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Magnetic Sensor Based Liquid-Phase Immunoassays for the Detection of Biological Targets

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Abstract: We have developed a liquid-phase immunoassay technique for the detection of biological targets using magnetic markers. In this method, Brownian relaxation of magnetic markers in solution was used to perform the liquid-phase detection. Biological targets were fixed on the surface of large polymer beads whose size was typically a few μm. When the magnetic markers were bound to the targets, their Brownian relaxation time was dominated by that of the polymer bead, becoming much longer than that of unbound (free) markers. The resulting difference between the magnetic properties of the bound and free markers was detected by relaxation measurements. Therefore, we can magnetically distinguish between the bound and free markers, i.e., we can omit a time consuming washing process called bound/free separation. We developed a detection system using magneto-resistive (MR) and flux gate sensors. We could detect 1.4×10^7 and 8.3×10^6 bound markers in 60 μl of solution for the cases of MR and flux gate sensors, respectively. If we assume that a single marker is bound to a single target, this sensitivity can be expressed as 3.8×10^{-16} mol/ml and 2.3×10^{-16} mol/ml in terms of the molecular-number concentration. We also demonstrated the detection of biological targets called biotins, which were conjugated on the surface of the polystyrene beads with a diameter of 3.3 μm. A strong relationship was obtained between the number of bound markers and the number of biotin-conjugated polymer beads, which confirmed the validity of the present method.

Keywords: Bionanotechnology, Magnetic sensors, Nanoparticles, Brownian relaxation, MR sensor, Flux gate sensor

1. Introduction

Immunoassay is the detection of biological targets such as disease-related proteins and cells. Magnetic immunoassay techniques that utilize magnetic markers have recently been developed.[1-13] One of the merits of this magnetic method is that we can perform immunoassays in the liquid phase; that is, we can magnetically distinguish bound markers from unbound (free) markers. Using this function, we can omit the time-consuming washing process used to separate them, i.e., the so-called bound/free separation.

This function can be realized by exploiting the difference in the Brownian relaxation time between the bound and free markers. For this purpose, several methods have been proposed to prolong the relaxation time of the bound markers. The resulting difference between the magnetic properties of the bound and free markers has been detected using a relaxation or susceptibility measurement.[4-13]

We have developed a liquid-phase detection technique employing large polymer beads to immobilize the bound markers.[4-7-13] In this method, biological targets such as proteins are fixed on the surface of large polymer beads whose size is typically a few μm. When the markers are bound to the targets, the Brownian rotation of the markers is dominated by that of the polymer beads. Because the diameter of the polymer bead is much larger than that of the marker, the Brownian relaxation time of the bound markers becomes much longer than that of the free markers.

In this paper, we report on a liquid-phase immunoassay using this method and magnetic sensors such as magneto resistive (MR) sensor and flux gate sensor. We first describe a measurement system that uses these magnetic sensors. We show that we could detect $N_m = 1.4 \times 10^7$ and 8.3×10^6 bound markers for the cases of MR and flux gate sensors, respectively. If we assume that a single marker is bound to a single target, this sensitivity can be expressed as 3.8×10^{-16} mol/ml and 2.3×10^{-16} mol/ml in terms of the molecular-number concentration. Next, we demonstrate the detection of biological targets called biotins. In the experiment, we used biotins that were conjugated on the surface of the polystyrene beads with a diameter of $d_p = 3.3$ μm. A

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strong relationship was obtained between the number of bound markers and the number of biotin-conjugated polymer beads, which shows the validity of the method presented here.

2. Detection Principle without Bound Free Separation

In order to perform the liquid-phase immunoassay, we used a magnetic marker whose Neel relaxation time is much longer than its Brownian relaxation time. **Figure 1** shows a schematic of the detection principle of the liquid-phase immunoassay. As shown, we used a large polymer bead to fix the biological targets. When the markers are added, some of the markers are coupled to the targets, whereas others remain uncoupled. The former and the latter are called bound and free markers, respectively. These bound and free markers coexist in the solution.

The bound and free markers can be magnetically distinguished by using the difference in their Brownian relaxation time, as shown below. When an external field B_{ex} is applied to the sample, as shown in **Fig. 1(a)**, the magnetic moments m of both the bound and free markers are aligned with the direction of B_{ex}. Then, the excitation field is set to zero. In this case, Brownian rotation of the markers occurs, as shown in **Fig. 1 (b)**.

