



Electrochemical immunosensor for anti-tissue transglutaminase antibodies based on the in situ detection of quantum dots

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ABSTRACT

A miniaturized electrochemical biosensor array with in situ detection of quantum dots (QDs) was developed for the detection of anti-transglutaminase IgG antibodies (a celiac disease biomarker) in human sera. For the fabrication of the sensor, a 8-channel screen-printed carbon electrochemical arrays were used as transducers and modified with tissue-transglutaminase by adsorption. The immunologic reaction was carried out in a few simple steps: reaction with human serum, which contains the analyte of interest, followed by the immunoreaction with anti-human IgG labeled with CdSe/ZnS QDs and electrochemical detection of Cd^{2+} released from QDs. All steps were performed on the screen-printed arrays as the solid support, and the detection of Cd^{2+} was performed in situ after acid attack of the QDs without a transfer step by voltammetric stripping. The electrochemical response was correlated with the anti-transglutaminase IgG concentration. The developed electrochemical immunosensor is a trustful screening tool for celiac disease diagnosis discriminating between positive and negative sera samples with high sensitivity.

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1. Introduction

Nowadays, electrochemical biosensors are playing an important role as clinical diagnostic platforms [1,2]. Due to the versatility and high sensitivity, electrochemical biosensors are placed in the lead to become a future detection platform at different medical centers for disease diagnosis. To achieve this goal, the development of new detection labels that improve the ones employed so far, which are mainly enzymatic, is a constant concern in this scientific field [3–6].

Quantum dots (QDs) are semiconductor nanoparticles widely used in several applications, mainly on optical and electrochemical biosensing [7–9]. QDs are used in bioassays with fluorescence detection due to their interesting luminescent properties [10]. Metallic components of QDs can be measured electrochemically after an acid attack to break the nanoparticle and release metal cations to the solution. Normally, both the bioassay and the subsequent acid attack are all performed outside of the detection platform [11–14]. Our research group has developed an innovative methodology in which the bioassay as the acid attack and the detection are performed directly on the screen-printed electrodes [15]. This methodology is much easier and similar to performing bioassays with enzymatic labels. Furthermore, using screen-printed

electrodes as the biosensor platform provides important benefits over the use of other kind of electrodes, such as low sample volume, low cost device, the ability to perform in-situ analysis, besides being disposable avoiding tedious cleaning steps of the electrodes. The combination of the biosensor methodology developed by our group and the use of QDs as electrochemical label results in an attractive platform capable to compete with enzymatic labeling systems but saving the time of the enzymatic reaction and reagents.

Celiac disease (CD) is a gluten-sensitive enteropathy triggered by dietary gluten in genetically susceptible individuals. CD patients, normally, experience immune reaction and the body produces autoantibodies causing the destruction of intestinal mucous [16]. The main criteria for the diagnosis of CD is a biopsy, but in recent years, the serological tests for the detection of biomarkers are being imposed, allowing to avoid the more invasive analysis [17]. Some of the autoantibodies produced in a CD patient react specifically with tissue transglutaminase (tTG), being an important biomarker of this autoimmune condition. Although, anti-tTG IgA antibodies seems to be a more specific biomarker, sometimes when the patient has IgA deficiency, the determination of anti-tTG IgG antibodies is crucial in order to make a diagnosis. Therefore, the detection of anti-tTG IgG is also conducted in medical facilities for the serological detection of CD. The most common methodology for the detection of CD autoantibodies is an enzyme-linked immunosorbent assay (ELISA). ELISA tests have been used for the detection of anti-tTG antibodies with good sensitivity and specificity [18,19]. An alternative to ELISA tests are the electrochemical immunosensors that have some

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advantages such as increased sensitivity due to the electrochemical detection, besides the use of lower sample volumes, resulting in cost savings [20–23].

