Heterogeneous immunoassays in microfluidic format using fluorescence detection with integrated amorphous silicon photodiodes

A. T. Pereira,^{1,2} P. Novo,¹ D. M. F. Prazeres,^{2,3} V. Chu,¹ and J. P. Conde^{1,3}

¹*INESC Microsistemas e Nanotecnologias and IN-Institute of Nanoscience and Nanotechnology, R. Alves Redol, 9, 1000-029 Lisbon, Portugal*

²*IBB-Institute for Biotechnology and Bioengineering, Instituto Superior Técnico, Av. Rovisco Pais, 1, 1049-001 Lisbon, Portugal*

³*Department of Chemical and Biological Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1, 1049-001 Lisbon, Portugal*

(Received 1 October 2010; accepted 7 January 2011; published online 15 February 2011)

Miniaturization of immunoassays through microfluidic technology has the potential to decrease the time and the quantity of reactants required for analysis, together with the potential of achieving multiplexing and portability. A lab-on-chip system incorporating a thin-film amorphous silicon (a-Si:H) photodiode microfabricated on a glass substrate with a thin-film amorphous silicon-carbon alloy directly deposited above the photodiode and acting as a fluorescence filter is integrated with a polydimethylsiloxane-based microfluidic network for the direct detection of antibody-antigen molecular recognition reactions using fluorescence. The model immunoassay used consists of primary antibody adsorption to the microchannel walls followed by its recognition by a secondary antibody labeled with a fluorescent quantum-dot tag. The conditions for the flow-through analysis in the microfluidic format were defined and the total assay time was 30 min. Specific molecular recognition was quantitatively detected. The measurements made with the a-Si:H photodiode are consistent with that obtained with a fluorescence microscope and both show a linear dependence on the antibody concentration in the nanomolar-micromolar range. © 2011 American Institute of Physics.

[doi:[10.1063/1.3553014](https://doi.org/10.1063/1.3553014)]

I. INTRODUCTION

Quantitative immunologic assays have been the essential tools in the detection of a wide range of analytes of clinical, medical, biotechnological, and environmental significance since the late 1960s.¹ The ability of antibodies to react strongly and specifically to a particular antigen is exploited in these assays. Immunoassays are currently a multistage, labor-intensive, and time consuming process. Automation of microtiter plate immunoassays can be achieved by the use of complex and bulky robotic systems for fluid manipulation. Microfluidic techniques allow the manipulation of small quantities (10^{-9} – 10^{-18} L) of fluids in channels with dimensions typically in the range of 10–100 μm .² Development of immunoassays in a microfluidic format started in the late 1990s³ with increasing interest being devoted to this topic in subsequent years.^{3–5} Miniaturization of immunoassays in a microfluidic system has the potential to provide fast, simple, sensitive, automated, and multiplexed immunoassays, with reduced consumption of sample and reagents and the possibility of bringing the analysis to the point-of-care.⁴

Typical applications of immunoassays in the microfluidic format have been reviewed and summarized in the literature,^{3–5} and include detection of different analytes such as small peptides, antibodies, toxins, and antigens of clinical interest. The first report of immunoassays in the microfluidic format was performed in glass structures using an optical band pass filter, an objective and photomultiplier tube (PMT) for fluorescence detection.^{6,7} Examples for both homogeneous⁸

and heterogeneous^{9–11} immunoassays have been reported. Magnetic^{12–15} and nonmagnetic^{16–19} bead-based immunoassays are widely exploited, taking advantage of their increased surface-volume ratio. Reduction in total reaction time has been achieved with several reports of assays with duration of 30 min or less.^{14,18,20–23} Ranges of sensitivity achieved are becoming comparable to those typically obtained in large scale size.^{9,24,25} For example, 1.56 pg ml^{−1} was the limit of detection achieved for electrochemical detection of interleukin-6,²⁶ and 10 pM of *Staphylococcal* enterotoxin B could be detected using fluorescence detection with a PMT.²¹ Other important advantages demonstrated in microimmunoassays were the small sample volume consumption^{19,21,26} and assay automation.^{15,23}

The use of microfluidic immunoassays coupled with integrated miniaturized detection systems would allow the miniaturization of the full immunoassay. Miniaturization implies a reduction in the detection volume. This also means that the total number of molecules of each analyte present for detection in the miniaturized system is reduced. Thus, it is crucial to choose an appropriated detection method with high sensitivity and scalable to smaller dimensions.²⁷ The most common form of miniaturized detection is the use of electrochemical detection^{9,14,19,28–32} because of the ease of electrode miniaturization and integration in the microfluidic system. Optical detection has also been used in microfluidic immunoassays, such as fluorescence detection^{7,15,20,21,33,34} using diode lasers coupled with PMT and appropriate wavelength filters. Examples of chemiluminescence detection by the use of charged coupled device (CCD) camera^{24,25,35} and PMT³⁶ can also be found in the literature. Photodiodes have also been used for microfluidic immunoassay detection, both for fluorescence^{37,38} and colorimetric³⁹ measurement. Although PMTs are very sensitive to light and can give high frequency response, it is not possible to miniaturize and integrate on-chip. The use of CCD cameras for optical detection has the disadvantage that the resulting image requires further analysis to obtain a quantitative answer. The use of photodiodes can potentially overcome these drawbacks since these devices are characterized by high photosensitivity, low dark current, and high frequency response and could be easily integrated on a chip.

The methods of injection most commonly used are either syringe injection pumps or electrophoresis. Electrophoresis has the advantage of not requiring an external instrument for injection. Both injection methods can be automated and precisely controlled. Electrophoresis is easier to miniaturize whereas the miniaturization of syringe pump injection is limited by syringe size. Also, reactant volumes are higher with a syringe pump. With electrophoretic volume manipulation, since the volume is smaller, the total analysis time is faster. However, electrophoresis is restricted to glass microfluidic structures. Microfluidic structures are fabricated by wet etching of glass, a technique that is laborious and costly. Polydimethylsiloxane (PDMS) is cheaper than glass and microfluidic structures are fabricated by soft lithography, which is a simpler and cheaper fabrication method. PDMS also has the advantage of being fully biocompatible.

