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Liquid phase immunoassay utilizing magnetic marker and high T_c superconducting quantum interference device

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We have developed a liquid phase immunoassay system utilizing a magnetic marker and a superconducting quantum interference device (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 biological material called IgE without BF separation. At present, we could detect the IgE down to 7 pg (or 39 amol).
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I. INTRODUCTION

Immunoassays are widely used to detect disease-related proteins for medical diagnosis. These proteins are generically called antigens, and the so-called binding reaction between antigen and its antibody is used for the immunoassay. The antibody is labeled with a marker, and the binding reaction is detected by measuring a signal from the marker. In making immunoassay, sufficiently large quantity of antibodies is put into a solution containing antigens. Then, some of them are bound to the antigens, but others remain unbound. The former and the latter are called bound and free markers, respectively. In a conventional immunoassay, the free markers need to be washed out after finishing the binding reaction in order to detect only the bound markers. This washing process, which is called bound/free (BF) separation, is time consuming and makes the high-speed detection difficult.

Recently, magnetic immunoassays utilizing a magnetic marker and magnetic sensors have been developed.[1–13] In this case, the antibody is labeled with the magnetic marker, and the binding reaction is detected by measuring the magnetic field from the marker. One of the merits of this magnetic method is that immunoassay can be performed in liquid phase without using the BF separation process. The key idea is to use Brownian rotation of the free markers in a solution.[3–9] Since the free markers make Brownian rotation in a solution, magnetic moment of the marker rotates with time. As a result of this Brownian rotation, the signal from the free markers becomes zero with exponential time dependence. On the other hand, the bound markers are fixed to the antigen and generate the signal field. Therefore, we can distinguish the bound markers from the fee ones without using the BF separation process.

In the previous studies, magnetic markers that show Néel relaxation were mainly used.[3–5] Immunoassays without BF separation were performed by utilizing the difference in the relaxation time between the Néel relaxation of the bound markers and the Brownian relaxation of the free markers. In this case, however, the signal from the bound markers was small. In order to increase the signal, therefore, magnetic markers that do not show the Néel relaxation but generate a remanence have also been used.[1,10–13] In this case, remanence field of the bound markers gives the signal. Since the marker can generate a high remanence field and the remanence measurement method is useful for low noise measurement, we can expect to improve the sensitivity of the immunoassay.

In this paper, we show the immunoassay without BF separation using the remanence method. A high T_c superconducting quantum interference device (SQUID) was used to detect the remanence field from the marker. We first studied the magnetic signal from the free markers. Although the signal from the free markers should be zero in ideal case, spurious signal occurred in practical case due to imperfectness of the Brownian rotation of the free markers in a solution. Since this spurious signal limited the sensitivity of the immunoassay without BF separation, its possible origins were studied. Next, we conducted the detection of the antigen called human IgE without BF separation. Good relationship was obtained between the detected signal and the weight of IgE. The minimum detectable weight of IgE was 7 pg (or 39 amol).

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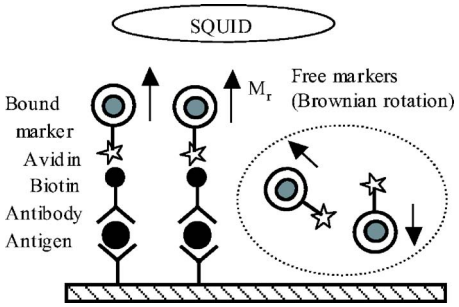

FIG. 1. (Color online) Schematic figure of the magnetic immunoassay utilizing magnetic marker and SQUID. The immunoassay is performed in liquid phase without using the so-called bound/free (BF) separation process. The Brownian rotation of the free markers is used to distinguish the bound markers from the free markers.

