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ORIGINAL PAPER

Allergen microarrays on high-sensitivity silicon slides

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Abstract We have recently introduced a silicon substrate for high-sensitivity microarrays, coated with a functional polymer named copoly(DMA-NAS-MAPS). The silicon dioxide thickness has been optimized to produce a fluorescence intensification due to the optical constructive interference between the incident and reflected lights of the fluorescent radiation. The polymeric coating efficiently suppresses aspecific interaction, making the low background a distinctive feature of these slides. Here, we used the new silicon microarray substrate for allergy diagnosis, in the detection of specific IgE in serum samples of subjects with sensitizations to inhalant allergens. We compared the performance of silicon versus glass substrates. Reproducibility data were measured. Moreover, receiver-operating characteristic (ROC) curves were plotted to discriminate between the allergy and no allergy status in 30 well-characterized serum samples. We found that reproducibility of the microarray on glass supports was not different from available data on allergen arrays, whereas the reproducibility on the silicon substrate was consistently better than on glass. Moreover, silicon significantly enhanced the performance of the allergen microarray as compared to glass in accurately identifying allergic patients spanning a wide range of specific IgE titers to the considered allergens.

Keywords Protein microarrays · Diagnosis · Fluorescence · Allergy · Sensitivity · Specificity · ROC curves

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Introduction

Allergy is a systemic disease whose pathogenic basis is the recognition of innocuous antigens by specific antibodies of the IgE isotype, followed by the release of inflammatory mediators by cells expressing type I Fc epsilon receptor. On this basis, no matter whether clinical expression of allergy concerns airways, gastrointestinal tract, or skin, the detection of specific IgE to relevant allergens in the serum is mandatory for its diagnosis.

Specific IgEs to one or more allergens are necessary but not sufficient markers to make a clinical diagnosis of allergy, since the expression of symptoms in the presence of the sensitizing source must also occur. In this scenario, the performance of available diagnostic tests aimed to measure allergen-specific IgE can be considered under two different perspectives. On one side, strictly sero-analytical performance is measured with parameters such as sensitivity, specificity, inter- and intra-assay variability, linearity, spiking recovery, etc. This approach, in spite of being legitimate on the basis of the comparison of new versus previously available assays, does not provide information on the actual clinical usefulness, or practical value, of the considered assay. On the other side, to discriminate between two different states of health (allergy/no allergy), a given assay has to be considered over the whole spectrum

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of operating conditions (high/low specific titers) and strictly defined gold standard criteria have to be applied to clearly discriminate patients versus controls. In this context, the receiver operating characteristic (ROC) plots are recognized tools which provide a real index of accuracy and clinical usefulness by demonstrating the limits of a given test ability to discriminate health from disease [1].

In the correct diagnosis of IgE-mediated diseases, a further level of complexity is provided by the fact that naturally occurring allergens vary significantly in their composition [2]. Many patients raise IgE not only to allergen components which define the specific antigenic composition of a given allergen source ("marker" molecules) but also to allergenic components which, being evolutionarily conserved, are structurally similar and immunologically cross-reactive (panallergens). IgE to panallergens typically cause false-positive results in extract-based diagnosis and are not clearly associated with clinically relevant symptoms. Component-resolved diagnosis (CRD) is a modern approach which allow us to overcome this and other critical limits of extract-based diagnosis, by dissecting the sensitization profile of single patients [3–6].

Recently, protein microarrays have emerged and have been combined with the CRD approach to allergy diagnosis for the simultaneous monitoring of IgE antibodies towards a large number of allergy-eliciting molecules [7–9]. The Immuno Solid-phase Allergen Chip (ISAC)® by Phadia, an array of 103 allergen components, is entering into clinical practice for the detection of allergen-specific IgE and/or IgGs. ISAC makes use of a commercial laser-induced fluorescence scanner for detecting the fluorescence of secondary antibodies against patient's allergen-specific immunoglobulins.

In the wide panorama of protein microarray tests of clinical significance, detection of IgE is a challenging task requiring sensitivity down to the nanogram per milliliter level. In a recent paper [10], we have introduced a silicon substrate for high-sensitivity microarrays, coated with a functional polymer named copoly (DMA-NAS-MAPS) [11, 12].

Here, we present the application of the new silicon microarray substrate to the detection of allergen-specific IgE in serum samples and compare the clinical performance of the silicon and the glass substrates both functionalized by the innovative functional copolymer copoly(DMA-NAS-MAPS). We matched by ROC plot analysis the results obtained with microarrays with gold standard criteria for defining specific allergy, including a consistent patient history, results of skin prick test, and results of in vitro measurement of allergen-specific IgE by the ImmunoCAP® assay (Pharmacia Diagnostics, Uppsala, Sweden), an industrial quasi-standard [13].

Materials and methods

Reagents and equipments

TRIS, BSA, Tween, phosphate-buffered saline (PBS) tablets, *N*,*N*-dimethylacrylamide (DMA), and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) were purchased from Sigma (St. Louis, MO). As the secondary antibodies, goat anti-human IgG labeled with Cy3 from Jackson Immuno Research and monoclonal anti-human IgE from Pharmingen (clone G7-26) in house labeled by Cy3 were used. *N*,*N*-acryloyloxysuccinimide (NAS) was from Polysciences (Warrington, PA).

Silicon slides were a gift from Prof. Selim M. Unlu, Optical Characterization and Nanophotonics Lab, Electrical and Computer Engineering Department, Boston University Photonics Center.

SuperEpoxy 2 and MirrorEpoxy substrates were from Arrayit (Sunnyvale, CA).