It is well known that the relaxation time of the Brownian rotation of the particle is given by,

$$\tau = 3\eta V_h / k_B T , \qquad (1)$$

where $V_h = (\pi/6) d_h^3$ is the hydrodynamic volume of the particle, d_h is the hydrodynamic diameter, and η is the viscosity of the carrier liquid.

Due to the Brownian rotation, the directions of the magnetic moments start to become random. As a result, the magnetization M of the assembly of the markers decays with time as

$$M(t) = M_0 \exp(-t/\tau) . \qquad (2)$$

Figure 2 shows the calculated results of the Brownian relaxation of both the free and bound markers. When we use a marker with a diameter d_h = 110 nm, the relaxation time of the free marker becomes τ_F = 0.5 ms. Therefore, free markers show rapid relaxation, as shown in **Fig. 2**. On the other hand, the Brownian relaxation of the bound markers is dominated by the volume of the polymer beads. If we use a polymer bead with a diameter of d_h = 3.3 μm, the relaxation time of the bound markers becomes τ_B = 10 s. Therefore, the bound markers show very slow relaxation compared to the free markers. This means that the signal from the bound markers continues for much longer time, compared to that from the free markers.

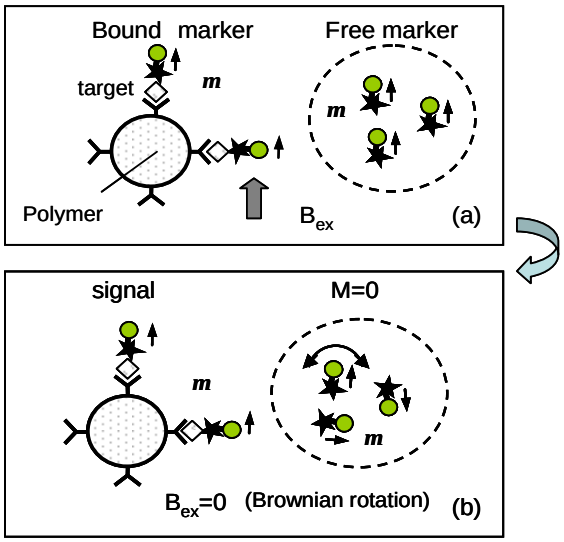

Fig. 1 Principle of liquid-phase immunoassay using the Brownian relaxation of magnetic markers in solution. (a) In the case when B_{ex} is applied, and (b) when B_{ex}=0.

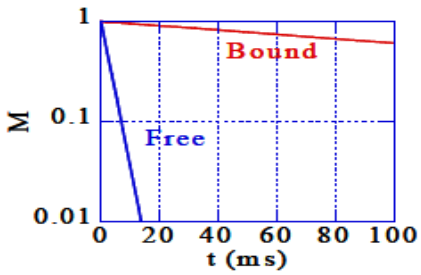

Fig. 2 Decrease of magnetic signal due to the Brownian relaxation. Signal from the free markers decays much faster than that from the bound markers.

We measure the signal from the sample at time T after the excitation field was set to B_{ex} = 0. The time T is chosen so as to satisfy the condition $\tau_F << T << \tau_B$, e.g., T = 1 s. At this time, the Brownian relaxation of the free markers will be completed, and the signal M from the free markers becomes zero, as shown in **Fig. 2 (b)**. On the other hand, the signal from the bound markers continues. Therefore, we can detect the signal only from the bound markers; that is, we can magnetically distinguish the

bound markers from the free ones.

3. Magnetic Marker

In the present experiment, commercial magnetic markers made of Fe_3O_4 nanoparticles were used (MagCellect particles, R&D Systems, U.S.A.). The size of single Fe_3O_4 particles was measured by transmission electron microscopy and found to be typically 20–25 nm.

On the other hand, the size of the marker in pure water was measured by means of dynamic light scattering and found to be typically 110 nm. Because the single Fe_3O_4 particle size was typically 20–25 nm, it follows that the aggregation of Fe_3O_4 particles occurred in the making of the magnetic markers; that is, the markers consisted of aggregated Fe_3O_4 particles.

The magnetic moment m of the marker was estimated from the M-H curve. In **Fig. 3**, the M-H curve of the markers that were diluted in solution is shown. The circles are the experimental results, whereas the solid line was calculated with the Langevin function $L(\xi) = \coth(\xi) - 1/\xi$ Here, $\xi = mH/k_BT$, and m is the magnetic moment of the marker. In the calculation, the value of m was taken as an adjustable parameter and was determined as $m = 4.85 \times 10^{-24}$ Wbm so as to obtain the best fit between the experimental results and the calculations.

