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

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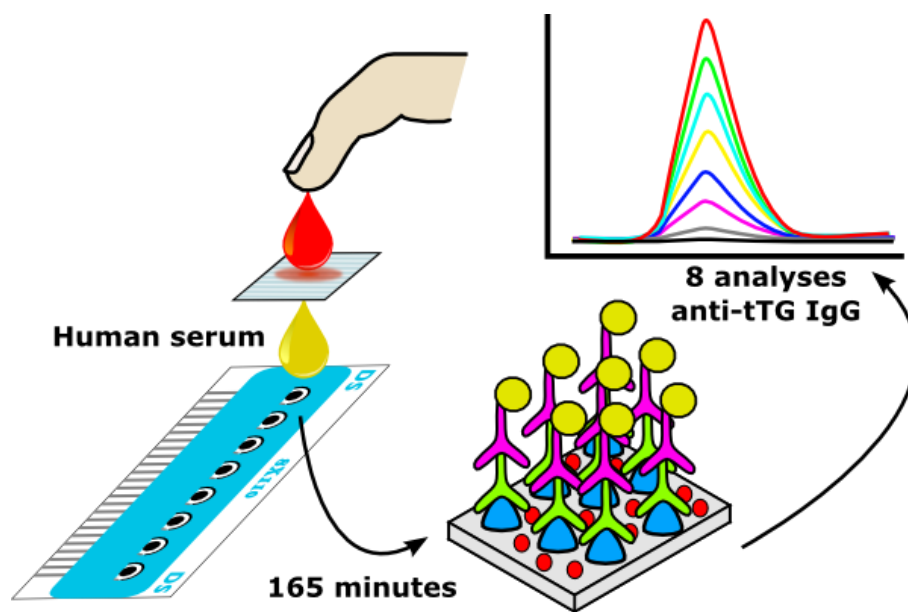
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Highlights

- An electrochemical biosensor for detection of anti-transglutaminase IgG antibodies was developed.
- In situ detection of quantum dots results in a simple and time-effective methodology.
- This biosensor is able to discriminate the key concentrations to diagnose the celiac disease.

Graphical abstract



ABSTRACT

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3 A miniaturized electrochemical biosensor array with in situ detection of quantum dots (QDs) was
4 developed for the detection of anti-transglutaminase IgG antibodies (a celiac disease biomarker) in
5 human sera. For the fabrication of the sensor, a 8-channel screen-printed carbon electrochemical
6 arrays were used as transducers and modified with tissue-transglutaminase by adsorption. The
7 immunologic reaction was carried out in a few simple steps: reaction with human serum, which
8 contains the analyte of interest, followed by the immunoreaction with anti-human IgG labeled with
9 CdSe/ZnS QDs and electrochemical detection of Cd²⁺ released from QDs. All steps were performed
10 on the screen-printed arrays as the solid support, and the detection of Cd²⁺ was performed in situ
11 after acid attack of the QDs without a transfer step by voltammetric stripping. The electrochemical
12 response was correlated with the anti-transglutaminase IgG concentration. The developed
13 electrochemical immunosensor is a trustful screening tool for celiac disease diagnosis
14 discriminating between positive and negative sera samples with high sensitivity.
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54 **KEYWORDS:** celiac disease, immunosensor, electrochemical biosensor, quantum dots, screen-
55 printed electrodes
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1. INTRODUCTION