II. DETECTION PRINCIPLE AND SQUID SYSTEM

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 marker was made of Fe_3O_4 nanoparticles with average diameter of $d=25$ nm so as to generate the remanence.[10,11] The Fe_3O_4 particle was coated with polymer, and the diameter of the polymer-coated Fe_3O_4 particles was typically 140 nm. Avidin was immobilized on the surface of the polymer, and the marker was coupled to the antibody through the biotin-avidin connection. The binding reaction between the antigen and its antibody was detected by measuring the remanence field M_r from the bound marker.

When the markers are added, bound and free markers coexist 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 in the solution, as shown below. First, an external field of 0.1 T is applied to the sample in order to magnetize the bound and free markers. When the external field is reduced to zero, 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 becomes zero. On the other hand, the magnetic moment of the bound marker is fixed and generates the remanence signal M_r. These properties were used to distinguish the bound markers from the free ones.

The magnetic property of the marker was measured with variable 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 present SQUID system after the excitation field of 0.1 T was applied. We obtained the signal flux of 5 $m\Phi_0$ for 1 ng of the marker, where $\Phi_0=2.07\times10^{-15}$ Wb is the flux quantum.

The SQUID system was described elsewhere.[11,14] Briefly, disk-shape reaction chamber (sample disk) was used in order to measure many samples. Twelve reaction cells were formed along a concentric circle with a diameter of 150 mm. The size of each reaction cell was chosen as 5 mm in diameter. Corresponding to this cell size, a directly coupled high T_c SQUID gradiometer with two 5×5 mm^2 pickup coils was used. The distance between the sample and the SQUID was set to be 1.5 mm.

The SQUID was installed in a cylindrical magnetic shield made of three layers of Permalloy. The sample disk was inserted inside the magnetic shield through a slit hole of the shield and was rotated by an ultrasonic motor. By rotating the sample disk, reaction cells pass through above the SQUID one after the other, and the signal from each reaction cell was measured serially. Thus, 12 samples could be measured in one rotation cycle. The rotation speed was typically 1/3 rotation/s. In the experiment, 100 times measurements were averaged in order to improve the signal to noise ratio. The system noise with the average was 0.2 $m\Phi_0$ in terms of flux noise.

The whole measurement system was also set in an additional shield box in order to enhance the magnetic shielding. The residual magnetic field $\mu_0 H_{res}$ at the position of the SQUID was $\mu_0 H_{res}=40$ nT in the present system, which was measured with the flux gate sensor.

Since we use the remanence method, remanence field from the reaction chamber must be kept as small as possible after the excitation field of 0.1 T is applied. Unfortunately, commercial chambers have a large magnetic contamination and generate a large remanence signal. Therefore, we have developed a reaction chamber with very few magnetic contamination by improving the fabrication process of the chamber.[11] The remanence signal from the reaction chamber was measured with the SQUID and was typically 0.2 $m\Phi_0$.

III. SPURIOUS SIGNAL FROM FREE MARKERS

In ideal case, the signal from the free markers should be perfectly zero due to the Brownian relaxation. However, we note that some spurious signal is generated from the free markers in practical case. This spurious signal will be caused by imperfectness of the Brownian rotation of the free markers. Since this spurious signal becomes an offset (or background signal) in the immunoassay, it limits the sensitivity of the immunoassay without BF separation. We also note that the quantity of the bound markers is very much smaller than that of the free markers. For example, we have to detect 1 ng bound markers in the presence of 5 μg free markers. Therefore, the spurious signal from the free markers must be reduced as much as possible.

