Bleed-to-read Disposable Microsystems for the Genetic and Serological Analysis of Celiac Disease Markers with Amperometric Detection

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Abstract

Coeliac disease is an auto-immune disorder induced by ingestion of gluten in genetically predisposed individuals. Its diagnostics is more accurate using a combination of immunologic and genetic tests to detect of high levels of certain auto-antibodies and the presence human leukocyte antigen HLA-DQ2 or HLA-DQ8 genetic markers. In this work, we report the design and testing of automated microsystems combining sample treatment, storage, fluidic transport and detection in a single platform able to carry out genetic or serologic analysis for detection of celiac disease markers. These microsystems share a common footprint and many fluidic features and are thus able to perform a complete assay. The microsystem for the genetic assay extracts and amplifies the DNA prior to detection, while the serology microsystem contains a filter and chamber for the generation and subsequent dilution of plasma. The performance of both platforms is demonstrated and compared with reference methods with an excellent correlation, which makes the developed platform amenable for clinical studies.

Introduction

The multiplexed detection of proteic or genetic disease biomarkers is a topic of intense research in the molecular diagnostics of human disorders as they notably increase the accuracy via the parallel detection of several of molecules [1,2]. Among the many existing detection technologies, electrochemical techniques offer a low-cost and high sensitivity alternative to traditional optical detection with the advantage of low-instrumentation cost and relative simplicity, being easily miniaturisable and compatibility with biological samples [3,4]. This facilitates integration in point-of-care or laboratory instruments allowing high throughput multiplexed bioassays in relatively short periods of time with low sample volumes [5].

Celiac disease is an example of a condition where diagnostics is more accurate using a combination of immunologic and genetic tests [6]. This disorder is associated with the ingestion of gluten by susceptible individuals, which are mainly diagnosed by small intestinal biopsy [7,8]. In recent years, serological screening tests that look for antibodies specific to celiac disease have become increasingly more specific and sensitive. If abnormally elevated levels of immunoglobulin A tissue transglutaminase (tTG) antibodies are found, a person has a high probability of have celiac disease [9,10]. High levels of IgA antibodies against gliadin (GLI) may also be detected and are useful to monitor compliance to a gluten-free diet [11]. A person suspected of having celiac disease should also be checked for total IgA deficiency to ensure that the specific test for celiac disease would not be a false negative result as a consequence of IgA deficiency [12]. On the other hand, celiac disease has a genetic component associated with the presence of human leukocyte antigen (HLA) HLA-DQ2 or HLA-DQ8 genetic markers [13]. Thus, genetic tests can only indicate predisposition to develop the condition. HLA typing indicating lack of DQ2 or DQ8 genes can exclude the probability of developing celiac disease which may help, for example, to provide more

accurate information to parents with a child with celiac disease about the risk for another child to develop the disease [14]. The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recently published new guidelines for CD diagnosis based on the presence of a high titre level of anti-tTG IgA antibodies combined with HLA typing [15]. Currently, there is no cost-effective, easy to-use technology that can be used at the point of care for the screening of celiac disease.

Recently, several electrochemical assays for the detection of auto-antibodies and genetic markers associated with celiac disease have been developed by one of our groups. Highly stable and electron permeable self-assembled monolayers of a carboxylic acid terminated dithiol scaffold on gold [16] or supramolecular polymeric assemblies [17] were used to covalently link tTG [18], digested gliadin [19,20] or IgA [21] to develop sandwich-type amperometric immunosensors to detect the corresponding autoantibodies in serum samples of celiac patients with low limits of detection and the results were in all cases compared with an standard ELISA with excellent correlation. In addition, we have reported a multiplexed electrochemical genosensor on ten probes specific to DQA1 and DQB1 alleles immobilised on an array of 36 electrodes. This sensor showed an excellent selectivity and correlation with standard HLA-typing techniques, demonstrating the efficiency of the developed genosensor array for the rapid and cost-effective screening of celiac disease predisposition [22].

The integration of bioanalytical assays into a single device capable of performing sampling, separation, dilution, storage and detection is a challenging task. In this sense, the use of microfluidic systems has many advantages such as low sample and reagent requirements, rapid analysis and portability and several examples have been reported [23,24]. This allows the construction of modular systems that can be matched and customised on demand, which results in a great flexibility and expands the application of integrated devices.

