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The Application of the PASA System in the Field of Allergy Diagnosis

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Introduction

An increasing number of patients are suffering from allergies such as hey fever, asthma or food allergies. Since 1967, when the radio-allergo-sorbent assay (RAST) for serum immunoglobulin E (IgE) and allergen-specific antibodies in serum was described [1], many various assay procedures have been developed, but only a few have become routine methods in clinical chemistry, immunologic laboratories or in doctor´s offices. Many of these assays are based on ELISA techniques [1, 2, 3]. Although it is possible to measure 96 samples with one microtiter plate, a complete determination of the allergen-specific IgE level in the patients serum is expensive and time-consuming. In addition, to measure *n* allergen-specific IgE antibodies the *n*-fold volume of serum sample is needed. Even if mixtures of different allergens in one assay are used, the single components have to be broken down after a positive test result. Therefore, it is important to develop a fast and economic screening technology for the allergen-specific IgE antibodies in serum samples.

Miniaturization of the test system can be achieved either using 384/1536 instead of 96-well microtiter plates [4] in combination with robot systems or by transferring the test on a planar surface using micro spotting techniques and the PASA system [5]. The aim of the study is to examine the suitability of the PASA system (parallel affinity sensor array) for highly parallel screening of allergen-specific IgE antibodies.

Experimental

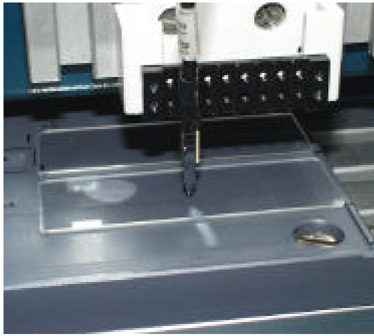
Materials

The support material (microscopic glass slides, Merck, Darmstadt, Germany) was cleaned using a 1:1 mixture of methanol and HCl (36%) for 30 min at room temperature (*RT*), washed with water (HPLC grade, Millipore, Milli-Q Plus 185) and immersed for 30 min in H_2SO_4 (98%). The glass slides were rinsed with water, dry methanol and dried under a nitrogen stream. Activation of the glass slides was done with a solution of 1% glycidyloxypropyltrimethoxysilane (Sigma-Aldrich, Deisenhofen, Germany) in dry toluene (Sigma-Aldrich) for 18 h at RT or with a solution of 3.5% trimethylchlorosilane (Merck) in dry isopropanol for 1 h at RT. After washing steps with toluene/isopropanol and methanol the glass slides were dried under nitrogen and stored until use in a desiccator. The 3-D-Link amino binding

slides were purchased from Surmodics (Minnesota, USA), the Xenobind slides from Dunn (Asbach, Germany). The allergen extracts were purchased from Immunotec (*IT*, Marl, Germany), the prick tests from Allergopharma (*AP*, Reinbeck, Germany) and kindly supplied by r-biopharm (*r-bio*, Darmstadt, Germany). The raw pollen material was purchased from Allergon (Ängelholm, Sweden). Apis mellifera (honey bee) was purchased from Allergon. Bee Venom and Phospholipase A_2 (PLA_2) from Sigma. The allergen extracts were purified with a gel-chromatographic column from Pharmacia (PD-10, cut-off: 10000 MW; Freiburg, Germany) and the dialysis cassettes (cut-off: 10000 MW) from Pierce (KMF, Sankt Augustin, Germany). The protein tests were performed with a BCA-assay (Sigma) and a micro-BCA-assay from Pierce. Myeloma-IgE was purchased from Calbiochem-Novabiochem (Bad Soden, Germany), the monoclonal rat-anti-human-IgE antibodies from Technopharm (Paris, France), the horseradish-peroxidase (*HRP*) labeled goat-anti-human-IgE antibody from KPL (Dunn). Monoclonal and polyclonal antibodies against Bet v1 and the recombinant Bet v1 (r Betv1) were kindly supplied by ALK Abell■ (Hørsholm, Denmark). Avidin-HRP/Streptavidin-HRP was purchased from Sigma. SeaBlock (*SB*) blocking solution was purchased from Pierce. Monoclonal and polyclonal antibodies against Der p1 and the natural affinity purified Der p1 were purchased from Indoor Biotechnologies (Cardiff, UK). 5´Aminomodified oligonucleotides from *Enterococci gallinarum* (E. gal.-NH_2, 18-mer: 5´-CACAACTGTGTAACATCC-3´) and the digoxigenin-labeled E. gal. complementary oligonucleotide was purchased from MWG Biotech (Ebersberg, Germany). Commercial goat-anti-digoxigenin-HRP Fab-fragment stock solution was purchased from Roche Diagnostics (Mannheim, Germany). The reference measurements were kindly performed by our project partners at the Department of Allergy and Clinical Immunology (Am Biederstein, München, Germany) using the Pharmacia-CAP specific IgE FEIA and the MAST CLA allergen-specific IgE-assay.

General Setup

The pipetting system consisted of a 3-dimensional stage, a controller (multi-dos), a personal computer, piezoelectric nanoliter pumps (standard micro pipette SPIP, manufactured by silicon etching) and a liquid handling system (syringe pumps) for priming and washing of the silicon pumps (Fig. 1, Gesim, Großerkmannsdorf, Germany). A standard 96-well microtiter plate was used as reagent reservoir. The fast evaporation of the small drops was delayed by the use of 1% glycerol.



