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PAPER IN FOREFRONT

IgG anti-gliadin determination with an immunological microfluidic system applied to the automated diagnostic of the celiac disease

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Abstract In the present article, a novel microfluidic immunosensor coupled with electrochemical detection for anti-gliadin IgG antibody quantification is proposed. This device represents an important tool for a fast, simple, sensitive, and automated diagnostic for celiac disease, which is carried out through detection of anti-gliadin IgG antibodies present in human serum samples. Celiac disease (CD) is an autoimmune disease generated by gluten protein fractions called prolamins. This pathology affects about one in 250 people around the world, produces intestinal inflammation, villous atrophy, and crypt hyperplasia, which causes a range of symptoms including altered bowel habits, malnutrition and weight loss. Our immunosensor consists of a Plexiglas device coupled to a gold electrode, with a central channel containing 3-aminopropyl-modified controlled pore glass (AP-CPG). The quantification of anti-gliadin IgG antibodies was carried out using a heterogeneous, non-competitive enzyme-linked immunosorbent assay (ELISA) in which IgG antibodies bound to gliadin protein, immobilized on AP-CPG, were determined by alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. The *p*-aminophenyl phosphate (*p*-APP) was converted to *p*-aminophenol (*p*-AP) by AP, and the electroactive product was quantified on a gold electrode at 0.250 V. The calculated detection limits for electrochemical detection and the ELISA procedure were 0.52 and 2.72 UR mL^{-1}, respectively, and the within- and between-assay coefficients of variation were below 5.8%. The optimized procedure was applied to the determination of anti-gliadin IgG antibodies in human serum samples.

Keywords Celiac disease · Microfluidic system · Enzyme immunoassays · Gold electrode · Gliadin · Immunosensor

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Introduction

Celiac disease (CD) is an autoimmune disease triggered by gluten proteins of certain cereals, which affects the small intestine of genetically susceptible individuals. It is estimated that about one in 250 people suffer from this immunological disease [1] that is characterized by intestinal inflammation, villous atrophy, and crypt hyperplasia [2]. These symptoms appear after exposure to gluten proteins fractions called prolamins; harmful prolamins include gliadins and glutenin subunits, as well as hordeins and secalins, the alcohol-soluble proteins of barley and rye, respectively [3, 4]; these proteins have high proline and glutamine content. Due to especially these amino acids, these proteins can provoke CD in susceptible individuals that posses T-cells with HLA-DQ2 or HLA-DQ8 receptors [5–7]. Gluten proteins from diet interact with these HLA molecules to activate an abnormal mucosal immune response and induce tissue damage [8]. The only treatment for CD is life-long avoidance of gluten proteins, but it is difficult to sustain [9].

At present, new therapeutic strategies are being considered, among them: decreasing intestinal permeability toward gluten [10], blocking the deamination of gluten [11], shifting to Th2 inflammatory reaction [12], antagonizing proinflammatory cytokines [13], inducing gluten tolerance, and others [14].

Regarding the diagnostic field, there is no functional test to diagnose celiac disease. Among the most widely used techniques are: endoscopy with biopsy, which is currently, considered the standard of celiac disease diagnostic and serologic techniques that detect the presence of specific antibodies as anti-gliadin, anti-endomysial, anti-tissue transglutaminase [15].

Recently, with the development of sensitive serological tests, it has become possible to evaluate the presence of autoantibodies in the patients' blood as the basis for the medical diagnosis at an early stage of the disease [16].

In the case of patients that suffer symptoms but present negative serological tests, IgA class antibodies deficiency should be taken into account. Clinical studies have estimated that the risk for CD increases 10–20-fold in IgA-deficient subjects [17–19]. As a result of this deficiency, the specific IgA class autoantibodies against gliadin (AGA), endomysium (EMA) and tissue transglutaminase (tTG) are not produced. In such cases, testing for the IgG-class autoantibodies has been suggested [20].

As mentioned above, one of the tools available for serologic diagnostic of CD is the determination of anti-gliadin IgG antibodies which is performed using enzyme-linked immunosorbent assay (ELISA) in human serum samples to provide an early diagnosis to the celiac patients [21, 22].

