Microfluidic immunoassay for detection of allergen-specific IgE in an enhanced magnetic field gradient
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detection of allergen-specific IgE in an
enhanced magnetic field gradient

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Department of BioSystems

Daejeon, Korea

Approved by

[Signature]
Professor Je-Kyun Park
강화된 자기장 구배하에서 알레르겐 특이 항체 검출을 위한 미세 유체 기반의 면역 측정

한 영 기

위 논문은 한국과학기술원 석사 학위논문으로 학위논문 심사위원회에서 심사 통과하였음.

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Abstract

This paper reports a novel immunoassay method using superparamagnetic nanoparticles and an enhanced magnetic field gradient in a microfluidic device. In this detection scheme, microbeads are deflected from their own pathway as much as analytes are conjugated with microbeads and superparamagnetic nanoparticles by sandwich immunoreactions. Since the deflection velocity of microbeads is proportional to the magnetic field gradient, lower detection limit can be controlled by the higher magnetic field gradient. In this study, a ferromagnetic microstructure connected with a permanent magnet is used to increase the magnetic field gradient (~10^4 T m^-1) in a microfluidic device. The microfluidic device with the microchannel is fabricated by poly(dimethylsiloxane) (PDMS) molding process and bonded on the glass wafer with the electroplated Ni microstructure. We successfully apply this method to detect an allergen-specific IgE antibody in human serum. House dust mites, Dermatophagoides pteronyssinus and Dermatophagoides farinae, are used as the sources for allergen. For allergen-specific IgE detection, we use fluorescent polystyrene beads conjugated with two types of mite allergens, superparamagnetic nanoparticles conjugated with anti-human IgE, and mite-specific IgE antibodies. The detectable range of rabbit IgG is 200 pg mL^-1 to 1 pg mL^-1, and those of the mite-specific human IgE are 19.47 ng mL^-1 to 104 pg mL^-1 (D. pteronyssinus) and 10.68 ng mL^-1 to 151 pg mL^-1 (D. farinae), respectively. With this platform technology, the multiplexed immunoassay could be possible when the specific allergen proteins are immobilized with the fluorescently encoded microbeads.
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# Nomenclature

## Alphabetic letters

- **B**  
  Magnetic field
- **B<sub>r</sub>**  
  Remanence of magnetic field of a permanent magnet
- **F<sub>D</sub>**  
  Stokes’ drag force
- **F<sub>sm</sub>**  
  Magnetic force on a superparamagnetic nanoparticle
- **F<sub>tsm</sub>**  
  The total magnetic force of superparamagnetic nanoparticles on a microbead
- **H**  
  External magnetic field
- **M**  
  Magnetization of a superparamagnetic nanoparticle
- **m**  
  Magnetic dipole moment
- **N<sub>sm</sub>**  
  The number of superparamagnetic nanoparticle conjugated to a microbead
- **R<sub>M</sub>**  
  Radius of a microbead
- **v**  
  Velocity of a microbead
- **V<sub>sm</sub>**  
  Volume of a superparamagnetic nanoparticle
Greek letters

\( \eta \)  
Viscosity of aqueous medium

\( \mu_0 \)  
Vacuum permeability

\( \chi_{aq} \)  
Susceptibility of aqueous solution

\( \chi_{sm} \)  
Susceptibility of a superparamagnetic nanoparticle

Abbreviations

BSA  
Bovine Serum Albumin

CCD  
Charge Coupled Device

CFD  
Computational Fluid Dynamics

DIW  
Deionized Water

\( D.\text{farinae} \)  
\textit{Dermatophagoides farinae}

\( D.\text{pteronyssinus} \)  
\textit{Dermatophagoides pteronyssinus}

EDC  
[1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide Hydrochloride]

FEMM  
Finite Element Method Magnetics
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PR</td>
<td>Photoresist</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>N-Hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>Sulfo-SMCC</td>
<td>Sulfosuccinimidyl 4-((N-maleimidomethyl)cyclohexane-1 carboxylate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
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Chapter 1 Introduction

1.1 Backgrounds of allergy detection methods

IgE-mediated type I allergic diseases are amongst the most common causes of chronic illness in the populations of industrialized countries. In the last few decades, the prevalence of allergy has increased dramatically and accordingly the costs for diagnosis and treatment represent a major burden for the health care systems of the affected nations [1, 2]. As immunoglobulin E (IgE) has been discovered as a key mediator of the allergic response in the immune system [3], the routine determination of IgE levels has improved the diagnosis of allergy significantly. While in vivo testing may cause the risk of adverse reactions such as anaphylactic shock, in vitro testing of allergen-specific IgE reduces them.

Determining IgE levels in serum is often sufficient to reliably identify the causative agent of allergy. Up to date, a large number of commercial diagnosis methods have been developed (Table 1). Because the IgE levels are very low in serum, the diagnosis method with the high sensitivity is requisite [4]. In 1967, the first radioallergosorbent test (RAST) was described [5]. Later, the radioisotype used in RAST was substituted by
chromogenic enzyme immunoassay (EIA) or fluorescence enzyme immunoassay (FEIA) [6]. At the present, the most widely used diagnosis method is Pharmacia CAP system (CAP) for total and allergen-specific IgE. The CAP system uses a cellulose polymer conjugated with allergens. Then, IgEs in serum are injected and the bound IgEs to allergens are detected by anti-IgE conjugated with β-galactosidase. Although it is possible to measure the allergen-specific IgE levels by these methods, these tests are expensive, time-consuming and need the large volume of reagents and serum sample [7]. In the case of CAP system, the analysis time of one test is about 3-4 hours and the volume of serum sample is about 50 μL/well [8]. Considering these points, it is desirable to develop not only sensitive and fast but also multiplexing assay method.

In this study, a novel allergy detection method enough to satisfy the requisite mentioned above was developed. As a model system, two types of mite allergens were used. The results of magnetic force-based microfluidic immunoassay using Ni microstructure were obtained and compared with the results by traditional diagnosis assay, CAP.
Table 1. A summary of allergy detection methods.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st generation</td>
<td></td>
</tr>
<tr>
<td>RAST</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td>RIA</td>
<td>Large quantity of specimen and blood</td>
</tr>
<tr>
<td></td>
<td>High cost</td>
</tr>
<tr>
<td></td>
<td>Difficult to detect of multiple IgE in one reaction</td>
</tr>
<tr>
<td>2nd generation</td>
<td></td>
</tr>
<tr>
<td>EIA,</td>
<td>Time-consuming</td>
</tr>
<tr>
<td>FEIA,</td>
<td>High cost</td>
</tr>
<tr>
<td>CAP</td>
<td>Difficult to detect of multiple IgE in one reaction</td>
</tr>
<tr>
<td>IFA</td>
<td>Multiple IgE detection in one reaction</td>
</tr>
<tr>
<td>MAST</td>
<td>No quantitative analysis</td>
</tr>
</tbody>
</table>
1.2 Reviews on magnetic particle-based assay systems

Recently, magnetic particle-based analytical applications have been reported [9-11]. Table 2 shows some magnetic particle-based immunoassay system. Since magnetic nanoparticles have biocompatibility, stability and easily modifiable surface with biomolecules [12], they are suitable for separating biomolecules. With these advantages, various detection methods had been developed [13-19].