Allergens

A list of allergens used in this work is reported in Table 1.

Allergens Bet v 1 (Bet v 1.0101), Phl p 1 (Phl p1.0101), Phl p 5 (Phl p 5.0101), and Alt a 1 (Alt a 1.0101) were recombinant allergens from Biomay (Vienna, Austria) whereas allergens Bet v 2 (Bet v 2.0101), Phl p 7, nDer p 1, nDer p 2, and nFel d 1 were recombinant (or native, when prefix "n" is used) allergens from Indoor Biotechnologies Ltd. (Warminster, UK).

The allergenicity of each component was validated by monoclonal antibodies and serum IgE antibody binding, according to information provided by the manufacturer.

Microarray slides coating by copoly(DMA-NAS-MAPS)

Copoly(DMA-NAS-MAPS) was synthesized and characterized as described in Pirri et al. [14]. Microscope glass slides and silicon slides were pre-treated with oxygen plasma for 10 min. After pre-treatment, glass and silicon slides were immersed for 30 min in a copoly(DMA-NAS-MAPS) solution (1% *w*/*v* in 0.9 M $(NH_4)_2SO_4$ water solution). Slides were finally rinsed with water and dried under vacuum at 80 °C.

Microarray experiments

An array of rabbit IgG labeled with Cy3 (reference), PBS (negative control), and nine allergens at 1 mg/ml concentration was patterned using an SciFlexArrayer S5 spotter from Scienion (Berlin, Germany) according to the scheme reported in Fig. 1. Every allergen was spotted in pure PBS except for Phl p 1 (Phl p1.0101) which was dissolved using

Table 1 List of allergens used in this work

Allergen	Abbreviation	Species	Description
Bet v 1 (Bet v 1.0101)	BV1	*Betula verrucosa*	Major *Fagales* tree pollen allergen
Bet v 2 (Bet v 2.0101)	BV2	*Betula verrucosa*	Profilin (panallergen)
Phl p 1 (Phl p1.0101)	PP1	*Phleum pratense*	Major grass pollen allergen
Phl p 5 (Phl p 5.0101)	PP5	*Phleum pratense*	Major grass pollen allergen
Phl p 7	PP7	*Phleum pratense*	Calcium binding protein (panallergen)
Alt a 1 (Alt a 1.0101)	ALT	*Alternaria altern.*	Major *Alternaria* allergen
nDer p 1	DP1	*Dermat. Pteron.*	Major mite allergen
nDer p 2	DP2	*Dermat. Pteron.*	Major mite allergen
nFel d 1	FD1	*Felis domesticus*	Major cat allergen

1 μM beta mercaptoethanol and Phl p 7 which was spotted in PBS with 0.15 M trehalose to improve the spot morphology.

In the experimental conditions used, the volume of the spotted drops is 400 pl. Printed slides were placed in a humid chamber and incubated at room temperature overnight. The slides were then blocked by 50 mM ethanolamine in Tris/HCl 1 M pH 9 for 1 h, washed with water, and dried by a stream of nitrogen. Arrayed slides were incubated for 2 h with serum samples (20 μl), washed by the washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05% *v*/*v*) for 10 min under stirring, rinsed with water, and incubated with the labeled secondary antibody at 0.01 mg/ml in the incubation buffer for 1 h. Slides were washed with PBS (10 min) and water (10 min). Scanning for fluorescence evaluation was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA); glass slides were analyzed using 60%, 78%, and 82% of photomultiplier (PMT) gain and laser power whereas silicon supports were analyzed using 50%, 60%, and 78% PMT gain and laser power. Fluorescence intensities of 12 replicated spots were averaged.

Patients

A cohort of 30 adult patients (age range 18–53, average 32.3±10.2 years) who were sent for advice to the allergist by their general practitioner with a diagnosis of allergic rhinitis and/or asthma was recruited from July 2008 to October 2009 at the outpatient clinic of the San Raffaele Hospital in Milan. Each patient underwent chest auscultation and prick testing by a professional allergist. Prick testing was performed with commercial extracts of *Phleum pratense* (as representative allergen for grass pollen), *Betula verrucosa* (as representative allergen for *Fagales* tree pollen), *Alternaria alternata*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, and *Felis domesticus* (ALK, Hørsholm, Denmark).

Inclusion criteria were the following: (a) prick test yielding a wheal diameter of ≥3 mm to at least one of the allergens included in the screening panel (prick testing was performed according to EAACI guidelines) [15]; (b) diagnosis and staging of rhinitis and/or asthma performed according to ARIA and GINA guidelines, respectively [16, 17]; (c) unambiguous correspondence between seasons and/