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3 Nowadays, electrochemical biosensors are playing an important role as clinical diagnostic
4 platforms[1,2]. Due to the versatility and high sensitivity, electrochemical biosensors are placed in
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6 the lead to become a future detection platform at different medical centers for disease diagnosis. To
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8 achieve this goal, the development of new detection labels that improve the ones employed so far,
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10 which are mainly enzymatic, is a constant concern in this scientific field[3–6].
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14 Quantum dots (QDs) are semiconductor nanoparticles widely used in several application, mainly on
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16 optical and electrochemical biosensing[7–9]. QDs are used in bioassays with fluorescence detection
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18 due to their interesting luminescent properties[10]. Metallic components of QDs can be measured
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20 electrochemically after an acid attack to break the nanoparticle and release metal cations to the
21
22 solution. Normally, both the bioassay and the subsequent acid attack are all performed outside of
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24 the detection platform[11–14]. Our research group has developed an innovative methodology in
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26 which the bioassay as the acid attack and the detection are performed directly on the screen-printed
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28 electrodes[15]. This methodology is much easier and similar to performing bioassays with
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30 enzymatic labels. Furthermore, using screen-printed electrodes as the biosensor platform provides
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32 important benefits over the use of other kind of electrodes, such as low sample volume, low cost
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34 device, the ability to perform in-situ analysis, besides being disposable avoiding tedious cleaning
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36 steps of the electrodes. The combination of the biosensor methodology developed by our group and
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38 the use of QDs as electrochemical label results in an attractive platform capable to compete with
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40 enzymatic labeling systems but saving the time of the enzymatic reaction and reagents.
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52 Celiac disease (CD) is a gluten-sensitive enteropathy triggered by dietary gluten in genetically
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54 susceptible individuals. CD patients, normally, experience immune reaction and the body produces
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56 autoantibodies causing the destruction of intestinal mucous[16]. The main criteria for the diagnosis
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58 of CD is a biopsy, but in recent years, the serological tests for the detection of biomarkers are being
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1 imposed, allowing to avoid the more invasive analysis[17]. Some of the autoantibodies produced in
2 a CD patient react specifically with tissue transglutaminase (tTG), being an important biomarker of
3 this autoimmune condition. Although, anti-tTG IgA antibodies seems to be a more specific
4 biomarker, sometimes when the patient has IgA deficiency, the determination of anti-tTG IgG
5 antibodies is crucial in order to make a diagnosis. Therefore, the detection of anti-tTG IgG is also
6 conducted in medical facilities for the serological detection of CD. The most common methodology
7 for the detection of CD autoantibodies is an enzyme-linked immunosorbent assay (ELISA). ELISA
8 tests have been used for the detection of anti-tTG antibodies with good sensitivity and
9 specificity[18,19]. An alternative to ELISA tests are the electrochemical immunosensors that have
10 some advantages such as increased sensitivity due to the electrochemical detection, besides the use
11 of lower sample volumes, resulting in cost savings[20–23].
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28 Among the published electrochemical immunosensors for the determination of anti-tTG IgG, this is,
29 to the best of our knowledge, the first work employing QDs as electrochemical label. Moreover, in
30 this work we use an innovative methodology where both the bioassay and the detection of QDs are
31 performed in the same platform (i.e. screen-printed carbon electrodes). As explained above, this
32 methodology highly simplifies the ones previously published using QDs electrochemical detection,
33 being easier to perform with less number of steps. Another advantage of this work is the use of 8-
34 channel screen-printed electrochemical arrays (8xSPCEs), which further simplifies the procedure
35 and drastically improves the time of analysis.
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50 **2. MATERIALS AND METHODS**

51 **2.1. APPARATUS AND ELECTRODES**

52 Voltammetric measurements were performed with a μ Stat 8000 (DropSens, Spain)
53 potentiostat interfaced to a Pentium 4 2.4 GHz computer system and controlled by DropView 8400
54 2.0 software. All measurements were carried out at room temperature. 8-channel screen-printed
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1 electrochemical arrays were purchased from DropSens (Spain). Each array is formed by eight 3-
2 electrode electrochemical cells (30 μL volume) with carbon-based working and counter electrodes,
3 whereas pseudoreference electrodes and electric contacts are made of silver. This device has
4 dimensions of 4.0 x 7.9 x 0.06 cm (length x width x height) and the diameter of the working
5 electrodes is 2.56 mm. 8-channel arrays were connected to the potentiostat through a specific
6 connector, DRP-CAST8X.
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12 2.2. REAGENTS AND SOLUTIONS