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 – 2 µm</td>
<td>About 1 hour 30 min</td>
<td>2 – 250 ng/mL (IL-5)</td>
<td>A solid phase</td>
<td>Permanent magnet</td>
<td>Fluorescence</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>2.8 µm</td>
<td>21 min</td>
<td>50 – 100 ng/mL (IgG)</td>
<td>A solid phase</td>
<td>Electromagnet</td>
<td>Electrochemical</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>2.8 µm</td>
<td>About 17 hours</td>
<td>1 – 4 µg/mL (human transferrin)</td>
<td>A label</td>
<td>None</td>
<td>Electrical signal</td>
<td>[18]</td>
</tr>
</tbody>
</table>
There are two types of immunoassays using magnetic particles: the magnetic beads as solid phases and magnetic nanoparticles as a label. The magnetic beads as solid phases were used to maximize surface-to-volume ratios and only to separate the reacted microbeads easily. The detection range of interleukin-5 (IL-5) was 2 – 250 ng / mL [13] and that of mouse IgG was 50 – 100 ng / mL [16]. All of two cases did not show low limit of detection. The magnetic beads as a label were used to detect their physical properties or indicate the completion of the immunoreaction. In their report, the system detected 1 – 4 μg / mL of the human transferrin [18]. This system showed not only high limit of detection but also the very long analysis time. To overcome the low sensitivity of the sensor, the giant magnetoresistive (GMR) and superconducting quantum interference device (SQUID) [14, 15] were also employed to construct the immunoassay measurement system. Although these systems show a very high sensitivity, the fabrication processes of these sensors are complex and they need high cost equipments.

Recently, a novel immunoassay scheme by magnetic force in a microfluidic device was developed [26]. In this detection scheme, microbeads are deflected from their own pathway as much as analytes are
conjugated with microbeads and superparamagnetic nanoparticles by sandwich immunoreactions. Since the deflection velocity of microbeads is proportional to the magnetic field gradient, lower detection limit can be controlled by the higher magnetic field gradient.

In this study, a ferromagnetic microstructure connected with a permanent magnet is used to increase the magnetic field gradient in a microfluidic device. Generally, separating the target magnetic microbeads or nanoparticles conjugated with biomolecules using this technique is referred as “high gradient magnetic separation (HGMS)” [20]. The important feature is that a magnetized wire attracts paramagnetic particles to part of its surface and repels diamagnetic particles at other locations [21]. Using this principle, various particles are separated efficiently as their locations or by the direction of external magnetic field gradient [22]. Also, micro- or nano-sized ferromagnetic materials concentrate the magnetic flux density and enhance the magnetic field gradient. With Ni wire, red blood cells (RBCs) having native magnetic properties were successfully separated in the microchannel [29]. Table 3 shows some applications of HGMS methods.
Table 3. Review of high gradient magnetic separation (HGMS).

<table>
<thead>
<tr>
<th>Scheme of device</th>
<th>Schematic view of the concentrated magnetic flux density</th>
<th>Contours of magnetic flux around a single wire (simulation)</th>
<th>Magnetic field horizontal to Ni wire</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference No.</td>
<td>[22]</td>
<td>[21]</td>
<td>[29]</td>
</tr>
</tbody>
</table>

1.3 Research objectives

In this study, we have developed a novel immunoassay method for detection of allergen-specific IgE based on the magnetic force in a microfluidic system. For this novel platform, microbeads and magnetic nanoparticles are used in constructing a magnetophoretic sandwich immunoassay system. To increase the sensitivity of the immunoassay system, ferromagnetic material such as Ni microstructure is introduced into the microfluidic system, which increases magnetic field gradient by concentrating the magnetic flux density.

Until now, the existing allergy diagnostic methods have a lot of
disadvantages. Although a CAP system is known to have the highest sensitivity and reliability, it shows the shortcomings in that the CAP system cannot analyze the multiple targets and the analysis time is very long.

To overcome these disadvantages, we apply a high sensitive immunoassay method based on the magnetophoretic sandwich immunoassay system for detection of allergen-specific IgE. In this paper, the fabrication and optimization of the magnetophoretic sandwich immunoassay system which has potential to perform multiplexed assays is described, and the multiple simultaneous analysis is performed in a microfluidic device. In addition, the obtained results are compared with a commercial CAP method.
1.4 Thesis outline

The thesis consists of six chapters. In Chapter 1, backgrounds and research objectives are mentioned. Chapter 2 describes the concept of magnetic force-based immunoassay in a microfluidic channel and theory of magnetism. Chapter 3 describes the device design and microfabrication. Chapter 4 talks about materials, assay method, and experimental setups. Chapter 5 is about the results and discussion. The last chapter is conclusions of this thesis.
2.1 Concept

In this paper, we attempted to develop a novel detection platform based on magnetic force in a microfluidic channel. Proposed detection scheme is that specific microbeads conjugated with superparamagnetic nanoparticles can only switch their path in a microchannel due to magnetic field. As shown in Figure 1, the buffer solution is injected into the lower inlet and the sample solution is injected into the upper inlet. The microbeads conjugated with magnetic nanoparticles are hydraulically focused to the sidewall of the microchannel. Among the focused microbeads, the only microbeads conjugated with superparamagnetic nanoparticles consequently move to the high gradient magnetic field under the specific applied magnetic field. In general, the flow in a microfluidic channel remains laminar and the diffusion effects of the micro-sized beads are negligible. Therefore, the microbeads conjugated with superparamagnetic nanoparticles can change their flow path in an applied magnetic field.

The deflected velocity of microbead-magnetic nanoparticle complex can be different due to the number of attached magnetic nanoparticles. If the
number of superparamagnetic nanoparticles on a microbead increases, the velocity of a microbead will increase because the velocity of microbeads is proportional to the total volume of the magnetic nanoparticles on a microbead and magnetic field gradient [23]. What the number of superparamagnetic nanoparticles on a microbead increases means that the concentration of target analyte is high. This implies that the concentration of target analyte can be analyzed by measuring the deflected velocity of microbead-magnetic nanoparticle complex. In addition, the deflected microbeads can be identified by their own optical properties of fluorescent microbeads which can be encoded [24]. This makes it possible to develop a multiplexed assay system.

Figure 1. Principle of microfluidic immunoassay using microbead conjugated with superparamagnetic nanoparticles and an enhanced magnetic field gradient in a microfluidic device.
2.2 Theory

An aqueous flow in a microchannel is generally not turbulent but laminar. Therefore, there is no force on the microbead at the perpendicular direction against the flow direction. However, when the microbeads conjugated with the superparamagnetic nanoparticles are exposed to an applied magnetic field in a microchannel, they will move against the flow direction due to the magnetic force on microbead-superparamagnetic nanoparticle complex. The magnetic force, $F_{sm}$, on a superparamagnetic nanoparticle in the aqueous solution is given by equation (1) [23]:

$$\vec{F}_{sm} = \frac{1}{2} \frac{V_{sm} \Delta \chi_{sm}}{\mu_0} \nabla B^2$$  \hfill (1)

