On-chip antibody immobilization for on-demand and rapid immunoassay on a microfluidic chip

Toshinori Ohashi,¹ Kazuma Mawatari,² and Takehiko Kitamori^{1,2,a)}

¹*Institute of Microchemical Technology, 3-2-1 Sakado, Takatsu, Kawasaki, Kanagawa 213-0012, Japan*

²*Department of Applied Chemistry, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan*

(Received 6 April 2010; accepted 6 May 2010; published online 30 September 2010)

Immunoassay is one of the important applications of microfluidic chips and many methodologies were reported for decreasing sample/reagent volume, shortening assay time, and so on. Micro-enzyme-linked immunosorbent assay (micro-ELISA) is our method that utilizes packed microbeads in the microfluidic channel and the immunoreactions are induced on the beads surface. Due to the large surface-to-volume ratio and small analytical volume, excellent performances have been verified in assay time and sample/reagent volume. In order to realize the micro-ELISA, one of the important processes is the immobilization of antibody on the beads surface. Previously, the immobilization process was performed in a macroscale tube by physisorption of antibody, and long time (2 h) and large amount of antibody (or high concentration) were required for the immobilization. In addition, the processes including the reaction and washing were laborious, and changing the analyte was not easy. In this research, we integrated the immobilization process into a microfluidic chip by applying the avidin-biotin surface chemistry. The integration enabled very fast (1 min) immobilization with very small amount of precious antibody consumption (100 ng) for one assay. Because the laborious immobilization process can be automatically performed on the microfluidic chip, ELISA method became very easy. On-demand immunoassay was also possible just by changing the antibodies without using large amount of precious antibodies. Finally, the analytical performance was investigated by measuring C-reactive protein and good performance (limit of detection <20 ng/ml) was verified. © 2010 American Institute of Physics. [doi:10.1063/1.3437592]

I. INTRODUCTION

Interest in miniaturized chemical systems on microchips has been increasing. Advances in integration of various chemical processes (including mixing, chemical reactions, separation, and so on) have progressed rapidly in a past decade.^{1,2} 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 microchip technology to applications in various analytical procedures and chemical syntheses. Especially, immunoassay is one of the important applications for medical research and clinical diagnosis. The conventional immunoassay required relatively long assay time, troublesome liquid-handling procedures, large amount of analytes and expensive antibody reagents, which limited the wide use in many laboratories and clinics. Currently, point-of-care testing is increasingly required but is difficult and impractical using the conventional methods. Microchip-based systems have the potential to overcome these problems.

^{a)} Author to whom correspondence should be addressed. Electronic mail: kitamori@iclt.u-tokyo.ac.jp. FAX: +81-3-5841-6039.

To date, many groups reported the integrations of immunoassay on microfluidic chips: rapid diffusion immunoassay,³ immunoassay using flow cytometer,⁴ multiplex immunoassay on capillary-assembled microchip,⁵ micromosaic immunoassay,⁶ and so on. A recent review can be referred.⁷ Excellent performances were reported in reduced sample, analyte volume and reduced analysis time. We have also successfully integrated the immunoassay into a microchip^{8,9} and also integrated enzyme-linked immunosorbent assay (ELISA) method.^{10,11} Our original sensitive detector [thermal lens microscope^{12,13} (TLM)] for nonfluorescent molecules was utilized for the detection, which enabled us to use a wide range of conventional ELISA reagents with increased sensitivity. In the micro-ELISA method, microbeads are coated with capture antibodies through physisorption and introduced to a microfluidic channel. A dam structure in the microfluidic channel stops the microbeads and length of the microbead area can be controlled. Immunoreactions with antigens are then induced on the surface of the microbeads. Enzyme-conjugated secondary antibodies are then introduced and capture the antigens. Finally, substrates are applied and dye molecules produced by the enzymatic colorimetric reaction are detected by our sensitive TLM. For new measurement, the microbead suspension is changed to new one, and same microchip can be used repeatedly. In a comparison with conventional methods, excellent performances in analysis time (10–20 min) and sample volume (several microliters) were also demonstrated. In addition, the practical systems were realized and the application to clinical diagnosis (allergy) was performed by using patient's serum samples.¹⁴ The good correlation with a conventional method was also demonstrated. The compact and automated systems may enable the wide use of immunoassay (ELISA) in many laboratories and clinics.

