Micropillar array chips toward new immunodiagnosis†

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In this paper, we demonstrate the possibility to use a micropillar array to perform molecular immunodiagnosis. A polydimethylsiloxane (PDMS) microdevice consisting of a rectangular array of micropillars (45 μm in height, 100 × 100 μm square cross section) was used to replace microchannels or gels (polyacrylamide or agarose) to perform electrokinetic separation. This microarray was used to mimic highly diluted gel and to maintain electrolyte within the pillar zone by capillary effect. The electrolyte composition (glycerol and agarose content) was investigated in order to improve protein separation by isoelectric focusing (IEF). The influence of glycerol on focusing time and on the different evaporative contributions was further evaluated. In order to perform an immunodiagnostic of milk allergy, different surface treatments were optimized to prevent milk allergen adsorption on PDMS surface. Poly(dimethylacrylamide)-*co*-allyl glycidyl ether (PDMA-AGE) as well as gelatin led to a satisfactory signal to noise ratio. Finally the possibility to perform protein mixture separation using this micropillar array chip followed by immunoblotting was demonstrated by using the serum from an allergic individual, confirming the great potential of this analytical platform in the field of immunodiagnosis.

Introduction

Almost 30% of the population in western countries is affected by allergy. In developed regions, cow's milk allergy is now suspected to affect at least 10% of infants, and has been confirmed in more than 5% of children.[1–3] Besides classical gastrointestinal, respiratory and cutaneous symptoms, dramatic and extreme situations can eventually be encountered such as anaphylactic shock. Allergy is an "abnormal" immune reaction due to sensitization to molecules (mainly proteins) called allergens. In a first step the exposure to allergens may lead to immunoglobulin E (IgE) production. In further contact with these specific allergens, mast cells and basophiles may be activated and release inflammatory and immune mediators leading to allergic symptoms. IgE concentration can thus be considered as an excellent marker of allergy. Besides the clinical examination (*in vivo*), allergy diagnosis can be performed *in vitro*. The most common test is based on the enzyme-linked immunosorbent assay (ELISA) to determine IgE specificity and concentration. Commercial *in vitro* tests such as ImmunoCap® can be performed within 3 h using about 50 μL of serum for the detection of IgE to one single allergen, or to a given mixture or crude extract of an allergenic source. Nevertheless some limitations can be mentioned especially in the case of haptens, minor or non-hydrosoluble allergens. Finally, there is also an *ex-vivo* approach called Basophile Activation Test (BAT) involving cells. Basophiles from allergic patients are incubated in the presence of allergens and may release mediators that can be detected by cytometry. This technique is time-consuming, expensive to run and requires the presence of the patient to be tested. So there is still space for new *in vitro* immunoassays dedicated to allergy diagnosis that should detect antibodies with high sensitivity, specificity, high-throughput using only low patient blood and reagent volumes.

The miniaturization and integration of analytical and bioanalytical procedures on microdevices, also called μ-TAS, is considered as a relevant answer to this challenge.[4–9] Indeed, the use of microchips should decrease analysis time as well as sample and reagent volume. A first step toward miniaturization in allergy diagnosis has already been reached with microarray technologies. Fall *et al.* have shown that this technology is suitable for specific IgE measurements using glass slides activated and allergen extracts or recombinant allergens. Chemiluminescence detection allows achieving rather low limit of detection of IgE ranging from 0.16 to 1.9 ng mL^{-1}.[10] Cretich *et al.* have improved microarray sensitivity using a crystalline silicon substrate coated with thermal silicon oxide functionalized by a polymeric coating.[11] Combining reflective substrate and specific surface chemistry allows a great improvement of the microarray performance. An approach based on magnetophoretic immunoassay has been recently developed. The IgE measurement is based on the magnetophoretic deflection velocity of microbeads that is proportional to the number of associated magnetic nanoparticles under magnetic field in the microchannel.[12] Despite the low limit of detection obtained, this method remains complex and laborious. In parallel, there have been many papers about miniaturization of immunoassays but most of them deal with capillary electrophoresis miniaturization.[13,14] In cow milk allergy diagnosis context, Busnel *et al.* have developed a method

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combining magnetic beads based immunoaffinity to capillary electrophoretic separation.[15] This fast and automated method requires a minimal sample volume. Nevertheless, it is dedicated to total IgE measurement and not to the specific ones. Kitamori *et al.* have introduced a bead-bed immunoassay system that was structured in a microdevice to determine carcinoembryonic antigen (CEA).[16] They used polystyrene beads grafted with antibody and they demonstrated that the reduction of the diffusion distance as well as the increase of the specific interface allows a drastic decrease in the time required to complete an assay. These investigations were continued using this micro-ELISA system for sensitive and rapid allergy diagnosis.[17] Due to the immobilization process, only water-soluble allergens can be grafted onto the beads. The correlation between micro-ELISA and conventional ImmunoCap system shows a good agreement using human serum of 85 patients. These papers have evidenced the potential of microdevices for allergy diagnosis. However, most of them require a preliminary step of allergen grafting and are limited to water-soluble allergens. In the 80s, Peltre *et al.* introduced immunoblotting for allergy studies.[18] It allows to study IgE binding from a great number of patient sera and to compare their individual ability to recognize molecular allergens. Immunoblotting has been first used in a view of allergen characterization[19] and standardization. Concerning cow's milk allergy, the role of different cow's milk proteins in the pathogenesis is still controversial and a change in prevalence of patient sensitization to different milk proteins has been observed during the last few years. Natale *et al.* have thus used two-dimensional immunoblotting and mass spectrometry to identify cow's milk allergens.[20] More recently Righetti *et al.* have shown that combining combinatorial peptide ligand libraries and the coupling of gel IEF to immunoblotting with sera of allergic patients allowed the identification of new minor allergens.[21] These papers have confirmed the usefulness of immunoblotting especially in order to elucidate allergy mechanisms, to prepare new therapeutic treatments as well as to improve molecular diagnosis.