where $V_{sm}$ is the volume of the superparamagnetic nanoparticle, $\Delta \chi_{sm} = \chi_{sm} - \chi_{aq}$ is the net magnetic susceptibility of a superparamagnetic nanoparticle in aqueous solution, $B$ is the magnetic field and $\mu_0$ is the vacuum permeability. This equation is originally stated from $\vec{F}_{sm} = (m \cdot \nabla) B$, where $m$ is magnetic dipole. The total moment on a particle can be written $m = V_{sm} M$. The magnetization of a superparamagnetic nanoparticle can be converted to $M = \Delta \chi_{sm} H$, where $H$ is external magnetic field. Using $B = \mu_0$
$\mathbf{H}$, equation (1) is obtained. This equation is valid for magnetic fields which do not saturate the value of magnetization of a superparamagnetic nanoparticle. If the external magnetic field is enough to saturate magnetization of a superparamagnetic nanoparticle, magnetic force is not proportional to $\nabla B^2$ but proportional to $\nabla B$. When the superparamagnetic nanoparticles are conjugated with the microbeads, the applied magnetic field will induce the magnetic force on superparamagnetic nanoparticles, and then the total magnetic force of the superparamagnetic nanoparticles on the microbeads will make the movement of the microbeads. The total magnetic force, $F_{\text{sm}}$, of the superparamagnetic nanoparticles on the microbead is the sum of the magnetic forces acting on each superparamagnetic nanoparticle on the microbead:

$$\vec{F}_{\text{sm}} = N_{\text{sm}} \vec{F}_{\text{nm}} = \frac{1}{2} N_{\text{sm}} \frac{V_{\text{sm}} \Delta \chi_{\text{sm}}}{\mu_0} \nabla B^2 \tag{2}$$

where $N_{\text{sm}}$ is the number of the superparamagnetic nanoparticle conjugated with a microbead. If the superparamagnetic nanoparticles are a specific size the volume ($V_{\text{sm}}$) and the magnetic susceptibility ($\chi_{\text{sm}}$) of each superparamagnetic nanoparticle have the same value because $V_{\text{sm}}$ and $\chi_{\text{sm}}$
are the variables of particle size. Therefore the $F_{tsm}$ is determined by the number of the superparamagnetic nanoparticle in the specific magnetic field gradient ($\nabla B^2$).

When the microbeads are moved by the total magnetic force, the Stoke’s drag force ($F_D$) is generated against the moving microbeads in the opposite direction. The $F_D$ is represented by the following equation (3):

$$\vec{F}_D = -6\pi R_M \eta \vec{v} \quad \text{.........................................................(3)}$$

where $R_M$ is the radius of the microbead, $\eta$ is the viscosity of the aqueous medium and $\vec{v}$ is the velocity of the microbead. The velocity of the microbead results from the magnetic force. Since the direction of the magnetic force is the perpendicular direction of fluid flow and the microbeads move in the laminar flow as shown Figure 2, the $F_D$ equals the $-F_{tsm}$:

$$\vec{F}_D = -\vec{F}_{tsm} \quad \text{.........................................................(4)}$$

Combining equation (2) and (3) into equation (4), the velocity of the
microbeads in the aqueous medium is represented by the following equation (5):

\[ \tilde{\nabla} = \frac{N_{sm} V_{sm} \Delta \chi_{sm}}{12 \pi R_M \eta \mu_0} \nabla B^2 \] .............................................................. (5)

Therefore, when the size of the superparamagnetic nanoparticle and the microbead is assumed to be uniform respectively, the velocity of the microbead is determined by the number of the superparamagnetic nanoparticle conjugated on the microbead \( N_{sm} \) and the magnetic field gradient.

![Diagram](image)

Figure 2. The microbeads conjugated with the superparamagnetic nanoparticles in a microchannel. The total magnetic force \( F_{tm} \) and Stokes’ drag force \( F_D \) are shown.
Chapter 3 Device Design and Microfabrication

3.1 Device design

Figure 3 shows the configuration of microfluidic device. The microfluidic device consists of two inlets and one outlet. The upper inlet is for the reacted sample solution and the lower inlet is for the buffer solution to focus microbeads of the sample solution hydrodynamically. The hydrodynamically focused microbeads of the sample solution will flow along by one sidewall of the microchannel. The permanent magnet is placed 2 mm apart from the microchannel. When the microbeads conjugated with superparamagnetic nanoparticles pass through the microchannel, the microbead-superparamagnetic nanoparticle complexes are exposed to magnetic field gradient. So the microbeads with superparamagnetic nanoparticles are deflected to the high magnetic field gradient and the microbeads with no superparamagnetic nanoparticles are not deflected. Also there is a Ni microstructure 15 μm apart from the microchannel. The role of Ni microstructure is to concentrate the magnetic flux density and to enhance the magnetic field gradient. The width and length of Ni microstructure is 50 μm, 12 mm respectively.
Figure 3. Design of the microfluidic device. (a) Dimension of the device. (b) Layout for PDMS channel part. (c) Layout for Ni microstructure.

3.2 Microfabrication

The microfluidic device with microchannel was fabricated by conventional PDMS (polydimethylsiloxane) (Sylgard 184, Dow Corning) molding processes and the Ni microstructure was electroplated on the Pyrex glass wafer. The fabrication process is simply illustrated in Figure 4. For the fabrication of microfluidic device, multi-exposure method was used. The negative photoresist (PR) SU-8 (Microlithography Chemical Co., MA) was spin-coated on the bare Si wafer and exposed to the first UV light for
pattern-transfer. The part exposed to the first UV light was hardened in bake oven and the part which was not exposed to the UV light, so not cross-linked was developed in the SU-8 developer. This SU-8 mold was for the microchannel. Next, for the Ni microstructure, second SU-8 was spin-coated on the first SU-8 layer. The second mask was aligned with the first SU-8 layer and the second SU-8 layer was exposed to UV light. Like the preceding, the second SU-8 layer was hardened and developed in the SU-8 developer. After the patterning, the prepared mixture of PDMS was degassed under the vacuum, poured onto the mold and cured for 2 hours at 100°C on the hot plate.

For the Ni microstructure, 300 Å of chrome and 3000 Å of gold were deposited successively on the glass wafer by sputtering. AZ 1512 positive PR (Clariant Corp., Someville, NJ) was spin-coated and exposed to UV light for pattern-transfer. The latent image was developed and hardened in bake oven. Next, the chrome-gold layer which was not covered with PR was exposed to the gold and chrome etchant in order and the PR was removed. For the mold of Ni electroplating, THB-151N negative PR made by JSR was spin-coated on the gold patterned wafer. Soft bake was performed to harden the PR. After soft-baking, the wafer was loaded on the
aligner upside down to let the gold pattern play a role as a mask. The wafer was exposed to UV light and developed in THB-D2 developer. Then, Ni was electroplated on the gold pattern. The Ni microstructure was about 50 μm in height. After Ni electroplating, PR was stripped by JSR THB-S1 stripper for about 2 hours at 60°C.

After the fabrication of PDMS microfluidic device and Ni microstructure, they were aligned and bonded. The glass wafer with Ni microstructure was treated with the heptane and dried. The surfaces of cured PDMS and glass wafer were treated by oxygen plasma (Harrick Science, Ossing, NY) for 11 sec. And then, the PDMS and glass wafer with Ni microstructure were bonded immediately. The fabricated device was shown in Figure 5. The dimension of the microchannel and Ni microstructure were shown in Figure 6.
Figure 4. Fabrication process of microfluidic device.

Figure 5. A photograph of the microfluidic device with a Ni microstructure.
Figure 6. Optical microscope images of the microchannels; (a) Inlet part, (b) Outlet part (expansion part), (c) Microchannel 15 μm apart from the Ni microstructure.
Chapter 4 Experimental

4.1 Materials

NaCl, KCl, Na₂HPO₄ and KH₂PO₄ were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Casein, bovine milk was obtained from CALBIOCHEM, EMD Biosciences, Inc. (Darmstadt, Germany). Tween®20 was obtained from Aldrich (St. Louis, MO, USA). One liter of 137 mM phosphate buffered saline (PBS) contained 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄ and the pH of the PBS buffer was adjusted to 7.2 and 7.4. All solutions were prepared using deionized water with Water Purification System (Human Corp., Korea) and autoclaved water. All chemicals used were of analytical grade.