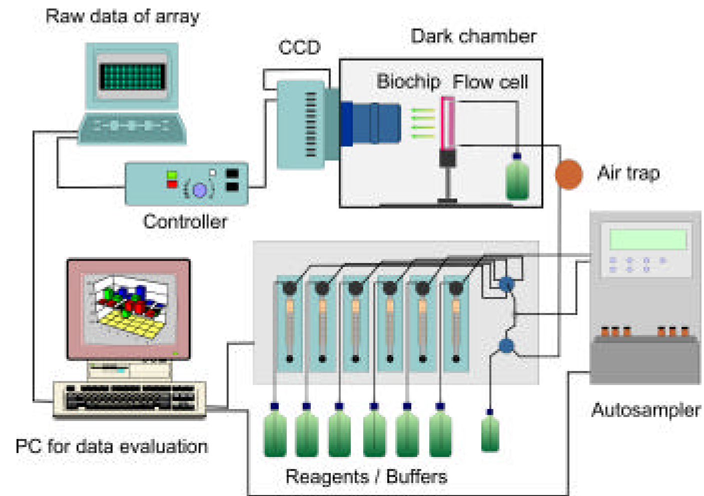


Fig. 1: Non-contact piezo system *Fig. 2: General setup of the PASA system*

After immobilization of the reagents on the modified glass slides, the biochip was washed and placed in a flow cell (Fig. 2) located in a dark chamber. Six different pumps allow the automatic filling of the flow cell (500-1000 µL) with blocking solution, serum sample, anti-human-IgE antibody and substrate (SuperSignal West Dura Chemiluminescence Substrate, Pierce). The chemiluminescence of the reaction between the peroxidase labeled anti-human-IgE antibody and the substrate is detected with a peltier-cooled, back-illuminated CCD-camera. For some tests the glass slides have been placed in the flow cell only for the detection of the chemiluminescence reaction. After immobilization of the corresponding reagents on the chip surface, the slides were washed with washing buffer, water and dried under a nitrogen stream. Incubation with serum samples was performed with chamber slides (200 µL, Sigma). The reaction with the HRP-labeled antibody was done using a cover slip (Hybri-slip, 22 x 40 mm, Sigma). After a washing step the slide was placed in the flow cell of the PASA-system and 500 µL of chemiluminescence substrate was filled into the flow-cell. Light emission was accumulated for a specified time using a 2x2 binning. Data evaluation was performed by the program "*imagetool"* [6] and the presentation of the test results by the program "*avis"* [7].

Methods

Extraction of allergens and destination of the Betv1/protein content

Allergen extracts were prepared using phosphate-buffered saline (*PBS*, pH 7.8) as extraction medium. After suspending the pollen for 1 h in PBS, the medium was centrifuged (14.000 g, 10 min, 4 °C). The supernatant was collected and the insoluble residue was extracted again for 1 h. After a filtration step (cellulose acetate, 0.45 µm, Sartorius, Göttingen, Germany) the supernatant was dialyzed (Slide-A-Lyzer dialysis cassettes) against water/PBS (10:1) for 24 h and then lyophilized. 10 mg dry substance was subsequently diluted with water/PBS-buffer. To characterize the obtained allergen extracts the protein content was determined using the BCA assay and the Bet v1 content using a sandwich-ELISA (*SW-ELISA*) with two different antibodies against Bet v1.

The Bet v1 assay was performed by coating the MTP with a polyclonal goat anti-Bet v1 antibody *AB* (1:10000 in carbonate buffer *CB*, 100 µL, 18 h, 4°C). After a blocking step using 1% BSA/0.5% SB-PBS the calibration was performed by a dilution (1:100-1:10^{-8}) of r Bet v1 (6.3 g/L) with BSA/SB-PBS. The different birch allergen extracts (IT, AP, r-bio, IWC) were diluted 1:100-1:10000 with BSA/SB-PBS. After 1 h at RT the bound Bet v1 was reacted with a monoclonal mouse anti-Bet v1 AB (1:10000 in PBS, 100 µL, 1 h, RT). The MTP was then incubated with anti-mouse IgG-HRP AB (1:40.000 in PBS, 100 µL, 1 h, RT, ICN) and the color development was stopped after 5 minutes using 5% H_2SO_4 solution. The absorbance was read at 450 nm.

To obtain the protein content, the allergen extracts were diluted 1:100-1:1000 with PBS. 150 µL of this solution was added to the MTP. 150 µL of a BSA calibration solution (0.125-200 µg/L) was filled into the MTP. By addition of 150 µL of the MicroBCA solution a color change to green-purple occurs. After an incubation time of 30 min at 36 °C the purple color was measured at 550 nm.

Immobilization of the reagents on glass slides

The different reagent solutions (crude allergen extracts [5-10 g/L], 1:40; myeloma-IgE [1 g/L], 1:300-1:3000 and anti-human-IgE antibodies LO-HE-10, LO-HE-17 [1 g/L], 1:100) were diluted with CB (pH 9.6, 1% glycerol) and dispensed with a piezoelectric pipetting system (Fig. 1, GeSIM, Grosserkmannsdorf, Germany). The chosen conditions were 5 droplets, spot diameter about 200 µm, U = 60 V, f = 300 Hz. The coating was performed for 18 h at 4°C.

On-chip biotinylation

After immobilization of the extracts, the chip was blocked with an Tris-ethanolamine buffer (50 mM ethanolamine, 0.1 M Tris, pH 9.0, 1h, RT). After a washing step with washing buffer (PBS-Tween-20 pH 7.6) the remaining free amino groups of the immobilized allergen extracts were biotinylated for 1 h at RT using a solution of biotin NHS ester (Sigma, 10 g/L in dry DMSO, 300 µL of the solution in DMSO were mixed with 2 mL CB, 1.5 mL/slide, 1 h, RT). After an incubation time of 1 h the glass slides were washed and blocked again with a solution of 1% BSA in PBS (1 h, RT). The slide was washed and the biotin groups were reacted with avidin-HRP (1:10000 in PBS/0.5% BSA/0.1% Tween, 30 min, RT, 250 µL, cover slip). The slide was placed in the flow cell of the PASA system and the reaction between the HRP-labeled AB and the chemiluminescence substrate was detected by accumulating the light emission for 60 s.

