Bioelectrochemistry
Volume 105, October 2015, Pages 88-94

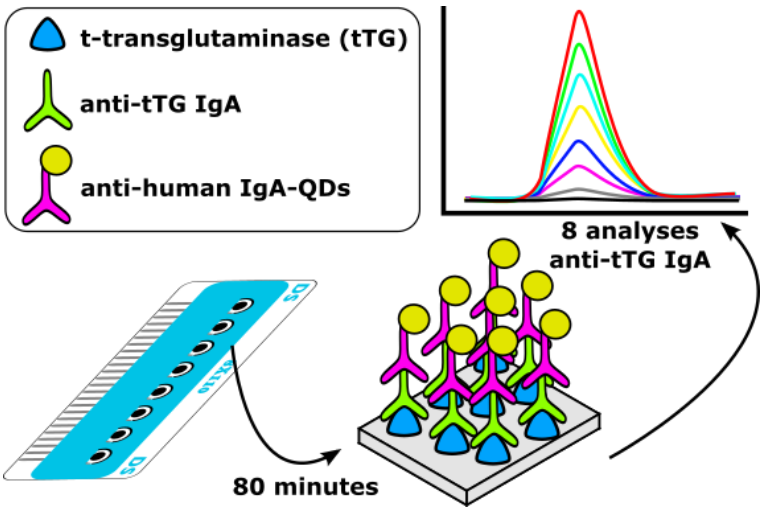
Towards a blocking-free electrochemical immunosensing strategy for anti-transglutaminase antibodies using screen-printed electrodes

Daniel Martín-Yerga, Agustín Costa-García

Show more

https://doi.org/10.1016/j.bioelechem.2015.05.014

Graphical abstract



Highlights

- A blocking-free one-step assay using screen-printed electrodes was developed.
- Detection of anti-transglutaminase IgA antibodies was performed in 80 min.
- A linear range from 3 to 40 U mL^{-1} was obtained using human serum controls.
- A limit of detection of 2.7 U mL^{-1} was estimated.
- The immunosensor was stable at least for 1 month stored at 4 ºC.

Towards a blocking-free electrochemical immunosensing strategy for anti-transglutaminase antibodies using screen-printed electrodes

*Daniel Martín-Yerga and Agustín Costa-García**

Nanobioanalysis group

Department of Physical and Analytical Chemistry

University of Oviedo

* Corresponding author: Agustín Costa García

Nanobioanalysis group

Department of Physical and Analytical Chemistry

University of Oviedo

8 Julián Clavería St., Oviedo 33006 (Spain)

E-mail: costa@uniovi.es

Telephone: (+34) 985103488

ABSTRACT

A blocking-free one-step immunosensing strategy using 8-channel screen-printed arrays for the detection of anti-transglutaminase IgA antibodies, celiac disease biomarkers, was developed. A simple but novel immobilization approach to efficiently modify the surface of screen-printed electrodes with a recognition element was employed in order to minimize the non-specific adsorption on the electrode surface, and the optimization of a methodology without a blocking step was carried out. After the functionalization of the electrode surface with tissue-transglutaminase, two different immunoassays, using multi-step and one-step strategies, were optimized. Serum controls from a commercial ELISA kit, anti-human IgA labelled with biotin and streptavidin labelled with CdSe/ZnS quantum dots were employed as bioreagents for the immunoassay. Screen-printed arrays were used as the solid support for the immunosensor and the detection of Cd(II) was performed *in situ* by anodic stripping voltammetry after an acid attack of the QDs. The electrochemical response from Cd(II) was correlated with the anti-transglutaminase IgA antibodies concentration. The analytical characteristics obtained for the multi-step and one-step electrochemical immunosensors allow to discriminate between positive and negative sera controls, establishing this biosensor as an useful tool for the determination of celiac disease biomarkers.

KEYWORDS: celiac disease, immunosensor, electrochemical biosensor, quantum dots, screen-printed electrodes

1. INTRODUCTION

Point-of-care (POC) testing is defined as medical testing at or near the site of patient care[1]. Ideal POC analytical devices should allow simple, rapid, sensitive and selective detection of one or more analytes. They should also be small and inexpensive devices to facilitate their usage by the patient himself. The most known POC devices are pregnancy tests and electrochemical glucometers for glucose monitoring[2]. Electrochemical biosensors (EBs) are being widely researched in recent years as a clinical diagnostic platform[3–5]. Such biosensors could turn into the basic tools for POC devices, due to their interesting properties such as high sensitivity and rapid analysis time. In addition, if screen-printed electrodes are used as the transducers, further advantages are obtained such as low cost, low reagent consumption and the ability to perform decentralized tests[6]. Moreover, novel screen-printed multichannel devices are being employed to perform multiple analysis simultaneously[7,8]. Although there has been substantial progress in the development of EBs, the way to POC diagnostic is still hard and long.

Many complex EBs involving several biological reactions and washing steps have been described in the literature [9–11]. Such procedures differ from the ideal concepts of a POC device. Most of these bioassays, and specially for immunoassays, require a blocking step of the electrode surface to reduce nonspecific adsorption of interfering proteins from reagents or samples, which may lead to high background signals decreasing the signal/noise ratio and hence the sensitivity. This blocking step is usually carried out with inert proteins such as bovine serum albumin (BSA) or casein, which increases the procedure complexity and the analysis cost and time. However, this blocking step is usually accepted by immunosensing researchers and only few EBs without incorporating a blocking step have been reported previously, typically replaced by complex washing strategies[12]. On the other hand, the modification of screen-printed electrodes with the recognition element is frequently performed by modifying the working electrode[13,14]. The hydrophobic character of untreated screen-printed electrodes makes difficult a complete coating

of the surface by the recognition element. Consequently, the rest of the electrochemical cell remains available for non-specific adsorption. The complex methodologies involving several reactions (including blocking of non-specific adsorption) and washing steps is one of the main drawbacks for the introduction of electrochemical immunosensors in real-world applications. The development of really simple and fast approaches is expected to have a remarkable significance in the future of these devices.

The development of methodologies employing direct electrochemical labels to replace the most used enzymatic labels may derive in several advantages such as the possibility to perform easy multiplexing detection[15] or avoid the often time-consuming enzymatic reaction. For these reasons, the development of simple electroactive labels, especially based on nanomaterials, is a constant concern. Quantum dots (QDs), semiconductor metallic nanoparticles, are being widely used as biosensing labels due to their optical and electrochemical properties[16,17]. The possibility to synthesize QDs with different metal composition or sizes provides a great versatility for biosensing applications[18]. In addition, the modification with different coatings allows a simple functionalization with biomaterials and to improve the stability[19]. For the electrochemical detection, typically an acid attack is carried out to release metal cations to the solution. It has been found that the appropriate selection of the quantum dots size may have an important effect on the number of released metal atoms and the electrochemical signal obtained[20]. In most of the published electrochemical biosensors using QDs, both the bioassay and the subsequent acid attack are performed outside of the detection platform[21–24]. However, the recent development by our group of a methodology for the in situ detection of QDs, in which the bioassay as well as the acid attack and the detection are performed directly on the screen-printed electrodes, has significantly simplified the use of QDs as electrochemical label for biosensors[25].