We also studied the relationship between the signal field B_s from the bound markers and the value of the excitation field B_{ex}. In this experiment, dried markers were used to simulate the bound markers. The result is shown in **Fig. 4**. As shown, the signal B_s was almost zero when $B_{ex} = 0$.

The value of B_s increased with B_{ex} and reached saturation above $B_{ex} > 40$ mT. We note that a large field B_{ex} is necessary to align the moment m of the bound markers. This is because the moment m of the bound markers must rotate inside the particle. In the following experiment, therefore, we set the excitation field to $B_{ex} = 40$ mT.

4. Measurement System

Figure 5 illustrates the measurement system. A disk-shaped sample plate, which has 12 reaction wells, was used. The well was 5 mm in diameter, and the sample (bound and free markers) was diluted in 60 μl of pure water. The detection of the bound markers was performed by the following three steps. (1) First, an

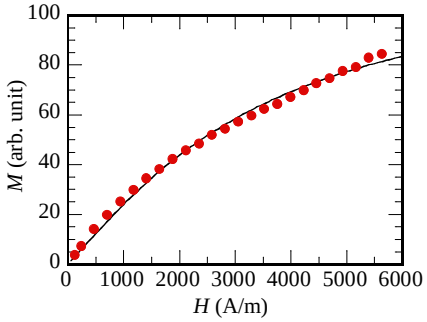

Fig. 3 M-H curve of the magnetic markers diluted in pure water.

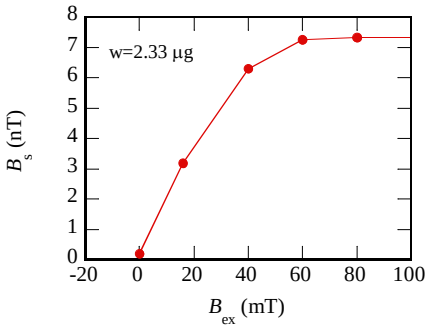

Fig. 4 Relationship between the signal field B_s and the excitation field B_{ex}. The sample was 2.33 μg of magnetic markers (MagCellect particles, R&D Systems, U.S.A.).

excitation field B_{ex}=40 mT was applied to the sample to align the magnetic moments m of both the bound and the free markers. (2) Then, the sample plate was rotated by an ultrasonic motor. In this case, $B_{ex} = 0$, and the Brownian relaxation occurs. (3) After $T = 1.5$ s, the reaction well comes above the magnetic sensor. The MR and the flux gate sensors were installed 2 mm and 4 mm under the sample plate, respectively. A signal field B_s, which was produced by the circular flux due to M of the bound markers, was detected.

For the magnetic sensors, we used a commercial MR sensor (HMC1001, Honeywell, USA) and a Flux gate sensor (Bartington, UK). Here, the MR sensor consisted of a resistive bridge made by 4 thin films, which were arranged in an area of 1 mm × 1 mm. The nominal sensitivity of the MR and flux gate sensors was 160V/T and 14.3×10^6 V/T, respectively.

The sensitive axis of the MR and flux gate sensors was set to a direction parallel to the sample plate, and the

signal field B_s was measured, as shown in **Fig. 5.**

Figure 6 shows an waveform of the detected signal when the sample plate was rotated at a speed of 20 rpm. In each reaction well, markers with different weights were set, and the excitation field B_{ex} = 40 mT was applied, as shown in **Fig. 5**. In this experiment, dried markers were used to simulate the bound markers. As shown in **Fig. 6 (a)**, we obtained the signal when the sample passed above the MR sensor. The amplitude of the signal decreased with the decrease in the weight of the markers. Similar result was obtained when we used the flux gate sensor, as shown in **Fig. 6(b)**.

We discuss the sensitivity of the present system. As can be seen from **Fig. 6 (a)**, the frequency components of the detected signal existed mainly around f = 8 Hz in the present experimental setup. Therefore, the output of the MR sensor was band-pass filtered between 2 and 16 Hz. The measurement was performed 40 times, and the data was averaged to decrease the system noise.

In this case, the measured peak-to-peak noise of the MR sensor system was 90 pT. Therefore, we could measure a signal field B_s larger than 90 pT. In the case of the flux gate sensor, on the other hand, the measured peak-to-peak noise was 30 pT. Therefore, the noise of the flux gate sensor was about 1/3 of that of the MR sensor.