The red fluorescent microbeads (excitation 580 nm / emission 605 nm) immobilized with NeutrAvidin molecules was purchased from Molecular Probes (Eugene, OR). The Luminex xMAP® Carboxylated Microspheres (dye 1: excitation 532 nm / emission 575 nm, dye 2: excitation 633 nm / emission 700 nm) was purchased from Luminex Corporation (Austin, Texas). The diameters of the red fluorescent microbeads and the Luminex xMAP® Carboxylated Microspheres were 1 μm and 5.6 μm, respectively.
The red fluorescent microbeads were brought into solution in 0.4 mL of aqueous suspensions containing 1 % solids, 50 mM sodium phosphate, 50 mM NaCl, pH 7.5, 0.02 % Tween® 20 and 5 mM azide. Luminex xMAP® Carboxylated Microspheres were provided in purified water containing 0.01 % merthiolate as a preservative. The concentration of the red fluorescent microbeads and Luminex xMAP® Carboxylated Microspheres were $1.4 \times 10^{10}$ beads / mL and $1.25 \times 10^7$ beads / mL, respectively.

Two kinds of superparamagnetic nanoparticles were used for these experiments. One of them was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The superparamagnetic nanoparticles conjugated to goat polyclonal anti-rabbit IgG (H+L) F(ab')2 fragments were supplied as 0.5 mL solution containing 0.1 % BSA and 0.05 % sodium azide. The superparamagnetic nanoparticles consist of iron oxide and their size was about 50 nm diameter including polymer coating and proteins on the surface. The other was kindly obtained from Professor Jinwoo Cheon (Department of Chemistry, Yonsei University, Korea). The material of the superparamagnetic nanoparticles was Fe₃O₄ and the diameter of the core was 9 nm. 2, 3-dimercaptosuccinic acid (DMSA) ligand was exchanged onto the core. After intermolecular disulfide cross-linkages between the
ligands, the remaining free thiol groups of DMSA ligand were used for the attachment of target-specific antibodies (anti-human IgE). The obtained superparamagnetic nanoparticles with the DMSA ligands were supplied in PBS buffer solution [25].

Normal rabbit IgG was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-rabbit IgG (γ-chain specific) clone RG-96 biotin conjugates were obtained from Sigma Chemical Company (St. Louis, MO, USA). Goat anti-human IgE was purchased from CHEMICON international, Inc (Temecula, CA). Sera from patients and mite allergens were obtained from Ajou University Hospital (Suwon, Korea).

4.2 Preparation of microbeads for the sandwich immunoassay of rabbit IgG

The red fluorescent microbeads immobilized with NeutrAvidin molecules were prepared for the detection of rabbit IgG. The solution of the red fluorescent microbeads were washed two times using a centrifuge at 13,000 g, 4 °C for 5 min in pH 7.4 PBS containing 0.02 % Tween®20 and diluted into pH 7.4 PBS before immunoassay. For blocking non-specific binding, the red fluorescent microbeads immobilized with NeutrAvidin
molecules were treated with casein. The solution of the red fluorescent microbeads was diluted by $1.4 \times 10^7$ beads / 100 μL in pH 7.4 PBS. And 1 % casein solution was prepared into the pH 7.4 PBS. The diluted solution of the red fluorescent microbeads was added to the 1 mL of 1 % casein solution. The mixture was incubated for 12 hours at room temperature with gentle mixing. After incubation, the red fluorescent microbeads blocked with casein were pelleted by a centrifuge at 13,000 g, 4°C for 5 min. The supernatant was removed and the pelleted microbeads were resuspended by vortex and sonication for 15 seconds in pH 7.4 PBS in order to wash the microbeads. These washing steps were repeated three times. The washed microbeads were diluted by $1.4 \times 10^7$ beads / 100 μL in pH 7.4 PBS. For preparation of anti-rabbit IgG conjugated microbeads, the prepared red fluorescent microbeads were reacted with mouse monoclonal anti-rabbit IgG biotin conjugates. The mouse monoclonal anti-rabbit IgG biotin conjugate solution was diluted to the concentration of 250 nM in pH 7.4 PBS. Then, 100 μL of the red fluorescent microbeads solution and 100 μL of the 250 nM mouse monoclonal anti-rabbit IgG biotin conjugate solution were mixed. After the mixture was incubated for 30 min at room temperature with gentle mixing, the reacted microbeads were pelleted by a
centrifuge at 13,000 g, 4°C for 5 min. The supernatant was removed and the pelleted microbeads were resuspended by vortex and sonication for 15 seconds in pH 7.4 PBS containing 0.02 % Tween®20 in order to remove the unbounded anti-rabbit IgG biotin conjugates. These washing steps were repeated three times. The prepared microbead solutions were stored at 4°C in the dark before use.

4.3 Conjugation of mite lysate to Microspheres

For the sandwich immunoassay of the allergen-specific human IgE, Luminex xMAP® Carboxylated Microspheres were conjugated with mite lysate. 6.25 x 10⁵ of carboxyl terminated microspheres were activated with 20 mM EDC / 60 mM sulfo-NHS in 100 mM sodium phosphate buffer, pH6.2 for 30 min at room temperature. 200 mM of beta-mercaptoethanol was added to quench free EDC not reacted with sulfo-NHS. Microspheres were washed with 100 mM MES buffer, pH 6.0 for three times, and then collected by a centrifuge at 10,000 g, 4°C for 1 min. Activated microspheres were incubated with 1.5 mg / mL of clear mite lysate for 3 hours at room temperature under gentle mixing. After reaction, microspheres were collected by a centrifuge at 10,000 g, 4°C for 1 min and incubated in PBS-TBN buffer containing 0.05 % Tween®20, 1 % BSA and
0.05 % azide under gentle mixing at room temperature for 1 hour. Then microspheres were washed three times and stored in PBS-TBN buffer. Microspheres were counted by inverted microscope using traditional hematocytometry method.

4.4 Conjugation of goat anti-human IgE / BSA to superparamagnetic nanoparticles (MNPs)

440 μg of BSA and 200 μg of goat anti human IgE were subjected to 100 μM of sulfo-SMCC cross linker and incubated in 10 mM sodium phosphate buffer, pH 7.2 for 30 min at room temperature. Free linkers were removed by using 5 mL desalting column saturated with 10mM sodium phosphate buffer, pH 7.2. Activated proteins were incubated with 510 μg of 9 nM MNPs under gentle rotation at 4°C overnight. After blocking of unconjugated sulfo-SMCC with 1mM beta-mercaptoethanol for 15 min at room temperature, protein-conjugated MNPs were collected using superdex-200 resin packed column. Conjugates were stored in 10 mM sodium phosphate buffer, pH 7.4 at 4°C.

The quantitative analysis of protein-MNP conjugates was performed. Conjugation ratio of the proteins (goat anti-human IgE / BSA) was
estimated by using spectrophotometric measurement method. Protein concentration was determined by traditional Bradford assay at 590 nm wavelength. And MNP concentration was measured by using its extinction coefficient (ε = 8.77 L / g·cm at 450 nm wavelength). The results showed that about 3 goat anti-human IgE molecules and 15 BSA molecules were conjugated with one MNP. The concentration of superparamagnetic nanoparticle solution was $2.613 \times 10^{11}$ MNPs / µL.