Celiac disease (CD) is an autoimmune enteropathy produced by intolerance to gluten. An effect of CD is the production of autoantibodies that cause the destruction of the intestinal mucosa[26]. A small intestinal biopsy is still considered the reference method for the diagnosis of CD, but serological tests for the detection of biomarkers are being of significant help to avoid the most invasive methods[27]. Anti-tissue transglutaminase (tTG) antibodies, particularly IgA antibodies, are serological biomarkers that provide important information concerning the disease with high sensitivity and specificity. The most common method for the detection of CD biomarkers is an enzyme-linked immunosorbent assay (ELISA)[28,29]. However, electrochemical immunosensors appear as an interesting alternative since they have several advantages such as the increase of sensitivity due to the electrochemical detection, lower sample volumes and, typically, shorter analysis time[3,5,30–32].

In this work, we developed a simple methodology for electrochemical immunosensors eliminating the usual blocking step and performing the *in situ* detection of QDs as electrochemical label. This methodology was employed for the determination of anti-tTG IgA antibodies, a celiac disease biomarker. Both, a multi-step bioassay procedure with intermediate washing steps, and a one-step bioassay procedure where all the biological reagents react simultaneously, were optimized. The one-step strategy is much simpler and is carried out in a shorter time than other biosensors published for the determination of celiac disease biomarkers. Another advantage of this work is the use of 8-channel screen-printed electrochemical arrays (8xSPCEs) that further simplifies the procedure and drastically improves the time of analysis. With this work, we get closer to the ideal POC device for celiac disease detection.

2. MATERIALS AND METHODS

2.1. APPARATUS AND ELECTRODES

Voltammetric measurements were performed with a μStat 8000 (DropSens, Spain) potentiostat interfaced to a Pentium 4 2.4 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 (ref.8x110). Each array is formed by eight 3-electrode electrochemical cells (30 μL volume for each individual cell) with carbon-based working and counter electrodes, whereas quasireference electrodes and electric contacts are made of silver. This device has dimensions of 4.0 x 7.9 x 0.06 cm (length x width x 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. A graphical scheme and a real image of the 8-channel array are shown in **Schematic S1** and **Figure S1**, respectively. Contact angle measurements were performed using a CAM 200 contact angle goniometer (KSV Instruments Ltd).

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), β-casein (98%, from bovine milk), peroxidase from horseradish (HRP), potassium ferrocyanide trihydrate, potassium ferricyanide and potassium chloride were purchased from Sigma-Aldrich. Human tissue transglutaminase (recombinantly produced in insect cells) was purchased from Zedira (Germany). Qdot® 655 streptavidin conjugate (QD-STV), biotinylated goat anti-human IgA (anti-H-IgA-BT) and Qdot® 655 goat F(ab')2 anti-human IgG conjugate (anti-H-IgG-QD) were purchased from Life Technologies. Varelisa Celikey tissue transglutaminase IgA and IgG ELISA kits were purchased from Phadia (Germany). Each kit contained six human serum calibrators (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, QD-STV, anti-H-IgA-BT,

anti-H-IgG-QD, BSA, and casein were prepared in 0.1 M pH 7.4 Tris-HCl buffer (hereafter called Tris buffer).

2.3. IMMUNOASSAY PROCEDURE

2.3.1. Immunosensor preparation

The 8-channel SPCEs were rinsed with Tris buffer and left to dry to generate an hydrophilic surface. Then, an aliquot of 4 µL of tTG solution (in Tris buffer) with a concentration of 0.1 µg/µL was dropped to cover completely the surface of the electrochemical cell of each electrode (including counter and quasireference electrodes). It was stored overnight until complete dryness at 4ºC.

2.3.2. Multi-step methodology

Firstly, the prepared immunosensor was washed with Tris buffer. The detection of anti-tTG IgA antibodies was carried out by incubating the immunosensor with 25 µL of serum solutions (1:2 in Tris buffer) for 60 min followed by a washing step with Tris buffer. Then, 25 µL of 7.5 µg/mL anti-H-IgA-BT (with 5 µg/µL of BSA) were added to the sensor for 60 minutes followed by another washing step with Tris buffer. Finally, 25 µL of QD-STV (10 nM in terms of QDs) were added and left to incubate for 30 minutes. A last washing step was carried out with ultrapure water. Then, the electrode was connected to the potentiostat for the electrochemical analysis. In **Schematic 1A**, a diagram of the immunoassay using this methodology is shown.

2.3.3. One-step methodology

In the first place, the prepared immunosensor was washed with Tris buffer. The detection of anti-tTG IgA antibodies was carried out by incubating the immunosensor for 80 minutes with 25 µL of a mixture of human serum calibrator (10 µL), anti-H-IgA-BT (1 µL, 190 µg/mL), QD-STV (1 µL, 250 nM in terms of QDs) and Tris (13 µL). A last washing step was carried out with ultrapure water. Then, the electrode was connected to the potentiostat for the electrochemical analysis. In **Schematic 1B**, a diagram of the immunoassay using this methodology is shown.

2.3.4. Electrochemical detection

The measurement step follow a methodology previously developed[25]. After the biological reaction, 1 µL of HCl 1.0 M was added on the working electrode to release Cd^{2+} from QDs and 25 µL of 0.1 M acetate buffer solution pH 4.5 with 1.0 mg L^{-1} Bi (III) was added. A constant potential of +1.00 V was applied for 60 s to activate the working electrode. The application of this potential may help to the activation of the carbon electrode surface, creating oxygenated functional groups and increasing the electroactive area, as previously described in the literature[33–35]. Furthermore, it is likely that the oxygen gas detaches part of the protein adsorbed on the electrode surface, leaving a more available surface for the subsequent deposition of cadmium. Cadmium was preconcentrated on the electrode surface by applying a potential of -1.10 V for 300 s (400 s for the one-step methodology), simultaneously a bismuth film was also formed during this step. Bismuth films have been proven as an excellent amplification component for the sensitive detection of heavy metals such as lead and cadmium due to the capability of Bi to form fused alloys with these metals. Its advantages have been extensively studied[36,37]. The potential was swept from -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).

2.4. ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY MEASUREMENTS

Electrochemical impedance spectroscopy (EIS) was performed with an Autolab PGSTAT12 (Metrohm Autolab) potentiostat/galvanostat controlled by Autolab FRA 4.9 software. Individual screen-printed carbon electrodes (SPCEs) (DropSens, ref. 110) were used for EIS measurements. The novel modification of SPCEs was performed following the methodology described in section 2.3.1 but using 10 µL of tTG solution (as usual for individual SPCEs since it is the necessary volume to fully cover the working electrode surface). Conventional modification (SPCEs are not washed before tTG immobilization) was performed by dropping an aliquot of 10

μL of tTG solution (in Tris buffer) with a concentration of 0.1 μg μl^{-1} on the surface of the working electrode and was left overnight. After washing the SPCEs with Tris buffer, 40 μL of $Fe(CN_6)^{3-/4-}$ solution (5 mM) prepared in KCl 0.1 M was dropped on the electrochemical cell and EIS measurements were carried out by applying a potential of +0.1 V and a AC amplitude of 10 mV. The impedance data was fitted to an equivalent circuit (**Figure S2**), and the value of the resistance to charge transfer (Rct) was chosen to compare both modification methodologies.