In a previous paper, we have developed a PDMS micropillar array dedicated to electrokinetic separation.[22] The geometry of this uncovered microdevice has been optimized to limit evaporation and to achieve satisfying separation by isoelectric focusing (IEF). This device has shown the ability to perform model protein separation with resolving power similar to mini-gels, but with shorter analysis time and reusability. In this work, the potential of this micropillar array has been investigated to perform molecular immunodiagnosis of allergy. For that purpose we have evaluated its ability to be used for allergen separation coupled to immunoblotting. The influence of agarose and glycerol content on protein separation was studied. Working with uncovered microchip necessitates taking evaporation into account. The role of glycerol in evaporative process has thus been studied. Finally, to work on cow's milk allergy requires a drastic PDMS surface treatment to avoid non specific allergen adsorption. Different surface treatments were developed and characterized to avoid major whey milk allergen (β-lactoglobulin) adsorption on the microdevice. Human sera of patients allergic to cow's milk, especially to β-lactoglobulin, have been tested and the results obtained demonstrated the great potential of this microchip for molecular diagnosis.

Methods and materials

Device microfabrication

The PDMS microdevice, which consists of a rectangular zone of PDMS micropillars protruding on a PDMS block, was fabricated as described in the previous paper.[22] A mixture of base polymer and curing agent in mass ratio of 10: 1, called PDMS prepolymer, was poured into a designed master fabricated by photolithography. Then the master filled with the liquid prepolymer was placed in a vacuum desiccator for degassing. The mixture was cured at 75 °C for 1 h. After cooling down to room temperature, the PDMS was peeled from the master, yielding a PDMS chip with micropillars protruding on it.

IEF in conventional mini-gels

To compare the IEF performances of the micropillar array chip with those of conventional gels, the protein β-lactoglobulin A from bovine milk, (Sigma, St Louis, USA) has been focused in the polyacrylamide mini-gel (PhastGel, Dry IEF, GE Healthcare) with the electrophoretic apparatus Phast System (GE Healthcare). The separations are performed with a Phast system apparatus comprising a flat Peltier cooling plate in an enclosed and water saturated chamber. The Phast system is set at 15 °C to limit evaporation and permits an efficient Joule heating dissipation. The gel was reswollen overnight with an aqueous solution of 5% (v/v) carrier ampholytes Servalyt 4–6 (Serva, Germany). The IEF was carried out at a constant temperature of 15 °C, using a stepwise gradient of electric field. Prefocusing of the ampholytes was done for 20 min at 250 V maximum. IEF marker proteins from BDH ranging from 2.4 to 5.65 were used as standards. The sample and the pI markers were applied for 15 min at 1.2 mA. The separation was then allowed to run for 20 more min. The sample, β-lactoglobulin, was loaded with the automatic sample applicator comb for 8 lanes at a concentration of 1.8 μg for one μL and for one single lane. After focusing, proteins were transferred onto a nitrocellulose membrane for immunoblotting.

IEF on micropillar chips

As previously described the Phast System was cooled down to 15 °C, and wet paper (Whatman n°1) was put inside the separation chamber to saturate it for the duration of the IEF separation. The PDMS micropillar chip was put into an oxygen plasma cleaner and the plasma treatment was performed at 300 mTorr chamber pressure for 30 s[7,8] just before being filled with the electrolyte.

A mixture of proteins were used as pI markers in IEF ranging from pI 4.45~9.6 (from Bio-Rad, USA) to be focused on bare micropillar chips. The electrolyte solution contained 5% (v/v) carrier ampholytes Servalyt 2–11 (Serva Electrophoresis GmbH, Heidelberg, Germany), 30% (v/v) glycerol and 0.2% (w/v) agarose (Agarose IEF, Pharmacia, Sweden) in water. 3 μL of either pI markers or the sample were mixed with the 7 μL electrolyte solution mentioned above. Then 7.25 μL of this mixture was pipetted at one end and the micropillar area (5 cm length, 3.9 mm width) was then filled by capillarity.

Before running an IEF separation, the micropillar chip was pre-treated with coating materials. For poly(dimethylacrylamide-*co*-allyl glycidyl ether) PDMA-AGE (kindly provided by Dr Marcella Chiari, CNR, Milano, Italy) coating, 0.1 M NaOH solution was filled into the micropillar zone and the chip was covered and kept at 4 °C for 10 min. Then the chip was rinsed with H_2O and dried by compressed air. 0.1% (w/v) PDMA-AGE aqueous solution was filled into the micropillar zone and the chip was covered and kept at 4 °C for another 10 min. After being rinsed with H_2O and dried by compressed air, the chip was loaded with 1.8 μg β-lactoglobulin mixed with electrolyte solution in ratio of 1:4 (v/v). The electrolyte solution for PDMA-AGE coated chip contained 2% (v/v) carrier ampholytes (pI 4–6), 37.5% (v/v) glycerol, 0.25% (w/v) agarose and 0.02% (w/v) PDMA-AGE.

