Biological Immunoassays without Bound/Free Separation Utilizing Magnetic Marker and HTS SQUID

K. Enpuku, K. Soejima, T. Nishimoto, T. Matsuda, H. Tokumitsu, T. Tanaka, K. Yoshinaga, H. Kuma and N. Hamasaki

***Abstract*— We have developed a magnetic immunoassay system utilizing a magnetic marker and HTS SQUID. In this system, the magnetic marker was used to detect the biological material called antigen. The magnetic marker was designed so as to generate a remanence, and the remanence field of the markers that bound to the antigens was measured with the SQUID. The measurement was performed in a solution that contained both the bound and free (or unbound) markers, i.e., without using the so-called Bound/Free (BF) separation process. The Brownian rotation of the free markers in the solution was used to distinguish the bound markers from the free ones. Using the system, we conducted the detection of the biological material called human IgE. Good relationship was obtained between the detected signal and the weight of IgE. Minimum detectable weight of IgE was 2.4 pg (or 15 atto-mol). This sensitivity was limited by the background signal from the free markers.**

***Index Terms*— HTS SQUID, Immunoassay, BF separation, magnetic marker, remanence, Brownian rotation**

I. Introduction

Recently, magnetic immunoassays utilizing a magnetic marker and SQUID have been developed [1]-[8]. In this application, binding reaction between an antigen and its antibody is magnetically detected by using the magnetic marker. The magnetic field from the marker that bound to the antigen is detected with the SQUID. Utilizing highly sensitive SQUID, we can detect very small amount of antigen [8].

Another merit of this magnetic method is that immunoassay can be performed in liquid phase, i.e., even when the bound and free (or unbound) markers coexist in a solution [2]-[6]. We can distinguish the bound markers from the fee ones by using the Brownian rotation of the free markers. Due to this property, we can eliminate a time-consuming washing process to separate the bound and free markers, i.e., BF (Bound/Free) separation process.

In the previous studies, immunoassays without BF separation have been demonstrated by using the magnetic markers that show the super-paramagnetic property [2]-[6]. However, the signal from the super-paramagnetic particle was small. In order to increase the signal, we have been developing a magnetic maker that can keep remanence [7]. In this case, large remanence field from the marker can be used.

Using the remanence method, we demonstrated highly sensitive immunoassay when we used the BF separation process [8]. In this paper, we develop the remanence method for the immunoassay without BF separation. First, we show the properties of the maker. Next, we discuss the background signal from the free makers in the solution. Finally, we show the detection of biological material without using the BF separation.

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II. Marker and Detection Method

In Fig. 1, magnetic immunoassay utilizing the magnetic marker and the SQUID is schematically shown. The so-called biotin-avidin system was used for the immunoassay. In this system, an antibody that was conjugated by a biotin was coupled to an antigen. Then, the antibody was coupled to the magnetic marker that was conjugated by an avidin. The remanence field M_r from the bound marker is detected with the SQUID.

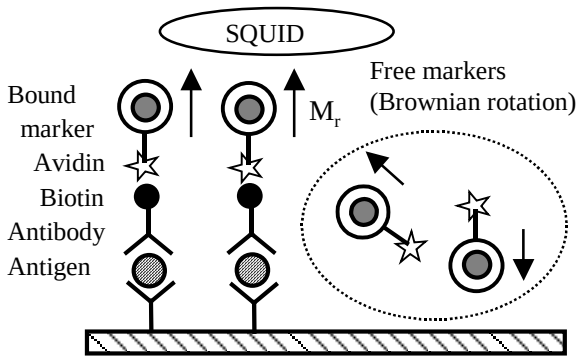

Fig. 1. Schematic figure of the magnetic immunoassay utilizing magnetic marker and SQUID.

A. Magnetic Marker

The marker was made of polymer-coated Fe_3O_4 nanoparticles. The Fe_3O_4 particle was designed so as to generate the remanence. From the Transmission Electron Microscope (TEM) measurement, the shape of Fe_3O_4 particle was cubic-like, and the size was typically 25 nm. The Fe_3O_4 particle was coated with polymer, and avidin was immobilized on the surface of the polymer. The hydrodynamic diameter of the marker was 220 nm, which was measured with the Dynamic Light Scattering (DLS).

The magnetic property of the marker was measured with vibrating sample magnetometer (VSM). From the *M-H* curve measured from the powder of the Fe_3O_4 particles, we obtained the saturation magnetization $\mu_0 M_s$=440 mT and the remanence $\mu_0 M_r$=40 mT. The apparent coercive field that gave M=0 was 9 mT. The remanence of the marker was also measured with the SQUID system after the excitation field of 0.1 T was applied. We obtained the signal flux of 5 mΦ_0 for 1 ng of the marker, where Φ_o=2.07x10^{-15} Wb is the flux quantum.