3. RESULTS AND DISCUSSION

3.1. Blocking-free immunosensing strategy for the determination of anti-tTG IgA antibodies

Bearing in mind the nonspecific adsorption issues on biosensor applications and the frequent blocking step applied in the great majority of the ~~published~~ electrochemical immunosensors, our approach to address this issue was to improve the modification of the sensing surface with the recognition element. The electrochemical cell was washed completely with the working buffer, and was left to dry at room temperature. This way, the electrochemical cell surface, previously hydrophobic, becomes hydrophilic. Screen-printed electrodes are hydrophobic due to the ink components used in the manufacture (typically polyvinyl chloride and organic resins), normally forming an hydrogen-terminated electrode surface. In order to create an hydrophilic surface, other authors employ time-consuming electrochemical pretreatments to generate oxygenated groups[35] or even ultrasonic polishing with alumina[38]. In the latter case, the improvement on the immobilization of catechol derivatives was described after the activation. In our work, the hydrophilic improvement was obtained after drying Tris molecules on the surface, which increase the hydrophilicity and the subsequent coating with the aqueous solution. The hydrophobicity of the surface would form a barrier limiting the access of the protein aqueous solution to the microscopic electrode surface with the outcome of producing an inferior coating. In contrast, if the surface is hydrophilic, an improved modification seems to occur because the aqueous solution with the recognition element covers the entire electrochemical cell achieving a

surface effectively modified. Therefore, just a negligible amount of the surface should remain available for nonspecific adsorption of interfering species. The higher hydrophilicity of the surface, comparing the contact angle of a tTG solution (in Tris buffer), is shown in **Figure 1A**. An aqueous droplet on a hydrophobic surface tends to exhibit a high contact angle. The shape of the tTG solution droplet was round on the untreated SPCE with a contact angle of 103±6º. The contact angle was reduced to 18±1º after the washing-drying steps with the Tris buffer solution, allowing a better coating efficiency and an increased protein load. This fact was corroborated by the increment on the resistance to charge transfer (Rct) using electrochemical impedance spectroscopy (**Figure 1B**). Values of R_{ct} of 395±12, 3935±440 and 8521±631 Ω were found for bare SPCE, conventionally-modified SPCE and SPCE modified with the novel strategy, respectively. The higher Rct obtained for the latter evidences the higher and more effective load of protein employing this strategy.

Voltammograms obtained for the positive and negative controls of the immunosensor involving a blocking step (30 min, 20 µg/µL of BSA) and conventional modification of the working electrode with tTG are shown in **Figure 2a**. Clearly, a high non-specific adsorption can be noticed by the background signal using this blocking agent. In consequence, for a more efficient surface blockage, a longer time, a higher concentration or a different blocking agent should be employed. For instance, in our previous work [39] for the determination of anti-tTG IgG antibodies using a similar platform, 20 µg/µL of casein was employed during 45 min in order to reduce non-specific binding. After removing the blocking step and using our novel modification of the electrochemical cell with tTG a significant background signal was still obtained (**Figure 2b**) but the signal/noise ratio was highly improved. A study of the different steps of the immunosensor was performed to prevent the non-specific adsorption of the immunoreactives. A small amount of BSA was added to the solution of each immunosensing step (serum control, anti-H-IgA-BT and STV-QD) and the effect on the signal/background ratio was measured. No changes were found

when BSA was added to the solutions of the serum controls or STV-QD. However, a smaller background signal was found when BSA was added to the solution of anti-H-IgA-BT, improving the signal to noise ratio due to the minimization of the nonspecific adsorption of this reagent. Thus, the effective electrode modification proposed in this work in combination with the addition of a small amount of BSA in the bioreagent solution, proned to produce the higher nonspecific binding, minimizes the nonspecific adsorption and improves the signal/background ratio, as shown in the **Figure 2c**. To confirm that the immunoreagents does not exhibit non-specific adsorption, 8-channel SPCEs were modified with HRP, a non-specific recognition element for anti-tTG IgA antibodies, and the previous methodology was carried out. In the **Figure 2d** is shown as the signal for the positive control is not significantly different than the signal for the negative control. Therefore, it seems that the analyte (anti-tTG IgA antibodies) as well as the immunoreagents (anti-H-IgA-BT and QDs-STV) are not adsorbed on the electrode surface in a non-specific way. On the other hand, the elimination of the blocking step produces a decrease in the capacitive currents (data not shown), probably, due to the fact that most of the inert protein blockage is eliminated, increasing the surface area for the immunoreaction of interest, confirmed by the increasing analytical signal obtained for the positive serum control (**Figure 2b**).

After the development of the blocking-free methodology, several parameters affecting the anti-tTG IgA biosensor functionality such as tTG, anti-H-IgA-BT and STV-QDs concentrations or immunological reaction times were optimized. Serum controls (positive and negative) from the ELISA kit were also employed for this optimization (diluted 1:2 in Tris buffer), and the biosensor was carried out varying the studied parameters. Considering the signal/background relation, the most suitable values for these parameters were described in section 2.3.2. With these optimized parameters the highest signal/background relation was obtained for the serum controls samples.

Voltammetric signals for different concentrations of anti-tTG IgA antibodies using human serum calibrators from the commercial ELISA kit are shown in **Figure 3A**. The anodic strripping peak current increased with the increment of the anti-tTG IgA concentration, as can be observed in the calibration curve of the same figure. The analytical signal was linearly dependent on the concentrations of anti-tTG IgA antibodies in the range of 3 to 100 U mL^{-1} following the linear regression equation: $i_p(\mu A) = 0.149\ (\pm 0.002) \cdot [\text{anti-tTG-IgA}] + 0.45\ (\pm 0.09)$, $R^2 = 0.9992$. The reproducibility between the slopes of the calibration curves was 5.5 % (in terms of RSD, n=4). The detection limit, calculated as the concentration corresponding to three times the standard deviation of the estimate, was 2.4 U mL^{-1}. According to the specifications of the commercial ELISA kit, the cut-off values to evaluate the results are the following ones: negative if the concentration is less than 5 U mL^{-1}, indeterminate between 5 and 8 U mL^{-1} and positive if the concentration is above 8 U mL^{-1}. Therefore, the developed sensor is able to differentiate these key concentrations. Using the calibration curve previously obtained, the concentration of anti-tTG IgA antibodies for the positive and negative control sera was measured. For the positive control, a concentration of 32±3 U mL^{-1} was obtained, which is fairly consistent with the data specified in the commercial kit (26±7 U mL^{-1}). For the negative control, the result obtained is below the detection limit of the method (< 3 U mL^{-1} indicated in the certified data of the commercial kit).