For interpretation of anti-gliadin IgG antibodies test results, it must be taken into account that the sera from healthy individuals (without any pathology gastroenteric) have values of anti-gliadin IgG included within variables limits in function of age. Recommended normal values are: cut-off of 0–5 years: ≤60 U ml^{-1} and for >5 years: ≤30 U ml^{-1} [23].

Immunoassays are currently the predominant analytical technique for the quantitative determination of a broad variety of analytes of clinical, medical, biotechnological, and environmental significance [24, 25]; among the most important advantages of immunoassays are their sensitivity, selectivity, and cost effectiveness [26–28]. Currently, ELISA is carried out in polystyrene microtiter plates, but it is a multistage labor-intensive process that requires long incubation periods and multiple incubations and washes. As a result, new schemes of immuno-analysis are being explored to provide fast, sensitive, and automated immunoassays [29].

Alternatively, the microfluidic technology is well suited to these tasks. Relevant information about these technologies that have the potential for immunoassays can be found in several reviews [30–33]. Microfluidic technology have many advantages with regard to conventional scale immunoassay platforms because it seeks to improve analytical performance by reducing the consumption of reagents, decreasing the analysis time, increasing reliability and sensitivity through automation, and integrating multiple processes in a single device. For these features, microfluidic systems had become a tool particularly suitable for many applications in immunoassay [30].

With respect to detection methods, electrochemistry is the second most commonly used [34] due to its features, among which are included: easy miniaturization and low power requirements. These features combine to make electrochemistry suitable, especially when compactness and portability are important [35].

In this article, we report the development of a microfluidic immunosensor coupled to a gold layer electrode for the quantification of human serum IgG antibodies to gliadin protein. Antibodies determinations in serum samples were carried out using a non-competitive immunoassay based on the use of antigens that are immobilized on 3-aminopropyl-modified controlled pore glass (AP-CPG), which was packed into the central channel of the device, antigens on AP-CPG reacted with the antibodies present in samples and the same were quantified by alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. The *p*-aminophenyl phosphate (*p*-APP) was converted to *p*-aminophenol (*p*-AP) by AP, whose back electrochemical oxidation was detected on gold electrode. The response current obtained from the product of enzymatic reaction is directly proportional to the activity of the enzyme and, consequently, to the amount of IgG antibodies to gliadin protein in serum samples. The results indicated that our device, designed for the quantification of IgG antibodies against gliadin protein can provide a fast, sensitive, and automated tool for the diagnosis of celiac disease.

Materials

Reagents and solutions

All reagents used were of analytical reagent grade. AP enzyme-labeled second antibodies specific to human γ-chain and gliadin from wheat were purchased from Sigma Chemical (St. Louis, MO, USA). 3-aminopropyl-modified controlled pore glass (AP-CPG; 1,354 Å mean pore diameter, 19.7 $m^2 g^{-1}$ surface area was purchased from Pure Biotech LLC), 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) was provided by Fluka Chemie (Steinheim, Switzerland). Glutaraldehyde (25% aqueous solution) was purchased from Merck, Darmstadt

The ELISA test kit for the quantitative determination of anti-gliadin IgG-class antibodies was purchased from RADIM S.p.A**.** Pomezia (Roma) Italia and was used in accordance with the manufacturer's instructions**.** All buffer solutions were prepared with Milli-Q water.

Instrumentation

Amperometric measurements were performed using the BAS LC 4 C (Bioanalytical Systems, West Lafayette, IN,

USA). The BAS 100 B (electrochemical analyzer Bioanalytical Systems) was used for cyclic voltammetric analysis.

The gold layer electrode was deposited at the central channel (CC) by sputtering (SPI-Module Sputter Coater with Etch mode, Structure Probe Inc., West Chester, PA) and the thickness of the gold electrode was measured using a Quartz Crystal Thickness Monitor model 12161 (Structure Probe Inc., West Chester, PA) [36, 37]. The syringe pumps system (Baby Bee Syringe Pump, Bioanalytical Systems) was used for pumping, sample introduction, and stopping flow.