One of the most important points to realize micro-ELISA is immobilization of antibody with high density on the surface of the microbeads. In our standard condition, a microbead suspension with volume of several hundreds of microliters is mixed with the antibody solution in a tube for immobilizing the antibodies on the microbead. For high-density immobilization, large amount of the antibody (several hundreds of $\mu\text{g}/\text{ml}$ after mixing) is required in addition to the long reaction time (2 h), while microbead suspension of just several microliters is required for micro-ELISA. In addition, the preparation processes of the reaction and washing are laborious. The prepared microbead suspensions cannot be frozen for preservation and the functions of precious antibodies are lost in long-term preservation. When the immobilization processes are integrated into a microfluidic chip, these problems will be solved and new immunoassay is realized with advantages of the less consumption of precious antibody, easy operation (automated from microbeads preparation), and on-demand (flexible choice of analytes just by changing the antibodies and rapid assay).

In this paper, we demonstrated the integration of the immobilizing process into a micro-ELISA chip. Avidin-biotin technique was applied for the surface chemistry. Microbead suspension with streptoavidin group was introduced into the microfluidic chip and biotin-labeled antibodies were introduced for immobilization afterward. Because no dilution process of the antibody was included, very small amount of antibody solution (10 $\mu\text{g}/\text{ml}$ and 10 μl) was used, which was a large advantage of this method. The reaction time of the immobilization was also decreased to 1 min due to the large surface-to-volume ratio of the microspace. Finally, an assay of C-reactive protein (CRP) was demonstrated and excellent performances were shown in small sample volume (3 μl), short assay time (13.5 min in total) and detection limit (<20 ng/ml). The concept of this new methodology was successfully verified.

II. EXPERIMENTAL SECTION

A. Chemicals

In this method, the material of microbeads was changed from polystyrene¹² to polydivinylbenzene with a diameter of 40 μm (Sekisui Chemical Co. Ltd., Japan) due to the low cost (about 1/30). The procedure of surface modification of streptoavidin on the microbeads is as follows. The 25 mg of biotin-longchain-BSA (Thermo Fisher Scientific, Inc., USA) and 8.0 g of the microbeads were mixed in a phosphate buffered saline (PBS) at the final volume of 100 ml. The suspension was mixed gently for 2 h at room temperature and stocked in a refrigerator for overnight. Then,

FIG. 1. Pictures of micro-ELISA system and micro-ELISA chip. A rod lens connected to an optical fiber (not shown) is inserted to a holder on the chip for sensitive detection. The size of the micro-ELISA system was $W253 \times D200 \times H222$ mm³.

the stock suspension was introduced to a separation column (Econo-Pac Chromatography Column, Bio-Rad Laboratories Inc., USA) and the microbeads were washed by 10 ml PBS solution (three times). The microbeads were washed out by 1% BSA solution to the final volume of 40 ml. The suspension was mixed gently for 2 h at room temperature and stocked in a refrigerator overnight. The stock suspension was concentrated at twofold by wasting the supernatant and the suspension was mixed with streptoavidin solution (5 mg in 10 ml 1% BSA solution) for 1 h. The suspension was introduced to the same separation column and the microbeads were washed by 10 ml PBS solution (three times). Finally, the microbeads were washed out by PBS solution containing 1% BSA to the final volume of 30 ml, which was 2.5% suspension. For immunoreaction, mouse monoclonal anti-CRP IgG was used and biotinylated with a kit (Dojindo Laboratories, Japan). The analyte was recombinant human CRP (Oriental Yeast Co. Ltd., Japan). As a secondary antibody, HRP-labeled rabbit anti-CRP IgG was prepared by a labeling kit (Dojindo Laboratories, Japan).