In a previous work, we presented the fabrication of a polymer-based microsystem integrating an electrochemical array with reagent storage, which was used for the simultaneous amperometric detection of breast cancer markers in serum [25]. The reproducibility and sensitivity of this microfluidic cell was further improved by interfacing to an automated pumping system requiring minimal end-user intervention [26]. Herein, we report the design and testing of automated microsystems combining sample treatment, storage, fluidic transport and detection in a single platform able to carry out genetic or serologic analysis for detection of celiac disease markers. These microsystems share a common footprint and many fluidic features and are thus able to perform a complete assay. The microsystem for the genetic assay extracts and amplifies the DNA prior to detection, while the serology microsystem contains a filter and chamber for the generation and subsequent dilution of plasma. The performance of both platforms has been compared with reference methods with excellent correlation.

Materials and Methods

Reagents and materials

Dithiol 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadoco-sanoic acid NHS ester (DT2-NHS) and dithiol 10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol (DT1) were purchased from SensoPath Technologies (Bozeman, MT). Stock solutions (1 mM) were prepared in acetonitrile, purged with argon and kept at –20ºC when not in use. Gliadin, IgA, anti-human IgA, anti-IgA-peroxidase conjugate, Tris buffer saline and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma. Commercial ELISA kits (Gliatest S IgA Chromo and Eu-tTG IgA) and recombinant tissue transglutaminase (tTG) were kindly supplied by Eurospital SpA, Trieste, Italy. Synthetic DNA probes were purchased from Biomers (Ulm, Germany). The following sequences were used:

3b probe: 5'-GTCGTGACTGGGAAAAC-TEG-SH-3',

4y probe: 5'-TCCTGTGTGAAATTGTTATCCGCT-TEG-SH-3',

5z probe: 5'- GGGCATAAGTCGGACAC-TEG-SH-3',

HGH probe: 5'-TAGCGGTGAGTCGATTCTGCCT-TEG-SH-3'

HRP reporter probe: 5'-ACTGGCCGTCGTTTTACA-HRP-3'

Fabrication of platforms and instrumentation

The electrode arrays and microfluidic platforms were fabricated using essentially the same procedure as reported elsewhere [24] using borofloat glass and polycarbonate substrates, respectively. Electrochemical detection was carried out using a hybrid multichannel potentiostat able to interface up to 48 electrodes. Details of its construction and specifications have been recently described [27]. The microfluidic platforms were first loaded with the appropriated reagent and positioned in a prototype control instrument integrating port and vent valves, a syringe pump, valve motors and heaters and interfaced to a personal computer using a dedicated program to allow the automated control of assays.

Electrochemical genetic assay

The working electrodes were functionalized via co-immobilization method by spotting 0.5 μL of a solution containing 1 μM of corresponding thiolated probe and 100 μM of DT1 for 3 hours followed by rinsing with copious amounts of water. The modified arrays were then assembled in the microfluidic platforms by means of a double-sided adhesive gasket. The next steps were carried out on the microfluidic platform. The blood sample plus water were pumped to the lysis chamber containing magnetic beads and the cells were lysed by mixing with a chemical lysing agent for 7 min. After washing with water (loaded in tank *a*, Figure 2), the DNA was eluted with Washing buffer (loaded in tank *b* Figure 2) and transferred to the PCR reagents reservoir and amplified. On-chip Boyle-Mariotte PCR was carried out using an

initial activation step of 1 min at 98°C, followed by 40 cycles of denaturation at 98°C for 1 second and annealing/elongation at 62°C for 25 seconds. After amplification, the sample was taken to the detection area and incubated for 2 min over the electrodes, followed by washing with 10 mM Tris buffer containing 500 mM of NaCl pH 7.4 (loaded in tank *e* Figure 2). A 10 nM solution of HRP-labeled detecting probe (loaded in tank *c* Figure 2) was flowed over the electrodes and incubated for 2 min. The amperometric measurements were carried out by injection of TMB Liquid Mixture from Sigma (loaded in tank *d*, Figure 2) at -0.2 V for one minute.