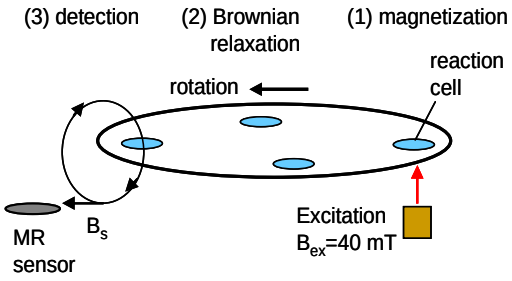

Fig. 5 Detection system using relaxation measurement.

5. Experimental Results

We first studied the relationship between the detected signal B_s and the weight w of the bound markers. In the experiment, we used the dried markers in order to simulate the bound markers. The experimental results are shown in **Fig. 7.** The results for the MR and the flux gate sensors are shown in **Figs. 7(a)** and **7(b)**, respectively.

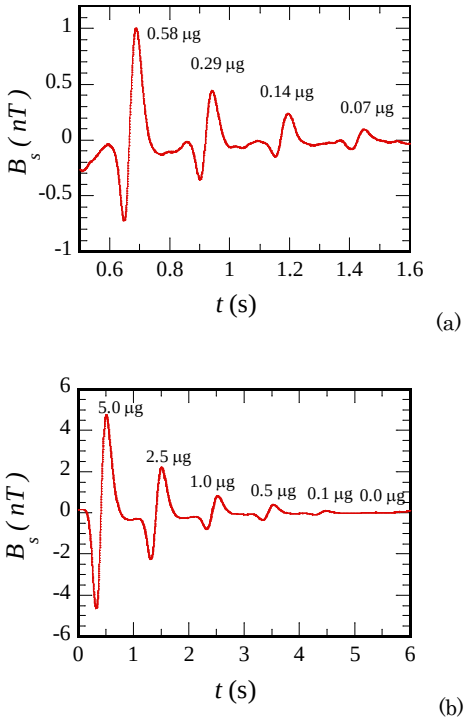

Fig. 6 Waveform of the detected signal when the sample plate was rotated. **(a)** Signal detected with the MR sensor, and **(b)** signal detected with the flux gate sensor. A marker, whose weight ranged from 0.1 to 5 μg, was set in each well on the plate.

As shown, a linear relationship was obtained between B_s and w. The minimum detectable weight of the marker was 50 ng and 30 ng for the case of the MR and the flux gate sensors, respectively.

We note that the signal field B_s detected with the flux gate sensor was smaller than that of the MR sensor, as can be seen from **Fig. 7.** This was because the distance between the flux gate sensor and the sample was larger than the case of the MR sensor; the distance was 4 mm and 2 mm in the case of the flux gate and the MR sensor, respectively. Due to the long distance, the signal from the magnetic marker decays at the sensor position.

Because the mean diameter of the marker was d_h = 110 nm, we can estimate the weight of the single marker as $w_1 = 3.6 \times 10^{-15}$ g, where we used the specific gravity of Fe_3O_4 as 5.2. Therefore, we can estimate that 50 ng corresponds to the $N_m = 1.4 \times 10^7$ markers. This means that we can expect to detect $N = 1.4 \times 10^7$ biological targets using the MR sensor if we assume that a single

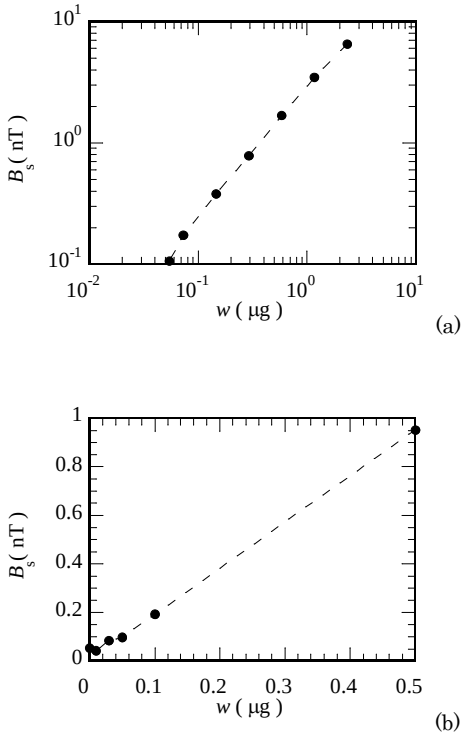

Fig. 7 Relationship between the detected signal B_s and the weight w of the marker. An excitation field B_{ex} = 40 mT was applied. (a) Result obtained with the MR sensor, and (b) flux gate sensor.

marker is bound to a single target. Because the volume of the sample was 60 μl as shown later, this sensitivity can be expressed as 3.8×10^{-16} mol/ml (or 0.38 fmol/ml) in terms of the molecular-number concentration.