For gelatin coating, the plasma treated PMDS chip was immersed in 0.1% gelatine from swine (Labosi, France) solution at room temperature for 2.5 h. After being rinsed with H_2O and dried by compressed air, the chip was loaded with 1.8 μg β-lactoglobulin A, mixed with electrolyte solution in a ratio of 1:4 (v/v). The electrolyte solution for gelatin coated chip contained 2.5% (v/v) carrier ampholytes (pI 4–6), 37.5% (v/v) glycerol and 0.25% (w/v) agarose.

Focusing was carried out under an electric field of 250 V cm^{-1} for 1 min and then 500 V cm^{-1} for 20 min. The focused protein bands were finally transferred onto a nitrocellulose membrane.

Immunoblotting

A piece of nitrocellulose membrane activated with cyanogen bromide (CNBr)[9] was placed onto the surface of the gel or the micropillar zone and three sheets of Whatman n°1 paper were put on top of them. Then a uniform weight of 150 g was added for 1 h. The membrane was then lifted off from the gel or micropillar zone surface and dried by exposure to a fan for 15 min. The membrane was blocked with PBS-0.3% Tween 20. When commercial antibodies were used, the membrane was incubated with the primary antibody solution, *anti*-β-lactoglobulin polyclonal antibody solution (antibodies produced in rabbit, 1000 times diluted with PBS-0.1% Tween 20), for 1 h and washed 3 times for 10 min with the NaCl-0.1% Tween 20 solution. Then the membrane was incubated with the secondary antibody solution, an alkaline phosphatase-labeled anti-rabbit IgG (Sigma, Germany), 5000 times diluted with PBS-0.1% Tween 20, for 1 h, followed by 3 times washing for 10 min with the NaCl-0.1% Tween 20 solution to limit the non specific adsorption of antibodies on the membrane. Finally, the membrane was stained as incubated with the substrate mixture, BCIP (5-bromo-4-chloro-3-Indolyl phosphate, Sigma) and NBT (nitro blue tetrazolium chloride, Sigma) solution following the manufacturer instructions. When IgE binding from an allergic patient serum was analyzed, the membrane was incubated with patient serum (diluted 10 times with PBS-0.1%Tween 20) as the primary antibody solution, overnight at room temperature, then incubated with Alkaline Phosphatase-labeled *anti*-human IgE antibody (ε-chain specific, produced in goat, Sigma) solution diluted 700 times with PBS-0.1%Tween 20 as secondary antibody solution for 1 h and finally stained as described previously. Three washes were performed between each incubation step. The immunoblots were scanned (GE Healthcare scanner) and processed *via* the GeneTools software (Synege, Cambridge UK) into an electropherogram.

Results and discussion

Optimisation of electrolyte composition

In a previous paper, we demonstrated the ability of a micropillar array to be used to perform electrokinetic separation of model proteins. This microdevice is an array of square pillars (45 μm height, 100 μm side) with square packing and an interpillar distance of 100 μm (Fig. 1). The array layout has been optimized in a previous paper.[22] The pillar height has been set at 45 μm as it correspond to the thickness of the thinner agarose gel casting we have previously performed.[23] Lower height has not been evaluated as decreasing pillar height involves a decrease of electrolyte volume and evaporation will be more critical using lower electrolyte volume. Pillar arrangement (square or triangular arrangement), pillar shape (cylindrical or square pillar) as well as pillar width and pillar distance have been studied. Comsol simulation of the isopotential lines, pI marker separation and evaporation evaluation have been used as critical parameters to design the optimal pillar layout. Nevertheless, in the case of real world samples, special attention has to be paid to the electrolyte composition in order to achieve a high resolution separation. Thus, in this paper, the influence of two additives on protein separation by isoelectric focusing was evaluated in a micropillar array. Agarose has first been investigated. This polysaccharide obtained from agar is commonly used for different life science applications, especially for gel electrophoresis as an alternative to polyacrylamide.[18,24] In parallel, glycerol has also been investigated. Glycerol has been chosen because of its ability to decrease electroosmotic flow.[25] Moreover, it has been demonstrated that glycerol leads to better solubilization of proteins and thus limits their adsorption onto solid surface.[26–28] In addition, glycerol can stabilize protein structures to maintain protein functions.

