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Lab-on-chip system combining a microfluidic-ELISA with an array of amorphous silicon photosensors for the detection of celiac disease epitopes

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ABSTRACT

This work presents a lab-on-chip system, which combines a glass-polydimethilsiloxane microfluidic network and an array of amorphous silicon photosensors for the diagnosis and follow-up of Celiac disease. The microfluidic chip implements an on-chip enzyme-linked immunosorbent assay (ELISA), relying on a sandwich immunoassay between antibodies against gliadin peptides (GPs) and a secondary antibody marked with horseradish peroxidase (Ig-HRP). This enzyme catalyzes a chemiluminescent reaction, whose light intensity is detected by the amorphous silicon photosensors and transduced into an electrical signal that can be processed to recognize the presence of antibodies against GPs in the serum of people affected by Celiac syndrome.
The correct operation of the developed lab-on-chip has been demonstrated using rabbit serum in the microfluidic ELISA. In particular, optimizing the dilution factors of both sera and Ig-HRP samples in the flowing solutions, the specific and non-specific antibodies against GPs can be successfully distinguished, showing the suitability of the presented device to effectively screen celiac disease epitopes.

1. Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine that affects genetically susceptible individuals. This disease is unique in that the critical etiologic factor has been identified as the ingestion of gluten found in grains of wheat, barley, rye and triticale. Upon ingestions of those proteins, the symptoms showed by patients affected by CD can be severe, thus the diagnosis and treatment must be fast [1]. The diagnostic procedure is performed through serological tests and histological examination of at least one biopsy [23].

In the recent years, the area of serological testing for CD has developed greatly, since it is less invasive than biopsy [26]. Among all serological tests, recently, a lot of attention has been given to the analysis of antibodies against the deamidated gliadin peptides (GPs) due to their promising performance as compared to the most commonly used antigliadin antibodies (AGA), endomysial antibodies (EMA) and tissue transglutaminase antibodies (tTG). Recent studies have demonstrated that the detection of antibodies for GPs can be applied for the diagnosis of celiac disease and for monitoring the adherence of CD patients to gluten free diet (GFD). The determination of antibodies is generally based on enzyme linked immunosorbent assay (ELISA) based screening tests [28].

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The ELISA process includes a series of washing, mixing, and incubation steps, which are labor intensive and time consuming, which often takes several hours, sometimes even up to 2 days to perform one single assay. Most of the time required in a long immunoassay is mostly because of the long incubation time attributed to inefficient mass transport for the immunoagents to move from a solution to the surface, where the conjugation occurs because the immunoreaction itself is relatively rapid [17]. Moreover, the immunoagents used in immunoassays are relatively expensive. To improve the throughput of these processes, robotic systems can be used for fluid handling, but this solution is only available to wealthy laboratories and requires significant maintenance efforts and a large laboratory footprint. Therefore, there is a demand to develop an automated and miniaturized platform for immunoassay.

Miniaturized analytical equipment based on lab-on-chip device (LoC) [18], has been growing due the possible advantages which can be gained by the used of these devices compared to standard analytical equipment, [13] such as (i) consumption of low sample volume, (ii) rapidity of analysis, (iii) easy handling and (iv) multiple target analysis. These advantages can potentially improve the performance and reduce the operating cost of conventional immunoassays.

In particular, LoC have been extensively studied to develop point-of care (POC) devices in the field of health-care and diagnostic. In these devices, biological components are usually immobilized on a solid-

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state surface, which interacts with the analyte. The interactions are detected by using electrochemical or, more often, optical methods [14,21]. In this context, surface chemistry treatments of microchip play a critical role because they deeply influence the biomolecular recognition in terms of selectivity and sensitivity [13]. These chemical treatments may negatively affect the functionality of detection elements such as photodiodes, when these are integrated on the microsystem. As a consequence, microfluidic chips for POC are often based on off-chip detection (e.g. using CCD or CMOS sensors) with difficulty in producing low-cost and portable systems.

Recently, we reported the development of an ELISA-on-chip device for the detection of GPs [10]. Nevertheless, in this ELISA-on-chip device, the serum to be analyzed was handled manually, by spotting a few microliter of sample on the array. As a consequence, the analysis is time consuming and more exposed to inaccuracies of the personnel.

In order to improve this device, we combined a microfluidic network, chemically functionalized to anchor GPs (probe) for the biochemical recognition of GP antibodies, based on an ELISA, with an array of silicon amorphous photosensors (a-Si:H) for the detection of the biochemical reaction. This microfluidic ELISA relies on a sandwich immunoassay between antibodies against GPs (target) and a secondary antibody marked with horseradish peroxidase (HRP), which is used to obtain a chemiluminescence signal detected by the array of a-Si:H photosensors.

Among the optical detection technique, chemiluminescence, in which light is produced as a result of a chemical reaction, is particularly suited for its application in miniaturized assays, offering high specificity and detectability [25] combined with rapidity and simplicity, avoiding the need for additional optical systems as radiation sources and optical filters. On the other hand, the advances in microfabrication have enabled high density, chip-scale integration of optical components, such as photodetectors, and integrated waveguides [29] in lab-on-chip system. In particular, the integration of hydrogenated amorphous silicon (a-Si:H) [16] photodetectors within a lab-on-chip can lead to a very compact system. Indeed, the a-Si:H technology, thanks to its low-temperature processing (below 250 °C), enables the fabrication of high performance photosensors on glass and polymers, ideal supports for lab-on-chip device. The possibility to use transparent substrates reduces the distance between the photosensors and the site where the luminescence takes place [2,6,27] while the very low dark current in reverse bias condition coupled with a quite high photosensitivity in the ultraviolet [4], visible [19,24] and near infrared [3] determine an effective competitiveness of a-Si:H photosensors with respect to cooled CCD detectors [20] and prove their wide applicability in a variety of analytical formats.

