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A lateral flow paper microarray for rapid allergy point of care diagnostics†

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There is a growing need for multiplexed specific IgE tests that can accurately evaluate patient sensitization profiles. However, currently available commercial tests are either single/low-plexed or require sophisticated instrumentation at considerable cost per assay. Here, we present a novel convenient lateral flow microarray-based device that employs a novel dual labelled gold nanoparticle-strategy for rapid and sensitive detection of a panel of 15 specific IgE responses in 35 clinical serum samples. Each gold nanoparticle was conjugated to an optimized ratio of HRP and anti-IgE, allowing significant enzymatic amplification to improve the sensitivity of the assay as compared to commercially available detection reagents. The mean inter-assay variability of the developed LFM assay was 12% CV, and analysis of a cohort of clinical samples (n = 35) revealed good general agreement with ImmunoCAP, yet with a varying performance among allergens (AUC = [0.54–0.88], threshold 1 kU). Due to the rapid and simple procedure, inexpensive materials and read-out by means of a consumer flatbed scanner, the presented assay may provide an interesting low-cost alternative to existing multiplexed methods when thresholds >1 kU are acceptable.

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Introduction

In recent decades, there has been a gradual increase in allergic diseases and atopic disorders such as asthma, particularly in Western countries.[1–3] It has been suggested that *in vitro* testing of specific IgE-reactivities can substantially improve the diagnostic accuracy and management particularly in primary care.[4] While specific IgE-testing may not be sufficient to predict clinical manifestations of allergy, it can be helpful for general practitioners, pediatricians and clinical allergy specialists to assess a patient's sensitivity profile and risk for developing severe symptoms such as anaphylaxis. Further, novel multiplexed strategies allowing the measurement and monitoring of a multitude of reactivities to correctly reveal a patient's sensitization profile may be helpful in improving epidemiological surveillance, examination of causal risk factors and assessment of individual disease management.[5] In order for such investigations of a broad sensitization profile of patients to be performed routinely in primary care, there is a need for assays that are rapid, inexpensive, easy to use yet with high multiplexing ability and retained assay performance. Today, gold standard methods for highly sensitive and accurate detection of specific IgE reactivities are for instance the single-plexed affinity chromatographic ImmunoCAP system and multiplexed array-based ImmunoCAP ISAC (Thermo Fisher), both requiring considerable investment in sophisticated instrumentation and a moderate-high cost/assay. In contrast, the ImmunoCAP "Rapid" lateral flow test for point of care application allows analysis of up to 10 specific IgE reactivities within <20 minutes but can hardly be scaled further up in multiplexing ability.[6] There is an active research of novel assays that may provide more rapid, more sensitive or improved multiplexed detection. For instance, a multiplex fluorescent suspension bead array assay was developed to sensitively measure the IgE levels in the serum of seven allergens within 6 hours.[7] Another recent suspension bead study demonstrated that fluorescent activated cell sorting (FACS) analysis could allow simultaneous analysis of IgE-reactivities towards up to 30 different allergens.[8] Further, recently a rolling circle amplification strategy has been used with a glass slide planar microarray setup to achieve improved sensitivity of three common allergy reactivities.[9] The costs associated with gold standard multiplexed tests or lab-based novel assays based on sophisticated equipment will likely be unrealistic for the majority of healthcare systems worldwide, particularly where cost-shared care is not available.[10]

In efforts to improve sensitivity in clinical immunology assays, a range of nanomaterial-enhanced methods have been