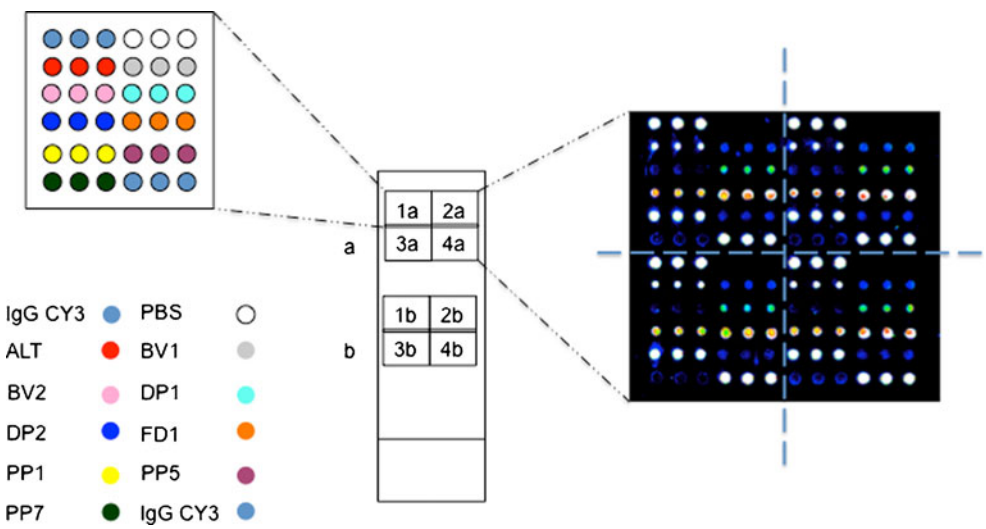

Fig. 1 Spotting scheme and typical result of the allergen microarray. Two arrays (each one made of four sub-arrays) were arrayed in two different zones of the slide (*a* and *b*), each allergen was spotted in triplicate in each sub-array. In each sub-array, PBS was used as negative control and Cy3-labeled IgG as a fluorescent reference

or conditions where symptoms were triggered and exposure to the relevant allergen; (d) specific IgE level determined in serum by ImmunoCAP® (Phadia, Uppsala, Sweden) above 0.35 kU/l to the same allergen of the panel which scored positive at prick testing; (e) willingness to participate to the study, after reading an informed consent approved by the Ethic Committee of the clinical institution participating in this study (San Raffaele Hospital, Ethic Committee, Protocol BBIA 10-10-05). When all inclusion criteria were simultaneously verified for one given allergen, the subject served as a gold standard for allergy to that allergen. Reciprocally, as negative controls for each allergen were recruited, those subjects scored negative for all inclusion criteria, except for signing the informed consent.

In the sub-group of patients who were simultaneously sensitized to *Phleum* and *Betula* at in vivo and in vitro extract-based diagnosis (criterion *a* and *d*, respectively), in order to discriminate those with bona fide sensitizations from subjects with sensitizations that could be referred to IgE binding to (highly cross-reacting, evolutionarily conserved) profilins and/or calcium binding proteins (CBP), Phl p 1 and/or Phl p 5 levels >0.35 kU/l (ImmunoCAP®, Phadia) served as markers for bona fide sensitization to grass pollen, whereas Bet v 1 levels >0.35 kU/l served as marker for bona fide sensitization to *Fagales* tree pollen. Serum IgE to Phl p 7 and Bet v 2 were also measured to interpret cross-reactivity. Subject characteristics and sensitization patterns of patients and controls are shown in Table 2.

Table 2 Patients characteristics (N=30)

	Number
Demography	
Rhinitis total	28
Rhinitis only	19
Rhinitis and asthma	9
OAS	8
Asthma total	11
Asthma only	2
Age range, years	18–53
Clinical characteristics of asthma and rhinitis	
Rhinitis	
Mild intermittent	5
Mild persistent	6
Moderate/severe intermittent	7
Moderate/severe persistent	10
Asthma	
Intermittent	6
Mild	4
Moderate	1
Severe	0
Sensitized (patients)	
Mono-sensitized	7
Sensitized to grass pollen	25
Sensitized to *Fagales* tree pollen	13
Sensitized to house dust mites	13
Sensitized to *Alternaria*	7
Sensitized to cat	15
Non-sensitized (controls)	
Non-sensitized to grass pollen	5
Non-sensitized to *Fagales* tree pollen	15
Non-sensitized to house dust mites	17
Non-sensitized to *Alternaria*	6
Non-sensitized to cat	14

ROC curve-based analysis of test performance

In order to compare the performance of allergen microarrays on glass versus silicon microarray slides, ROC curves were designed for each allergen [1] by plotting absolute fluorescence intensity values (proportional to IgE binding to the allergen components of grass pollen, *Fagales* tree pollen, *Alternaria*, cat, or house dust mites, which were included in the microarray) in sera from gold standard allergic patients versus negative controls.

Among the allergen components included in the compared allergen microarrays, Fel d 1 [18], Alt a 1 [19], and Bet v 1 [20] served as single markers for allergy to cat, *Alternaria*, and *Fagales* tree pollen, respectively, since they are known to be highly representative components of the corresponding allergen source. In the case of allergy to house dust mites and grass pollen, two values were used, namely Der p1/Der p 2 and Phl p 1/Phl p 5, respectively, to account for the relatively frequent possibility that only one of the allergen components of each pair [21, 22] is actually responsible for the considered sensitization. When comparing the performance of an IgE binding assay based on allergen components, it is necessary to compare binding signals obtained with allergen extracts with the binding signals to components which are representative of the repertoire of allergens included in that whole extract. Even if single elements could be poorly recognized, or negative, this would not indicate per se poor performance of the test but could be a physiological consequence of the qualitative characteristics of the immune response of the tested serum. On this ground, the highest absorbance value of each pair of the selected allergen components in each patient was taken as marker for allergy to house dust mite and grass pollen, respectively.

Statistical analysis

ROC curves were plotted using the Prism software (GraphPad, La Jolla, CA, USA), which calculates the sensitivity and specificity using each value of the data

table, where readings of fluorescence intensity of patients and controls were separately recorded. This program generates pairs of sensitivity and specificity cutoff values and the 95% confidence interval for each of them [23]. ROC analysis does not provide the better tradeoff of sensitivity versus specificity, a result which was beyond the scope of the present study. However, ROC analysis allowed us to quantify the area under the curve (AUC), which expresses the overall ability of the test to discriminate between the compared clinical statuses. AUC values range between 0.5 (truly useless test) and 1.0 (perfect test with zero false positives and zero false negatives). In the present situation, the AUC represents the probability that a randomly selected allergic patient will have a higher test result than a randomly selected control. This software also reports a p value testing the null hypothesis (that the AUC equals 0.5).