Among the published electrochemical immunosensors for the determination of anti-tTG IgG, this is, to the best of our knowledge, the first work employing QDs as electrochemical label. Moreover, in this work we use an innovative methodology where both the bioassay and the detection of QDs are performed in the same platform (i.e. screen-printed carbon electrodes). As explained above, this methodology highly simplifies the ones previously published using QDs electrochemical detection, being easier to perform with less number of steps. Another advantage of this work is the use of 8-channel screen-printed electrochemical arrays (8 × SPCEs), which further simplifies the procedure and drastically improves the time of analysis.

2. Materials and methods

2.1. Apparatus and electrodes

Voltammetric measurements were performed with a μ Stat 8000 (DropSens, Spain) potentiostat interfaced to a Pentium 42.4 GHz computer system and controlled by DropView 8400 2.0 software. All measurements were carried out at room temperature. 8-channel screen-printed electrochemical arrays were purchased from DropSens (Spain). Each array is formed by eight 3-electrode electrochemical cells (30 μ L volume) with carbon-based working and counter electrodes, whereas pseudoreference electrodes and electric contacts are made of silver. This device has dimensions of $4.0 \times 7.9 \times 0.06$ cm³ (length $\times$ width $\times$ height) and the diameter of the working electrodes is 2.56 mm. 8-channel arrays were connected to the potentiostat through a specific connector, DRP-CAST8X.

2.2. Reagents and solutions

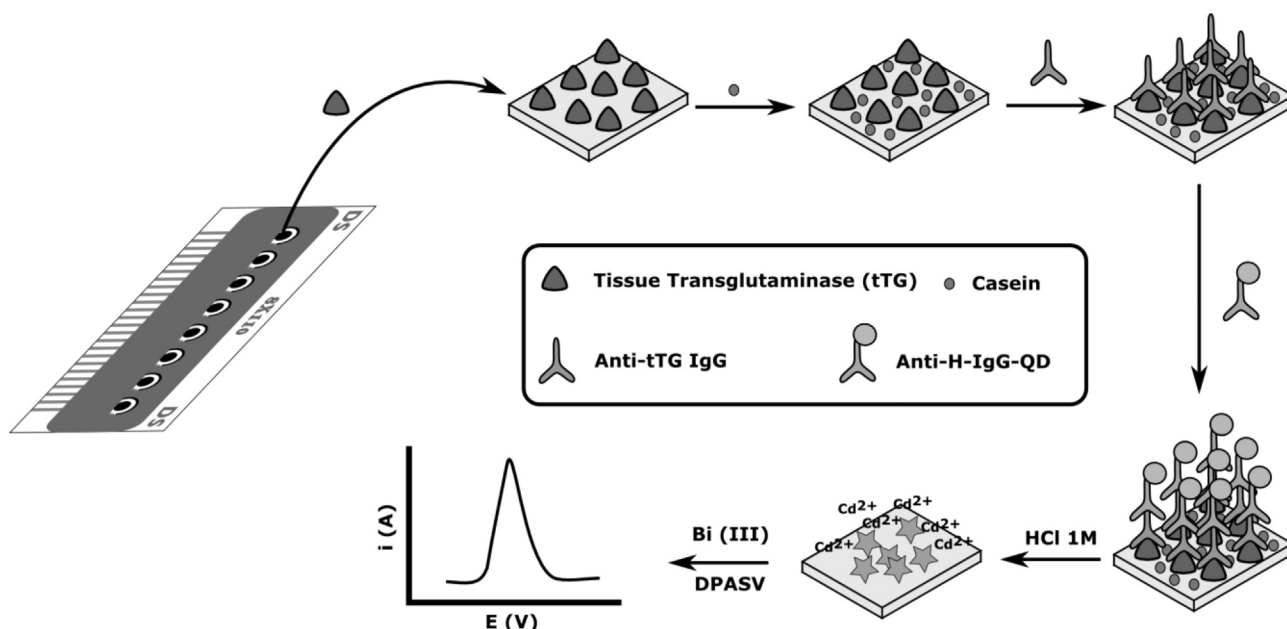
Sodium hydroxide, acetic acid (100%), fuming hydrochloric acid (37%) were purchased from Merck. Bismuth(III) standard, Tris(hydroxymethyl)aminomethane (Tris), bovine serum albumin