Agarose is a thermoreversible hydrogel. In this study, it has been used at a concentration ranging from 0.1 to 0.3% (w/v). Ogston and De Gennes have predicted agarose gel pore size according to the gel concentration, and atomic force microscopy confirmed the variation of pore size with agarose concentration.[29] We are thus able to estimate in our conditions a pore size higher than 400 nm. In these conditions, sieving mechanisms can be neglected and the diluted agarose gel will act to increase the electrolyte viscosity. Consequently the diffusion coefficient and the electroosmotic flow will decrease limiting cathodic drift.[30] Higher concentration has not been used to avoid

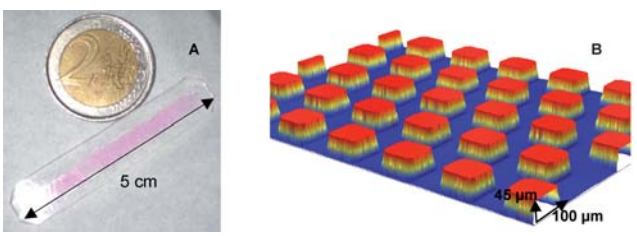

Fig. 1 **(A)** Picture of micropillar array filled by a coloured solution. **(B)** Profilometer picture of micropillar array.

difficulties related to pillar filling. The influence of agarose concentration at a given glycerol percentage on protein separation in micropillar array is shown in Fig. 2A. In the absence of agarose, no separation was achieved between myoglobin and hemoglobin proteins (brown bands) whereas increasing the agarose percentage improved resolution; three brown bands could be distinguished. The results evidenced that the presence of agarose even at low concentration was crucial to improve resolution. For IEF separation, the resolving power can be expressed by ΔpI the difference in pI of two analytes for separation:

$$\Delta(pI) = 3\sqrt{\frac{D(dpH/dx}{E(-d\mu/dpH)}}$$

Where D is the analyte diffusion coefficient, E the electric field, dμ/dpH is the analyte mobility derivative relative to pH and dpH/dx is the pH derivative relative to the distance x. It can thus be deduced that higher resolution will be achieved for analytes presenting low diffusion coefficient and high mobility slope at their isoelectric point. Agarose, by increasing electrolyte viscosity, should certainly decrease protein diffusion coefficients, leading to this increase in resolution. Finally agarose percentage of 0.1% (w/v) was sufficient to separate all the proteins, even those two differing by only 0.1 pI unit.

Thereafter the influence of glycerol has been evaluated using electrolyte containing 0.1% (w/v) agarose. Fig. 2B presents the influence of glycerol content on protein separation. As expected, higher resolution was obtained with the higher glycerol percentages. In the absence of glycerol, the band distortion is stronger and the separation of myoglobin and hemoglobin is not resolved. Whereas an increase of glycerol percentage allows

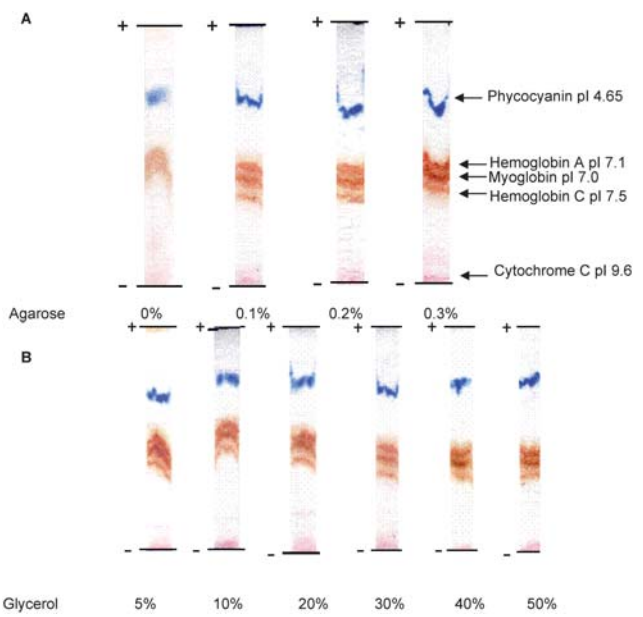

Fig. 2 Influence of **(A)** agarose percentage (w/v) and **(B)** glycerol percentage (v/v) on model protein resolution. IEF separation of model protein using PDMS micropillar array. **(A)** Electrolyte: 30% (v/v) glycerol and agarose at various percentages, **(B)** glycerol at various percentages and 0.1% (v/v) agarose, 5% (v/v) ampholytes, 571 V cm^{-1} for 21 min, temperature 15 °C.

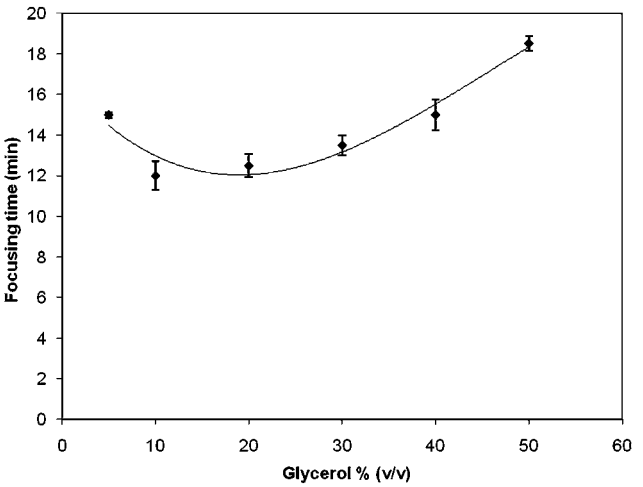

Fig. 3 Influence of glycerol content on focusing time using micropillar array IEF separation of model protein using PDMS micropillar array. Electrolyte: 0.1% (v/v) agarose, glycerol at various percentages, 5% (v/v) ampholytes, 571 V cm^{-1}, temperature 15 °C. Three repetitions.