The device presented in this paper overcomes the integration issues deriving from combining the on-chip sensor fabrication and the surface functionalization based on polymer brushes [7–9]. In addition, the proposed device permits the simultaneous detection of multiple GPs antibodies. The development of a LoC based on a microfluidic ELISA, with integrated optical sensing for the detection of antibodies against GPs was never reported before. This LoC system is engineered to reuse the optical sensing part of the chip for multiple patients, while the chemically functionalized microfluidic network can be easily removed after the analysis of one serum sample. This LoC is valuable to (i) verify adherence of CD patients to the GFD, (ii) permit the screening of multiple antibodies in one analysis, (iii) perform the analysis using a little amount of serum, thus resulting less invasive for the patient, (iv) and more importantly represents a novel instrument for the diagnosis of CD.

2. Experimental

2.1. Materials

All reagents were purchased by Aldrich Chemicals. 2-Hydroxyethyl methacrylate (HEMA) was distilled prior to use, whereas the other chemicals were used without further purification. 2-Bromo-2-methyl-propionic acid 3-trichlorosylanyl-propyl ester was synthesized following a reported procedure [15]. Methanol (analytical reagent grade) was used without further purification, while toluene was distilled over sodium.

Synthetic gliadine peptides PQPQLPYPQ (VEA) or FPGQQQPFPPQQP (31–43), anti-VEA and anti-DEC rabbit antisera and secondary antibody anti-rabbit marked with HRP were provided by Primm (Milan, Italy).

Chemiluminescent substrate SuperSignal® ELISA Pico was purchased by Thermo Scientific.

All the gases utilized for the deposition of the amorphous silicon layers (silane, diborane, methane and phosphine) and the dry etching processes were provided by Rivoira.

2.2. Equipments

Water was purified with a Milli-Q pulse (MILLIPORE, R = 18.2 MΩ·cm) ultra-pure water system.

The a-Si:H layers were deposited by a Glasstech Solar Inc. (GSI) Plasma Enhanced Chemical Vapor Deposition system working at 13.56 MHz constituted by three ultra high-vacuum (UHV) chambers sharing a load-lock chamber for the sample transportation. Each UHV chamber uses a turbomolecular pump coupled with a mechanical pump for the achievement of an ultra-high pre-deposition vacuum and a roots pump coupled with a backing pump during the deposition process.

All the microelectronic processes for the fabrication of the a-Si:H photosensor array have been performed in a clean room, which includes a vacuum evaporation system (Balzers 510) and a sputtering system (Materials Research Corporation, Orangeburg, New York, USA) for the deposition of the metal layers and the Indium Tin Oxide (ITO) film acting as contact electrodes. The photolithographic processes were performed using TAMARACK 152R mask-aligner for mask reproduction, a Reactive Ion Etching system (from IONVAC PROCESS, Italy) for dry etching of the amorphous silicon and ITO films and a chemical bench for wet etching of the metal films.

The current–voltage (I–V) curves of the photosensors were acquired by means of a Keithley 236 Source Measure Unit, utilized in the Voltage-Source/Current-Measure mode.

The sensor quantum efficiency was measured by using a dual arm optical set-up, which includes a tungsten lamp acting as light source, a monochromator (model Spex 340E from Jobin-Yvon) able to transmit a narrow band of wavelengths chosen from the wide range of wavelengths available at its input, a beam-splitter located at the monochromator output to minimize the effect of a lamp instability, a UV-enhanced crystalline silicon diode (model DR 2550-2BNC from Hamamatsu) used as reference, lenses (from Melles-Griot) acting as focusing optics and two Keithley 236 Source Measure Units, which acquire the currents of the reference diode and the a-Si:H sensor.

A custom low-noise readout electronic board [22] measures the photosensor currents that are induced by the chemiluminescent reactions occurring inside the microfluidic channels. The circuit operation relies on a charge sensitive amplifier (the DDC118 Current-Input Analog-to-Digital Converter from Texas Instruments), which integrates the currents flowing through the a-Si:H diodes and converts it to a 20-bit digital signal. The chip has eight identical input channels to simultaneously monitor eight sensors of the photosensor array. The DDC118 output signal is acquired by a PIC18F4550 microcontroller from Microchip that also provides the communication interfaces, both USB and UART, to a personal computer for the subsequent signal elaboration.

2.3. Methods

2.3.1. Synthesis of the PHEMA brush layer and its functionalization

Glass and oxidized silicon substrates were immersed in a Piranha solution (H_2SO_4: H_2O_2 3:1) for 20 min, copiously rinsed with Milli-Q water and then dried with a stream of nitrogen. Subsequently, the substrates were incubated in a solution of 0.2% of 2-bromo-2-methyl-

propionic acid 3-trichlorosylanyl-propyl ester (BMPTS) in toluene over night [9].