4.5 Pooling of serum sample

Mite allergen-specific IgE level of each sample was measured by UniCAP (Pharmacia Diagnostics, Uppsala, Sweden), a commercial diagnostic kit measuring the allergen-specific IgE level. Serum samples of the 44 patients (age; 2~62) were obtained from the Ajou University Hospital (Suwon, Korea). Serum samples were grouped into 5 categories according to their mite allergen-specific IgE levels. To minimize sample to sample variation of different categories in their protein contents, 4 or 5 samples in each group are mixed in the same volume. For *D. pteronyssinus* and *D. farinae*, 5 pools were made, respectively. IgE level represented in Table 4 is the average value of included sera using UniCAP.
Table 4. List of pooled sera used in the experiment.

<table>
<thead>
<tr>
<th>Pool</th>
<th>D. pt. 1</th>
<th>D. pt. 2</th>
<th>D. pt. 3</th>
<th>D. pt. 4</th>
<th>D. pt. 5</th>
<th>D. fa. 1</th>
<th>D. fa. 2</th>
<th>D. fa. 3</th>
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<th>D. fa. 5</th>
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<tr>
<td>IgE level (U / mL)</td>
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<td>4.495</td>
<td>22.25</td>
<td>75.72</td>
<td>0.482</td>
<td>2.168</td>
<td>8.36</td>
<td>43.65</td>
<td>81.14</td>
</tr>
<tr>
<td>IgE level (ng / mL)</td>
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<td>4.30</td>
<td>10.79</td>
<td>53.4</td>
<td>181.73</td>
<td>1.16</td>
<td>5.20</td>
<td>20.06</td>
<td>104.76</td>
<td>194.74</td>
</tr>
<tr>
<td>Number of included serum</td>
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<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5. Classification of allergy according to IgE unit

<table>
<thead>
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<th>class</th>
<th>IgE level (U / mL)</th>
<th>IgE level (ng / mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 0.35</td>
<td>&lt; 0.85</td>
</tr>
<tr>
<td>1</td>
<td>0.35 ~ 0.70</td>
<td>0.85 ~ 1.69</td>
</tr>
<tr>
<td>2</td>
<td>0.70 ~ 3.50</td>
<td>1.69 ~ 8.47</td>
</tr>
<tr>
<td>3</td>
<td>3.50 ~ 17.5</td>
<td>8.47 ~ 42.4</td>
</tr>
<tr>
<td>4</td>
<td>17.5 ~ 50.0</td>
<td>42.4 ~ 121</td>
</tr>
<tr>
<td>5</td>
<td>50.0 ~ 100</td>
<td>121 ~ 242</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 100</td>
<td>&gt; 242</td>
</tr>
</tbody>
</table>
4.6 Measurement set-up

As shown in Figure 7, the microfluidic device was placed on an inverted microscope (Zeiss Axiovert 25; Carl Zeiss, Germany) with a 50 W mercury lamp of light source for excitation of fluorescent microbeads. A CCD camera was integrated on the inverted microscope to capture images of the movement of fluorescent microbeads. The microfluidic device had two inlets and one outlet. The tubing was inserted into the holes to connect the 10 µL and 50 µL microsyringes (1700 series gastight syringes; Hamilton Company, NV). The microsyringes were connected with another side of tubing to pump the aqueous medium by a dual syringe pump (Pump 11 Pico Plus; Harvard Apparatus, Inc., MA). The reacted sample solutions were injected through one of the inlets and the buffer solution (137 mM PBS, pH 7.4) was injected through another inlet. In order to apply magnetic field, we used NdFeB 35 permanent magnet (Magtopia, Korea) which was 50 x 25 x 10 mm$^3$ and $B_r = 12000$ gauss. The permanent magnet was placed 2 mm apart from the microchannel. The movements of fluorescent microbeads conjugated with the superparamagnetic nanoparticles were occurred at the hydrodynamic focused region of the microchannel in applied magnetic field. The images of the movement of fluorescent microbeads were recorded by a
CCD camera. Then, recorded movies were analyzed to measure the velocities of fluorescent microbeads. To measure the velocities of fluorescent microbeads, the commercial measurement program, i-Solution (IMT; Image and Microscope Technology, Korea) was used.

Figure 7. Schematic diagram of a magnetic force-based immunoassay apparatus.
4.7 Sandwich immunoassay procedure for detection of rabbit IgG

The schematic procedure of sandwich immunoassay method for rabbit IgG is shown in Figure 8. All reactions were performed in 1.5 mL microcentrifuge tubes. $2.55 \times 10^5$ beads / 70 μL of the red fluorescent microbeads conjugated with mouse monoclonal anti-rabbit IgG were aliquoted in a microcentrifuge tube. After the 2-fold serial dilutions of normal rabbit IgG in pH 7.4 PBS, 10 μL of antigen sample solution was added to each aliquot 70 μL of the microbead solution in microcentrifuge tubes. In order to observe backgrounds, the control experiment was carried out by adding 10 μL of pH 7.4 PBS instead of analyte solution. The mixture solution was mixed and incubated for 10 min at room temperature. Then, 5 μL of the solution containing the superparamagnetic nanoparticles conjugated with goat anti-rabbit IgG was added to each mixture solution in

![Figure 8](image-url)  
**Figure 8.** The sandwich immunoassay scheme for detection of rabbit IgG using superparamagnetic nanoparticles as labels.
order. The volume and concentration of the solution of superparamagnetic nanoparticles were fixed. The mixture solution was mixed and incubated for 10 min at 4°C. After sandwich immunoassay, the solution with microbead-superparamagnetic nanoparticle complexes was injected into the microchannel using syringe pump to measure the velocity of microbeads. The flow rate was 3 μL / hr. The velocities were measured only in the x-direction perpendicular to flow direction. The permanent magnet was fixed at 2 mm apart from the microchannel.

4.8 Sandwich immunoassay procedure for detection of mite allergen-specific human IgE

The schematic procedure of sandwich immunoassay method for detection of mite allergen-specific human IgE is shown in Figure 9. All reactions were also performed in 1.5 mL microcentrifuge tubes. We used two types of mite allergens: Dermatophagoides pteronyssinus, Dermatophagoides farinae. House dust mites were obtained from the Ajou University Hospital (Suwon, Korea). We prepared 5,649 beads / 70 μL of the Luminex microbeads conjugated with D. farinae and 5,130 beads / 70 μL of the Luminex microbeads conjugated with D. pteronyssinus in 1M
PBS, pH 7.4, respectively. And we diluted the human serum in 1M PBS, pH 7.4, too. Because there were not only mite allergen-specific IgE but also many other proteins in human serum, we tried to neutralize the charge of other proteins by increasing the salt concentration of buffer solution. With this work, we successfully prevent the 9 nm superparamagnetic nanoparticles conjugated with anti-human IgE from binding other proteins. After the 2-fold serial dilutions of human serum in 1M PBS, pH 7.4, 10 μL of diluted serum sample solution was added to each aliquot 70 μL of the microbead solution in microcentrifuge tubes. In order to observe backgrounds like the preceding experiments, the control experiment was carried out by adding 10 μL of pH 7.4 PBS instead of analyte solution. Also, the negative control experiments were performed. The negative control experiments were for showing no cross-reaction of other human IgE in serum with mite allergen. For the negative control experiments, 1 μg / mL of the purified human IgE solution containing no mite allergen-specific human IgE was used. The mixture solution was mixed and incubated for 10 min at 4 °C. For the separation of microbeads conjugated with mite allergen-specific human IgE and other proteins, the solution was centrifuged at 13,000 g, 4 °C for 5 min. Then, the supernatant was discarded and the same
volume of PBS-TBN containing 1% BSA, 0.02% Tween®20 and 0.05% azide, pH 7.4 was filled. The pellet was resuspended and 5 μL of the solution containing the 9 nm superparamagnetic nanoparticles conjugated with anti-human IgE was added to each mixture solution. The concentration of the superparamagnetic nanoparticles solution was $1.31 \times 10^{10}$ / μL. The mixture solution was mixed and incubated for 10 min at 4°C. After sandwich immunoassay, the solution with microbead-superparamagnetic nanoparticle complexes was injected into the microchannel using syringe pump to measure the velocity of microbeads. The flow rate was 3 μL / hr. The velocities were measured only in the x-direction perpendicular to flow direction. The permanent magnet was fixed at 2 mm apart from the microchannel.
Figure 9. The sandwich immunoassay scheme for detection of mite allergen-specific human IgE using superparamagnetic nanoparticles as labels.
Chapter 5 Results and Discussion