Results

Silicon substrates

Here, we report on the development of a highly sensitive microarray for the detection of allergen-specific IgE in serum samples. The allergen array is developed using a crystalline silicon slide coated by a thermally grown silicon dioxide (SiO_2) layer and functionalized by a polymeric coating. The silicon dioxide thickness has been optimized to produce a fluorescence intensification due to the optical constructive interference between the incident and reflected lights of the fluorescent radiation. The condition of constructive interference at the substrate surface is nowadays fulfilled in several types of glass slides coated with layers of dielectric or metal films [24]. However, the strategy involved in producing such complex multi-layer structures often suffers low reproducibility and difficult process control.

A simple configuration to achieve a fluorescent enhancement close to that provided by multi-layer slides consists of a silicon planar reflector coated with a thin film of SiO_2 [25].

Silicon slides coated by an 80 nm thick layer of thermally grown SiO_2 provide highly pure and flat substrates for microarrays; the surface was functionalized by adsorption of copoly(DMA-NAS-MAPS), a ter-copolymer based on DMA, NAS, and MAPS, originally developed for glass-based DNA microarrays [14]. This functional polymer has been widely applied to the biosensor field for the coating of nanobeads [26], silicon microcantilevers [27], polydimethylsiloxane [28], and nitrocellulose [29].

On the supports, the polymer forms a thin layer (few nanometers) whose morphology exactly copies the one of the underlying silicon, which is characterized by a very low surface roughness. The backbone of the polymer is made of the DMA monomer which forms hydrogen bonds with the silicon surface and constitute a hydrophilic interface able to resist non-specific adhesion of proteins; the NAS functional monomer binds the bio-probes, whereas MAPS monomers contribute to film stability by reacting with surface silanols.

The coating procedure is simple and reproducible, as compared to organo-silanization procedures that require harsh conditions and suffer poor reproducibility.

Importantly, the copoly(DMA-NAS-MAPS) film does not alter the optical properties of the optimized SiO_2 layer, thus allowing us to produce microarray supports able to amplify the fluorescent signals from four to six times [10] and characterized by a very low non-specific background.

This is an essential requirement when fluorescence intensification strategies are in place since, without efficient background reduction, the sensitivity gain is limited.

Allergen-specific IgE detection on microarrays

The set of nine allergens reported in Table 1 was spotted on copoly(DMA-NAS-MAPS)-coated silicon and glass slides at a concentration of 1 mg/ml in PBS-based buffers. The addition to the spotting buffer of 1 μM beta mercaptoethanol was necessary to improve the solubility of Phl p 1 (Phl p1.0101). Trehalose at a concentration of 0.15 M was added to Phl p 7 to improve its spot morphology. To perform a comparative evaluation of glass and silicon/SiO_2 supports, allergens arrayed on the different slides following the scheme depicted in Fig. 1; 2 arrays (each one made of four sub-arrays) were spotted in two different zones of the slide (a and b), each allergen was spotted in triplicate in each sub-array. In each sub-array, PBS was spotted as negative control and Cy3-labeled IgG as a fluorescent reference.

Following the steps described in "Microarray experiments," antibodies from the patient's serum recognize the immobilized proteins on the array surface. An anti-IgE secondary antibody, labeled with Cy3, is used for the detection of allergen-specific IgE.

In the first set of experiments, a pool of sera from allergic patients characterized by ImmunoCAP®-System from Phadia was analyzed. The pool was formed as a sero-analytical tool by mixing equal amounts of serum from four subjects with elevated IgE levels to Alt a 1, Bet v 1, Fel d 1, and Phl p 1 and scored 18.25, 4.12, 32.50, and 98.54 kU/l IgE levels at ImmunoCAP® analysis, respectively. Four sera from atopic individuals not sensitized (specific IgE<0.35 kU/l at ImmunoCAP® analysis) to any of the allergens included in the microarray were used as negative controls.

We found that negative controls did not display any detectable fluorescent signal (data not shown) whereas several fluorescent spots were clearly detectable on glass and on silicon slides reacted with the pool of sera. Figure 1 shows a typical result of the assay performed on the pool of sera.

Study of reproducibility of IgE detection

The pool of positive sera was used to assess the inter-assay, within-slide, and batch-to-batch reproducibility on glass and on silicon slides coated by copoly(DMA-NAS-MAPS). Silicon and glass supports underwent the same protocol except for the analysis of the fluorescent signals when different PMT gains and laser power were used to avoid saturation of the signals on silicon.

Coefficients of variation (CV, standard deviation in percentage of the mean fluorescence value for each allergen) were calculated, and the results are shown in Table 3 for glass slides (top) and silicon supports (bottom).

The inter-assay CV for each allergen varied from 6% to 16% on glass slides and from 3% to 11% on silicon. The within-slide variation was assessed for the individual allergens by calculating the variation of eight sub-arrays on the same slide and ranged from 8% to 22% on glass and from 3% to 19% on silicon. The batch-to-batch imprecision was determined by testing three different batches of slides on three consecutive days and varied from 3% to 23% for glass slides and from 3% to 16% for silicon.

The reproducibility data of glass supports are comparable with previously published data on allergen array reproducibility [30]. The reproducibility data obtained on silicon are consistently performing more than that on glass. These data indicate that the enhancement of fluorescent signals provided by the use of silicon can improve the precision of diagnosis performed by microarrays.

