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Rapid and reagent-saving immunoassay using innovative stirring actions of magnetic beads in microreactors in the sequential injection mode

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Abstract

We developed new ELISA techniques in sequential injection analysis (SIA) mode using microreactors with content of a few microliters. We immobilized antibodies on magnetic beads 1.0 μm in diameter, injected the beads into microreactors and applied rotating magnetic fields of several hundred gauss. Magnetic beads, suspended in liquid in density of approximately 10^9–10^{10} particles per millilitre, form a large number of thin rod clusters, whose length-wise axes are oriented in parallel with the magnetic field. We rotate the Nd magnets below the center of the microreactor by a tiny motor at about 2000–5000 rpm. These rotating clusters remarkably accelerate the binding rate of the antibodies with antigens in the liquid. The beads are trapped around the center of the rotating magnetic field even in the flowing liquid. This newly found phenomenon enables easy bead handling in microreactors. Modification of reactor walls with selected blocking reagents was essential, because protein-coated beads often stick to the wall surface and cannot move freely. Washing steps were also shortened.

Keywords: SIA; Microreactor; Magnetic beads; Rotating field

1. Introduction

Immunoassay in the sequential injection analysis (SIA) mode was developed by Pollema et al. [1] in early 1990s. They used magnetic beads and electromagnets to construct an automatic SIA system to realize rapid replacement of the magnetic beads with specific immobilized biomolecules. Sample volumes injected into their reaction coil was 25 μL, which was an order of magnitude smaller than the volume of wells of standard microplates. Since that time demands for more reagent-saving and rapid analysis remain strong and advanced various technical progresses. Pollema et al. used magnetic beads 4.5 μm in diameter (sheep anti-mouse IgG coated Dynabeads M-450), but today variously and reliably activated magnetic beads 1 μm in diameter or smaller, that is, beads with higher specific area and fluidity are commercially available (for example, Dynabeads MyOne®). Highly sensitive immunoassay using a few μL of reagents and samples became possible owing to development of microfluidics, microreactors, and detectors. For example, Aguilar et al. [2] reported electrochemical immunoassay system which can detect 9 fg of mouse IgG using 1 μL for the antigen and 1 μL for the secondary antibody–enzyme conjugate within 30 min.

Rapid analysis still remains important and challenging. For example, finishing reliable clinical analysis within a few minutes is desirable for patients and doctors. We studied on speed-up of the immunoassay using antibodies immobilized on surfaces of magnetic beads in rotating magnetic fields [3,4]. Without magnetic field, magnetic beads are freely and separately suspended in buffer solution. But when magnetic field applied, they form rod-like clusters whose length-wise axes, typically 10 μm long, are oriented in parallel with the magnetic field. When rotating magnetic fields are impressed, each rod cluster rotates locally around its center. Proper rotations of magnetic field, 10^1–10^2 rotations per second, proved very effective in agitating locally and microscopically the bead-suspending liquid and increase the collision frequencies and the reaction rates between free solute ligands and receptors immobilized on magnetic beads either in microreactors or in test tubes of common sizes [3,4]. Such a mode of bead utilization is quite different from traditional one, in which individual magnetic beads are moved together with their surrounding liquid or attracted and fixed to the container wall by a quasistatic magnetic field. Rotating magnetic fields reveal another characteristic behavior of magnetic beads: their

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clusters are as a whole attracted to the center of the rotating magnetic field [5].

In the present investigation, we have applied those two behaviors of magnetic beads to speed-up of reactions in microreactors and to retaining magnetic bead clusters within microreactors, while allowing solution flowing thorough. Those innovative applications and manipulations will give new freedoms to SIA or FIA using magnetic beads. We carried out a sandwich ELISA as a model experiment to confirm the speed-up effect of magnetic bead agitation and retaining of magnetic beads against flowing buffer solutions.

2. Experimental

2.1. Chemicals and reagents

We used one kind of magnetic beads, Dynabeads MyOne^{TM} Streptavidin C1 (carboxylated). They were supplied as a suspension of 10 mg (approximately 7–12 $\times$ 10^9 beads) per millilitre, dissolved in phosphate buffered saline (PBS) pH 7.4, containing 0.01% Tween®-20 and 0.09% NaN_3 as a preservative.