5.1 Effect of Ni microstructure (simulation results)

Movements of microbeads conjugated with superparamagnetic nanoparticles are affected by the gradient of magnetic fields. The velocity is proportional to the magnetic field gradient. What the velocity increases means that the limit of detection lowers. Therefore, the magnetic field gradient should be higher. For this purpose, we applied the ferromagnetic material, Ni microstructure, to the microfluidic device. The ferromagnetic material concentrates the magnetic flux density under external magnetic field [20, 21]. To confirm this effect, a FEMM program (Finite Element Method Magnetics, Aladdin Enterprises, Menlo Park, California) and a commercial CFD solver (CFD-ACE; CFDRC, Huntsville, Alabama) was used. All simulation conditions were identical with experimental ones. The height and length of the NdFeB 35 permanent magnet were 10 mm and 50 mm, respectively. The height and width of the Ni microstructure were 50 μm and 50 μm, respectively. The distance between the permanent magnet and the Ni microstructure were 2 mm. The properties of permanent magnet were followings: relative recoil permeability - 1.05, density - 7.4 g / cm³,
specific resistivity - 144 $\mu\Omega\cdot$cm.

First of all, the FEMM simulation results of a permanent magnet and Ni microstructure under external magnetic field were compared. Figure 10 (a) shows the simulated result of Ni microstructure under the external magnetic field and Figure 10 (b) shows the simulated result of the permanent magnet. The simulated magnetic field gradient of a permanent magnet was about 200 T / m in a concerned region which is about 2 mm apart from the permanent magnet. However, that of Ni microstructure under the external magnetic field was about $\sim 10^4$ T / m, which was about 50 times as large as that of the permanent magnet. From these results, the phenomenon that the

![Graphs](image)

Figure 10. Comparison of simulated results between a permanent magnet and Ni microstructure under external magnetic field. (a) The plot of simulated magnetic field gradient with Ni microstructure under external magnetic field. (b) The plot of simulated magnetic field gradient with only permanent magnet.
ferromagnetic material concentrates the magnetic flux density was confirmed numerically.

Based on the results above mentioned, the simulations under various conditions were performed. From these results, the dimension of microfluidic device with Ni microstructure was determined. The results are shown in Figure 11 and 12. Figure 11 shows the simulated magnetic field gradient from Ni microstructure under the external magnetic field. Figure 12 (a) shows the calculated magnetic field gradient according to the height of Ni microstructure and Figure 12 (b) shows the calculated magnetic field gradient according to the section of Ni microstructure. From two results, we

Figure 11. The simulated magnetic field gradient from Ni microstructure under the external magnetic field by FEMM simulation.
determined the gap between the microchannel and Ni microstructure and the height of Ni microstructure. We designed the gap between the microchannel and Ni microstructure as 15 µm and the height of Ni microstructure as 50 µm. In this case, the calculated magnetic field gradient \( \frac{dB}{dx} \) was \( \sim 10^4 \) T/m.

We also got the simulation results by CFD-ACE. The simulation was performed with the same conditions as FEMM. The simulated magnetic field gradient is shown in Figure 13 and the calculated \( \frac{dB_z}{dx} \) from Ni microstructure is shown in Figure 14. Like the preceding simulation results, we confirmed that the magnetic field gradient \( \frac{dB_x}{dx} \) around Ni microstructure were highly increased.

![Figure 12](image_url)

Figure 12. Plot of the calculated magnetic field gradient from Ni microstructure. (a) Magnetic field gradient according to the height of Ni microstructure. (b) Magnetic field gradient according to the section of Ni microstructure.
Figure 13. The simulated magnetic field gradient from Ni microstructure under the external magnetic field by CFD-ACE.

Figure 14. The calculated $dB_x / dx$ from Ni microstructure by CFD-ACE. The area around Ni microstructure (dashed line) shows a sudden $dB_x / dx$. 
5.2 Sandwich immunoassay using Ni microstructure

We carried out sandwich immunoassay for rabbit IgG using Ni microstructure which was confirmed to enhance the magnetic field gradient. For this experiment, microbeads conjugated with monoclonal goat anti-rabbit IgG, normal rabbit IgG and 50 nm superparamagnetic nanoparticles conjugated with polyclonal goat anti-rabbit IgG were used. The concentration of the red fluorescent microbeads was $2.55 \times 10^5$ beads / 70 μL. A volume of rabbit IgG solution was 10 μL with different concentrations. Laminar flow stream was in the direction of the positive y-axis and magnetic field gradient was in the direction of the x-axis. The velocity of microbeads conjugated with superparamagnetic nanoparticles was dependent upon the concentration of superparamagnetic nanoparticles as expected by theory.

First of all, the control experiment was performed. In the control experiment, 10 μL of PBS solution was used instead of rabbit IgG solution. The background velocity was about 0.087 μm / s. Based on the control experiment result, immunoassay of rabbit IgG was carried out. The mean velocity of the lowest concentration (1 pg / mL) of rabbit IgG was $0.8 \pm 0.17 \mu m / s$. This concentration came under 6.65 fM. The mean velocity at
the 200 pg / mL of rabbit IgG was 6.22 ± 0.33 μm / s. These results are shown in Figure 15.

As shown in Figure 16, the detectable range of concentration of rabbit IgG was far lower than that of the preceding experiment. While the detection limit of result without Ni microstructure was about 244 pg / mL [26], that of result using HGMS with Ni microstructure was about 1 pg / mL. The detection limit was about 250 times lower than that of previously reported results. The velocities of the microbeads were measured over a range of concentration of rabbit IgG from 200 pg / mL to 1 pg / mL.

Figure 15. CCD images of movements of reacted fluorescent microbeads under an enhanced magnetic field gradient. (a) The analyte was 10 μL PBS for a background. The velocity of the control experiment was about 0.087 μm / s. (b) The analyte was 10 μL of 4 pg / mL rabbit IgG. The velocity was 1.31 ± 0.35 μm / s. (c) The analyte was 10 μL of 200 pg / mL rabbit IgG. The velocity was 6.22 ± 0.33 μm / s.
Figure 16. Result of sandwich immunoassay for detection of rabbit IgG using the Ni microstructure. The detectable concentration of rabbit IgG was lower to the range from 200 pg/mL to 1 pg/mL.