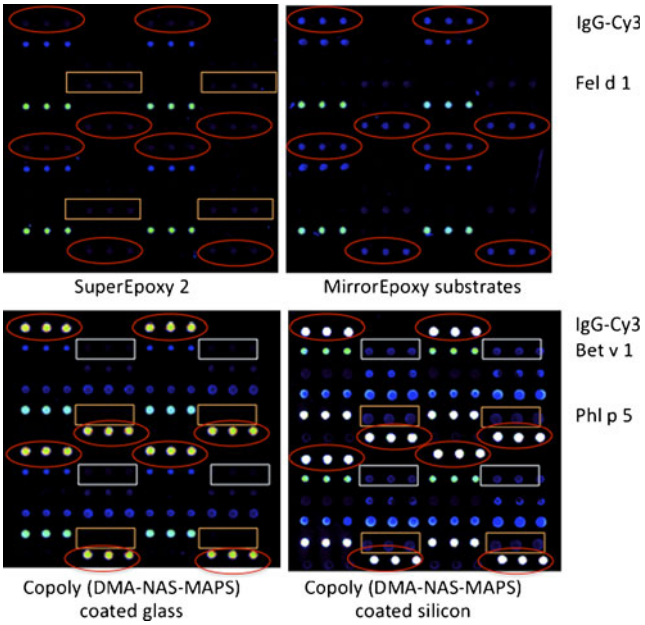

Fig. 2 Results of the assay for allergen-specific IgE detection in the pool of sera on microarrays analyzed at 60% laser power and PMT gain. The *upper panel* reports the results of the analysis of the pool of sera on SuperEpoxy 2 and MirrorEpoxy substrates; the *lower panel* reports the results on copoly(DMA-NAS-MAPS)-coated glass and silicon. In the detection conditions used, MirrorEpoxy substrates reveal the presence of IgE specific to the allergen Fel d1 that was not detected on SuperEpoxy 2. The superior performance of MirrorEpoxy substrates is clearly visible from the enhanced fluorescence of the reference Cy3-labeled IgG (*red circles*) and is due to the fluorescence enhancement produced by its reflective backside mirror coating. Similarly, signals of IgE specific for Bet v 1 and Phlp 5 are detectable on silicon substrates but not visible on glass slides, and the intensity of fluorescence of the reference Cy3-labeled IgG (*red circles*) is higher on silicon than on glass

Comparison of the performance with commercial supports for microarrays

In the second set of experiments, the same pool of sera and protocol described above were used to compare copoly (DMA-NAS-MAPS)-coated glass and silicon supports with two of the most performing commercially available supports for microarrays.

Figure 2 shows the results of the assay for allergen-specific IgE detection on microarrays analyzed at 60% laser power and PMT gain. In the upper panel, the results of the analysis of the pool of sera on SuperEpoxy 2 and MirrorEpoxy substrate from Arrayit (both based on a highly reactive epoxy surface chemistry) are reported. In the detection conditions used, MirrorEpoxy substrates reveal the presence of IgE specific to the allergen Fel d1 that was not detected on SuperEpoxy 2. Surface chemistry being equal, the superior performance of MirrorEpoxy

Table 3 Reproducibility of microarray testing for allergen-specific mmunoglobulin E on slides coated by copoly(DMA-NAS-MAPS) expressed as CV (standard deviation in percentage of mean value)

	ALT	*BV1*	*BV2*	*DP1*	*DP2*	*FD1*	*PP1*	*PP5*	*PP7*
Glass									
Inter-assay	11	8	12	13	6	10	6	12	16
Within slide	14	13	17	18	11	14	8	14	22
Batch to batch	4	11	19	8	16	3	9	7	23
Silicon									
Inter-assay	7	8	11	3	4	7	3	8	11
Within slide	12	3	18	12	3	11	5	14	19
Batch to batch	4	8	13	8	3	6	6	6	16

substrate is clearly visible from the enhanced fluorescence of the reference Cy3-labeled IgG (red circles) and is due to the fluorescence enhancement produced by its reflective backside mirror coating. Similarly, signals of IgE specific for Bet v 1 and Phlp 5 are detectable on silicon substrates but not visible on glass slides; likewise, the intensity of fluorescence of the reference Cy3-labeled IgG (red circles) is higher on silicon than on glass. In this comparative experiment, silicon supports provide a fluorescence intensity which is higher than each detected on the other supports and is the only surface able, at 60% laser power and PMT gain, to correctly diagnose, in the pool of sera, the presence of IgE specific to the six allergens Alt a 1, Bet v 1, Der p 1, Del p 2, Fel d 1, and Phl p 1.

One could object that the sensitivity and precision of this diagnosis would be simply improved by using higher laser power and PMT gain. However, there are conditions in which the highest laser power of commercial scanners is not sufficient to detect low abundant biomarkers. More generally, the use of slides capable of enhancing the fluorescence signals allows the use of less sophisticated and powerful lasers, making possible the development of cheaper, simpler, and smaller detection setups.

Clinical evaluation of allergen microarrays on glass and silicon substrate by ROC curves

An extensive characterization of copoly(DMA-NAS-MAPS)-coated glass and silicon slides was performed, analyzing 30 well-characterized serum samples.

In Fig. 3, as an example, a comparison of the analysis (78% laser power and PMT gain) of eight sera performed on glass and silicon supports is shown. Only one sub-array is reported for each substrate; for all the serum samples, silicon provided higher signals as compared to glass whereas on the negative sera no signals were detected neither on glass nor on silicon.