fraction V (BSA) and β -casein (98%, from bovine milk) were purchased from Sigma-Aldrich (Spain). Human tissue transglutaminase (recombinantly produced in insect cells) was purchased from Zedira (Germany). Qdot[®] 655 goat F(ab')₂ anti-human IgG Conjugate (H+L) (anti-H-IgG-QD) was purchased from Life Technologies (Spain). Varelisa Celikey IgG ELISA kits were purchased from 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 Q5[™] purification system from Millipore Ibérica S.A. (Madrid, Spain) was used throughout this work. All other reagents were of analytical grade. Working solutions of tTG, anti-H-IgG-QD, BSA, and casein were prepared in 0.1 M pH 7.4 Tris–HCl buffer.

2.3. Immunoassay procedure

An aliquot of 4 μ L of tTG solution with a concentration of 0.1 mg mL⁻¹ was dropped on the surface of each working electrode and was left overnight. After washing the screen-printed electrode with 0.1 M pH 7.4 Tris–HCl buffer, a blocking step was carried out placing 25 μ L of casein blocking buffer (20 μ g μ L⁻¹ of casein in 0.1 M pH 7.4 Tris–HCl) solution for 45 min. The detection of anti-tTG IgG antibodies was accomplished by incubating the immunosensor with human serum samples for 60 min followed by a washing step with 0.1 M Tris–HCl pH 7.4 buffer. Finally, 25 μ L of anti-H-IgG-QD (10 nM in terms of QDs) solution (with 1 μ g μ L⁻¹ of BSA) was dropped on the modified electrode for 60 min. A last washing step was carried out with ultrapure water. Then, the electrode was connected to the potentiostat for the electrochemical analysis. In the Scheme 1, a diagram of the immunoassay using the biosensor array is presented.

The measurement step was performed according to a methodology previously developed [15]. After the biological reaction, 1 μ L of HCl 1.0 M was added on the working electrode to release Cd²⁺ from QDs, followed by the addition of 25 μ L of 0.1 M acetate buffer solution pH 4.5 with 1.0 mg L⁻¹ Bi (III). A constant potential of +1.00 V was applied for 60 s to activate the working electrode. Cadmium was preconcentrated on the electrode surface by applying a potential of –1.10 V for 300 s, simultaneously a bismuth film was also formed during this step. The potential was swept from



Scheme 1. Schematic diagram of the electrochemical biosensor array. The bioassay is carried out using the working electrodes of the array as transducers and in situ electrochemical detection of QDs.

–1.10 V to –0.65 V using differential pulse voltammetry (DPV) with optimized parameters (0.05 V for amplitude, 0.01 V for step potential, 0.01 s for modulation time and 0.1 s for interval time).

3. Results and discussion

3.1. Study of non-specific adsorption and sensor biofunctionality

The initial working conditions chosen for the evaluation of the biofunctionality of the transducer modified with tTG were: 0.2 mg mL^{–1} of tTG, 12 nM of anti-H-IgG-QD, 20 mg mL^{–1} of BSA as blocking reagent, 30 min as blocking time and 60 min incubations for the immunoreactions. The analytical signal obtained by the biosensor with the initial working conditions was evaluated using positive and negative serum samples for anti-tTG IgG detection. The obtained results (Fig. 1(A)) show how the biosensor worked properly because the peak current for the positive control was significantly higher than the peak current for the negative control.

Both real serum samples and serum controls contain a large amount of immunoglobulins and other proteins that may produce nonspecific adsorption and yield an unrealistic result. Therefore, the reduction of the nonspecific adsorption is crucial to obtain low background signals and improve the sensitivity of the biosensor. The high signal obtained with negative controls for the initial studies is due to this nonspecific adsorption.

