

An electrochemical deamidated gliadin antibody immunosensor for celiac disease clinical diagnosis

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The first electrochemical immunosensor (EI) for the detection of antibodies against deamidated gliadin peptides (DGP) is described here. A disposable nanohybrid screen-printed carbon electrode modified with DGP was employed as the transducer's sensing surface. Real serum samples were successfully assayed and the results were corroborated with an ELISA kit. The presented EI is a promising analytical tool for celiac disease diagnosis.

Celiac disease (CD) is caused by a genetically determined specific immune response to antigens present in gluten. Gliadins, namely α -gliadins, are considered as 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).² tTG catalyzes gliadin deamidation in the intestinal mucosa of CD patients, resulting in deamidated gliadin peptides (DGP) which are recognized by the 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 tTG.²

By mimicking the enzymatic gastrointestinal digestion of a representative α -gliadin it was found that a 33-mer peptide (LQLQFPQPQLPYP QPQLPYPQPQLPYPQPQPF; α 2-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 frequency of anti-gliadin antibody (AGA) detection for CD diagnosis has declined because AGA tests lack specificity in comparison with anti-tTG IgA antibody assays. This disadvantage was overcome by the introduction of DGP as antigen.⁵ Deamidated gliadin antibodies, especially class G immunoglobulins, are specific for CD and, therefore, their detection is of the utmost interest.⁶

Electrochemical immunosensors (EIs) are interesting alternatives to the conventional immunoassays, such as enzyme-linked immunosorbent assay (ELISA). EIs are simple, easy to handle and cost-effective analytical tools. The aim of this work was to develop an EI for the detection of anti-DGP antibodies in human serum samples. For this purpose, DGPs were immobilized on a single-use carbon/gold nanostructured screen-printed carbon electrode (SPCE) surface, where they served as the capture element of the sensing phase. A carrier protein linked with DGP was chosen instead of a deamidated α -gliadin, because this peptide, with only 33 amino acids (aa56–88), is very small (approximately 4 kDa), making its immobilization onto a solid transducer surface very difficult. Despite the presence of nanomaterials that contribute to the amplification of the analytical signal, which is extremely useful when small amounts of protein material are employed, the referred peptide is too small to address efficient immunosensing. A carrier fusion protein with approximately 33 kDa is more likely to be useful, ensuring the success of the immobilization strategy. In this work a DGPx4 protein was used as the capture element for the detection of antibodies specific for deamidated gliadin. This protein is obtained through the fusion of 2 different deamidated gliadin peptides and 2 DQ2-epitopes, linked to a carrier protein.⁷ The use of a metal nanoparticle–carbon nanostructure hybrid as the transducer surface allows a synergic effect on each material property since the hybrid system may have collective characteristics that are drastically different from the individual components.⁸

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At the moment there are three publications regarding the use of nanostructured screen-printed electrodes for CD diagnosis.^{9–11} However, to the best of our knowledge, at the moment, there are not any publications regarding the use of EIs for CD diagnosis using anti-DGP antibodies as serological markers.

Voltammetric analysis was performed with an Autolab PGSTAT12 (Metrohm Autolab, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266 MHz computer system and controlled by GPES 4.8 software (version for Windows 98). SPCEs and a specific connector were purchased from DropSens (Spain). Carboxyl modified multi-walled carbon nanotubes (MWCNTs) were purchased from Nanocyl (Belgium). Standard 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- γ 1-DQ2- γ 2) 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 serum samples collected in the "Hospital Universitario Central de Asturias" (Spain), which were previously analysed with a VARE-LISA Gliadin IgG Antibodies ELISA kit from 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.

Single-use SPCEs were modified with 4 μL of a 0.1 mg mL^{-1} MWCNT dispersion and the SPCEs were left to dry at room temperature until complete evaporation of the solvent. Then, the SPCE-MWCNT-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 $-5 \mu\text{A}$ for 60 s in an acidic 0.1 mM AuCl_4^- solution,¹² resulting in a nanohybrid transducer surface (SPCE-MWCNT-NPAus).

The immunosensor's construction and detection strategy is presented in Fig. 1 and consisted of the following steps:

- The SPCE-MWCNT-NPAus working electrode was coated with 10 μL of a 0.30 $\mu\text{g mL}^{-1}$ DPGx4 solution and left to incubate overnight at 4 °C.
- The electrode was then washed with a 0.1 M Tris- HNO_3 pH 7.2 buffer.
- Free surface sites were blocked with 40 μL of a 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 $\text{mM Mg}(\text{NO}_3)_2$.

- The immunosensor was incubated with an anti-human immunoglobulin G labeled with alkaline phosphatase (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 $\text{mM Mg}(\text{NO}_3)_2$.

- 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 enzymatic deposition of metallic silver catalyzed by alkaline phosphatase (AP) has already been reported.¹³

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

The influence of the concentrations of the capture element and the secondary labeled antibody on the analytical signal was investigated. The best relationship between the analytical and background signal was achieved for a DPGx4 concentration of $0.30 \mu\text{g mL}^{-1}$ and a dilution of 1 : 50 000 of the anti-H-IgG-AP. Free surface sites were effectively blocked with BSA (2%). These optimized experimental conditions were used for the detection of the serological marker in real serum samples. Fig. 2 shows typical cyclic voltammograms obtained for the analysis of anti-DGP antibodies in positive and negative samples, using the optimized experimental conditions. There is a significant difference between the analytical signals obtained, especially for positive samples, in the presence and in the absence of the capture element (*i.e.* DGPx4) on the electrode surface, which indicates that it was correctly immobilized.