Thin-film photodiode based on hydrogenated amorphous silicon is a well-established technology allowing large area fabrication for applications such as digital x-ray imagers⁴⁰ and image sensors. a-Si:H has been used in biosensors for fluorescence detection of proteins and DNA, using *p-i-n* photodiodes^{41–44} and photoconductors.^{45,46} DNA multicolor detection was achieved using a *p-i-n* and a *p-i-n-i-p* amorphous silicon stacked structure.⁴⁷ A trichromatic biosensor was developed using *n-i-p-i-i-n* a-Si:H photodiodes.⁴⁸ Also, a-Si:H *p-i-i-n* photodiodes have been used to detect the growth of thin protein layers by reflectometric interference spectroscopy.⁴⁹ Other applications of a-Si:H photodiodes in biosensing include chemiluminescent and colorimetric detection in immunoassays using reaction volumes in the 10–50 μ L range.^{50–52} Preliminary results have also been reported combining colorimetric detection with a-Si:H photodiodes and microfluidic immunoassays.⁵³

However, until now, a bioanalytical system that includes the optical detection system and a disposable PDMS microfluidic handling unit fully integrated on a glass lab-on-chip has not been, to the best of our knowledge, reported in the literature. In this platform, the antigen is first immobilized on the microchannel walls, followed by the antibody-antigen molecular recognition reaction. The antibody is labeled with a fluorescent tag, either fluorescein isothiocyanate (FITC) or quantum dots. The emitted fluorescence intensity is measured by the photodiode. Each step of the

FIG. 1. Schematic representation of the miniaturized immunoassay: (a) adsorption of the primary (probe) antibody to the microchannel walls; (b) blocking of the surface with BSA (represented by the yellow cylinders); (c) specific recognition of the probe antibody with a secondary antibody (target), labeled with a fluorescent tag; (d) alignment of the microchannel with the integrated photodiode and with the laser light beam for the fluorescence detection of the molecular recognition event which occurred in (c).

immunoassay, namely, (i) the washing conditions in the microfluidic setup, (ii) the conditions for probe antibody adsorption in the microfluidic channel walls, (iii) the conditions for antibody/antigen molecular recognition, and (iv) the detection of antibody-antigen molecular recognition using the integrated photodiode, requires optimization, which is described in detail. The combination of microfluidics with micron-sized photodiodes allows full system miniaturization and points toward a miniaturized, multiplex system that will permit fast and inexpensive point-of-care analysis of molecules with biological and biomedical relevance.

II. GENERAL DESCRIPTION OF THE MODEL MICROFLUIDIC IMMUNOASSAY SYSTEM

A model immunoassay in which the antigen is itself an antibody, immunoglobulin G (IgG), was selected for simplicity. Detection of antibodies (probes) present in the human serum by immunoassay recognition using an antibody (target) which is specific against the probe is very common in clinical analysis when, for example, the diagnostic of an infection is made by quantifying the immunological response of the patient. Examples of such diseases are HIV, toxoplasmosis, rubella, hepatitis, and cytomegalovirus.⁵⁴

The first step of the model immunoassay consists in the adsorption of the probe antigen (IgG) to the microchannel walls [Fig. 1(a)]. The number of probe molecules that are bound to this solid surface is critical for the sensitivity of the reaction and can be adjusted by varying the concentration of antigen in solution and immobilization conditions, such as exposure time, flow rate of the solution containing the target antibodies, and functionalization of the channel inner walls. In a microtiter plate assay, this step is usually performed overnight at 4 °C to allow full binding of the target antibody. Next, a surface blocking step is usually required to minimize nonspecific adsorp-

FIG. 2. (a) Schematic of the measurement setup with the microfluidic channel aligned with the integrated photodiode and the fluorescence excitation laser beam reflecting in a semitransparent mirror and focused on the photodiode/microfluidic channel assembly. For alignment, a stereo microscope is used and a PMMA support (not represented) keeps the PDMS microfluidic network in place during the measurement. (b) Micrograph showing the photodiode array aligned with the microfluidic channel. The sensing region, where the microchannel is aligned with the photodiodes, is highlighted by the light lines. The dark square indicates an individual photodiode (top view).

tion of the target antibody. In this work, bovine serum albumin (BSA) is used [Fig. 1(b)]. In the following step, a specific antibody (anti-IgG) is allowed to react with the first antibody that is immobilized in the microchannel [Fig. 1(c)]. Each of the steps described above requires at least 1 h when the analysis is carried out in a microtiter plate format. In this macroscopic format, the complete assay, including the several washing steps required, can take up to 24 h.⁵⁴ For integrated fluorescence detection, the second antibody (target) is labeled with a fluorescent marker [in this case, quantum dots Qdot[®] 625 Goat F(ab')₂ Anti-Mouse IgG conjugate (H+L) (Invitrogen)]. When excited at the appropriate wavelength ($\lambda_{\text{exc}}=405$ nm), the Qdot625 will emit light ($\lambda_{\text{em}}=625$ nm) that is detected by the integrated photodiode. An integrated optical filter is optimized to block light at the excitation wavelength and to maximize the transmission of light at the emission wavelength, allowing it to reach the photodiode [Fig. 1(d)]. Figure 2(a) shows an overall schematic of the measurement setup.

Two main reactions contribute to the level of the signal obtained in the assay: (i) the immobilization of the antigen to the microchannel walls and (ii) the antibody-antigen molecular recognition reaction. Two factors control the extent of the antibody-antigen reaction: (i) the diffusion of antigens to the microchannel wall where the antibodies are immobilized and (ii) the equilibrium of the reaction of formation of the antibody-antigen complex. Since the affinity constant is usually high, varying from 10^5 to 10^{12} mol⁻¹, the antibody-antigen reaction is expected to be diffusion controlled.⁴ Therefore, due to the small dimensions of the microchannels (20 μm height), this molecular recognition reaction is expected to reach completion within minutes. Another important factor to be taken into account in the system is the ability of the integrated photodetector to efficiently capture the emitted light and reject the excitation light: this involves the optimization of the performance of the fluorescence filter and of the overall geometry of the system.