a satisfactory separation of these proteins especially at 30% of glycerol since three brown bands can be well distinguished. In parallel, due to its high viscosity, varying glycerol percentage should also influence the focusing time. The proteins are considered as focused when the band location and its width remain constant. In order to discuss further the contribution of glycerol content on the focusing mechanism, focusing time is reported as a function of glycerol content in Fig. 3. In parallel, the variation of water/glycerol mixture viscosity has been measured elsewhere demonstrating that the viscosity of this binary mixture increases continuously with glycerol content.[25] Given that the analyte mobility is related to the inverse of electrolyte viscosity and assuming that the eventual temperature increase due to heat dissipation by Joule effect was negligible, it can be expected that the focusing time should increase continuously as a function of glycerol percentage. Nevertheless, the results obtained using our microdevice were quite different: for low glycerol percentages (Gly < 20%), focusing time decreased with glycerol content whereas for high content (Gly > 20%), an opposite trend was observed. Finally the minimal focusing time was obtained with glycerol content of about 20% (v/v).

In order to investigate further the mechanisms involved, capillary isoelectric focusing (CIEF) experiments were performed with a mixture of model protein and the influence of glycerol content on focusing was studied. For this purpose, the mobilisation pressure was modified as a function of the electrolyte viscosity to avoid any bias due to different mobilisation rate. These experiments showed that for constant focusing time and mobilisation rate, increasing glycerol content led to decreased signal to noise ratio due to incomplete protein focusing (ESI Fig. S1†). These results confirm that, as expected, in a closed device, the focusing time required to complete protein focusing increases with glycerol content. It thus highlights that the different trend observed using micropillar array is certainly due to evaporative phenomena.

The contribution of passive evaporation was thus evaluated by weighing the microdevice after being filled by electrolyte when

varying glycerol content. The room humidity inside the enclosure was simultaneously controlled; it should increase in the case of electrolyte evaporation. Fig. 4A shows the evolution of the remaining liquid within pillars as a function of time for glycerol content ranging from 0 to 50%. This procedure has been successfully used in a previous paper to determine the most suitable pillar layout to limit evaporation.[22] According to the glycerol percentage, two trends were observed. For glycerol percentages lower than 20%, the liquid mass decreased drastically in a short time, whereas a slight increase was observed over longer times. This dramatic decrease was certainly due to water evaporation as it has already been shown for binary mixture drops.[31] It was demonstrated that in the early stages, the more volatile component evaporated principally. The properties of water and glycerol are reported in Table 1 and the values of vapour pressure and boiling point confirm that water should mostly evaporate. In the case of electrolyte containing higher percentage of glycerol, a slight decrease was observed in the initial few minutes whereas significant increase was observed at the longer time. As the experiments were performed in a water saturated enclosure, this weight increase could be explained by the hygroscopic character of glycerol.

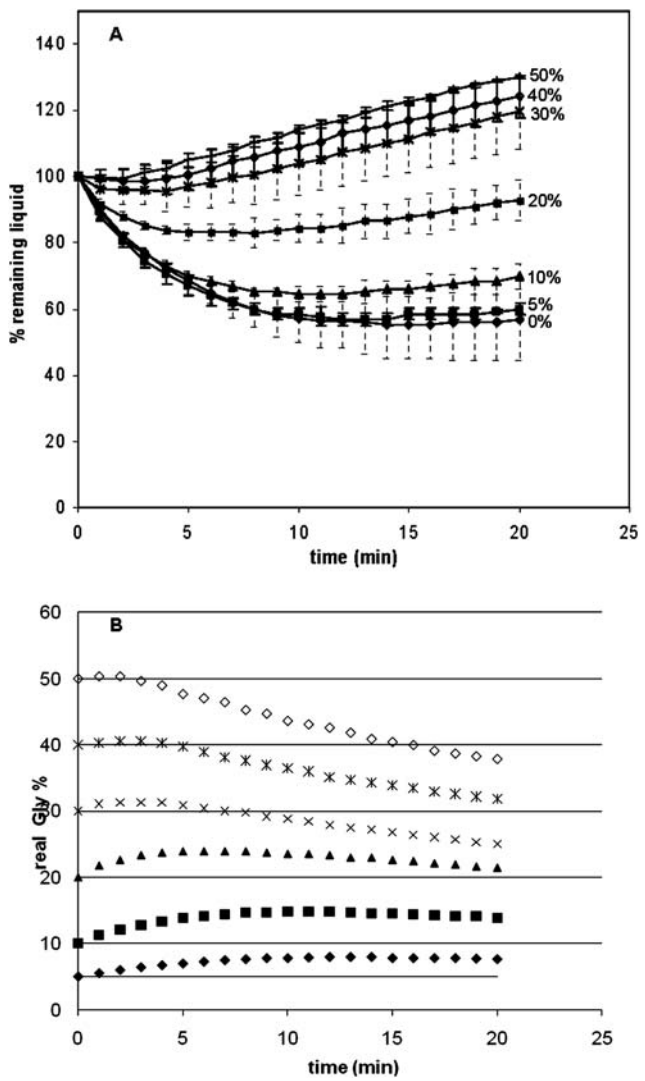

Fig. 4 Evaluation of passive evaporation as function of glycerol content within micropillar array. **(A)** Variation of the remaining liquid within the microdevice for different glycerol content. **(B)** Comparison of initial (—) and real glycerol percentage **(---)** remaining in the microdevice. Time scale corresponds to time after microdevice filling. Measurements are performed at 20 °C in saturated enclosure.