All solutions and reagent temperatures were conditioned before the experiment using a Vicking Masson II laboratory water bath (Vicking SRL, Buenos Aires, Argentina).

Absorbance was detected by Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 general UV/VIS spectrophotometer.

All pH measurements were made with an Orion expandable ion analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.)

Methods

Immunosensor: design concept

Figure 1 presents a generalized schematic of the design concept employed for the development of our microfluidic immunosensor, where accessory channel (AC), cleaning channel (CLC), CC, and detection system are shown. The main body of the sensor was made of Plexiglas. This device has a CC containing AP-CPG with a diameter of 100 μm; accessory channels and cleaning channel with diameters of 50 μm and a gold layer electrode as a part of electrochemical detector in the outlet of the device. AP-CPG was packed in the CC and then kept in a previous site of the detection zone because the CC diameter decreases, preventing the escape of AP-CPG.

Modification and immobilization procedure

To carry out the process of modification, 1 mg of AP-CPG was allowed to react with 1 ml of an aqueous solution of 5% *w*/*w* glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After three washes with 0.10 M phosphate buffer of pH 7.00, a 10 μg mL^{-1} of gliadin solution was coupled to the residual aldehyde groups overnight at 5 °C. The immobilized antigens preparation was finally washed three times with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C. The immobilized gliadin preparation was perfectly stable for at least 1 month.

Detection principle

The fundamental objective of this work is the detection of IgG antibodies against gliadin protein. Scheme 1 shows the complete procedure. These antibodies present in human serum samples, were allowed to react with gliadin protein, which was immobilized on AP-CPG located into of the CC of the immunosensor. Bounded antibodies were quantified by the action of the AP enzyme-labeled second antibodies specific to human IgG. The *p*-aminophenyl phosphate (*p*-APP) was converted to *p*-aminophenol (*p*-AP) by AP, whose back electrochemical oxidation was detected on gold electrode at 0.250 V, this potential was selected by the electrochemical study of the behavior of the *p*-AP, by cyclic voltammetry (CV) at the gold electrode. The response current obtained from the product of enzymatic reaction is directly proportional to the activity of the enzyme and, consequently, to the amount of IgG antibodies to gliadin protein in serum samples.

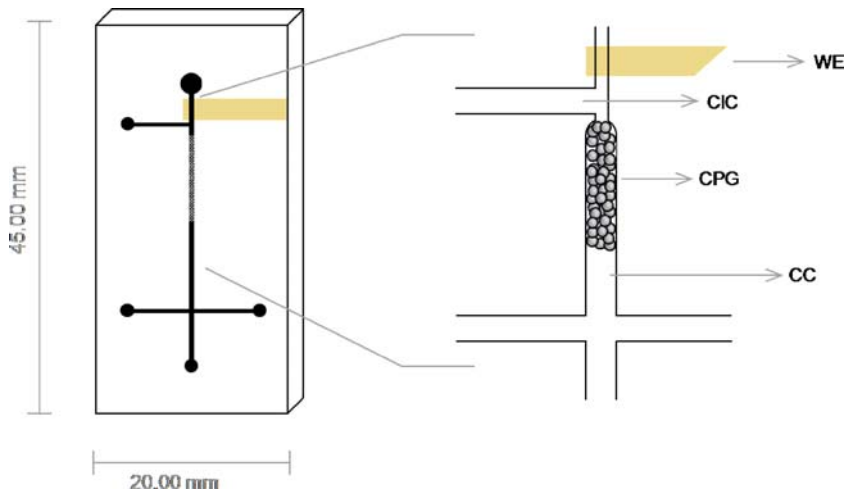
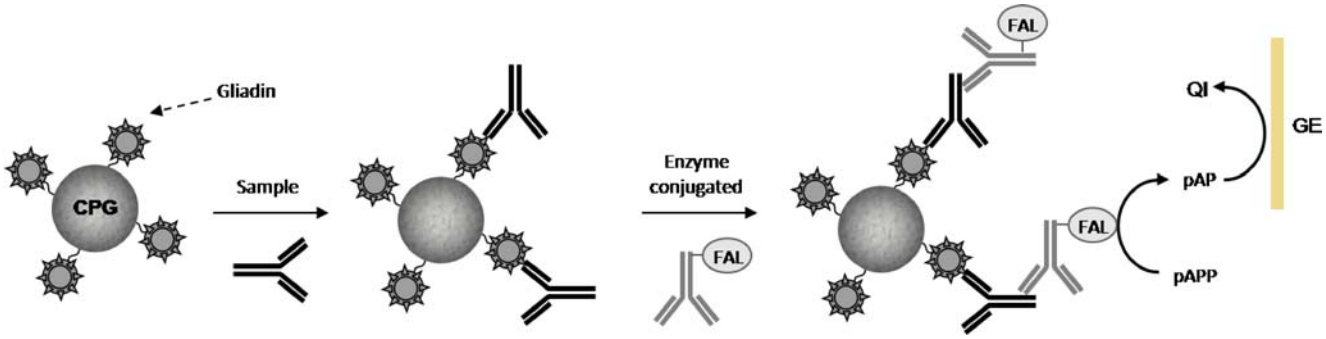