III. MATERIALS AND METHODS

A. Integrated microfluidic/photodiode system fabrication

1. Photodiode fabrication

200 $\mu\text{m} \times 200$ μm a-Si:H *p-i-n* photodiodes were microfabricated on glass substrates.⁵¹ An Al bottom electrode is deposited on Schott AF45 glass and patterned using wet chemical etching. Next, a *p-i-n* a-Si:H structure is deposited by rf (13.56 MHz) plasma enhanced chemical vapor deposition (rf-PECVD) at 250 °C at a power density of 50 mW/cm². Phosphine (5 sccm, diluted to 2% in hydrogen) and diborane (5 sccm, diluted to 2% in hydrogen) were added to pure silane to obtain *n*-type and *p*-type films, respectively. A diode mesa structure is patterned by photoli-

thography and reactive ion etching. A 100-nm-thick insulating layer of silicon nitride (SiN_x) deposited by PECVD is used as a sidewall passivation layer and a via is opened to allow electrical contact between the top transparent conductive oxide (indium-tin-oxide) and the top a-Si:H *p*-layer. A 2 μm -thick thin-film amorphous silicon-carbon alloy (a-SiC:H) deposited by rf-PECVD at 100 °C and 300 mW/cm^2 is used as an absorption filter layer to cut the excitation light in the fluorescence measurements. The bandgap of the a-SiC:H film depends on the gas flow ratio between silane and ethylene, and for the fluorophore labels used in this application, a filter with a bandgap of 2.04 (E_{04} of 2.25 eV), which corresponds to a gas flow rate ($\text{C}_2\text{H}_4/\text{C}_2\text{H}_6 + \text{SiH}_4$) of 0.08, was used.⁵⁵ The finished devices were wirebonded to a printed circuit board (PCB) plate. The final die has dimensions of 15×13 mm and contains a linear array of 23 photodiodes separated by a distance of 200 μm , as shown in Fig. 2(b). The dimensions of the die allow the alignment of the microfluidic device on top of every photodiode of the array. An absorption filter was chosen to cut the excitation light due to ease of fabrication and because the absorption filter can be deposited in the same deposition system used for the a-Si:H deposition. The use of an interference filter may improve the system performance because of its sharper cutoff. However, the fabrication of an interference filter would require the sequential deposition of multilayers of two materials with different refractive indices, for example, SiO_2 and TiO_2 in a dedicated and calibrated system. In addition, the performance of interference filters is strongly dependent on the incidence angle of the light, which would impair its performance when the channel and the detector are of comparable characteristic dimensions and placed at close proximity.⁵⁶

2. Photodiode characterization

The photoresponse of the device was measured either in dc using a picoammeter (Keithley 237) or in ac (13 Hz) using a lock-in amplifier (EG&G Princeton Applied Research 5209), a current preamplifier (Ithaco I211), and a light beam chopper (HMS). In the dc measurement, the photoresponse is calculated by subtracting the diode current under illumination from the dark current. The light source was a 250 W tungsten-halogen lamp, coupled to a monochromator (McPherson 2035) to select the wavelength of the incident light. The incident wavelengths of interest were 625 or 405 nm, respectively [the emission and excitation wavelengths of the quantum dots used to label the antibodies (see Sec. III B)]. The incident light flux was determined using a calibrated silicon photodetector (Advanced Photonix, Inc., SD100-11-11-021). The measurements were conducted under zero bias voltage conditions.

3. PDMS microfluidic channel fabrication

Rectangular section microchannels, with a height h of 20 μm and a width w of 200 μm , were fabricated by soft lithography in PDMS using an SU-8 mold on a crystalline Si base substrate. An Al-on-quartz shadow mask was used for the UV patterning of the SU-8-2015 (Microchem) mold. PDMS (Sylgard 184—Dow Corning) was prepared by mixing the curing agent and the base in a 1:10 weight ratio followed by vacuum deairing. To prepare molds which can resist repetitive usage, PDMS structures formed from the SU-8 were used as molds to make permanent masters made of epoxy (Permabond ES562). The epoxy-PDMS assembly was left degassing overnight and cured at 120 °C for 30 min. To make the microfluidic network, PDMS was injected over the SU-8 or epoxy molds and cured for 2 h at 60 °C. The base of the channels was a 50 μm -thick flat PDMS base plate that was prepared by spinning the PDMS mixture at 500 rpm for 5 s and then at 2230 rpm for 20 s.

The PDMS was chemically treated to extract the unreacted oligomers from the bulk phase.⁵⁷ This treatment results in a stable PDMS surface with higher retention of hydrophilic properties after oxidization. The PDMS pieces were sequentially immersed in triethylamine, ethyl acetate, and acetone, each for 2 h at room temperature with stirring. After this treatment, the PDMS pieces were washed with de-ionized water and left to dry at 60 °C overnight. The channel was sealed to the base plate after surface oxidation with a corona discharge (Electro-Technic Products).

B. Optimization of the microfluidic immunoassay

The conditions for the microfluidic operations described in Sec. II were optimized to (i) achieve molecular recognition in the model immunoassay; (ii) improve the overall signal obtained; (iii) minimize reagent consumption; and (iv) reduce the total time of analysis. For this optimization procedure antibodies labeled with fluorescein (FITC) and detection by fluorescence microscopy were used. The optimized conditions for the microfluidic operations thus obtained were then used with Qdot625-labeled antibodies using the system with integrated photodiodes and are reported in Sec. IV C. FITC fluorescence detection is not possible using the integrated system described in Sec. III C because the fluorescence filter does not have the optical characteristics required to reject sufficiently the excitation wavelength of FITC (595 nm).

Fluorescence microscopy (LEICA DMLM) was used to measure the fluorescence of the adsorbed antibodies after wash using a digital camera (LEICA DFC300FX). The fluorescence micrographs were taken using an exposure time of 510 ms, color saturation of 1.5 $\times$, and gain of 1.0 $\times$. For color correction, gamma was 0.60, black 0 and white 100, and RGB values were set at 0, 0, 0, respectively. For the analysis of fluorescence intensity photos were converted to 32 bit gray scale images. The values of gray intensity were measured inside the microchannel using the software IMAGEJ. The gray intensity outside the channel was subtracted to account for the background fluorescence. A calibration of the fluorescence was performed every day by flowing at a constant rate solutions with different concentrations of FITC dissolved in carbonate buffer (CB) (from 0.05 to 4 mg mL⁻¹) to correct for possible day-to-day variability of the lamp intensity.²

1. Optimization of the washing conditions in the microfluidic setup

Phosphate buffer saline (PBS) was used as the washing solution which was flowed through the system with varying flow rate (Q) and washing times to remove the weakly bound probe antibodies adsorbed on the microchannel walls. Probe antibodies were Anti-Mouse IgG FITC-labeled (SIGMA) diluted in CB 50 mM, pH 9.6 with concentration of 100 mg L⁻¹ injected for 10 min with a flow rate (Q) of 0.5 μ L min⁻¹. A syringe pump (New Era Syringe Pump) was used to provide a constant fluid flow.

2. Optimization of the conditions for antibody adsorption in the microfluidic setup

Anti-Mouse IgG FITC-labeled (SIGMA) diluted in CB 50 mM, pH 9.6, in concentrations from 10 mg L⁻¹ (66.7 nM) to 1000 mg L⁻¹ (6.67 μ M) was injected with a Q of 6.9 nL min⁻¹ for different immobilization times. After adsorption of the probe antibodies, washing was performed by flowing CB at a Q =69 nL min⁻¹ for 10 min. Fluorescence intensity was monitored every 30 s during the wash process as described above (Sec. III B 1).

3. Optimization of the conditions for antibody/antigen molecular recognition in the microfluidic setup

The primary antibody, Goat IgG or Mouse IgG (both from SIGMA) 100 mg L⁻¹ in CB, was injected in the microchannel with Q =6.9 nL min⁻¹ for 10 min for adsorption in the microchannel walls. The washing solution used [0.1% (w/v) BSA (Eurobio) and 0.05% Tween 20 (SIGMA)] in PBS was flowed with Q =69 μ L min⁻¹ for 3 min to wash the channel and to block the surface decreasing nonspecific interactions. The secondary antibody, Anti-Mouse IgG FITC-labeled or Anti-Goat IgG FITC-labeled (both from SIGMA), 100 mg L⁻¹ in PBS was then injected in the channel with Q =6.9 nL min⁻¹ for 10 min. After the molecular recognition reaction, the channel was washed by flowing PBS with Q =69 nL min⁻¹ for 3 min.

FIG. 3. Characterization of the integrated a-Si:H photodiode/a-SiC:H fluorescence filter used for integrated detection of molecular recognition reactions. (a) Photodiode current density as a function of the incident photon flux using incident light at 625 and at 405 nm (corresponding, respectively, to emission and excitation wavelengths of the fluorophore Qdot625). (b) External quantum efficiency, EQE, of the photodiode with (circles) and without (squares) the integrated a-SiC:H filter.

C. Detection of antibody-antigen molecular recognition using the integrated photodiode

A series of immunoassays was performed using the optimized conditions as specified in Sec. III B 3. The laser light, reflected by a semitransparent mirror, is optically aligned with the microchannel and with the integrated photodiode using a stereomicroscope (Nikon 75519), as shown schematically in Fig. 2(a).

To obtain correlation between the fluorescence signal emitted by antibodies labeled with Qdot625 and the number of antibodies present, separate experiments were performed in which a microchannel was filled with different concentrations of Qdot625-labeled antibody diluted in PBS. The fluorescence signal was then measured with the photodiode as described above.

IV. RESULTS AND DISCUSSION

A. Integrated photodiode characteristics

The photocurrent density of the a-Si:H photodiode with the integrated a-SiC:H fluorescence filter shows a linear dependence with the photon flux, as shown in Fig. 3(a). A decrease of approximately four orders of magnitude in the photocurrent density is obtained, for an excitation light of 405 nm relative to 625 nm. The external quantum efficiency, EQE, of the a-Si:H photodiode and of a-Si:H photodiode/a-SiC:H filter tandem is plotted as a function of the wavelength in Fig. 3(b), showing that the a-SiC:H filter efficiently cuts the light below 480 nm.^{43,58} The sensitivity of measurements made with a voltage bias of 0 V is limited by the light rejection characteristics of the a-SiC:H filter used and not by the dark current of the a-Si:H photodiode.

B. Probe antibody immobilization

The conditions used to perform immunoassays with large volumes (in the range of hundreds of microliters) of solution in a microwell are well established and the use of integrated a-Si:H photodiode detection has been reported.^{51,52} The challenge is to develop integrated fluorescence detection in a microfluidic immunoassay in which four main steps require definition and optimization: (i) immobilization of the first (probe) antibody; (ii) surface blocking to reduce nonspecific adsorption; (iii) antibody-antigen probe-target molecular recognition reaction; and (iv) stringent washes between each step. To simplify the process optimization for reaction in a microfluidic channel, antibodies labeled with FITC were used and the fluorescence levels were assessed using fluorescence microscopy.

Transposing immunoassays from a batch format using volumes of solution in the microliter range and above, to the continuous flow format in a microfluidic system, where reactions occur mostly in a flow-through mode, requires the combination of three interconnected parameters: flow

rate Q , time of flow t , and concentration C . The values of Q and t control the volume V of solution that passes through the microchannel since $Q=V/t$. V is related with the number of molecules n that pass through the microchannel because $C=n/V$, where C is the concentration of the molecule of interest. In a batch system, the fraction of n that adsorbs on the surface n_{ads} depends on the diffusion of the molecule toward the surface and on the reaction rate constant of the adsorption reaction. In a continuous system, since the fluid is moving with a constant Q , the molecules are also moving due to convection. The Péclet number, Pe , relates the diffusion time τ_D with the convection time τ_c due to fluid velocity v_0 :⁵⁹ $Pe=v_0h/D$, where h is the characteristic distance for diffusion (in this case, the height of the microchannel) and D is the diffusion constant. To favor efficient adsorption of the molecules (antibodies) on the microchannel walls, as well as the surface blocking and the antibody-antigen recognition reaction, a diffusion-dominant regime where the molecules of interest have time to diffuse from the interior of the microchannel to the walls would be favored (Pe small). In the case of a convection dominated regime (Pe large), most of the molecules will flow through the length of the microchannel without having time to diffuse to the microchannel walls. For a fixed microchannel geometry (fixed h) and a particular molecule (fixed D), a low Pe will require a low v_0 . In the antibody adsorption [Fig. 1(a)] and in the antibody-antigen reaction [Fig. 1(c)] a $Q=6.9 \text{ nL min}^{-1}$ was used. Using a typical diffusion coefficient for an antibody molecule⁶⁰ of $\sim 4 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$, $Pe \sim 6.5$, with $\tau_D \sim 1 \text{ s}$ and $\tau_C \sim 0.8 \text{ s}$. Since τ_D and τ_C are of the same order of magnitude antibody adsorption will be favored.

1. The effect of washing on adsorbed antibodies

Washing is one of the most important steps of this assay since it controls the specificity, sensitivity, and reproducibility of both the probe immobilization and molecular recognition steps of the immunoassay. In a microfluidic format, washing stringency can be varied by using different flow rates and/or washing times. Here, washing was optimized by immobilizing first Anti-Mouse IgG FITC-labeled on the microchannel surface using a fixed set of conditions and then analyzing the effect of the washing parameters on the overall fluorescence intensity (see experimental details in Sec. III B 1). First the effect of the Q of the washing solution on the concentration of adsorbed antibodies was studied. Figure 4 shows that the decrease in the density of adsorbed antibodies during washing (as shown by the decrease of the measured fluorescence) is faster for higher values of Q of the washing solution. The final surface coverage is approximately the same for all the values of Q of the washing solution ($\sim 50\%$ of the initial). This observation suggests that an approximately constant amount of antibody adsorbs strongly on the microchannel walls which is independent of the washing conditions used.

The shear stress in the microchannel surface (and hence on the adsorbed antibody layers) due to the flow of the washing solution can be estimated using as an approximation the Poiseuille flow in an infinite parallel plate configuration. The shear stress τ is related with Q , channel dimensions and fluid viscosity (μ) by⁵⁹ $\tau=-(6Q\mu)/(h^2w)$. Assuming that the solution has the viscosity of water ($\mu=0.001 \text{ Pa s}$), τ varies between 0.625 and 12.5 Pa for values of Q between 0.5 and 10 $\mu\text{L min}^{-1}$. Since constant levels of fluorescence intensity are reached even for the highest values of Q tested (Fig. 4), the shear stress is below the threshold level required to remove the strongly adsorbed antibodies.

2. Definition of flow and wash conditions for antibody immobilization

Immobilization of antibodies to solid surfaces can be achieved by covalent immobilization using cross-linking molecules, by a molecular specific interaction (such as using protein A which binds to the Fc region of the antibody), or by physical-chemical adsorption.⁶¹ From an experimental point of view, direct adsorption of antibodies to a solid surface is the simplest and fastest method. Antibody molecules are prone to adsorb to plastic surfaces by hydrophobic interactions, a property that has been exploited for decades in conventional immunoassays by using standard polystyrene microtiter plates.⁵⁴ In the model immunoassay used in this work, simple adsorption was used to facilitate the adaptation of the protocols developed to other antigen-antibody pairs of biological or clinical relevance. Since the probe antibody is polyclonal and is playing the role of

FIG. 4. Effect of the flow rate of the washing solution on the concentration of adsorbed antibodies on the surface of the microchannel. Probe antibodies labeled with FITC were adsorbed to the microchannel walls prior to the washing experiment. The error bars refer to the standard deviation obtained from three distinct samples. The dashed line shows the 50% fluorescence level which corresponds to the strongly adsorbed probe antibodies.

an antigen, random positioning of the probe antibody after adsorption is advantageous, because several different possible antigenic sites are thus exposed for recognition.

Figure 5(a) shows the effect of the washing time for different concentrations of probe antibodies in solution. A higher concentration of antibodies in solution results in higher concentrations of adsorbed antibodies both before and after wash. After the initial large decrease, the fluorescence remains approximately constant. The ratio of fluorescence decrease due to washing depends on the initial concentration of antibodies in solution. When comparing relative fluorescence levels [rather

FIG. 5. Effect of the concentration of antibody in solution on the density of strongly adsorbed antibody on the microchannel walls. (a) Fluorescence from adsorbed Anti-Mouse IgG-FITC as a function of time. At time $t=0$ s, the initial fluorescence is measured after flowing different concentrations of Anti-Mouse IgG-FITC at 6.9 nL min^{-1} for 30 min. Each individual curve is measured on a single sample. (b) Fluorescence intensity of adsorbed antibodies as a function of antibody concentration in the initial solution. The different symbols show the signal obtained for different flow times of antibody Anti-Mouse IgG-FITC at 6.9 nL min^{-1} . Washing was performed for 3 min at 69 nL min^{-1} with CB. The lines are guides to the eye. Each point is measured on a different sample.

TABLE I. Summary of the conditions used for the immunoassay recognition reaction sequence.

Step	Solution	Time (min)	Flow rate (nL min ⁻¹)
1. Adsorption of antigen	100 mg L ⁻¹ Mouse IgG or Goat IgG in CB	10	6.9
2. Washing/blocking	0.1% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS	3	69
3. Recognition reaction	100 mg L ⁻¹ Anti-Mouse IgG-FITC or 2 nM Qdot 625 labeled in PBS	10	6.9
4. Washing	PBS	3	6.9

than the absolute values shown in Fig. 5(a)] one observes that the higher the concentration of antibody in solution, the larger the decrease of fluorescence after the wash. For the concentration of 1000 mg L⁻¹ of antibody in solution, the fluorescence level rapidly decreased upon washing to ~20% of the initial value after 30 s, whereas for the concentration of 100 mg L⁻¹ the fluorescence level shows a slower decrease upon washing to ~60% of the initial value after 10 min. This observation suggests that for higher concentrations of antibodies in solution, the first layer of antibodies at the surface is covered by a higher number of successive layers of antibodies that are kept together via weak bonding interactions. The appropriate washing procedure is required to remove the weakly adsorbed molecules. Using the results shown in Fig. 5(a), a flow of washing solution of 6.9 nL min⁻¹ for 3 min was selected.

Figure 5(b) shows the density of strongly adsorbed probe antibodies (after wash) as a function of the antibody concentration in the solution for different flow times. For the flow times studied (between 6 and 30 min), the immobilized antibody density shows a fast rise between 10 and 100 mg L⁻¹. For concentrations above 1000 mg L⁻¹ there was observed a decrease in fluorescence level regardless of the flow time used, as can be verified in Fig. 5(b). This decrease could be due to steric interference effects that limit the access of antibodies in solution to the free binding sites in the surface for high concentration of surface antibodies. From the results shown in Fig. 5(b), the concentration of the probe antibody selected to be used in the immunoassays was 100 mg L⁻¹ and the time of flow at 6.9 nL min⁻¹ was 10 min. These values represent a compromise between reactant consumption and the duration of the adsorption step.

C. Probe-target (antibody-antigen) molecular recognition detection

A series of immunoassays was performed using the optimized conditions as specified in Secs. III B 3 and III C and Table I. Assays were performed with antibodies labeled with Qdot625 for integrated photodiode detection and also with antibodies labeled with FITC for comparison and as a link to the optimization of conditions described in Sec. IV B. The results are summarized in Fig. 6 and are qualitatively similar regardless of whether the fluorescence was detected with the integrated a-Si:H *p-i-n* photodetector/a-SiC:H fluorescence filter device or with a fluorescence microscope. The fluorescence level obtained for the specific reaction between Mouse IgG and Anti-Mouse IgG is significantly higher than that for the nonspecific reaction between Goat IgG-Anti-Mouse IgG demonstrating that a specific recognition reaction was achieved and successfully detected. In addition, the results shown in Fig. 6 validate the assumption that the conditions optimized for miniaturized immunoassays using FITC-labeled antibodies can be transferred to Qdot625-labeled antibodies.

The results shown in Fig. 6 demonstrate that an immunoassay can successfully perform in a microfluidic format with an integrated optical detection of fluorescence using a thin-film silicon microphotodiode. To calibrate the current measured by the photodiode with respect to the number of antibody molecules detected with the immunoassay, the microchannel, the microchannel was filled with a known concentration of Qdot625-labeled antibodies. For the calculations, it was assumed that all the molecules detected were located directly on top of the photodiode, i.e., within a volume of 200×200×20 μm centered on top of the photodiode. It was assumed that the

FIG. 6. Microfluidic immunoassay performed with a specific molecular recognition reaction, Mouse IgG-Anti-Mouse IgG, and a nonspecific reaction, Goat IgG-Anti-Mouse IgG. A comparison is made between the fluorescence detected using the integrated a-Si:H *p-i-n* photodiode with an a-SiC:H fluorescence filter (scale on the left) and using fluorescence microscopy (scale on the right). The error bars are calculated from measurements on at least three different samples.

fluorescence emission efficiency of the antibody-label assembly is the same when the antibody is in solution and when the antibody is adsorbed. The molecular density was calibrated by dividing the total number of molecules in solution by the area of the microchannel top and bottom surfaces. An optical model⁴⁴ was used that relates the ratio of fluorescence collected by the photodiode with the geometry of the photodiode and the relative position between the emitting species and the photodiode. Using this model and the details of the present experiment, the fraction of photons collected by the photodiode was calculated at $\sim 18\%$ of the light emitted isotropically by the quantum-dot-labeled antibodies in the microchannel. Figure 7 shows that the photodiode has a linear response to the number of quantum-dot-labeled antibodies in the channel. Applying the calibration curve of Fig. 7 to the current density measured in Fig. 6 allows one to estimate that for the specific Mouse IgG-Anti-Mouse IgG reaction, the molecular density of target antibodies is 4.4×10^{12} molecules m^{-2} ($\sim 3.5 \times 10^5$ target molecules on top of the photodiode). This surface density corresponds to only 0.14% of complete packing (3.18×10^{15} molecules m^{-2} considering that the diameter of the quantum-dot antibody complex is 20 nm as stated by the manufacturer). Note that full packing of the Qdot625 antibody complex is not expected to happen due to steric interferences. For the antibodies the complex footprint area is 100 times larger than the area of the antibody molecule.⁶²

V. CONCLUSIONS

Integrated detection of the molecular recognition reaction between an immobilized probe antigen and a target antibody was performed using an integrated photodiode making the system a potential platform for miniaturization and multiplexing for point-of-care analysis. The current sensitivity of the fluorescence measurement using the integrated *p-i-n* a-Si:H photodiode and a-SiC:H absorption filter allows the detection of a minimum of $\sim 10^5$ quantum-dot-labeled biomolecules. The flow, concentration, and duration conditions for probe antibody immobilization, the molecular recognition reaction, and the several washes involved in a complete immunoassay performed inside a microchannel were optimized. The total assay, including immobilization, recognition reaction, and detection, took approximately 30 min.

FIG. 7. Dependence of the response of a *p-i-n* a-Si:H photodiode with an integrated a-SiC:H fluorescence filter on the surface density and volume concentration of antibodies labeled with fluorescent quantum dots Qdot625. The equivalence between molecular density with both molecular concentration in solution and the number of molecules present in the surface is shown in the upper scales. The error bars from three different measurements are smaller than the point size.

