

A micro-ELISA system for the rapid and sensitive measurement of total and specific immunoglobulin E and clinical application to allergy diagnosis†

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We have developed a novel, practical micro-ELISA system for sensitive and rapid allergy diagnosis. The enzymatic reactions occurred under stopped-flow conditions, resulting in both high precision and high sensitivity. A BSA-biotin-avidin linker was introduced for the immobilization of water-soluble allergens on polystyrene microbeads, enabling immobilization of allergens in sufficient density to provide high sensitivity. Evaluation of the system's performance showed a good detection limit (2 ng/mL) for total IgE measurement. In addition, a good correlation with a conventional method (CAP method) was demonstrated using human serum samples from 85 allergy patients. Importantly, sample volumes (5 µL) were 10 times smaller and analysis time (12 min) was >20 times faster than the conventional method. All procedures were automatically regulated with our simple microfluidic system, and all the fluidic, optic and electronic components were integrated for portability. We believe that our system has the potential to become a very powerful tool, particularly for point-of-care diagnosis.

Introduction

In recent years the number of patients suffering from allergies has increased, and the importance of developing test systems measuring IgE levels in serum samples is now widely recognized. Allergies can have serious health consequences, and developing point-of-care (POC) diagnostic methods is imperative. In addition, a decrease in sample volumes and analysis time is advantageous, especially for pediatric allergy diagnosis.

During the last 20 years, a wide range of instrumental methods has been used for the determination of IgE as radioallergosorbent test (RAST), enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA) and fluorescence enzyme immunoassay (FEIA).¹ The majority of allergy testing methods are based on antibody receptor interactions. Specifically, FEIA and ELISA have been widely used as a sensitive and selective method for specific IgE measurement.^{2,3} Actually, the CAP system (Pharmacia) employing FIEA has been used in clinical routine.⁴ While it is possible to measure allergen-specific IgEs in the patient's blood, these tests suffer from a number of limitations.⁵ They are usually expensive, time-consuming (approx. 3–4 hours), requiring large volumes of reagents and serum sample (50 µL) as

well as complex liquid handling; thereby limiting their application for POC allergy diagnosis.

Miniaturized chemical systems on microchips are garnering great interest. Advances in integration of various chemical processes (including mixing, chemical reactions and separation) have progressed rapidly in the past decade.^{6,7} The benefits of miniaturization and integration include smaller sample and reagent volumes, more efficient reactions due to large surface-to-volume ratios, smaller space requirements and decreased cost. These advantages have led to microchip technology applications in various analytical procedures and chemical syntheses. For allergy diagnosis, a microsystem utilizing magnetophoretic immunoassay for measurement specific IgE was reported, and an excellent limit of detection (LOD) was demonstrated.⁸ However, the method needs complex and laborious bulk scale pretreatment as dilution of serum, reaction, centrifugation and temperature control due to the utilization of magnetic nanoparticles, and the automation is difficult. Microchip technology was only utilized for detection, and a relatively long time (30 min) was required.

To date, we have developed basic concepts for micro-integration by micro unit operations (MUO) and continuous-flow chemical processing (CFCP).⁹ These basic concepts have led to the successful integration of various analytical systems on microchips for environmental analysis, food analysis and single cell analysis,^{10–13} as well as continuing to incorporate our innovative and sensitive detector of non-fluorescent molecules, the thermal lens microscope (TLM).^{14,15} Targeting the development of clinical diagnostic tools, we have successfully integrated the immunoassay into a microchip,^{16,17} applying basic ELISA methodology to a micro-ELISA system.^{18,19} In micro-ELISA, polystyrene micro beads are coated with capture antibodies through physisorption and introduced to a microchannel. A dam

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structure in the microchannel functions to stop the micro beads. Immunoreactions with antigens are then induced on the surface of the beads. Enzyme-conjugated secondary antibodies are then introduced and capture the antigens. Finally, substrates are applied and dye molecules produced by the enzymatic reaction are detected by our sensitive TLM under continuous flow conditions. A comparison with conventional methods demonstrated excellent performance in analysis time (10–20 min) and sample volume (several microliters).

We attempted to apply our micro-ELISA method to the measurement of total and specific IgEs. However, two problems were encountered. First, for allergy diagnosis, antigens (not antibodies) should be immobilized on the beads. Among the many kinds of allergens, some were water-soluble and could not be immobilized by physisorption alone, due to the hydrophobicity of the polystyrene surface. The second problem was the system precision and sensitivity for enzymatic reactions. In this micro-ELISA, enzymatic reactions were performed under continuous-flow conditions. The liquid volume in the packed-beads area was in the order of ≈ 50 nL. In order to increase sensitivity, a longer contact (reaction) time (> 30 s) was required. Therefore, a low flow rate of $0.02 \mu\text{L}/\text{min}$ (contact time of 2–3 min) was utilized. However, the low flow rate was not stable for long-term or repeated measurements, and signal intensity occasionally fluctuated, deteriorating measurement precision. For practical application, the flow rate should be on the scale of $\approx 1 \mu\text{L}/\text{min}$; however, with a reaction time of several seconds, the resultant low sensitivity negatively influenced the lower LOD.

In this paper, a rapid and sensitive micro-ELISA method for allergy diagnosis was developed. A linker was utilized to immobilize water-soluble allergens on a hydrophobic polystyrene surface with sufficient density to realize high sensitivity. Additionally, stopped-flow conditions were utilized for the enzymatic reactions. Dye molecule accumulation was detected as a peak and a good lower LOD was achieved. Instrumentation for easy operation was also one of the important achievements. A practical and portable ELISA system was developed. All the procedures were automatically processed by a personal computer (PC), simplifying the liquid handling and interface from macro to microspace. Testing of the micro-ELISA system was performed using human serum samples from 85 allergy patients. We demonstrated a good correlation with a conventional method; requiring much smaller sample and reagent volumes as well as faster analysis times. The advantages and applicability of the developed micro-ELISA system for practical POC diagnosis were verified.

Experimental section

Principle of micro-ELISA for allergy diagnosis

The basic principle of micro-ELISA was reported in our previous paper.¹⁷ Here, we improved upon the method, focusing on two points for the achievement of sensitive allergy diagnosis. Firstly, the immobilization method of allergens was modified. Linkers of BSA-biotin-avidin were immobilized on the polystyrene surface by physisorption, with BSA functioning to ameliorate non-specific adsorption. Biotinylated allergens were then introduced and connected to BSA-biotin-avidin linkers. Secondly, the

conditions of the enzymatic reaction to produce dye molecules were improved. Previously, enzymatic reactions were performed under continuous flow conditions. The contact time (reaction time) was ~ 1 s, which was insufficient for achieving equilibrium. Adversely, lower flow rates increase fluctuation in flow velocity. In this method, we utilized stopped-flow conditions to optimize the enzymatic reaction. By utilizing stopped-flow conditions, dye molecules accumulate on the bead-packed area for several minutes and are detected by TLM as a peak subsequent to driving the liquid again.

Micro-ELISA system

In this paper, the development of a practical and automated micro-ELISA system was targeted. Fig. 1 illustrates the basic system including fluidic and optical components. The samples and reagents are stocked in tubes on an automated rotating stage. An injector of PEEK tubing (i.d. = $200 \mu\text{m}$), fixed on another automated stage, sucks a specified volume of sample and reagents under the control of a syringe pump. A small-volume (inner volume $< 1 \mu\text{L}$) and simple micro valves (custom-made) with high pressure resistance (> 1 MPa) were used in this micro-ELISA system, which enabled fast (< 1 s) response times for stopping solutions. The injector moves downward and is connected to an ELISA microchip through a Teflon connector by inserting the injector to the hydrophobic connector hole. The samples and reagents are then driven to a bead-packed area at a determined flow rate by the syringe pump. The length of PEEK tube (i.d. = $200 \mu\text{m}$) from the Teflon connector to the microchip was set as short as 20 mm to prevent broadening of the sample plug (dilution) with a resultant decrease in sensitivity. The same procedures (sucking and inserting) are utilized for washing solution, enzyme-conjugated secondary antibodies and substrates. A glass microchip with a Y shaped microchannel and a dam structure was utilized in this experiment. The size of microchannel was $200 \mu\text{m}$ wide $\times$ $200 \mu\text{m}$ deep, and the gap size at the dam structure was $20 \mu\text{m}$. Microbeads ($45 \mu\text{m}$) were introduced to the microchannel and a precise 5 mm-long bead-packed area was formed by introducing more beads and flushing the extra length with a syringe and valve. Blue dye molecules produced by the enzymatic reaction were detected by a TLM probe fixed on a microchip 15 mm downstream from the dam

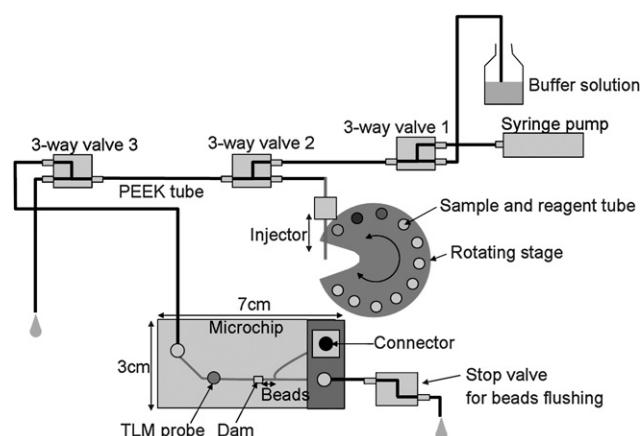


Fig. 1 Block diagram of micro-ELISA system.

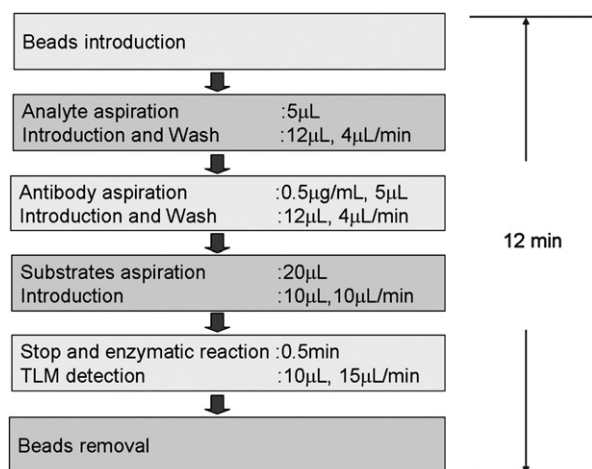


Fig. 2 A flow chart of the measurement protocol and conditions.

structure. The wavelengths of the excitation and probe beams were 658 nm and 785 nm, respectively. The details are explained in a previous paper.²⁰ All the stages, valves and syringe pump are controlled by original software on a PC. TLM signals were analyzed using the PC. All procedures were automated, from bead introduction to TLM detection and bead removal. For all the experiments, a single microchip was utilized repeatedly by changing the microbeads. All the components were integrated into a portably sized unit (W 253 mm × D 200 mm × H 222 mm).

The measurement protocol established for allergy diagnosis is illustrated in Fig. 2. The time from bead introduction to bead removal was just 12 min. In order to measure new analytes, a new bead solution was introduced and the same procedure was repeated.

Chemicals

Total IgE was chosen due to its frequent use for screening, and Cedar specific IgE was also selected for its popularity especially in Japan. Goat anti-human polyclonal IgE (ε) antibody, HRP-labeled goat anti-human IgE (ε), TMB kit (SureBlue) and blocking solution (10% BSA) were purchased from KPL. Human IgE calibrator was obtained (Bethyl Laboratories, Inc.). Cedar pollen allergen (Japanese) Cry j1, Cedar pollen allergen (Japanese) Cry j2, biotin-labeled cedar pollen allergen (Japanese) Cry j1 and biotin-labeled cedar pollen allergen (Japanese) Cry j2 were used for allergens (Asahi Food & Healthcare Co., Ltd.). HRP-labeled antibody was diluted with 1% BSA buffer solution. BSA buffer solution (0.2%) was used as the micro-ELISA system carrier solution. Polystyrene microbeads (45 μm) (Polyscience, Inc.) were utilized after removing the microbeads with a larger diameter than 53 μm. The polystyrene beads containing a linker of BSA-biotin-avidin were prepared by adding BSA-biotin (Thermo Fisher Scientific, Inc., ImmunoPure Biotinylated Bovine Serum Albumin) and streptavidin (Thermo Fisher Scientific, Inc., ImmunoPure Biotinylated Streptavidin) sequentially.

Results and discussion

We evaluated our micro-ELISA system using a standard total IgE solution (200 ng/mL). The typical time-course signal for

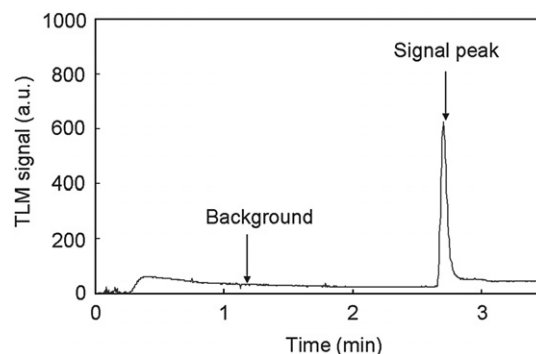


Fig. 3 Typical time-course signals of the micro-ELISA method.

TLM detection is illustrated in Fig. 3. A clear peak signal following enzymatic reaction in stopped-flow conditions was observed as expected. A small background signal was observed due to enzymatic reaction during the introduction of substrate solution (10 μL/min). In order to obtain the signal intensity of the peak, signals at 30 s before and after the peak position were averaged to define the value as a background signal, and the peak height was subtracted by the averaged background signal, which was defined as a micro-ELISA signal. Air bubbles or dust can sometimes scatter the excitation or probe beam of TLM, generating false peak signals. The width of the false peak signals are much narrower than the true peak signals because of the longer bead-packed area (5 mm) compared with dust or bubbles, and are automatically removed by our original software during analysis.

Next, we investigated stop times for use in stopped-flow conditions. A range of stop times (10 to 150 s) was selected and signal intensities were measured. Sample concentrations used were 200 ng/mL and 1000 ng/mL. The results are illustrated in Fig. 4(a). At a sample concentration of 1000 ng/mL, the signal increased linearly until 30 s, and leveled off due to the equilibrium of dye molecule production and diffusion. At a sample concentration of 200 ng/mL, the signal intensity increased until 150 s, with an observed tendency to level off. As a result, a measurement range of up to 1000 ng/mL with a stop time of 30 s was chosen. The lower LOD was evaluated using a standard solution of total IgE and three repeated measurements. The concentration was changed from 0 to 1200 ng/mL, and average (S) and standard deviation (σ) were measured. The calibration curve is shown in Fig. 4(b). The lower LOD was derived by evaluating the concentration where the $S - 2\sigma$ of the positive control is higher than $S + 2\sigma$ of blank and was calculated to be 2 ng/mL (0.8 IU/mL). This value was quite smaller than the cut-off level of 648 ng/mL (270 IU/mL) for total IgE measurement.

Finally, the micro-ELISA system was tested using human serum samples obtained from 85 allergy patients. Signal intensity was measured by the micro-ELISA system, and the concentration was calculated by utilizing 4-parameter logistics for improving the accuracy. Total IgE and Cedar specific IgE were selected. For comparison, the same serum samples were measured by the CAP method (conventional method). The correlation was evaluated as shown in Fig. 5. A good correlation was observed between the two methods with a correlation coefficient of 0.939 for total IgE and 0.923 for Cedar IgE. The

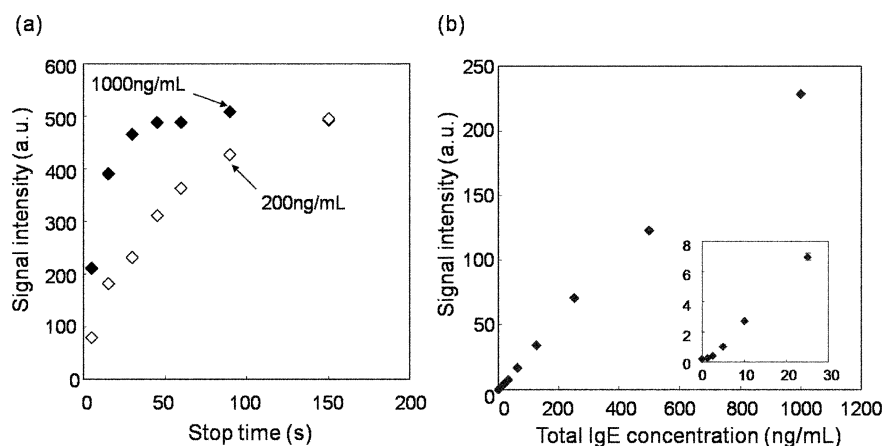


Fig. 4 Investigation stopped flow condition; (a) dependence of signal intensity on stop time and (b) calibration curve for the determination of standard total IgE solution. The inset figure shows the expanded graph in the range of 0–30 ng/mL with error bars of $\pm \sigma$.

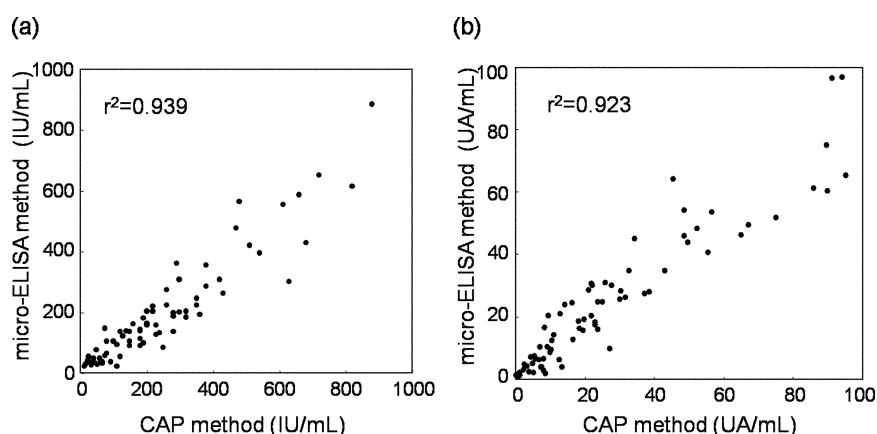


Fig. 5 Correlation of signals between the micro-ELISA method and the CAP method: (a) total IgE and (b) cedar IgE determined using human serum from 85 allergy patients.

immobilization method of antibodies was also effective for protecting nonspecific adsorption, and good correlation was obtained even for human serum samples. Therefore, based on the results, the applicability of our system for practical POC clinical diagnosis is verified with a much smaller (1/10) sample volume and shorter (1/20) analysis time. Parallel measurement is also possible by increasing the number of the microchannels. The compact and fully automated nature of the system is especially suitable for POC clinical diagnosis.

Conclusion

We have developed a micro-ELISA system for sensitive allergy diagnosis. A stopped-flow condition was created during the enzymatic reaction. A linker was utilized for the immobilization of water-soluble allergens on a polystyrene surface and resulted in the immobilization of acceptable allergen density. The lower LOD was sufficient to meet the requirement for allergy diagnosis. Good correlation with a conventional method was demonstrated using a smaller sample volume (1/10) and shorter analysis time (1/20). All the processes were automatically applied with our original microfluidic system, extending the applicability of the

microsystem to real clinical diagnostic situations. Our system is flexible and can be also applied to a wide range of targets simply by changing the immobilized antigens or antibodies on the polystyrene beads. We believe our system has the potential to become a very powerful diagnostic tool, especially for POC analysis.

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