This blocking-free strategy was also applied to a system previously published by our group for the determination of anti-tTG IgG antibodies, where a conventional modification procedure and a blocking step was used[39]. With the new approach (specific details are given in the supporting information), an improvement in the sensitivity (calibration slope) is achieved (**Figure S3**). The following linear relation was obtained for the previously published methodology: $i_p(\mu A) = 0.106\ (\pm 0.006) \cdot [\text{anti-tTG-IgG}] + 0.7\ (\pm 0.1)$, $R^2 = 0.994$, while that for the novel strategy the following linear relation was obtained: $i_p(\mu A) = 0.151\ (\pm 0.004) \cdot [\text{anti-tTG-IgG}] + 0.64\ (\pm 0.09)$, $R^2 = 0.997$.

Therefore, the blocking-free strategy developed in this work is able to eliminate one bioassay step, saving significant time and improving the analytical characteristics of the method.

3.2. One-step immunosensing methodology for detection of anti-tTG IgA antibodies

Another valuable goal in the development of biosensors as POC devices is minimizing the immunoreaction steps in order to simplify the analytical procedure avoiding washing steps and reducing the total time of analysis. Therefore, the biosensor optimization was performed to reduce the number of immunological steps, carrying out a simultaneous reaction between all bioreagents (serum controls, anti-H-IgA-BT, QDs-STV and BSA) onto the immunosensing surface without a separate blocking step. Optimization of the parameters affecting the signal/background ratio such as the concentration of reagents and the reaction time was performed (data not shown). Serum controls (positive and negative) from the ELISA kit were also employed for this optimization. The higher signal/background ratio was obtained using concentrations of 6.75 μg μL^{-1} for anti-H-IgA-BT, 9.1 nM for QDs-STV (in terms of QD) and a 1:1.2 ratio for the serum controls. In this case, the effect of BSA in this solution was detrimental for the signal/background ratio, and better results were obtained without BSA. The total time of the immunological reaction was 80 minutes. Even with these optimized conditions, the sensitivity was lower than for the multistep methodology, and to minimize this effect the deposition time of Cd(II) was increased, achieving the higher signal/noise ratio using 400 s of deposition time.

Voltammetric signals and the calibration curve for different concentrations of anti-tTG IgA antibodies using human serum calibrators from the commercial ELISA kit are shown in **Figure 3B**. Saturation of the sensor from higher concentrations than 40 U/mL was observed. Linear relationship between the peak current and the antibody concentration followed the equation: $i_p(\mu A) = 0.068\ (\pm 0.002) \cdot [\text{anti-tTG-IgA}] + 0.86\ (\pm 0.03)$, $R^2 = 0.996$. The reproducibility between the slopes of the calibration curves was 11.2 % (in terms of RSD, n=4). The detection limit from

the calibration curve (calculated as the concentration corresponding to three times the standard deviation of the estimate) was 2.7 U/mL. The lower sensitivity and the smaller linear range compared with the multistep methodology is coherent with the simultaneous immunological reactions, which result in smaller reaction kinetics and a higher electrode surface impediment yielding smaller immunosensing capacity. However, the developed sensor is able to differentiate the clinical relevant concentrations (previously described) with a one-step immunosensing strategy, and performing 8 simultaneous analyses in less than 90 minutes. Using the calibration curve previously obtained, the concentration of anti-tTG IgA antibodies for the positive and negative control sera was measured. For the positive control, a concentration of 30±4 U mL^{-1} was obtained, which is fairly consistent with the data specified in the commercial kit (26±7 U mL^{-1}). For the negative control, the result obtained is lower than the detection limit of the method, consistent with the data provided by the commercial kit (< 3 U mL^{-1}). These results are also consistent with the data obtained for the multistep methodology, confirming that both methodologies could be useful for the detection of anti-tTG IgA antibodies in serum samples.

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 IgA 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 12.1% for the negative samples and 8.1% for the positive samples. The result indicates that the one-step methodology provides precise results. To evaluate the stability of the biosensor, several immunosensors were modified with tTG as explained in section 2.3.1 and stored at 4 ºC 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 **Figure S4**, a significant change was not 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.

These results and analytical characteristics are comparable to those obtained by other electrochemical immunosensors using similar detection platforms but with longer assay times and performing a blocking step[30,40,41]. In other cases, where shorter analysis times are achieved, the procedure is more complicated with several steps[42–44]. Only a blocking-free strategy was published by Pividori et al.[45] using graphite-epoxy composite electrodes, but a complex methodology with several time-consuming (20 min) washing steps to prevent nonspecific adsorption is carried out. Therefore, in the latter case, the elimination of the blocking step complicates the experimental procedure. We consider that this work has critical advantages versus the other electrochemical immunosensors published for the detection of anti-tTG IgA antibodies due to the straightforward methodology developed, the elimination of the blocking step and the further simplification of the immunological reactions, carried out in one step.

4. CONCLUSIONS

A new electrochemical biosensor for the detection of anti-tissue transglutaminase IgA antibodies was developed. Tissue transglutaminase was immobilized on the surface of the electrochemical cell of 8-channel screen-printed arrays using a novel strategy achieving an effective surface coverage and minimizing the electrode surface available for non-specific adsorption. As a result, a one-step immunoassay following a simple and blocking-free methodology was performed. CdSe/ZnS QDs are used as electrochemical label with an in-situ detection on the electrode surface previously used to carry out the immunoassay. The developed biosensor is able to differentiate the clinical relevant concentrations with this one-step immunosensing strategy performing 8 simultaneous analyses in less than 90 minutes. The good results obtained for serum calibrators and controls from a commercial ELISA kit indicate that the developed analytical platform is a valid tool for the determination of celiac disease biomarkers.

ACKNOWLEDGEMENTS

This work has been supported by the MICINN-12-CTQ2011-24560 project from the Spanish Ministery of Economy and Competitiveness (MEC) and by the FC-15-GRUPIN-021 project from the Asturias regional Government. Daniel Martín-Yerga thanks the MEC for the award of a FPI grant (BES-2012-054408).

REFERENCES

[1] G.J. Kost, N.K. Tran, R.F. Louie, Point-of-Care Testing: Principles, Practice and Critial-Emergency-Disaster Medicine, in: Encycl. Anal. Chem., John Wiley & Sons, Ltd, 2008.

[2] V. Gubala, L.F. Harris, A.J. Ricco, M.X. Tan, D.E. Williams, Point of care diagnostics: status and future., Anal. Chem. 84 (2012) 487–515.

[3] J. Wang, Electrochemical biosensors: towards point-of-care cancer diagnostics., Biosens. Bioelectron. 21 (2006) 1887–92.

[4] J. Wang, Electrochemical glucose biosensors., Chem. Rev. 108 (2008) 814–25.

[5] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Electrochemical biosensors., Chem. Soc. Rev. 39 (2010) 1747–63.

[6] Z. Taleat, A. Khoshroo, M. Mazloum-Ardakani, Screen-printed electrodes for biosensing: a review (2008–2013), Microchim. Acta. 181 (2014) 865–891.

[7] M.M.P.S. Neves, M.B. González-García, D. Hernández-Santos, P. Fanjul-Bolado, Screen-Printed Electrochemical 96-Well Plate : a High- Throughput Platform for Multiple Analytical Applications, Electroanalysis. 26 (2014) 2764–2772.

