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 tran- glutaminase (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 car- bon electrodes (SPCE) nanostructurized with carbon nanotubes and gold nanoparticles were used as the transducer surface. This transducer exhibits the excellent characteristics of carbon–metal nanoparticlehybrid 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 dysfunc- tion (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 sen- sitivity 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 his- tological examination of at least one biopsy that demonstrates mucosal villous atrophy while the patient consumes gluten con- taining 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 present- ing extraintestinal syntomatology (Briani et al., 2008; Lindfors et al., 2009). Another important aspect to consider in a serological diag- nosis is the selective IgA deficiency of the patient. If an individual presents a negative serological test but exhibits suggestive symp- toms, the possibility of IgA deficiency should be considered and an IgG test must be performed. The identification of tissue transg- lutaminase (tTG) as the antigen against which the autoantibodies are directed, has led to a greater understanding of the pathogen- esis 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 serologi- cal 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 inter- actions 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 indi- rect 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 dispos- able 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 sec- ond 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 pro- posed. 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 analyti- cal signal and the amount of antibodies present in the samples was established. More recently, an electrochemical immunosen- sor 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 meth- ods 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 electro-chemical transducer surface consists of a screen-printed carbon electrode (SPCE) modified with multiwalled carbon nanotubes (MWCNTs) and gold nanoparticles (NPAus). Carbon nanostruc-ture/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 con- sisted 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 biorecogni- tion 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 con- trolled by Autolab GPES 4.8 (software version for Windows 98). All the measurements were carried out at room temperature. Dis- posable screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Spain). These electrodes incorporate a conven- tional three-electrode configuration, printed on ceramic substrates (3.4 × 1.0 cm). Both the working- (disk-shaped 4 mm diameter) and counter electrodes are made of carbon inks, whereas the pseudoref- erence 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 pro- duced in insect cells) was purchased from Zedira (Germany). Goat anti-human IgG (Fc specific) conjugated with alkaline phos- phatase (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 con- tained 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 sam- ples: 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 nega- tive or positive, respectively, for CD. Ultrapure water obtained with a Millipore Direct-Q^{TM} 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 phos- phatase 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 4 °C. The MWCNTs solutions were prepared by dilution of the concentrated solution with a mixture of DMF:water (1:1). The gold tetrachloroau- rate solution was prepared in 0.1 M HCl.

2.3. Electrode nanostructuration

The modification of the screen-printed electrodes with nanohy- brid 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* elec- trochemical 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.

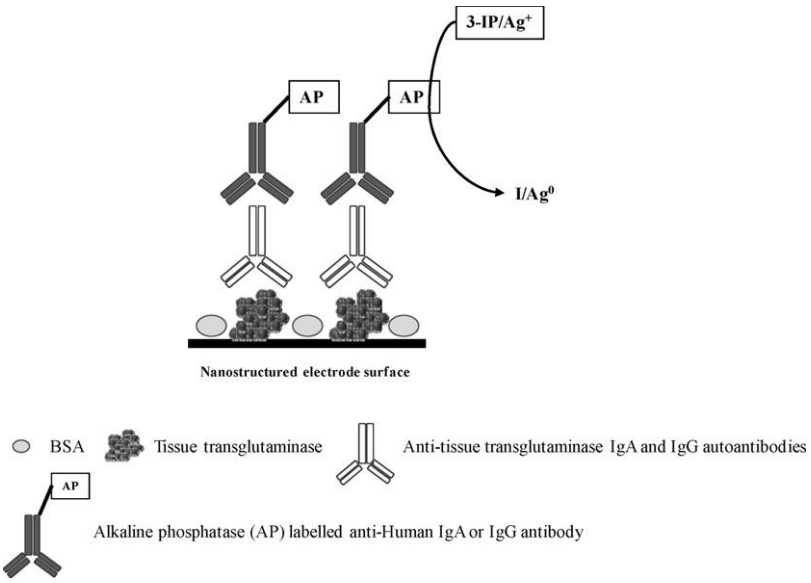

Fig. 1. Schematic representation of the immunosensor architecture used in this study.

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 4 °C. 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 0.1 M Tris pH 9.8 buffer containing 20 mM 2 mM $Mg(NO_3)_2$. The enzymatic reaction was carried out by dropping a 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 phos- phate 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). 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.