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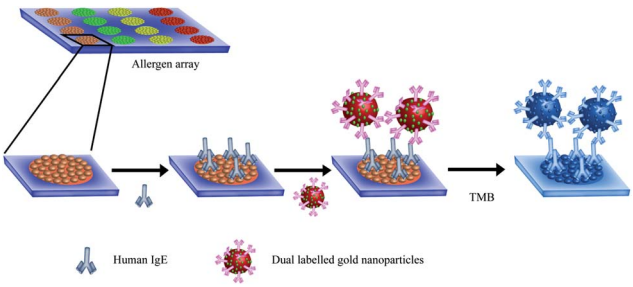
described.[11–14] More effective detection of IgE reactivities in clinical samples has for instance been demonstrated in a flow cytometric bead array setup.[3,8] Further, a metal enhanced fluorescent probe using silver nanoparticles for IgE detection was presented by Xia *et al.*[15] and Liang and colleagues have recently investigated a superparamagnetic nanoparticle-based lateral flow immunoassay for rapid detection of shellfish major allergen tropomyosin.[16] Finally, Bruno and colleagues reported that microchip integrated core–shell magnetic nanoparticles could be employed to detect specific serum IgE at a concentration of 1 ng mL^{-1} with a very low sample volume,[17] and similarly an allergen functionalized magnetic nanoparticle based diagnostic assay was developed to detect IgE-reactivity towards peanut in serum samples with sensitivity close to the ImmunoCAP assay.[18] Though the reported analytical methods may detect specific IgE at the ng mL^{-1} level, several disadvantages such as high cost, long assay times and single/low-plexed operation are evident. Consequently, we believe there is a need for point of care diagnostic equipment capable of comprehensive, multiplexing and scalable determination of a patient's sensitization profiles. In recent years a number of research groups have investigated a possible integration of planar microarray technology with microfluidic and micro/nanotechnology so as to provide assays amenable for point of care. In our group, we have previously developed a lateral flow protein microarray assay for rapid detection of specific IgG-response associated with bovine pleuropneumonia as well as in a highly multiplexed antigen microarray where we evaluated binding specificities of immuno purified polyclonal rabbit IgG.[19,20] While the lateral flow protein microarray assays developed in our group presented encouraging performance in terms of specificity, assay procedure simplicity, assay time and cost/assay, the sensitivity has been limited. Further, signal amplification in enzymatic catalytic reaction based immuno assays has been demonstrated by other groups, although not for multiplexed clinical applications.[21,22] Here, we present a scalable lateral flow allergen microarray assay for determination of patient specific IgE reactivities, with enhanced sensitivity due to the application of in-house synthesized dual labelled (horseradish peroxidase (HRP) and anti-IgE) gold nanoparticles (GNPs). Due to a ratio of 6 : 1 for HRP : anti-IgE, each bound GNP offers a significant amplification for the catalytic reaction. As a clinical model system, 35 pre-characterized human serum samples were analyzed using the LFM assay system with 15 component allergens in detecting specific IgE-reactivities. The schematic lateral flow microarray (LFM) assay procedure and amplification strategy is presented in Scheme 1. The assay is simple and convenient to perform, requires less than 10 minutes of time to complete and the gold/HRP-based colorimetric patterns may be detected using a consumer-grade flatbed scanner or a smartphone camera. Hence, we believe the presented assay could in the future find use as a bridging option where comprehensive testing of IgE reactivities is needed, but when cost-efficiency, portability and ease of use are of essence.



Scheme 1 Schematic overview of paper based lateral flow allergen microarrays. The clinical serum sample is flown through the different allergens spotted nitrocellulose membrane and IgE antibodies will attach to specific allergen spots. Followed by washing, exposed with dual labeled GNP and finally substrate TMB is added to improve the signal intensity of the different allergen spots.

Experimental section

Materials

Chloroauric acid ($HAuCl_4$), trisodium citrate, HRP, 3,3′,5,5″-tetramethylbenzidine liquid substrate for membrane (TMB), Tween20, bovine gamma globulin, bovine serum albumin (BSA), sucrose, trehalose, and NaCl were purchased from Sigma Aldrich. Nitrocellulose membrane HF090MC100 was obtained from Millipore. All used chemicals were of analytical grade and were applied without further purification.

The complete LFM assay was performed using assay buffer containing 0.5% Tween20, 0.45 M NaCl, 0.05% bovine gamma globulin, together with 3% BSA and 1% sucrose in 0.1 M phosphate buffer (PB) at pH 7.4. Finally, other buffers such as storage buffer (1% BSA, 5% sucrose, and 5% trehalose in 0.1 M PB) and washing buffer (0.1% BSA, in 0.1 M PB, pH 7.4) were used in this study.

All the human sera used in this study ($n = 35$) were selected for good sensitization profile coverage and kindly provided by Thermo Fisher Scientific (Uppsala, Sweden-division, formerly Phadia) with corresponding ImmunoCAP data. Validated purified allergen components and monoclonal anti-human IgE were also provided by Thermo Fisher Scientific.

Synthesis of gold nanoparticles

25 nm GNPs were prepared using a standard citrate reduction method.[23] In brief, 50 mL of 0.01% aqueous $HAuCl_4$ solution was heated to 100 °C under refluxing conditions. During vigorous stirring, 750 μL of 1% sodium citrate was rapidly added, such that the yellow solution became finally dark red indicating the end point. The mixture was then kept at 100 °C for 15 minutes, followed by continuously stirring at room temperature until cooling. The prepared nanoparticles were characterized by UV-vis spectral analysis (The SpectraMax® M5 Multi-Mode Microplate Reader) and High Resolution Transmission Electron Microscopy (HR-TEM, JEM-2100F instrument).

Preparation and optimization of anti-human IgE and HRP labelled GNP conjugates

A salt induced GNP aggregation test was performed as described earlier in order to optimize the assay conditions for the

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preparation of the antibody and HRP conjugated GNP.[24] Different pH-adjusted GNPs (at a concentration of 0.4 A530 units per mL) were mixed with antibodies of 10 μg mL^{-1} in a 96-well microplate. The mixture was kept at room temperature for 30 minutes with stirrer and subsequently 20 μL of 10% sodium chloride was added to each well and the reactants were agitated and kept at room temperature for 10 minutes. The final colour change was monitored by the absorbance at 530 and 620 nm (by means of a SpectraMax® M5 Multi-Mode Microplate Reader) and the optimum pH was observed (see ESI†). Similarly, the concentration of the antibody needed for full saturation of the GNP surface was quantified by adding 10 μL of serial concentrations (0–100 μg mL^{-1}) of anti-human monoclonal IgE antibody in a 96 well plate containing 100 μL of GNPs adjusted to an optimal pH of 8.2. Following the incubation, 20 μL of 10% sodium chloride was added to each well and finally the differential absorbance of the reactant was measured. In addition, the concentration of HRP required to saturate the remaining sites on partially antibody-coated GNP (using 50% of the optimum antibody concentration) surface was analogously investigated.

Dual labelled GNP conjugate preparation

The dual labelled anti-human IgE + HRP GNPs were synthesized under optimized conditions (see ESI, Fig. S1†). In brief, 1 mL of (0.4 A530 units per mL) GNP was mixed with 100 μL of 30 μg mL^{-1} anti-human IgE antibody kept at room temperature for 30 minutes on gentle stirring. Subsequently 100 μL of 0.2 mg mL^{-1} HRP was added and the suspension was kept at room temperature for 2 hours. The conjugate suspension was then centrifuged at 10 000 rpm at 4 °C for 10 minutes and the supernatant was discarded. The pellet was resuspended in washing buffer and centrifuged at 10 000 rpm at 4 °C for another 10 minutes. The washing and centrifugation procedure was repeated twice. Finally, the supernatant was removed and the end volume adjusted to 100 μL with storage buffer and the conjugate suspension was kept at 4 °C prior to use.

Paper based LFM preparation

The LFM was prepared as described by Gantelius and colleagues.[19] Briefly, as surface materials for the microarray patterning, nitrocellulose strips High Flow Plus HF090MC100 (Millipore) were used. The strips were prefabricated with a cardboard backing and were cut into smaller segments of 1 cm width and 2.5 cm length and each strip was attached with a shelf-glue on a microscopic glass slide. For patterning, a Nanoplotter 2.0 (Gesim) was used, and one drop of approximately 300 pL was deposited on each spot. The array layout consists of 15 rows of allergens with four downstream identical spots for each allergen and three rows of human IgE as a positive control with four serial dilutions spotted on both the corner and middle of the array strip (see ESI, Fig. S2†). Prior to performing the assay, a strip of adhesive tape of approximately 2 mm width and 10 mm length was placed on one end of the strip to create a flow barrier, with room for a sample application window which could harbor at any time no more than 30–50 μL of sample (in form of a liquid bulge). Thus, the sample could only travel below the flow barrier through the membrane and not on top of the strip. A patch of coarse membrane (Whatman) was placed at the other end of the strip and held in place with the help of a small metal weight of 100 g to provide a sink boundary for the capillary flow.

LFM assay for the detection of allergens

Initially, 30 μL of assay buffer was applied in the sample application zone in order to block the membrane and reduce non-specific binding. Subsequently, 30 μL of serum sample was applied, followed by a washing step with assay buffer. When the sample application area lost all visual signs of wetting, which was observed as a clear visible transition, 30 μL of dual labelled GNPs (OD 2.0 A530) was applied. In comparison, in a parallel experiment 30 μL of commercial anti-human IgE gold conjugates (BBI) was applied (OD 2.0 A530). Following another washing step with assay buffer, the strip was finally exposed to 30 μL of TMB chromogenic substrate for HRP amplification. One pooled positive control and one clinical serum sample were used and analyzed in triplicate to investigate the reproducibility and efficiency of dual labelled GNP based LFM assay.

Data analysis

Once the LFM was dried, a grayscale (600 dpi) scan of the strip was made using a HP-scanjet 8270 in combination with Vuescan software (Hamrick). The scanned image was inverted and subsequently loaded in Genepix 5.0 (Axon laboratories) to analyze intensities of all spots (spot size 120 μm). Subsequently, the data were imported in Matlab (Mathworks). The mean intensities of each allergen spot were extracted and the average of the four replicate allergen spots was taken. Since background intensities varied, the mean intensity of each allergen spot was divided by its background. After that the average of the four spots were taken and further analysis was performed.

Results

Characterization of dual labelled GNP conjugates

Spectrophotometric analysis of GNPs before and after coupling with the antibody and HRP indicated that the absorption maximum shifted from 530 nm to 540 nm upon coupling (Fig. 1A). Hence, UV-spectral analysis confirms the coupling of the antibody with the nanoparticles. The FE-TEM image shows that the GNPs have an average diameter of 26 ± 0.2 nm and after the antibody and HRP coupling the average diameter was 29 ± 0.2 nm (Fig. 1B and C). Also grayish halos around the modified GNPs were observed, indicating that biomolecules were bound to the GNP surface.[24] From GNP conjugation optimization it was found that a starting concentration of 3 μg mL^{-1} of the anti-human IgE antibody could be fully conjugated on 1 mL of GNPs, followed by coating of remaining GNP surface sites with 20 μg mL^{-1} enzyme HRP (ESI, Fig. S1†).

Efficiency of dual labelled GNPs

The detection efficiency of the dual labelled GNPs based LFM was analyzed as compared with commercially available 40 nm

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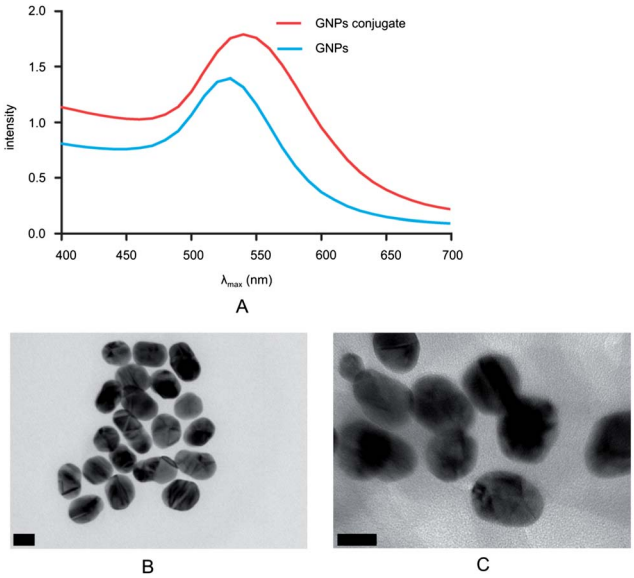

Fig. 1 UV and FE-TEM analysis of GNP conjugates. (A) UV spectra of GNPs (530 nm) and dual labelled gold conjugates (540 nm). (B) FE-TEM images of GNPs (26 ± 2 nm) and (C) dual labelled GNPs (29 ± 2 nm). The scale bar is 20 nm.

anti-IgE gold conjugates (OD 2 at 530 nm British Biocell International (BBI)). In Fig. 2A, the results from a LFM-experiment with different beads are shown. Due to the addition of the substrate, the background of LFM with homemade beads with the added substrate (HMBS) for enzyme activation is higher than it is for solely homemade beads (HMB) or commercial beads (CB). After addition of the substrate, an increase in the mean intensities of the allergen spots was found, with a bias towards amplification of allergen spots with a previously weak signal (Fig. 2B and C), whereas for allergens with higher ImmunoCAP values the differences between HMBS and CB were small. This is possibly due to partial saturation of the spot intensities at higher ImmunoCAP values.

The coefficient of variation (CV) was calculated as the standard deviation divided by the background-subtracted mean of signal intensities for the different bead types, employing one clinical sample and one pooled positive control sample (Table 1). Since the backgrounds were not the same for the LFMs with different beads, normalization of the mean intensity signal was done by dividing the spot intensity by the background of that spot (as determined by Genepix). The measured average CV values of HMBS observed at an acceptable range (less than 15%) as compared to CVs of the HMB and CB were 13 and 14, respectively.

Evaluation of clinical samples

Initially, the LFM-assay was evaluated using a pooled positive control and a negative control sample. In Fig. 3A, the relationship between the LFM analysis and two other methods (ImmunoCAP and ImmunoCAP Rapid, both products of Thermo Fisher Scientific) for the pooled positive control sample is shown. The mean intensity value corresponds with both methods and shows a linear relationship.

Besides the positive and negative control samples, 35 clinical samples from a variety of patients with different specific IgE reactivities were tested with the LFM. Fig. 3B shows a scatter plot between the mean intensities and ImmunoCAP values of the samples. A linear relationship between the ImmunoCAP values and mean intensities can be seen for ImmunoCAP values above 1 kU (average of allergens R^2-value 0.51). The low average R^2-value could be explained by the differences in assay principles and immobilization strategy between the LFM and the ImmunoCAP, as well as by the variation in performance among allergens, varying between 0.20 (allergen t9) and 0.83 (allergen m6) (ESI, Table S1†). For ImmunoCAP values lower than 1.0 kU, no relationship can be found between the values, indicating that the LFM assay is as expected less sensitive than the ImmunoCAP. ROC curves were made using the corrected mean intensity value and the area under each curve (AUC) for each allergen was measured for different thresholds of the

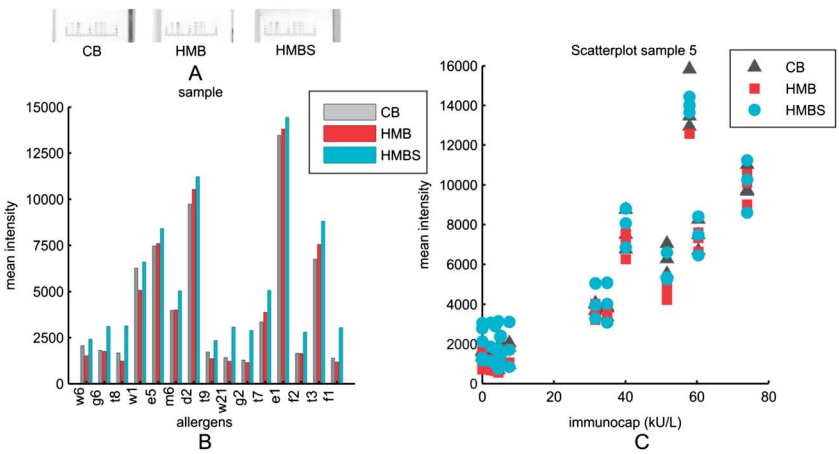

Fig. 2 The influences of different beads on the signal intensity. (A) The lateral flow tests from left to right with the commercial beads (CB), home-made beads (HMB) and home-made beads with substrate addition (HMBS). (B) The mean intensity of the lateral flow tests measured as a function of the allergen. (C) Scatter plot showing the mean intensity of the sample with respect to the ImmunoCAP values (triplicate).

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Table 1 The coefficient of variation of lateral flow microarray allergy assay using different beads[a]

	Allergen															
	w6	g6	t8	w1	e5	m6	d2	t9	w21	g2	t7	e1	f2	t3	f1	Average
Positive control																
CB	0.08	0.07	0.04	0.06	0.11	0.04	0.05	0.06	0.04	0.03	0.02	0.04	0.05	0.05	0.06	0.05
HMB	0.04	0.01	0.11	0.05	0.04	0.05	0.04	0.04	0.12	0.04	0.04	0.05	0.04	0.06	0.07	0.05
HMBS	0.05	0.01	0.08	0.14	0.04	0.05	0.03	0.03	0.10	0.08	0.00	0.06	0.03	0.07	0.10	0.06
Sample 5																
CB	0.20	0.23	0.20	0.17	0.11	0.12	0.13	0.08	0.08	0.17	0.13	0.11	0.06	0.13	0.23	0.14
HMB	0.01	0.22	0.06	0.22	0.17	0.16	0.16	0.08	0.09	0.14	0.15	0.17	0.09	0.15	0.05	0.13
HMBS	0.06	0.09	0.23	0.19	0.08	0.03	0.09	0.10	0.15	0.15	0.06	0.12	0.09	0.14	0.23	0.12
Average	0.07	0.11	0.05	0.18	0.13	0.11	0.14	0.07	0.05	0.13	0.15	0.14	0.09	0.14	0.03	

[a] CB = commercial beads, HMB = home-made beads and HMBS = home-made beads with the addition of a substrate.

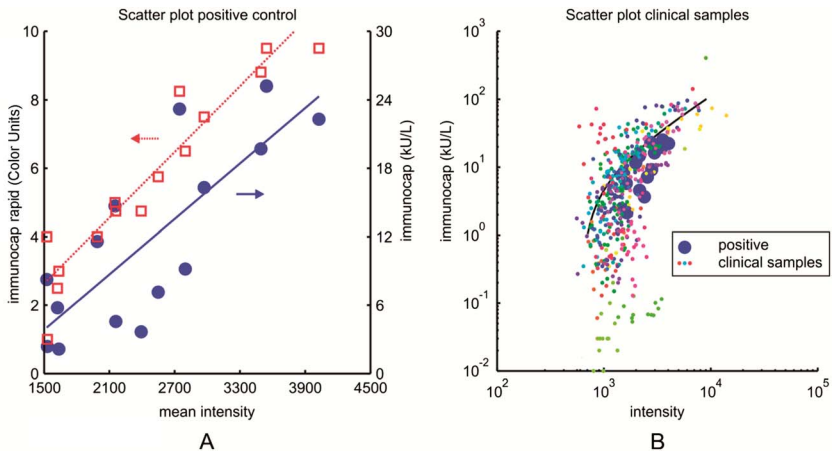

Fig. 3 Scatter plots of the commercial results and the mean intensity obtained with the lateral flow test. (A) Scatter plot of the positive control samples performed with LFM and compared with both ImmunoCAP rapid (left *y*-axis, squares) and ImmunoCAP (right axis, circles). (B) Relationship between 35 clinical samples obtained with the commercial test and the lateral flow test. Each colour shows a different sample.

Table 2 The area under the ROC for the different allergens using different thresholds

	Threshold					
Allergen	0.1	0.5	1	5	10	20
w6	0.652	0.728	0.679	0.744	0.788	0.588
g6	0.833	0.837	0.879	0.940	0.969	0.931
t8	0.852	0.850	0.768	0.711	0.902	0.779
w1	0.852	0.750	0.750	0.849	0.873	0.823
e5	0.618	0.618	0.600	0.863	0.788	0.826
m6	0.710	0.677	0.806	0.926	1.000	1.000
d2	0.648	0.734	0.748	0.910	0.920	0.899
t9	0.689	0.692	0.665	0.725	0.815	0.734
w21	0.719	0.656	0.535	0.568	0.745	0.941
g2	0.839	0.665	0.804	0.794	0.829	0.788
t7	0.717	0.755	0.755	0.792	0.851	0.781
e1	0.858	0.818	0.753	0.872	0.842	0.823
f2	0.583	0.649	0.638	0.745	0.701	0.813
t3	0.744	0.731	0.731	0.786	0.870	0.883
f1	0.542	0.739	0.738	0.775	0.788	0.794

ImmunoCAP value (Table 2 and Fig. 4). The correlation in terms of AUC ([0.54–0.88], average 0.72) varied considerably depending on the individual allergen performance, and higher thresholds yielded improved AUC-values as expected.

Discussion

In this work, we have constructed and evaluated a 10 minute inexpensive lateral flow allergen microarray assay with enhanced sensitivity due to the implementation of dual-labelled GNPs and an enzymatic amplification step. A panel of 15 allergen components was used for the construction of the array and 35 clinical samples with varying sensitivity patterns were analyzed and the results compared with ImmunoCAP. Further, a novel in-house synthesized dual-labelled GNP reagent carrying anti-IgE as well as HRP for enhanced signal amplification was evaluated in comparison with a commercial alternative. Encouragingly, the in-house made particles generated improved signals overall, and particularly resulted in higher sensitivity among the low intensity reactivities. While the

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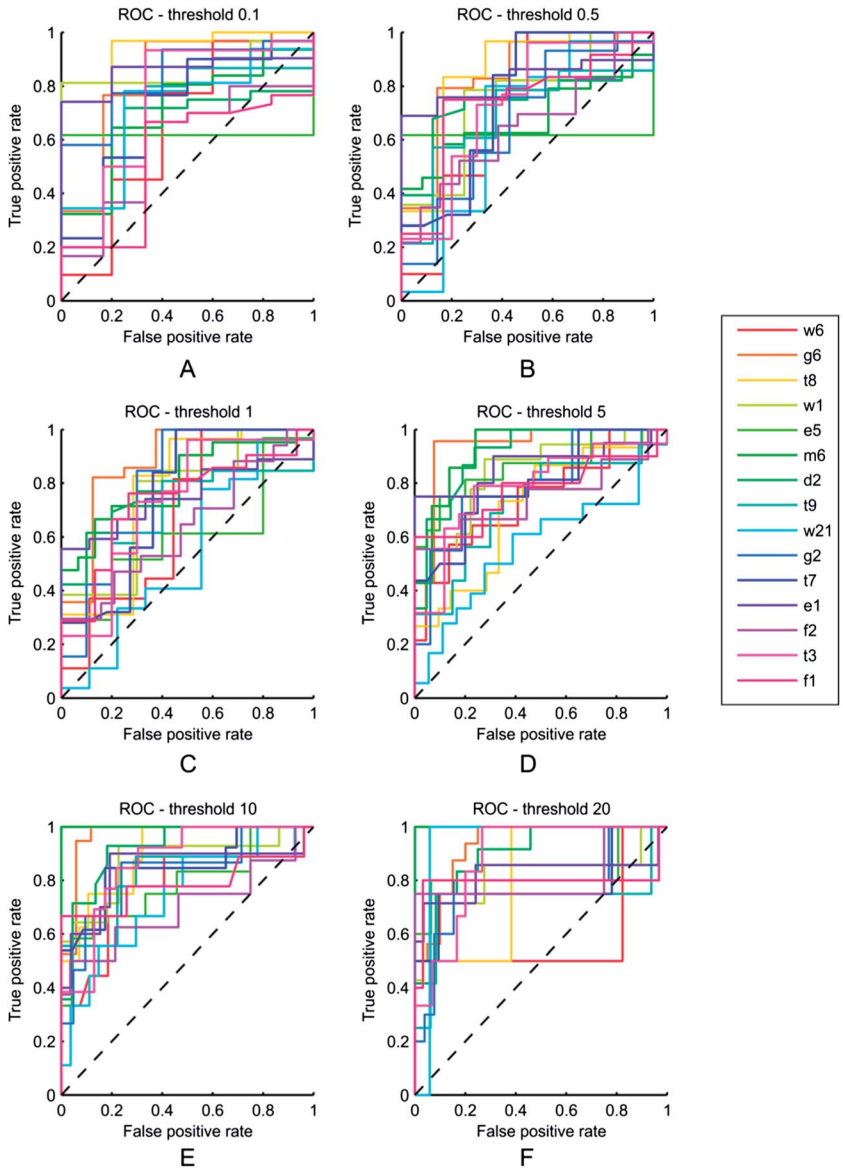

Fig. 4 The ROC curve of the clinical samples with varying thresholds: (A) 0.1, (B) 0.5, (C) 1.0, (D) 5, (E) 10 and (F) 20 kU L^{-1}.

ImmunoCAP results were as expected in general the most sensitive, a moderate/good correlation was found (average R^2-value = 0.51, average AUC = 0.72) when a threshold value of 1 kU was used. Importantly, the correlation was dependent on the type of allergen tested, with some allergens performing much better than others (R^2-value varies between 0.20 and 0.83). The considerable difference in performance between allergens has also been reported in other comparative studies such as for a recently presented fluorescent multiplex array[7] where the reported R^2-values were between 0.19 and 0.77. The excellent performance for some allergens in this study suggests that future optimization in for instance the printing buffer composition and allergen concentration for the weak-performing allergens could be valuable. Further, as the presented LFM assay studies on different assay principles as compared with the ImmunoCAP, and since variability in the deposition efficiency and functional performance of deposited allergens is expected, we believe that an excellent linear regression cannot be expected and that it is most relevant to observe the binary correlation pattern from the ROC-analysis. While it is likely that some clinical applications will require better sensitivities than 1 kU, the demonstrated performance would still provide valuable clinical support in for instance primary care patient management.[25] For the enzymatically amplified results, the mean variability between experiments was found to be lower than 15%, which should allow at least a semi-quantitative assessment of the specific IgE reactivities. Previous studies have suggested that microarray-based detection of specific IgE-reactivities with an even higher mean variability (15 to 33%) can be deemed highly reproducible and valuable options in the clinical diagnostics of allergies.[26,27] In this study, the array was limited to 15 allergen components. However, as demonstrated previously by

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our group, the LFM may be easily scaled to at least 384 different affinity binders, and in a future study we will attempt to incorporate full ISAC panels to assess the very high multiplexing ability of the assay.

Conclusion

We have successfully constructed and evaluated a novel scalable lateral flow allergen microarray with enhanced sensitivity based on an in-house developed nanoparticle-based amplification strategy. Here, the assay was easy to perform and the results were ready within 10 minutes of assay time. An agreement with ImmunoCAP data was found when a threshold of 1 kU was applied. Due to the portability, simplicity, rapid procedure, convenient read-out options and low materials cost, we hope that the assay may find use in contexts that require comprehensive measurements of IgE reactivities but where resources cannot stand the costs and requirements of gold standard technologies.

Acknowledgements

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Electronic Supplementary Material (ESI) for Analyst.
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Supporting Information

A lateral flow paper microarray for rapid allergy point of care diagnostics

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Figure S1

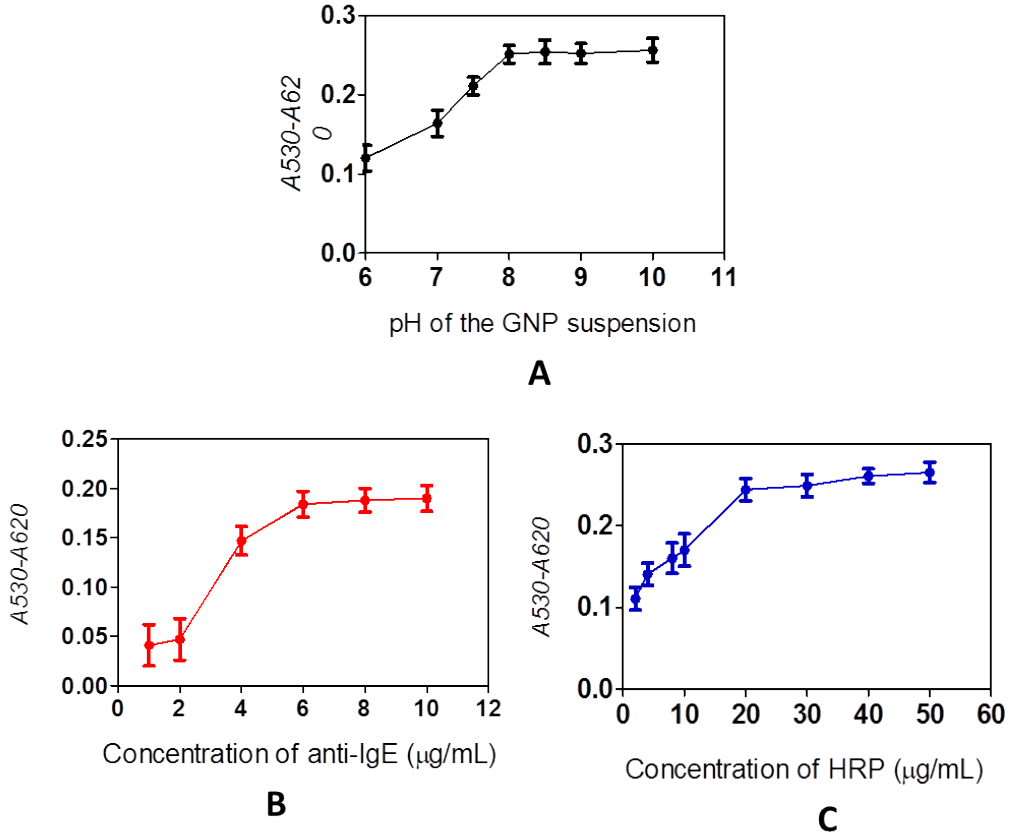

Figure S1. Optimization of assay condition for preparation of dual labelled GNPs conjugate. (A), pH optimization for preparation of gold nanoparticles conjugates. (B), Quantification of the anti-Human IgE antibody and (C), HRP saturated on GNPs.

The optimum pH for efficient conjugation of biomolecules on GNPs was obtained by measuring the differential absorbance (A530–A620) of GNPs conjugates (see figure S1). The maximum rate of A530–A620 was reached at pH 8, whereas at pH 6 and 7 the absorbance rate was less than obtained at pH 8 and there was no measurable change observed from pH 8 to 10. Correspondingly, the amount of anti-human IgE antibody saturation of the GNP solution was quantified. It was observed that 6 μg/mL of anti-human IgE antibody was high enough to saturated 1 mL of GNPs, simultaneously for the conjugation used and examined was 3 μg/mL of anti-human IgE antibody. The remaining uncoated GNPs surface was coated with enzyme HRP and the concentration was determined that 20 μg/mL of HRP saturated completely GNPs surface.

Figure S2. The layout of the spots on the LFM

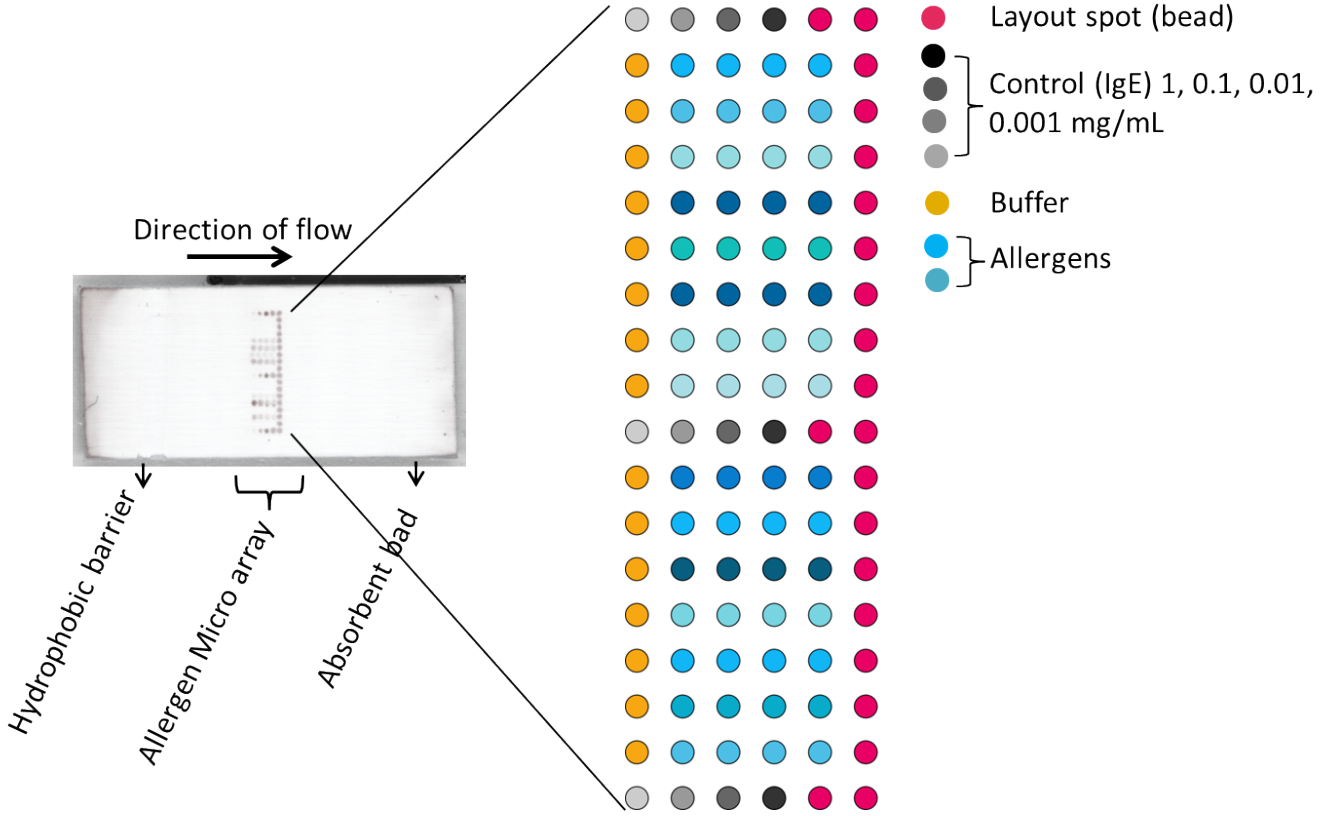

Figure S2. The layout of the spots on the LFM, the fluid is flown from left to right. A schematic overview of the assay layout is given. It consists of buffer, control, frame and allergen spots. The frame spots are useful to indicate where the spot array is positioned on the strip, making the Genepix analysis easier. The control spots should give a visible signal, otherwise the test was not successful. The buffer spots are placed at the beginning of the array and should not contain any signal after the analysis.

Table S1. The R^2 values of the scatter plot for the different allergens based on mean intensity.

	Allergen														
	w6	**g6**	**t8**	**w1**	**e5**	**m6**	**d2**	**t9**	**w21**	**g2**	**t7**	**e1**	**f2**	**t3**	**f1**
R^2-value	0.33	0.74	0.26	0.77	0.70	0.83	0.54	0.23	0.10	0.36	0.38	0.52	0.81	0.45	0.62
R^2-value*	0.37	0.73	0.21	0.79	0.70	0.83	0.51	0.20	0.23	0.36	0.36	0.50	0.82	0.451	0.59

* only for positive samples (ImmunoCAP > 1)