[8] J. Biscay, M.B.G. García, A.C. García, Electrochemical biotin determination based on a screen printed carbon electrode array and magnetic beads, Sens. Actuators B. 205 (2014) 426–432.

[9] D. Du, Z. Zou, Y. Shin, J. Wang, H. Wu, M.H. Engelhard, et al., Sensitive immunosensor for cancer biomarker based on dual signal amplification strategy of graphene sheets and multienzyme functionalized carbon nanospheres., Anal. Chem. 82 (2010) 2989–95.

[10] D. Zheng, Q. Wang, F. Gao, Q. Wang, W. Qiu, F. Gao, Development of a novel electrochemical DNA biosensor based on elongated hexagonal-pyramid CdS and poly-isonicotinic acid composite film., Biosens. Bioelectron. 60 (2014) 167–74.

[11] S. Patris, P. De Pauw, M. Vandeput, J. Huet, P. Van Antwerpen, S. Muyldermans, et al., Nanoimmunoassay onto a screen printed electrode for HER2 breast cancer biomarker determination., Talanta. 130 (2014) 164–70.

[12] H. V Tran, B. Piro, S. Reisberg, L. Huy Nguyen, T. Dung Nguyen, H.T. Duc, et al., An electrochemical ELISA-like immunosensor for miRNAs detection based on screen-printed gold electrodes modified with reduced graphene oxide and carbon nanotubes., Biosens. Bioelectron. 62 (2014) 25–30.

[13] G. Lai, H. Zhang, J. Yong, A. Yu, In situ deposition of gold nanoparticles on polydopamine functionalized silica nanosphere for ultrasensitive nonenzymatic electrochemical immunoassay., Biosens. Bioelectron. 47 (2013) 178–83.

[14] R.C. Alves, F.B. Pimentel, H.P.A. Nouws, R.C.B. Marques, M.B. González-García, M.B.P.P. Oliveira, et al., Detection of Ara h 1 (a major peanut allergen) in food using an electrochemical gold nanoparticle-coated screen-printed immunosensor., Biosens. Bioelectron. 64C (2014) 19–24.

[15] J.-J. Shi, T.-T. He, F. Jiang, E.S. Abdel-Halim, J.-J. Zhu, Ultrasensitive multi-analyte electrochemical immunoassay based on GNR-modified heated screen-printed carbon electrodes and PS@PDA-metal labels for rapid detection of MMP-9 and IL-6., Biosens. Bioelectron. 55 (2014) 51–6.

[16] R. Gill, M. Zayats, I. Willner, Semiconductor quantum dots for bioanalysis., Angew. Chem., Int. Ed. 47 (2008) 7602–25.

[17] F. Lisdat, D. Schäfer, A. Kapp, Quantum dots on electrodes-new tools for bioelectroanalysis., Anal. Bioanal. Chem. (2013) 3739–3752.

[18] J. Drbohlavova, V. Adam, R. Kizek, J. Hubalek, Quantum dots - characterization, preparation and usage in biological systems., Int. J. Mol. Sci. 10 (2009) 656–73.

[19] D. Hynek, K. Tmejova, V. Milosavljevic, A. Moulick, P. Kopel, V. Adam, et al., Electrochemical Characterization of Various Synthesized Quantum Dots and the Effect of Aging and Storage Way, Int. J. Electrochem. Sci. 10 (2015) 1117–1127.

[20] D. Martín-Yerga, D. Bouzas-Ramos, M. Menéndez-Miranda, A.R.M. Bustos, J.R. Encinar, J.M. Costa-Fernández, et al., Voltammetric determination of size and particle concentration of Cd-based quantum dots, Electrochim. Acta. 166 (2015) 100–106.

[21] J. Wang, G. Liu, A. Merkoçi, Electrochemical coding technology for simultaneous detection of multiple DNA targets., J. Am. Chem. Soc. 125 (2003) 3214–5.

[22] J.A. Hansen, J. Wang, A.-N. Kawde, Y. Xiang, K. V Gothelf, G. Collins, Quantum-dot/aptamer-based ultrasensitive multi-analyte electrochemical biosensor., J. Am. Chem. Soc. 128 (2006) 2228–9.

[23] K. Pinwattana, J. Wang, C.-T. Lin, H. Wu, D. Du, Y. Lin, et al., CdSe/ZnS quantum dots based electrochemical immunoassay for the detection of phosphorylated bovine serum albumin., Biosens. Bioelectron. 26 (2010) 1109–13.

[24] D. Du, J. Ding, Y. Tao, H. Li, X. Chen, CdTe nanocrystal-based electrochemical biosensor for the recognition of neutravidin by anodic stripping voltammetry at electrodeposited bismuth film., Biosens. Bioelectron. 24 (2008) 869–74.

[25] D. Martín-Yerga, M.B. González-García, A. Costa-García, Biosensor array based on the in situ detection of quantum dots as electrochemical label, Sens. Actuators B. 182 (2013) 184–189.

[26] K. Lindfors, K. Kaukinen, M. Mäki, A role for anti-transglutaminase 2 autoantibodies in the pathogenesis of coeliac disease?, Amino Acids. 36 (2009) 685–91.

[27] M.M.P. da Silva Neves, M.B. González-García, H.P.A. Nouws, C. Delerue-Matos, A. Santos-Silva, A. Costa-García, et al., Celiac disease diagnosis and gluten-free food analytical control., Anal. Bioanal. Chem. 397 (2010) 1743–53.

[28] K. Teesalu, D. Agardh, M. Panarina, M. Utt, O. Uibo, R. Uibo, A modified ELISA for improved detection of IgA, IgG, and IgM anti-tissue transglutaminase antibodies in celiac disease, Clin. Chim. Acta. 403 (2009) 37–41.

[29] D. Basso, G. Guariso, D. Bozzato, E. Rossi, M. Pescarin, P. Fogar, et al., New screening tests enrich anti-transglutaminase results and support a highly sensitive two-test based strategy for celiac disease diagnosis., Clin. Chim. Acta. 412 (2011) 1662–7.

[30] M.M.P.S. Neves, M.B. González-García, H.P.A. Nouws, A. Costa-García, Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes., Biosens. Bioelectron. 31 (2012) 95–100.

[31] M.M.P.S. Neves, M.B. González-García, C. Delerue-Matos, A. Costa-García, Multiplexed electrochemical immunosensor for detection of celiac disease serological markers, Sens. Actuators B. 187 (2013) 33–39.

[32] D. Martín-Yerga, A. Costa-García, Electrochemical Immunosensors for Celiac Disease Detection, Int. J. Celiac Dis. 2 (2014) 142–143.

[33] J. Wang, M. Pedrero, H. Sakslund, O. Hammerich, J. Pingarron, Electrochemical activation of screen-printed carbon strips, Analyst. 121 (1996) 345.

[34] R.O. Kadara, N. Jenkinson, C.E. Banks, Characterisation of commercially available electrochemical sensing platforms, Sens. Actuators B. 138 (2009) 556–562.

[35] M. Moreno, A.S. Arribas, E. Bermejo, M. Chicharro, A. Zapardiel, M.C. Rodríguez, et al., Selective detection of dopamine in the presence of ascorbic acid using carbon nanotube modified screen-printed electrodes., Talanta. 80 (2010) 2149–56.

