has impacted the rate of diagnosis as well as the understanding of the epidemiology of this autoimmune condition (Rubio-Tapia and Murray, 2010). Current diagnostic routines include serology in combination with the histological examination of at least one biopsy that demonstrates mucosal villous atrophy while the patient consumes gluten containing food. When a decline of CD-related autoantibodies and the remission of the symptoms are observed when the patient is on a gluten-free diet, the second biopsy may not be needed (Hill et al., 2005). Therefore, serological tests, without surrogating the histological exam, have been used as a front-line strategy for screening candidates for the need of a duodenal biopsy (Sugai et al., 2010), avoiding such unnecessary invasive exams. Moreover many patients have little or no gastrointestinal symptoms while presenting extraintestinal syntomatology (Briani et al., 2008; Lindfors et al., 2009). Another important aspect to consider in a serological diagnosis is the selective IgA deficiency of the patient. If an individual presents a negative serological test but exhibits suggestive symptoms, the possibility of IgA deficiency should be considered and an IgG test must be performed. The identification of tissue transglutaminase (tTG) as the antigen against which the autoantibodies are directed, has led to a greater understanding of the pathogenesis of CD and to the development of improved serological tests (Alaedini and Green, 2005; Hill and McMillan, 2006; Armstrong et al., 2011). The common methodology for CD clinical serological diagnostic purposes is an enzyme-linked immunosorbent assay (ELISA). ELISA tests that measure the antibodies directed against tTG have high diagnostic sensitivity and specificity for the detection of CD. In an ELISA, antibodies are detected after several incubation, washing

and separation steps. An alternative to these high-cost and laborious assays could be electrochemical immunosensors (EIs). EIs combine the specificity inherent to antigen–antibody interactions with the high sensitivity of electrochemical transduction (Alonso-Lomillo et al., 2010), offering new exciting alternatives to the conventional immunochemical tests which are based on indirect detection, compromising real-time analysis.

There are few publications concerning the development of EIs to aid the diagnosis of CD. As far as we are concerned, regarding the use of EIs for the detection of autoantibodies directed against tTG, only 3 studies are published. An impedimetric immunosensor for the detection of antibodies against transglutaminase in human serum was presented by Balkenhohl and Lisdat (2007). The immunosensor was based on the immobilization of transglutaminase onto disposable screen-printed gold electrodes which were modified with a polyelectrolyte layer of poly(sodium 4- styrenesulfonic acid). Using impedance spectroscopy a qualitative analysis was carried out. According to the authors, an acceptable sensitivity was achieved but the results suggest a lower precision than ELISA. In a second study (Pividori et al., 2009) an amperometric electrochemical immunosensor based on the physical adsorption of tTG from guinea pig liver onto graphite–epoxy composite (GEC) electrodes was proposed. For 10 positive and 10 negative processed serum samples a sensitivity of 70% and a specificity of 100% were achieved when compared with the commercial ELISA method. Only a qualitative study was performed and no correlation between the analytical signal and the amount of antibodies present in the samples was established. More recently, an electrochemical immunosensor for the detection of human anti-tissue transglutaminase IgA and IgG antibodies in real samples by the covalent attachment of tTG to gold electrodes by the use of self-assembled monolayers of a group carboxylic-terminated bipodal alkanethiol was developed (Dulay et al., 2011). The results obtained with the immunosensor showed a good correlation with those obtained using a commercial ELISA.

Baring in mind that one of the big challenges of analytical chemistry is the development of accurate and sensitive methods that allow rapid *in situ* analyses, a disposable electrochemical immunosensor for CD clinical evaluation through the detection of antibodies to tTG in human serum is proposed. The electrochemical transducer surface consists of a screen-printed carbon electrode (SPCE) modified with multiwalled carbon nanotubes (MWCNTs) and gold nanoparticles (NPAus). Carbon nanostructure/metal nanoparticle hybrids have been widely exploited in the last years for electrochemical sensing applications since the conjugation of each material enhances their individual properties (Agüí et al., 2008). Therefore the immunosensing strategy consisted on the immobilization, by physical adsorption, of human tTG on a screen-printed carbon

electrode modified with carbon nanotubes and gold nanoparticles. The nanomaterials are used as an efficient amplification interface between the biorecognition process and the electrochemical transduction event. After the reaction with the patient's serum a secondary enzyme-labelled antibody is used to obtain electrochemical detection using 3-indoxyl phosphate and silver ions. The results indicate that the proposed immunosensor can be competitive with the standard methodology.

2. Materials and methods

2.1. Instrumentation

Voltammetric experiments were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266 MHz computer system and controlled by Autolab GPES 4.8 (software version for Windows 98). All the measurements were carried out at room temperature. Disposable screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Spain). These electrodes incorporate a conventional three-electrode configuration, printed on ceramic substrates (3.4x1.0 cm). Both the working- (disk-shaped 4 mm diameter) and counter electrodes are made of carbon inks, whereas the pseudoreference electrode and the electric contacts are made of silver. A ring-shaped layer printed around the working electrode constitutes the reservoir (50 μL) of the electrochemical cell. The SPCEs were easily connected to the potentiostat through a specific DropSens connector (Spain).

2.2. Chemicals and immunochemicals

Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate, bovine serum albumin fraction V (BSA), were purchased from Sigma (Spain). Carboxyl modified multiwalled carbon nanotubes (MWCNTs) were purchased from Nanocyl (Belgium). Standard gold tetrachloroaurate ($AuCl_4^-$), silver nitrate, hydrochloric acid (37%) and nitric acid (HNO_3) were obtained from Merck (Germany). Biosynth (Switzerland) supplied the 3-indoxyl phosphate disodium salt (3-IP). Human tissue transglutaminase (recombinantly produced in insect cells) was purchased from Zedira (Germany). Goat anti-human IgG (Fc specific) conjugated with alkaline phosphatase (anti-H-IgG-AP) was purchased from Sigma (Spain) and goat anti-human IgA (alpha chain specific) labelled with alkaline phosphatase (anti-H-IgA-AP) was provided by Invitrogen (Spain). The validation of the developed procedure was performed with anonymous sera samples analysed by Varelisa Celikey and Varelisa Celikey IgG ELISA kits supplied by Phadia (Germany). Each kit contained six standard serum samples (0, 3, 7, 16, 40, 100 U mL^{-1}) and a positive and a negative control. The kit's

manufacturers propose a range of values with clinical significance for the analysis of the samples: an equivocal result lays between 5 and 8 U mL^{-1} in the case of IgA detection and between 7 and 10 U mL^{-1} for IgG determination. Below and above these values the samples are classified as negative or positive, respectively, for CD. Ultrapure water obtained with a Millipore Direct-Q™ purification system from Millipore Ibérica S.A. (Spain) was used throughout this work. All chemicals employed were of analytical reagent grade.
Working solutions of tTG were prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer. Working solutions of the secondary alkaline phosphatase labelled antibodies were prepared in 0.1 M Tris-HNO_3 pH 7.2 containing 2 mM $Mg(NO_3)_2$. A solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate was prepared daily in 0.1 M Tris-HNO_3 pH 9.8 and 20 mM $Mg(NO_3)_2$ and stored in opaque tubes at 4ºC. The MWCNTs solutions were prepared by dilution of the concentrated solution with a mixture of DMF:water (1:1). The gold tetrachloroaurate solution was prepared in 0.1 M HCl.

2.3. Electrode nanostructuration

The modification of the screen-printed electrodes with nanohybrid structures was previously studied in our group (Neves et al., 2011). Single-use SPCEs were modified with 4 µL of a 0.1 mg mL^{-1} MWCNTs dispersion and the solution was left to dry at room temperature until its complete evaporation. Then, the MWCNTs-modified electrode was carefully washed with water and dried at room temperature. The coating process was followed by *in situ* electrochemical deposition of gold nanoparticles (NPAus) by applying a constant current intensity of -5 µA for 60 s in an acidic solution of 0.1 mM $AuCl_4^-$. The resulting SPCE-MWCNT-NPAus were rinsed with water and were ready to use.

2.4. Immunosensor for the detection of anti-tTG antibodies

The following procedure (Fig. 1) describes an optimized assay.The SPCE-MWCNT-NPAus working electrodes were coated with 10 µL of a 0.1 µg $µL^{-1}$ tTG solution and left to incubate overnight at 4ºC. After the incubation step the electrode was washed with 0.1 M Tris-HNO_3 pH 7.2 buffer. Free surface sites of the tTG-modified electrodes were blocked with a BSA solution (2% in 0.1 M Tris-HNO_3 pH 7.2) during 30 min. The detection of anti-tTG antibodies was accomplished by incubating the immunosensors with human serum samples for 60 min followed by a washing step with a 0.1 M Tris-HNO_3 pH 7.2 buffer containing 2 mM $Mg(NO_3)_2$. Finally, the immunosensor was incubated with an anti-H-IgA-AP (1:30,000) or an anti-H-IgG-AP (1:50,000) solution for 60 min and washed with a 0.1M Tris pH 9.8 buffer containing 20mM 2 mM $Mg(NO_3)_2$. The enzymatic reaction was carried out by dropping a 40 µL aliquot of a solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate on the immunosensor's surface. The enzymatically deposition of silver

catalyzed by alkaline phosphatase (AP) was studied previously and the work was already reported (Fanjul-Bolado et al., 2007). For the generation of the analytical signal an alkaline phosphatase labelled secondary antibody was employed. AP worked as the enzymatic label and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^+) as the substrate. AP hydrolyzes 3-IP resulting in a indoxyl intermediate that will reduce the silver ions presents in solution resulting in metallic silver (Ag^o) and indigo blue (I). Thus, the silver enzymatically deposited on the electrode surface can be detected through the anodic peak of the silver when an anodic stripping scan is carried out. After 20 min of reaction, a cyclic voltammogram was recorded from - 0.002 V to + 0.4 V, at scan rate of 50 mV s^{-1}, to obtain the electrochemical oxidation current of the enzymatically deposited silver.

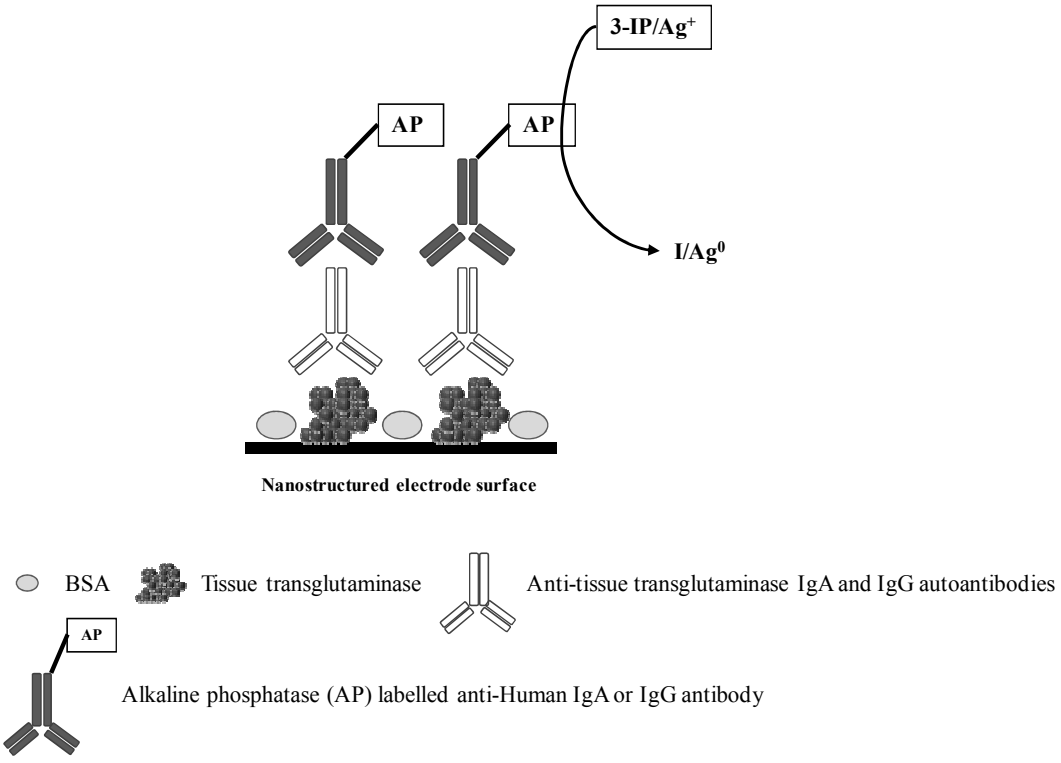


Fig. 1. Schematic representation of the immunosensor architecture used in this study.

3. Results and discussion

3.1. Optimization of the immunosensing strategy

The working area of the SPCE-MWCNT-NPAus (see Supplementary material) was coated with different amounts of tTG. The highest analytical signals and the best reproducibility were obtained using 10 μL of a 0.1 μg μL^{-1} tTG solution when left overnight

at 4ºC and avoiding the evaporation to dryness of the solution. Non-specific adsorption was effectively blocked with the addition of 40 µL BSA (2%) during 30 min. The sensing phase of the immunosensor was then completed. The analytical signals obtained with a nanohybrid-modified electrode and a non-nanostructured electrode were compared. Fig. 2 shows cyclic voltammograms obtained for the detection of anti-tTG IgA antibodies using a tTG-modified SPCE as well as a tTG-modified SPCE-MWCNT-NPAus. The obtained results indicate that the presence of the nanomaterials improves the faradaic/capacitive current ratio. The conjugation of MWCNTs and NPAus has already demonstrated to possess important analytical properties for biosensors due to the metal nanoparticles' ability to adsorb proteins without compromising their bioactivity, and the excellent electrocatalytical properties of the carbon nanotubes (Agüí et al., 2008; Neves et al., 2011). The differences observed in the peaks potential can be explained due to the presence of gold nanoparticles on the transducer surface. Gold nanoparticles facilitate the reduction of silver and, as a consequence of that, the anodic peak of the silver is shifted toward less positive potentials (Hernández-Santos et al., 2000; de la Escosura-Muñiz et al., 2004; Lee et al., 2004).

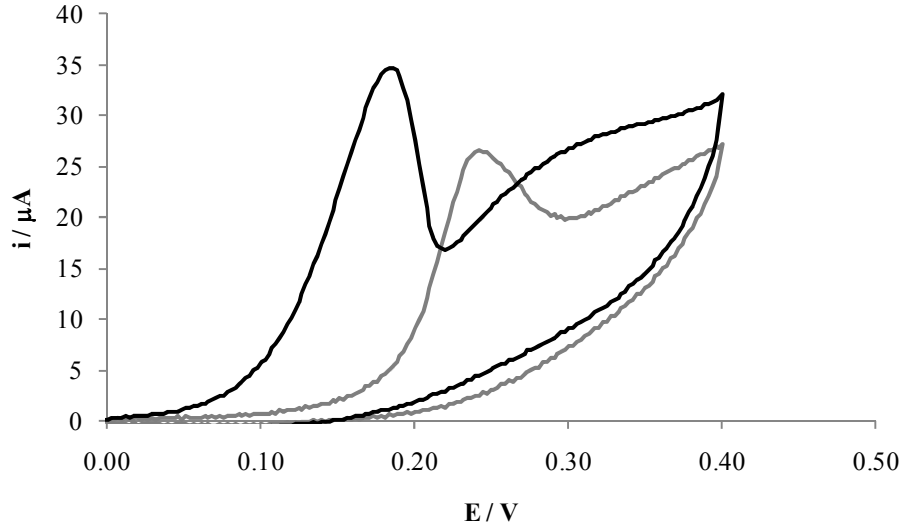


Fig. 2. Effect of the transducer surface on the analytical signal for the detection of anti-tTG IgA autoantibodies using a tTG-modified SPCE (grey line) and a tTG-modified SPCE-MWCNT-NPAus (black line). Experimental conditions: tTG 0.1 µg μL^{-1}; BSA 2%; control samples diluted 1:2; anti-H-IgA-AP 1:30,000; 3-IP, 1.0 mM; Ag^{+}, 0.4 mM. Cyclic voltammetric scans from - 0.002 V to + 0.4 V at a scan rate of 50 mV s^{-1}.

In Fig. 3 voltammograms are shown for the analysis of a positive and a negative sample using a tTG-modified SPCE-MWCNT-NPAus and non-tTG-modified SPCE-MWCNT-NPAus. The data show that the stripping peak current intensity of silver depends on the concentration of the alkaline phosphatase labelled antibodies, which in turn depends of the amount of autoantibodies in the analysed samples. Therefore the measured analytical signals for a positive and a negative sample analysed with the complete sensing platform (electrode with tTG) can be perfectly distinguished, the highest peak corresponding to the determination of autoantibodies in the positive sample. When the same samples were analysed with an incomplete immunosensing strategy (i.e. electrode without antigen) two similar peaks with current intensities of approximately 8 μA were obtained. Furthermore, these current values are comparable to those obtained with a negative sample assayed using a tTG-modified electrode. These results indicate that these peaks correspond to the sensor's background signal resulting from residual non-specific protein binding that occur despite of the effective surface blocking and are due to the presence of several different immunoglobulins and other proteins in the serum samples. Moreover, the obtained current difference for a positive sample analysed in the presence or absence of tTG on the electrode surface, indicates that the immunological reaction between antigen and autoantibodies was successfully performed.