Electrochemical serological assay

The working electrodes were spotted with 5 μL of a stock solution of DT2-NHS for 3 hours followed by rinsing with copious amounts of acetonitrile. The capture antigens were covalently immobilized on NHS-activated SAM by spotting with 5 μL of a 0.5 mg/mL solution of the antigen in 10 mM acetate buffer pH 5.0 for one hour at 37ºC. The remaining NHS active ester sites were blocked with 1.0 M ethanolamine pH 8.5 for 30 minutes at 37ºC. The modified arrays were then assembled in the microfluidic platform by means of a double-sided adhesive gasket.

The next steps were carried out on the microfluidic platform. The blood samples were on-chip filtered, diluted 1:100 in PBS pH 7.2 (loaded in tank *a*, Figure 4) and incubated for 2 minutes over the electrodes. The calibrators (loaded in tanks *d* and *e*, Figure 4) were diluted in PBS pH 7.2. For detection, a mixture of 1 μg/mL HRP-labeled detecting antibody in PBS pH 7.2 (loaded in tank *b*, Figure 4) was flowed over the electrodes and incubated for 2 min. Intermediate washing steps with PBS pH 7.2 ensured removal of unbounded material. The amperometric measurements were carried out by injection of a liquid mixture of TMB Liquid Mixture from Sigma (loaded in tank *c*, Figure 4) at -0.2 V for one minute.

Results & Discussion

The microfluidic platforms for both DNA and serological analysis were designed so as to have a common footprint of the size of a 96-well standard ELISA plate (128 × 86 mm) with a thickness of 3 mm (Fig. 1a,b) as well as common valving points and detection zones, so that one single instrument could be used to interface with both microsystems. Both microsystems also share several other features such as a 5-inlet tank holder to store the reagents used in the assays, a 2 mL waste reservoir and a detection area opposite to the tank area. In addition, and to facilitate the operation and fluidic actuation, both microsystems have common pumping and ventilation interfaces and three turning valves are used to drive the liquids to the appropriate sections of the chips.

The detection zone is divided in one (genetic) or three (serology) microfluidic channels addressing selected electrodes for the measurement of samples and calibrators depending on the assay to be realised. The electrode arrays consist of 36 gold working electrodes (1 × 1 mm^2) in a 3 × 12 arrangement placed between a silver pseudo reference (0.2 × 1 mm^2) and a gold counter electrode of the same size in order to create 36 planar electrochemical cells [22,27]. The electrode array is integrated on the fluidic microsystem using a double-sided medical grade adhesive foil of 50 μm thickness previously laser machined to generate microchannel structures of 2 mm width and is connected via pressure-sensitive connectors to a prototype PCB-based multichannel potentiostat for real-time measurement of the amperometric signal of the 36 electrodes [27].

The fluidic storage part of the platforms consists of five tanks fabricated on polypropylene with the appropriate volume to run a single assay per cartridge. They are integrated in the microsystem using piercing structures located on one side and are manually filled. The liquids from the reservoirs are actuated by applying a positive air pressure (i.e. via a

programmable syringe pump) through the septa and are driven to the detection zone via three turning valves. Figures 1c and 1d show photographs of the platforms inserted in the control instrument.

Microsystem for genetic analysis

Figure 2 shows a picture of a fabricated DNA microsystem and a flow diagram of its operation. The microsystem contains three well-defined areas for sample preparation (including DNA isolation), PCR amplification and electrochemical detection.

The assay starts when the blood sample (~20 μL) is introduced in the luer inlet 1 and moved to the cell lysis chamber 2 using a cap to push the sample. The lysis chamber contains modified magnetic beads able to break the cells and capture the DNA. This process is accelerated by means of a rotating magnetic mixer located under the microsystem in this area. The captured DNA is then washed twice on this chamber with Tris buffer and the DNA is extracted using ultrapure water. This step also requires magnetic stirring and the buffers are previously metered in chamber **3**. Once the captured DNA has been extracted, the solution is transferred to the pre-PCR chamber **4**, which contains the required reagents for PCR amplification in dried form. After dissolution of the lyophilized PCR reagents and mixing of the buffer solution, the sample is transferred into the PCR module **6** for amplification. For this purpose, the fluid is first metered in chamber **5** before being redirected and oscillated into the meandering PCR channel positioned over two heating blocks. The oscillating actuation is carried out by sequentially pushing the liquid against the dead end and releasing the pressure to move the liquid back. After the required number of cycles, the amplified DNA sample is then taken to section **D** and incubated over the electrodes for a given time to start the detection process based on a sandwich assay in which the electrodes are modified with a capture probe (CP). Detection is carried out amperometrically after incubation of a HRP-labelled detection probe (DP)

and injection of an enzymatic substrate/mediator that generates an electroactive product. The microsystem has the possibility to store in chamber **7** and **8** the detection probe and substrate/mediator mixture, respectively, in dried form, which can be reconstituted using ultrapure water from the corresponding tanks.