[36] A. Economou, Bismuth-film electrodes: recent developments and potentialities for electroanalysis, TrAC, Trends Anal. Chem. 24 (2005) 334–340.

[37] N. Serrano, J.M. Díaz-Cruz, C. Ariño, M. Esteban, Ex situ Deposited Bismuth Film on Screen-Printed Carbon Electrode: A Disposable Device for Stripping Voltammetry of Heavy Metal Ions, Electroanalysis. 22 (2010) 1460–1467.

[38] Y.-L. Su, C.-Y. Tai, J.-M. Zen, A Simple Method to Tune Up Screen-Printed Carbon Electrodes Applicable to the Design of Disposable Electrochemical Sensors, Electroanalysis. 25 (2013) 2539–2546.

[39] D. Martín-Yerga, M.B. González-García, A. Costa-García, Electrochemical immunosensor for anti-tissue transglutaminase antibodies based on the in situ detection of quantum dots., Talanta. 130 (2014) 598–602.

[40] T. Balkenhohl, F. Lisdat, Screen-printed electrodes as impedimetric immunosensors for the detection of anti-transglutaminase antibodies in human sera., Anal. Chim. Acta. 597 (2007) 50–7.

[41] M. Giannetto, M. Mattarozzi, E. Umiltà, A. Manfredi, S. Quaglia, M. Careri, An amperometric immunosensor for diagnosis of celiac disease based on covalent immobilization of open conformation tissue transglutaminase for determination of anti-tTG antibodies in human serum., Biosens. Bioelectron. 62C (2014) 325–330.

[42] S. Dulay, P. Lozano-Sánchez, E. Iwuoha, I. Katakis, C.K. O'Sullivan, Electrochemical detection of celiac disease-related anti-tissue transglutaminase antibodies using thiol based surface chemistry., Biosens. Bioelectron. 26 (2011) 3852–3856.

[43] G. Adornetto, G. Volpe, A. De Stefano, S. Martini, G. Gallucci, A. Manzoni, et al., An ELIME assay for the rapid diagnosis of coeliac disease., Anal. Bioanal. Chem. 403 (2012) 1191–4.

[44] S. V Kergaravat, L. Beltramino, N. Garnero, L. Trotta, M. Wagener, M. Isabel Pividori, et al., Electrochemical magneto immunosensor for the detection of anti-TG2 antibody in celiac disease., Biosens. Bioelectron. 48 (2013) 203–9.

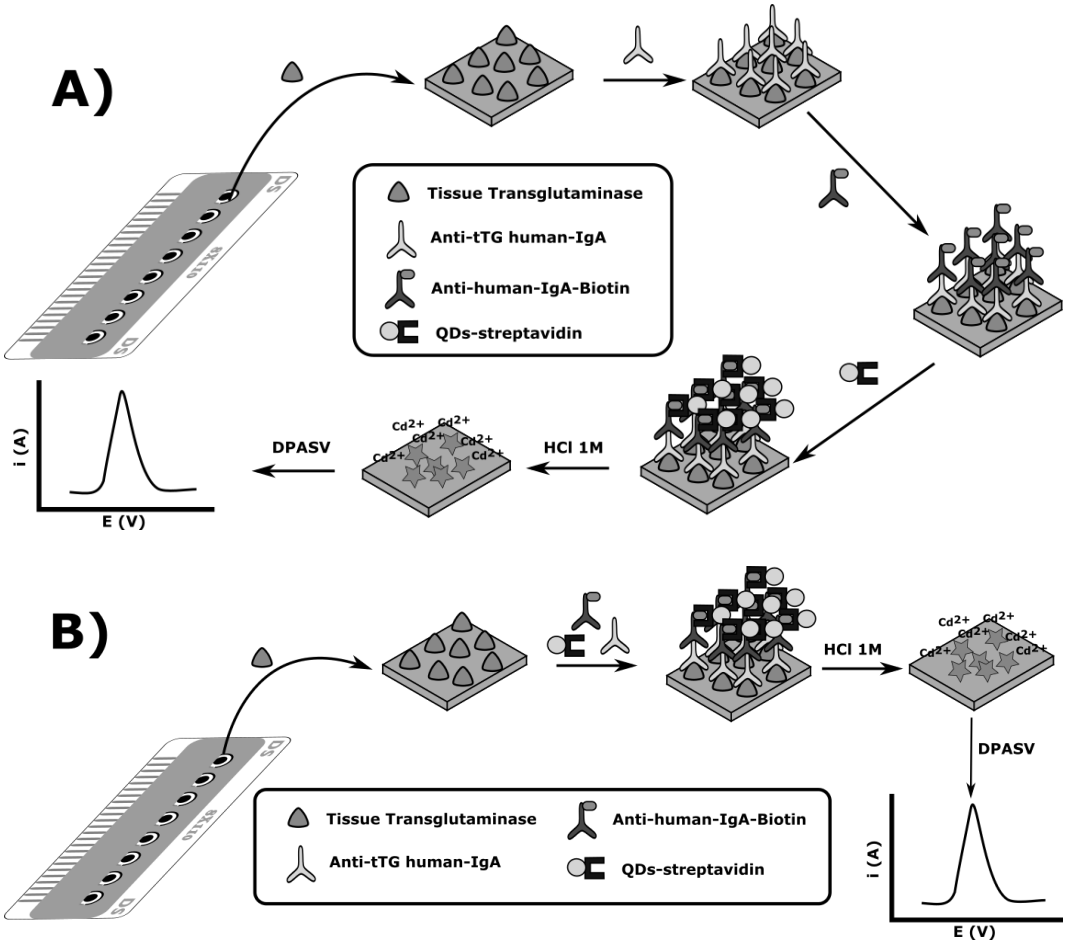
[45] M.I. Pividori, A. Lermo, A. Bonanni, S. Alegret, M. del Valle, Electrochemical immunosensor for the diagnosis of celiac disease., Anal. Biochem. 388 (2009) 229–34.

VITAE

Agustín Costa-García obtained his B.Sc. degree in chemistry, focus in analytical chemistry, in 1974 (University of Oviedo) and the Ph.D. in chemistry in 1977 (University of Oviedo). Since February 2000 he is professor in analytical chemistry (University of Oviedo). He leads the Nanobioanalysis Research Group of the University of Oviedo and has been supervisor of several research projects developed at the electrochemistry laboratories of the Department of Physical and Analytical Chemistry of the University of Oviedo. Nowadays his research is focused on the development of nanostructured electrodic surfaces and its use as transducers for electrochemical immunosensors and genosensors employing both enzymatic and non-enzymatic labels.