To lower the nonspecific adsorption several parameters that could affect this process were optimized. A study of the blocking effect of BSA and casein was performed, testing the effect of different concentrations (5, 10 and 20 µg µL^{–1}) of these blocking agents. Casein blocked the surface more effectively than BSA, achieving the best signal/background relation with 20 µg µL^{–1} of casein. With lower concentrations of casein the blocking effect was not enough, while that with higher concentrations the blocking effect was excessive, negatively affecting the analytical signal. After this optimization, a study of the blocking time was carried out. In this case, the nonspecific adsorption decreased with the blocking time. The maximum signal/background ratio was obtained for 45 min of blocking time. For higher times, the specific binding appeared to be compromised with the blockade of the surface and the analytical signal decreased. Therefore, an optimum blocking time of 45 min was chosen.

However, after the blocking agent was optimized, the results still showed a slightly high background signal, above 1 µA, while the positive control signal was about 3.7 µA. Therefore, some non-specific adsorption was taking place, which could be originated by the adsorption of the detection antibody (anti-H-IgG-QD). Then, the

effect of a small concentration of BSA (1, 5 and 10 mg mL^{–1}) in this solution was tested. With a 1 mg mL^{–1} BSA in this solution, a background signal considerably lower than 1 µA was obtained, decreasing the nonspecific adsorption, as shown in Fig. 1(B).

3.2. Optimization of the experimental conditions

Several parameters affecting the biosensor functionality were optimized. Serum controls (positive and negative) from the ELISA kit were also employed for this optimization, and the biosensing was carried out varying the studied parameters.

tTG concentration was changed between different values (0.01, 0.05, 0.1 and 0.2 mg mL^{–1}) and the response of the biosensor was tested. Results showed that the most suitable concentration of tTG was 0.1 mg mL^{–1}. Lower concentrations decreased the signal for the positive control (without a noticeable change in the negative control signal) reducing the signal/background relation. Higher concentrations did not improve the signal/background relation compared to that obtained with 0.1 mg mL^{–1}.

Other parameters such as the incubation time of the serum and the incubation time and concentration of anti-H-IgG-QD solution were also optimized (data not shown). Optimal values were obtained with 60 min for both incubation times and 10 nM (in terms of QDs) for the concentration of anti-H-IgG-QD. With these optimized parameters the highest signal/background relation was obtained.

3.3. Analytical performance

3.3.1. Calibration plot

The developed sensor was used with the optimized conditions to establish a relationship between the analytical signal and the concentration of anti-tTG IgG antibodies using human serum calibrators from the commercial ELISA kit. Fig. 2(A) shows the calibration curve for the tested concentration of anti-tTG IgG antibodies. Saturation of the sensor for 100 U mL^{–1} was observed, therefore, the superior limit of the linear range obtained for the biosensor was 40 U mL^{–1}. A linear relationship between the peak current and the antibody concentration was achieved according to the following equation: $i(\mu\text{A}) = 0.106 (\pm 0.006) [\text{anti-tTG-IgG}] + 0.7 (\pm 0.1)$, $R^2 = 0.994$. The reproducibility between the slopes of the calibration curves ($n = 4$) was 5.9% (in terms of RSD). The voltammograms obtained are shown in the Fig. 2(B). The detection limit from the calibration curve (calculated as the concentration corresponding to three times the standard deviation of the estimate) was 2.2 U mL^{–1}. According to the specifications of the commercial ELISA kit, the cutoff values to evaluate the results are the following: negative if the concentration is less than 7 U mL^{–1},