Determination of total and allergen-specific IgE antibodies

The GOPS-modified glass slides were blocked with 1% BSA/0.5% Seablock (*SB*) for 1 h at RT. The serum sample was diluted 1:5 (allergen specific IgE-level) or 1:10 (total IgE-level) with BSA (1%)/Tween-20 (0.1%) PBS (pH 7.8) and incubated for 30 min at RT using a chamber slide (200 µL). After several washing steps with washing buffer (PBS-Tween pH 7.6) the slides were incubated with a polyclonal, affinity-purified HRP-labeled goat anti-human-IgE AB (1:500 in PBS/BSA/Tween, 250 µL,

cover slip) for 30 min at RT. The slide was placed in the flow cell and the chemiluminescence was accumulated for 90 s (allergen specific-IgE level) or 30 s (total IgE level).

Calibration with immobilized myeloma-IgE

Myeloma IgE (0.1-3.3 mg/L) and the monoclonal anti-human-IgE antibodies (LO-HE-10/LO-HE-17 [1 mg/L], 1:100) were immobilized for 18 h at 4°C on GOPS-slides. The chip was blocked with 1% BSA in PBS for 1 h at RT and placed in the flow cell of the PASA system. The serum samples were incubated for 30 min at RT (1:10 in PBS/BSA/Tween) following an incubation with HRP-labeled polyclonal anti-human-IgE AB in a 1:500 dilution in 1% BSA/0.5% Tween-PBS. 500 µL of chemiluminescence substrate was pumped into the flow cell and the signal was accumulated for 30 s.

Determination of the Der p1 concentration in different mite extracts

The MTP was coated with a monoclonal mouse-anti-Der p1 antibody M5H8 ([2 g/L], 1:1000 in CB, 100 µL, 18 h, 4 °C). After a blocking step with 1% BSA in PBS (300 µL, 1h, RT) a calibration curve was obtained by dilution of natural purified Der p1 ([2500 µg/L], 1:10-1:80, 100 µL, 2 h, RT). The mite extracts (prick test r-bio, IT) were diluted 1:100-1:10000 in PBS. Bound Der p1 was detected by filling the MTP first with a solution of a biotinylated monoclonal mouse-anti-Der p1 AB (4C1, 1:1000 in PBS, 100 µL, 1h, RT) and second with HRP-labeled Streptavidin (1:10000 in PBS/BSA/0.5 % Tween, 100 µL, 45 min, RT). The color development was stopped after 5 minutes and the absorbance was read at 450 nm. For the indirect/direct immobilization tests the monoclonal anti-Der p1 AB (M5H8, 1:100, 20 droplets)/the mite extracts (1:40, 5 droplets) were immobilized on different GOPS chips. After a blocking step with 1% BSA in PBS, the glass slides were washed with washing buffer/water and dried under a nitrogen stream. Using the indirect test format, the mite extracts were immobilized on the antibody-coated area as described before. After an incubation time of 2 h at RT the bound Der p1 was measured for both test formats with the biotin-anti-Der p1 antibody 4C1 (1:100 in PBS/BSA/Tween, 200 µL, 30 min, RT) using the chamber slide system. The slides were washed, incubated for 30 min at RT with HRP-labeled streptavidin (1:500 in PBS%BSA/Tween, 250 µL, cover slips) and after another washing step placed in the flow cell of the PASA system. Chemiluminescence was accumulated for 60 s.

DNA-Hybridization on GOPS modified slides

E. gal.-NH_2 oligonucleotide (5 pmol/µL) was immobilized as capture oligo on different modified glass slides. After a washing step with Tris washing solution (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, pH: 7.5) the hybridization reaction was performed with digoxigenin-(DIG)-labeled E. gal. complementary oligonucleotide (0.1 pmol/µL in hybridization buffer (5 x saline-sodium-citrate-buffer, *SSC*) with 0.5% blocking reagent (Roche Diagnostics, Germany), chamber slides) in a Thermomix Incubator (2h, 50 °C). After washing with 0.5 SSC 0.1% Tween, the hybrids were visualized with HRP-labeled goat-anti DIG antibody (1:100 in PBS, 20 min, RT, 200 µL, cover slip).

Results and Discussion

Allergen Extracts

Most allergens are small proteins [8, 9]. Bet v1, the main allergen of birch pollen has a molecular weight of 18 kDa [10]. On the other hand, allergen extracts are mixtures of different allergenic and non-allergenic proteins. As it can be seen from allergen lists [a], a birch pollen extract contains at least 6 different allergenic proteins: The major allergen Bet v1 (17 kDa), the minor allergen Bet v2 (a profilin with 15 kDa), Bet v3, Bet v4 (8 kDa) and Bet v6 (a isoflavone reductase homologous protein with 33.5 kDa) and Bet v7 (a cyclophilin with a molecular weight of 18 kDa). For a reproducible and controllable immobilization of such extracts on planar glass slides, it is important to standardize the used allergen extracts. The immobilization of some commercial extracts on the modified glass slides did not work properly. Therefore, we compared different commercial birch pollen extracts with a self-made extract considering the Bet v1 and total protein content. In the first step, we performed a purification of commercial birch pollen extracts by a gelchromatographic PD-10 column. The purification process was monitored collecting the eluate in a MTP (4 droplets/well). The different fractions have been analyzed for their protein contents as shown in Fig. 3.