3.2. Reproducibility

A precision study of the immunosensor's performance was carried out by evaluating the reproducibility of two interday immunosensing assays of positive as well as negative human anti-tTG antibodies sera. Three measurements using three separate sensors were preformed on each day and the obtained results indicate a good reproducibility of the immunosensor, with an average relative standard deviation (RSD) of 9.32 % for the negative samples and 2.01% for the positive samples.

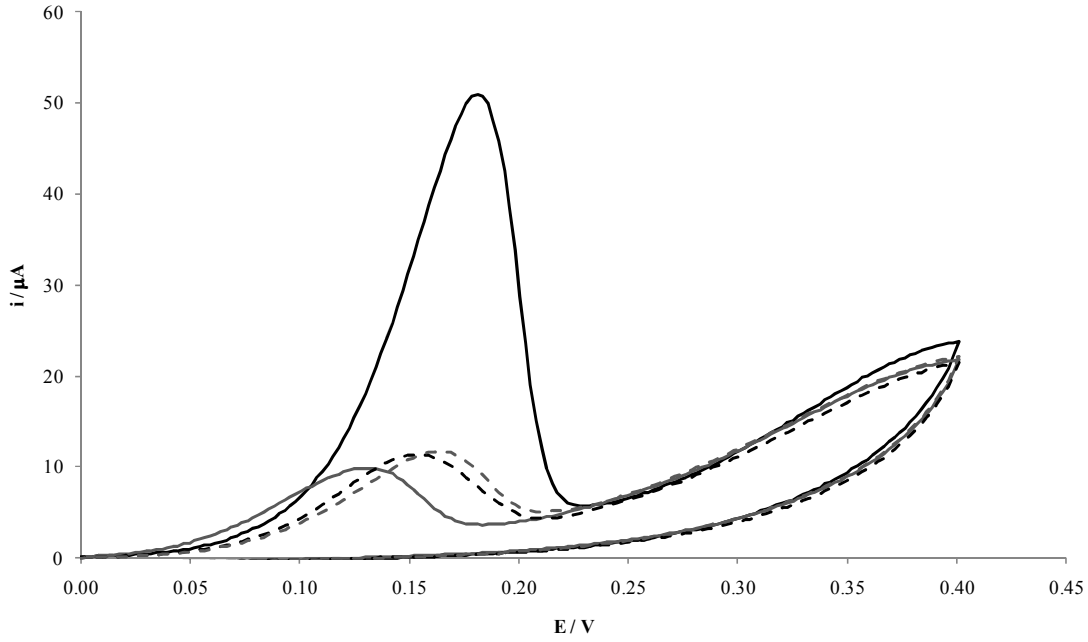
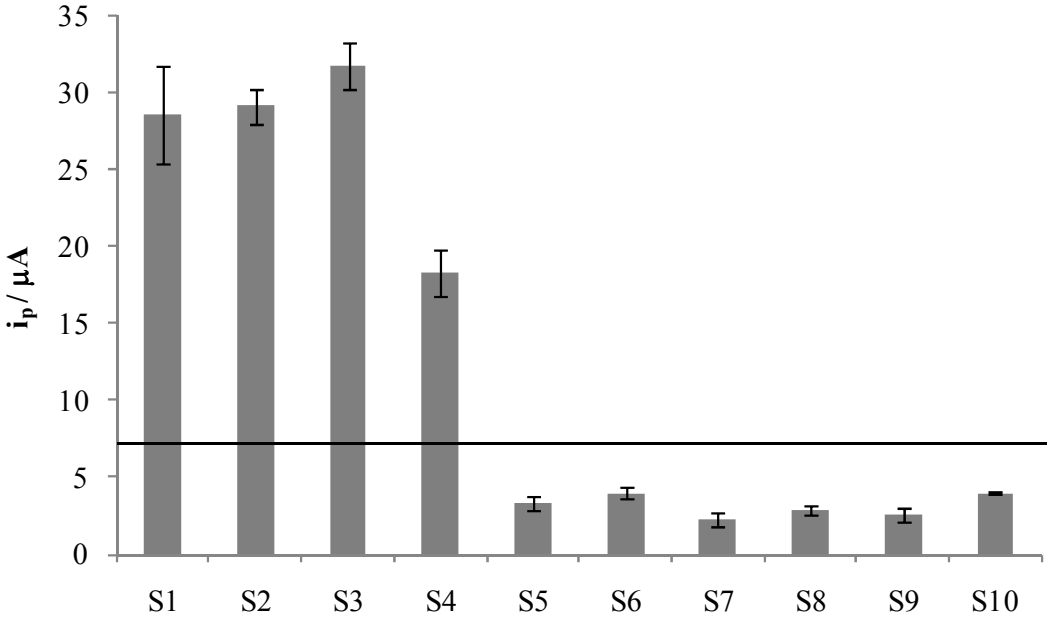


Fig. 3. Cyclic voltammograms obtained in the analysis of positive (black lines) and negative (grey lines) serum samples with tTG-modifed (solid lines) and non-tTG-modified nanohybrid electrodes (dashed lines). Experimental conditions: tTG 0.1 μg μL^{-1}; BSA 2%; serum samples diluted 1:200; anti-H-IgA-AP 1:30,000; 3-IP 1.0 mM; Ag^{+} 0.4 mM. Cyclic voltammetric scans from - 0.002 V to + 0.4 V at a scan rate of 50 mV s^{-1}.

3.3. Evaluation of real patient's samples

To be able to classify samples as being positive, negative or ambiguous, a cut-off value was defined as the average peak current intensity plus three times the standard deviation obtained for 8 negative samples. These values are 7.3 μA and 12 μA for anti-tTG IgA and IgG antibodies, respectively. The developed immunosensor was then applied to the analysis of 10 different human sera from real patients for the detection of anti-tTG class A and G antibodies. The results are presented in Fig. 4 and show that four samples were positive for celiac disease (S1–S4), presenting both IgA and IgG anti-tTG antibodies, sample 5 was only positive for IgG detection, which indicates that the donor is a celiac patient with selective IgA deficiency, and the remaining five samples correspond to healthy individuals (S6–S10).

a



b

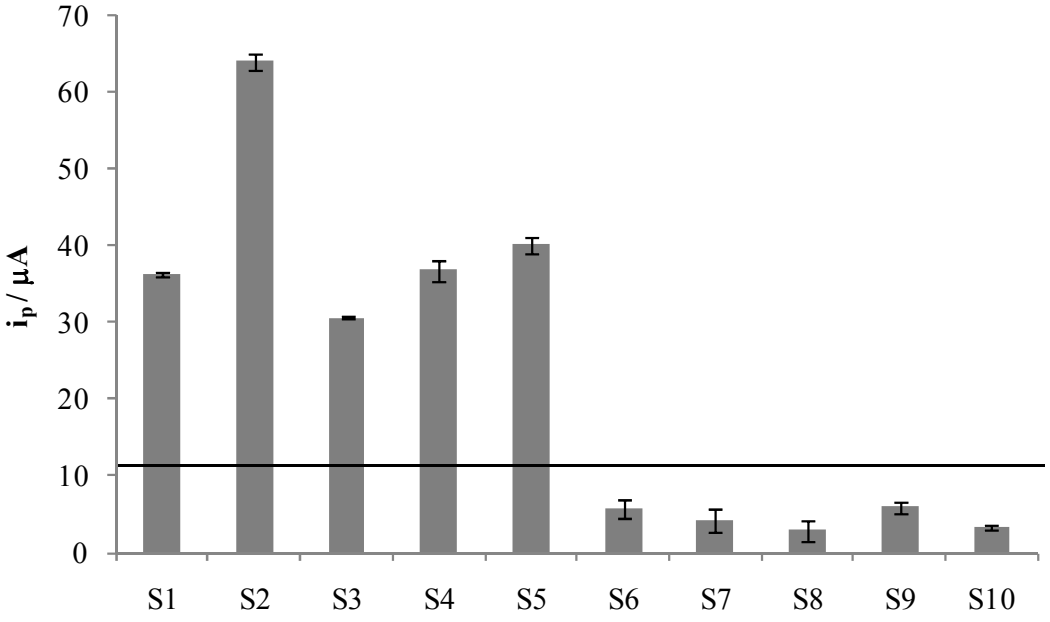
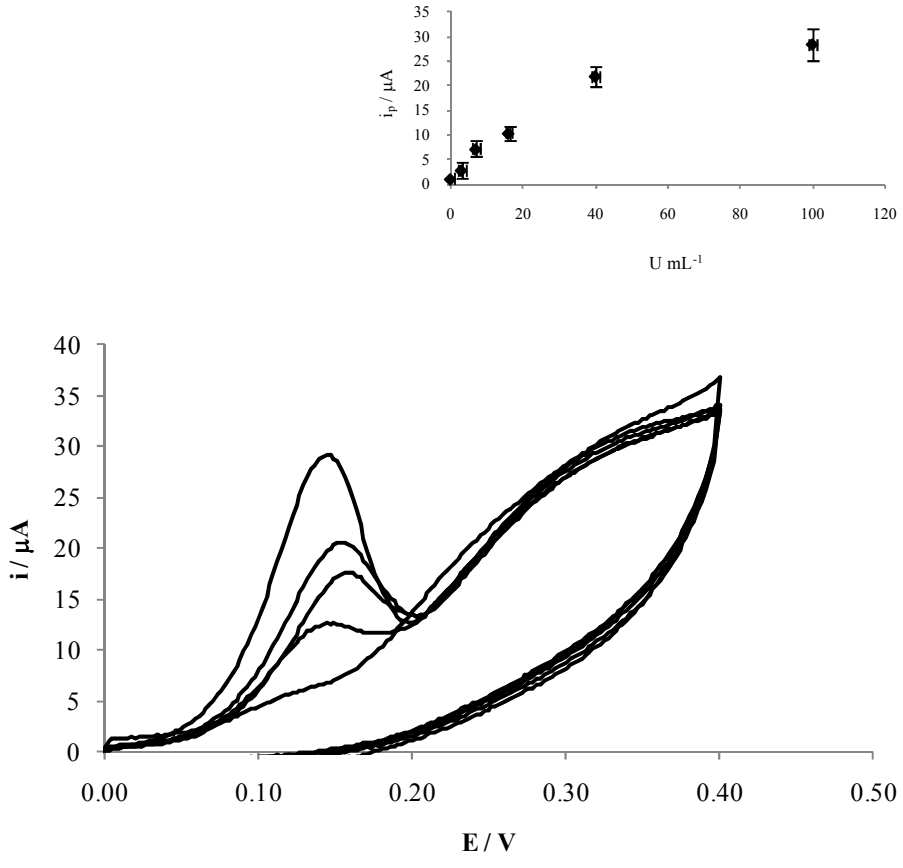


Fig. 4. Peak current intensities obtained in the determination of (a) IgA and (b) IgG anti-tTG autoantibodies in real patient's samples using the optimized electrochemical immunosensor. Experimental conditions: tTG 0.1 μg μL^{-1}; BSA 2%; serum samples diluted 1:200; anti-H-IgA-AP 1:30,000 (a) and anti-H-IgA-AP 1:50,000 (b); 3-IP 1.0 mM; Ag^{+} 0.4 mM (average data ± standard deviation are indicated (n=3)).

3.4. Calibration plot for the determination of anti-tTG antibodies in human serum samples

SPCE-MWCNT-NPAus modified with tTG were used to establish a relationship between the stripping peak current intensity and the concentration of anti-tTG antibodies, in arbitrary units, using the calibrators of the commercial ELISA kits. The peak current intensities were extrapolated to the calibrator solutions plot to convert the results in arbitrary units to be able to compare the results, in quantitative terms, with the results obtained using the ELISA. The insets (calibration plots) shown in Fig. 5 present the relationship between the peak current intensity and the concentration of anti-tTG antibodies present in the assayed calibrators. As can be observed the voltammetric response of the sensor is not linear within the entire analysed concentration range. Saturation is achieved after 40 U mL^{-1}. Using the optimized conditions linear regression equations of i_p (μA) = (0.53 ± 0.02) [antibody U mL^{-1}] + (1.66 ± 0.48) (r = 0.994) and i_p (μA) = 0.47 ± 0.01 [antibody U mL^{-1}] + 1.93 ± 0.03 (r = 0.999) were achieved for the determination of anti-tTG IgA and IgG autoantibodies, respectively. Examples of cyclic voltammograms obtained within the linear ranges are also shown in Fig. 5.

a



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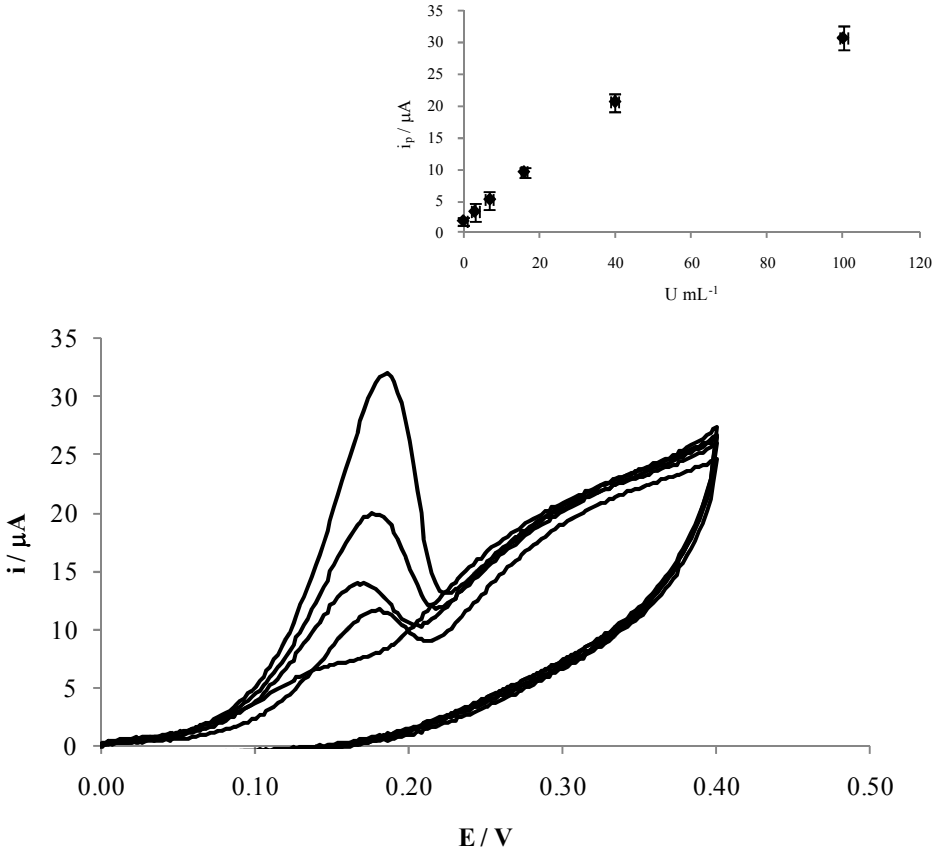

Fig. 5. Cyclic voltammograms of different calibrator solutions in the linear range. (a) [Anti-tTG IgA] (U mL^{-1}), (b) [anti-tTG IgG] (U mL^{-1}): 0, 3, 7, 16 and 40. Experimental conditions: tTG 0.1 µg $µL^{-1}$; BSA 2%, serum samples diluted 1:200; anti-H-IgA-AP 1:30,000 (a) and anti-H-IgG-AP 1:50,000 (b); 3-IP 1.0 mM; Ag^{+} 0.4 mM. Inset: relationship between i_p and antibody concentration (U mL^{-1}) (average data ± standard deviation are indicated (*n*=3)).

3.5. Comparison of the developed immunosensor with a commercial ELISA

Data given in Table 1 indicate that the results of a quantitative analyses obtained with the electrochemical immunoassay do not perfectly match the ELISA results. However, the qualitative results (i.e. positive or negative) match perfectly. Moreover to the clinical diagnostic of this autoimmune condition the most significant aspect is the qualitative detection of the serological markers. For this reason the results achieved with the immunosensor were supported by those obtained with the commercial ELISA assay.

Table 1. Comparison of the analyses of anti-tTG antibody concentration in different human sera using the developed electrochemical immunosensor and a commercial ELISA.

