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Multiplexed Electrochemical Immunosensor for Detection of Celiac Disease Serological Markers

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Abstract:
Celiac disease is a gluten-induced autoimmune enteropathy characterized by the presence of antibodies against gliadin (AGA) and anti-tissue transglutaminase (anti-tTG) autoantibodies. 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. A screen-printed carbon electrode with two working electrodes nanostructured with a carbon-metal hybrid system 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 followed by the electrochemical detection of the human antibodies present in the serum samples assayed. 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. 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.

Key words: Celiac disease, tissue transglutaminase, gliadin, electrochemical immunosensors, nanomaterials, multiplexed detection.

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]. CD presents a prevalence of up to 1% in the world population, yet a high percentage of cases remain underdiagnosed [3]. In spite of efforts towards the development of new therapeutic strategies 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. The diagnostic criteria for this autoimmune condition, which require endoscopy with small bowel biopsy, have been changing over the last few decades, especially due to the advent of serological tests with high sensitivity and specificity [1]. 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. The immunoglobulin A (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 IgG class of antibodies is considered [5]. Electrochemical immunosensors (EIs) combining the specificity inherent to antigen–antibody interactions with the high sensitivity of electrochemical transduction [6], can be an excellent alternative to conventional immunochemical tests. Therefore, an electrochemical dual immunosensor for the simultaneous and point-of-care detection of AGA and anti-tTG antibodies was developed. A dual screen-printed carbon electrode nanostructured with a carbon-metal hybrid system 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 followed by the electrochemical detection of the human antibodies present in the serum samples assayed. 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. The analytical signal was based on the anodic redissolution of enzymatically generated silver by cyclic voltammetry. The results indicate that the proposed immunosensor can be competitive with the standard methodology.

Experimental

Apparatus and reagents

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 controlled by Autolab GPES 4.8 software (version for Windows 98). Dual screen-printed carbon electrodes (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). 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). 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 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. 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.

Procedure

Single-use dual SPCEs (Fig. 1) 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 $AuCl_4^-$ [7].

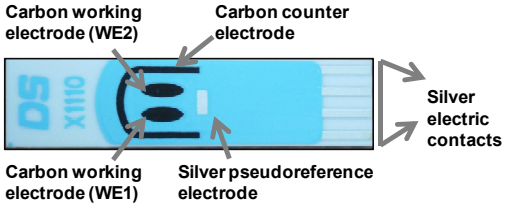

Fig. 1. Model of the screen-printed electrode used. Ref. X1110- Screen-Printed Carbon Dual sensor.

The immunosensing strategy comprises 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 µg.µL^{-1} tTG solution (WE2) 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 60 µL of a casein solution (in case of anti-IgA detection) or with 60 µL of bovine serum albumin (BSA) solution (in case of anti-IgG detection) 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-IgA-AP (1:25000) or an anti-H-IgG-AP (1:50000) 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 catalyzed by alkaline phosphatase (AP) was already reported [8].

- 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

Each variable involved in the construction of an immunosensor can influence the analytical response; therefore aspects such as the antigen concentration, the employed blocking agent and the concentration of the secondary labelled antibody were optimized.

Regarding the concentration of the antigen immobilized on the nanostructured surface, a gliadin concentration of 1 mg mL^{-1} and a tTg concentration of 0.1 µg $µL^{-1}$ revealed to be the most adequate for achieve the best relation between sensitivity and reproducibility. In order to minimize nonspecific adsorption, the effect of different blocking agents was studied. In the case of the detection of the immunoglobulins of class A, the best analytical/background signal ratio was obtained with casein (2%). In the case of the detection of IgG immunoglobulins the free surface sites were effectively blocked with BSA (2%). The influence of the secondary labeled antibody on the analytical signal was also investigated. The best relation between analytical and background signal was achieved for a dilution of 1:25000 in the case of anti-H-IgA-AP and 1:50000 for anti-H-IgG-AP. These optimized conditions were used throughout the work. Figure 2 shows 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. Figure 3 shows 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.

These results demonstrates the usefulness of this new electrochemical immunosensor for the simultaneous detection of antibodies against gliadin and tissue transglutaminase. This multiplexed sensor is a promising strategy for future transference to a point-of-care analytical device for celiac disease diagnosis.