A solution of 20 mL 2-hydroxyethyl methacrylate (HEMA) and 20 mL water was degassed by bubbling through dry nitrogen (N_2) for 30 min and transferred in a schlenk tube where it was stored under argon. Copper(I) chloride (0.110 g), copper(II) bromide (0.072 g) and 2.2′-dipyridyl (0.488 g) were added. To dissolve the solid, the mixture was stirred for 10 min (while degassing), which yielded a dark brown solution. The solution was then sonicated until complete dissolution of the solid was achieved. Subsequently the solution was transferred using a cannula into a schlenk tube containing the glass substrates previously reacted with BMPTS [10,11]. After polymerization (3 h at room temperature, in the dark), the samples were removed and washed with methanol and Milli-Q water. The PHEMA polymer films were treated (18 h, at room temperature) with a solution of succinic anhydride (100 mg) (SA) and triethylamine (100 μL) in 2 mL of dry tetrahydrofuran (THF) to obtain PHEMA-SA. Subsequently, they were rinsed with THF and Milli-Q water and dried with a stream of nitrogen.

2.3.2. Fabrication of the microfluidic chip

The microfluidic chip includes (i) a 4×4 cm^2 glass substrate covered with PHEMA brush layer functionalized with SA and (ii) a polydimethylsiloxane (PDMS) microfluidic network bonded together. The top view and cross section of the microfluidic chip are reported in Fig. 1.

The microfluidic network (4×4 cm^2) consists of 4 parallel channels (1500 μm width × 50 μm height × 2.85 cm length) having separate inlets and outlets. The mold for the polydimethylsiloxane (PDMS) channels was fabricated by standard photolithography using SU-8 2050 (Microchem) on silicon wafers, with a final thickness of ≈50 μm, following the recommended procedure by the manufacturer. Briefly, a silicon wafer was cleaned with HF (2%) for 2 min and copiously rinsed with Milli-Q water. Thereafter, the wafer was spin-coated with a layer of SU-8 2050 at 500 rpm for 5 s and 3000 rpm for 30 s. The wafer was prebaked at 65 °C for 2 min and at 95 °C for 7 min and exposed for 1.5 min for total exposure energy of 240 mJ cm^{-2}. The wafer was then post-baked at 65 °C for 2 min and at 95 °C for 6 min, followed by 5 min development and hard-baking at 200 °C for 30 min.

In order to fabricate the microfluidic channels, 15 g PDMS (Dow corning USA) mixture at a 10 (base material):1 (curing agent) ratio was poured on the mold and cured at 80 °C for 25 min. Afterwards, the PDMS slab was left to cool and access holes were made using a 304 SS TiN coated round punch (0.021″ ID × 0.028″ OD × 1.5″ long) purchased from Syneo LLC (Angleton, TX, USA). Subsequently, the cured PDMS slab was removed from the mold.

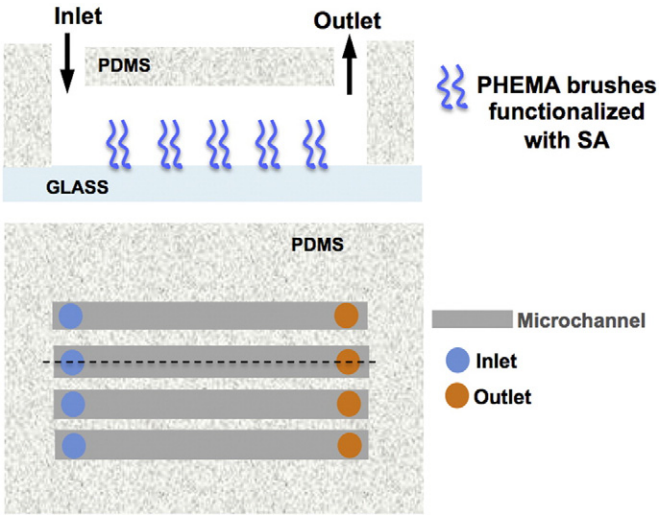

Fig. 1. Cross section (top) and top view (bottom) of the microfluidic chip. The cross section is taken along the dashed line. Dimensions are not in scale.

The PDMS was then bonded to the glass slide previously functionalized with PHEMA-SA, using the transfer bonding technique with uncured PDMS as the adhesive. 1–2 mL of PDMS mixture 10 (base material):1 (curing agent) ratio was poured on a glass slide (5×5 cm^2), which was then spin-coated at 4500 rpm for 4 min. This resulted in a thin layer of uncured PDMS. The previously fabricated PDMS slab was placed on this glass slide and left for 1–2 min. Then, the PDMS cured slab was lifted off, leaving a layer of uncured PDMS on it. A glass slide already bearing PHEMA-SA brushes was prepared for bonding, cleaning it with a solution of Milli-Q water. The PDMS slab with the thin layer of uncured PDMS was then placed on the brush functionalized glass slide and cured at 90 °C for 15 min. The inlet and outlet connections were made by inserting stainless steel pins (0.013″ ID × 0.025″ OD × 0.5″ long) purchased by New Ingland Small Tube Corporation (Litchfield, NH, USA) in the inlet/outlet hole previously formed. Reaction mixture and buffer solutions were flowed in/out of the microfluidic channels by connecting tygon tubing (0.020″ ID × 0.060″ OD) purchased by Generalcontrol S.p.A. (Milan, Italy) to the pins inserted in the device.