3. **Results anddiscussion**

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 4 °C 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 analyti- cal 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 pres- ence of the nanomaterials improves the faradaic/capacitive current ratio. The conjugation of MWCNTs and NPAus has already demon- strated to possess important analytical properties for biosensors due to the metal nanoparticles' ability to adsorb proteins without compromising their bioactivity, and the excellent electrocatalyt- ical 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-Mun˜ iz et al., 2004; Lee et al., 2004). In

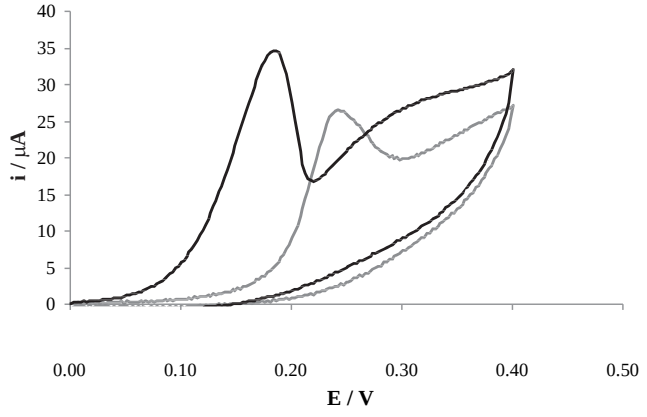

Fig. 2. Effect of the transducer surface on the analytical signal for the detec- tion of anti-tTG IgA autoantibodies using a tTG-modified SPCE (grey line) and a tTG-modified SPCE–MWCNT–NPAus (black line). Experimental conditions: tTG 0.1 µg μL^{-1}; BSA 2%; control samples diluted 1:2; anti-H-IgA-AP 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}.

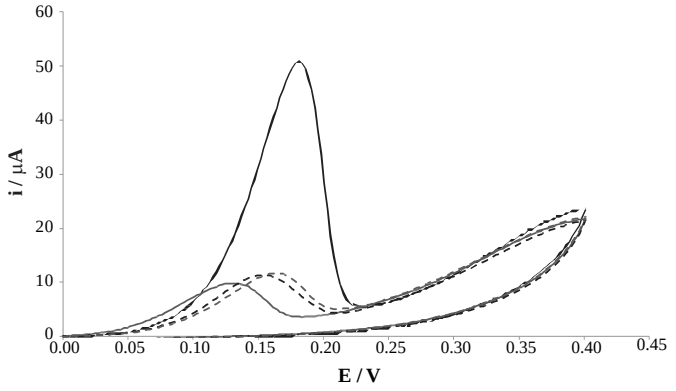

Fig. 3. Cyclic voltammograms obtained in the analysis of positive (black lines) and negative (grey lines) serum samples with tTG-modified (solid lines) and non- tTG-modified nanohybrid electrodes (dashed lines). Experimental conditions: tTG 0.1 µg µL^{-1}; BSA 2%; serum samples diluted 1:200; anti-H-IgA-AP 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}.

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 distin- guished, 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 inten- sities 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 indi- cate 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.

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 cur- rent 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