ROC analysis

Highly satisfactory sensitivity and specificity yields were obtained in the diagnosis of mite allergy by measuring IgE reactivity to Der p 1 and/or Der p2 with both glass- and silicon-microarrayed allergens, since a tradeoff value was identified (>240 for both Der p 1 and Der p 2) which allowed us to segregate 100% of patients from 100% of controls (Fig. 4). This was reflected by AUC which equaled unit in both conditions ($p<0.0001$).

In the case of diagnosis of allergy to *Fagales* tree pollen with Bet v 1 and to grass pollen with Phl p 1 and Phl p 5, allergen components on silicon slides allowed us to reach high performances (AUC=1, $p<0.00019$ in both cases), whereas allergens arrayed on glass yielded slightly less accurate, although still highly significant results (AUC 0.9 and 0.9167, respectively, with $p<0.0001913$ in both cases; ibid.).

A lower performance was observed in the diagnosis of allergy to cat with Fel d 1 with both type of supports (ibid.). However, also in this case, allergens arrayed on silicon scored better than glass-coated allergens (AUC 0.9576, $p<0.0001$ versus 0.8438, $p=0.001378$, respectively).

In diagnosis of allergy to *Alternaria*, Alt a 1 scored as the less accurate marker among those considered in this

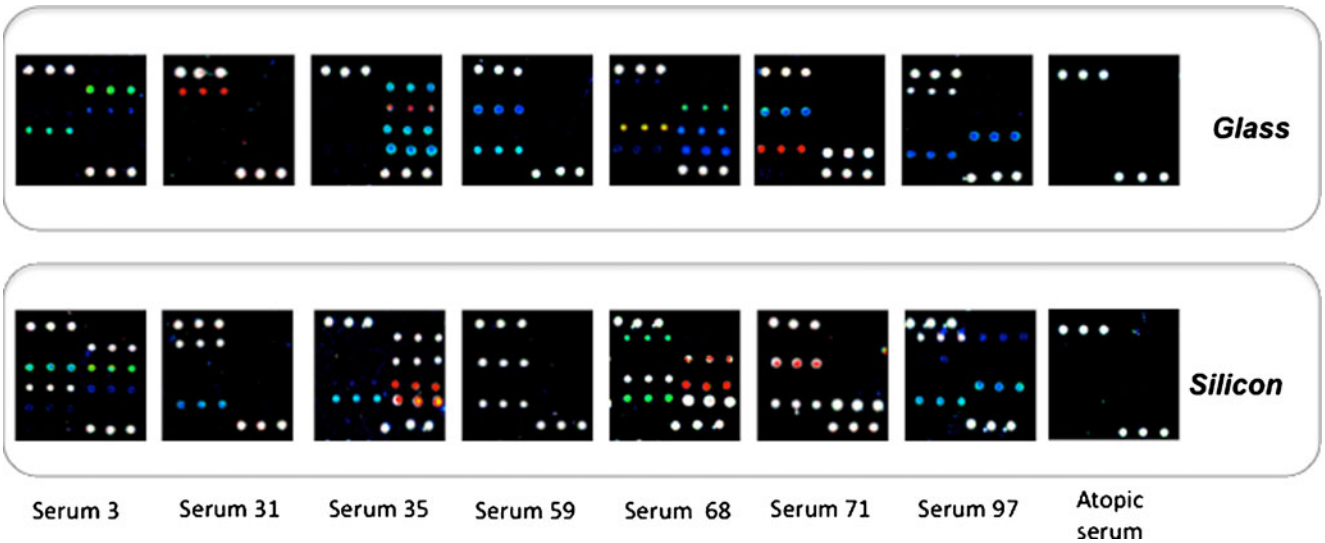

Fig. 3 Comparison of the analysis (78% laser power and PMT gain) of eight sera performed on glass and silicon supports coated with copoly(DMA-NAS-MAPS). Only one sub-array is reported for each substrate; for all the serum samples, silicon provided higher signals as compared to glass whereas on the negative serum no signals were detected neither on glass nor on silicon