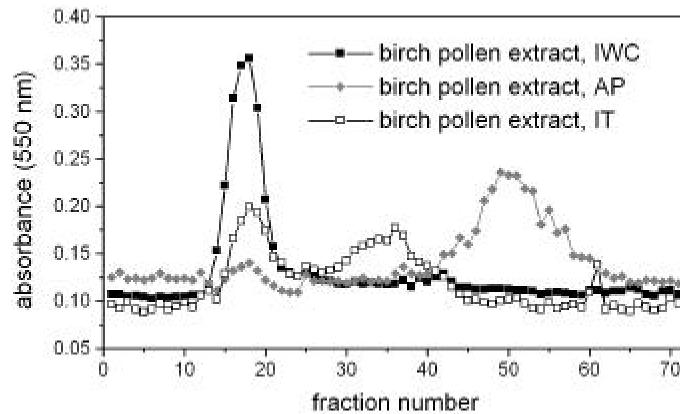


Fig. 3: Purification of different birch pollen extracts with a gelchromatographic column. BCA protein test.

The PD-10 column was equilibrated with 20 mL PBS (mobile phase). 500 µL allergen extracts were applied to the column. 4 droplets/well were collected. 10 µL of the plate was transferred to a new MTP using an 8-channel pipette. 200 µL BCA reagent were added to each well. After a reaction time of 30 min at 37 °C the purple color was measured at 550 nm.

In contrast to the commercial extracts, the self-made dialyzed extract (Fig. 3) showed only one protein peak in the expected region (fraction number 12-18). Using the commercial extracts another broad peak appeared in later fractions (fraction number 42-60). The Bet v1 SW-ELISA of the collected fractions (Fig. 4) showed that the main allergen of the birch pollen extract, Bet v1, is found in the fractions number 12-21.

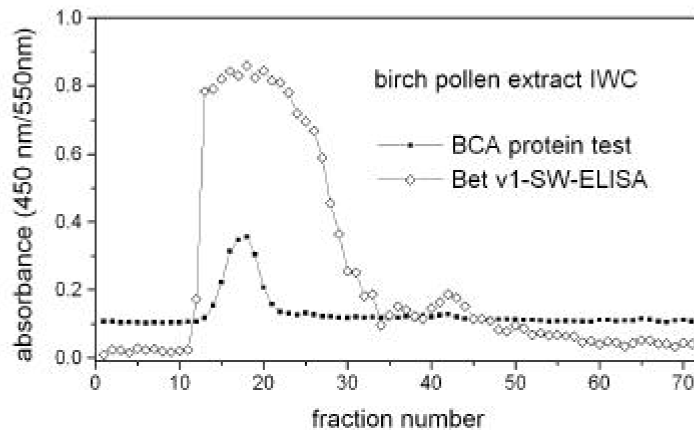


Fig. 4: Comparison between BCA and Bet v1-assay of the purification of the self-made birch pollen extract using a PD-10 column. 4 droplets/well were collected.

BCA-assay: 10 µL of the plate was transferred to a new MTP, 200 µL BCA reagent were added to each well. After a reaction time of 30 min at 37 °C the purple color was measured at 550 nm.

Bet v1-SW-ELISA: A MTP was coated overnight with a polyclonal anti-Bet v1-AB. After a blocking step with 1% BSA 90 µL PBS and 10 µL of the fractions were filled in the MTP and incubated for 1h at RT. The bound Bet v1 was detected using the mouse-anti Bet v1 and the anti-mouse-IgG-HRP system. The reaction was stopped after 5 minutes and the absorbance read at 450 nm.

As it can be seen from Fig. 4, the curve for the protein peak and the Bet v1 peak correlate in the case of the self-made extract. There is also a "real" protein peak in the protein-corresponding region. The prick test AP (Fig. 3) showed low signal intensity for the protein peak and a higher signal in the later fractions. Cross-reactivity of the BCA protein test with some additives in the extract solutions (e.g. phenol) might be the reason for the second peak in the BCA-protein trace. The very low intensity of the protein peak itself was due to a higher dilution (Tab. 1) of the commercial allergen extracts in comparison to the self-made one. To determine the Bet v1 and real protein content, 2 mL of commercial extracts were dialyzed under the same conditions used for the self-made extract and lyophilized. 200 µL PBS was added and the protein content was determined using the micro-BCA-assay. The protein content in the original 2 mL solution was recalculated from the 200 µL solution. The following Bet v1 and total protein contents (Tab. 1) could be obtained:

Table 1: Bet v1/total protein ratio in birch pollen extracts of different origin

Extract	**Bet v1 [1] [mg/L]**	**Protein Content [2] [g/L]**		**Bet v1/Protein Ratio [%]**	
		No Dialysis	Dialysis	No Dialysis	Dialysis
IWC	41 (28-53)	---	3.00	---	1.3
IT	2.1 (1.8-2.5)	3.6 ± 0.1	0.30	0.06	0.7
r-bio	7.5 (5.6-9.8)	7.4 ± 0.4	0.20	0.11	3.8
AP	5.2 (4.1-6.3)	3.9 ± 0.3	0.16	0.15	3.2

1: Sandwich-ELISA
2: Micro-BCA-assay

After dialysis all extracts have similar Betv1/protein ratios. Regarding the prick tests, it could be expected that these extracts are more diluted for their use in the *in-vivo* allergy diagnosis. The Bet v1/total protein ratio should be nearly the same for different birch pollen extracts presupposed that no additional protein (e.g. BSA) was added. Comparing the results with literature, the Betv1/total protein content with 0.7-3.8% is in the lower range of the literature results of 1-10% [11]. For long-term storage of allergen extracts, it is necessary to add some additives, like glycerol (50%), phenol (10%) or azide (1%). It is known that these additives have an influence on most protein tests and perhaps on the later covalent attachment on planar glass slides. Our experiments demonstrated that immobilization of amino-modified oligonucleotides [Fig. 5, 50 µM, *E.gal*-NH_2, 18-mer] and some allergen extracts (e.g. mite extracts, Fig. 7) on an epoxysilane-modified glass surface could be inhibited by addition of glycerol to the printing medium.