5.3 Sandwich immunoassay for mite allergen-specific human IgE

In this experiment, sandwich immunoassay for mite allergen-specific human IgE was performed. Two types of mite allergens were used. One was *D. farinae* and the other was *D. pteronyssinus*. The microbeads (5.6 μm Luminex bead) were different from the microbeads (1 μm red fluorescent microbead) for detection of rabbit IgG. The experiments were performed with the same methods as those for detection of rabbit IgG. First, the
control experiment and the negative control experiment were performed for a background. The purpose of the negative control experiment was to show no cross-reaction between mite allergens and other human IgE's not mite allergen-specific human IgE. So, 1 μg / mL of the purified human IgE containing no mite allergen-specific human IgE was used for the negative control experiment. And for the control experiment, the PBS buffer solution was used like preceding experiments. Each volume of the solution used was 10 μL. The velocity of the microbeads in the control experiment case was about 0.3 μm / s for the microbeads conjugated with D.farinae allergens and about 0.06 μm / s for the microbeads conjugated with D.pteronyssinus allergens, respectively (Figure 17). Figure 18 shows the results of the negative control experiment. The velocity of the microbeads in the negative control experiment case was about 0.65 ± 0.36 μm / s for the microbeads conjugated with D.farinae allergens and about 0.36 ± 0.07 μm / s for the microbeads conjugated with D.pteronyssinus allergens, respectively.
Figure 17. CCD images of movements of reacted microbeads in the control experiment case under an enhanced magnetic field gradient. The analyte was 10 μL PBS for a background. (a) Microbeads conjugated with *D. farinae* allergens. The mean velocity was about 0.3 μm / s. (b) Microbeads conjugated with *D. pteronyssinus* allergens. The mean velocity was about 0.06 μm / s.

Figure 18. CCD images of movements of reacted microbeads in the negative control experiment case under an enhanced magnetic field gradient. The analyte was 10 μL of 1 μg / mL of the purified human IgE containing no mite allergen-specific human IgE for a background. (a) Microbeads conjugated with *D. farinae* allergens. The mean velocity was about 0.65 ± 0.36 μm / s. (b) Microbeads conjugated with *D. pteronyssinus* allergens. The mean velocity was about 0.36 ± 0.07 μm / s.
With this background, the sandwich immunoassay for the mite allergen-specific human IgE was performed. The analyte was the diluted serum from 5 groups of patients’ samples. Firstly, the experiments for *D. farinae* allergen were performed. As shown in Figure 19, the velocities of the microbeads were measured over a range of concentration of the *D. farinae* allergen-specific human IgE from 19.47 ng / mL to 104 pg / mL. The lowest concentration of human IgE that was measured over the background was about 104 pg / mL. The mean velocity of the lowest concentration (104 pg / mL) of human IgE was $1.78 \pm 0.36 \mu m / s$, Figure 20 (a). The mean velocity at the 19.47 ng / mL of human IgE was $14.6 \pm 0.8 \mu m / s$, Figure 20 (b).
Figure 19. Result of sandwich immunoassay for detection of *D. farinae* allergen-specific human IgE using the Ni microstructure. The detectable concentration of human IgE was from 19.47 ng / mL to 104 pg / mL.

Figure 20. CCD images of movements of microbeads conjugated with *D. farinae* allergens under an enhanced magnetic field gradient. (a) The analyte was 104 pg / mL of *D. farinae* allergen-specific human IgE. The mean velocity was about 1.78 ± 0.36 μm / s. (b) The analyte was 19.47 ng / mL of *D. farinae* allergen-specific human IgE. The mean velocity was about 14.6 ± 0.8 μm / s.
Secondly, the experiments for \textit{D.pteronyssinus} allergen were performed. As shown in Figure 21, the velocities of the microbeads were measured over a range of concentration of the \textit{D.pteronyssinus} allergen-specific human IgE from 10.68 ng / mL to 151 pg / mL. The lowest concentration of human IgE that was measured over the background was about 151 pg / mL. The mean velocity of the lowest concentration (151 pg / mL) of human IgE was 1.0 \( \pm \) 0.48 \( \mu \text{m} \) / s, Figure 22 (a). The mean velocity at the 3.64 ng / mL of human IgE was 9.29 \( \pm \) 0.26 \( \mu \text{m} \) / s, Figure 22 (b). The velocity was almost saturated at about 10 ng / mL. The velocity at 10 ng / mL of human IgE was 10.0 \( \pm \) 0.33 \( \mu \text{m} \) / s.
Figure 21. Result of sandwich immunoassay for detection of *D. pteronyssinus* allergen-specific human IgE using the Ni microstructure. The detectable concentration of human IgE was from 10.68 ng/mL to 151 pg/mL.

Figure 22. CCD images of movements of microbeads conjugated with *D. pteronyssinus* allergens under an enhanced magnetic field gradient. (a) The analyte was 151 pg/mL of *D. pteronyssinus* allergen-specific human IgE. The mean velocity was about 1.0 ± 0.48 μm/s. (b) The analyte was 3.64 ng/mL of *D. pteronyssinus* allergen-specific human IgE. The mean velocity was about 9.29 ± 0.26 μm/s.
In this study, two types of the sandwich immunoassays for the detection of the *D. farinae* allergen-specific human IgE and the *D. pteronyssinus* allergen-specific human IgE were successfully performed. However, the results of *D. farinae* and *D. pteronyssinus* allergens were different each other. The velocities of microbeads in the cases of not only immunoassay experiments but also control experiments and negative control experiments showed different values. This difference was caused by two allergens themselves. We conjugated the same concentration of mite lysates to the microbeads. Of common knowledge, the *D. farinae* has 7 allergens and the *D. pteronyssinus* has 11 allergens, respectively. Therefore, the amounts of allergens in two mite lysates were different and that of conjugated allergens to the microbeads were also different. This resulted in the difference of two results.

Like the results mentioned above, this detection platform for allergen-specific IgE made up for the weak points in the existing allergy detection methods. Compared with the CAP systems which are known to have the highest sensitivity and reliability, the new detection platform has a lot of advantages over that (Table 6).
Table 6. Advantages of the new detection platform over CAP

<table>
<thead>
<tr>
<th></th>
<th>CAP</th>
<th>New platform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detectable range</td>
<td>0.85–242 ng / mL</td>
<td><em>D. farinae</em>: 1.05 ~ 20 ng / mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. pteronyssinus</em>: 0.43–10 ng / mL</td>
</tr>
<tr>
<td>Time</td>
<td>More than 3 hours</td>
<td>20 min</td>
</tr>
<tr>
<td>Cost</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Volume of serum</td>
<td>50 µL/well</td>
<td>10 µL</td>
</tr>
<tr>
<td>Equipment</td>
<td>Large scale</td>
<td>Microchip</td>
</tr>
<tr>
<td>Simultaneous multiple analysis</td>
<td>Impossible</td>
<td>Possible</td>
</tr>
</tbody>
</table>

In this detection platform, Luminex microbeads were used as the supporting microbeads. The Luminex microbeads consists of two types of dyes, so they can be encoded by the ratios of two different dyes. This was the reason that we chose the Luminex microbeads. If this sandwich immunoassay scheme is used, multiplexing assays can be possible in a single reaction without the consumption of time and a tedious labor.

We applied the ferromagnetic materials, Ni microstructure, into the microfluidic device. So, the magnetic field gradient was enhanced remarkably. However, the permanent magnet was incongruent for the
automation, miniaturization and integration. To do so, a microelectromagnet should be introduced. The manipulation of magnetic microbeads or nanoparticles in the microsystem was already studied by other groups [9, 27]. In those reports, the magnetic field gradient of the microelectromagnetic chips was similar to that of our device system. High magnetic field gradient, $10^3 - 10^4$ T / mm, can be generated by specific design of materials [28].