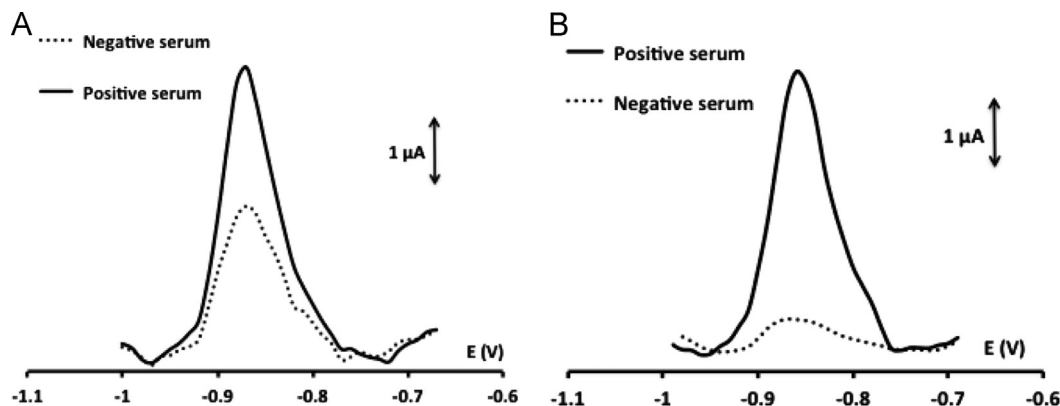


Fig. 1. (A) Differential-pulse voltammetry of the analytical signal for positive and negative sera using the electrochemical immunosensor without optimization. (B) Differential-pulse voltammograms of the analytical signal for positive and negative sera after the optimization of the electrochemical immunosensor.

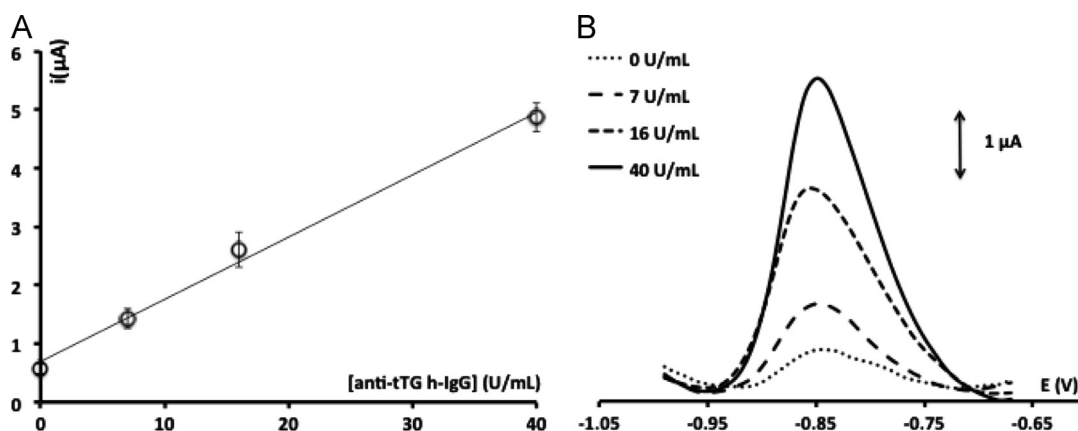


Fig. 2. (A) Linear response of the sensor for the different calibrators of the ELISA kit according to anti-tTG IgG antibody concentration (0, 7, 16 and 40 U mL⁻¹). (B) Differential-pulse voltammograms for the same calibrators of the ELISA kit using the electrochemical immunosensor.

uncertain between 7 and 10 U mL⁻¹ and positive if the concentration is above 10 U mL⁻¹. Therefore, the developed sensor can differentiate these key concentrations as indicated. Using the calibration curve previously obtained, the concentration of anti-tTG IgG antibodies for the positive and negative control sera was measured. For the positive control, a concentration of 29 ± 1 U mL⁻¹ was obtained, which is fairly consistent with the data specified in the commercial kit (30.4 U mL⁻¹). For the negative control, the result obtained is below of the detection limit of the method (< 3 U mL⁻¹ indicated in the quality data of the commercial kit).