2.3.3. Functionalization of the microchannels with peptides

The functionalization of each microfluidic channel with peptide was obtained by flowing a water solution containing 13 mg of n-hydroxysuccinimide (NHS) and 75 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and leaving the solution inside the microchannel in stop flow for 60 min. Each channel was rinsed with Milli-Q water at the flow rate of 5 μL/min for 5 min. Subsequently, a Blocking buffer (pH 7.5 NaH_2PO_4 2.5 mM; Na_2HPO_4 7.5 mM; bovine serum albumin (BSA) 4%) was flowed into the channels and left inside for 10 min. The channels were rinsed with Milli-Q water at the flow rate of 5 μL/min for 5 min and dried with a stream of nitrogen. Afterwards a solution of Reaction buffer (pH 7.5 NaH_2PO_4 2.5 mM; Na_2HPO_4 7.5 mM) containing 0.3 mg/mL of synthetic gliadin peptides PQPQLPYPQ (VEA) or FPGQQQPFPPQQP (31–43) was flowed into the channels and left inside over night at 4 °C, rinsed with Milli-Q water for 10 min at 5 μL/min and dried with a stream of nitrogen. A Reaction buffer solution containing 10 mM of ethanolamine (pH 8) was flowed into the channels and left inside for 30 min, the channels were subsequently rinsed with Milli-Q water at 5 μL/min for 10 min and dried with a stream of nitrogen. A qualitative description of the microchannel inside at the end of this functionalization is reported in Fig. 2a.

2.3.4. Analytical procedure

Reaction buffer solutions of anti-VEA or anti-31-43 rabbit antisera were flowed into the channels and incubated for 10 min. Subsequently the channels were rinsed with Milli-Q water, 5 μL/min for 5 min and dried with a stream of nitrogen.

A Reaction buffer solution of secondary antibody anti-rabbit marked with HRP was flowed into the channels and incubated for 10 min.

Subsequently, the channels were rinsed with Milli-Q water at the flow rate of 5 μL/min for 5 min, with Rinsing buffer (NaH_2PO_4 2.5 mM; Na_2HPO_4 7.5 mM; 0.01% of tween 20, pH 7.5) for 20 min, with Milli-Q water at the flow rate of 5 μL/min for 5 min and dried with a stream of nitrogen and used for the chemiluminescent reaction. A qualitative description of the microchannel inside, during this analytical procedure, is reported in Fig. 2b and c.

2.3.5. Fabrication of the a-Si:H photosensor array

The photosensor array has been fabricated on a 5×5 cm^2 glass substrate uniformly covered with an Indium Tin Oxide layer (ITO) [12], which acts as transparent front electrode of the a-Si:H photodiodes. The array consists of 5×6 elements. Each photosensor is p-type/intrinsic/n-type stacked structure grown by Plasma Enhanced Chemical Vapor Deposition (PECVD). A metal stack constitutes the bottom electrode. A qualitatively top view and cross section of the fabricated array is shown in Fig. 3.

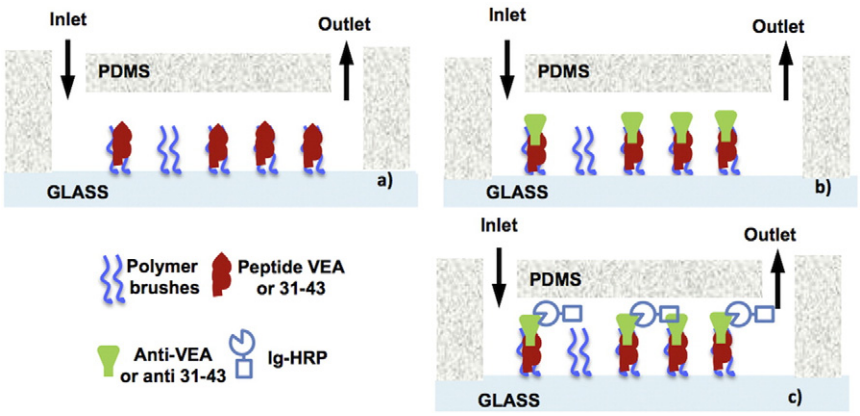

Fig. 2. Functionalization of the microchannels with **a)** gliadin peptides (VEA or 31-43), **b)** anti-VEA or anti-31-43 rabbit antisera and **c)** secondary anti-rabbit antibody marked with HRP.

Light impinges on the glass and slips unabsorbed through the ITO front contact. Due to the designed thicknesses and energy gaps of the a-Si:H layers [Caputo et al. 2007, [6]], the incoming radiation that is mostly absorbed in the intrinsic region of the p-i-n structure is the intrinsic layer, while the role of the doped layers is limited to the creation of the built-in voltage. The photogenerated electron-hole pairs are swept out toward the doped layers, thanks to the electric field existing in the i-layer, and collected at the metal electrodes.

The technological steps leading to the fabrication of the photosensor array are:

a. deposition by magnetron sputtering of a 180 nm-thick ITO layer and its pattering for the definition of the front electrode of the diodes;
b. deposition by PECVD of the p-i-n a-Si:H layers with 10 nm/400 nm/50 nm thickness, respectively;
c. sputtering of a three-metal layer stack (10 nm-thick Cr/150 nm-thick Al/30 nm-thick Cr) acting as top electrode of the sensors;
d. mesa patterning of the device structure by wet and reactive ion etchings for the metal stack and a-Si:H layers respectively;
e. deposition of a 5 μm-thick SU8-3005 (from Micro-Chem, MA, U.S.A.) layer acting as insulation layer;
f. opening of via holes over the diodes on the SU8 layer;
g. sputtering of a 150 nm-thick TiW metal top layer for the definition of the rear common electrode.

The area of each diode is 2×2 mm^2, while the pitch array is 5 mm.