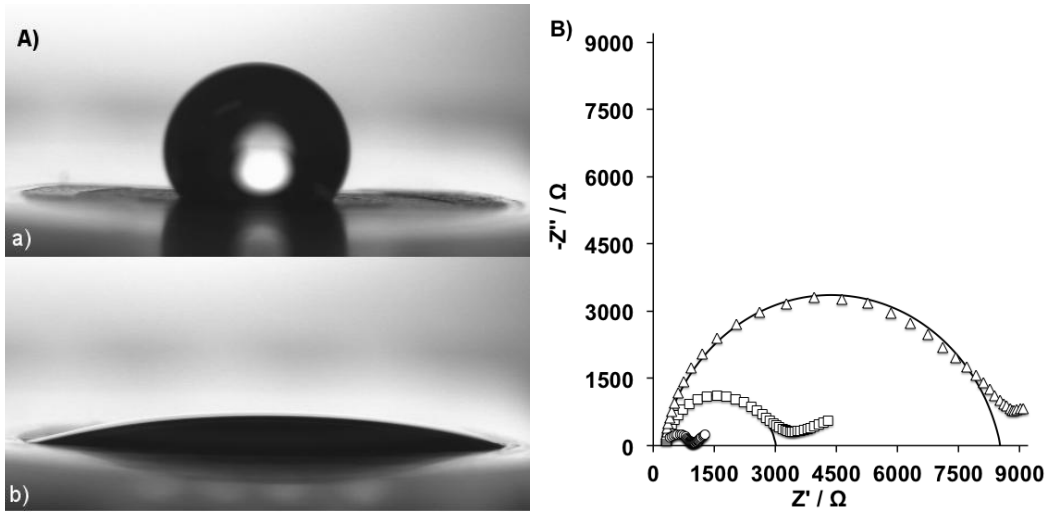
Daniel Martín-Yerga obtained his B.Sc. degree in chemistry, focus on analytical chemistry in 2010 (University of Oviedo) and the M.Sc. degree in analytical and bioanalytical chemistry in 2011 (University of Oviedo). At present, he is a PhD student at the Nanobioanalysis research group of the University of Oviedo, supervised by Prof. A. Costa-García. He has been working with electrochemical detection of metals and metal-based nanoparticles and its application in biosensing devices using screen-printed electrodes.

Schematic 1. **A)** Schematic diagram of the electrochemical immunosensor for anti-tTG IgA antibodies detection with the multi-step bioassay procedure. **B)** Schematic diagram of the electrochemical immunosensor for anti-tTG IgA antibodies detection with the one-step bioassay procedure.



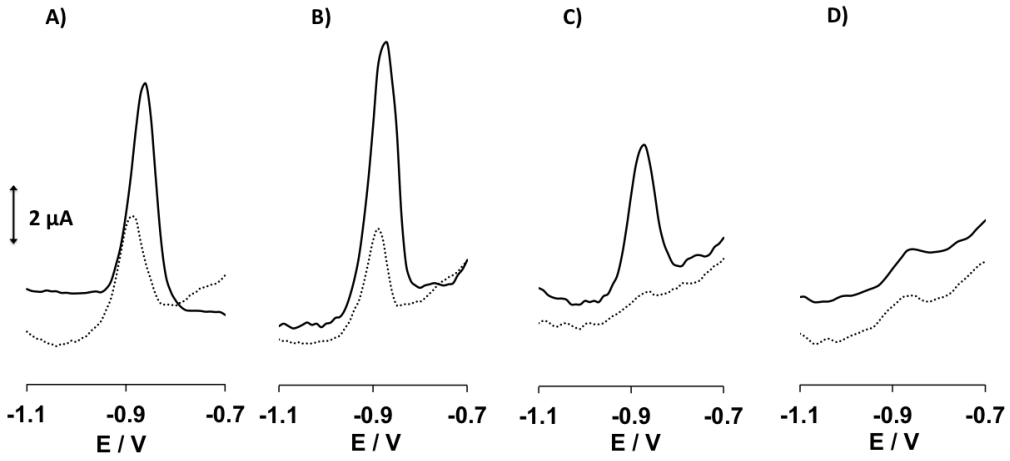
(image preferred size: 1.5 column)

Figure 1. A) Picture of tTG solution droplets and its contact angles using a conventional modification procedure (a) and the novel modification procedure developed in this work (b). **B)** Nyquist plots for EIS measurements for bare SPCE (circles), SPCE after conventional modification with tTG (squares) and SPCE after the novel modification with tTG developed in this work (triangles).



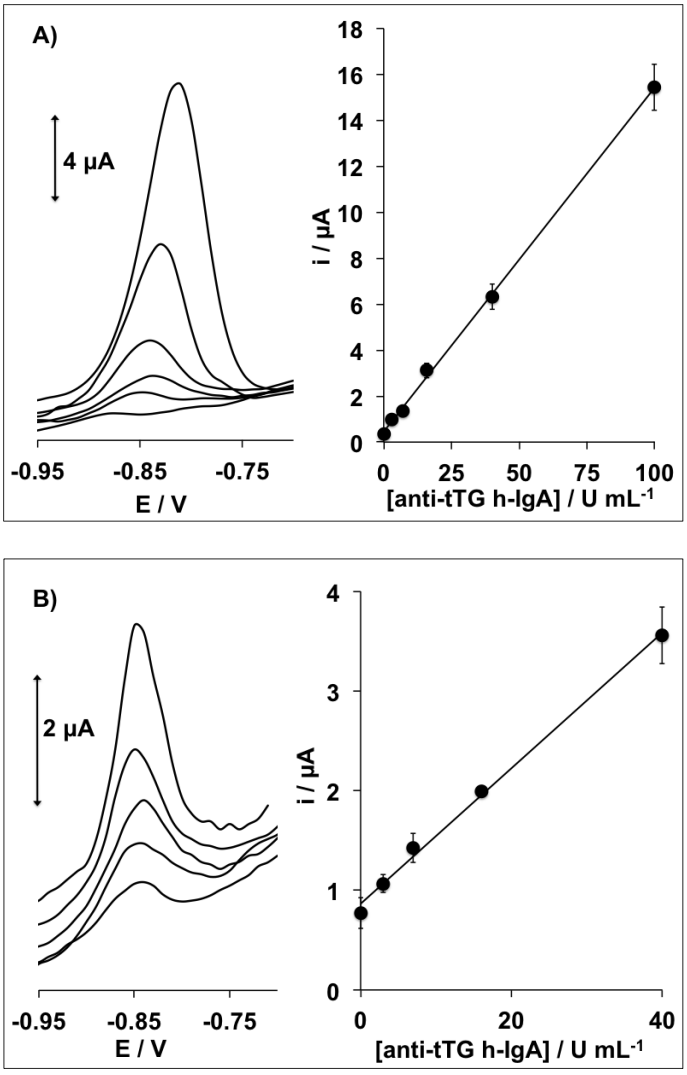
(image preferred size: 1.5-column)

Figure 2. Differential-pulse voltammetry of the analytical signal for positive (solid line) and negative (dashed line) sera controls using the electrochemical immunosensor with a blocking step (A), without blocking step and without BSA (B), without blocking step but with BSA in the anti-H-IgA-BT step (C), using HRP as non-specific recognition element of the immunosensor (D).



(image preferred size: 1.5-column)

Figure 3. A) Differential-pulse voltammograms and calibration plot for the developed immunosensor following the multi-step procedure (0, 3, 7, 16, 40, 100 U/mL for anti-tTG IgA concentrations). **B)** Differential-pulse voltammograms and calibration plot for the developed immunosensor following the one-step procedure (0, 3, 7, 16, 40 U/mL for anti-tTG IgA concentrations).