Afterwards, the immunosensor was applied for the detection of the antibodies of interest in real serum samples. For this purpose, a *cut-off* line was set 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), which were previously analyzed using an ELISA kit, were assayed with the immunosensor for the detection of anti-DGP IgG antibodies. The results, presented in Fig. 3, revealed that the EI developed presents a good qualitative correlation with the reference methods for CD diagnosis.

The results obtained in this study demonstrate the usefulness of this new electrochemical immunosensor for the detection of anti-DGP IgG antibodies, a new serological marker for CD.

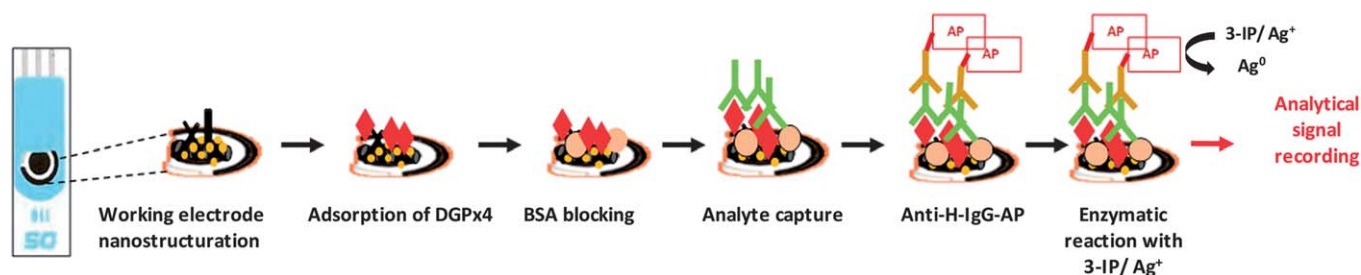


Fig. 1 Schematic representation of the immunosensing strategy for the detection of anti-DGP antibodies.

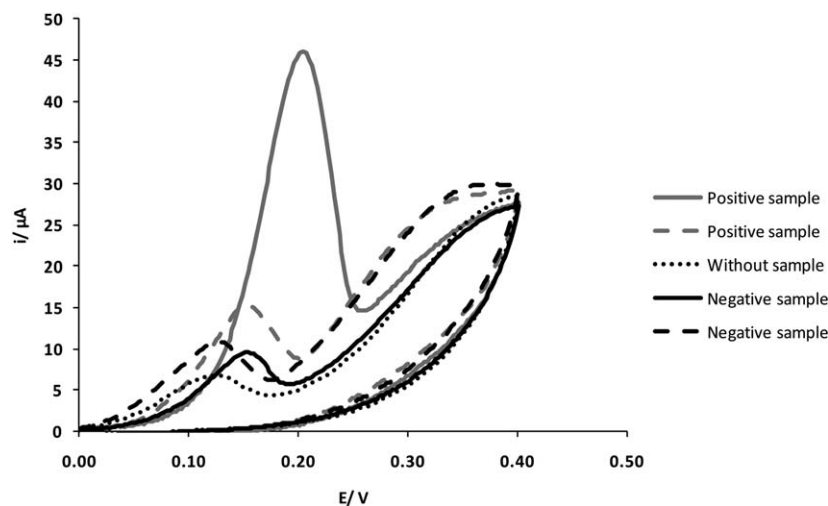


Fig. 2 Cyclic voltammograms obtained for the detection of anti-gliadin deamidated antibodies in positive and negative samples, using a transducer surface with (solid lines) and without (dashed lines) the capture element (DGPx4) immobilized on the electrode surface. The analytical signal obtained in the absence of a sample was also investigated. Experimental conditions: DGPx4 $0.30 \mu\text{g } \mu\text{L}^{-1}$, serum samples diluted 1 : 200; anti-H-IgG-AP diluted 1 : 50 000; 3-IP 1.0 mM; Ag^+ 0.4 mM. Cyclic voltammetric scans from -0.002 V to $+0.4 \text{ V}$ at a scan rate of 50 mV s^{-1} .

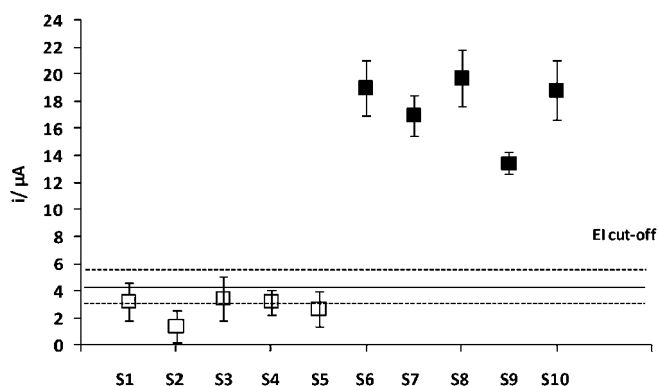


Fig. 3 Evaluation of 5 negative (□) and 5 positive (■) human serum samples for the detection of IgG immunoglobulins generated against deamidated gliadin peptides, using the optimized electrochemical immunosensor. Experimental conditions as in Fig. 2 (average data \pm standard deviations are indicated ($n = 3$)).

In comparison with the commercial ELISA kits, this reliable new EI is a more cost-effective analytical tool. Once the steps of the immunosensing procedure are reduced, this sensor can be used directly in a point-of-care testing approach.

In future studies it would also be interesting to use dual-screen-printed electrodes to endorse the simultaneous detection of antibodies against DPG (IgG) and tissue transglutaminase (IgA) in order to facilitate diagnostic work-up and the follow-up of celiac disease.

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