As for reagents for ELISA, primary antibody, anti-rat IgG biotin conjugate, Sigma B7139, was supplied as a solution in 0.01 M phosphate buffered saline, pH 7.4, containing 1% BSA with 15 mM sodium azide as a preservative. Its antibody content is 0.1–1.0 mg/mL (prior to the addition of BSA). This antibody was immobilized on Dynabeads through avidin–biotin bonding. The antigen, rat IgG, was supplied as lyophilized powder (Sigma I4131). The secondary antibody, anti-rat IgG horseradish peroxidase conjugate (Sigma A9037), was provided as a solution in 0.01 M phosphate buffered saline, pH 7.4, containing 1% BSA with 0.01% thimerosal as a preservative. As substrate for horseradish peroxidase, ready-to-use ABTS solution containing ABTS and H_2O_2 in glycin/citric acid buffer was purchased from Roche Diagnostic GmbH (Germany). We estimated its ABTS concentration at 65 μm on the basis of the observed absorption coefficient of 2.4 cm^{-1} and the reported molar absorption coefficient [6] of 36,000 M^{-1} cm^{-1} at the peak wavelength 340 nm. Polymer of 2-methacryloyloxy-ethylphosphoriline (MPC) were purchased from NOF Corporation (Tokyo, Japan) and used to modify wall surfaces of the microreactor to prevent sticking of protein-immobilized magnetic beads.

2.2. Sequential injection system using magnetic beads agitation

Fig. 1 shows the set-up of the sequential injection system and Table 1 summarizes the SIA protocol. Sequential injections were manually conducted. Phosphate buffered solution (0.01 M, pH 7.4) was pumped into the microreactor through Teflon tubing (360 μm o.d., 100 μm i.d.) at a flow rate of 10 μL/min using a precision micropump (Ultra-Plus II MicroLC System, Micro-Tech Scientific Inc.). The magnetic beads coated with the primary antigen, samples, and reagents except the substrate solution were sequentially injected via 5 μL sample loops of a six-port injection valve (M-485 Nanopeak Injection valve with port-to-port volume of 50 nL, Scivex and Upchurch Scientific), which was inserted between the pump and the microreactor. The substrate solution for the label enzyme was injected by a microsyringe pump (KD Scientific Inc. Type KDS210) into the microreactor. Either one of two streams from the micropump and the microsyringe pump was selected by a 3-way valve (Rheodyne #7030).

Fig. 2 shows the microreactor with content of 2 μL. It was in house molded by casting poly(dimethyl siloxane) (PDMS hereafter) into an SU-8 (http://www.microchem.com) mold, which was prepared by standard electron-beam patterning and UV lithography.

Rotating magnetic fields were produced by small neodimium magnets (or magnets; 10Ø $\times$ 5 mm, surface magnetic flux density 420 mT, Seiko Sangyo Co., Japan), which were held in

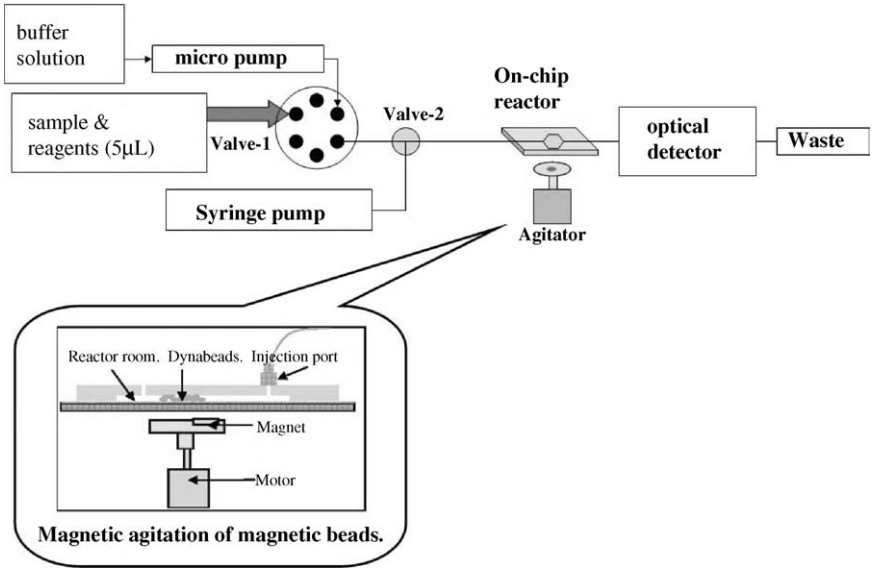

Fig. 1. The set-up of the sequential injection system.