The susceptibility signal from the free markers in the solution was also measured with a homemade magnetometer using the MR sensor. In Fig. 2, *M-H* curve of the free makers at low fields is shown. As shown, the value of M increased and then began to saturate with the increase of the external field H_{ex}. Although this behavior is similar to that of the maker showing super-paramagnetic property [9], we note that the saturation of M occurred at low value of H_{ex} in the present case.

The *M-H* curve of the free makers in the solution can be given by [9]

$$M = M_S\left[\coth\left(\frac{mH}{k_B T}\right) - \frac{k_B T}{mH}\right] \qquad (1)$$

where m is the magnetic moment of the magnetic particle. Note that Eq. (1) is similar to the *M-H* curve of the paramagnetic particle.

The solid line in Fig. 2 shows the *M-H* curve calculated from Eq. (1). In the calculation, we took m=3.6x10^{-23} Wb m so as to obtain the best fit. As shown, the magnetic field dependence of M was explained well with Eq. (1).

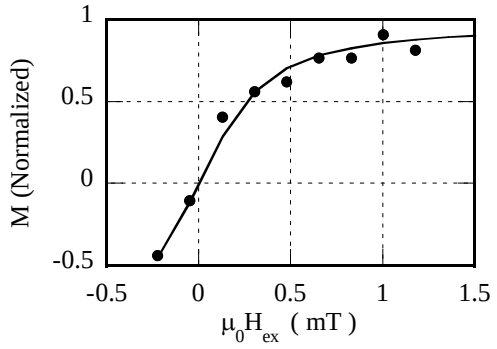

Fig. 2. *M-H* curve of the free markers in the solution at low fields

Using the expression of $m=\mu_0 M(\pi d^3/6)$, we estimated the diameter d of the magnetic particle. In the estimation, we tentatively assumed two cases when $M=M_s$ and $M=M_r$. The diameter was estimated as d=52 nm and d=116 nm when we used $\mu_0 M_s$=440 mT and $\mu_0 M_r$=40 mT, respectively. These estimated values are larger than the size of Fe_3O_4 particles measured with TEM, i.e., 25 nm. This difference may suggest that aggregation of Fe_3O_4 particles occurred in the fabrication process of the magnetic marker.

B. Detection Method without BF Separation

When the markers are added, bound and free markers co-exist in the solution as shown schematically in Fig. 1. In order to make immunoassay without BF separation, we use Brownian rotation of the free markers, as shown below. First, an external field of 0.1 T is applied to the sample in order to magnetize the bound markers. In this case, the free markers are also magnetized. When the external field is reduced to zero, however, Brownian rotation of the free markers occurs. Due to the Brownian rotation, the magnetic moment of the free marker also rotates with time in a random manner, and the signal from the free markers decay with time.

The Brownian relaxation time is given by $\tau_B=3\eta V/k_B T$, where η is the viscosity of the liquid, V is the hydrodynamic volume of the marker, k_B is the Boltzmann's constant and T is the temperature [10]. Taking T=300 K and η=10^{-3} kg/ms, we find τ_B=4 ms for the present marker with diameter of 220 nm. Therefore, if we wait 2 minutes after the external field became zero, the Brownian relaxation will completely finish and the signal from the free markers becomes zero. On the other hand, the magnetic moment of the bound marker is fixed, and keep the remanence signal M_r. Therefore, we can detect the bound marker even in the presence of the free markers.

III. BACKGROUND SIGNAL

In ideal case, the signal from the free markers should be perfectly zero due to the Brownian relaxation. However, we note that some background signal was generated from the free markers in practical case. In Fig. 3, the background signal is shown. In the experiment, 5 μg of free markers were added to a solution of 50 μl, as shown in Fig. 3(a). An external field of 0.1 T was applied to the sample *outside* the SQUID system. Then, the external field was reduced to zero. Waiting 2 minutes after the field becomes zero to complete the Brownian rotation, we inserted the sample into the SQUID system, and measured the signal from the free markers, as shown in Fig. 3(a). Details of the SQUID system were described elsewhere [11].

Figure 3(b) shows the waveform of the detected signal when the sample passed through above the SQUID. Peak-to-peak value of the waveform gives the signal flux. As shown, the background signal of 1.5 mΦ_0 was measured, where the flux of 1.5 mΦ_0 roughly corresponds to the signal field of 15 pT.

We note that the background signal was not caused by a magnetic contamination of a reaction chamber. The signal from

the reaction chamber was checked with the same experimental procedure as shown in Fig. 3(a) without adding the free markers. The signal from the chamber was 0.2 $m\Phi_0$, which was much less than the background signal from the free markers.