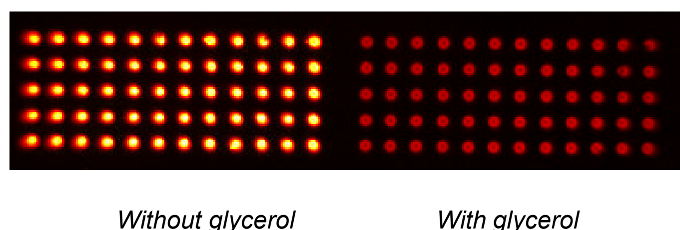


Fig. 5: Signal reduction by adding glycerol to the printing medium. 3-D-Link slide.

3-D-Link-slide. 50 µM E. gal-NH_2, 18-mer. Hybridization with DIG-labeled E. gal complementary oligonucleotide (0.1pmol/µL). Detection with anti-DIG-HRP AB. Chemiluminescence was accumulated for 30 s. Shown area approx. 2.5 x 0.6 cm, Spot distance 1.00 mm. Signal intensities shown in Fig. 6.

As seen in Fig. 5 and Fig. 6, addition of glycerol to the printing medium leads to a significant signal decrease with GOPS or 3-D-link slides. In comparison, the signal reduction using xenobind slides (Fig. 6) occurred only at higher pH-values.

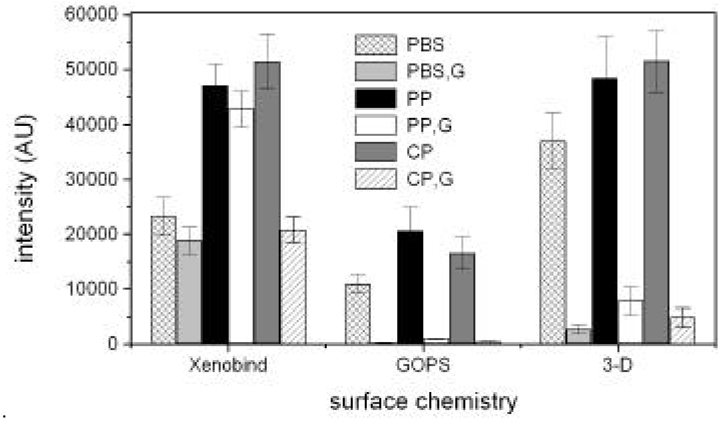


Fig. 6: Signal reduction by adding glycerol to the printing medium. Comparison between different glass slides and printing buffers. Test performance see Fig. 5.

PBS: phosphate buffered saline (pH 7.8), PP: phosphate buffered saline (pH 8.5), CB: carbonate buffer (pH: 9.6), G: glycerol 1%. Xenobind: aminobinding slide from Xenapore. GOPS: glycidyloxypropyltrimethoxysilane. 3-D: 3-D-link-aminobinding slide

Although the printing medium contained a certain concentration of glycerol (1%), most of the water evaporated and the substance was trapped in the concentrated glycerol droplet. Glycerol (viscosity of 954 $kgm^{-1}s^{-1}$, 25 °C [12]) has a 1000fold higher viscosity as water (0.8904 $kgm^{-1}s^{-1}$, 25 °C [12]) and therefore the diffusion of the substances to the surface is hampered. The pH-dependent signal reduction could also be caused by traces of impurities in glycerol.

As mentioned above, *in-vivo* allergy diagnosis often is performed by using prick tests. The respective solutions are relatively diluted and often contain 50% glycerol and 10% phenol. The direct immobilization of mite prick test solutions (r-bio) on 3-GOPS modified glass slides resulted in very low signals (Fig. 7) in comparison with the mite extract from IT (without glycerol), although the concentration of Der p1 in the prick test is higher according to the SW-ELISA for Der p1 (Fig. 8).

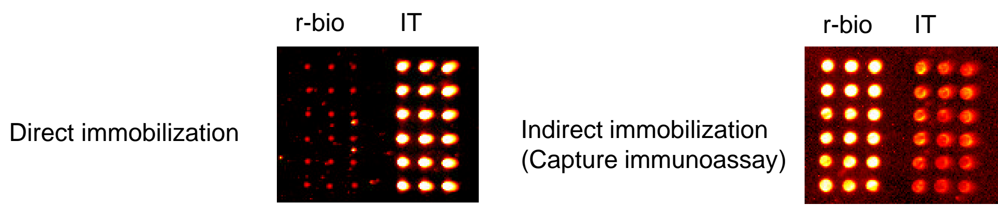


Fig. 7: Comparison between the chemiluminescence signal of direct and indirect immobilization of two different mite extracts (r-bio: prick test, IT: mite extract without glycerol) on GOPS modified glass slides.

Spot distance 1.00 mm. Shown area approx 0.8 x 0.6 cm. Direct test format: Mite extracts were immobilized on GOPS slides (5 droplets). Blocking: 1% BSA in PBS. Biotin-labeled mouse anti-Der p1 AB (4C1)-HRP-Streptavidin.

Indirect test format: Immobilization of monoclonal anti-Der p1 AB M5H8 (1:100 in CB, 20 droplets). Mite extracts were immobilized at the same position used for AB immobilization (5 droplets). Biotin-labeled mouse anti-Der p1 AB (4C1)-HRP-Streptavidin. Chemiluminescence was accumulated for 60 s.