(image preferred size: 1-column)

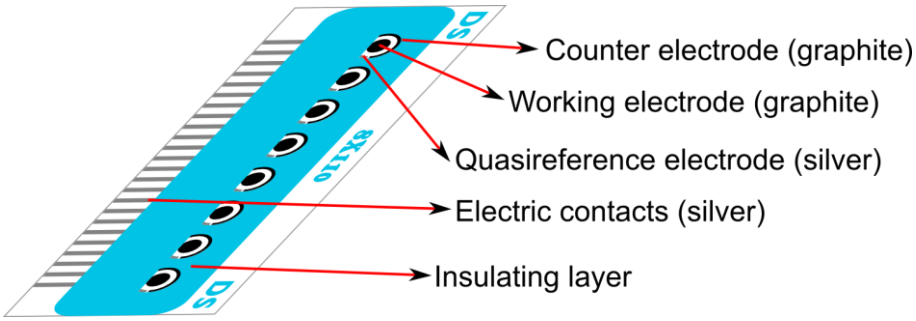
SUPPORTING INFORMATION

Towards a blocking-free electrochemical immunosensing strategy for anti-transglutaminase antibodies using screen-printed electrodes

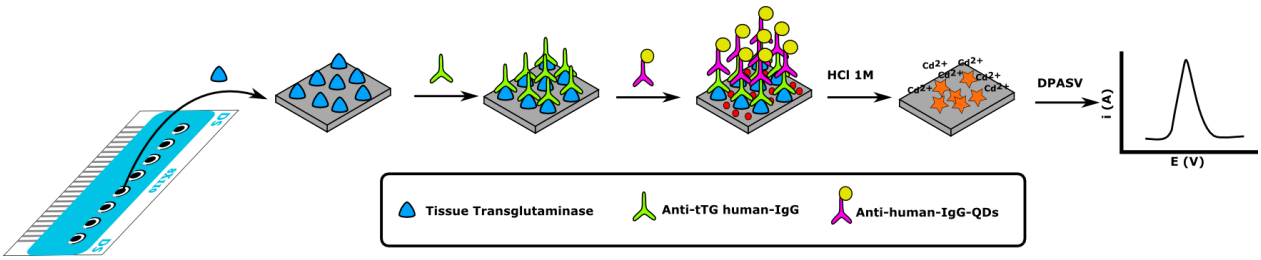
Daniel Martín-Yerga and Agustín Costa-García

Immunoassay procedure for the determination of anti-tissue transglutaminase IgG antibodies

An aliquot of 4 µL of tTG solution (in 0.1 M pH 7.4 Tris buffer) with a concentration of 0.1 µg μl^{-1} was dropped on the surface of each working electrode and was left overnight at 4 ºC. After washing the screen-printed electrode with Tris buffer, the immunosensor was incubated with human serum controls for 60 min followed by a washing step with Tris buffer. Finally, 25 µL of anti-H-IgG-QD (10 nM in terms of QDs) solution (with 10 µg μL^{-1} of BSA) was added 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 **Schematic S2**, a diagram of the immunoassay using this biosensor is presented. The measurement step was performed according to the methodology described in section 2.3.4. An scheme of the methodology with a blocking step (casein 20 µg μL^{-1}, 45 min) is presented in a previous work(Martín-Yerga et al., 2012).



Schematic S1. Schematic diagram of the 8-channel screen-printed electrochemical array (Dropsens 8x110).



Schematic S2. Schematic diagram of the electrochemical immunosensor for anti-tTG IgG antibodies detection with a blocking-free multi-step bioassay procedure.

Figure S1. Image of the 8-channel screen-printed electrochemical array and the corresponding connector to the Dropsens µStat 8000 potentiostat.

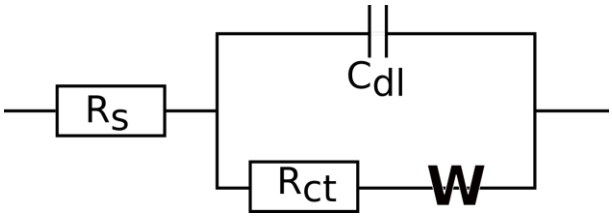


Figure S2. Equivalent circuit employed to fit the EIS data. Rs is the uncompensated solution resistance; Cdl the double-layer capacitance, Rct is the resistance to charge transfer and W is the Warburg diffusion coeficient.

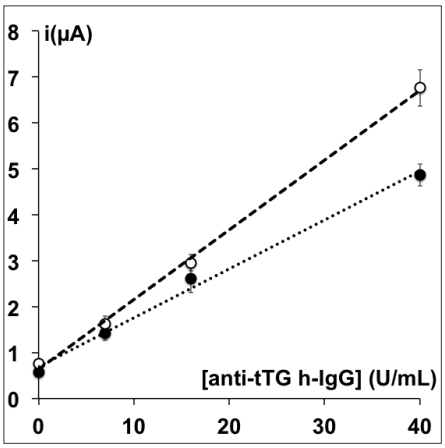


Figure S3. Calibration plot for the concentration of anti-tTG IgG antibodies with a blocking step (point line) and with a blocking-free methodology (dashed line).

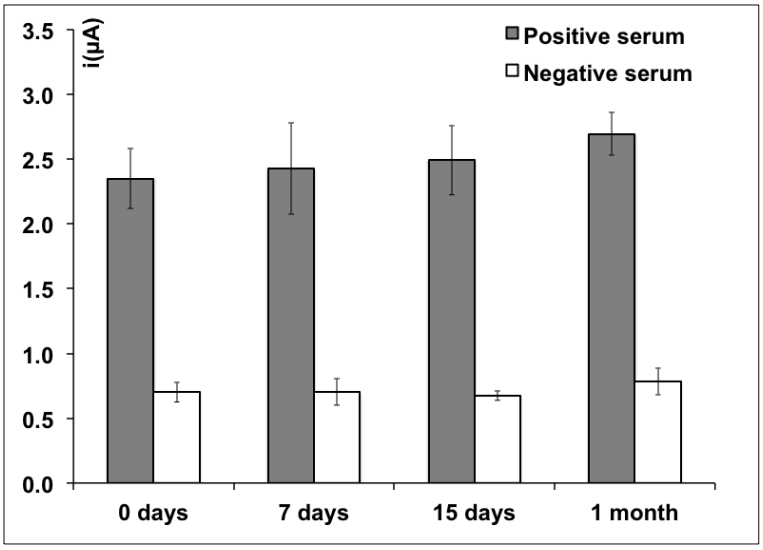


Figure S4. Stability study of the electrochemical immunosensor following the one-step bioassay procedure for the detection of anti-tTG IgA antibodies.

REFERENCES

Martín-Yerga, D., González-García, M.B., Costa-García, A., 2014. Electrochemical immunosensor for anti-tissue transglutaminase antibodies based on the in situ detection of quantum dots. Talanta 130, 598–602.

Agustín Costa-García:

Daniel Martín-Yerga: