



Microfluidic biochip for chemiluminescent detection of allergen-specific antibodies

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ABSTRACT

Protein microarrays for allergen-specific antibodies detection were integrated in microfluidic chips, with imaging chemiluminescence as the analytical technique. This paper demonstrates the feasibility of miniaturized chemiluminescent ELISA by presenting rapid, reproducible and sensitive detection of protein antibodies using microfluidics.

Three different proteins, β -lactoglobulin, peanut lectin and human IgG were immobilized via a “macro-molecules to polydimethylsiloxane elastomer (PDMS) transfer” protocol and used as capturing agent for the detection of specific antibodies. A convenient and reversible procedure was used to bond the PDMS microarray substrate to complimentary SU-8/glass microfluidic reaction chambers.

The hydrodynamic behaviours of the three proteins interactions within the micro-chambers were investigated to select the most efficient flowing parameters (come to terms with the assay time and performances).

The use of optimized conditions led to the concomitant detection of three specific antibodies at pM level in 300 μ L and using 6 min sample incubation time.

Finally, sera from allergic patients were assayed using the microfluidic device modified with apple hazelnut and pollen allergen. The results obtained compared favourably with those obtained with the classical Pharmacia CAP system.

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1. Introduction

The increased demand for proteins analysis, both diagnostic and research targeted, has initiated the development of new tools in the technological field of protein microarrays (Angenendt, 2005). Immunoassays are the most widespread analytical technique, involving protein–target interactions, and used to monitor and to evaluate clinical diseases, biotechnological threats or allergic profiles. Among the employed techniques (Pang et al., 2005), well-established enzyme-linked immunosorbent assay (ELISA) tests in 96-well microtitre plates still remain the most common technique for the detection of antigen–antibody interactions because of

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their robustness, sensitivity and reproducibility (Elshal and McCoy, 2006). In order to increase the integration and parallelization of these protein assays and, lowering their cost and sample consumption, especially in the diagnostic field, research over the last decade has concentrated on the miniaturization of bio-analytical systems (Templin et al., 2002). Following the rapid expansion of miniaturization technologies (Samuel and Sia, 2003) and continued development of the micro total analysis system (μ TAS) concept (Manz et al., 1990), an intense work has been underway to produce microsystems integrating protein microarrays (Pawlak et al., 2002; Sia et al., 2004; Wassaf et al., 2006) composed of all the necessary fluid handling and transportation components.

The advantages of such microfluidic systems for protein biochips include low reagent volumes, low sample and energy consumption and large surface to volume ratios (Cesaro-Tadic et al., 2004; Luo et al., 2005) which could, depending of the flow rate, enable more efficient mass transport by reduction of the diffusion paths (Eteshola and Leckband, 2001; Myszkowski et al., 1997; Vijayendran et al., 2003).

Conventional two-dimensional microarray technologies – i.e. Affymetrix®, Agilent® and Luminex® – employ different

technological approaches to create a compact, regular array of probe sites immobilized to a solid-phase support, usually a silicon chip or a glass slide (Sundberg et al., 2000). Although these conventional two-dimensional arrays represent the current benchmark technology, polydimethylsiloxane elastomer (PDMS) has become an interesting candidate for protein immobilization, due to its wide use for microdevices fabrication (Dodge et al., 2006; Ebara et al., 2006; Goral et al., 2006).

Nevertheless, even though PDMS exhibits several advantages (transparency, low toxicity and soft lithography processing) (Lottersy et al., 1997) over other polymeric materials, major drawbacks exist when working with bare PDMS, these include: intrinsic highly hydrophobic properties (Efimenko et al., 2002), relative permeability to solvent (Duineveld et al., 2002; Efimenko et al., 2002; Muzzalupo et al., 1999) and bio-fouling tendency, leading to non-specific adsorption of proteins (Linder et al., 2001; Mata et al., 2005).

When used as a bio-interface for biochip and microarray development, the most disadvantageous property of the PDMS is its chemical inertness (Mata et al., 2005). This property has an adverse effect on the ability to directly immobilize biomolecules on PDMS substrate and several strategies have been proposed to introduce reactive chemical functions on PDMS surfaces to control immobilization of target analytes (Goda et al., 2006; Hu et al., 2002; Makamba et al., 2005; Mao et al., 2002; Papra et al., 2001; Wang et al., 2005).

In the last 4 years, our research group developed a novel and convenient method called “Macromolecules to PDMS Transfer” for PDMS surface patterning (Heyries et al., 2007; Marquette and Blum, 2004a,b; Marquette et al., 2005, 2006a,b).

Beads or proteins were spotted and dried on a 3D master, covered with Sylgard® 184, cured and recovered, after peeling off, as spots of beads or proteins entrapped at the surface of the bare PDMS.

Although the procedure seems drastic for proteins, such non-conventional biomolecule treatment was found to have no major negative consequence on their integrity (Heyries et al., 2007).

In this paper, we demonstrate the feasibility of miniaturized chemiluminescent immunosensors by combining this original protein–PDMS microarrays (Marquette et al., 2006a,b) with an integrated microfluidic system and a highly sensitive chemiluminescent imaging system (Fig. 1b).

Confirmation of concomitant protein detection using the present system was realized with three different proteins: two allergenic food proteins – i.e. peanut lectin and milk β -lactoglobulin – and one human immunoglobulin. The biomolecules were spotted and used to detect the corresponding specific antibodies. The influence of the fluidic parameters on the hydrodynamic behaviour of the biochip was first characterized and then the analytical potentialities of the system were demonstrated and discussed.

Finally, to demonstrate the potentialities of the developed system, we managed to immobilize concomitantly two different recombinant allergenic proteins (rMal d4 and rCor a1 from apple and hazelnut, respectively) and one allergen extract from *Dactylis glomerata* pollen. Sera from allergic patients were assayed using the microfluidic device and the results compared favourably with those obtained with the classical Pharmacia CAP system.

2. Materials and methods

2.1. Reagents

Arachis hypogaea lectin (from peanut), anti-*A. hypogaea* lectin antibodies developed in rabbit, bovine serum albumin (BSA),

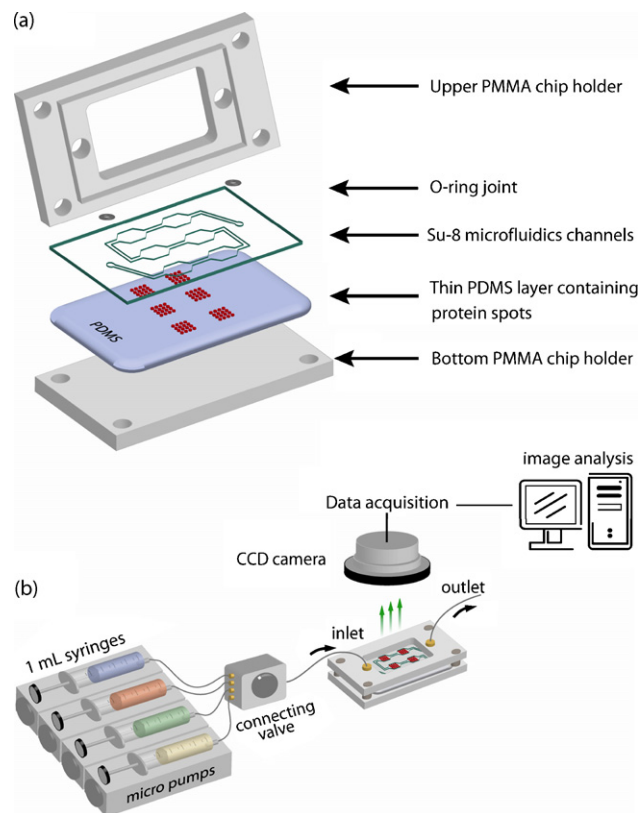


Fig. 1. (a) Detailed description of the microfluidic cartridge and (b) the complete micro-pumping and imaging detection systems.

human IgG, anti-human IgE (ϵ -chain specific) peroxidase-labelled and luminol (3-aminophthalhydrazide) were purchased from Sigma (France).

Peroxidase-labelled polyclonal anti-human IgG (A and M) antibodies developed in goat and peroxidase-labelled polyclonal anti-rabbit IgG(H + L) antibodies developed in mouse were supplied by Jackson Immuno Research (USA). PDMS precursor and curing agent (Sylgard® 184) were supplied by Dow Corning. All buffers and aqueous solutions were prepared with distilled, demineralized water.

Recombinant allergenic proteins rMal d4 (from apple, 1.22 mg/mL) and rCor a1 (from hazelnut, 0.55 mg/mL), and reactive sera were kindly provided by Dr. Stephan Scheurer (Paul Ehrlich Institute, Germany) whereas *D. glomerata* pollen extract and reactive serum was provided by Pr Gabriel Peltre (CNRS, France).

2.2. Protein immobilization procedure

The biochips were prepared by arraying 1.3 nL drops of spotting solutions with a BioChip Arrayer BCA1 (PerkinElmer). Spotting solutions were prepared, when not mentioned, with protein concentration of 1 mg/mL in 0.1 M carbonate buffer, pH 9. Each of the six arrays was composed of 16 spots (identical or not, diameter = 150 μ m, S.I. 1) that were deposited on the surface of a cleaned glass slide. After spotting, the drops were dried and the arrays were transferred to the PDMS interface, by pouring 2 mL of Sylgard® 184 (precursor and curing agent, 10:1) onto the glass substrate, and curing for 10 min at 100 °C.

Peeling off the PDMS polymer then completed the biochip preparation process within less than 30 min.

2.3. Microfabrication

The microfluidic channels of the microarray were fabricated in SU-8 polymer. The commercial NanoTM SU-8 50 polymer (Micro-Chem, MA, USA) was spin-coated and the customized microchannel pattern developed by photolithography techniques on a glass substrate. The resultant microchannel was 74- μ m deep and 400- μ m wide. At six locations, corresponding to the protein microarray areas, the microchannel widened to 2-mm wide reaction chambers. The access holes were mechanically drilled to allow fluidic access from the top of the microchannel.

Each chamber of the microfluidic system has a volume of 0.5 μ L and the entire volume of the system is 5 μ L. Thus, using a flow rate of 1 μ L/min leads to the replenishment of the microfluidic system in 5 min whereas at higher flow rates, 20 μ L/min, only 15 s will be required to achieve the chip filling.

2.4. On chip protein assays

Immediately after being peeled off, the protein-PDMS microarrays were aligned with the corresponding SU-8 microfluidic system and inserted in a PMMA chip holder (Fig. 1a). Sealing of the protein chip cartridge was performed by tightly and carefully screwing the plastic holder and the connection tubing (PTFE tube natural 0.3 mm in diameter with an Omnifit Gripper Fitting System).

Four 1-mL syringes, connected to a 6-way connection valve and regulated with a Bee Hive Controller (Baby Bee syringe drive, Bioanalytical Systems, Ltd.) were used to control flow of assay solutions within the reaction chamber (Fig. 1b). All the experiments were performed at room temperature (25 °C).

Before each experiment, a saturating solution of VBSTA (veronal buffer 30 mM, NaCl 0.2 M, pH 8.5 with addition of Tween-20 0.5% (v/v) and BSA 1% (w/v)), used to prevent non-specific binding, was pumped into the assembled biochip for 20 min at a flow rate of 5 μ L/min.

For concomitant detection of anti-lectin, anti- β -lactoglobulin and anti-human IgG, all the necessary proteins – i.e. specific antibodies and peroxidase-labelled secondary antibodies – were injected together and flowed in the microfluidic system after a pre-incubation step of 15 min at 37 °C.

Washing the excess reagents was achieved using a 20- μ L/min flow of VBS (veronal buffer 30 mM, NaCl 0.2 M, pH 8.5) for 20 min.

At that time, the chip was ready to be measured and the microfluidic system was filled with the chemiluminescence measuring solution (VBS added of 220 μ M of luminol, 200 μ M of *p*-iodophenol and 500 μ M of hydrogen peroxide). The biochip was immediately placed in the CCD camera (Las-1000 Plus, Intelligent Dark Box II, FUJIFILM) measurement chamber for light integration during 10 min. Quantification of the signal was achieved using a FUJIFILM image analysis program (Image Gauge 4.0).

3. Results and discussion

We recently developed a new approach (Heyries et al., 2007) to directly modify Sylgard[®] 184 surface with spots of protein and demonstrated its use for microarray-based immunoassays.

The mechanisms involved in this direct protein immobilization have been investigated and experimental results suggested that both chemical bounding and moulding effect were implicated (Heyries et al., 2007). Chemical bounding between the protein and the PDMS elastomer structure was believed to be mainly due to a poisoning of the Kardstedt catalyst used during the polymer curing. This poisoning was demonstrated to be related, with reference to previous works (Bartzoka et al., 1998; Perutz et al., 1998; Quirk

et al., 2005), to the presence of free amino or thiol groups in the immobilized molecules.

The method consists of a direct transfer of dried protein/salt solutions to the PDMS interface during the polymer curing. Such non-conventional treatment of proteins was found to have no major negative consequence on their integrity (Heyries et al., 2007; Quist et al., 2005).

3.1. System suitability for biochip development

In the present work, protein biochip microsystems were realized by coupling bare PDMS supporting matrices of protein spots with a customized SU-8 microfluidic chip inserted in a complementary plastic chip holder to ensure reproducible alignment of microfluidic inter-connects for enhanced introduction of sample and reagents.

Six sequential reaction chambers (Fig. 2) were carefully designed to enable uniform distribution of sample flow over the entire area of the protein array. Modelling of the microchip geometry with Coventor Software (Coventor Inc., NC, USA) provided an assessment of the optimal design and necessary alignment for efficient fluid transfer. Simulations were conducted for each flow rate used in the experiments. Optimal flow transport was evidenced within the entire reaction chambers (examples are shown in Fig. 2a–c).

The homogeneity of the sample distribution within the microfluidic chambers was observed experimentally through the interaction of peroxidase-labelled anti-human IgG onto a biochip composed of 6 identical microarrays of 16 spots of human IgG. An incubation protocol with continuous flowing of the chemiluminescent assay reagents (i.e. saturation solution, antibodies solution and washing solution) was performed to ensure a simplified and reproducible experimental procedure.

The chemiluminescent image obtained is shown in Fig. 2d. The observed signal variation R.S.D. was 9.5% for the 96 measured spots, which confirmed the homogeneous distribution of liquid within the microfluidic cartridge system during the incubation of antibodies.

The absence of non-specific binding within this microsystem was also confirmed by the discrete spots chemiluminescent signals obtained (Fig. 2d). In this respect, no other measurable signal was achieved except for the individual 150 μ m protein spots. Interference signals or non-specific adsorption did not occur either on the glass or SU-8 surfaces of the microfluidic channels or the micro-chambers walls. These observations confirm that pre-incubation with a saturation solution (Tween-20 0.5% (v/v) and BSA 1% (w/v)), inside the chip, at a flow rate of 5 μ L/min prevented any non-specific binding, even at antibody concentrations higher than 1 μ g/mL.

3.2. Optimization of the fluidic parameters

The present system, characterized by its low non-specific binding and its interesting homogeneous distribution of the injected reagent was then applied to the concomitant detection of three anti-protein (human IgG, peanut lectin and β -lactoglobulin) antibodies.

In order to decrease the time allocated to the incubation of the different antibodies – i.e. specific and secondary – a pre-mixing approach was chosen. Consequently, anti-human IgG, anti-peanut lectin, anti- β -lactoglobulin and peroxidase-labelled anti-species antibodies were mixed together (Fig. 3) and incubated 15 min at 37 °C prior their injection in the microsystem.

The hydrodynamic behaviour of the proteins interactions within the micro-chambers were studied with microarrays of immobilized peanut lectin, β -lactoglobulin and human IgG. The influence

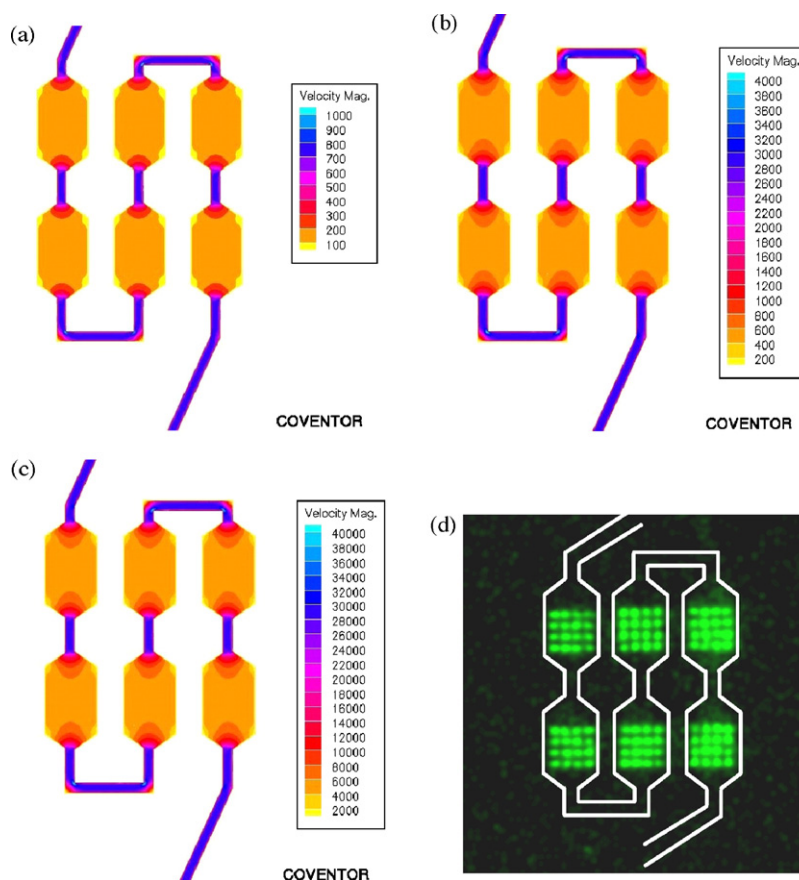


Fig. 2. Computer simulations with Coventorware® software. (a) Flow rate: 1.25 µL/min. (b) Flow rate: 5 µL/min. (c) Flow rate: 50 µL/min (velocity magnitude is µm/s). (d) Chemiluminescent image of a homogeneous human IgG biochip (peroxidase-labelled anti-human IgG: 1 µg/mL, flow rate: 0.5 µL/min, 30 min).

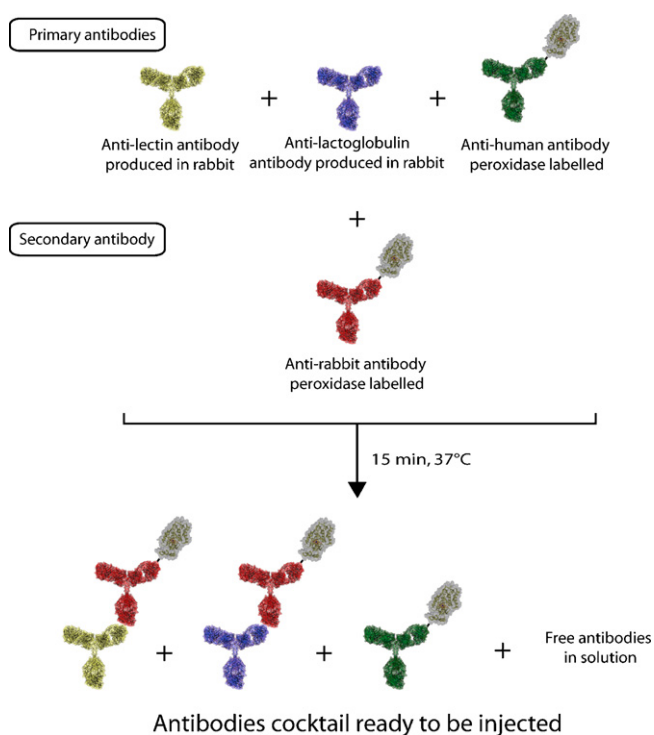


Fig. 3. Strategy for the pre-incubation of primary and secondary antibodies cocktail before injection in the microsystem. Primary and secondary antibodies were incubated 15 min at 37 °C together prior injection in the chip.

of the injected volume, the incubation time and the flow rate on the chemiluminescent signal obtained after each interaction was assessed at a constant concentration of injected antibodies (1 µg/mL each).

Fig. 4a and b presents the variations of the chemiluminescent signals with the incubation flow rate, obtained for each protein interaction, at either constant incubation time (60 min) or constant incubation volume (300 µL), respectively. Such parameters are of critical importance since they are conditioning the operating conditions and subsequent performance of the biochip with respect to sensitivity and limit of detection.

As shown in Fig. 4a, increasing the injection flow rate at constant injection time (i.e. increasing the injected volume), from 0.8 µL/min to 20 µL/min, induced a fivefold increase of the observed chemiluminescent signal. The binding kinetic, observed for each protein, suggests that a higher number of interactions occurred between the immobilized proteins and the injected antibodies.

This observation is in good agreement with a mass transport limitation model. Indeed, if diffusion is the only mechanism for transporting the target toward the sensor surface, replenishment of target near the sensor surface following binding of target to immobilized capture molecules is subject to mass transport limitations (Golden et al., 2007). Thus, flowing sample at sufficiently high flow rates over the sensor surface can minimize the diffusion limitation (Myszka et al., 1997; Vijayendran et al., 2003) and then increase the analytical signal.

At higher flow rate, a decrease of the chemiluminescent signal occurs, related to a reduce number of interactions. At these high flow rates (above 20 µL/min) the system starts then to be driven by a reaction-limited model.

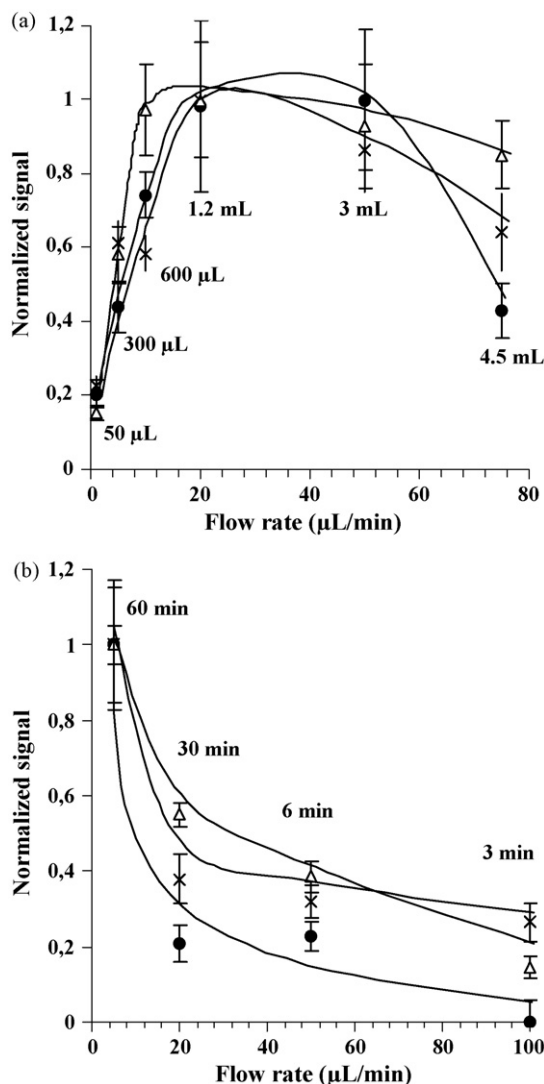


Fig. 4. Influence of the flow rate used during the antibodies cocktail injection. Two sets of experiments were carried out at room temperature (25 °C). (a) Using constant 1 h incubation, different flow rates were applied, modifying the injected volume in the microfluidic system or (b) injecting a constant 300 µL volume varying the flow rate, and thus the incubation time. (●) Peanut lectin, (×) human IgG and (Δ) β-lactoglobulin.

Table 1
Analytical performances of the chemiluminescent protein biochip

System characteristics	Analytical performances (detection range)
60 min 50 µL flow rate: 0.8 µL/min	Peanut lectin: 66 pM–6.6 nM, β-lactoglobulin: 6.6 pM–12 nM and human IgG: 66 pM–30 nM
6 min 300 µL flow rate: 50 µL/min	Peanut lectin: 33 pM–3.3 nM, β-lactoglobulin: 6.6 pM–3.3 nM and human IgG: 66 pM–3.3 nM
60 min 1.2 mL flow rate: 20 µL/min	Peanut lectin: 6.6 pM–3.3 nM, β-lactoglobulin: 0.6 pM–3.3 nM and human IgG: 6.6 pM–3.3 nM
ELISA assay 60-min incubation 50 µL	Peanut lectin: 6.6 pM–6.6 nM, β-lactoglobulin: 0.6–666 pM and human IgG: 6.6 pM–6.6 nM

ELISA assays used for comparison with the microfluidic chip were performed using the same incubation times and antibodies concentrations at 37 °C. Chemiluminescent signal was recorded with a CCD camera for the microfluidic system whereas a dedicated luminometer was used for the ELISA assays.

Each of the three proteins studied here exhibited similar responses to flow rate changes. This observation is consistent with the homogeneous protein structure and mass (antibodies) which is responsible for similar diffusion coefficient ($\sim 10^{-7}$ cm²/s) (Jøssang et al., 1988) and the homogeneous dissociation constants determined for each of the different protein interaction (S.I. 2).

The analytical performances of the system were evaluated at two critical flow rates, the lower (0.8 µL/min) and the optimum (20 µL/min) (Table 1). As expected, when using a constant injection time while increasing the injection flow rate, more sensitive protein detections were achieved, with a typical one order of magnitude decreasing of each detection limit.

For each injection flow rate used, the value of the injected volume is indicated in Fig. 4a. These experimental parameters indicate that the required sample volume drastically increases, from 50 µL to 1.2 mL, when using the optimum flow rate. Such injected volume does not fulfil the requirements of lowering the time and the chemical consumption of the assay. Indeed, for rapid diagnostic testing, usually performed on capillary blood, the available sample volume is in the 5–100 µL range. To account for the necessary dilution of the injected sample, a maximum injection volume of 300 µL should be considered. This volume was used to assess the influence of the injection time within the microfluidic biochip.

Injecting a constant volume through the microfluidic channel at variable flow rates caused a corresponding difference in incubation time (indicated in Fig. 4b). The results obtained for the concomitant injection of the three different antibodies in the system are displayed in Fig. 4b.

As the flow rate increases, i.e. the incubation time decreases, the chemiluminescent signal rapidly diminishes, in accordance with the expected mass transport limitations (Kusnezow et al., 2005; Zimmermann et al., 2005).

Interestingly, for a 50 µL/min flow rate (6 min incubation time), the chemiluminescent signal obtained for the three proteins was still 40% of the maximum signal whereas the incubation time was one order of magnitude lower. Despite the fact that such a 50-µL/min flow rate remains very high, this observation led us to consider the analytical performances of the microfluidic system using such conditions.

As can be seen in Table 1, detection limits of 33 pM, 6.6 pM and 66 pM were found for anti-peanut lectin, anti-β-lactoglobulin and anti-human IgG, respectively. These results compare well with the detection limits obtained using 60 min incubation at a flow rate of 0.8 µL/min (an injected volume of 50 µL). Thus, in the present three proteins system, and according to the fluidic optimization curves (Fig. 4a and b), the flowing of a 6-time larger volume during a 10-time lower time leads to unchanged performances.

These results were compared to standard microtitre plate-based chemiluminescent assays (Table 1) performed with similar volumes and times, but under static conditions. The performances of the present optimized microfluidic assay (6 min, 300 µL) were found to be acceptable, with a lowering of the detection limit of only one order of magnitude when compared to the standard assay onto microtitre plate.

3.3. Type E immunoglobulins detection in human serum sample

In order to demonstrate the usefulness of the proposed microfluidic device, real biological samples were tested for allergy diagnosis. Two different recombinant proteins, rMal d4 and rCor a1 from apple and hazelnut, respectively, together with a pollen extract from *D. glomerata* were immobilized at the PDMS–air interface using the “macromolecules to PDMS transfer” procedure. These allergenic proteins are expected to be good candidates for allergy diagnosis (Lauer et al., 2004; Son et al., 1999) in human sera.

Table 2

Analytical performances of the microfluidic chip using human sera samples diluted 10 times

Serum reference	CAP system assay	Microfluidic assay results
PEI 17	Class 3 against hazelnut extracts (rCor a1 protein)	Positive
PEI 131	Class 4 against apple extracts (rMal d4 protein)	Positive
CNRS 17	Class 6 against <i>D. glomerata</i> pollen extracts	Positive
Control (non-allergic patient serum)	Not reactive	Negative

Samples were considered positive when the measured signal was at least three times the standard deviation of the assay.

Three different sera, each reactive toward one of the three immobilized allergenic proteins, were assayed using the microfluidic biochip. As biological sample consumption is of critical importance, only 5 μ L of the initial serum were used and diluted 10 times in the saturating buffer before injection in the chip. During the experiment, 45 μ L of the diluted sera were flown through the chip for 30 min at room temperature with a flow rate of 1.5 μ L/min. This flow rate was chosen as a compromise between time consumption of the assay and the necessary residence time of the sample onto the arrays. Peroxidase-labelled anti-IgE antibodies were then flowed for 30 min but at a higher flow rate of 20 μ L/min (optimized flow rate) and the chemiluminescent signals emitted from the microfluidic chip measured for 10 min.

The results obtained were in good agreement with the sera reactivity displayed by the standard Pharmacia CAP system (Table 2). For each serum, the expected specific reactivity (CAP system) was recovered using our microfluidic system without any cross-reactivity between the sensing layers. As a control experiment, a non-allergic patient serum (regarding the tested allergens) was also assayed for the quantification of the non-specific interactions of the sera with both the immobilized proteins and the biochip material. As a proof of the very low non-specific adsorption of proteins within our PDMS microfluidic system, no detectable signal was obtained using this control serum.

4. Conclusions

The present immobilization method through the direct entrapment of protein at the PDMS interface enabled the rapid prototyping of protein biochips. Indeed, PDMS surfaces modified with protein spots were produced in 30 min and using low amounts of PDMS polymer and proteins.

We demonstrated herein the integration of an original protein immobilization technique within a microfluidic system. The integrated microfluidic chip enabled reproducible and concomitant chemiluminescent detection of anti- β -lactoglobulin, anti-peanut lectin and anti-human IgG antibodies. The detection limits obtained, using sample flowing time within the system as low as 6 min, compared well with those obtained with standard ELISA on microtitre plate.

The potential of the actual method was emphasized through its comparison to the high standard CAP method for allergy diagnostic. As a matter of fact, down to class 3 samples (corresponding to the average reactivity level of allergenic patient) were detected using the proposed system.

A fully automated and integrated microfluidic device for allergy diagnosis is being developed and we believe that the presented procedure will become an interesting alternative for various macromolecules immobilization in PDMS for biochip developments. Increasing the number of analytical parameters within the pre-

sented system, through the immobilization of a larger number of allergen proteins, and systematic quantification of specific IgE antibodies in human sera samples for allergy diagnosis are tasks currently under study.

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Appendix A. Supplementary data

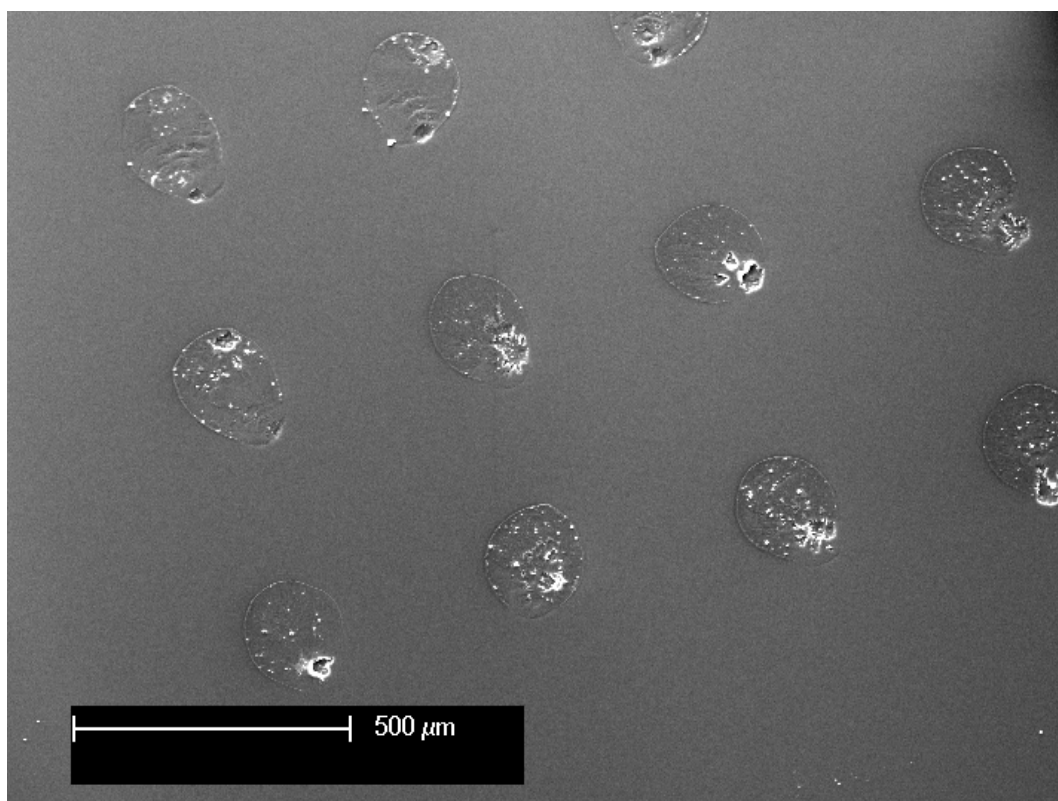
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.02.025.

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Electronic supplementary material 1



SEM image of a proteins microarray at the surface of the PDMS.

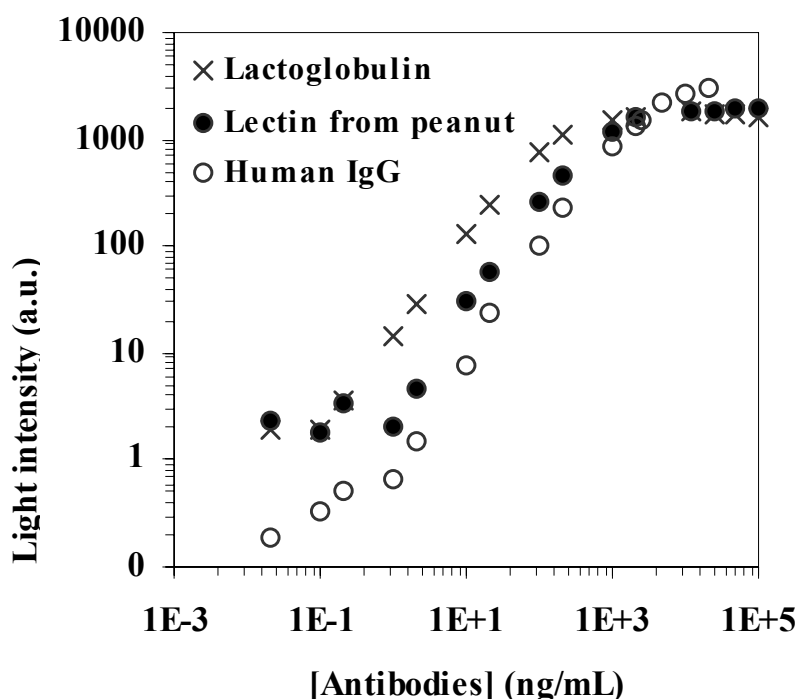
Electronic supplementary material 2

Kd determination assay protocol

Lectin from peanut, β -lactoglobulin and human immunoglobulin proteins were dissolved in carbonate buffer (Na_2CO_3) 0.1 M pH 9 at a concentration of 50 $\mu\text{g/mL}$. 50 μL of each protein solutions were coated in a 96-wells micotiter plate (White Nunc MaxisorpTM) for 3 hours at 37°C. After three washing with 150 μL of VBS, coated wells were saturated with 100 μL of VBSTA for 1 hour at 37°C. Afterwards, 50 μL of primary anti-lectin and anti- β -lactoglobulin antibodies diluted in VBSTA at various concentrations were incubated for 1 hour at 37°C. The wells were then washed three times with 150 μL of VBS. Primary anti-human peroxidase labelled antibodies at various concentrations and secondary anti-rabbit peroxidase labelled at a concentration of 1 $\mu\text{g/mL}$ were incubated for 1 hour at 37°C before being washed three times with 150 μL of VBS. Finally the microtiter plate was introduced in a luminescent plate reader (Labsystem) and light emission was recorded after injection of 100 μL of VBS containing in addition 200 μM of luminol, 500 μM of hydrogen peroxide and 200 μM of p-iodophenol.

Sigma plot software using dedicated fitting curve equations was used to estimate each antigen-antibody Kd.

	Estimated Kd (nM)
Lectin from peanut	4.2
Human IgG	23.5
β -lactoglobulin	0.78



ELISA curves used to estimate the Kd of the involved antibodies.