13 Sodium hydroxide, acetic acid (100 %), fuming hydrochloric acid (37%) were purchased
14 from Merck. Bismuth(III) standard, Tris(hydroxymethyl)aminomethane (Tris), bovine serum
15 albumin fraction V (BSA) and β -casein (98%, from bovine milk) were purchased from Sigma-
16 Aldrich (Spain). Human tissue transglutaminase (recombinantly produced in insect cells) was
17 purchased from Zedira (Germany). Qdot® 655 goat F(ab')₂ anti-human IgG Conjugate (H+L)
18 (anti-H-IgG-QD) was purchased from Life Technologies (Spain). Vareliisa Celikey IgG ELISA kits
19 were purchased from Phadia (Germany). Each kit contained six standard serum samples (0, 3, 7, 16,
20 40, 100 U mL⁻¹) and a positive and a negative control. Ultrapure water obtained with a Millipore
21 Direct Q5™ purification system from Millipore Ibérica S.A. (Madrid, Spain) was used throughout
22 this work. All other reagents were of analytical grade. Working solutions of tTG, anti-H-IgG-QD,
23 BSA, and casein were prepared in 0.1 M pH 7.4 Tris-HCl buffer.
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44 2.3. IMMUNOASSAY PROCEDURE

45 An aliquot of 4 μL of tTG solution with a concentration of 0.1 mg mL⁻¹ was dropped on the
46 surface of each working electrode and was left overnight. After washing the screen-printed
47 electrode with 0.1 M pH 7.4 Tris-HCl buffer, a blocking step was carried out placing 25 μL of
48 casein blocking buffer (20 μg μL^{-1} of casein in 0.1 M pH 7.4 Tris-HCl) solution for 45 minutes.
49 The detection of anti-tTG IgG antibodies was accomplished by incubating the immunosensor with
50 human serum samples for 60 min followed by a washing step with 0.1 M Tris-HCl pH 7.4 buffer.
51 Finally, 25 μL of anti-H-IgG-QD (10 nM in terms of QDs) solution (with 1 μg μL^{-1} of BSA) was
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dropped on the modified electrode for 60 minutes. 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.

[SCHEME 1]

The measurement step was performed according to a methodology previously developed[15]. After the biological reaction, 1 μL of HCl 1.0 M was added on the working electrode to release Cd^{2+} from QDs, followed by the addition of 25 μL 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 -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 minutes as blocking time and 60 minutes 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 (**Figure 1A**) 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.

[FIGURE 1]

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3 Both real serum samples and serum controls contain a large amount of immunoglobulins and other
4 proteins that may produce nonspecific adsorption and yield an unrealistic result. Therefore, the
5 reduction of the nonspecific adsorption is crucial to obtain low background signals and improve the
6 sensitivity of the biosensor. The high signal obtained with negative controls for the initial studies is
7 due to this nonspecific adsorption.
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18 To lower the nonspecific adsorption several parameters that could affect this process were
19 optimized. A study of the blocking effect of BSA and casein was performed, testing the effect of
20 different concentrations (5, 10 and 20 $\mu\text{g } \mu\text{L}^{-1}$) of these blocking agents. Casein blocked the surface
21 more effectively than BSA, achieving the best signal/background relation with 20 $\mu\text{g } \mu\text{L}^{-1}$ of
22 casein. With lower concentrations of casein the blocking effect was not enough, while that with
23 higher concentrations the blocking effect was excessive, negatively affecting the analytical signal.
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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 minutes 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 minutes was chosen.