Figure 3 shows the results obtained in the detection of three alleles related with celiac disease in patient samples previously genotyped using Luminex One Lambda Labtype® SSO HLA Class II DQA1/DQB1 Typing Test, and Olerup SSP typing kits. The electrode arrays were modified according with the modification map showed in Figure 4a where three DNA thiolated capture probes (3b, 4y and 5z) as well as with a positive control sequence corresponding to the human growth hormone (HGH) were immobilized in the electrode surface in triplicate. The HGH target sequence is present in all samples and serves to assess the efficiency of the on-chip amplification while 3b, 4y and 5z correspond to the HLA-DQ allele family present in celiac patients. As can be seen, there is an excellent correlation between the results obtained with the electrochemical microsystem and the reference genotyping. Samples of patients 2 and 4 are not celiac and are thus positive only to HGH while patients 5, 7 and 8 present all three celiac alleles. The ability of the developed microsystem to selectively detect one or two alleles is evidenced in samples 1, 3 and 6, thus making possible a low resolution genotyping.

Microsystem for serologic analysis

Figure 4 shows a picture of a fabricated serology microsystem and a flow diagram of its operation. The microsystem has been designed to detect proteins contained in the blood plasma and therefore contains an area to filter the blood sample and dilute the generated plasma, as well as an electrochemical detection area.

The assay starts when the blood sample (~20 μL) is introduced in the Luer inlet 1' and moved to the blood filtration chamber 2' using a cap to push the sample. This chamber contains a membrane filter able to retain all 'solid' components of the blood, generating a fresh plasma sample used for analysis. The plasma is then metered in channel 3' and transferred to the dilution chamber 4' where it can be diluted up to a factor of 1:100. For this, the dilution buffer is metered in chamber 5' and then transferred to the dilution chamber 4'. After homogenisation the diluted sample is incubated over the electrodes. As in the case of the DNA microsystem, detection is based on a sandwich assay using a secondary antibody labelled with HRP.

Figure 5 shows a comparison of the results obtained in the detection of three autoantibodies related with celiac disease in patient samples using the microsystem and commercial ELISA kits. The electrode arrays were modified as shown in the map with capture antigens (IgA, tTG and digested gliadin) by crosslinking with a COOH-terminated thiolated bipodal scaffold. In this case, a quantitative measurement was carried out by interpolation using two calibrator solutions containing a mixture of the three target autoantibodies in low and high concentrations. These calibrators were injected in two separate channels parallel to the sample. As can be seen, there is an excellent correlation between the autoantibody levels found in the eight samples using both methods. In this case, samples of patients 2 and 3 are not celiac but show IgA deficiency while the rest are typical celiac patients with high anti-tTG and anti-gliadin levels above the clinical cut-off of 20 U/mL and IgA deficiency.

Conclusions

In this paper, the design and evaluation of automated microsystems for genetic and serologic analysis of celiac disease markers is presented. These microsystems are able to run a complete assay as they integrate sample treatment, storage, fluidic transport and detection in a single

platform. The fluidic operations can be totally automated, requiring only the intervention of the patient to introduce a small blood sample. The performance of both platforms is demonstrated and compared with reference methods with an excellent correlation, which makes the developed platform amenable for clinical studies.