Fig. 1 Schematic representation of inmunomagnetic sensor. *CPG* controlled pore glass, *WE* working electrode, *CLC* cleaning channel, *CC* central channel



Scheme 1 Schematic representation of the immune reaction. *CPG* controlled pore glass, *FAL* alkaline phosphatase, *pAPP* *p*-aminophenyl phosphate, *pAP* *p*-aminophenol, *QI* *p*-benzoquinone imine, *GE* gold electrode

Immunosensor setup

A diagram of the flow-through CC of the microfluidic immunosensor and detection system is shown in Fig. 1. This device has: CC, CLC, AC; the diameter of the CC decreases before detection zone, this reduction of the diameter prevents the loss of modified AP-CPG. A gold layer electrode of 80 nm thickness was deposited in the central channel by sputtering. The gold layer electrode was cleaned and preconditioned using cyclic voltammetry in 0.5 M sulfuric acid by threefold cycling in the potential range between −300 and 1,500 mV at 100 mV s^{-1} scan rate.

The immunosensor coupled to a gold electrode was connected to a syringe pump system which forces a liquid stream to flow through the channel of the device; this system has a command that allows control and selects the flow rate. Also, our device was electrically connected to an electrochemical detector and a recorder to perform the amperometric measurements.

Measurement procedure

The procedure for the quantification of anti-gliadin IgG antibodies involves the following stages (Table 1). Firstly, gold electrode was cleaned and preconditioned every five determinations, using cyclic voltammetry in 0.5 M sulfuric acid by threefold cycling in the potential range between −300 and 1,500 mV at 100 mVs^{-1} scan rate, this procedure was made through CLC without effect on modified ACPG and has vital importance because the accumulation of reaction products affecting the sensibility of the work electrode occurs on the gold surface electrode. Then, a suspension of modified AP-CPG (10 μl in 100 μl of PBS buffer) was injected using syringe pumps at a flow rate of 10 μL min^{-1} for 5 min, modified AP-CPG was packed into the CC, in a previous position to the detection place of the device. The carrier buffer was 0.01 M PBS, pH 7.2. Later, the immunosensor was exposed to a flow with desorption buffer (0.1 M glycine–HCl, pH 2) at a flow rate of 2.0 μL min^{-1} for 5 min and then was rinsed with 0.01 M PBS (pH 7.2) for 4 min.

Unspecific binding was blocked by a 5-min treatment at room temperature with 3% skim milk in a 0.01 M phosphate-buffered saline (PBS), pH 7.2 at flow rate of 2.0 μL min^{-1} and then was rinsed with 0.01 M PBS (pH 7.2) for 4 min at a flow rate of 2.0 μL min^{-1}. After that, serum sample, firstly diluted 100-fold with 0.01 M PBS (pH 7.2), was injected into the PBS carrier stream at flow rate of 2.0 μL min^{-1} for 5 min. The IgG-specific antibodies to gliadin protein present in the serum sample reacted with gliadin protein immobilized on AP-CPG. The microfluidic device was washed with 0.01 M PBS (pH 7.2) at a flow rate of