In Fig. 4, change of the background signal is shown after the free markers were added into the solution. The horizontal axis represents the time after the marker was added. The vertical axis shows the background signal Φ_{sF} at each time. Here, the sample was magnetized by the field of 0.1 T *outside* the SQUID system in each measurement, and the background signal from the free markers was measurement after 2-min. waiting time.

As shown in Fig. 4, behavior of the background signal changed from sample to sample. In sample A, the background signal was large. In sample B, the background was small at the start, but increased with time. In sample C, the background was kept small. We note that the remanence signal from the dried sample was the same between the samples. Therefore, difference in the background signal will be caused by the occurrence of aggregation or precipitation of the free markers.

Another possible origin of the background signal will be residual dc magnetic field H_{res} existing in the SQUID system. In the present system, the residual field of $\mu_0 H_{res}$=40 nT exists. When the residual field H_{res} exists, the susceptibility signal is generated from the free makers as shown in Eq. (1). In order to study the effect, we set a small coil inside the SQUID system, and applied a small dc field H_{ex} in the measurement. In Fig. 5, the relationship between the background signal and the dc field is shown. The horizontal axis represents the sum of the residual and external field. As shown, linear relation was obtained between the background signal and the dc field, where the sensitivity was roughly given by 62.5 $m\Phi_0/\mu T$.

When the external field H_{ex} is small, the susceptibility signal given by Eq. (1) can be approximated as $M/M_s=mH_{ex}/3k_BT$. From this relation, we can expect that the background signal from the free markers increase with the magnetic moment m. When aggregation of the marker occurred, the value of m increases due to the increase of the volume, and results in the increase of the background signal. Therefore, it is expected that the background signal can be much decreased by avoiding the aggregation of the marker.

IV. Experimental results

We conducted an experiment to detect the antigen called IgE using the remanence method. The sample shown in Fig. 1 was prepared using the following standard procedure [8]. First, a substrate was coated with capturing antibody called A116UN for IgE. Secondly, a blocking material (Block Ace) was coated to prevent nonspecific binding of the antigen to the substrate. Thirdly, serially diluted IgE was added and incubated at room temperature for 1 hour. Then, the antibody conjugated by the biotin was added, and incubated for 30 min. Finally, the magnetic marker conjugated by the avidin was added. The quantity of the marker was 5 μg, and a 50 μl solution of the marker was used in the experiment.

When the markers were added, binding reaction between markers and antibodies begins to occur. We waited 60 min or 120 min so as to complete the binding reaction. Then, some of the markers were bound to the antibodies, but others remained unbound, i.e., bound and free markers co-existed in the solution as shown schematically in Fig. 1.

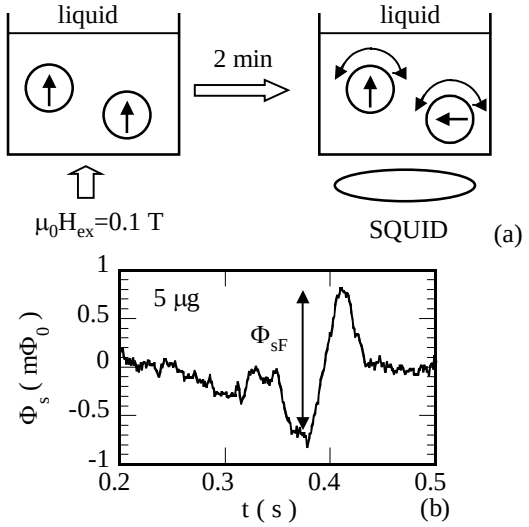

Fig. 3. Background signal from the free markers in the solution. (a) Experimental procedure, and (b) Waveform of the background signal detected with the SQUID system. Peak-to-peak value of the waveform gives the background signal Φ_{sF}.

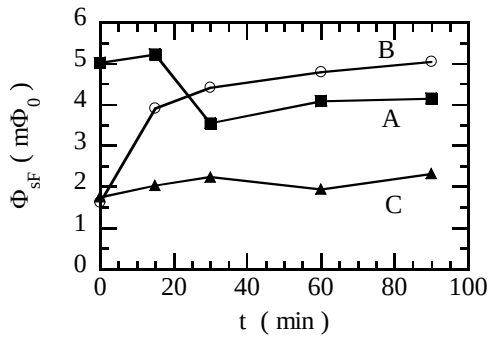

Fig. 4. Background signals of different samples. Changes of the background signal with time are shown after the free markers were added into the solution.