	Electrochemical immunosensor		ELISA	
Real patient's sera	Anti-tTG IgA antibodies[a]	Anti-tTG IgG antibodies[a]	Anti-tTG IgA antibodies[b]	Anti-tTG IgG antibodies[b]
S1	52.05 ± 6.22	36.28 ± 0.71	82.96 ± 0.29	35.21 ± 1.00
S2	53.24 ± 2.32	64.06 ± 2.24	81.95 ± 0.18	87.59 ± 1.14
S3	58.39 ± 2.91	30.68 ± 0.50	59.91 ± 0.01	53.95 ± 0.36
S4	31.94 ± 2.99	36.85 ± 2.82	61.75 ± 0.11	91.97 ± 0.65
S5	2.59 ± 1.32	40.21 ± 2.27	0.030 ± 0.001	35.48 ± 0.39
S6	3.92 ± 0.72	5.74 ± 2.36	0.086 ± 0.003	0.314 ± 0.001
S7	0.44 ± 0.91	4.22 ± 3.26	0.088 ± 0.007	0.409 ± 0.014
S8	1.65 ± 0.58	2.99 ± 2.87	0.030 ± 0.003	0.242 ± 0.007
S9	1.10 ± 0.84	5.93 ± 1.44	0.035 ± 0.012	0.426 ± 0.001
S10	3.91 ± 0.24	3.28 ± 0.66	0.100 ± 0.002	0.532 ± 0.050

[a] Concentrations are given in U mL^{-1} (Average data ± standard deviation are indicated (n=3))

[b] Concentrations are given in U mL^{-1} (Average data ± standard deviation are indicated (n=2))

4. Conclusions

Combining the advantages of the strong antibody–antigen interaction with the sensitivity of the electrochemical techniques, a new disposable electrochemical immunosensor for the detection of gluten-induced celiac disease-specific anti-tissue transglutaminase IgA and IgG autoantibodies in real patients' samples was developed. Tissue transglutaminase was successfully immobilized on a nanohybrid (carbon nanotube/gold nanoparticle) screen-printed carbon electrode surface and 10 different human sera were assayed. The results obtained were corroborated with a commercial ELISA kit indicating that the electrochemical immunosensor is a trustful analytical screening tool. The proposed miniaturized electrochemical immunoassay can be a good alternative to the conventional optical screening assays. Moreover the employment of disposable SPCEs as electrochemical transducer surfaces, in detriment of classical solid electrodes, avoids some problems as memory effects and tedious cleaning processes. SPCE-based biosensors allows to perform a point-of-care diagnostic and as a consequence the decentralization of clinical applications. A biosensor developed on a screen-printed electrode can be a portable and ready-to-use device, able to be competitive not only with traditional methods, but also with other biosensors.

Acknowledgments

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Supplementary material

Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes

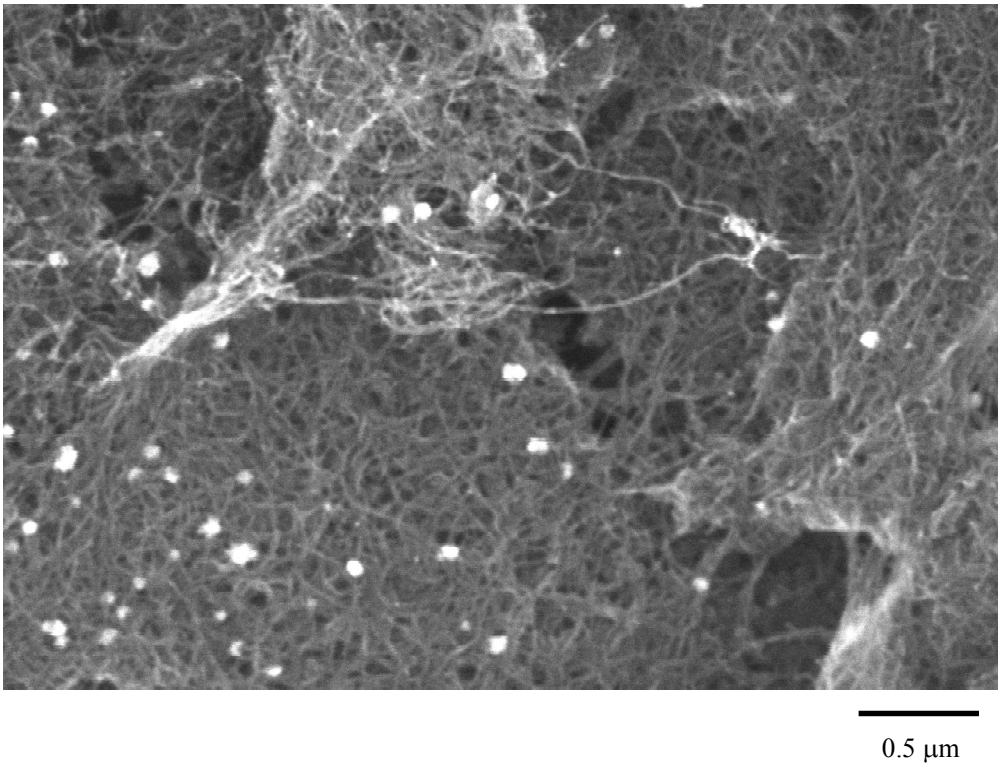

Fig. S1. Scanning electron microscope (SEM) image of the optimized SPCE-MWCNT-NPAus working electrode (amplification factor 3×10^4).

Voltammetric immunosensor for the diagnosis of celiac disease based on the quantification of anti-gliadin antibodies

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Abstract

Antibodies against gliadin are used to detect celiac disease (CD) in patients. An electrochemical immunosensor for the voltammetric detection of human anti-gliadin antibodies (AGA) IgA and AGA IgG in real serum samples is proposed. The transducer surface consists of screen-printed carbon electrodes modified with a carbon nanotube/gold nanoparticle hybrid system, which provides a very useful surface for the amplification of the immunological interactions. The immunosensing strategy is based on the immobilization of gliadin, the antigen for the autoantibodies of interest, onto the nanostructured surface. The antigen–antibody interaction is recorded using alkaline phosphatase labelled anti-human antibodies and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^+) was used as the substrate. The analytical signal is based on the anodic redissolution of the enzymatically generated silver by cyclic voltammetry. The electrochemical behaviour of this immunosensor was carefully evaluated assessing aspects as sensitivity, non-specific binding and matrix effects, and repeatability and reproducibility. The results were supported with a commercial ELISA test.

Keywords: Electrochemical immunosensors. Gliadin. Celiac disease. Point-of-care analysis. Screen-printed electrodes. Nanomaterials.

Abreviations

CD – celiac disease

EIs – clectrochemical immunosensors

AGA – human anti-gliadin antibodies

Anti-H-IgA-AP – anti-human IgA antibodies conjugated with alkaline phosphatase

Anti-H-IgG-AP – anti-human IgG antibodies conjugated with alkaline phosphatase

SPCEs – screen-printed carbon electrodes

MWCNTs – carboxylated multiwalled carbon nanotubes

NPAus – gold nanoparticles

1. Introduction

Celiac disease (CD) is an autoimmune enteropathy characterized by an inappropriate T-cell mediated immune response to the ingestion of gluten in genetically susceptible individuals [1]. Gluten is a complex mixture of wheat storage proteins that can be insoluble in alcohol, the glutenins, or soluble in alcohol, the gliadins. The alcohol-soluble fractions have analogous toxic proteins that are present in barley, the hordeins, in rye, the secalins, and in oats, the avenins [2]. It is generally accepted that gliadins are the major triggering factors in CD [3]. The mechanism underlying CD pathogeneses can be explained by the ingestion of these alcohol-soluble proteins. The deamidation of these gluten peptides is mediated by tissue transglutaminase (tTG) creating epitopes with increased immunostimulatory potential. The deamidated epitopes are then presented, in association with the human leucocyte antigens DQ2 and DQ8 of antigen-presenting cells, to CD4+ T cells [4]. The humoral autoimmune response leads to an abnormal intestinal mucosa characterized by villous atrophy and crypt hyperplasia [4] resulting in malabsorption related problems. Common serological changes include the appearance of antibodies against gliadin, tTG and endomysium, which are specific indicators of the disease.

In spite of efforts towards the development of new therapeutic strategies [5] the recovery of the intestinal mucosal still requires the total elimination of gluten proteins from the patient's diet. Therefore, an early and accurate diagnosis of CD is extremely important to control the gastrointestinal damage and to ensure the patient's quality of life.

Small bowel biopsy has been the gold standard diagnostic test for CD during the last 30 years. However, the different clinical presentations of this condition can complicate the diagnosis and, as a result, delay the treatment [6]. Recent guidelines from the European Society of Paediatric Gastroenterology and Nutrition [7] and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition [8] have suggested serological tests as the front-line test to clear clinical suspicion of celiac disease in patients presenting characteristic symptoms or in those who belong to a risk group. In the case of patients that have the symptoms but present negative serological tests, IgA class antibodies deficiency should be considered. The determination of the IgG class of antibodies against gliadin, tTG and endomysium has been suggested as an alternative [9].

The advances in the efficacy of serological antibody testing potentiate the possibility of accurate screening programs in the community, serving as a first-line method to clarify clinically suspicious cases in an underdiagnosed stage and also to manage the follow-up of this multifactorial disease [10]. Currently, enzyme-linked immunosorbent assay (ELISA) is the most used methodology for clinical serological CD diagnosis; nonetheless the development of more rapid, sensitive, and cost-effective strategies that allow point-of-care analysis are required.

Electrochemical immunosensors (EIs) are specific, fast, portable and low cost devices which offer exciting opportunities for numerous decentralized clinical applications, ranging from “alternative-site” screening to home self- testing [11]. The nature of the electrochemical sensor’s surface and the strategies for the antibody or antigen immobilization have been one of the major issues in immunosensing development. The transduction of the biorecognition events requires innovative approaches that couple different sensing platforms. The use of hybrid structures formed by combining carbon and metal nanomaterials has demonstrated to improve the electrocatalytic efficiency of many electrochemical processes allowing a synergic enhancement of each of the material properties [12, 13, 14].

In the recent years, the electrochemical biosensors field have witnessed a growing interest in the development of analytical devices to celiac disease diagnosis. The employment of EIs for the detection of specific antibodies for CD was first reported in 2007 by Balkenhohl and Lisdat [15, 16]. These authors developed an impedimetric immunosensor for the detection of antibodies directed against tTG using screen-printed gold electrodes modified with a polyelectrolyte layer of poly(sodium 4- styrenesulfonic acid) [15]. The same authors also developed a similar immunosensor for the determination of anti-gliadin antibodies (AGA), using 3-mercaptopropionic acid to modify the screen-printed gold electrode surface [16]. The amount of these autoantibodies present in positive and negative serum samples was evaluated. The high charge transfer resistance

values obtained by impedance spectroscopic measurements were related to the content of antibodies; however only a qualitative analysis was performed. Moreover, an acceptable sensitivity was achieved but the results suggest a lower precision, when compared with ELISA assays. On the other hand, Pividori et al. proposed an amperometric immunosensor based on the physical adsorption of tTG from guinea pig liver onto graphite–epoxy composite (GEC) electrodes [17]. For 10 positive and 10 negative processed serum samples a sensitivity of 70% and a specificity of 100% were achieved, compared with the commercial ELISAs, but this method was also based on a yes/no binary qualitative system and no direct correlation between the analytical signal and antibody concentration was achieved. Pereira et al. [18] have proposed a microfluidic immunosensor coupled with electrochemical detection for AGA IgG quantification. The methodology consists of a 30-min assay using a pore glass bed coated with the antigen gliadin on a bare gold electrode for the detection of the labelled anti-human IgG. The results obtained in this work were comparable with those obtained by commercial ELISA kits but the work lacks the detection of IgA-class antibodies and immunoglobulin A (IgA) isotype is considered to be the most specific [19]. An electrochemical supramolecular platform based on cyclodextrin-modified gold surface electrodes to detect antigliadin antibodies in real serum samples was also proposed [20]. In this work an amperometric detection of AGA IgA and AGA IgG in samples of CD patients in 3 stages of the disease was achieved and the results showed a good correlation with an ELISA assay. Two more EIs that provide a qualitative and semi-quantitative estimation of the antibody content in real sera were reported. One was developed for the detection of human anti-tTG antibodies [21] and the other for the detection of human AGA antibodies [22]. In the construction of both sensors, the antigen (i.e. tTG or gliadin) was covalently attached onto self-assembled monolayers of a carboxylic-terminated bipodal alkanethiol group which was immobilized on gold electrodes. The immunosensors performance was corroborated with a commercial ELISA kit and the results were in good agreement with those obtained with the conventional methodology. However, in almost every case, surface renewable electrodes and conventional electrochemical cells are used and this has serious disadvantages has memory effects and necessary cleaning processes. In spite of the good analytical results, the employed electrochemical devices do not allow to carry out a continuous analytical performance. Moreover, for the decentralization of the diagnostic towards a point-of-care analysis, the research was to be directed toward more simple, miniaturized and portable devices.

In our group, we already developed a disposable electrochemical immunosensor for the detection of IgA and IgG type anti-tTG autoantibodies in real patient's samples [23] using a screen-printed carbon electrode modified with a carbon nanotube (CNT)/ gold

nanoparticle hybrid system as transducer surface. That nanostructured electrodic surface was developed in a previous work [24] in order to be applied as a transducer surface in several biosensor devices. Due to the growing need to perform rapid "in situ" analyses, disposable screen-printed electrodes have been successfully employed in the development of several biosensors devices in many fields [25]. Furthermore, hybrid CNT-nanoparticle materials are interesting because the combination of the physical and chemical properties of the individual components leads to a robust and efficient transducer amplification platform [12].

In this work we propose the same single-use hybrid transducer platform [24] for the quantification of AGA IgA and AGA IgG in human serum. The antigen and secondary labelled antibodies' concentrations were optimized and non-specific binding was also studied. The evaluation of real patient's samples was performed with the developed immunosensor and the results were compared with commercial ELISA kits.

2. Materials and methods

2.1. Instrumentation

Voltammetric experiments were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266 MHz computer system and controlled by Autolab GPES 4.8 (software version for Windows 98). All the measurements were carried out at room temperature. Disposable screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Spain). These electrodes incorporate a conventional three-electrode cell configuration, printed on ceramic substrates (3.4×1.0 cm). Both the working- (disk-shaped 4 mm diameter) and the counter electrode are made of carbon inks, whereas the pseudo-reference electrode and the electric contacts are made of silver. A ring-shaped layer printed around the working electrode constitutes the reservoir (50 μL) of the electrochemical cell. The SPCEs were easily connected to the potentiostat through a specific DropSens connector (Spain).

2.2. Reagents and solutions

Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate, bovine serum albumin fraction V (BSA), β-Casein from bovine milk (casein) were purchased from Sigma (Spain). Carboxylated multiwalled carbon nanotubes (MWCNTs) were purchased from Nanocyl (Belgium). Standard gold (III) tetrachloro complex ($AuCl_4^-$), silver nitrate, hydrochloric acid (37%) and nitric acid (HNO_3) were obtained from Merck (Germany). Biosynth

(Switzerland) supplied the 3-indoxyl phosphate disodium salt (3-IP). Wheat gliadin and goat anti-human IgG (Fc specific) conjugated with alkaline phosphatase (anti-H-IgG-AP) were purchased from Sigma (Spain) and goat anti-human IgA (alpha chain specific) labelled with alkaline phosphatase (anti-H-IgA-AP) was provided by Invitrogen (Spain). Positive and negative controls (*EliA* CCP Positive Control 100 and *EliA* IgG/IgM/IgA Negative Control 100) were acquired from Phadia (Germany). The validation of the developed procedure was performed using anonymous sera samples, collected in the "Hospital Universitario Central de Asturias" (Spain), which were analyzed by VARELISA Gliadin IgA Antibodies and VARELISA Gliadin IgG Antibodies ELISA kits supplied by Phadia (Germany). Each kit contained six standard serum samples (0, 3, 7, 16, 40, 100 U mL^{-1}) and a positive and a negative control. The kit's instructions established a cut-off range regarding the serological recommended normal values to negative (< 11 U mL^{-1}), equivocal (11-17 U mL^{-1}) and positive (> 17 U mL^{-1}) determinations. Ultrapure water obtained from a Millipore Direct-Q™ purification system from Millipore Ibérica S.A. (Spain) was used throughout the work. All chemicals employed were of analytical reagent grade. Working solutions of gliadin were prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer (buffer 1). Working solutions of secondary alkaline phosphatase labelled antibodies were prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer containing 2 mM $Mg(NO_3)_2$ (buffer 2). A solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate was prepared daily in 0.1 M Tris-HNO_3 pH 9.8 and 20 mM $Mg(NO_3)_2$ (buffer 3) and stored in opaque tubes at 4ºC. BSA and casein lyophilized powder were reconstituted in buffer 1. All the dilutions of the control samples and real sera samples were performed with buffer 1. The MWCNTs working solutions were prepared by dilution of the concentrated solution with a mixture of the dimethylformamide(DMF):water (1:1) and gold tetrachloroaurate solutions were prepared in 0.1 M HCl as was reported in a previous work [24].