The immunosensor developed in this work has similar characteristics to the electrochemical biosensors that have been published in the literature. In the case of the two works published by Neves et al.[21,22], linear ranges of 0–40 U mL⁻¹ or 0–100 U mL⁻¹ and a detection limit of 2.95 U mL⁻¹ are obtained. The higher slope of the calibration plots shown by these biosensors may be due to two reasons mainly: the use of an enzymatic label, which amplifies the analytical signal and the use of screen-printed electrodes with higher surface area. However, it requires one step more to perform the bioassay, and therefore, the methodology becomes more complex. Besides, the use of larger sample volume and reagents make the mentioned biosensors less cost-effective compared to the biosensor presented in this work. In the work developed by Dulay et al.[20], in which an enzymatic label is also employed, due to the use of arbitrary units in the determination of anti-tTG IgG antibodies, the analytical characteristics of the biosensors are not easily comparable.

3.3.2. Precision and stability studies

A precision study of the immunosensor was carried out by evaluating the reproducibility of two interday assays of positive as well as negative human anti-tTG IgG antibodies sera. Three measurements using three different sensors were performed on each day and the obtained results indicate a good reproducibility of the immunosensor, with an average relative standard deviation (RSD) of 9.4% for the negative samples and 4.5% for the positive samples. The result indicates that the method provides precise results.

Besides the analytical performance, it is important to evaluate the stability of the biosensor. For this study, several immunosensors were modified with tTG as explained (overnight at 4 °C), washed with 0.1 M pH 7.4 Tris–HCl buffer the next day and stored at 4 °C for different time periods. The response of the biosensor was tested over a 1-month period using the positive and negative serum controls from the commercial ELISA kit. As shown in Fig. 3,

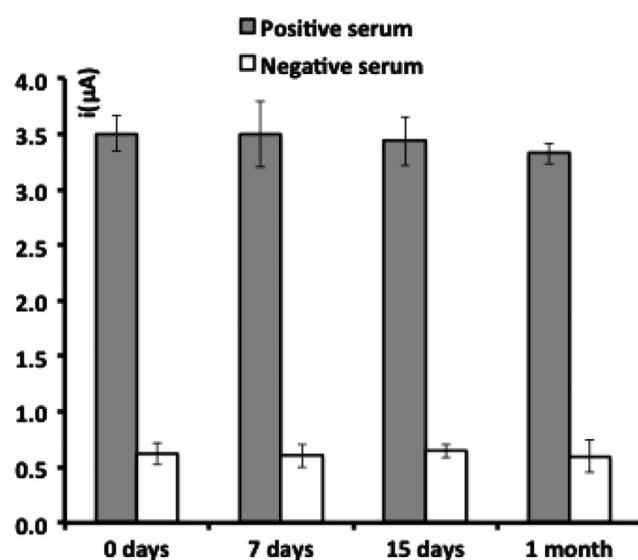


Fig. 3. Stability study of the electrochemical immunosensor for the detection of anti-tTG IgG antibodies.

no significant change was observed in the analytical response of the immunosensor, so it can be confirmed that they are stable for at least 1 month under these storage conditions.

4. Conclusions

For the first time a disposable electrochemical biosensor for the detection of anti-tTG IgG antibodies based on QDs detection was developed. QDs are used as electrochemical label and are detected directly on the biosensor platform, which are 8-channel screen-printed carbon electrochemical arrays. The methodology developed in this work simplifies previously published works where QDs are used as electrochemical label, requiring, generally, a transfer step of the solution to the detection platform. Therefore, the developed immunosensor system presents advantages such as low cost, low volume of reagents required, time saving due to the 8-channel platform, being a simple methodology. It was found that the developed biosensor worked successfully for the detection of anti-tTG antibodies in human serum controls. This work shows how a direct electrochemical label such as QDs could compete with enzymatic labels, approaching one more step to the ideal point-of-care system.

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