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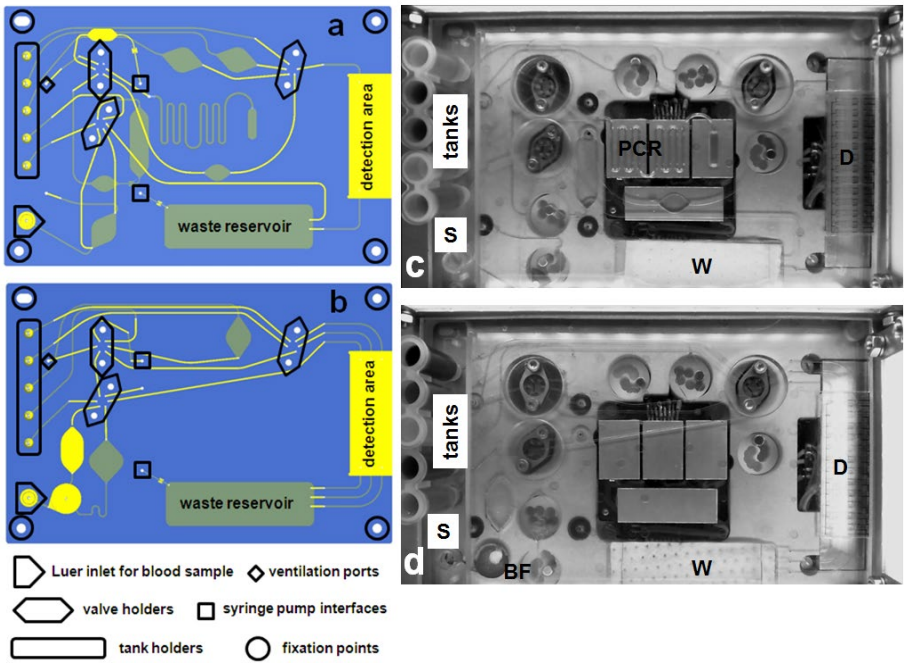

Figure 1. Left: CAD designs for DNA (a) and serology (b) microsystems indicating the common elements present in both microsystems. Right: Photographs of the genetic (c) and serological (d) platforms inserted in the control instrument. D: Detection area, W: waste reservoir, S: sample inlet, PCR: DNA amplification zone, BF: blood filter.