2.3.6. Analysis with the array of a-Si:H photosensors

The analysis was performed, at room temperature, positioning the microfluidic chip on the glass hosting the array of photosensors. Each channel was aligned with a column of photosensors (5 for each channel). The optical losses due to refraction were reduced interposing coupling oil between the two glasses. A qualitatively picture of the photosensor array/microfluidic chip is reported in Fig. 4.

The photosensor array was connected to a house-made electronic read out system, which in turn communicates via USB to a computer that displays the measured currents using dedicated software. The microfluidic chip, the photosensor array and the electronics were closed in a small box, provided with holes to allow microfluidic connection. The chemiluminescent cocktail was flowed into the channels at the flow rate of 50 μL/min. Once the channel was completely filled, the pump was stopped and the reaction was followed in stop-flow measuring the sensor photocurrent induced by the chemiluminescence signal.

3. Results and discussion

Previously, we reported an ELISA-on-chip device, where biochemical recognition and detection are all performed on a single glass substrate [5,10]. In particular, one side of the glass substrate hosts an array of amorphous silicon photosensors that act as optical transducer element, while the opposite side is functionalized with an array of poly(2-hydroxyethyl methacrylate) (PHEMA) brushes, aligned with the

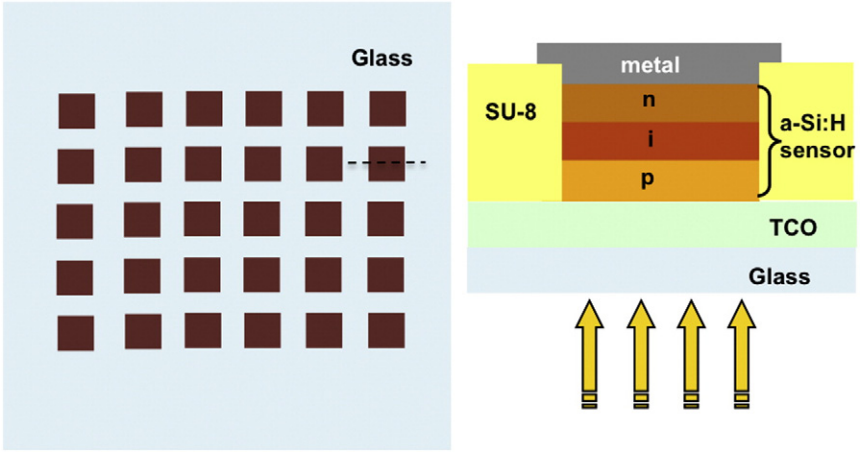

Fig. 3. Sketch of the top view (left) and cross section (right) of the a-Si:H photosensor array. The 5×6 brown squares represent the photosensors, whose cross section is taken along the dashed line.

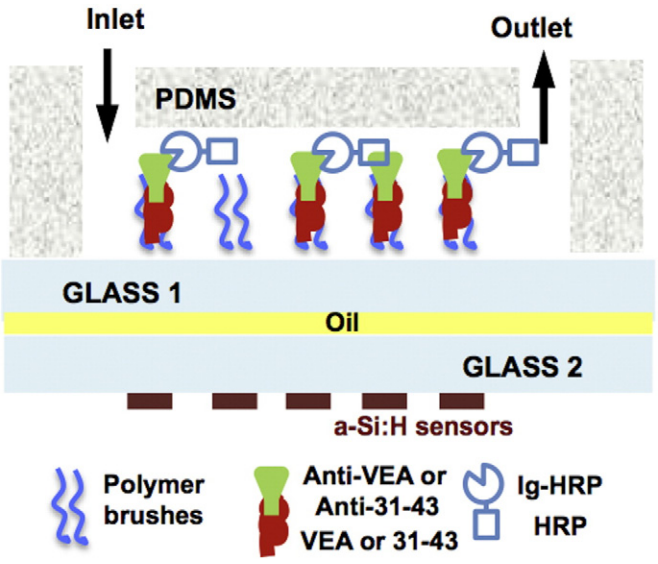

Fig. 4. Schematic representation of the coupling between the microfluidic chip and the photosensor array to perform the monitoring of the chemiluminescent reactions occurring inside the microfluidic channels.

photosensors, to immobilize GPs for the recognition of specific antibodies. In this system, the screening of GPs antibodies is performed by manually spotting antisera solutions on the array of GPs. The detection of specific and nonspecific antibodies was successful but the sample handling was time consuming and susceptible to inaccuracies of the personnel.

Thus, in this work we introduced a microfluidic-ELISA based on GPs functionalized microfluidic network, to overcome the issues deriving from the sample handling, combined with the array of a:Si-H photosensors for the detection of the chemiluminescent signal deriving from the interaction of GPs with the specific antibody.

The LoC was designed to have an array of 30 a-Si:H photosensors deposited on a glass substrate (Fig. 5).

The photosensors (the 30 gray squares in the picture) are organized as a 5×6 matrix. All the n-type a-Si:H layers of the photodiodes are connected together by the metal top electrode, which includes the six brown vertical lines and the brown C-shaped geometry. Each p-type a-Si:H layer is instead connected to its own electrical contact, which is individually brought to the edge of the glass substrate for the connection to the read-out electronics. These individual connections are not visible because, as described above, they are made in ITO. Indeed, the ITO used in this work presents a resistivity equal to $3.25 \cdot 10^{-4}\Omega$/cm and a transmittance greater than 80% for wavelength above 420 nm. This last result is particularly important because, since the peak emission spectra of the HRP occurs 425 nm, it ensures that mostly part of the chemiluminescence radiation passes unabsorbed through the ITO layer.