Table 1 Sequences required for the gliadin-specific IgG antibody immunoassay

Sequence	Condition	Time (min)
Blocking solution	3% skim milk in a 0.01 M phosphate buffer saline (PBS), pH 7.2	5
Washing buffer	Flow rate: 2 μL min^{-1}(PBS, pH 7.2)	4
Serum samples	diluted 100-fold 2 μl min^{-1}	5
Washing buffer	Flow rate: 2 μL min^{-1}(PBS, pH 7.2)	4
Enzyme conjugated	AP-conjugated (dilution of 1/2000) 2 μl min^{-1}	5
Washing buffer	Flow rate: 2 μL min^{-1}(PBS, pH 7.2)	4
Substrate	2 μl 2.7 mM *p*-APP in DEA buffer, pH 9.6	1
Signal analysis	LC-4C amperometric detector, 0.10 V	1

2.0 μL min^{-1} for 4 min to remove excess of sample. Bound antibodies were quantified using alkaline phosphatase enzyme-labeled second antibodies specific to human IgG (dilution of 1/1,000 in 0.01 M PBS, pH 7.2) injected at flow rate of 2.0 μL min^{-1} for 5 min. Then, the microfluidic device was washed with 0.01 M PBS (pH 7.2) for 4 min. Diethanolamine (DEA) buffer (100 mM diethanolamine, 50 mM KCl, 1 mM $MgCl_2$, pH 9.6) was used to prepare the *p*-APP solution (2.7 mM *p*-APP in a DEA buffer, pH 9.6), which was injected into the carrier stream at a flow rate of 2.0 μL min^{-1} for 1 min and the enzymatic product *p*-aminophenol (*p*-AP) was detected on the gold surface electrode.

The immunosensor was exposed after each analysis, to a flow of desorption buffer (0.1 M glycine–HCl, pH 2) at a flow rate of 2.0 μL min^{-1} for 5 min and then washed with PBS, pH 7.2. With this treatment, the anti-gliadin antibodies bound to immobilized gliadin were desorbed, allowing to start with a next determination.

Regarding the stability of immobilized gliadin preparations, these were perfectly stable for at least 1 month. This date must be taken into account because this represents the main factor limiting the useful life of our immunosensor.

A standard curve for the electrochemical procedure was produced following the protocol with a series of standards that covered the clinically relevant range (0–200 UR mL^{-1}) supplied with the ELISA test kit. The standards provided were ready to use, and consequently, were employed without any preparation, the blank was the buffer 0.01 M PBS, pH 7.2. Electrochemical measurements were performed and the resulting anodic current was measured. The stock solution of *p*-APP was prepared freshly before the experiment and stored under the exclusion of light for the duration of the experiment.

Results and discussion

Optimization

For the optimization procedure, many factors must be taken into account, particularly when repeat analysis is desired. These factors include: sample matrix, flow rate, incubation time, elution conditions, pH, operating and storage temperature, all affect the system's life expectancy [38].

Flow rate

In a microfluidic system, where samples and reagents are continuously flowing, the flow rate is an important factor because it affects the dispersion of the analyte, yield of the inmunologic reaction, and response of the detector [39]. The immobilized antibody may be damaged or removed from the stationary support in the presence of high flow and pressure [40], while a slower flow rate allows more time for antibody–antigen reactions. So, for the determination of anti-gliadin IgG antibodies, the flow rate was analyzed by studying the currents obtained for a standard of 100 UR mL^{-1} at different flow rates.1, 2, 3, 4, 5, 7, 9, 12, 15 μL min^{-1} (Fig. 2).

As result of this study, we can say that flow rates from 1 to 3 μL min^{-1} had a little effect over immunologic reaction and consequently over signals obtained. On the other hand, when the flow rate exceeded 4 μL min^{-1}, the signal was reduced. Consequently, the flow rate used throughout the experiment was 2 μL min^{-1}, except when the effect of flow rate was tested.

Sample volume

Another parameter studied was the sample size. This factor was evaluated using a standard of 100 UR mL^{-1}, in a range of 1–25 μL. The rate of response increased linearly and sensitivity was almost quadruplicated if the sample size rises from 1 to 10 μL, insignificant differences can be observed when sample size is greater than 3 μL (Fig. 3). Then, a sample size of 10 μL was used.