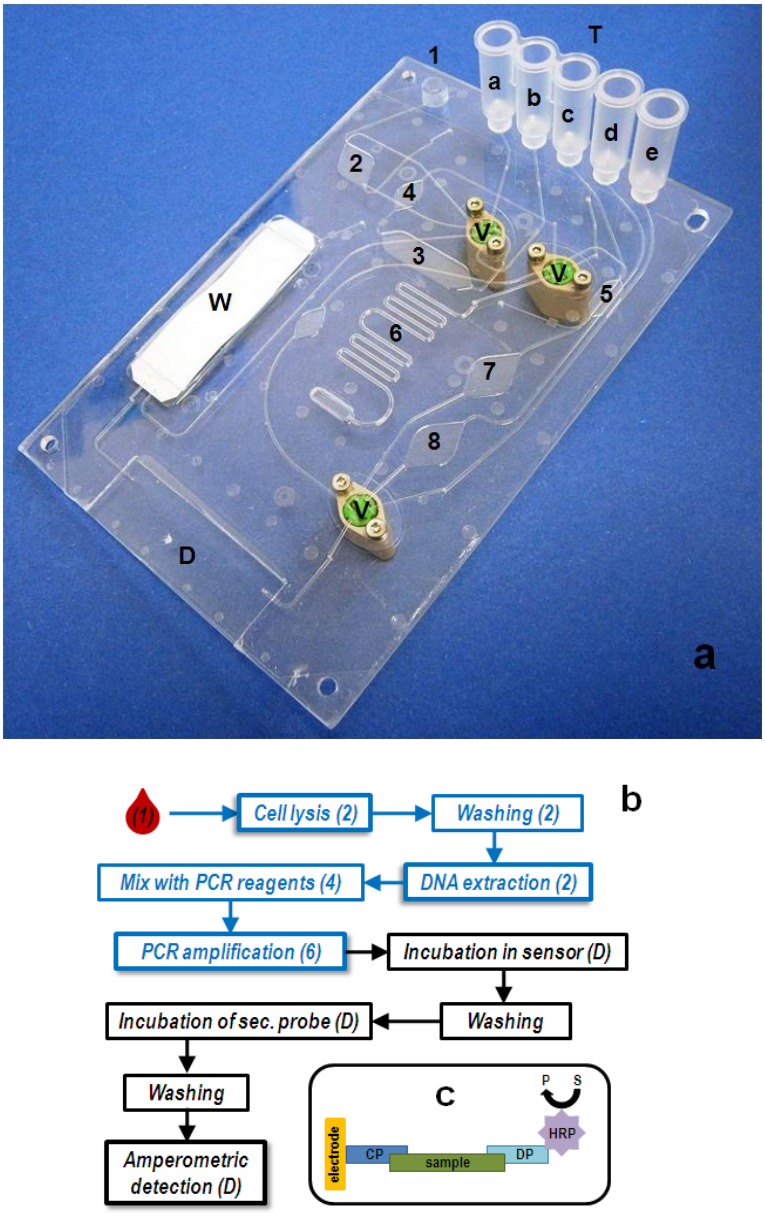

Figure 2. a) View of the DNA microsystem. 1: sample inlet, 2: lysis chamber, 3: metering chamber, 4: PCR reagents chamber, 5: PCR metering chamber, 6: PCR amplification chamber, 7: chamber for reconstitution of detection probe, 8: chamber for reconstitution of dried substrate, D: Detection area, V: valves, T: tanks (a: wash buffer for lysis, b: extraction buffer, c: detection probe, d: substrate/mediator for electrochemical detection, e: wash buffer for detection), W: waste reservoir. b) Flow diagram of DNA microsystem operation indicating in parenthesis in which area of the microsystem the steps are carried out. c) Detection principle based on a sandwich assay. CP: capture probe, DP: detection probe, HRP: peroxidase, S: substrate, P: product.