Table 1 Solvent properties

	Water	Glycerol
Density d (g/m^3)	0.9970	1.12582
Viscosisty η (cP)	0.89	945.0
Permittivity ε	78.39	42.5
Boiling temperature Tb/°C	100	290
Vapor pressure Vp (atm)	2.3 10^{-2}	0.22 10^{-6}

Finally, assuming that the evaporation process was mainly due to water evaporation, it was possible to estimate the real glycerol content as a function of time (Fig. 4B). Fig. 4B evidences that at low initial glycerol content (glycerol < 20%), the real glycerol percentage was higher than the initial one. Whereas for higher initial glycerol percentages (glycerol > 20%), the real glycerol percentage was lower than the initial one. Finally Fig. 4B shows that in case of passive evaporation, different real glycerol percentages are obtained for initial value of 5% and 40%, whereas these initial glycerol values lead to similar focusing time (Fig. 3). These results demonstrated that passive evaporation occurred, but they also evidenced that evaporation due to Joule heating at the beginning of ampholytes and protein focusing should not be neglected. Indeed, when applying an electric field on liquid, a Joule heating might be induced due to current flow through the solution. Sophisticated methods are described in many papers to evaluate the temperature gradient in microdevice of PDMS or glass/PDMS.[32–35] In this case, a micropillar array was used so the liquid/PDMS and liquid/gas interfaces have to be dealt with. Moreover as the conductivity was not constant during the focusing, the temperature gradient evaluation was tricky. As the buffer temperature is directly related to the electrolyte conductivity, it has been measured by capillary electrophoresis experiments in order to illustrate the Joule heating as a function of glycerol content (data not shown). As expected, dramatic decrease in electrolyte conductivity was observed when glycerol content increases; consequently the power dissipated was 10 times lower in electrolyte with 50% of glycerol than in one with 5%. These results confirm that Joule heating should play an important role in the evaporation process. Therefore, evaporation due to Joule heating and passive evaporation were both involved in the focusing process. The competition between both contributions resulted in the observed variation of focusing time as a function of glycerol content.

Surface treatment

A preliminary study was performed on the micropillar array chips to achieve optimized allergens separation and their immunoblotting, which is the basis of molecular allergen characterization and detection. Protein blotting, the key step in immunoblotting, consists of a fast transfer of proteins from a gel to a membrane to reduce protein diffusion and to allow their

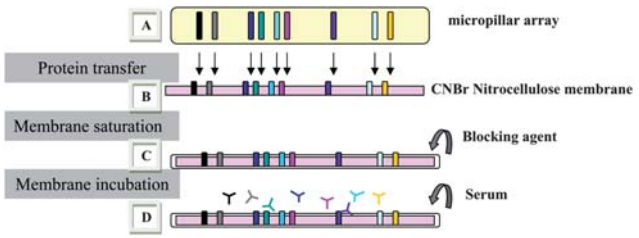

Fig. 5 Principle of immunoblotting on micropillar array. (A) Allergens separation by IEF within the pillars. (B) Allergens transfer on dry nitrocellulose membrane. (C) Membrane saturation. (D) Incubation with primary antibodies.

easier identification. The sketch of the immunoblotting process is presented in Fig. 5. After their separation within the pillars by IEF (Fig. 5A), allergens were transferred and immobilized onto a dry nitrocellulose membrane (Fig. 5B). In this work, a nitrocellulose membrane activated with cyanogen bromide (CNBr) was used to improve membrane binding capacity due to covalent binding and consequently to enhance sensitivity. The membrane was then saturated (Fig. 5C) to avoid subsequent non specific protein binding, and incubated with primary antibodies of interest (Fig. 5D), followed by incubation with enzyme labelled secondary antibodies and staining. In the context of milk allergy diagnosis, β-lactoglobulin, known as a major water-soluble milk allergen, has been used as a model.[2,36] Preliminary results from the micropillar array treated by oxygen plasma showed that β-lactoglobulin adsorbed on PDMS surface and that its focusing was not possible (Fig. 6A). To perform IEF of milk proteins without protein adsorption, a more drastic surface treatment was thus essential. Different approaches were tested. The preliminary ones consisted of different cellulose derivatives that are currently used for capillary or channel treatment.[37–39] Thermal treatments as well as dynamic coatings were tried; a slight improvement was obtained with low reproducibility certainly due to the high electrolyte viscosity that made the pillar array filling tricky.