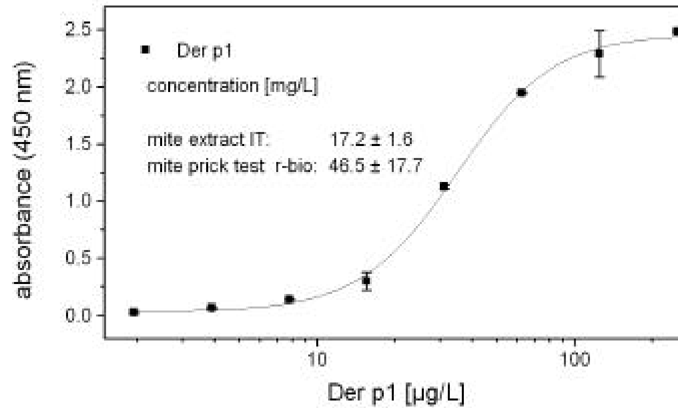
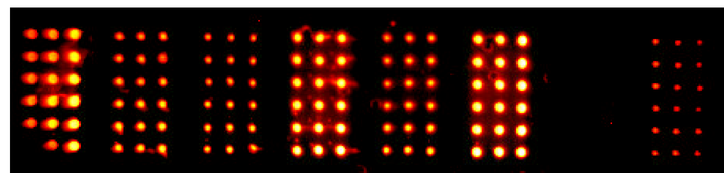


Fig. 8: Determination of the Der p1 contents of two commercial mite extracts using the mite Der p1 assay from Indoor Biotechnologies.

One possibility to use prick tests on planar modified glass slides is to use the sandwich-ELISA format on the chip. Unfortunately, not for all relevant allergenic proteins the corresponding monoclonal antibodies are commercially available. Nevertheless, the use of the capture immunoassay format is a good alternative for the immobilization of a few allergens that are difficult to immobilize by the direct way.

On-chip biotinylation

A blank spot on a multiallergen chip after the allergy test could be caused either by insufficient immobilization of the corresponding protein or by the lack of allergen specific IgE-ABs in the patient serum sample. The first step for a quality control of the allergen chip might be a visual inspection that proteins of all used allergen extracts had been immobilized on the modified glass surface. We have tried to detect the immobilized proteins using commercial protein tests (BCA, Coomassie, silver). All tests were too insensitive. Therefore, an on-chip biotinylation of the immobilized proteins was performed as described in materials and methods. The best test results considering signal to background ratio (S/B) could be obtained using the GOPS slides as shown in Fig 9.



Allergen	BSA	Birch	MW	TI	RW	OG	Apple	Kiwi
S/B:	3.6	3.6	3.5	4.9	3.7	5.3	1.2	2.6

Fig. 9: On-chip biotinylation of immobilized allergen extracts on a GOPS modified glass slide.

Chemiluminescence was accumulated for 60 s. BSA: bovine serum albumin, The following extracts were used: Birch: birch, MW: mugwort, TI: timothy, RW: ragweed, OG: orchard grass, Apple: apple, Kiwi: Kiwi.
Shown area approx. 3.1 x 0.8 cm. Spot distance 1.00 mm.

However, the apple extract showed a very low signal-to-background ratio. Either immobilization of apple allergens is not efficient enough under the chosen conditions or the extract had a lower amount of free amino groups available for biotinylation. Another point to consider is the fact that the protein content between the different allergen extracts differs, especially the concentration of relevant proteins. At present, it is only possible to adjust the total protein concentration. However, as a non-selective test, the on-chip biotinylation in combination with other selective tests (allergen-specific ELISAs) is a suitable approach for the quality control of an effective immobilization of proteins on the chip surface.

Determination of the total IgE level and calibration of the test system

The Pharmacia CAP system for total and specific IgE [14] uses human serum samples with known IgE levels (2, 10, 50, 200, 1000, 2000 kU/L calibrated against the WHO reference standard 75/702) to classify test results in different RAST classes. At the moment, the PASA system has only one reaction chamber. Therefore, it was not possible to measure several serum samples at a time. To have a Pharmacia-CAP equivalent calibration strategy, the reaction chamber would have been divided into at least 7 different chambers. Calibrating the test results by direct immobilization of different concentrations of myeloma IgE on the chip could be a solution.

Table 2: RAST-class system (14)

RAST	kU/L	IgE [µg/L]	Significance (Level of allergy)
0	< 0.35	< 0.8	Absent or undetectable
1	0.35 - 0.69	0.8 - 1.6	Low
2	0.70 - 3.49	1.6 - 8.3	Moderate
3	3.50 - 17.50	8.3 - 42	High
4	17.50 - 52.5	42 - 126	Very high
5	52.5 - 100	126 - 240	Very high
6	> 100	> 240	Very high

1 IU of IgE is defined as 2.4 ng IgE. RAST class 1 starts at 0.8 µg/L IgE.

Adapting the direct myeloma immobilization on GOPS modified glass slides the following CCD picture and calibration lines (Fig. 10/Fig. 10a) could be obtained

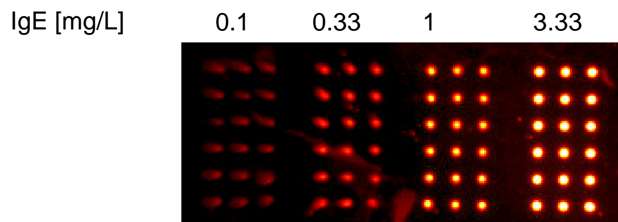


Fig. 10: CCD-Image of different myeloma-IgE concentrations immobilized on GOPS modified slides.