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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 nonspecific 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

1 **Figure 1B.**
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5 3.2. OPTIMIZATION OF THE EXPERIMENTAL CONDITIONS

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8 Several parameters affecting the biosensor functionality were optimized. Serum controls (positive
9 and negative) from the ELISA kit were also employed for this optimization, and the biosensing was
10 carried out varying the studied parameters.
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18 tTG concentration was changed between different values (0.01, 0.05, 0.1 and 0.2 mg mL^{-1}) and the
19 response of the biosensor was tested. Results showed that the most suitable concentration of tTG
20 was 0.1 mg mL^{-1} . Lower concentrations decreased the signal for the positive control (without a
21 noticeable change in the negative control signal) reducing the signal/background relation. Higher
22 concentrations did not improve the signal/background relation compared to that obtained with 0.1
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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 minutes 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.

66 3.3. ANALYTICAL PERFORMANCE

67 3.3.1. Calibration plot

68 The developed sensor was used with the optimized conditions to establish a relationship between
69 the analytical signal and the concentration of anti-tTG IgG antibodies using human serum
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calibrators from the commercial ELISA kit. **Figure 2A** 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: $i(\mu\text{A}) = 0.106 (\pm 0.006) \cdot [\text{anti-tTG-IgG}] + 0.7 (\pm 0.1)$, $R^2 = 0.994$. 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 **Figure 2B**. 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, 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 (<3U mL⁻¹ indicated in the quality data of the commercial kit).

[FIGURE 2]

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 **Figure 3**, 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.

[FIGURE 3]

4. CONCLUSIONS

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

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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.

Figure 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.