2.3. SPCEs nanostructuration with MWCNTs and NPAus

The modification of the SPCEs was carried out using the methodology described previously [24]. A 4 µL aliquot of the 0.1 mg mL^{-1} MWCNTs dispersion was deposited on the working electrode's surface. The solution was left to dry at room temperature until its complete evaporation. Then, the modified SPCEs were carefully washed with water and dried at room temperature. Gold nanostructures were generated "in situ" over the carbon nanotube-modified electrode (SPCE-MWCNT-NPAus) by applying a constant current intensity of -5µA for 60 s in an acidic solution of 0.1 mM $AuCl_4^-$. Finally, the nanostructured electrodes were rinsed with water.

2.4. Construction of the immunosensor and electrochemical detection

The following procedure (Fig. 1) describes an optimized assay:

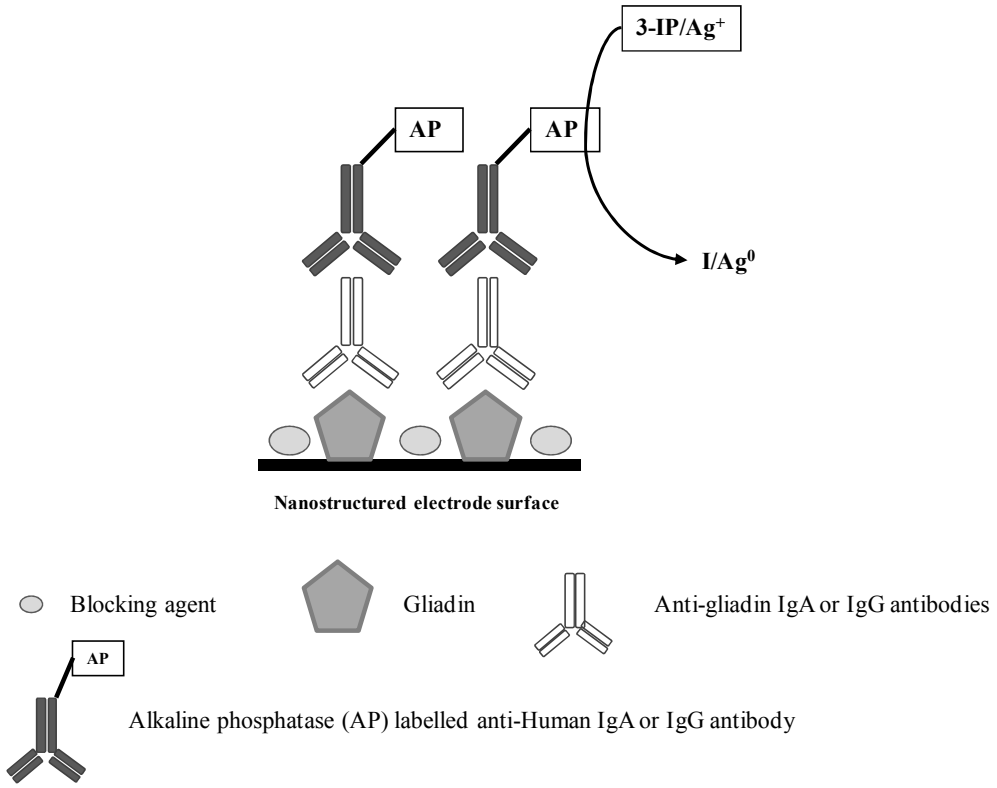


Fig. 1. Schematic representation of the immunosensor architecture used in this study.

The working area of the SPCE-MWCNT-NPAus was coated with 40 μL of a 1 mg mL^{-1} wheat gliadin solution and incubated overnight at 4 ºC. The immobilization of the gliadin on the electrode surface was achieved by physical adsorption. After the overnight incubation step the electrode was washed with buffer 1. Free surface sites of the gliadin-modified SPCE-MWCNT-NPAus were blocked with 40 μL of a casein (2%) or BSA (2%) solution during 30 min. After a washing step with buffer 1 the sensing part of the immunosensor was completed. The detection of anti-gliadin antibodies was accomplished by incubating the immunosensors with 40 μL of serum samples (1:200) for 60 min followed by a washing step with buffer 2. Finally, the immunosensor was incubated with 40 μL of anti-H-IgA-AP (1:25,000) or anti-H-IgG-AP (1:50,000) solutions for 60 min and

washed with buffer 3. The enzymatic reaction was carried out by placing a 40 μL aliquot of the 1.0 mM 3-IP / 0.4 mM silver nitrate solution on the sensor. AP was used as the enzymatic label and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^+) as the substrate. AP hydrolyzes 3-IP resulting in a indoxyl intermediate that will reduce the silver ions presents in solution resulting in metallic silver (Ag^0) and indigo blue (I) [26]. After 20 min, the redissolution peak of the silver enzymatically deposited on the electrode surface was recorded using an anodic stripping cyclic voltammetric scan from - 0.002 V to + 0.4 V at a scan rate of 50 mV s^{-1}.

2. **Results and discussion**

3.1. Optimization of the experimental conditions

Each variable involved in the construction of an immunosensor can influence the analytical response; therefore an optimization study was carried out using positive and negative control samples.

The adequate concentration of the antigen was evaluated in order to achieve the best relation between sensitivity and reproducibility. The data presented in Fig. 2 reveal that a gliadin concentration of 1 mg mL^{-1} is the most adequate because it presents the best compromise between the analytical and the background signals, and was selected for all subsequent studies.

In order to minimize nonspecific adsorption, the effect of different blocking agents was studied. In the case of the AGA IgA detection, the best analytical /background signal ratio was obtained with casein (2%). In the case of the AGA IgG analysis the free surface sites were effectively blocked with BSA (2%). With these blocking agents, matrix effects were found to be minimal. The influence of the secondary labelled antibody on the analytical signal was also investigated. The best relation between analytical and background signal was achieved for a dilution of 1:25,000 in the case of anti-H-IgA-AP (Fig. 3a) and 1:50,000 for anti-H-IgG-AP (Fig. 3b). These optimized conditions were used throughout the work. Fig. 4 shows typical cyclic voltammograms obtained for the detection of AGA IgA (Fig. 4a) and AGA-IgG (Fig. 4b) in positive control samples (solid lines) and negative control samples (dashed lines) in optimized conditions.

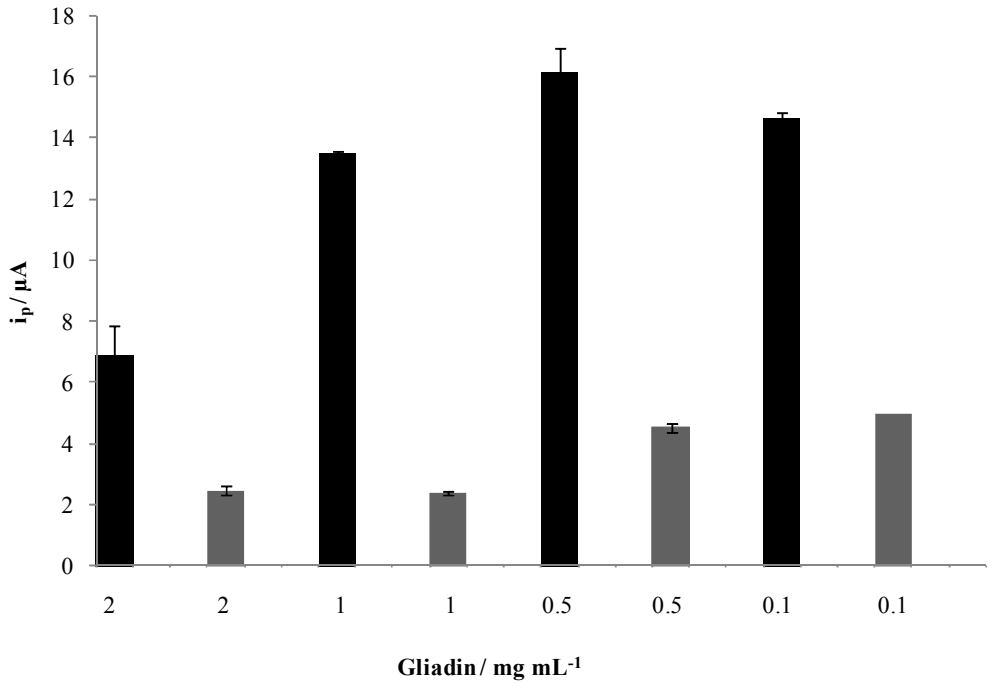
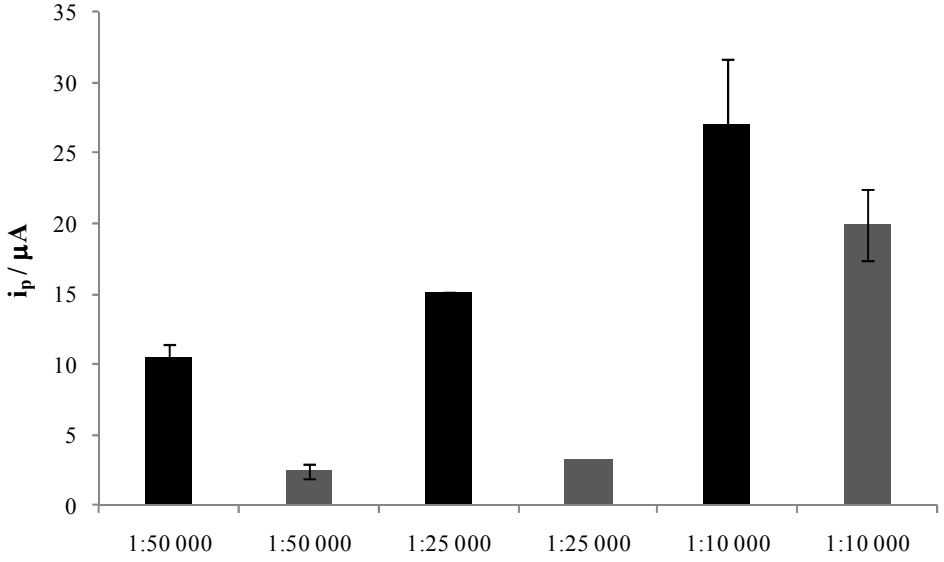


Fig. 2 Evaluation of the effect of the antigen concentration on the analytical signal using positive (black bars) and negative (grey bars) control samples. Experimental conditions: BSA 2%; anti-H-IgG-AP 1:150,000; 3-IP 1.0 mM; Ag^+ 0.4 mM. The positive and negative sera were diluted at 1:10. (Average data ± standard deviation are indicated (*n*=3))

a



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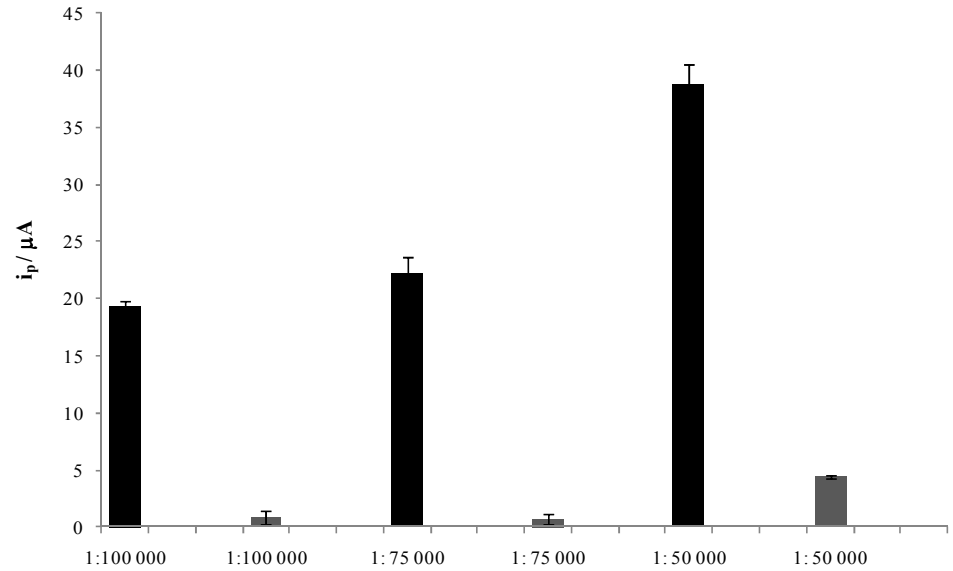
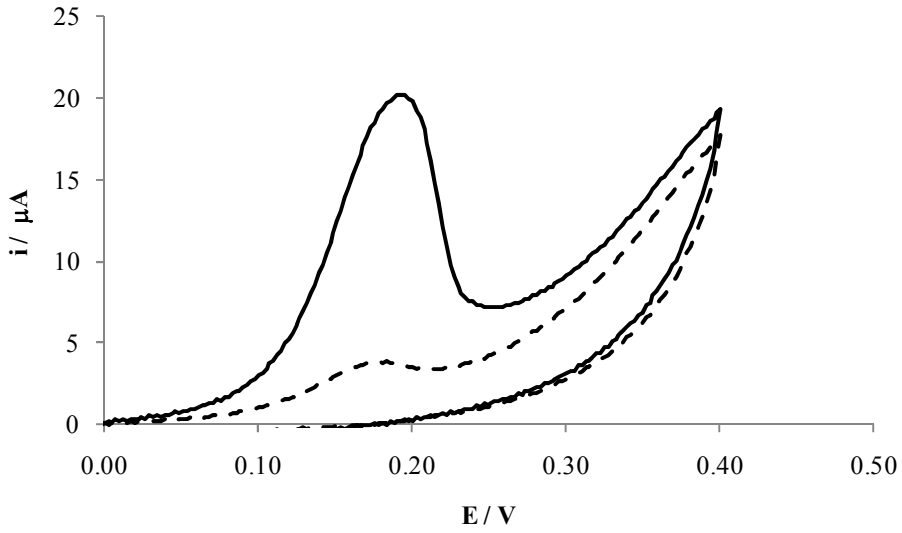


Fig. 3. Effect of the secondary labelled antibody on the analytical signal. (a) AGA-IgA positive (black bars) and negative (grey bars) control samples (1:2). (b) AGA-IgG positive (black bars) and negative (grey bars) control samples (1:2). Experimental conditions: 1 mg mL^{-1} gliadin adsorbed on the hybrid-modified electrode surface; casein 2% (a); BSA 2% (b); 3-IP 1.0 mM; Ag^{+} 0.4 mM. (Average data ± standard deviation are indicated (n=3)).

a



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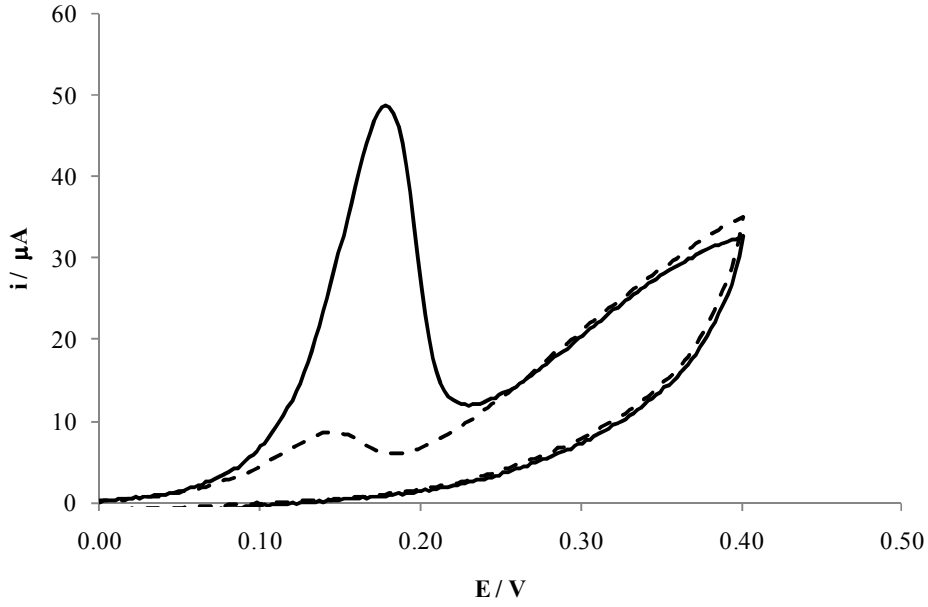
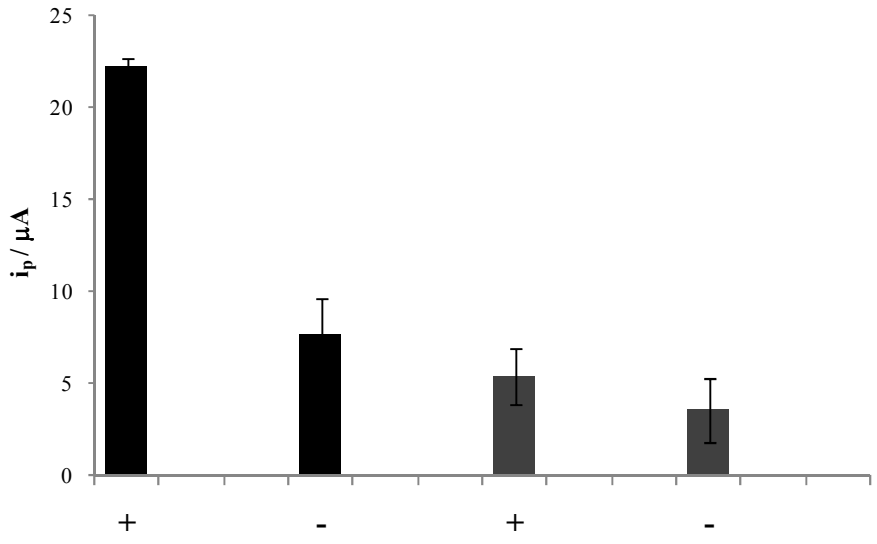


Fig. 4. Cyclic voltammograms obtained for an optimized assay. (a) AGA-IgA positive (solid line) and negative (dashed line) control samples (1:2). (b) AGA-IgG positive (solid line) and negative (dashed line) control samples (1:2). Experimental conditions: 1 mg mL^{-1} gliadin adsorbed on the hybrid-modified electrode surface; (a) casein 2% and anti-H-IgA-AP 1:25,000; (b) BSA 2% and anti-H-IgG-AP 1:50,000; 3-IP 1.0 mM; Ag^{+} 0.4 mM. Cyclic voltammetric scan from - 0.002 V to + 0.4 V at a scan rate of 50 mV s^{-1}.