The values of surface coverage could be improved by increasing the number of antibodies immobilized in the first step of the immunoassay. Stable protein adsorption on PDMS surfaces has been reported as having low efficiency. Improvements have been proposed using chemical modification of the PDMS surface by UV-grafting,⁶³ multilayer protein coating,⁶⁴ or dextran coverage,⁶⁵ for example. However, a careful cost-benefit analysis should be performed for surface modifications of the microchannels, keeping in mind that one goal is to obtain a disposable, low cost, and easy to use device for point-of-care analysis. Furthermore, a sharpening of the filter absorption characteristics would widen the applications of the proposed system to a broader range of fluorophores and consequentially broader range of analysis. Decreased light leakage of the filter would further improve the sensitivity of the system.

ACKNOWLEDGMENTS

The authors would like to acknowledge V. Soares and A. Joskowiak for their help in clean room processing and photodiode filter fabrication. This work was supported by Fundação para a Ciência e a Tecnologia (FCT) through research projects and Ph.D. grants and from the European Project OTASENS. The authors also acknowledge funding from FCT through the Associated Laboratories IN, Institute of Nanoscience and Nanotechnology, and IBB, Institute of Biotechnology and Bioengineering.

¹C. Self and D. Cook, *Curr. Opin. Biotechnol.* **7**, 60 (1996).

²H. Parsa, C. D. Chin, P. Mongkolwisetwara, B. W. Lee, J. J. Wang, and S. K. Sia, *Lab Chip* **8**, 2062 (2008).

³A. Bange, H. B. Halsall, and W. R. Heineman, *Biosens. Bioelectron.* **20**, 2488 (2005).

⁴T. G. Henares, F. Mizutani, and H. Hisamoto, *Anal. Chim. Acta* **611**, 17 (2008).

⁵S. Hartwell and K. Grudpan, *Mikrochim. Acta* **169**, 201 (2010).

⁶N. Chiem and D. J. Harrison, *Anal. Chem.* **69**, 373 (1997).

⁷N. H. Chiem and D. J. Harrison, *Clin. Chem.* **44**, 591 (1998).

⁸Y. Huang, S. Zhao, M. Shi, and Y. Liu, *Anal. Biochem.* **399**, 72 (2010).

⁹F. Arévalo, G. Messina, P. Molina, M. Zón, J. Raba, and H. Fernández, *Talanta* **80**, 1986 (2010).