In this scheme, the ferromagnetic material, superparamagnetic nanoparticles, were used as labels. This material has the tendency to be attracted toward the high magnetic field gradient. In opposition to the ferromagnetic material, the diamagnetic material has the characteristic to be repelled from the high magnetic field gradient. If the diamagnetic material is introduced to this scheme as labels, the lower limit of detection could be expected.

After taking the factors mentioned above into consideration, a highly sensitive, automated and multiplexing assay system could be realizable.
Chapter 6 Conclusions

We have performed the microfluidic sandwich immunoassays for the detection of mite allergen-specific human IgE in an enhanced magnetic field gradient. With the Ni microstructure, the limit of detection could be successfully lower than that of the preceding study. These results were expected by the simulation results. Compared with the traditional diagnosis assay, CAP, the obtained results were more sensitive than CAP. The movements of microbeads conjugated with magnetic nanoparticles were measured and analyzed in terms of the velocity. The measured velocity had relation to the concentration of human IgE. By the relationship, this system could be used to analyze the unknown concentration of target biomolecules such as human IgE in the solution, efficiently. This method may be suitable for use as a self-care type of allergy detection system that does not need special medical equipment and doctors. The advantage of this method is its safety as it does not require radio isotopes or in vivo tests used in other detection methods. Also, the multiplexed immunoassay could be possible because the encoded microbeads such as Luminex microbeads were used in this scheme. In addition, the permanent magnet can be substituted with the
microelectromagnet. If so, this detection scheme could be utilized in a lab on-a-chip or a μTAS system.
Summary in Korean

본 연구에서는 단백질과 같은 생체 분자를 감지하기 위해 강화된 자기장 구배와 상자성 미세 입자를 이용한 미세 유체 기반의 면역 측정법을 개발하였다. 또한, 이러한 자기력을 이용한 미세 유체 기반의 면역 측정법을 인간 혈청에 있는 진드기 알레르겐 특이 항체 (IgE) 검출 시스템에 성공적으로 적용할 수 있었다. 이러한 측정 방법의 기본적인 원리는 면역 반응에 의해 형광 미세 입자에 붕가된 상자성 미세 입자로 인해 형광 미세 입자가 높은 자기장 구배로 편향되는 현상을 이용한 것이다. 따라서 편향된 속도를 측정하게 되면 생체 분자의 농도를 정량화할 수 있게 된다. 즉, 생체 분자의 농도와 형광 미세 입자가 끌려오는 속도의 관계를 바탕으로 미지의 농도를 가진 생체 분자를 분석, 검출할 수 있다.

본 실험에서는 두 가지 실험이 수행되었는데 하나는 진드기 알레르겐 특이 항체 검출 시스템에 도입하기 위한 모델 실험으로써 토끼 IgG 검출 실험이 수행되었고, 다른 하나는 진드기 알레르겐 특이 항체 검출 실험이다. 첫 번째 실험에서는 polyclonal anti-rabbit IgG가 붓어 있는 50 nm 크기의 상자성 미세 입자, 1 μm의 적색 형광 미세 입자, 그리고 monoclonal rabbit IgG가 사용되었다. 두 번째 실험이에서는 5.6 μm의 형광 미세 입자, 9 nm의 상자성 미세 입자, 그리고 사람 혈청에 있는 진드기 알레르겐 특이 항체가 사용되었다. 실험 전에
준비 사항으로 5.6 µm의 형광 미세 입자는 D. farinae와 D. pteronyssinus 두 종류의 진드기 알레르겐을 결합시켰고, 9 nm의 상자성 미세 입자는 진드기 알레르겐 특이 항체를 인식할 수 있는 anti-human IgE를 결합시켜 놓았다. 소자의 구조 면에서는 강자성체인 니켈 미세 구조를 도입하여 영구 자석에서 나오는 자기력선을 집속시킴으로써 자기장 구배를 강화하였다. 니켈 미세 구조는 유리 기판 위에 도금으로 만들어졌고 미세 유체 채널을 가진 소자는 PDMS를 주형으로 하는 공정방법에 의해 제작되었다. 만들어진 두 소자는 플라즈마 처리를 통해 경질되고 접합되었다. 이렇게 제작되어진 소자에서의 자기장 구배는 영구 자석의 것보다 훨씬 높은 약 10⁴ T / m 가지 얻을 수 있었다. 이로 인해 이전 연구에서 얻은 결과를 훨씬 높은 높도보다 약 250배 가량 배출할 수 있었다. 검출 범위는 토끼 IgG의 경우는 약 200 pg / mL에서 1 pg / mL까지 측정이 가능하였고 진드기 알레르겐인 D. farinae와 D. pteronyssinus 특이 항체인 인간의 IgE는 각각 19.47 ng / mL에서 104 pg / mL, 10.68 ng / mL에서 151 pg / mL까지 가능하였다.

본 연구에서 사용한 5.6 µm 형광 미세 입자는 내부의 염료 비율을 달리함으로써 여러 가지의 형광을 얻을 수 있는 인코딩가 가능한 입자이다. 이 입자만 여러 가지로 바꿔주고 각각에 원하는 생체 분자를 결합하게 되면 이 시스템으로 다중 면역 검출이 가능하게 된다. 또한, 영구 자석보다 크기는 철전 작으면서 니켈 미세 구조를 도입한 경우만큼의 높은 자기장 구배를 실현할 수
있는 미세 전자석을 미세 소자에 통합한다면 본 연구과 같은 고감도이고 측정 한계가 낮은 분석 시스템이 구현될 수 있을 뿐만 아니라 집적화와 자동화가 가능해져 보다 효율적인 측정 센서 시스템을 구현할 수 있을 것으로 기대된다.
References


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제가 이렇게 연구를 할 수 있었던 것은 무엇보다도 우리 학원들의 힘이 도움이 되었습니다. 대학원부터 지금까지, 또 2년동안 같은 방을 쓰며 언제나 조언을 아껴서 없이 많이 실험을 해준 주현이에게 감사한다는 말을 하고 싶습니다. 고맙다. 강주현!! 제 연구의 선구자가 되어준 출업생 구성이에게도 고마운 마음을 전합니다. 뱅의 큰 힘으로써 날카로운 지적을 해준 성희이, 이제는 한 아이의 엄마가 된 주현이, 뱅의 바른 생활을 이끌어가는 원재, 언제나 우리에게 웃음을 주는 성희이, 같이 입학해서 같이 졸업하게된 동기 덕분이, 뱅의 막대로서 많은 일을 한 유진이 그리고 학원들에게 풍요로움을 채워준 마음 넉넉한 유창이형과 상필이 형, 모두에게 정말 감사드립니다.

실험을 하는데 있어서 정말 많은 도움을 주시고 총고를 해주신 김학성 교수님 연구실의 오은규씨와 김종문씨에게도 감사의 말씀을 전합니다.

대학원부터 지금까지 같이 고생하고 있는 창현과 종원, 지금은 여其它问题 회사원이 되어버린 준아와 동희, 그리고 뒤늦게 새로운 친구를 찾아 열심히 노력하고 있는 용준이에게도 고마운 마음 전합니다.

마지막으로 저를 남아주시고 지금까지 올바르게 자랄 수 있도록 해주신 사랑하는 우리 아버지, 어머니에게 감사합니다. 그리고 험한 대학원 생활을 잘 견딜 수 있도록 결에 있어준 해야에게 감사의 마음을 전합니다.
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