Figure 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

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

Scheme 1

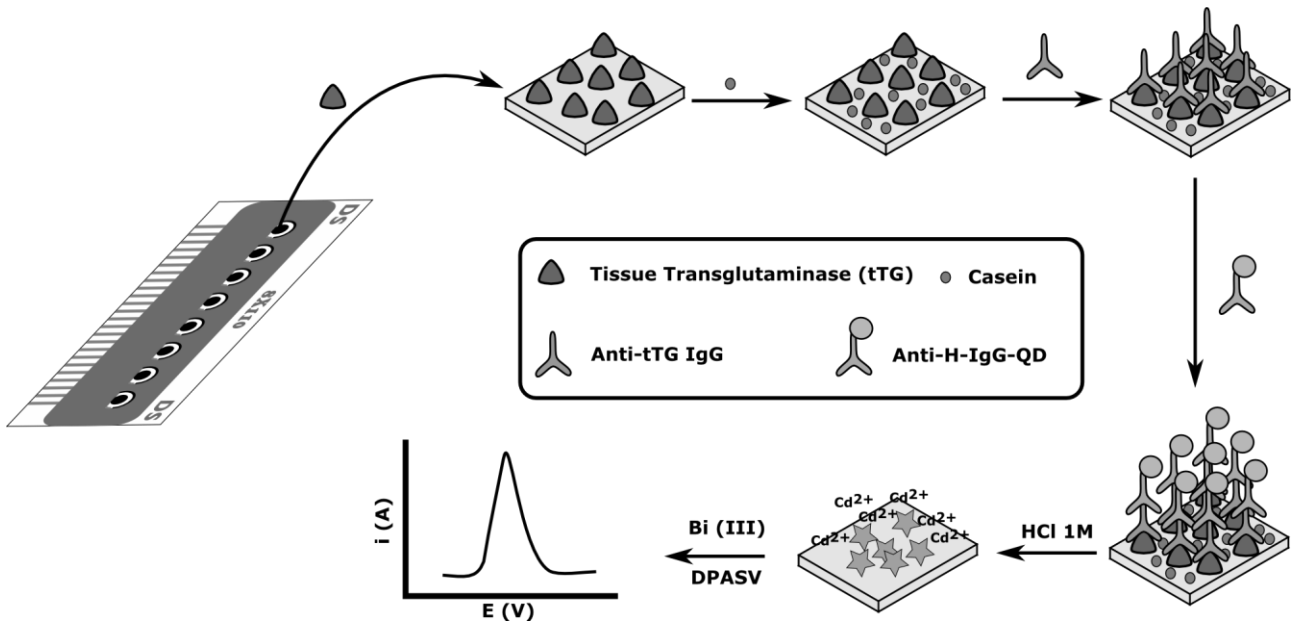


Figure 1

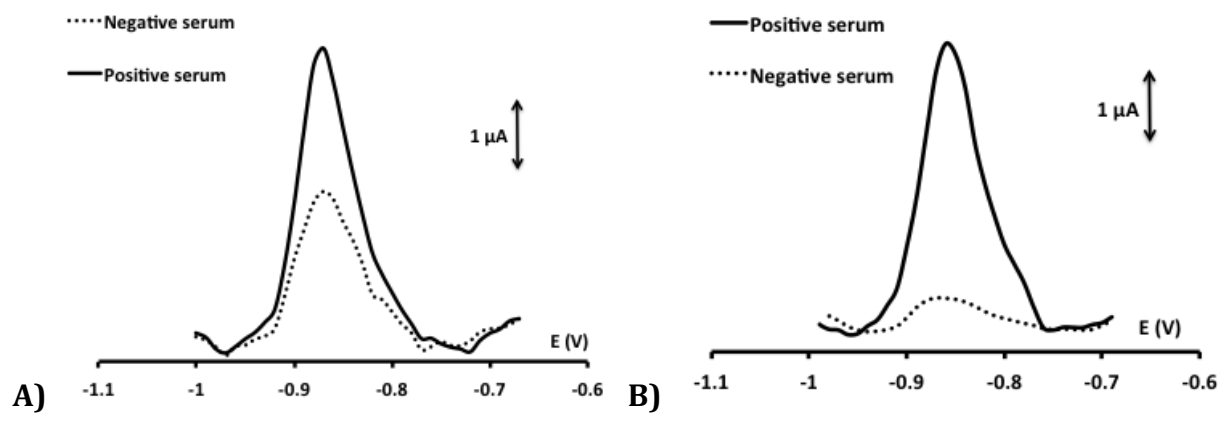


Figure 2

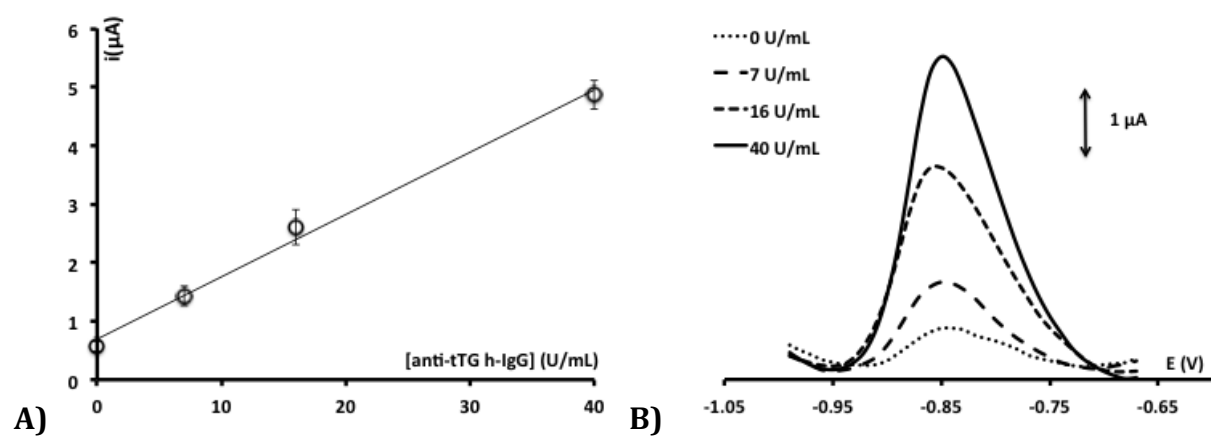


Figure 3