- ¹⁰ D. Lin, C. Taylor, W. French Anderson, A. Scherer, and E. Kartalov, *J Chromatogr B Analyt Technol Biomed Life Sci* **878**, 258 (2010).
- ¹¹ E. Ouellet, C. Lausted, T. Lin, C. Yang, L. Hood, and E. Lagally, *Lab Chip* **10**, 581 (2010).
- ¹² Y. Lee, K. Lien, H. Lei, and G. Lee, *Biosens. Bioelectron.* **25**, 745 (2009).
- ¹³ N. Martínez, R. Schneider, G. Messina, and J. Raba, *Biosens. Bioelectron.* **25**, 1376 (2010).
- ¹⁴ S. Pereira, G. Messina, and J. Raba, *J Chromatogr B Analyt Technol Biomed Life Sci* **878**, 253 (2010).
- ¹⁵ S. Yang, K. Lien, K. Huang, H. Lei, and G. Lee, *Biosens. Bioelectron.* **24**, 861 (2008).
- ¹⁶ M. Ihara, A. Yoshikawa, Y. Wu, H. Takahashi, K. Mawatari, K. Shimura, K. Sato, T. Kitamori, and H. Ueda, *Lab Chip* **10**, 92 (2010).
- ¹⁷ W. Laiwattanapaisa, T. Songjaroen, T. Maturos, T. Lomas, A. Sappat, and A. Tuantranont, *Sensors* **9**, 10066 (2009).
- ¹⁸ S. Pereira, J. Raba, and G. Messina, *Anal. Bioanal. Chem.* **396**, 2921 (2010).
- ¹⁹ S. Yoo, Y. Choi, J. Ju, G. Tae, H. Kim, and S. Lee, *Analyst (Cambridge, U.K.)* **134**, 2462 (2009).
- ²⁰ J. Kong, L. Jiang, X. Su, J. Qin, Y. Du, and B. Lin, *Lab Chip* **9**, 1541 (2009).
- ²¹ R. Meagher, A. Hatch, R. Renzi, and A. Singh, *Lab Chip* **8**, 2046 (2008).
- ²² N. Panini, G. Messina, E. Salinas, H. Fernández, and J. Raba, *Biosens. Bioelectron.* **23**, 1145 (2008).
- ²³ G. Suárez, Y. Jin, J. Auerswald, S. Berchtold, H. Knapp, J. Diserens, Y. Leterrier, J. Månson, and G. Voirin, *Lab Chip* **9**, 1625 (2009).
- ²⁴ K. Heyries, M. Loughran, D. Hoffmann, A. Homsy, L. Blum, and C. Marquette, *Biosens. Bioelectron.* **23**, 1812 (2008).
- ²⁵ S. Sun, M. Yang, Y. Kostov, and A. Rasooly, *Lab Chip* **10**, 2093 (2010).
- ²⁶ G. Messina, N. Panini, N. Martinez, and J. Raba, *Anal. Biochem.* **380**, 262 (2008).
- ²⁷ B. Kuswandi, Nuriman, J. Huskens, and W. Verboom, *Anal. Chim. Acta* **601**, 141 (2007).
- ²⁸ J. Choi, K. Oh, J. Thomas, W. Heineman, H. Halsall, J. Nevin, A. Helmicki, H. Henderson, and C. Ahn, *Lab Chip* **2**, 27 (2002).
- ²⁹ K. Liang, W. Mu, M. Huang, Z. Yu, and Q. Lai, *Biomed. Microdevices* **9**, 325 (2007).
- ³⁰ N. Nashida, W. Satoh, J. Fukuda, and H. Suzuki, *Biosens. Bioelectron.* **22**, 3167 (2007).
- ³¹ D. Tang, R. Yuan, and Y. Chai, *Clin. Chem.* **53**, 1323 (2007).
- ³² J. Zeravik, T. Ruzgas, and M. Fránek, *Biosens. Bioelectron.* **18**, 1321 (2003).
- ³³ A. Dodge, K. Fluri, E. Verpoorte, and N. F. de Rooij, *Anal. Chem.* **73**, 3400 (2001).
- ³⁴ J. Fruetel, R. Renzi, V. Vandernoot, J. Stamps, B. Horn, J. West, S. Ferko, R. Crocker, C. Bailey, D. Arnold, B. Wiedenman, W. Choi, D. Yee, I. Shokair, E. Hasselbrink, P. Paul, D. Rakestraw, and D. Padgen, *Electrophoresis* **26**, 1144 (2005).
- ³⁵ B. Lee, A. Tajima, J. Kim, Y. Yamagata, and T. Nagamune, *Biotechnol. Bioprocess Eng.* **15**, 145 (2010).
- ³⁶ E. Yacoub-George, W. Hell, L. Meixner, F. Wenninger, K. Bock, P. Lindner, H. Wolf, T. Kloth, and K. Feller, *Biosens. Bioelectron.* **22**, 1368 (2007).
- ³⁷ K. Shin, S. Lee, K. Han, S. Kim, E. Yang, J. Park, B. Ju, J. Kang, and T. Kim, *Biosens. Bioelectron.* **22**, 2261 (2007).
- ³⁸ S. Sia, V. Linder, B. Parviz, A. Siegel, and G. Whitesides, *Angew. Chem., Int. Ed. Engl.* **43**, 498 (2004).
- ³⁹ V. Srinivasan, V. Pamula, and R. Fair, *Lab Chip* **4**, 310 (2004).
- ⁴⁰ R. A. Street, *Hydrogenated Amorphous Silicon* (Cambridge University Press, Cambridge, UK, 1991).
- ⁴¹ T. Kamei, B. M. Paegel, J. R. Scherer, A. M. Skelley, R. A. Street, and R. A. Mathies, *Anal. Chem.* **75**, 5300 (2003).
- ⁴² T. Kamei, N. M. Toriello, E. T. Lagally, R. G. Blazej, J. R. Scherer, R. A. Street, and R. A. Mathies, *Biomed. Microdevices* **7**, 147 (2005).
- ⁴³ A. C. Pimentel, D. M. F. Prazeres, V. Chu, and J. P. Conde, *J. Appl. Phys.* **104**, 054913 (2008).
- ⁴⁴ A. C. Pimentel, A. T. Pereira, D. M. F. Prazeres, V. Chu, and J. P. Conde, *Appl. Phys. Lett.* **94**, 164106 (2009).
- ⁴⁵ F. Fixe, V. Chu, D. M. F. Prazeres, and J. P. Conde, *Nucleic Acids Res.* **32**, e70 (2004).
- ⁴⁶ A. C. Pimentel, D. M. F. Prazeres, V. Chu, and J. P. Conde, *J. Appl. Phys.* **106**, 104904 (2009).
- ⁴⁷ D. Caputo, G. de Cesare, A. Nascetti, R. Negri, and R. Scipinotti, *IEEE Sens. J.* **7**, 1274 (2007).
- ⁴⁸ K. Watty, C. Merfort, K. Seibel, L. Scholer, and M. Bohm, *Phys. Status Solidi A* **207**, 700 (2010).
- ⁴⁹ M. Samann, L. Steinle, and M. B. Schubert, *J. Non-Cryst. Solids* **354**, 2575 (2008).
- ⁵⁰ A. C. Pimentel, A. T. Pereira, V. Chu, D. M. F. Prazeres, and J. P. Conde, *IEEE Sens. J.* **7**, 415 (2007).
- ⁵¹ A. Gouvea, A. T. Pereira, A. C. Pimentel, D. M. F. Prazeres, V. Chu, and J. P. Conde, *Sens. Actuators B* **135**, 102 (2008).
- ⁵² A. T. Pereira, A. C. Pimentel, V. Chu, D. M. F. Prazeres, and J. P. Conde, *IEEE Sens. J.* **9**, 1282 (2009).
- ⁵³ A. T. Pereira, V. Chu, D. M. F. Prazeres, and J. P. Conde, *MRS Proceedings* **1191**, 73 (2009).
- ⁵⁴ S. S. Deshpande, *Enzyme Immunoassays: From Concept to Product Development* (Chapman and Hall, New York, 1996).
- ⁵⁵ J. P. Conde, A. Jóskowiak, B. Lipovšek, A. Pimentel, A. T. Pereira, M. Santos, J. Krč, M. Topič, D. M. F. Prazeres, and V. Chu, *Phys. Status Solidi C* **7**, 1156 (2010).
- ⁵⁶ M. Dandin, P. Abshire, and E. Smela, *Lab Chip* **7**, 955 (2007).
- ⁵⁷ J. Vickers, M. Caulum, and C. Henry, *Anal. Chem.* **78**, 7446 (2006).
- ⁵⁸ B. Lipovšek, A. Jóskowiak, J. Krč, M. Topič, D. M. F. Prazeres, V. Chu, and J. P. Conde, *Sens. Actuators, A* **163**, 96 (2010).
- ⁵⁹ H. Bruus, *Theoretical Microfluidics* (Oxford University Press, Oxford, 2008).
- ⁶⁰ D. Berk, F. Yuan, M. Leunig, and R. Jain, *Biophys. J.* **65**, 2428 (1993).
- ⁶¹ D. Wild, *The Immunoassay Handbook* (Elsevier, Amsterdam, The Netherlands, 2005).
- ⁶² E. Delamarche, in *Nanobiotechnology: Concepts, Applications and Perspectives*, edited by C. M. Niemeyer and C. A. Mirkin (Wiley, Weinheim, 2004), pp. 31–52.
- ⁶³ S. W. Hu, X. Q. Ren, M. Bachman, C. E. Sims, G. P. Li, and N. L. Allbritton, *Langmuir* **20**, 5569 (2004).
- ⁶⁴ W. C. Sung, C. C. Chang, H. Makamba, and S. H. Chen, *Anal. Chem.* **80**, 1529 (2008).
- ⁶⁵ L. Yu, C. M. Li, Y. S. Liu, J. Gao, W. Wang, and Y. Gan, *Lab Chip* **9**, 1243 (2009).