B. Micro-ELISA system

The basic principle of micro-ELISA was reported in our previous paper.¹² Stopped-flow conditions were applied for enzymatic reaction and the signals were obtained as peak. In this paper, an automated micro-ELISA system (IMT-501, Institute of Microchemical Technology Co. Ltd., Japan) was utilized. The picture of the system and microfluidic chip is shown in Fig. 1. 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 approximately $20 \mu\text{m}$. The procedures for micro-ELISA are shown in Fig. 2. Same microfluidic chip was used for all the measurement just by changing the bead suspensions. All the parameters and moving parts were controlled by a personal computer (PC). Sample and reagents in tubes were set on a rotating table and automatically introduced into the micro-ELISA chip via Teflon connector. The peak signals were detected by a TLM device¹⁵ and sent to the PC. The

FIG. 2. Protocol for micro-ELISA. Same chip can be used for new analytes by changing the microbead suspensions that can be automatically performed.

concentrations of the analytes were automatically obtained using the calibration curve. The wavelengths of the excitation and probe beams in the TLM device were 658 and 785 nm, respectively. A rod lens with optical fiber was inserted into a holder in Fig. 1 to focus the two laser beams into the microfluidic channel and no optical adjustment was required, which enabled easy use of the TLM device.

III. RESULTS AND DISCUSSION

For comparison, we prepared the microbead suspension by our conventional method using a macroscale tube and the analytes were analyzed by micro-ELISA method using the prepared microbeads. The microbead (2.5%) was mixed with mouse monoclonal anti-CRP IgG solution for 2 h to induce physisorption of the antibodies on the bead surface. The concentrations of the anti-CRP IgG after mixing with the microbead solution were adjusted in the range of 0–1000 $\mu\text{g/mL}$, which were simply calculated assuming no adsorption. By utilizing the microbeads, micro-ELISA signals of CRP (2000 ng/ml) were measured. The protocols for micro-ELISA were same as Fig. 2 except no second step included. The result is shown in Fig. 3. The high concentration (several hundred $\mu\text{g/ml}$) and long time (2 h) were required for immobilization reaction. Including the washing process and treatment by a blocking reagent (BSA), almost 3 h were required.

Then, we investigated to integrate the immobilization process into the microfluidic chips. Streptavidin-coated microbeads were introduced into the micro-ELISA chip. Then, biotinylated anti-CRP IgG solutions were introduced and CRP solutions (400 ng/ml) were analyzed in accordance with the protocol of Fig. 2. First, the concentration dependence of anti-CRP IgG was investigated for constant flow rate of 10 $\mu\text{L/min}$ and injection time of 1 min. The result is shown

FIG. 3. Results of measuring 2000 ng/ml CRP solution by utilizing our conventional antibody immobilization method.

in Fig. 4 (left). The signals increased by increasing the concentrations and leveled off from approximately 1 $\mu\text{g/ml}$. Compared to the results in Fig. 3, the concentration was largely decreased from several hundreds of $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ (10 $\mu\text{g/ml}$ for experiments below). This is because no dilution process of the antibody solution is needed and immobilization reaction at almost original concentration can be performed. The other advantage is sample volume. In this immobilization process, just 10 μl was required for anti-CRP IgG solution. It means that the total amount of anti-CRP IgG required for one assay was as small as 100 ng. The reaction time was also shortened from 2 h to 1 min due to the large surface-to-volume ratio. We also investigated the flow rate dependence for immobilization at 10 $\mu\text{g/ml}$ of anti-CRP IgG solution in the range of 2–10 $\mu\text{l/min}$. The total volume introduced was kept at 10 μl for all the conditions. The result is shown in Fig. 4 (right). Although the slight decrease in sensitivity was observed due to reduced residence time, the flow rate did not have large effect for the sensitivity. By considering the

FIG. 4. Results of measuring 400 ng/ml CRP solutions by developed immobilization method: dependence of antibody concentration (left) and flow rate for immobilization (right).

FIG. 5. Calibration curve for CRP determination.

process time required, we set the flow rate at $10 \mu\text{l}/\text{min}$. In this condition, the immobilization process finished in 1 min, which was very short compared with 2 h in our conventional immobilization method in a macroscale tube.

Finally, we investigated the analytical performance with the developed method. The concentrations of CRP solutions were changed in the range of 0–400 ng/ml and micro-ELISA signals were measured in accordance with the protocol in Fig. 2. The result is shown in Fig. 5. The signals increased with the concentration, which was reasonable. At lowest concentrations, 20 ng/ml could be detected with 13.5 min assay time. The analytical time and detection limit are almost comparable with our conventional micro-ELISA method considering the additional process of the antibody immobilization. This method is very sensitive and can be used for high sensitivity CRP analysis for diagnosis of chronic inflammation in cardiovascular system, which required sensitive detection below $1 \mu\text{g}/\text{ml}$. The repeatability of this method was also investigated. The CRP solution of 200 ng/ml was analyzed five times repeatedly. The result is summarized in a Table I. In spite of the low concentration condition, good repeatability (CV: 5.7%) was obtained. The applicability of the immobilization method was verified.