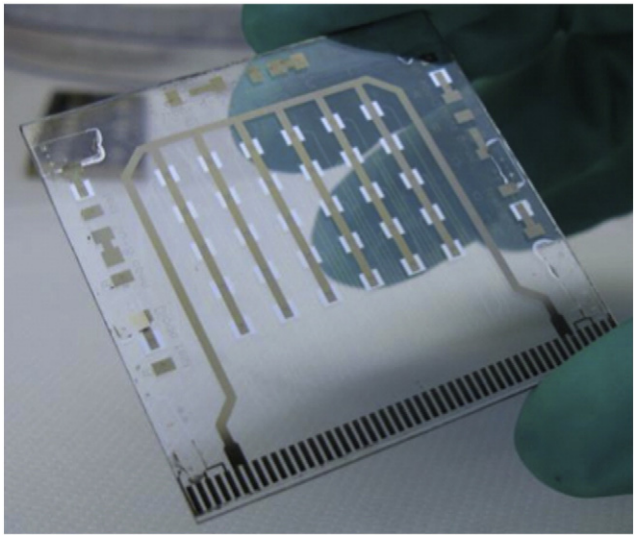

Fig. 5. Picture of the fabricated a:Si-H photosensors array.

We found that the current–voltage characteristics of the photosensors are quite uniform over all the array. The current density J measured in dark condition in the small reverse voltage region (below $+100$ mV applied to the n-region) shows a value around 10^{-11} A/cm^2. The shot noise theory, formulated by Schottky in 1919, establishes that the time-dependent fluctuations in electrical current density J caused by the discreteness of the electron charge determine a mean square value $\langle J^2 \rangle$ equal to $2qJB$, where q is the elementary electrical charge and B is the signal band. Taking into account that the current–voltage data are measured with a frequency band of 1 Hz, the measured dark current density determines a noise contribution around 1.6 fA. Furthermore, from the characterization of the custom read-out board [22], we found that the noise level introduced by the electronics in the same signal band is around 15 fA. Coupling these two data, we can state that the dark current noise contribution does not affect the minimum detectable signal and that the noise performances of our experimental read-out set-up are comparable with those of sophisticated and expensive state-of-the-art laboratory equipment.

From the quantum efficiency measurements, we found that the quantum yield at 425 nm is equal to 0.28 with a corresponding responsivity of 100 mA/W. This value is lower than that measured by Pereira et al. [30] on similar a-Si:H devices, however, the results that will be shown below demonstrate that its combination with the very low dark current density and the excellent noise characteristics of all the detection system fully satisfies the requirements of the analytical performances of the presented lab-on-chip application.

A functionalized microfluidic chip, having four parallel channels is aligned with the array of photosensors, which serves for the detection of the chemiluminescent signal deriving from the immunoassay occurring in the microfluidic chip. The use of two different glasses, for the biological part and the detection part, and their optical coupling through the scheme shown in Fig. 4 lead to some important benefits:

1. overcoming of the integration issues deriving from the interactions between the reagents utilized for the surface chemistry treatments and the detection elements (photosensors in this case); indeed in this way the two glasses can be processed separately avoiding negative influence of the surface chemistry treatments on the performances of the optical detection devices,
2. reduction, with respect to off-chip detection, of the radiation losses thanks to the directly coupling which reduces to about 2 mm the distance between the site where the chemiluminescence occurs and the detection site;
3. preservation of the compactness of the entire system.

Furthermore, once the immunoassay is performed for a patient, the microfluidic chip can be easily removed and a novel chip can be positioned to analyze the serum of another patient.

The microfluidic chip is functionalized with PHEMA polymer brushes by using the chemical procedure reported above. Briefly, PHEMA is grown on a glass substrate and functionalized with succinic anhydride to obtain carboxylic functional groups on the brush layer (PHEMA-SA). The glass substrate is then bonded with the array of four parallel channels made in polidimethylsiloxane PDMS. In each channel is flowed a water solution containing NHS-EDC and this solution kept inside for 1 h. After rinsing with purified water, a different peptide is flowed in each channel and left inside over night at 4 °C. Finally the channels are rinsed out using purified water.

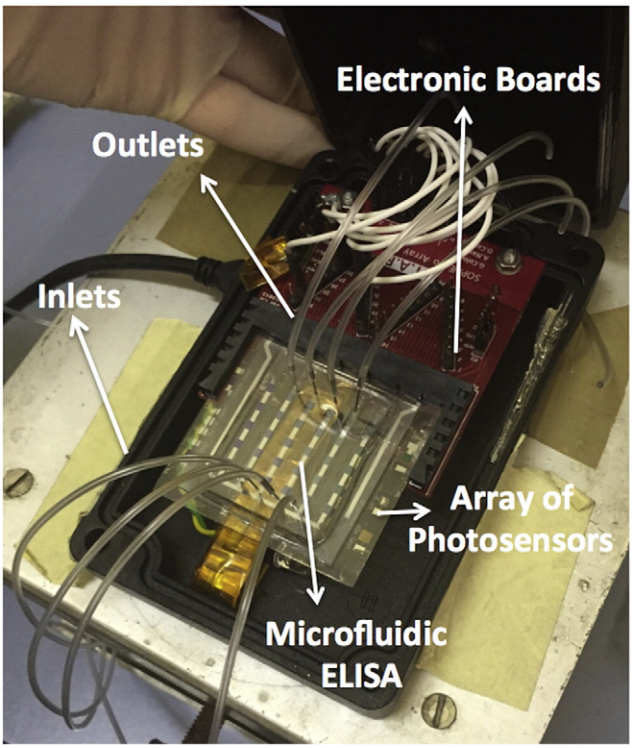

Fig. 6. Picture of the inside of the box containing the electronic read-out board, the photosensor array, the microfluidic chip and the microfluidic connections needed to perform the ELISA. The lid hosts the holes for the microfluidic connections and permits a shielding of the ambient light.