Shown area approx. 1.7 x 0.7 cm , Spot distance 1.00 mm

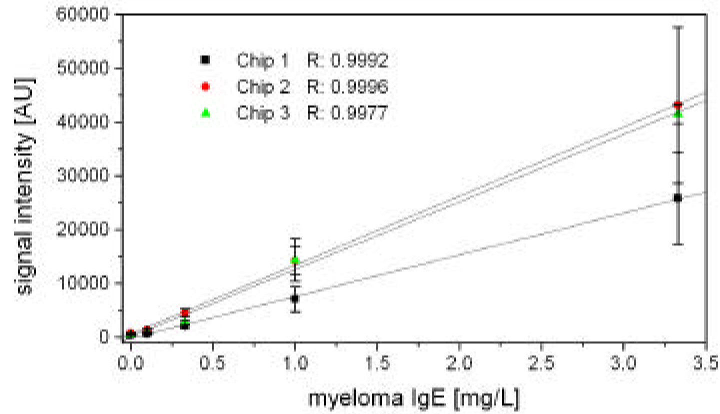


Fig. 10 a: Determination of the chip-to-chip variation based on directly immobilized myeloma IgE.

Myeloma-IgE (0.1 - 3.3 mg/L) was immobilized on GOPS modified glass slides. Blocking: 1% BSA in PBS. Detection: HRP-labeled goat-anti-human-IgE 1:500 in PBS/BSA/Tween. Chemiluminescence was accumulated for 30 s. Error bars 1s, 18 replicates/concentration.

The chip-to-chip variation based upon immobilized myeloma IgE (Fig. 10 a) was up to 50%, although there was a linear relationship between IgE concentration and signal intensity. Signal intensities obtained by reproducible immobilization of myeloma IgE could be used as an internal chemiluminescence signal classification of the test results. In addition immobilized myeloma IgE is, as seen in Fig. 10, a good quality control for the function and stability of the peroxidase labeled anti-human-IgE AB and the chip-to-chip variation of the allergen chip.

Another possibility to calibrate measurements is to measure serum samples of known RAST classes and to compare the result of the unknown sample with these results. As shown in Fig. 11 it is possible to distinguish between different total IgE concentrations.

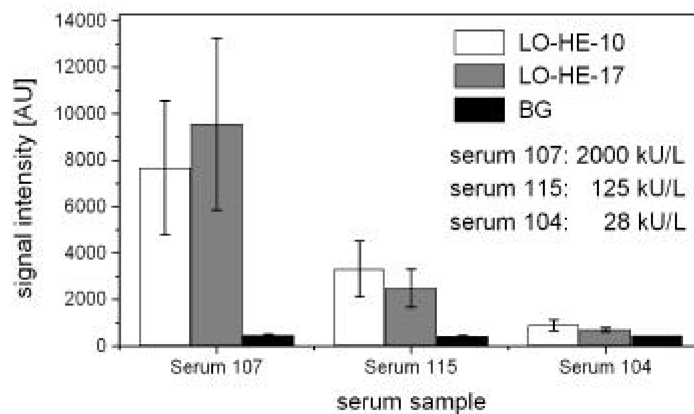


Fig. 11: Measurement of serum samples of different RAST classes.

Monoclonal anti-human-IgE-ABs (LO-HE-10/LO-HE-17) were immobilized on GOPS modified glass slides. Blocking: 1% BSA in PBS. Serum dilution: 1:10 in PBS/BSA/Tween. Detection: HRP-labeled goat-anti-human-IgE 1:500 in PBS/BSA/Tween. Chemiluminescence was accumulated for 30 s. BG: Background. Error bars 1s, 60 replicates/AB.

The lowest IgE concentration distinguishable from the background level was 28 kU/L total IgE. Although the signal intensities between the samples deviate from the expected ratio, it is common in immunological detection systems that the response factor is not linear. Nevertheless, it is possible to classify serum samples in low, medium and high IgE levels.

Determination of allergen-specific-IgE levels

Different self-made and purified allergen extracts of grass and birch pollen were immobilized on GOPS and TMS activated glass slides with and without addition of glycerol to the printing medium. Using this self-made extracts positive signals for all immobilized allergen extracts could be obtained for both cases as seen in Fig. 12.

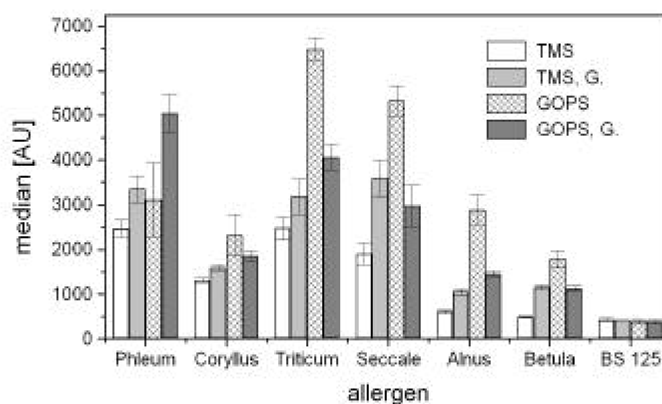


Fig. 12: Determination of allergen-specific IgE-ABs in a serum sample. Comparison between different surface treatments (TMS/GOPS). Influence of glycerol (G.) in the printing medium.

The following pollen extracts have been used: Phleum (timothy), Coryllus (hazel), Triticum (wheat), Seccale (rye), Alnus (alder), Betula (birch). A measurement of a negative control (serum sample negative against grass and birch pollen, BS 125, MAST) showed no positive signals on the corresponding chip. Extract concentration: 100 mg/L. Serum dilution 1:5. Detection with anti-human-IgE-HRP (1:500 in PBS/BSA/Tween). Chemiluminescence was accumulated for 90 s. Error bars 1s, 18 replicates/substance.