3.2. Study of non specific adsorption and sensor biofunctionality

Regardless of the blocking agents' efficiencies the presence of several different immunoglobulins and other proteins in the serum samples can lead to non specific binding (NSB) and difficult the analytical detection of the designed immunosensor. Moreover, it is also important to ensure that both the antigen and the secondary labelled antibody specifically react with the target immunoglobulin (i.e. AGA class A and G). Hence, the background signal was evaluated using positive and negative serum samples for both AGA-IgA and IgG detection. The results (Fig. 5) showed that the analytes of interest, the human antibodies, are effectively captured by the antigen since the obtained i_p for a positive sample assayed with a gliadin-modified electrode is clearly different from the i_p obtained with a BSA-modified electrode (without gliadin). The analytical signal obtained with the sensor in the absence of gliadin for positive and negative samples is approximately equal and is similar to a negative sample assayed with the complete immunosensing strategy. These current intensities, in the order of 5-10 μA, therefore result from NSB.

a



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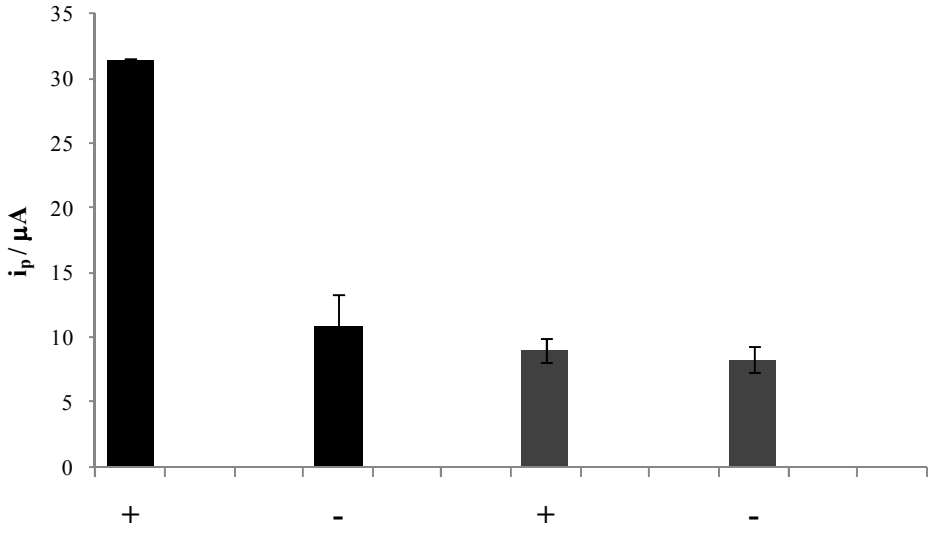


Fig. 5. Effect of non-specific adsorption on the analytical signal for the evaluation of (a) AGA IgA and (b) AGA IgG content in CD positive (+) and negative (-) serum samples with gliadin-modified (black bar) and BSA-modified (without gliadin) (grey bar) electrodes. Experimental conditions as in Fig. 3. (Average data ± standard deviation are indicated (n=3)).

3.3. Precision studies

The precision of the methodology was evaluated in terms of repeatability and reproducibility using a real positive sample. The repeatability was evaluated by performing five successive inter-electrodic measurements using separate immunosensors and the reproducibility was assessed through 2 inter-day evaluations (interval of one month). Relative standard deviation (RSD) values for the detection of AGA IgA (0.033) and for the detection of AGA IgG (0.035) were obtained when the repeatability was assayed. Regarding the reproducibility studies, the RSD values achieved for the detection of AGA IgA and AGA IgG were 0.025 and 0.002, respectively. The result indicates that the method provides precise results.

3.4. Calibration plot

Calibrator serum solutions provided by the acquired ELISA kits for AGA IgA and AGA IgG detection are real patient samples and contain antibody concentrations within the range of clinical interest. Under the optimized conditions and using these human serum sample calibrators (1:2 diluted), the following linear relationships between i_p and the immunonoglobulin content (in arbitrary units) were obtained:

i_p (μA) = (0.21 ± 0.13) [AGA IgA] + (5.06 ± 0.63) r = 0.997, n = 6

i_p (μA) = (0.49 ± 0.18) [AGA IgG] + (8.72 ± 0.87) r = 0.997, n = 6

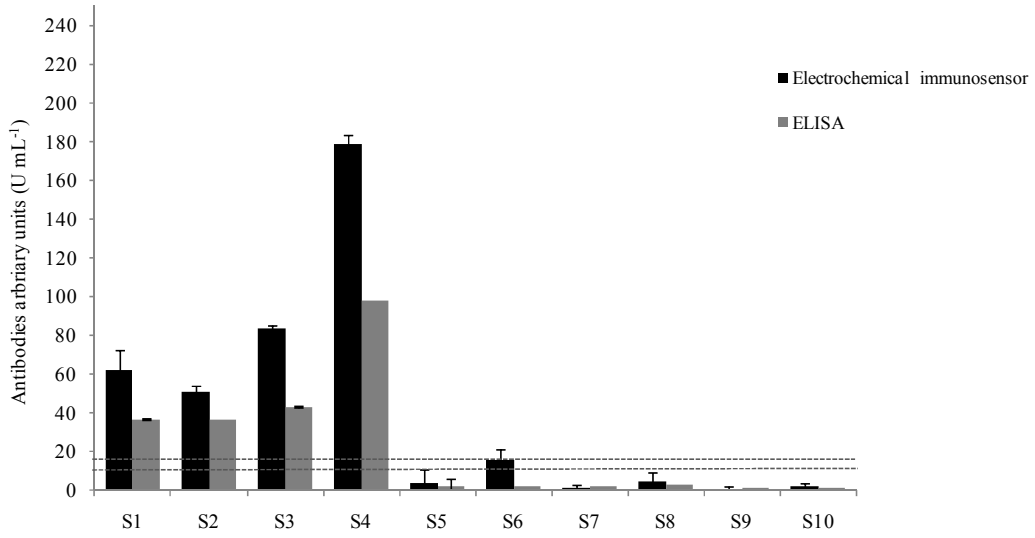
The limits of detection (LOD) and the limits of quantification (LOQ) were calculated from the calibration plots using the equations: LOD=3s/m and LOQ=10s/m (where s is the standard deviation of the intercept and m is the slope of the calibration plot) [27]. In case of AGA IgA detection, the LOD and LOQ values obtained were 9.1 U mL^{-1} and 30.4 U mL^{-1}, respectively. In case of AGA IgG detection, LOD and LOQ were found to be 9.0 U mL^{-1} and 31.2 U mL^{-1}, respectively.

3.5. Immunosensor assessment for the quantification of antigliadin antibodies in real samples

For the evaluation of the adequacy of the developed immunosensor in real samples, 10 different real serum samples were analyzed and the results were compared with those obtained with a commercial ELISA assay. According to the ELISA kit's instructions, serum calibrators are ready to use and patient's samples should be diluted 1:101. Serum calibrators assayed by the immunosensor were diluted 1:2. Therefore, in order to ensure the correct comparison of the results obtained with the immunosensor and the ELISA assay, patient's samples analyzed by the immunosensor were diluted 1:200. The i_p obtained with the immunosensor was extrapolated to the calibrator's linear ranges to convert the current data in arbitrary units. The recommended cut-off value by the kit fabricants was used to determine the clinical relevance of the results. The results obtained with the immunosensor were compared with those obtained with the ELISA test (Fig. 6). The data obtained indicate that samples 1, 2, 3 and 4 belong to CD patients, sample 5 to a CD patient with selective IgA deficiency but with positive results when class G antibodies were analyzed and the remaining samples correspond to healthy individuals. All the samples were qualitatively classified the same (positive or negative) by both analytical tools. Therefore, regarding the qualitative analysis both methods were in agreement. On the other hand, in the quantitative analysis of the immunoglobulin's content a straight correlation between the results was not observed. In fact in almost every case, except for

S7 and S9 (Fig. 6a) and S7 (Fig. 6b), the immunosensor measures a higher amount of AGAs. These differences could be due to the different sample dilutions. While ELISA is performed according to the instructions of the manufacturer, the electrochemical immunoassay is performed with similar conditions to those used in the ELISA, trying to mimic it. Possibly these parameters should be optimized for the immunosensor regardless of the ELISA's conditions. Nonetheless, the methodologies have different characteristics, so the similarity achieved in the measurements is very acceptable.

a



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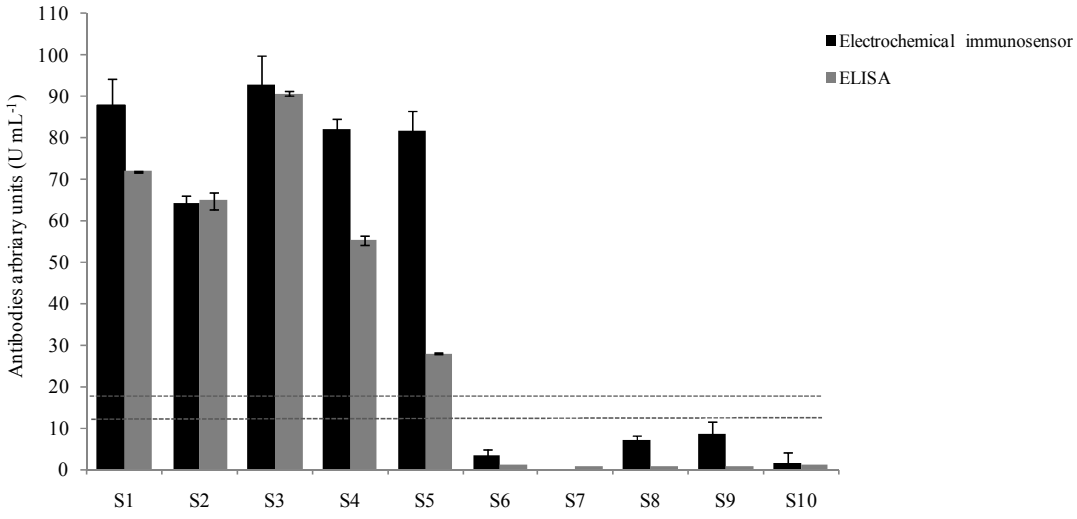

Fig. 6. Comparison of results obtained for real samples assayed with the proposed electrochemical immunosensors and with a standard ELISA kit. (a) AGA IgA and (b) AGA IgG. The presented cut-off levels (11-17 U mL^{-1}) are the recommended by the ELISA kits.

4. Conclusions

A novel electrochemical immunosensor based on a nanohybrid analytical transducer platform for the detection of human antibodies directed against gliadin in real samples was developed. Several parameters involved in the immunosensing strategy, such as the blocking agent and the antigen and AP labelled anti-human IgA and IgG antibody concentrations, were optimized leading to an excellent analytical performance with no significant non-specific adsorptions. Precision studies also indicate that the proposed methodology is reproducible and presents a good repeatability. Real patient's samples were analyzed with the electrochemical immunosensor and the results obtained were corroborated with a commercial ELISA kit. A correlation between the average values obtained with the sensor (in arbitrary units) and those obtained with the ELISA was also investigated. The results showed some disparity, since the content of AGA IgA and IgG in the sera obtained by the sensor was, in general, higher than that obtained with the ELISA. However, in a methodology that intends to be a first-line diagnostic test for an autoimmune disease the qualitative analysis is much more important than the quantitive analysis. Therefore, in comparison with the conventional ELISA-based analysis, the developed electrochemical immunosensor is a reliable, low cost and simple methodology, being a perfect option as first platform for future transference to a point-of-care analytical device for celiac disease diagnosis. The implementation of this methodology to the development of an electrochemical bi-immunosensor for the simultaneous detection of autoantibodies against gliadin and tissue transglutaminase will be evaluated.

Acknowledgments

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Multiplexed Electrochemical Immunosensor for Detection of Celiac Disease Serological Markers

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Abstract

Celiac disease (CD) is a gluten-induced autoimmune enteropathy characterized by the presence of antibodies against gliadin (AGA) and anti-tissue transglutaminase (anti-tTG) antibodies. A disposable electrochemical dual immunosensor for the simultaneous detection of IgA and IgG type AGA and anti-tTG antibodies in real patient's samples is presented. The proposed immunosensor is based on a dual screen-printed carbon electrode, with two working electrodes, nanostructured with a carbon-metal hybrid system that worked as the transducer surface. The immunosensing strategy consisted of the immobilization of gliadin and tTG (i.e. CD specific antigens) on the nanostructured electrode surface. The electrochemical detection of the human antibodies present in the assayed serum samples was carried out through the antigen-antibody interaction and recorded using alkaline phosphatase labelled anti-human antibodies and a mixture of 3-indoxyl phosphate with silver ions was used as the substrate. The analytical signal was based on the anodic redissolution of enzymatically generated silver by cyclic voltammetry. The results obtained were corroborated with commercial ELISA kits indicating that the developed sensor can be a good alternative to the traditional methods allowing a decentralization of the analyses towards a point-of-care strategy.

Keywords: Celiac disease; Tissue transglutaminase; Gliadin; Electrochemical immunosensors; Nanomaterials; Multiplexed detection.

Abbreviations

CD – celiac disease

EIs – electrochemical immunosensors

AGA – anti-gliadin antibodies

Anti-H-IgA-AP – anti-human IgA antibodies conjugated with alkaline phosphatase

Anti-H-IgG-AP – anti-human IgG antibodies conjugated with alkaline phosphatase

SPCEs – screen-printed carbon electrodes

MWCNTs – carboxylated multiwalled carbon nanotubes

NPAus – gold nanoparticles

1. Introduction

Celiac disease (CD) is a disorder of the small intestine caused by an inappropriate immune response to wheat gluten and similar proteins of barley and rye in genetically susceptible individuals [1]. The humoral autoimmune response leads to an abnormal intestinal mucosa characterized by villous atrophy and crypt hyperplasia, resulting in malabsorption related problems [2]. Common serological changes of this condition include the appearance of antibodies against gliadin (AGA) and anti-tissue transglutaminase (anti-tTG) antibodies; which are specific serological markers of the disease. Is reported that immunoglobulin A (IgA) anti-tTG antibody detection has sensitivity higher than 90% and specificity higher than 95%; IgA AGA detection presents a sensitivity of about 80% and a specificity ranging from 80 to 90% [3]. The IgA isotype is considered to be the most specific; however, selective IgA deficiency affects about 2–5% of patients diagnosed with CD [4]. In these cases, the determination of the immunoglobulin G (IgG) class of antibodies is considered [5].

The gold standard diagnosis for CD which relies on a intestinal biopsy has been changing over the last few decades, especially due to the advent of serological tests with high sensitivity and specificity [1]. Serological markers can be used in front-line detection strategy to rule out the pathology.