As seen for the quantification of anti-gliadin antibodies, small sample and reactive volumes are required; this point is very important because the cost of reagents can be very expensive and some samples are only available in trace amounts. Therefore, it has always been an aim to reduce sample volume and increase sensitivity in all biological assays.

pH range and substrate concentration

The rate of enzymatic response under flow conditions was studied in the pH range of 8–10 and shows a maximum

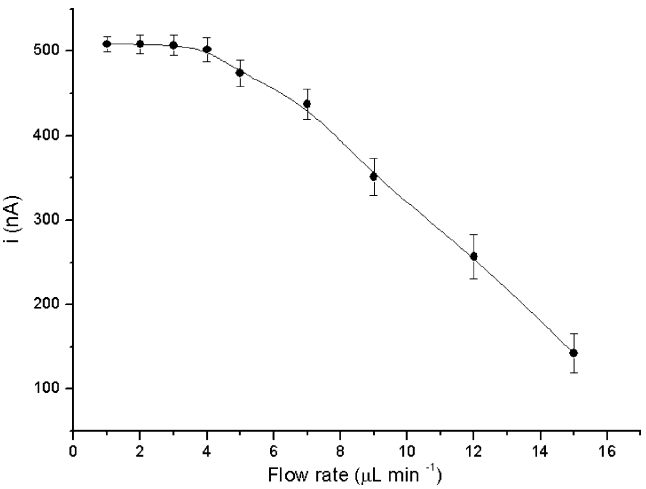


Fig. 2 Effect of flow rate analyzing a standard of 100 UR mL^{-1} gliadin-specific antibodies at different flow rates from 1 to 15 μL min^{-1}

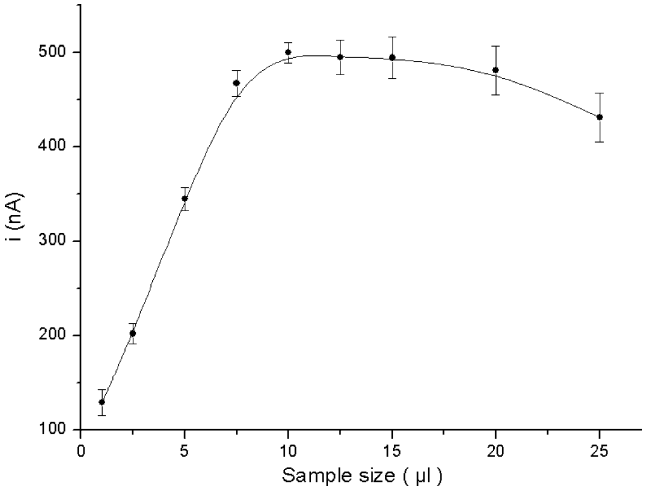


Fig. 3 Effect of sample size for a standard of 100 UR mL^{-1} gliadin-specific antibodies. Each value of current is based on five determinations

value of activity at pH 9.6. The pH value used was 9.6 in DEA buffer. The effect of varying pAPP concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum pAPP concentration found was 2.7 mM. That concentration was then used.

Quantitative test for the detection of anti-gliadin-specific IgG antibodies in the microfluidic immunosensor

The quantification of anti-gliadin IgG antibodies concentration was evaluated in 22 serum human samples, with the proposed method and under the conditions described above, where the electrochemical response of the enzymatic product is proportional to the concentration of anti-gliadin-specific IgG antibodies in serum samples of celiac patients.

The anti-gliadin IgG antibodies calibration plot was obtained by plotting *i* versus anti-gliadin IgG antibodies concentration. A linear relation, $i(\text{nA})=1.38+5.04\times C_{\text{IgG antibodies}}$, was observed between the *i* and the IgG concentrations in the range of 0.0 and 200 UR mL^{-1}. The correlation coefficient (*r*) for this plot was 0.999. The coefficient of variation (CV) for the determination of 100 UR mL^{-1}anti-gliadin-specific antibodies was below 3.6% (six replicates). These values demonstrate that our microfluidic immunosensor can be used to quantify the amount of anti-gliadin IgG-specific antibodies in unknown samples.