Using GOPS slides the obtained signal intensities were higher for birch, alder, wheat and rye. The other extracts showed equivalent test results. Addition of 1% glycerol to the printing medium had different effects depending on the surface chemistries. Using TMS slides the signal intensity increased, whereas using GOPS slides the signal intensity decreased. Working with grass or tree pollen extracts it is possible to use hydrophobic TMS slides and glycerol as stabilizer. Tests in our laboratory showed that direct immobilization of mite extracts could only be achieved using slides with aldehyde-, epoxy- or other activated groups for covalent attachment. The patient of the used serum sample was suffering under an atopical eczema and rhinitis allergica. The reference measurements showed that these symptoms are caused by a very high level of IgE against grass (e.g. Phleum, 11.5 kU/L = RAST class 4) and tree pollen (e.g. birch, 8.5 kU/L = RAST class 3) allergens. The hazel extract was not determined in the reference measurements. It is known that patients sensibilized against grass pollen allergens also show symptoms for cereal and tree pollen allergens [14]. Highly conserved profilins [15] expressed in nearly every pollen species were found to be the reason for this kind of cross-reactivity

between birch and other tree pollen allergens. Another point to consider is the lectin-carbohydrate cross reactivity [16]. Many plant proteins contain lectins reacting with the carbohydrate chains of the IgE-AB. In addition, sequential homologies have been found e.g. between the major birch pollen allergen Bet v1, the apple allergen Mal d1, the celery allergen Api g1, the hazelnut allergen Cor a 1 and different other allergens [17]. Signal intensities obtained by immobilization of recombinant (e.g. r Bet v1) or purified allergens on GOPS modified slides were higher and more reproducible as seen in Fig. 13 for honeybee-specific IgE tests. The negative control serum sample number MT 74 (tested negative against honeybee allergy) showed no signals in comparison to serum sample number MT 82 containing specific IgE ABs against honey bee.

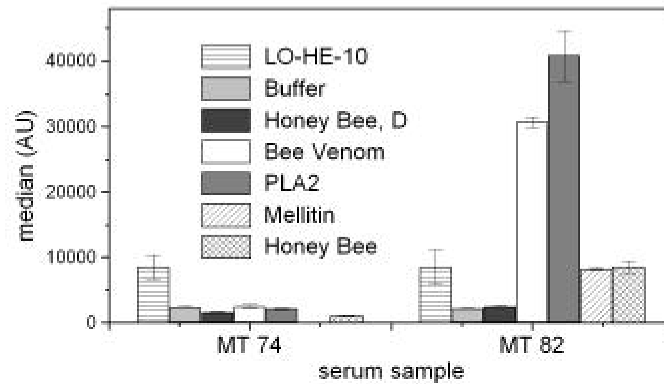


Fig. 13: Comparison between immobilization of honeybee extracts and purified proteins from honeybee using a positive IgE sample.

Phospholipase A_2 [20 mg/L], Honeybee extract: extracted with and without dialysis (D), Bee venom [100 mg/L], Mellitin [10 mg/L], Buffer: buffer substance without protein, LO-HE-10: anti-human-IgE-AB. Serum dilution 1:5 in PBS/BSA/Tween. Detection: anti-human-IgE-AB (1:500) in PBS/BSA/Tween. Chemiluminescence was accumulated for 90 s. Error bars 1s, 18 replicates/substance.

The detection limit of r Betv1-specific IgE in a birch pollen positive serum sample is shown in Fig. 14.

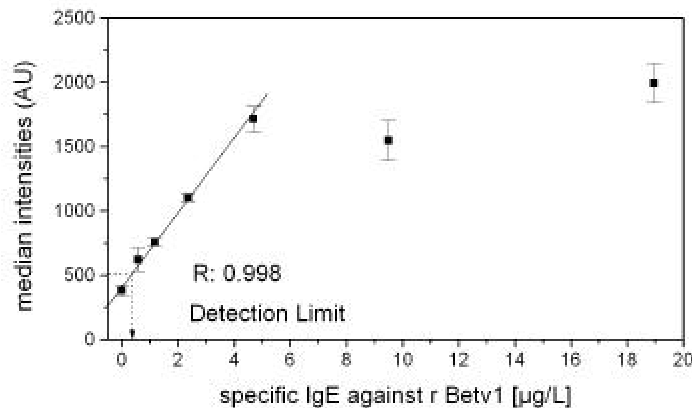


Fig. 14: Determination of the detection limit of r Betv1 specific IgE ABs in a patients serum sample.

r Bet v1 (67 mg/L) has been immobilized on GOPS slides. A birch pollen positive serum sample was diluted with the negative control BS 125. The chemiluminescence signal was accumulated for 60 s. Error bars 1s, 18 replicates.

A detection limit of 0.35 µg/L (3 s method) r Bet v1 specific IgE could be found. Therefore, it would be possible to distinguish between RAST class 0 and RAST class 1. Serum samples with higher IgE concentrations (> RAST 2) could not be classified directly, because the chip surface would be saturated. Appropriate dilution of samples would solve this problem.

Summary

Using self-made and purified allergen extracts, positive signals for tree and grass pollen, mite and honey bee specific IgE ABs have been obtained. The detection limit of Bet v1-specific IgE-ABs with recombinant Bet v1 was 0.35 kU/L. A combination of natural extracts and recombinant or purified allergens immobilized on one chip seems to lead to the best chip performance. This may allow a better diagnosis resulting in a better allergy treatment. Positive signals for allergen extracts could be broken down with the use of recombinant allergens. The immobilization of allergens could be controlled using the on-chip-biotinylation and allergen-specific ELISAs based on animal antibodies. At present, the PASA system can be used for a semi-quantitative screening of total and allergen-specific IgE levels in human serum samples. It is possible to distinguish between non-allergic and allergic patients and to classify them in three categories of low, medium and high IgE concentration. In addition, the volume of the serum sample for a test can be low (50-100 µL/test) and the assay can be performed within one hour. However, the surface chemistry and the test system (incubation time, dilution of the serum samples, calibration of the serum samples) have to be improved further.

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