Nowadays, analytical techniques are moving towards the development of more simple, fast, and point-of-care (POC) analyses. Therefore, electrochemical biosensors are playing a growing part in many fields in which a more accurate, sensitive, fast, low cost and, specially, *in situ* analysis is required [6]. Electrochemical biosensors are in many cases the best option for diagnostics given their high selectivity and sensitivity which allows early detection of many diseases. Among the different types of electrodes that are employed in the construction of electrochemical biosensors, screen-printed electrodes (SPEs) are of special interest. Their properties, such as simplicity and low cost, versatility of design, small dimensions and possibility of incorporation in portable systems, as well as adequate electroanalytical characteristics, have progressively introduced SPEs in fields such as environmental analysis, food quality control, and clinical diagnostics [7, 8].

In recent years, the development of electrochemical transducers that make use of electrode surface modifications with nanomaterials became an exciting area of development for modern analytical science [9, 10, 11]. The use of nanomaterials extends the potentialities of these electrodes by improving their original electrochemical properties. For this reason, carbon nanostructure/ metal nanoparticle hybrid systems have been recently exploited as last generation transducers for the development of biosensors [12, 13, 14], because a synergic effect of each material property is obtained [15]. Within the electrochemical biosensors field, electrochemical immunosensors (EIs) combining the specificity inherent to antigen–antibody interactions with the high sensitivity of electrochemical transduction [16], can be an excellent alternative to conventional immunochemical tests. Therefore, a dual immunosensor for the simultaneous detection of AGA and anti-tTG antibodies was developed. A dual screen-printed carbon electrode (SPCE) nanostructured with a carbon-gold hybrid system was employed as the transducer surface. The immunosensing strategy consisted of the immobilization of gliadin and tTG (i.e. CD specific antigens) on the nanostructured electrode surface followed by the electrochemical detection of the human antibodies present in real serum samples. The antigen-antibody interaction was recorded using alkaline phosphatase labelled anti-human antibodies and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^{+}) was used as the substrate [17]. The analytical signal was based on the anodic redissolution of enzymatically generated silver by cyclic voltammetry. As far as we are concerned, there is no record of an EI for the simultaneous detection of the referred antibodies.

The published works regarding EIs for CD clinical diagnosis that are currently available are focused on the detection of just one of the biomarkers. [12, 13, 18-25].

2. Materials and methods

2.1. Instrumentation

Voltammetric analysis was performed with an Autolab PGSTAT 12 (Eco Chimie B.V., The Netherlands) potentiostat interfaced to an AMD K-6 266 MHz computer system and ntrolled by Autolab GPES 4.8 software (version for Windows 98). Dual SPCEs and a specific connector were purchased from DropSens (Spain). The dual SPCEs comprises two ellipse-shaped carbon working electrodes (6.3mm^2 each one), a carbon counter electrode and a silver pseudo-reference electrode, all of them screen-printed on a ceramic substrate (3.4cm × 1.0 cm).

2.2. Reagents and solutions

Carboxyl modified multiwalled carbon nanotubes (MWCNTs) were purchased from Nanocyl (Belgium). Standard gold tetrachloroaurate ($AuCl_4^-$) and silver nitrate were obtained from Merck (Germany). Biosynth (Switzerland) supplied the 3-indoxyl phosphate disodium salt. Human tissue transglutaminase (recombinantly produced in insect cells) was purchased from Zedira (Germany). Wheat gliadin was provided by Sigma (Spain). Goat anti-human IgG (Fc specific) conjugated with alkaline phosphatase (anti-H-IgG-AP) was purchased from Sigma (Spain) and goat anti-human IgA (alpha chain specific) labelled with alkaline phosphatase (anti-H-IgA-AP) was provided by Invitrogen (Spain). The validation of the developed procedure was performed with anonymous sera samples collected in the "Hospital Universitario Central de Asturias" (Spain), which were analysed by Varelisa Celikey, Varelisa Celikey IgG, VARELISA Gliadin IgA Antibodies and VARELISA Gliadin IgG Antibodies ELISA kits, all supplied by Phadia (Germany). Each kit contained six standard serum samples (0, 3, 7, 16, 40 and 100 U mL^{-1}) and a positive and a negative control. The kit's manufacturers proposed a range of values with clinical significance for the analysis of the samples. According to the kit's instructions an equivocal result should be considered between 5 and 8 U mL^{-1} in the case of anti-tTG IgA detection (Varelisa Celikey); between 7 and 10 U mL^{-1} for anti-tTG IgG determination (Varelisa Celikey IgG) and for VARELISA Gliadin IgA Antibodies and VARELISA Gliadin IgG Antibodies the range of 11 and 17 U mL^{-1} was recommended as equivocal. Below and above these values the samples are classified as negative or positive, respectively, for CD. Ultrapure water obtained with a Millipore Direct-QTM purification system from Millipore Ibérica S.A. (Spain) was used throughout this work. All chemicals employed were of analytical reagent grade. Working solutions of tTG and gliadin were prepared in a 0.1 M

Tris-HNO_3 pH 7.2 buffer. Working solutions of the secondary alkaline phosphatase labelled antibodies were prepared in 0.1 M Tris-HNO_3 pH 7.2 containing 2 mM $Mg(NO_3)_2$. A solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate was prepared daily in 0.1 M Tris-HNO_3 pH 9.8 and 20 mM $Mg(NO_3)_2$ and stored in opaque tubes at 4ºC. The MWCNTs solutions were prepared by dilution of the concentrated solution with a mixture of DMF:water (1:1). The gold tetrachloroaurate solution was prepared in 0.1 M HCl.

2.3. Electrodes nanostructuration

Single-use dual SPCEs were modified with 2 μL of a 0.1 mg mL^{-1} MWCNTs dispersion and the solution was left to dry at room temperature until its complete evaporation. Then, the MWCNTs-modified electrode was carefully washed with water and dried at room temperature. The coating process was followed by *in situ* electrochemical deposition of gold nanoparticles (NPAus) by applying a constant current intensity of – 5 μA for 60 s in an acidic solution of 0.1 mM $AuCl_4^-$ [15].

2.4. *Construction of the dual immunosensor and electrochemical detection*

The immunosensing strategy (Fig. 1) comprise the following steps: the dual SPCE–MWCNT–NPAus working electrodes (WE) were coated with 4 μL of 1 mg mL^{-1} gliadin solution (WE1) and with 4 μL of a 0.1 $\mu g.\mu L^{-1}$ tTG solution (WE2) and left to incubate overnight at 4 ◦C. The electrodes were washed using 0.1M Tris–HNO_3 pH 7.2 buffer. Free surface sites were blocked with 60 μL of a bovine serum albumin (BSA) solution for 30 min, followed by a second washing step with the same buffer. Then, the immunosensor was incubated with human serum samples for 60 min followed by a washing step with a 0.1 M Tris–HNO_3 pH 7.2 buffer containing 2 mM $Mg(NO_3)_2$. Finally, the immunosensor was incubated with an anti-H-IgA-AP (1:25,000) or an anti-H-IgG-AP (1:50,000) solution (both volumes of 60 μL) for 60 min and washed with a 0.1 M Tris pH 9.8 buffer containing 20 mM 2 mM $Mg(NO_3)_2$. The enzymatic reaction was carried out by dropping a 60 μL aliquot of a solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate on the immunosensor's surface. The enzymatically deposition of metallic silver catalysed by alkaline phosphatase (AP) was already reported [17]. After 20 min of reaction, a cyclic voltammogram was recorded from – 0.002 V to + 0.4V, at scan rate of 50 mV s^{-1}, to obtain the electrochemical oxidation current of the enzymatically deposited silver.

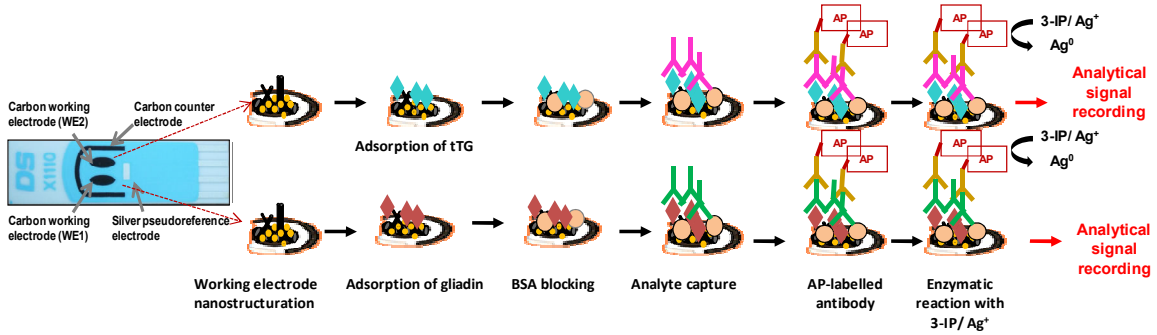

Fig. 1. Schematic representation of the immunosensing strategy followed for the analytical detection of CD serological markers.

3. Results and discussion

3.1. Optimization studies

For the optimization procedure were taken in account two important factors of the immunosensing strategy: the antigen´s concentration and the influence of the concentration of the labelled secondary antibody on the analytical signal. Regarding the detection of anti-tTG IgA (Fig. 2 a1) and AgA IgA (Fig. 2 b1) the optimal amount of tTG and gliadin to be immobilized on the surface was 0.1 μg μL^{-1} and 1 mg mL^{-1}, respectively, because for this values was verified the best relation between the analytical and the background signal. In case of anti-tTG IgG (Fig. 2 a2) and AGA IgG (Fig. 2 b2), equal optimum amounts of tTG and gliadin to be immobilized on the surface were selected for the same reasons.

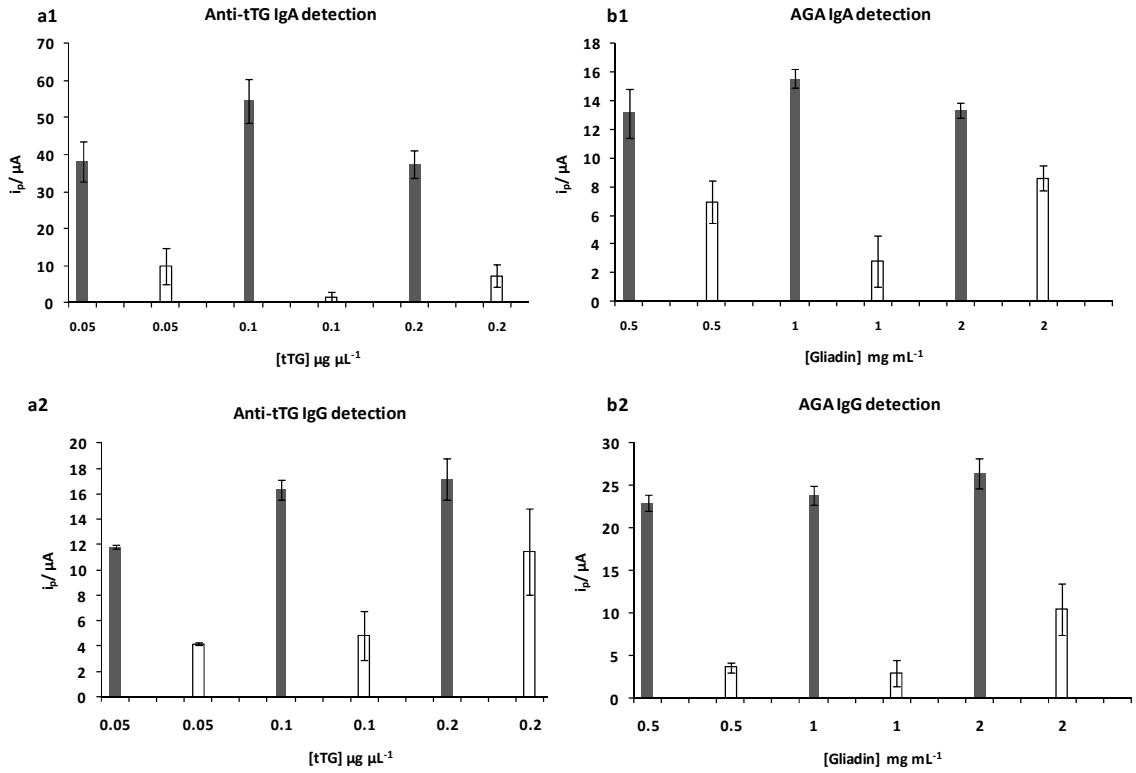

Fig. 2. Effect of the different concentrations of the antigen (tTG: a1 and a2; gliadin: b1 and b2) on the analytical signal for the detection of the corresponding immunoglobulins class A (a1 and b1) and class G (a2 and b2). Experimental conditions: BSA 2%; positive (grey bars) and negative (white bars) control samples (1:2); anti-H-IgA-AP 1:30,000 (a1 and b1) and anti-H-IgG-AP 1:50,000 (a2 and b2); 3-IP 1.0 mM; Ag^+ 0.4 mM. Average data ± standard deviations are indicated (n = 3).

The influence of the secondary labelled antibody on the analytical signal was also investigated. The influence of four different concentrations of anti-H-IgA-AP antibody (1:5000; 1:10,000; 1:25,000; 1:50,000) and anti-H-IgG-AP (1:10,000; 1:25,000; 1:50,000; 1:100,000) were investigated using a positive and a negative control samples. As can be observed in Fig. 3, in all cases, as the analytical signal corresponding to the assay with the positive control (grey squares) increases, the signal obtained with the negative control (white squares) also increases due to the augment of non-specific binding. Therefore, the best compromise between both signals was achieved for a dilution of 1:25,000 in the case of anti-H-IgA-AP and 1:50,000 for anti-H-IgG-AP. Finally, in order to minimize non-specific adsorption, albumin (2%) was employed as blocking agent (results not shown). These optimized conditions were used throughout the work. Typical cyclic voltammograms obtained for the detection of AGA IgA and anti-tTG IgA and AGA IgG and anti-tTG IgG in positive and negative samples, in optimized conditions, can be observed in

Fig. 4. This figure also gives the results obtained with a sample whose donor is a celiac patient with selective IgA deficiency. As can be observed the dual-sensor gives a signal corresponding to a negative sample in case of IgA detection and a positive result when immunoglobulin G are assayed.

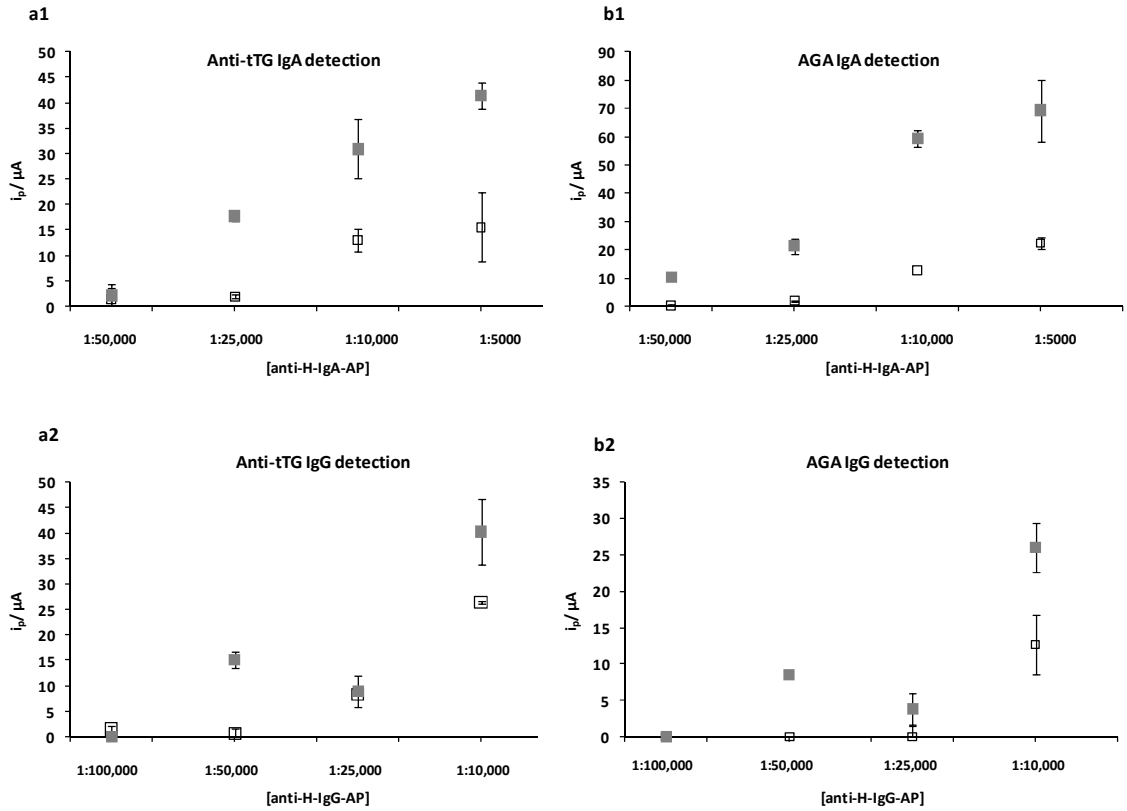

Fig. 3. Influence of the secondary AP-labelled antibody on the analytical signal. Experimental conditions: tTG 0.1 µg µL^{-1}; gliadin 1 mg mL^{-1}; BSA 2%; positive (grey squares) and negative (white squares) control samples (1:5); 3-IP 1.0 mM; Ag^+ 0.4 mM. Average data ± standard deviations are indicated (n = 3).