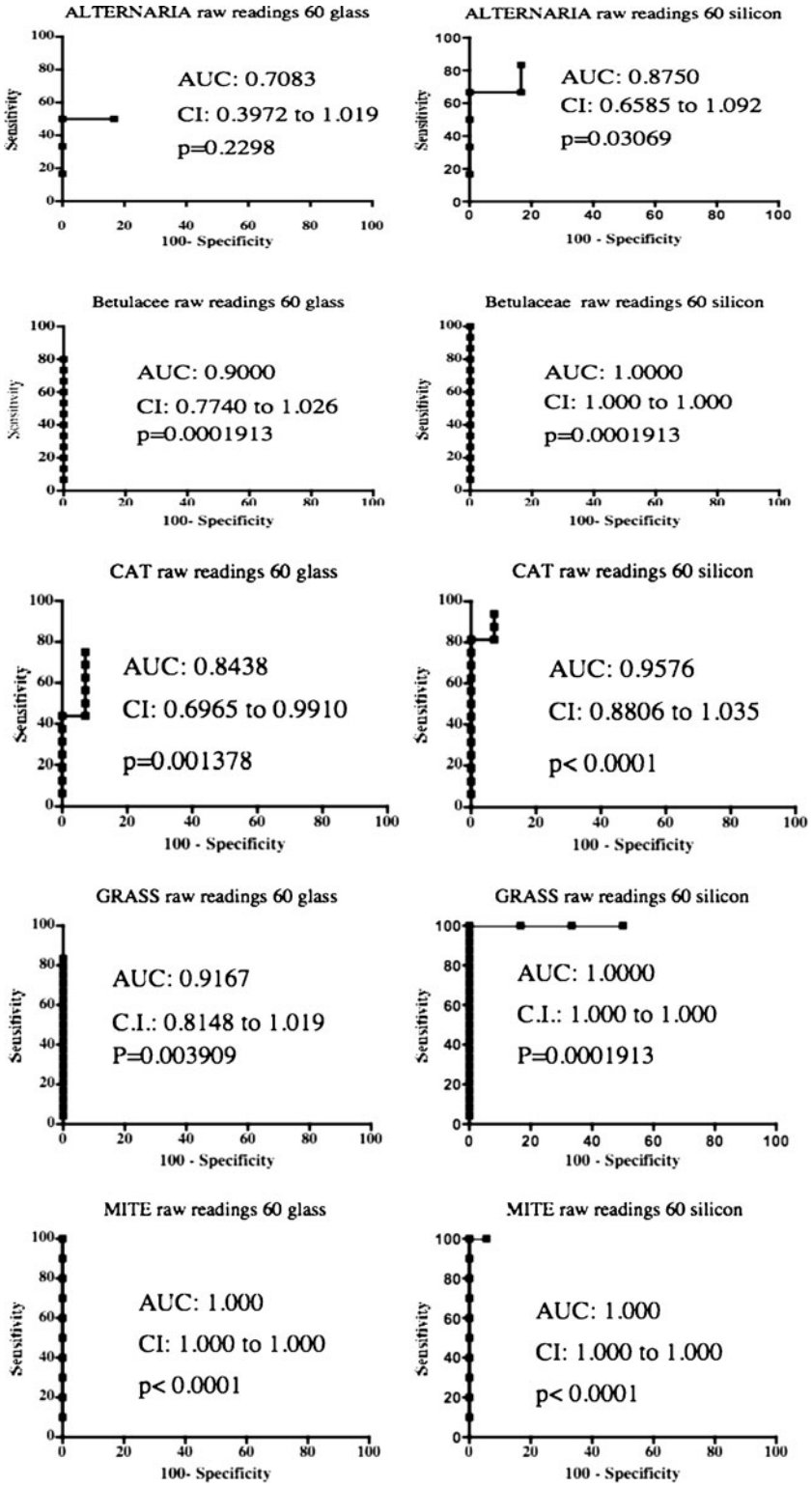

Fig. 4 ROC curve analysis of sensitivity (on the *y-axis*) and specificity (on the *x-axis*, expressed as "1-specificity") in the diagnosis of allergy to the indicated allergens. Results obtained with allergen microarray on glass (five *left panels*) versus silicon (five *right panels*) are separately depicted as plots. *Numbers* indicate area under the curve (*AUC*), confidence interval (*CI*), and *p* values for each ROC analysis

study since Alt a 1-based diagnosis barely reached significance only when the allergen was immobilized on silicon (AUC 0.8750, $p=0.03069$) (ibid.).

Several characteristics related to each single allergen component may account for these results in the present model. However, taken together, these data suggest that a significant improvement in overall performance of allergen component-based diagnosis with this kind of microarray can be achieved using silicon with fluorescence-enhancing properties.

Capability of the tested allergen microarray to discriminate bona fide sensitizations to grass pollen and/or *Fagales* tree pollen from the effects of cross-reactivity via panallergens

The rigorous recruitment criteria of study patients included the determination of IgE levels to defined grass pollen (Phl p 1 and Phl p 5) and *Fagales* tree pollen (Bet v 1) allergens, since in subjects who are simultaneously positive with extract-based diagnosis to *P. pratense* and *B. verrucosa*, sensitization to either allergen source cannot be distinguished from bona fide double sensitizations. This is a consequence of the quite common IgE reactivity to profilins [31] and to the less common reactivity to calcium binding protein [3], which are significantly represented in both *P. pratense* (Phl p 12 and Phl p 7, respectively) and *B. verrucosa* (Bet v 2 and Bet v 4, respectively) extracts. These molecules can be cross-recognized by IgE due to their important structural homology [32, 33].

The sensitization profile of 21 study patients with positive extract-based in vivo and in vitro testing for *P. pratense* and *B. verrucosa* is shown in Table 4. We found that eight of 21 patients would have been misdiagnosed with extracts as double positive, whereas they were either sensitized to *Fagales* tree pollen only (one patient) or to grass pollen only (seven patients). The sensitization profile of 21 study patients with positive extract-based in vivo and in vitro testing for *P. pratense* and *B. verrucosa* is shown in Table 4.

The distinction between positive results due to bona fide sensitization to major allergens of two allergen extracts (grasses and *Fagales*) from positive results due to IgE specific to corresponding, evolutionarily conserved panallergens present in both extracts is one of the crucial properties which was tested with the set of allergens which were included in our panel.

We found that eight of 21 patients would have been misdiagnosed with extracts as double positive to grass and *Fagales*, whereas they were either sensitized to *Fagales* tree pollen only (one patient) or to grass pollen only (seven patients). ImmunoCAP® analysis with allergen components allowed us to explain the observed cross-reactivity with IgE to profilins (Bet v 2 and Phl p 12) in all but one patient, who had IgE to calcium binding proteins (Bet v 4 and Phl p 7).

The comparison of the performance of Bet v 2 and Phl p 7 in the tested microarray versus ImmunoCAP® (Phadia) was beyond the scope of the present study. However, we evaluated whether in this real-life clinical context the limited panel of allergen component we included in the microarray allowed anyhow to attribute the outcomes of cross-reactivity to the panallergens included in the microarray. Moreover, this information worked as a double check in the attribution of bona fide sensitizations to *Fagales* tree pollen and grass pollen, since it allowed us to interpret extract-based cross-reactivity in subjects with IgE to either allergen extract. This analysis is in qualitative terms one of the most relevant performance parameter of component-resolved diagnosis.