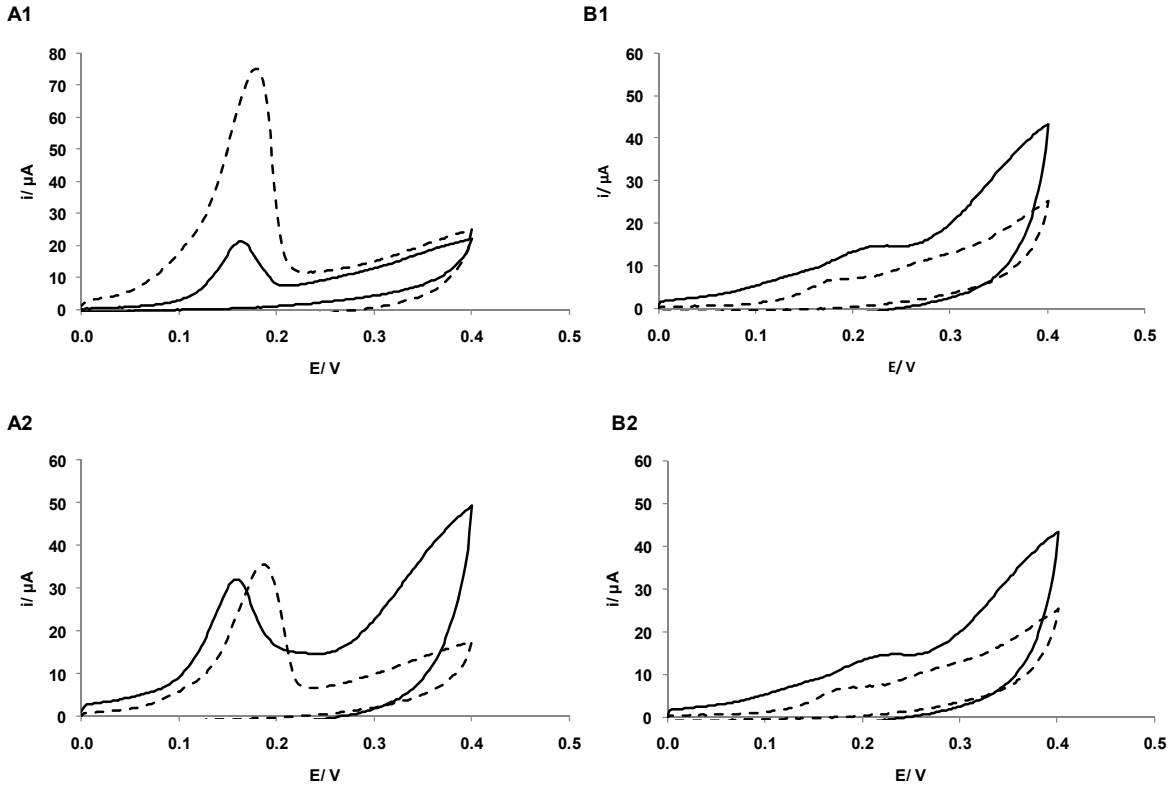

Fig. 2. Cyclic voltammograms obtained for an optimized assay: (A1) AGA-IgA positive (solid line) and anti-tTG IgA positive (dashed line) samples; (B1) AGA-IgA negative (solid line) and anti-tTG IgA negative (dashed line) samples. (A2) AGA-IgG positive (solid line) and anti-tTG IgG positive (dashed line) samples; (B2) AGA-IgG negative (solid line) and anti-tTG IgG negative (dashed line) samples.

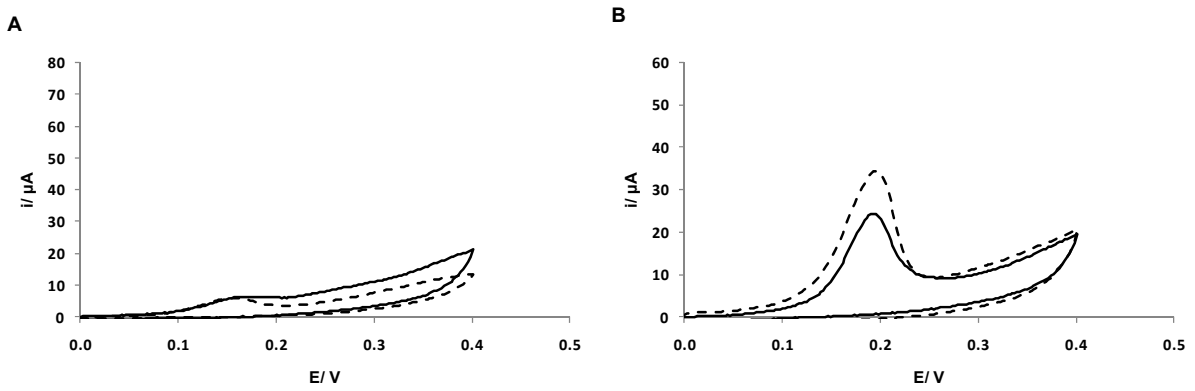

Fig. 3. Cyclic voltammograms obtained in the analysis of a sample of a CD patient with selective IgA deficiency. (A) AGA-IgA detection (solid line) and anti-tTG IgA detection (dashed line); (B) AGA-IgG detection (solid line) and anti-tTG IgG detection (dashed line) samples.

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