Table 1
SIA protocol for the model sandwitch-Elisa experiments

Step	Operation	Liquids	Time (min)	Flow rate (mL/min)	Volume (mL)
1	Injection of beads into MR	Suspension of beads bound with primary Ab	0.5	10	5
2	Injection of Ag into MR	RAT-IgG soln 1, 10, 100, ng/mL	0.5	10	5
3	Flow stop	Incubation of primary Ab–Ag binding	1	0	0
4	Washing	0.01 M PBS + 1%Tween-20	1	10	10
5	Injection of secondary Ab into MR	HRP-labeled anti-RAT IgG antibody (6 mg/mL)	0.5	10	5
6	Flow stop	Incubation of secondary Ab–Ag binding	1	0	0
7	Washing	0.01 M PBS + 1%Tween-20	1	10	10
8	Initial pumping of substrate to fill the dead volume	Ready-to-use ABTS solution containing H_2O_2	0.5	10	5
9	Enzymatic reaction in various residence time		> 1	2	>2
			> 2	1	>2
			> 4	0.5	>2
			> 8	0.25	>2
10	Optical detection	$ABTS^+$ produced by enzymatic reaction	0.5		

MR: microreactor.

holes in aluminum disc. The disc was rotated a few centimetre below the reactor by a tiny toy motor (Mabuchi Motor Co., RE-140, 25 mm × 21 mm). The rotating speed was at rates of 2000–5000 rpm, which were controlled by a dc power supply (1.5 V or below). The rotating axis passes through the center of the microreactor but the magnets were set 10 mm off the axis, so that they rotate below and around the microreactor. Strength of Magnetic fields was measured using a gauss-meter (#4048 hand-held digital gauss-meter, F.W. Bell). Clustering and movements of magnetic beads were recorded by microscopes with CCD cameras.

We measured the optical absorption spectra around the 414 nm absorption peak of the reaction product, $ABTS^+$, which was contained in the solution flowing out from the microreactor during the enzymatic reaction at a constant rate, 0.25, 0.5, 1.0, or 2.0 μL/min. At the exit of the microreactor, 2 μL of the solution was aspirated by a microsyringe and transferred to a NanoDrop® ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE 19810, USA). Pipeting the sample solution is directly pipetted to a 1 mm gap between the ends of two optical fibers of the spectrophotometer and form a thin liquid column by surface tension in a several seconds after the sampling aspiration. Thereafter, optical measurements are finished less than 10 s on the spectrophotometer.

3. Results and discussion

3.1. Gathering of magnetic beads and the enzymatic reaction

Fig. 3(a) shows a close up picture of the magnetic bead clusters, and Fig. 3(b) rod-like clusters whose length-wise axes are oriented in parallel with the magnetic field. Fig. 4(a) is a CCD camera image of the inside of the microreactor 10 s after injection of the magnetic beads and just after the change of the carrier liquid from the bead-suspending buffer to the ABTS substrate solution. A cloud of thin brown color suggests that magnetic beads begin gathering toward the center of the rotating magnetic field. Thirty seconds thereafter, there appears darker narrower area in the center as shown in image Fig. 4(b), suggesting that magnetic beads are gathering denser. The cloud of beads are surrounded by a wider cloud of green color due to ABTS cation formation by enzymatic reaction of peroxidase immobilized on the bead-surface. Another 30 s thereafter, the central area became quite dark, and green color of $ABTS^+$

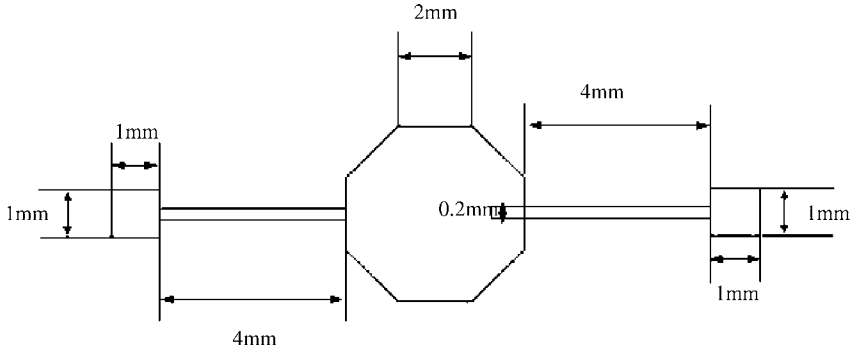

Fig. 2. The in-house made PDMS microreactor with content of 2 μL. Two squares 1 mm × 1 mm on the right and left sides are the inlet and outlet holes for solution.