The detection limit (DL) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For electrochemical detection procedure, the DL was 0.52 UR mL^{-1}.

The sensitivity (*S*) is defined as the slope of the regression line signal vs. concentration. *S* for electrochemical detection was 5.04 nA/UR mL^{-1}.

The precision of the electrochemical assay was checked with control serum at 5, 100, and 200 UR mL^{-1} anti-gliadin-specific antibody concentrations. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The anti-gliadin assay showed good precision; the CV within-assay values were below 3.6% and the between-assay values were below 5.5% (Table 2).

Table 2 Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated for three consecutive days)

Control sera[a]	Within-assay		Between-assay	
	Mean	CV %	Mean	CV %
5 U mL^{-1}	5.26	3.0	5.36	4.91
100 U mL^{-1}	99.06	3.6	102.10	5.50
200 U mL^{-1}	200.3	2.8	98.21	5.43

[a] UR mL^{-1} IgG-specific antibodies to gliadin protein

The ELISA procedure was also carried out, absorbance changes were plotted against the corresponding anti-gliadin IgG antibodies concentration and a calibration curve was constructed. The linear regression equation was $A=0.11+0.010\ C_{\text{IgG antibodies}}$, with the linear relation coefficient $r=0.992$, the CV for the determination of 100 UR mL^{-1} anti-gliadin-specific antibodies was 5.8 % (six replicates). For the EIA procedure, the DL and *S* were 2.72 U ml^{-1} and 0.010 Abs./U ml^{-1}, respectively.

Regarding the total assay time for the determination of the anti-gliadin IgG antibodies concentration, for the proposed method, the assay time was 29 min, much less than the 2.5 h normally used with conventional batch well ELISA, which is five times faster than the plate method.

The electrochemical system was compared with a commercial spectrophotometric system for the quantifi-

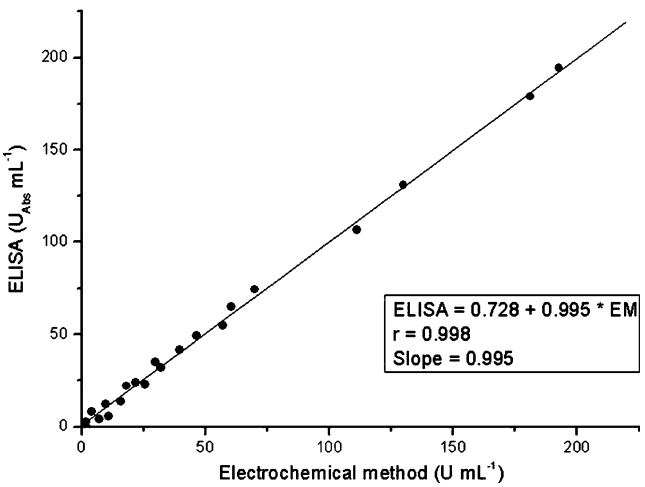


Fig. 4 Correlation between proposed method and commercial photometric assays

cation of anti-gliadin-specific IgG antibody in serum samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Fig. 4).

As seen; the statistical values show that the proposed method have a LD less than the cut-off values, so the microfluidic immunosensor could be used as an easy and fast alternative in order to take a diagnostic decision.

Conclusions

This work presents an automatic immuno-microfluidic sensor coupled with electrochemical detection for the quantification of anti-gliadin IgG antibodies in human serum samples. This device represents a significant tool for a sensitive and automated diagnostic of celiac disease.

In our automatic microfluidic system coupled to a gold layer electrode, gliadin protein was successfully immobilized on AP-CPG packed into the central channel. The use of AP-CPG as solid support increased the surface area-to-volume ratio and consequently the sensitivity; other benefits of the sensor were: faster response times of analysis (29 min), lower sample consumed than conventional immunoassay techniques, and also that the measurements can be carried out without any special sample pretreatment, making the system extremely useful for a fast delivery of the diagnostic information and able for many analysis adaptations. Therefore, a high-sensitivity microfluidic sensor has been developed and it is promising for many automatic immunoassay applications.

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