The microfluidic device was aligned on the array of photosensors and inserted in the base of a small box ($6 \times 8 \times 12$ cm^3) containing the electronic read-out and the microfluidic tubes (Fig. 6). Each channel has separated inlet and outlet for liquid handling. Holes drilled in the lid of the box (partially shown in the figure) allow the microfluidic connection to a peristaltic pump to perform the immunoassay and at the same time shielding of the photosensors from the ambient radiation.

As a proof of principle, for each microfluidic chip, we functionalized two channels with the GPs named VEA and two channels with 31-43 (see Fig. 2a). Reaction buffer solution of Rabbit sera containing primary antibodies against VEA (anti-VEA) or 31-43 (anti-31-43) (see Fig. 2b) were flowed into the four parallel channels with the following scheme:

i. a reaction buffer solution containing anti-VEA was flowed into the channel functionalized only with VEA;
ii. a reaction buffer solution containing anti-31-43 was flowed into the channel functionalized only with GP 31-43;
iii. a reaction buffer solution contain anti-VEA was flowed into the channel functionalized only with GP 31-43;
iv. reaction buffer solution without rabbit sera was flowed into the channel functionalized only with peptide VEA (peptide-only).

The reaction buffer sera solutions were incubated in stop-flow for 10 min and subsequently rinsed with water. Subsequently, in each channel was flowed a solution of a secondary antibody labeled with HRP (Ig-HRP) and incubated for 10 min (see Fig. 2c).

In the immunoassay analysis, a chemiluminescent (CK) cocktail based on luminol and hydrogen peroxide (H_2O_2) is flowed into the channels. The HRP catalyzes the oxidation of luminol by H_2O_2, yielding a chemiluminescent signal, which is detected by the photodiodes and read-out by the electronic board. In a typical experiment, the photocurrent signal detected by the photosensors increases, reaches a peak whose value depends on the type of interaction and then decreases.

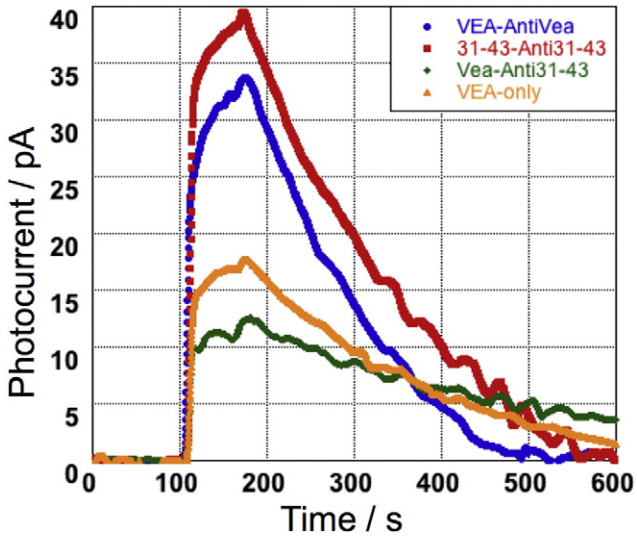

Fig. 7. Photocurrent signal versus time obtained by flowing the CK cocktail into the microchannels functionalized with rabbit antisera and Ig-HRP diluted 500 and 200 times, respectively.

This behavior is illustrated in Fig. 7, which reports the results obtained using reaction buffer solutions of rabbit antisera and Ig-HRP diluted 500 and 200 times, respectively. We observe that the photocurrent signal originated by the interaction between a GP and its specific antibody (i.e. GP with its specific rabbit antiserum) is always higher than that detected when the same GP reacts with the nonspecific rabbit antisera or when no antisera is flowed in to the functionalized channel. This implies that the LoC system can be successfully used to screen the antibodies in the serum.

We did found not significant changes in the photocurrent values, performing the experiments in different days with changes in the room temperature of about 5 °C. The optimized dilution conditions for both rabbit sera and Ig-HRP were investigated. In particular, reaction buffer solutions of rabbit sera were prepared diluting the serum between 50 and 500 times and the standard solution of Ig-HRP between 200 and 2000 times.

For this purpose, several microfluidic chips were functionalized with GPs, afterwards they were positioned on the top of the array of a-Si:H photosensors. The reaction buffer solutions of anti-VEA and anti-31-43 and Ig-HRP were flowed into the microchannel following the analytical procedure described in the Methods section. Finally, the ELISA assay was performed using the chemiluminescent cocktail.

The results obtained from this set of experiments are summarized in Fig. 8. In this figure, the photocurrent peaks measured during the different immunoassays are reported together with the error bars, calculated over three measurements for each combination of dilution. We observed that the intensity of the photocurrent signals for the three types of interactions strongly depends on both the dilution factors. In particular, when Ig-HRP was diluted 200 times (Fig. 8a), the intensity of the CK signal for the specific and non-specific interactions is strongly different only when the rabbit antisera solution is diluted 500 times. This may depend on the possible reactions of GPs with non-specific antibody, which may occur at high concentrations of rabbit serum. Also, the intensity of the chemiluminescent signal observed when Ig-HRP is flowed into channels functionalized only with GPs (peptide-only) suggests a possible physical absorption (non-specific absorption) of antibodies onto the PDMS and/or PHEMA polymer matrix.