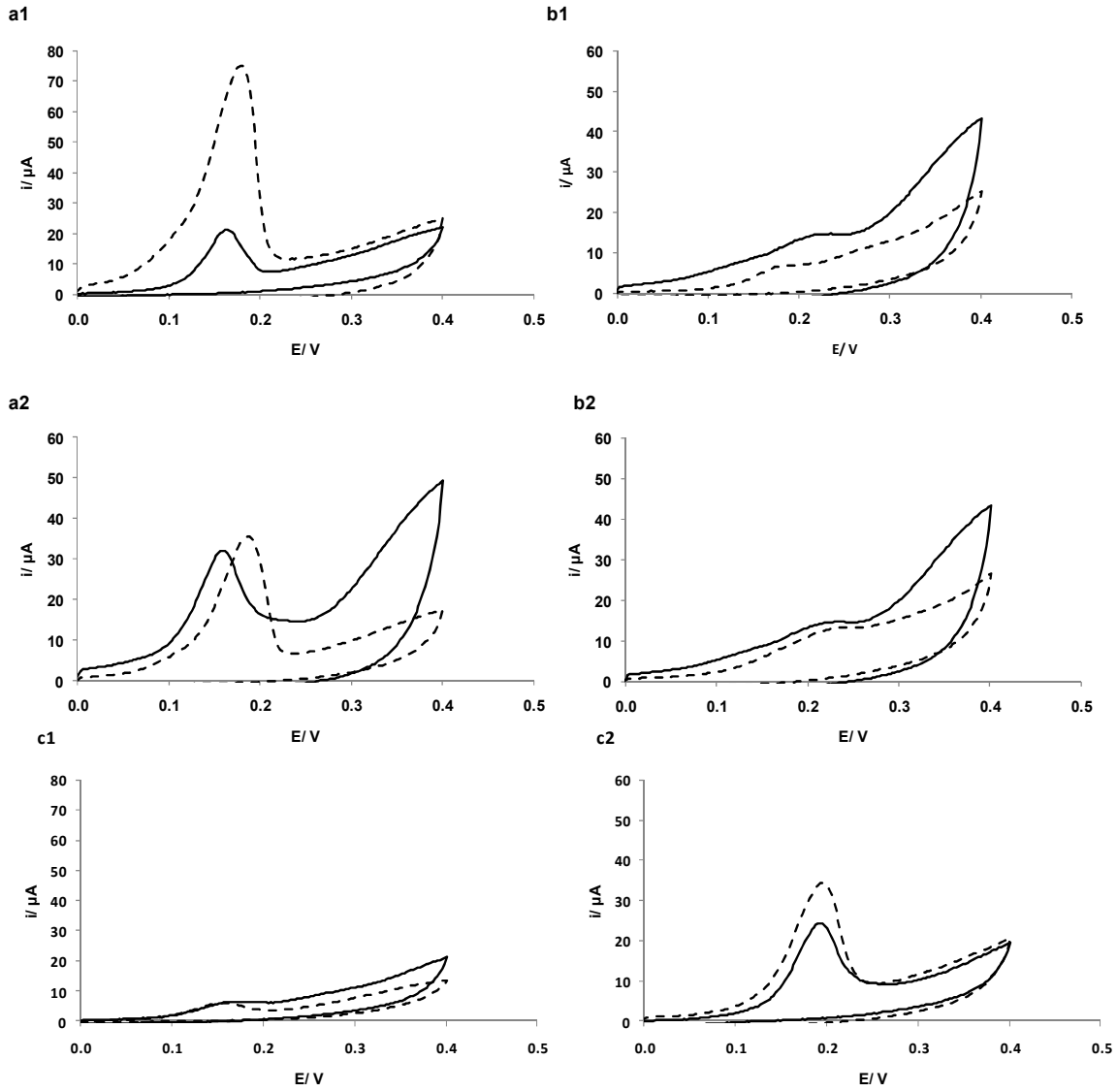

Fig.4. Cyclic voltammograms obtained for an optimized assay: (a1) AGA-IgA positive (solid line) and anti-tTG IgA positive (dashed line) samples; (b1) AGA-IgA negative (solid line) and anti-tTG IgA negative (dashed line) samples. (a2) AGA-IgG positive (solid line) and anti-tTG IgG positive (dashed line) samples; (b2) AGA-IgG negative (solid line) and anti-tTG IgG negative (dashed line) samples. In (c) are presented cyclic voltammograms obtained in the analysis of a sample of a CD patient with selective IgA deficiency: (c1) AGA-IgA detection (solid line) and anti-tTG IgA detection (dashed line); (c2) AGA-IgG detection (solid line) and anti-tTG IgG detection (dashed line) samples. Experimental conditions: tTG 0.1 µg µL^{-1}; gliadin 1 mg mL^{-1}; BSA 2%; serum samples diluted 1:200; anti-H-IgA-AP 1:25,000 (a) and anti-H-IgA-AP 1:50,000 (b); 3-IP 1.0 mM; Ag^{+} 0.4 mM.

3.2. *Precision studies*

The precision of the methodology was evaluated in terms of reproducibility which was assessed through 3 inter-day evaluations. In case of IgA detection, relative standard deviation (RSD%) values of 7.50 % and 3.72 % were obtained for AGA and anti-tTG detection, respectively. RSD (%) values of 7.18 % and 4.32 % were obtained for the detection of AGA and anti-tTG type G immunoglobulins, respectively.

3.3. Calibration plot

Calibrator serum solutions provided by the acquired ELISA kits are real patient samples and contain antibody concentrations within the range of clinical interest. Under the optimized conditions and using these human sera sample calibrators (1:2 diluted), a linearity between peak current intensity and the immunonoglobulin content (in arbitrary units) was achieved between 0 and 100 U mL^{-1}. The linear relationships of the method is given by the following equations:

$$_{Log}i_p(\mu A)=(4.7x10^{-1} \pm 8.5x10^{-4})_{Log}[\text{tTG IgA}] + (7.1\ x10^{-1} \pm 1.3\ x10^{-3})\ r=0.995,\ n=6$$

$$_{Log}i_p(\mu A)=(6.3x10^{-1} \pm 2.3x10^{-3})_{Log}[\text{tTG IgG}] + (3.2\ x10^{-1} \pm 5.6\ x10^{-3})\ r=0.998,\ n=6$$

$$_{Log}i_p(\mu A)=(6.5x10^{-1} \pm 5.2x10^{-3})_{Log}[\text{AGA IgA}] - (2.0\ x10^{-2} \pm 7.1\ x10^{-3})\ r=0.986,\ n=6$$

$$_{Log}i_p(\mu A)=(6.0x10^{-1} \pm 2.7x10^{-2})_{Log}[\text{AGA IgG}] - (3.3\ x10^{-1} \pm 4.0\ x10^{-2})\ r=0.994,\ n=6$$

The limits of detection (LOD) and the limits of quantification (LOQ) were calculated from the calibration plots using the equations: LOD = 3 s/m and LOQ = 10 s/m (where s is the standard deviation of the intercept and m is the slope of the calibration plot) [26]. In case of tTG IgA detection, the LOD and LOQ values obtained were 2.45 U mL^{-1} and 20.42 U mL^{-1}, respectively. In case of tTG IgG detection, LOD and LOQ were found to be 2.95 U mL^{-1} and 37.15 U mL^{-1} respectively. LOD and LOQ values were 3.16 U mL^{-1} and 46.77 U mL^{-1} for AGA IgA detection and 2.82 U mL^{-1} and 32.36 U mL^{-1}, respectively, for AGA IgG determination.

3.4. Comparison of the response of the dual sensor with the commercial ELISA

In order to establish an agreement between the dual sensor and a commercial methodology available, the performance of the electrochemical immunosensor was evaluated against the already referred ELISAs. Twelve different real samples were analysed with both methodologies. In order to correlate the results obtained with the sensor with the data obtained with the ELISA, the currents obtained with the electrochemical immunosensor for each sample were extrapolated to the corresponding calibration plot (Section 3.3.) to obtain equivalent arbitrary units to those found when analysing the same samples by commercial ELISA kits. The results, presented in Fig. 5, showed that three samples were positive for celiac disease (S1–S3) and the other three (S4-S6) were negative. The results revealed that with the dual sensor gave were obtained, in general, some slightly higher values than the obtained with the spectrofotometric assay. Nevertheless, the comparison of the results between the electrochemical immunosensor and commercial kits indicate an excellent degree of correlation between both methodologies.

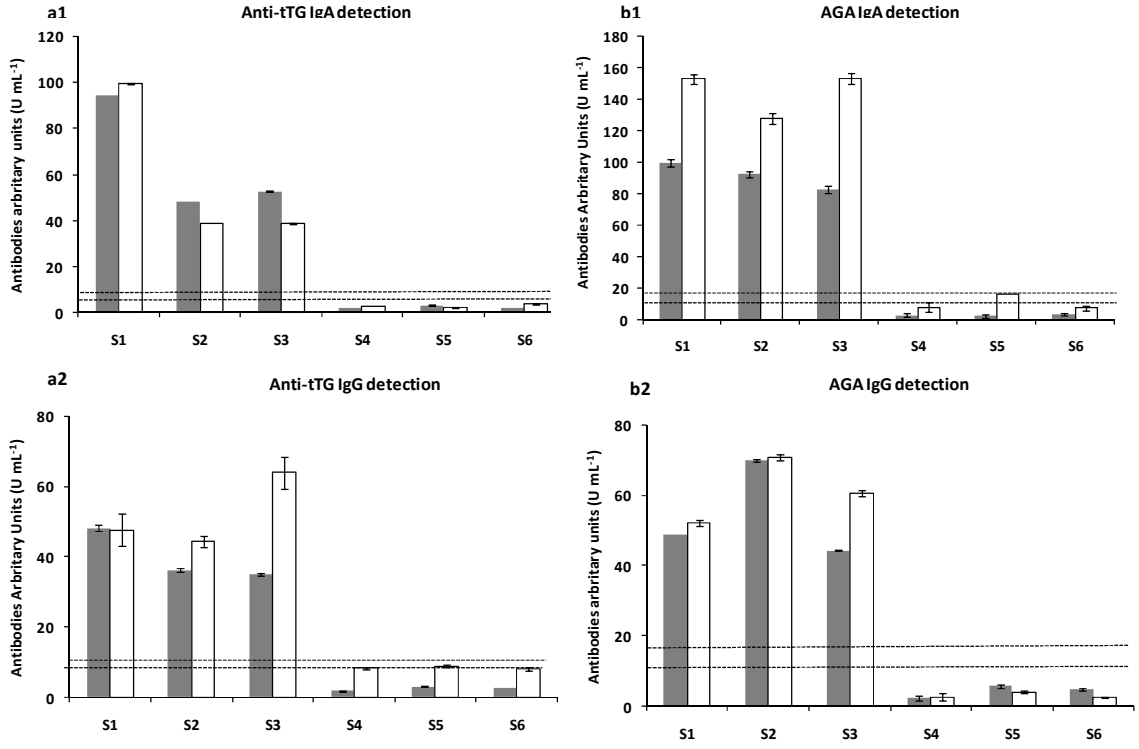

Fig.5. Comparison of results obtained for real samples assayed with a standard ELISA kit (grey bars) and the proposed dual-electrochemical immunosensor (white bars). The dual sensor performed a simultaneous detection of immunoglobulins type A (tTG (a1) and AGA (b1)) and immunoglobulins type G (tTG (a2) and AGA (b2)). The presented cut-off levels (a1: 5-8 U mL^{-1}; a2: 7-10 U mL^{-1}, b1 and b2: 11-17 U mL^{-1}) are the recommended by the ELISA kits. Sensor's experimental conditions as in Fig. 4.

3.5. Assessment of real serum samples with the dual-electrochemical immunosensor

In CD clinical diagnosis the most important analytical aspect is verifying the presence or the absence of CD serological markers in patient´s samples. Therefore a qualitative analysis based on a binary yes/no format is sufficient to determine the analytical parameters of this autoimmune condition. To achieved this goal, a cut-off line (see Fig. 6. horizontal dotted and dashed lines) was determine as the average peak current intensity plus three times the standard deviation obtained for 15 negative samples of healthy individuals. The dual immunosensor was then applied to the simultaneous analysis of 12 different human sera samples from CD patients and healthy individuals for the detection of the serological markers in study. The results are presented in Fig. 6 and show that six samples were positive for celiac disease (1–6) and the other six samples (7-12) assayed remained below the cut-off line. The results were corroborated with the commercial ElISA kits.

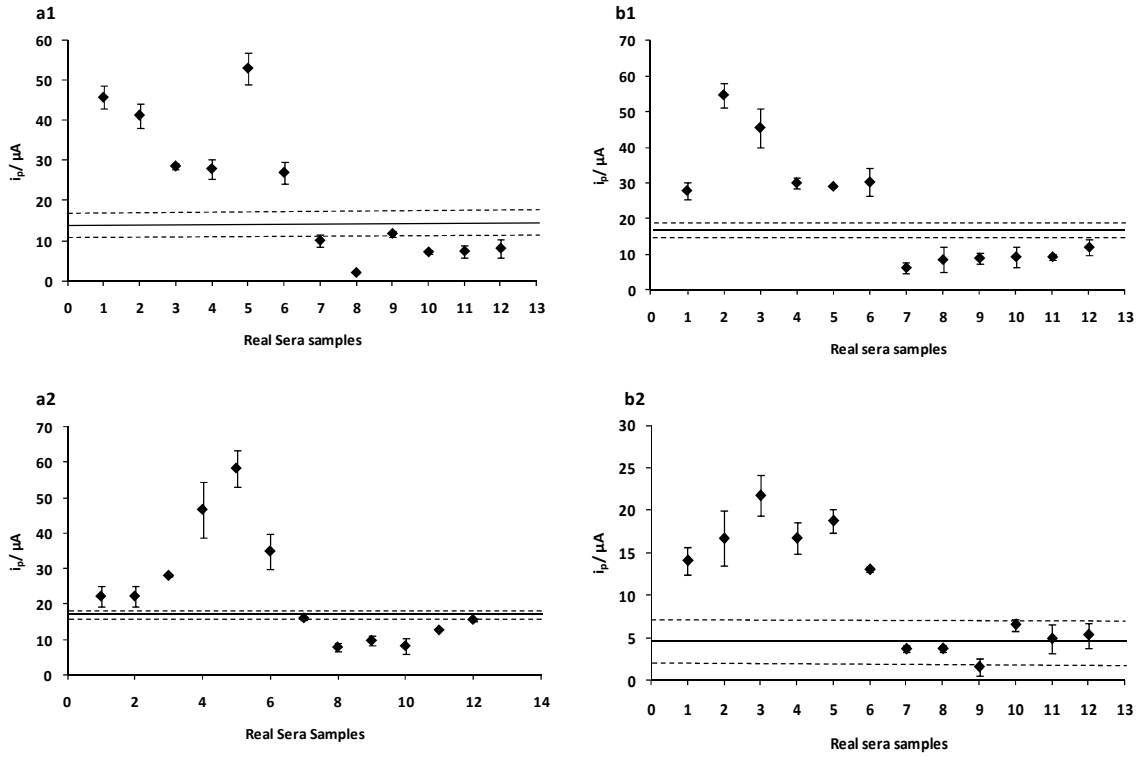

Fig.6. Evaluation of 6 positive and 6 negative human samples for the simultaneous detection of class A immunoglobulins, tTG IgA (a1) and AGA IgA (b1), and class G immunoglobulins, tTG IgG (a2) and AGA IgG (b2), using the optimized electrochemical immunosensor. Optimized conditions as in Fig. 4. (average data ± standard deviations are indicated (n = 3)).

4. Conclusions

The new tendencies in analytical chemistry are moving towards the development of simple, fast and *in situ* diagnostic devices. Electrochemical immunosensors offer exciting opportunities for numerous decentralized clinical applications and the development of disposable screen-printed electrodes was an important contribution due to their electrical properties and small size. Therefore, in this work, a disposable dual-electrochemical immunosensor for the simultaneous detection of two significant serological markers of celiac disease was developed. Aspects as the optimization of the recognition element (e.g. antigen), the detection labelled antibody and the reproducibility of the sensor were investigated. The sensor unequivocally assayed 12 real samples from healthy and celiac individuals, whose veracity was corroborated with a commercial ElISA Kit. Moreover, a correlation between the average values obtained with the sensor (in arbitrary units) and those obtained with the ELISA for the same samples was also achieved. The results show that both methodologies are in good agreement. The proposed sensor is a sensitive and effective methodology to the simultaneous determination of IgA and IgG antibodies against gliadin and tissue-transglutaminase. In future, the reduction of the steps in the construction of the sensors will be evaluated.