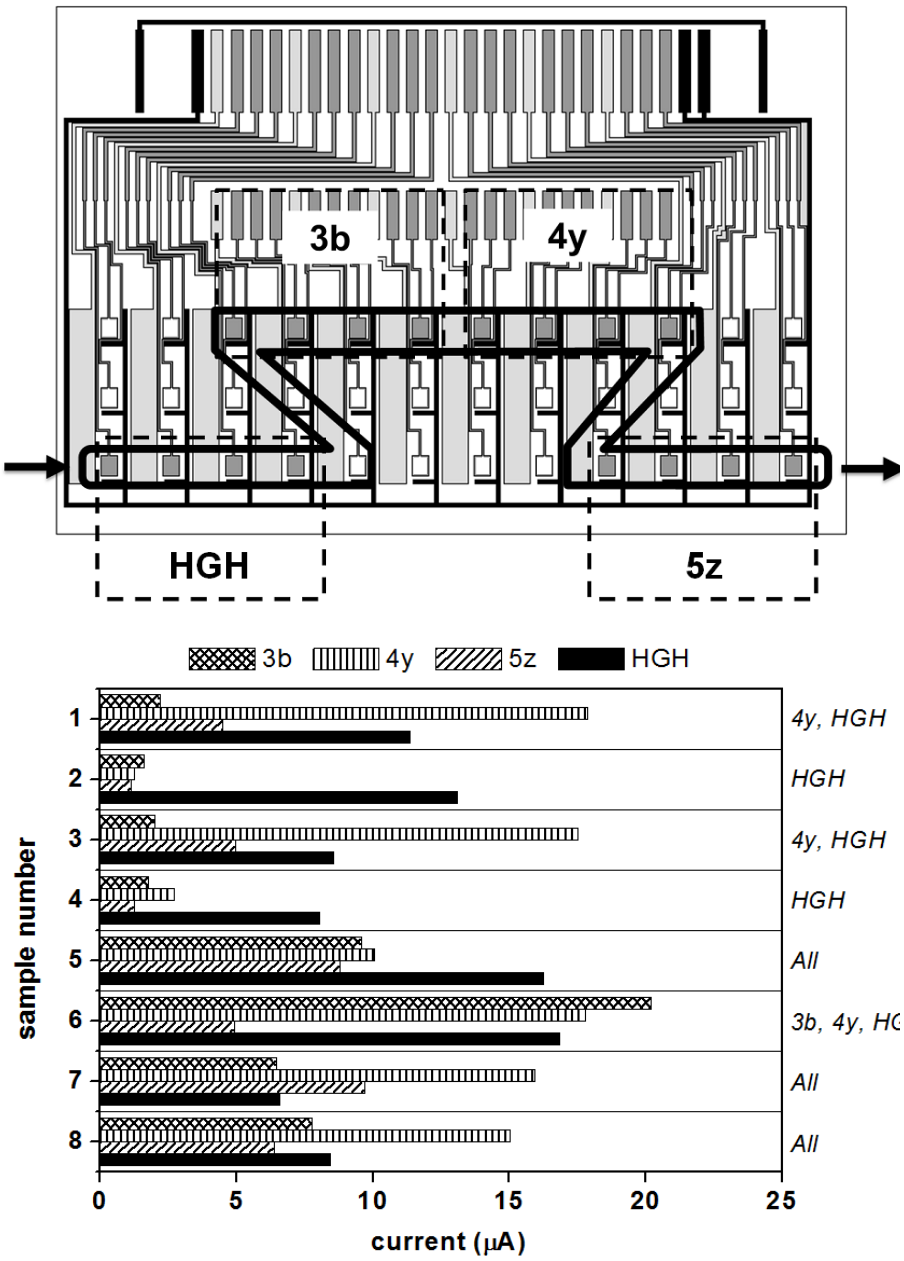

Figure 3. Top: Modification map of the electrode array for genetic analysis. Bottom: Electrochemical responses obtained on real samples for the detection of 3b, 4y, 5z and HGH. The column in italics on the right indicates the genotyping of the sample.

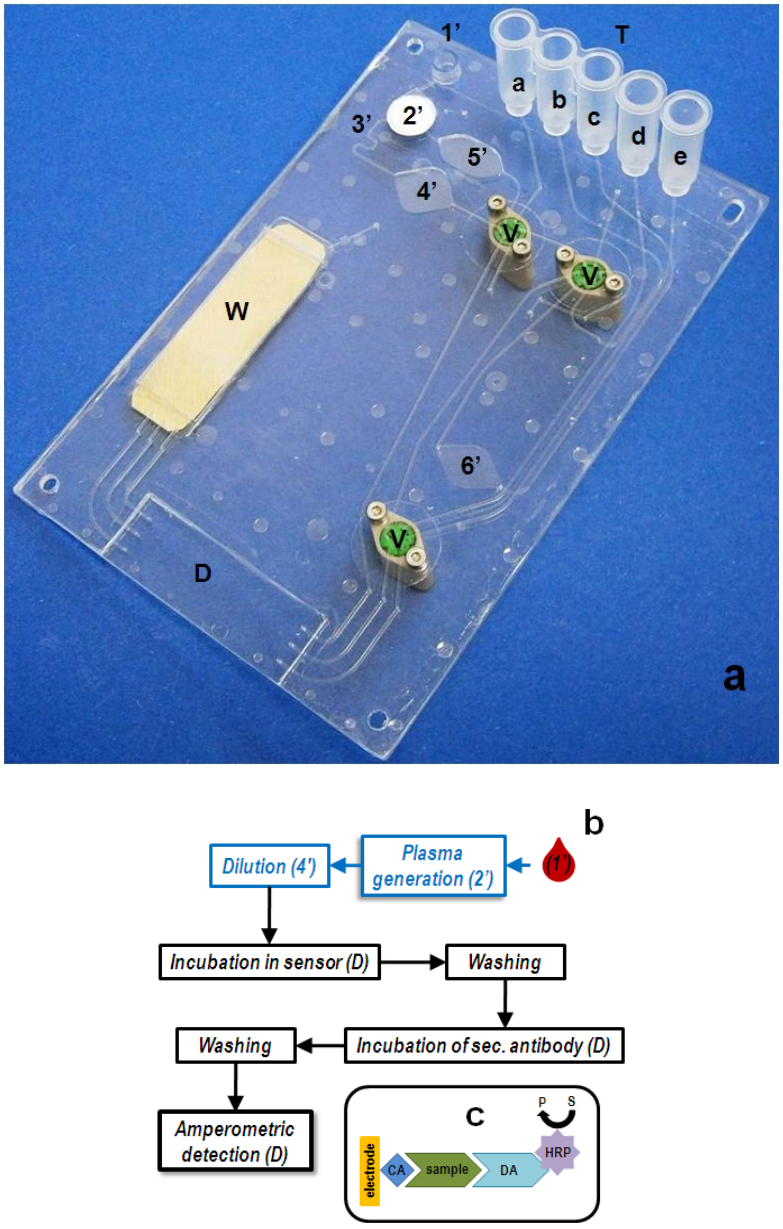

Figure 4. a) View of the serology microsystem. 1': sample inlet, 2': blood filter, 3': plasma metering channel, 4': sample dilution chamber, 5': dilution buffer metering chamber, 6': chamber for reconstitution of dried substrate, D: Detection area, V: valves, T: tanks (a: dilution and washing buffer, b and c: calibrators, d: secondary antibody, e: substrate/mediator for electrochemical detection, W: waste reservoir. b) Flow diagram of serology microsystem operation indicating in parenthesis in which area of the microsystem the steps are carried out. c) Detection principle based on a sandwich assay. CA: capture antigen, DA: detection antibody, HRP: peroxidase, S: substrate, P: product.