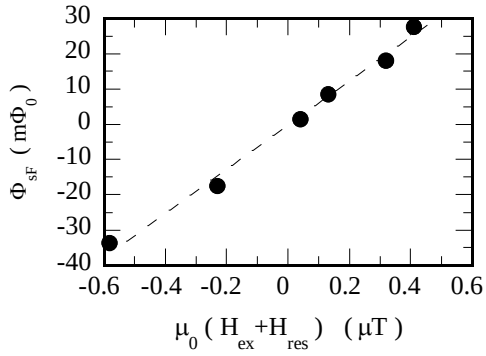

Fig. 5. Relationship between the background signal from the free markers and the dc field existing in the SQUID system.

The signal from the bound markers was detected without BF separation. In order to generate remanence of the bound marker, the external field of 0.1 T was applied *outside* the SQUID system. We waited two minutes to complete the Brownian rotation of the free markers. Then, we measured the signal flux Φ_s from the sample

The measured flux Φ_s is the sum of the signal flux Φ_{sB} from the bound markers and the background signal Φ_{sF} from the free markers, i.e., $\Phi_s=\Phi_{sB}+\Phi_{sF}$. In obtaining the signal flux Φ_{sB}, we regard the background signal as an offset, i.e., the signal flux Φ_{sB} was evaluated by subtracting the offset from the measured flux Φ_s. The value of the background signal was obtained from the measured flux in the absence of IgE.

In Fig. 6, the relationship between the signal flux Φ_{sB} from the bound markers and the weight *w* of IgE is shown. The triangles and circles show the results obtained after the reaction times of 60 min and 120 min, respectively. As shown, good relationship was obtained between the signal flux Φ_{sB} and the weight of IgE. When the weight of IgE was small, the values of Φ_{sB} were almost the same between the two reaction times. On the other hand, the signal flux became larger for the longer reaction time when the weight of IgE becomes large. This result may indicate that the reaction speed slightly depends on the amount of IgE, as pointed out in ref. 5.

For comparison, symbols (+) in Fig. 6 show the experimental results when we used the BF separation process, i.e., when the free markers were washed out [8]. As shown, the detected signals without BF separation agree well with those obtained with BF separation. This agreement indicates that the detection without BF separation was performed correctly.

In Fig. 7, mean values and standard deviations of the detected flux Φ_s measured without BF separation are shown. These results were obtained from the measurement of 8 samples. The signal at *w*=0 is the background signal. As shown, we could clearly detect the IgE down to 2.4 pg. On the other hand, the difference between the case of *w*=0 and *w*=0.72 pg was not clear when we took the standard deviation into account. Since the molecular weight of IgE is 180,000, 2.4 pg corresponds to 15 atto-mol. We note, however, that the minimum detectable weight of IgE was as small as 0.3 pg in the case with BF separation, as shown in Fig. 6. Therefore, it is necessary to decrease the background signal in order to improve the sensitivity in the case without BF separation..

V. Conclusion

We performed a magnetic immunoassay without BF separation. In this system, the magnetic marker was designed so as to generate a remanence, and the remanence field from the bound markers was measured with the SQUID. The Brownian rotation of the free markers in the solution was used to distinguish the bound markers from the free ones. At present, minimum detectable weight of IgE is 2.4 pg (or 15 atto-mol). In order to improve the sensitivity, it is necessary to decrease the background signal from the free markers.

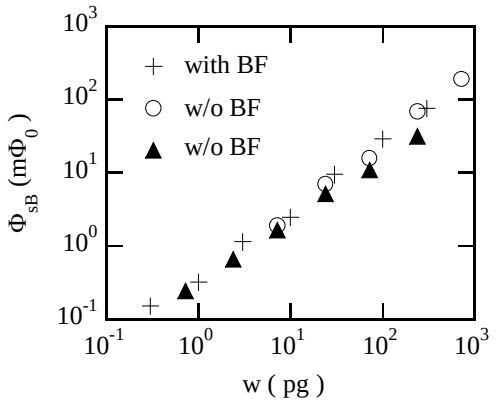

Fig. 6. Relationship between the signal flux Φ_{sB} from the bound markers and the weight *w* of IgE. The triangles and circles show the results obtained without BF separation, which were measured after the reaction time of 60 min and 120 min, respectively. The symbols (+) show the results obtained with BF separation.

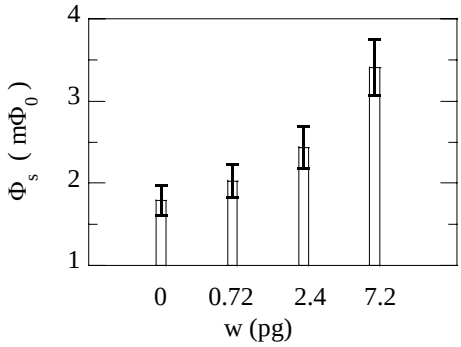

Fig. 7. Mean values and standard deviations of the detected flux Φ_s measured without BF separation.

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