We found that the allergen microarray on glass slides pinpointed five of the eight patients, which at ImmunoCAP® analysis reacted with Bet v 2 (four patient) and Phl p 7 (one patient). These five sera yielded a fluorescence intensity reading above zero. With microarrays on silicon supports, all eight patients were correctly identified by the tested microarray. The coated silicon slides were thus able to detect specific IgE levels at the concentration of 1 ng/ml in serum.

With both types of slides, sera which scored negative at ImmunoCAP® for Phl p 7 and Bet v 2 consistently yielded non-measurable signals (i.e., non-false-positive was observed). Thus, the use of silicon increased the performance of the tested allergen microarray in a significant fashion and made it overlapping with the industrial quasi-standard ImmunoCAP®.

Table 4 Sensitization profile of 21 patients with positive prick testing and in vitro specific IgE (>0.35 kU/l) for *P. pratense* and/or *B. verrucosa*

	Number
Phl p 1 and/or Phl p 5>0.35 AND Bet v 1>0.35	13
Phl p 1 and/or Phl p 5>0.35 only	7
Bet v 1>0.35 only	1
Bet v 2 and Phl p 12>0.35	9
Bet v 4 and Phl p 7>0.35	1

Table 5 Patients with low IgE reactivity level (at ImmunoCAP® analysis) to single minor allergens not identified by microarray performed with glass-coated allergens in five out of eight cases identified by microarray performed on silicon slides

Patient	Bet v 2			Phl p 7		
ID	CAP	Glass	Silicon	CAP	Glass	Silicon
24	1.1	0	340	0	0	0
32	1.59	0	723	0	0	0
59	7.59	497	6,280	0	0	0
71	5.04	372	3,807	0	0	0
74	1.2	0	800	0	0	0
79	2.09	106	934	0	0	0
91	0	0	0	3.04	333	294
29005690	21.33	611	9,257	0	0	0

"CAP" indicates ImmunoCAP® (Phadia) values (kU/l). "Glass" and "silicon" are absolute values of fluorescence intensity readings of allergen microarray performed with the indicated allergen component coated on glass- or silicon-pre-treated slides, respectively

Discussion

A number of studies with allergen component-based microarrays demonstrated that the sensitivity and specificity of these novel test systems are comparable with (and sometimes superior to) established state-of-the-art technologies [30, 34]. Room for improvement has been observed especially with regards to precision (within run, between run, batch to batch), as well as with reference to single allergens which raised specific problems (e.g., mugwort) [35]. Moreover, validations of this approach within a carefully established clinical context are eagerly awaited.

Here, we set up proof-of-principle allergen microarray produced on copoly(DMA-NAS-MAPS)-coated silicon with optical properties able to enhance fluorescent signals and explored its performance in a real-life clinical setting, where ROC plots were generated to establish its ability to discriminate between allergic and control individuals for each of the selected allergens.

We found that copoly(DMA-NAS-MAPS)-coated glass slides yielded results in allergen microarrays overlapping or higher than previously published data generated with a commercial microarray [30, 35]. Moreover, surface chemistry being the same, we introduced the use of silicon slides bearing a layer of thermally grown SiO_2 whose thickness has been optimized to exploit the optical constructive interference between the incident and reflected lights of the fluorescent radiation. The combination of silicon slides with the polymeric coating allows amplification of fluorescent signals, limiting the aspecific background and yielding results superior to that obtained on glass.

Several reasons may explain the unsatisfactory performance of allergen component-based diagnosis and even more so in the case of microarray, where the amounts of allergen that can be immobilized on the reaction slide are remarkably lower as compared to diagnostic tools exploiting enhanced binding surfaces, such as the industrial quasi-standard ImmunoCAP® from Phadia [13]. These include the lack of components which may be relevant in single subjects of the population sensitized to a given allergen source, the poor expression of conformational epitopes relevant for IgE recognition, the absent or different glycosylation pattern as compared to native allergen, etc. [36].

We also believe that, when using an array of a large set of different proteins, due to their differential binding vocations, a depth investigation on the real amount of surface-bound probes is mandatory.

Despite this watchfulness, our data suggest that silicon slides with fluorescence amplification capacity significantly improves the clinical efficacy on component-based diagnosis with allergen microarray as compared to the use of regular glass, since for all considered allergens the overall performance was higher in the former condition.

Moreover, patients with low IgE reactivity level (at ImmunoCAP® analysis) to single minor allergens of grass pollen and *Fagales* tree pollen, namely Phl p 7 and Bet v 2, were not identified by microarray performed with glass-coated allergens in five out of eight cases, whereas they were all identified by microarray performed on silicon slides (Table 5). Although anecdotal, this observation is relevant in clinical terms, since these molecules work as cross-reactive panallergens and allow us to interpret cross-reactivity observed with extracts in patients with bona fide sensitization to either allergen source. Notably, Phl p 7 behave differently probably due to its low molecular weight (8.8 kDa) which provides low reactivity with the polymeric surface. Indeed, we believe that, when using an array of different proteins for clinical purposes, a careful calibration of the immobilization yield of each protein is necessary to improve the reliability of the system.

In conclusion, microarray-based diagnosis of allergic diseases is significantly improved by the use of silicon planar reflectors coated with an optimized layer of SiO_2 able to enhance the fluorescence. This technological advancement further contributes to make this diagnostic approach a valuable alternative to traditional diagnostic tools for the testing of allergen-specific IgE.

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