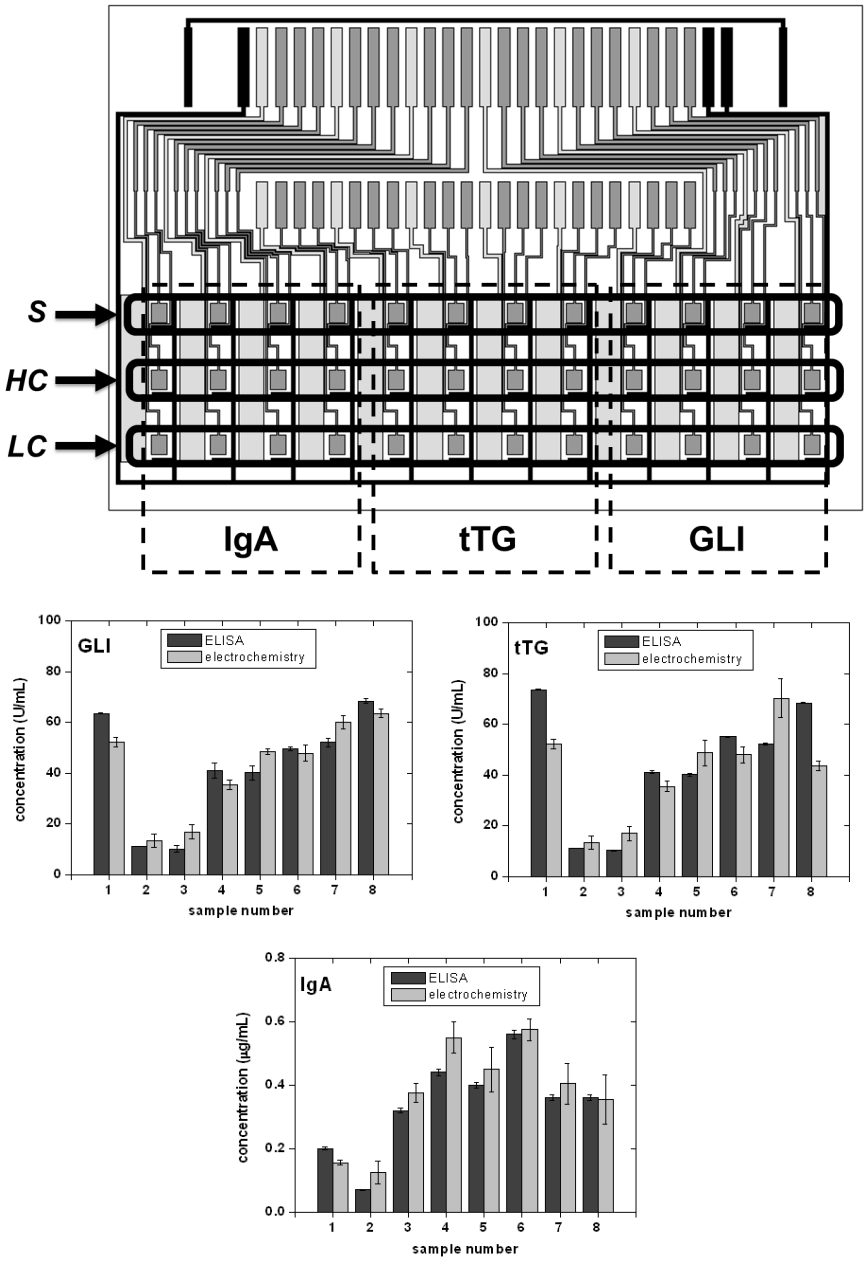

Figure 5. Top: Modification map of the electrode array for serological analysis. Bottom: Levels of GLI, tTG and IgA autoantibodies obtained with the electrochemical platform and comparison with the results obtained using a commercial ELISA kit.