In parallel, gelatin is commonly used to inhibit non-specific adsorption in ELISA experiments and it has already been used in surface treatment for PDMS microdevices, in particular in view of cell adhesion.[40,41] PDMS micropillar array was thus coated by gelatin to perform protein IEF. For that purpose, different parameters were optimized and the contact angle measured. The effect of O_2 plasma treatment prior to gelatin coating was first investigated. The results show that gelatin coating decreased PDMS contact angle and that the hydrophobicity of the coated PDMS was lower when interactions occurred between silanol groups and gelatin (θ° 48.8, RSD = 0.8, $n = 3$) than for interactions between siloxane groups and gelatin (θ° 87.5, RSD = 1.5, $n = 3$). Then, gelatin concentration (ESI Table S1†) and incubation time (ESI Table S2†) were optimised with micropillar array treated by O_2 plasma. These results showed that one hour of incubation with 0.1% (w/v) gelatin solution was sufficient to drastically decrease the PDMS hydrophobicity (θ° 45.3, RSD = 1.2, $n = 3$). The immunoblotting with *anti*-β-lactoglobulin polyclonal antibodies confirmed the results of contact angle measurements as one focused band of β-lactoglobulin was observed on the membrane (Fig. 6B).

Besides, another surface treatment using a polymer developed by Chiari *et al.* for capillary coating was investigated.[42] PDMA-AGE has already proven its ability to limit protein adsorption both for glass and PDMS chips as well as its stability over a wide pH range.[43,44] The coating procedure was optimized and pre-coating with PDMA-AGE at 0.1% (w/v) combined to dynamic coating with PDMA-AGE at 0.02% (w/v) ensured the lowest protein adsorption with the highest reproducibility, as the coefficient of variation was inferior to 10% on the position of the band of β-lactoglobulin identified by immunoblotting (Fig. 6C).

So both gelatin and PDMA-AGE coatings decrease the hydrophobicity of PDMS micropillar array surface and efficiently inhibit protein adsorption. The comparison of these two methods showed that PDMA-AGE coating led to a higher signal to noise ratio, indicating that PDMA-AGE coating decreased

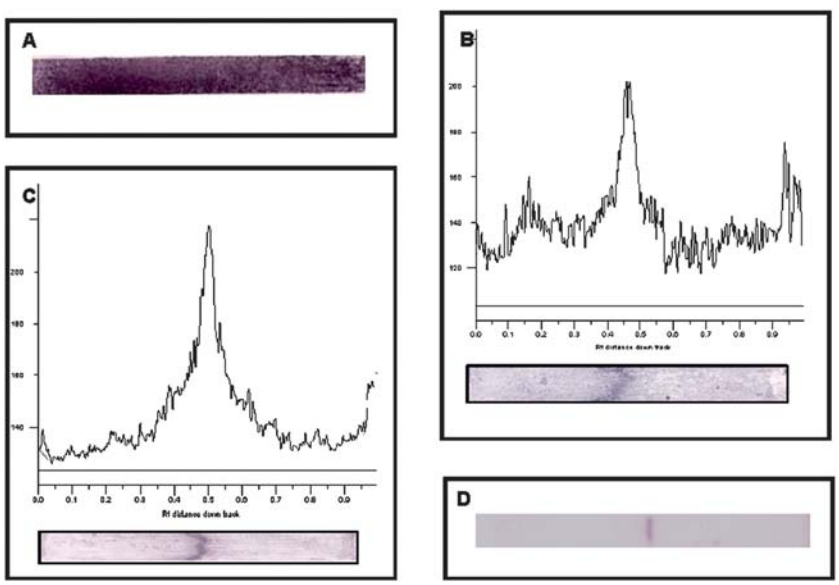

Fig. 6 β-lactoglobulin immunoblotting with rabbit antibodies after IEF on different supports. **(A)** On micropillar array treated by O_2 plasma. **(B)** On micropillar array coated by gelatin. **(C)** On micropillar array coated by PDMA-AGE. **(D)** On polyacrylamide mini-gel.

more efficiently the surface hydrophobicity. These results are confirmed by the contact angles obtained for gelatin coated PDMS (θ° 48.8, RSD = 0.8, $n = 3$) and PDMA-AGE coated PDMS (θ° 32.0, RSD = 1.2, $n = 3$).[43] The lower hydrophobicity induced by PDMA-AGE coating was not only due to the difference in molecular structures of the two coating materials, but also to the combination of pre- and dynamic coating with PDMA-AGE. Hence, PDMA-AGE coating was chosen for further experiments. The immunoblots obtained with micropillar array and mini-gel are similar, indeed in both cases a unique band corresponding to β-lactoglobulin was obtained (Fig. 6D). The band of protein obtained with micropillar array is less regular than the one obtained with mini-gel. This is certainly due to a more pronounced effect of evaporation on the chip edges. Nevertheless, Fig. 2 showed that the resolution is not dramatically affected by the band shape; indeed in a given axis a similar resolution is achieved. These results demonstrate that micropillar array with pillar height of 45 μm coated by PDMA-AGE can replace a 500 μm thick gel in order to perform an IEF separation followed by immunoblotting as part of a future molecular diagnosis.