In the case of the flux gate sensor, minimum detectable weight was 30 ng. Therefore, we can expect the sensitivity of 0.23 fmol/ml in terms of the molecular-number concentration.

Next, we performed the detection of biological targets called biotins. In the experiment, we used biotins that were conjugated on the surface of polystyrene beads with a diameter of d_p = 3.3 μm (Spherotech Inc, USA). N_p biotin-conjugated polymer beads and 2.33 μg of avidin-coated magnetic markers (MagCellect particles, R&D Systems, U.S.A.) were added to 60 μl of 10 mM phosphate buffer (pH = 7.4) solution. They were incubated for 20 min to complete the binding reaction; the magnetic markers coupled with the polymer beads through the binding reaction between avidin and biotin.

Figure 8 shows the scanning electron microscope (SEM) image of the polymer beads after the binding reaction between biotin and avidin was completed. We can see that the magnetic markers were uniformly bound on the surface of the polymer beads.

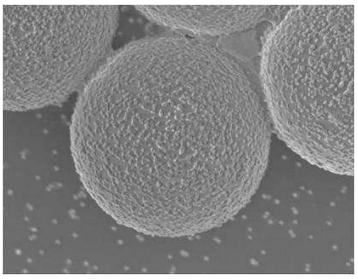

Fig. 8 SEM image of the polymer beads after the binding reaction. Magnetic markers were bound on surface of the polymer bead.

Figure 9 shows the detected signal B_s when the number of polymer beads N_p was changed. The results for the MR and the flux gate sensors are shown in **Figs. 9(a)** and **9(b)**, respectively. As shown, the signal increased almost linearly with the change of the number of polymer

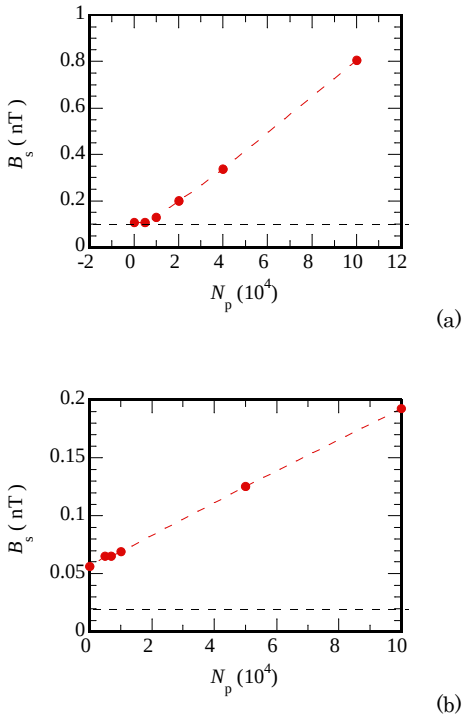

Fig. 9 Relationship between the detected signal B_s and the number of biotin-conjugated polymer beads N_p. **(a)** Result obtained with the MR sensor, and **(b)** flux gate sensor.

beads.

The minimum detectable number of beads was N_p = 10,000 and 5,000 for the case of the MR and the flux gate sensor, respectively. Since about 750 biotins were fixed on the single polymer bead, the minimum detectable number of biotins was estimated as $N_b = 7 \times 10^6$ and 3.5×10^6 for the case of the MR and the flux gate senor, respectively. These results are consistent with those obtained from **Fig. 7**.

6. Conclusion

We have developed a liquid-phase immunoassay technique using Brownian relaxation of magnetic markers. A relaxation measurement was used to distinguish the bound markers from the free ones. The measurement system using the MR sensor showed a sensitivity to detect 1.4×10^7 of the markers in 60 μl of solution. The sensitivity was improved as 8.3×10^6 when the flux gate sensor was used. We also demonstrated the detection of biological targets called biotins and confirmed the validity of the present method. The sensitivity of the present method was estimated as 3.8×10^{-16} and 2.3×10^{-16} mol/ml in terms of the molecular-number concentration for the MR and the flux gate sensor, respectively.

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