Acknowledgments

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Detection of antibodies against deamidated gliadin in human serum samples

2012

(Manuscript in preparation)

Brief introduction

Celiac disease (CD) is caused by a genetically determined specific immune response to antigens present in gluten. Gliadins, namely, α-gliadins, are considered the main agent in the autoimmune response [1]. This immune response may be focused on a limited region of the α-gliadin component of gluten, and previous studies have suggested that the generation of epitopes for recognition by CD4+ T cells requires deamidation of the protein by tissue transglutaminase (tTG). tTG catalyzes gliadin deamidation in the intestinal mucosa of celiac disease patients, resulting in deamidated gliadin peptides (DGP) which are recognized by human leucocyte antigen, HLA-DQ2, receptors of immune cells. However, it had not previously been shown that candidate epitope peptides could be generated from gluten *in vivo*, or that these epitopes were selective products of physiological digestion of gluten by tissue transglutaminase [2].

By mimicking the enzymatic gastrointestinal digestion of a representative α-gliadin was found that a 33-mer peptide (LQLQPFPQPQLPYP QPQLPYPQPQLPYPQPQPF; α2-gliadin 56–88) containing known peptide epitopes is generated by digestion with intestinal enzymes *in vivo* and *in vitro*, producing a highly stimulatory antigen for CD4+ T cells [3]. This fragment was resistant to further breakdown by luminal proteases and intestinal brush-border enzymes due to its high proline content. The 33-mer contains six partly overlapping copies of three DQ2-restricted T cell alpha epitopes [4].

The detection of anti- gliadin antibodies (AGA) begin to be in disuse because AGA test lacks of specificity in comparasion with anti-tTG antibodies assays. This disadvantage was overcome by the introduction of DGP as antigen. Therefore deamidated gliadin antibodies, especially class G immunoglobulins (IgG), are specific for CD and their detection of outmust interest [5]. At the moment, there are any publications in literature regarding electrochemical immunosensors for CD diagnosis using anti-deamidated gliadin antibodies as serological markers. Therefore, an experimental work to achieve this purpose has been started within the scope of this dissertation; however the results presented here still are preliminary.

Experimental

Apparatus and reagents

Voltammetric analysis was performed with an Autolab PGSTAT 12 (Eco Chimie B.V, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266 MHz computer system and controlled by Autolab GPES 4.8 software (version for Windows 98). Screen-printed carbon electrodes (SPCEs) and a specific connector were purchased from DropSens (Spain). Carboxyl modified multiwalled carbon nanotubes (MWCNTs) were purchased from Nanocyl (Belgium). Standard gold tetrachloroaurate ($AuCl_4^-$) and silver nitrate were obtained from Merck (Germany). Biosynth (Switzerland) supplied the 3-indoxyl phosphate disodium salt (3-IP). A fusion protein of 4 deamidated gliadin peptides (33 kDa) (DGPx4: carrier-33merDGP - 26merDGP - DQ2-γ1 - DQ2-γ2) was purchased from Zedira (Germany). Goat anti-human IgG (Fc specific) conjugated with alkaline phosphatase (anti-H-IgG-AP) was purchased from Sigma (Spain). The sensor was assayed with anonymous sera samples collected in the "Hospital Universitario Central de Asturias" (Spain), previously analysed with VARELISA Gliadin IgG Antibodies ELISA kit supplied by Phadia (Germany). Ultrapure water obtained with a Millipore Direct-Q™ purification system from Millipore Ibérica S.A. (Spain) was used throughout this work. All chemicals employed were of analytical reagent grade.

Procedure

Single-use SPCEs were modified with 4 μL of a 0.1 mg mL^{-1} MWCNTs dispersion and the solution was left to dry at room temperature until its complete evaporation. Then, the MWCNTs-modified electrodes were carefully washed with water and dried at room temperature. The coating process was followed by in situ electrochemical deposition of gold nanoparticles (NPAus) by applying a constant current intensity of −5 μA for 60 s in an acidic solution of 0.1 mM $AuCl_4^-$ [6].

The immunosensing strategy comprises the following steps:

- The SPCE–MWCNT–NPAus working electrodes (WE) were coated with 10 μL of 0.30 $\mu g.\mu L^{-1}$ DPGx4 solution and left to incubate overnight at 4 ◦C.
-Washing of the electrode using 0.1M Tris–HNO_3 pH 7.2 buffer.
- Free surface sites were blocked with 40 μL of 2% bovine serum albumin (BSA) solution for 30 min.

- The immunosensor was incubated with human serum samples for 60 min followed by a washing step with a 0.1 M Tris–HNO_3 pH 7.2 buffer containing 2 mM $Mg(NO_3)_2$.
- Finally, the immunosensor was incubated with an anti-H-IgG-AP (1:50,000) solution (40 μL) for 60 min and washed with a 0.1 M Tris pH 9.8 buffer containing 20 mM $Mg(NO_3)_2$.
- The enzymatic reaction was carried out by dropping a 40 μL aliquot of a solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate on the immunosensor surface. The enzymatically deposition of metallic silver catalyzed by alkaline phosphatase (AP) was already reported [7].

- After 20 min of reaction, a cyclic voltammogram was recorded from –0.002 V to +0.4V, at scan rate of 50 mV s^{-1}, to obtain the electrochemical oxidation current of the enzymatically deposited silver.

Results and discussion

The aim ofthis work was the development of an EI for the detection of anti-DGP in human serum samples. For that purpose, DGP were immobilized on the nanostructured electrode surface working as the capture element of the sensing phase. A carrier protein linked with DGP was chosen instead of an deamidated α-gliadin, because this peptide with only 33 amino acids (aa56-88) is too small (approximately 4 Da). Therefore, its immobilization onto a solid transducer surface is very difficult, even in spite of the presence of nanomaterials that amplify the analytical signal. In this way, a carrier fusion protein with approximately 33 kDa is more likely to be useful to this immobilization strategy, ensuring the success of the immobilization strategy. The DGPx4 is a fusion protein of 2 different deamidated gliadin peptides and 2 DQ2-epitopes, fused with a carrier protein to be used as antigen for the detection of antibodies specific for deamidated gliadin [8].

The influence of the concentrations of the capture element and of the secondary labeled antibody on the analytical signal and was investigated. The best relation between analytical and background signal was achieved for a DPGx4 concentration of 0.30 μg μL^{-1} and a dilution of 1:50000 was achieved for anti-H-IgG-AP. Free surface sites were effectively blocked with BSA (2%). These optimized experimental conditions were used to the assessment of serological markers in real serum samples. Figure 1 shows typical cyclic voltammograms obtained for the detection of anti-DGP antibodies in positive and negative samples, under optimized experimental conditions. There is a significant difference between the analytical signals obtained in the presence and in the absence of the capture element (i.e. DGPx4) in the immunosensing architecture.

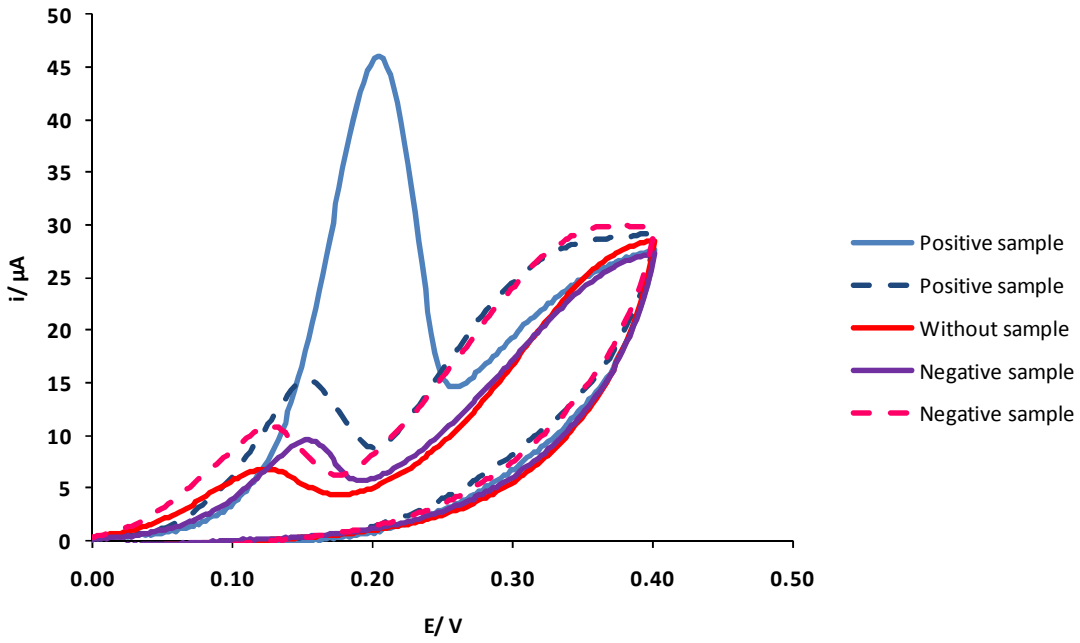

Figure 1. Cyclic voltammograms obtained for the detection of anti-gliadin deamidated antibodies in positive and negative samples, using a transducer surface with the capture element (DGPx4) immobilized (solid lines) and with no capture element immobilized (dashed lines). The analytical signal obtained with the absence of a sample was also investigated. Experimental conditions: DPGx4 0.30 µg μL^{-1}, serum samples diluted 1:200; anti-H-IgG-AP 1:50000; 3-IP 1.0 mM; Ag^{+} 0.4 mM. Cyclic voltammetric scans from −0.02 V to +0.4 V at a scan rate of 50 mV s^{-1}.

Afterwards, the immunosensor was applied in the assessment of the antibodies of interest in real serum samples. For that purpose, a cut-off line was determine as the average peak current intensity plus three times the standard deviation obtained for 15 negative samples of healthy individuals. Ten different serum samples (5 positive and 5 negative) previously analyzed with an ELISA kit, were assayed with the immunosensor for the detection of anti-DGPx4 class G antibodies. The results presented in Figure 2 revealed that the EI, in development, presents a good qualitative correlation with the reference methods for CD diagnosis.

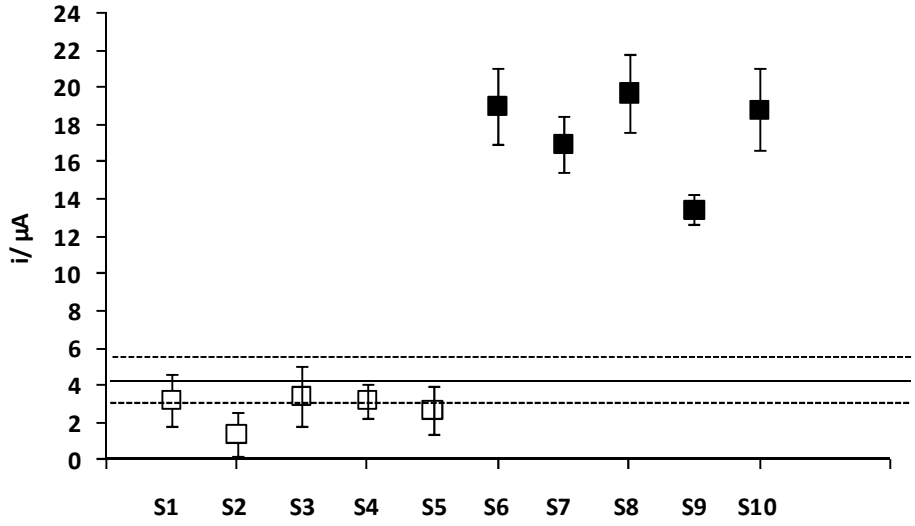

Figure 2. Evaluation of 5 negative □and 5 positive ■human serum samples for the detection of class G immunoglobulins generated against deamidated gliadin peptides, using the optimized electrochemical immunosensor. Optimized conditions as in Figure 1 (average data ± standard deviations are indicated (n = 3)).

Conclusions

These results demonstrate the usefulness of this new electrochemical immunosensor for the detection of anti-DPG antibodies. Nevertheless, these results are preliminary and a more complete validation procedure has to be carried out.

In future would be interesting to use dual-screen printed electrodes to endorse the detection of antibodies against IgG, DPG in combination with IgA tissue transglutaminase in order to facilitate diagnostic work-up and the follow-up of this autoimmune condition.

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FINAL CONSIDERATIONS

In this thesis, electrochemical immunosensors (EIs) for cealic desease (CD) diagnosis were developed through the combination of traditional immunochemical methods with modern electrochemical transducers. The association of favourable characteristics like the high specificity of the traditional immunochemical methods and the short analysis times and low limits of detection of the electroanalytical techniques resulted in faster, simpler and more sensitive analysis. In the pursued of achieving this main goal, several diferent partial goals were attained along the process.

Baring in mind that the development of the transducer surface is a crucial step in the construction of a biosensor device, several approaches were tested. Therefore, different transducer formats (SPCEs; SPCE-MWCNTs; SPCE-NPAus; SPCE-MWCNT-NPAus; SPCE-CNFs; SPCE-CNF-NPAus) were evaluated and their biofunctionality was tested by employing the highly specific strepatividin/biotin complex. The transducers modified with a hybrid conjugation of carbon nanotubes and gold nanoparticles showed the best results, and were used in all the subsequent studies. Moreover, this hybrid transducer surface, once modified with streptavidin, offered a stable surface for at least 30 days, which is ideal for use in solid-phase assays with biotinylated molecules (such as antibodies, enzymes, DNA, etc..), and is a direct response to requests from researchers in the biosensor field. The results of this study were important not only to decide which was the best transducer surface to work with, but also to increase the knowledge of the development and optimization of nanostructured screen-printed biosensors, providing a stronger basis for the development and optimization of EIs. Thus, besides the application of the resulting transducers in the subsequent steps of the present work they are also expected to be useful in (bio)analytical chemistry as a whole and in electroanalysis in particular.

After selecting the most adequate transducer surface, the sensing phases of the EIs were constructed relying on antigen-antibody interactions. The fact that antibodies can be produced specifically against many biological analytes of interest improves the EIs versatility.

EIs for clinical diagnosis of CD, through the detection of antibodies (classes A and G), directed against gliadin and tissue transglutaminase were the ones first developed. Because of sucessful results, the separated EIs were transfered to a multiplexed detection platform in order to perform a simultaneous detection of both CD serological markers. Dual-SPCE-MWCNT-NPAus were employed and a more complete screening of CD was acomplished. Finally, the study of an EI for the detection of anti- deaminated gliadin IgG antibodies was also inciated. The initial results are very promising. In all the developed sensors the recognition elements were successfully immobilized on the nanohybrid SPCE surface and different human serum samples were analyzed. Corroboration of the results

with commercial ELISA kits indicated that the proposed EIs are trustful analytical screening tools. Therefore, new disposable platforms for CD diagnosis were sucessfuly developed in this dissertation.

In comparison with other reported biosensors (optical or electrochemical), the EIs developed in this dissertation have the clear advantage of being constructed on disposable SPEs, which avoids some problems such as memory effects and tedious cleaning processes, typical of classical solid electrodes. Moreover, these SPEs were not homemade but industrially produced, which largely reduces the possibility of irreproducibility in their format. The use of SPEs as electrodic transducer surfaces, being small-sized, easy to handle and able to be incorporated in portable ready-to-use devices (e.g. similar to the ones of glucose sensors) is the best option, in this field, to decentralize clinical applications. Moreover, the studies presented in this dissertation were performed with human serum samples, which ensure the adequate evaluation of the sensitivity and specificity of the methodologies and also indicates that matrix effects are controlled. Moreover, to the best of our knowledge, these are the first studies regarding the employment of electrochemical immunosensors for simultaneous detection of AGA and anti-tTG antibodies as well as for the detection of anti-DGP antibodies.

Nevertheless, in future studies, it would be important to reduce the time of the immunosensing architecture steps. To achieve a point-of-care technology the analysis has to be performed in a simple and rapid way. In this dissertation the development of the methodology is presented, so now it would be interesting to pursue this line of research.

At the start of this doctoral research project, the development of an EI for detection of gluten traces in “gluten-free” food samples was also foreseen. However, this was not possible in the given time frame. Thus, the other future prespective would be the development of that EI to analyse gluten in foodstuffs which are naturally gluten-free or in products from which gluten has been eliminated, in order to ensure a safe diet for celiac patients. In this way, EIs can be useful analytical tools for the two biggest problems that are related to celiac disease: the diagnostic/follow-up and the quality control of gluten-free food. With the experimental work carried out in this doctoral thesis, new advances in the field of electrochemical biosensing were accomplished. The end of this dissertation is not necessarily a finalization of a project, instead it's a starting point for future challenges.