TABLE I. Investigation of repeatability by five times repeated measurements of 200 ng/ml CRP solution.

Average (ng/ml)	Standard deviation (ng/ml)	CV (%)
189.3	10.7	5.7

IV. CONCLUSION

We developed a micro-ELISA system for on-demand and rapid immunoassay. Immobilization process of antibodies was integrated into microfluidic chips by applying the avidin-biotin surface chemistry. The integration enabled very fast (1 min) immobilization with very small amount of precious antibody consumption (100 ng) for one assay. Because the laborious immobilization process can be automatically performed in the microfluidic channel, ELISA method became very easy and on-demand immunoassay was also possible by changing the antibodies. As for analytical performance, excellent lower limit of detection (<20 ng/ml) was verified for CRP measurement. In addition to the heterogeneous immunoassay, this method can be also used for homogeneous immunoassay, where the homogeneous immunoreaction is conducted first on microfluidic chip or in a macroscale tube, and bound-free separation and detection are performed using the developed immobilization method. This developed method will contribute to the wide use of immunoassay in many laboratories and clinics.

ACKNOWLEDGMENTS

This work was partially supported by the Program of Development of System and Technology for Advanced Measurement and Analysis (SENTAN) from the Japan Science and Technology Agency (JST).

- ¹D. R. Reyes, D. Iossifids, P.-A. Auroux, and A. Manz, *Anal. Chem.* **74**, 2623 (2002).
- ²P.-A. Auroux, D. Iossifids, D. R. Reyes, and A. Manz, *Anal. Chem.* **74**, 2637 (2002).
- ³A. Hatch, A. E. Kamholz, K. R. Hawkins, M. S. Munson, E. A. Schilling, B. H. Weigl, and P. Yager, *Nat. Biotechnol.* **19**, 461 (2001).
- ⁴D. Holmes, J. K. She, P. L. Roach, and H. Morgan, *Lab Chip* **7**, 1048 (2007).
- ⁵T. G. Henares, S. Funano, S. Terabe, F. Mizutani, R. Sekizawa, and H. Hisamoto, *Anal. Chim. Acta* **589**, 173 (2007).
- ⁶A. Bernard, B. Michel, and E. Delamarche, *Anal. Chem.* **73**, 8 (2001).
- ⁷T. G. Henares, F. Mizutani, and H. Hisamoto, *Anal. Chim. Acta* **611**, 17 (2008).
- ⁸K. Sato, M. Tokeshi, T. Odake, H. Kimura, T. Ooi, M. Nakao, and T. Kitamori, *Anal. Chem.* **72**, 1144 (2000).
- ⁹K. Sato, M. Yamanaka, H. Takahashi, M. Tokeshi, H. Kimura, and T. Kitamori, *Electrophoresis* **23**, 734 (2002).
- ¹⁰K. Sato, K. Yamanaka, T. Hagino, M. Tokeshi, H. Kimura, and T. Kitamori, *Lab Chip* **4**, 570 (2004).
- ¹¹M. Kakuta, H. Takahashi, S. Kazuno, K. Murayama, T. Ueno, and M. Tokeshi, *Meas. Sci. Technol.* **17**, 3189 (2006).
- ¹²K. Uchiyama, A. Hibara, H. Kimura, T. Sawada, and T. Kitamori, *Jpn. J. Appl. Phys., Part 1* **39**, 5316 (2000).
- ¹³T. Kitamori, M. Tokeshi, A. Hibara, and K. Sato, *Anal. Chem.* **76**, 52A (2004).
- ¹⁴T. Ohashi, K. Mawatari, K. Sato, M. Tokeshi, and T. Kitamori, *Lab Chip* **9**, 991 (2009).
- ¹⁵Y. Matsuoka, Y. Yoshida, M. Tokeshi, A. Hattori, T. Fukuzawa, J. Yamaguchi, K. Uchiyama, and T. Kitamori, *Proceedings of the Micro Total Analysis System*, Malmö, Sweden, 2004, pp. 452–454.