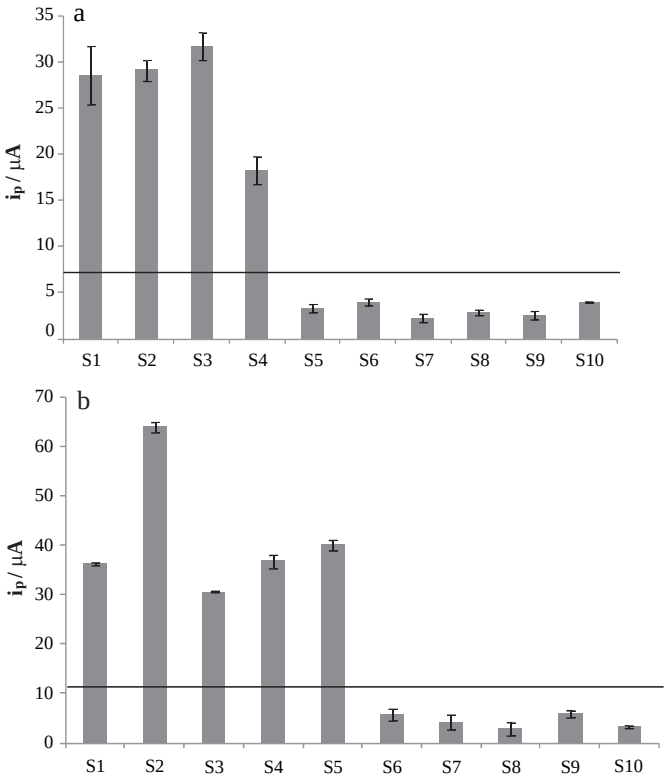

Fig. 4. Peak current intensities obtained in the determination of (a) IgA and (b) IgG anti-tTG autoantibodies in real patient's samples using the optimized electro- chemical immunosensor. Experimental conditions: tTG 0.1 µg µL^{-1} ; BSA 2%; serum samples diluted 1:200; anti-H-IgA-AP 1:30,000 (a) and 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)).

was then applied to the analysis of 10 different human sera from real patients for the detection of anti-tTG class A and G antibod- ies. 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).

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 intensi- ties were extrapolated to the calibrator solutions plot to convert the results in arbitrary units to be able to compare the results, in quan- titative 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 anti- bodies 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 voltam- mograms obtained within the linear ranges are also shown in Fig. 5.

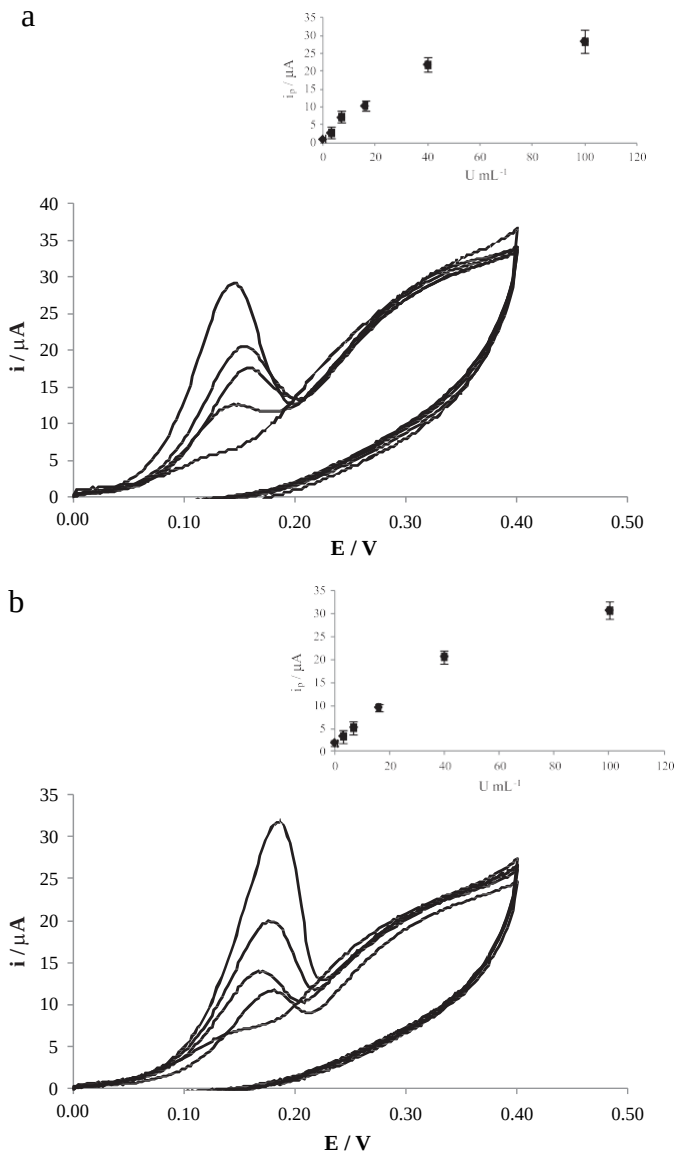

Fig. 5. Cyclic voltammograms of different calibrator solutions in the linear range. (a) [Anti-tTG IgA] (U mL^{-1}), (b) [anti-tTG IgG] (U mL^{-1}): 0, 3, 7, 16 and 40. Experimental conditions: tTG 0.1 µg µL^{-1}; BSA 2%, serum samples diluted 1:200; anti-H-IgA-AP 1:30,000 (a) and anti-H-IgG-AP 1:50,000 (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 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.

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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.09.044.

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

Real patient's sera	Electrochemical immunosensor		ELISA	
	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)).

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