Similar results are obtained when Ig-HRP is diluted 2000 times (Fig. 8b). Indeed, when the rabbit antiserum is diluted 50 or 250 times, the CK signal due to non-specific interactions and absorption on channel wall is still occurring, while this undesired signal strongly decreases for rabbit antisera diluted 500 times. From Fig. 5b, we deduce that the

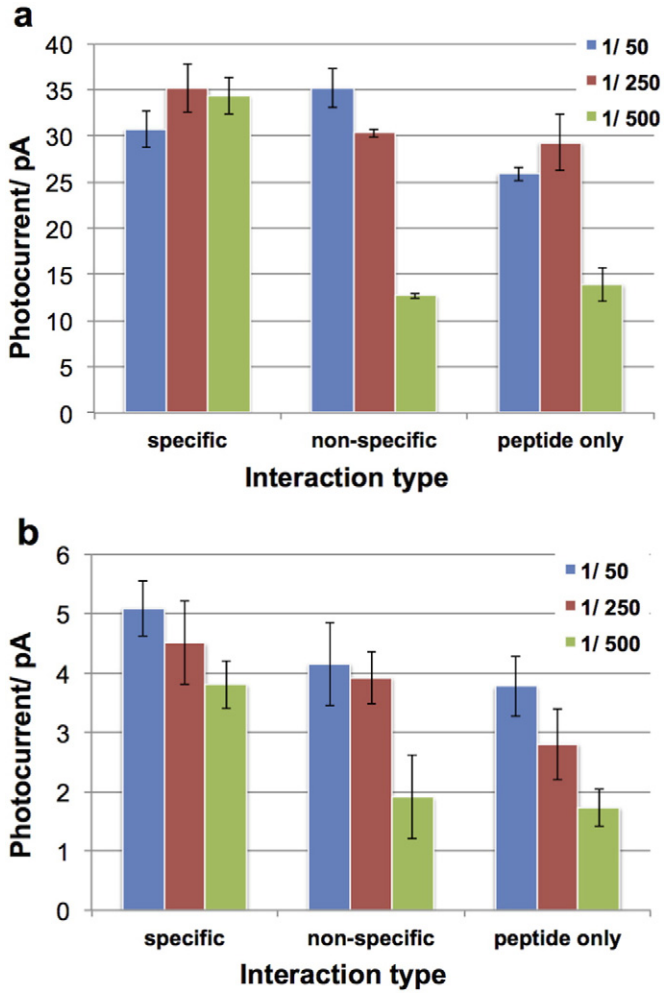

Fig. 8. Optimization of rabbit antisera and Ig-HRP concentrations. Three different rabbit antisera dilutions for each panel were tested on the microfluidic platform, using reaction buffer solutions of Ig-HRP diluted **a)** 200 times and **b)** 2000 times. Error bars indicate the standard deviations calculated over three independent measurements.

absolute chemiluminescent peak is much lower than that achieved for Ig-HRP dilution equal to 200 and that the standard deviation is much higher. Therefore, in the investigated ranges the optimized buffer dilutions are 500 and 200 for the rabbit antisera and Ig-HRP, respectively.

As a final remark, we would like to note that the total time of analysis, once the microfluidic chip has been functionalized with PHEMA-SA, is around 1 h. This time includes the analytical procedure and the analysis with the array of a-Si:H photosensors, both described in the Methods section. Therefore, the presented lab-on-chip system requires half of the time that is usually necessary for an ELISA analysis [17].

4. Conclusions

We have presented a lab-on-chip system, relying on a sandwich immunoassay between antibodies against gliadin peptides (GPs) and a secondary antibody marked with horseradish peroxidase (Ig-HRP), suitable for the diagnosis and follow-up of celiac disease. The system optically couples a glass-PDMS microfluidic network, where the immunoassay occurs, and a glass hosting an array of amorphous silicon photosensors, which detect the chemiluminescent reactions occurring inside the microfluidic channels. In particular, the microfluidic device is directly positioned and aligned over the array of photosensors and is inserted in a small metal box containing the electronic read-out board and the microfluidic connections to perform the immunoassay. The use of two different glasses for the biological part and the detection part is advantageous, because, while the direct coupling minimizes the radiation losses, once the immunoassay is performed for a patient the microfluidic chip can be easily removed and a novel chip can be positioned to analyze the serum of another patient. Furthermore, the developed analytical procedure allows a total analysis time less than half of the analysis time required by the standard ELISA immunoassay.

The system has been tested using rabbit serum in the microfluidic ELISA. We found that the dilution factors for the rabbit antisera and the Ig-HRP strongly affect the chemiluminescent signal. Dilution factor of 500 for the rabbit antisera showed the highest difference between specific and non-specific interaction between antisera and GPs independently on the Ig-HRP dilutions, while the Ig-HRP concentration affects the absolute value of the chemiluminescent peak. By using the optimized dilution condition, the intensity of the signals deriving by the specific interactions is much higher than those observed when nonspecific antisera and no-antisera (peptide-only) were flowed into the functionalized channels, demonstrating that the proposed lab-on-chip system can be successfully used to screen the antibodies in the serum.

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