Molecular immunodiagnosis

Analytical conditions have thus been optimized to perform an isoelectric focusing of a cow's milk allergen on PDMA-AGE coated micropillar array. This allergen has been detected by rabbit primary antibodies. Allergic patients sensitized to allergens produce *anti*-allergen IgE antibodies in their sera. So, an *in vitro* immunodiagnostic has to be done by using human sera from allergic patients sensitive to the allergen and producing allergen specific IgE antibodies. To test the specificity of our allergen detection we used a mixture of a non-allergenic protein (myoglobin) and β-lactoglobulin as a major milk allergen. They were separated by IEF on the coated micropillar array chip followed by immunoblotting performed using sera from patients sensitized to milk proteins, especially to β-lactoglobulin (as measured by ELISA, data not shown). The results obtained are shown before (Fig. 7A) and after the IgE immune reaction with 10 times diluted patient serum (10 μL) (Fig. 7B). Before immune reaction, only a red myoglobin protein band can be seen as it is a naturally coloured protein. After the immune reaction and staining, the band corresponding to β-lactoglobulin binding to patient IgE appeared in violet while the red band of myoglobin disappeared, certainly due to heme group modification. In fact, the band of myoglobin disappeared from the blot through the action of multiple washings. Indeed in order to avoid non specific adsorption of antibodies on the membrane, different washings are required. The presence of a band corresponding to β-lactoglobulin indicated that the patient was allergic to β-lactoglobulin, in agreement with the ELISA results. These results confirmed that the PDMA-AGE coating is efficient even when using human sera and above all, that this micropillar array chip, coupled to specific immunoblotting, can be successfully used to perform molecular immunodiagnosis.

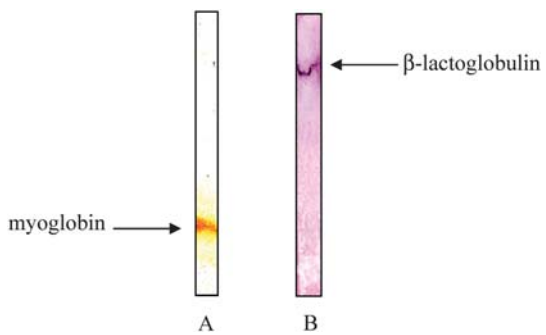

Fig. 7 IEF of a mixture of β-lactoglobulin and myoglobin on PDMS micropillar array coated by PDMA-AGE (A) immunoblotting with 10 μl of serum of allergic patient sensitized to β-lactoglobulin (B).

In order to evaluate further the micropillar array chip performance, different key parameters of this bioanalytical miniaturized method should be compared with a conventional method based on immunoblotting performed after protein separation by slab gel electrophoresis. Using a conventional format (10 × 10 cm) of slab gel, the focusing step lasts about 2 to 4 h and the patient serum volume required is about 300 μL to be used 10 times diluted. A first step toward a miniaturized device can be to use mini-gels (4 × 4 cm) fitting to the electrophoresis equipment Phast System from GE healthcare. In this case the focusing time ranged from 30 to 90 min and our experiments show that the serum volume required was about 100 μL, diluted 10 times. A brief comparison shows that a micropillar array chip of the same size but 10 times thinner than the conventional polyacrylamide gel allowed to drastically decrease the separation time by a factor ranging from 4 to 6, and uses 30 times less patient serum since only 10 μL were needed. The decrease in sample volume required could be due to the higher transfer rate of proteins from micropillar array than from gels. Indeed, the interpillar distance (100 μm) permitted a good access to electrolyte and the low agarose percentage led to a highly diluted gel, both of these conditions favoured the transfer of liquid onto the nitrocellulose membrane and thus the increase in the transfer rate of proteins, and consequently a higher detection sensitivity of this new type of immunoblotting. The low volume of serum required is a particular key issue when dealing with milk allergy as it mainly concerns infants and young children whose sera is extremely precious.

Another important result lies in the thickness of our micropillar chip: 45 μm. During the passive transfer by capillarity into a dry blotting membrane the liquid volume will be fully sucked up within the membrane thickness of 120 μm. We should consider that the blotting membrane is finally stained by the product of an enzymatic reaction and that this staining is mainly localized on the membrane surface. Its detection is quickly inhibited by the white absorbing membrane solid phase and limited to less than a depth of about 20 μm. A blotting of a "thick" gel, thicker than our micropillar array, is a waste of separated sample as only a part of it might be sticking to the blotting membrane and then only a part of what has been captured on the membrane is detectable by the usual enzymatic staining methods. So by choosing this height of the micropillars we are very close to the optimal efficiency of the immunoblotting technique.

Finally any further improvement of this one-dimension (1D) microarray contributes to the development of future 2D micropillar arrays. Coupled to immunoblotting they might pave the way to novel micro molecular immunodiagnosis.

Conclusion

In this work, electrolyte composition for IEF on micropillar array chips was optimized. The concentrations of agarose and glycerol as additives were optimized and the evaporation mechanisms during IEF were evaluated. In order to inhibit protein adsorption on the PDMS micropillar array, several coating approaches were investigated and PDMA-AGE coating led to the higher signal to noise ratio. Molecular immunodiagnosis of allergy based on PDMA-AGE coated micropillar array chips coupled to immunoblotting was established with allergic human sera. This micropillar array coupled to immunoblotting allowed performing molecular immunodiagnosis which was highly selective and sensitive, with low sample volume consumption. Further improvements could be performed by microfabricating multiple micropillar arrays on one chip which will improve the molecular immunodiagnosis with high throughput since multiple membranes can be incubated with multiple serum samples simultaneously.

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