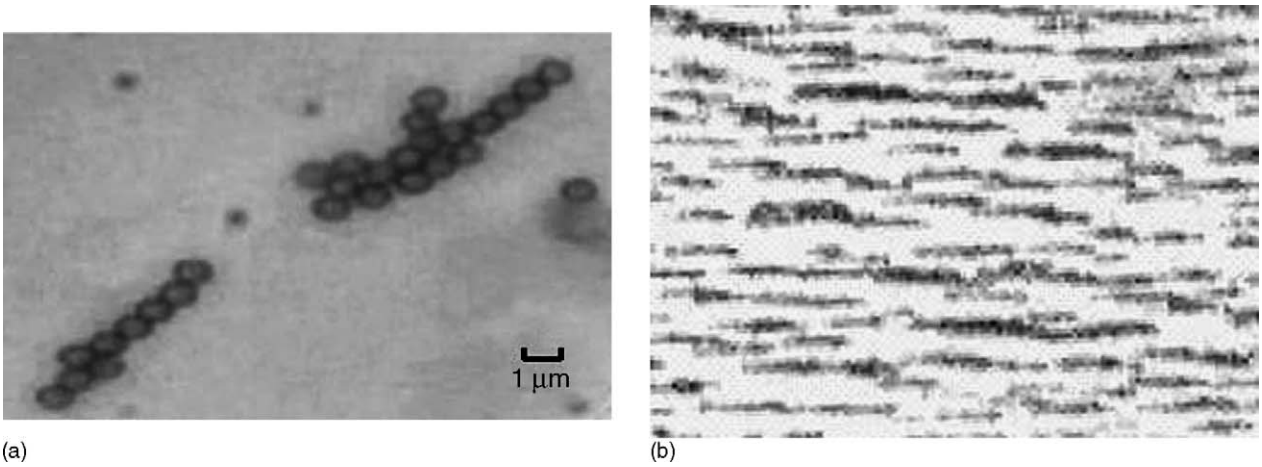

Fig. 3. (a) A close up picture of the magnetic bead clusters. (b) Rod-like clusters whose length-wise axes are oriented in parallel with the magnetic field.

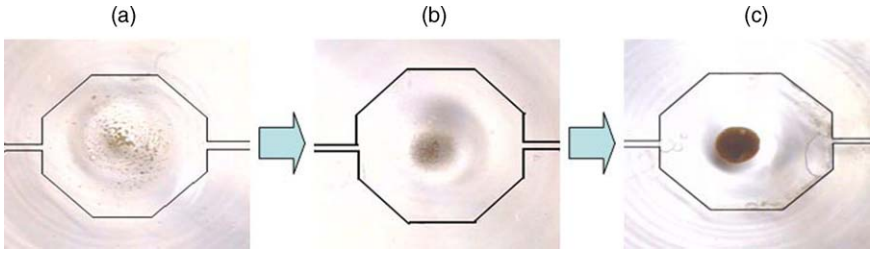

Fig. 4. (a) Magnetic beads begin gathering toward the center of the rotating magnetic field. (b) Magnetic beads gathering denser and they are surrounded by a wider cloud of green color due to ABTS cation formation (30 s after). (c) Quite dark beads area and green color of $ABTS^+$ reached the exit channel at the right (another 30 s after).

became thicker, which reached the exit channel at the right (Fig. 4(c)).

3.2. Quantitative measurements of production rate of $ABTS^+$

Fig. 5 shows corresponding changes in production rate of $ABTS^+$, which is proportional to the product, optical density × flow rate, against the flow rate of solution containing ABTS. The production rates increase with increasing flow rate, but are approximately constant between 4 and 6 μL/min. This range of flow rate was suitable for quantitative analysis, for example, ELISA.

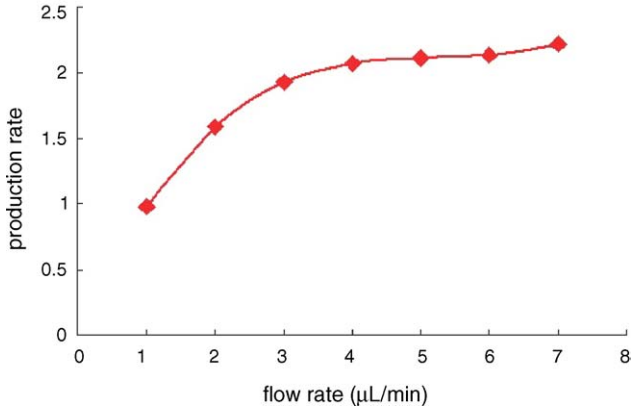

Fig. 5. Production rate of $ABTS^+$ against the flow rate of solution containing ABTS. The reaction rate is enhanced by peroxidase immobilized on surfaces of the magnetic Beads.

4. Conclusions

We succeeded in injecting magnetic beads as small as 1 μm in diameter into a microreactor, in agitating them vigorously, and in keeping them within the reactor even in flowing buffer solution by applying rotating magnetic field. Collision frequencies between solute molecules and biomolecules immobilized on beads increased considerably because of short diffusion distance between them and vigorous movements of magnetic beads. Until now "there are only a few examples in which beads are employed for heterogeneous assays on microfluidic devices, because of the difficulties associated with packing and handling these in etched microstructures"[4].

We confirmed confinement of magnetic beads within the microreactor thorough direct microscope observations and thorough stable enzyme reactions.

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