In Fig. 2, the spurious signal from the free markers is shown. In the experiment, 5 μg of free markers were added to a 0.1% Tween-20/phosphate buffer solution (PBST) of 50 μl, as shown in Fig. 2(a). An external field of 0.1 T was applied to the sample *outside* the SQUID system, so that the magnetic moments of the free markers were aligned in the direction of the magnetic field. Then, the external field was reduced to zero. When the field becomes zero, Brownian rotation of the free markers occurs. Waiting 2 min after the field becomes zero, we inserted the sample into the SQUID

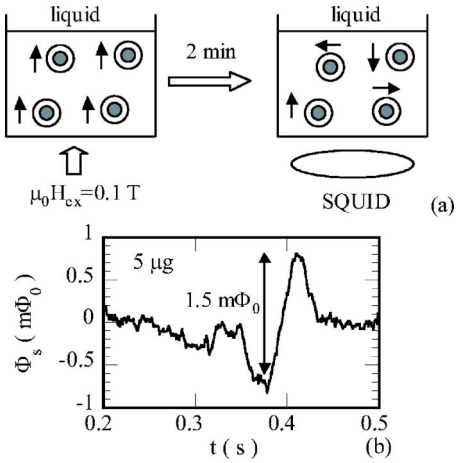

FIG. 2. (Color online) Magnetic signals from the free markers. (a) Experimental procedure. (b) Spurious signal from the free markers when 5 μg free markers exist in a 50 μl solution.

system and measured the signal from the free markers, as shown in Fig. 2(a).

Figure 2(b) shows the wave form of the detected signal when the sample passed through above the SQUID. Peak-to-peak value of the wave form gives the signal flux. As shown, the spurious signal of 1.5 mΦ_0 was measured, where the signal flux of 1.5 mΦ_0 roughly corresponds to the signal field of 15 pT.

We note that this spurious 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. 2(a) without adding the free markers. The signal from the chamber was 0.2 mΦ_0, which was much less than the spurious signal from the free markers shown in Fig. 2(b).

For comparison, we measured the signal when the sample was dried, which corresponds to the case of the bound markers. In this case, markers of 0.01 μg were dried and magnetized by the field of 0.1 T, as shown in Fig. 3(a). Then, the remanence field of the markers was measured with the SQUID. As shown in Fig. 3(b), the signal of 44 mΦ_0 was measured. The signal of the bound markers will become 44 $\times(5/0.01)=22\,000$ mΦ_0 when the weight of the markers is increased to 5 μg as in the case of the fee markers. Therefore, we can obtain the signal ratio between the bound and the free marker as $22\,000/1.5=14\,700$.

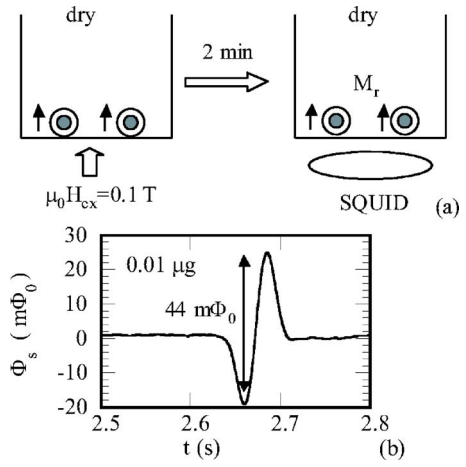

FIG. 3. (Color online) Magnetic signal from the bound markers. (a) Experimental procedure. (b) Remanence signal from the 0.01 μg bound markers.

In the following, we discuss the origin of the spurious signal from the free markers. The Brownian relaxation time is given by $\tau_B=3\eta V/k_BT$, where η is the viscosity of the liquid, V is the hydrodynamic volume of the marker, k_B is Boltzmann's constant, and T is the temperature.[15] Taking $T=300$ K and $\eta=10^{-3}$ kg/m s, we find $\tau_B=1$ ms for the present marker with diameter of 140 nm. Since the measurement was started 2 min after the external field became zero, the Brownian relaxation should be finished in ideal case, and the magnetic field from the free markers should be zero. The spurious signal of 1.5 mΦ_0 shown in Fig. 2(b) indicates the imperfectness of the Brownian relaxation in the present case.

We note that the value of the spurious signal from the free markers changed from sample to sample, though the remanence signal from the dried sample was the same between samples. Therefore, one possible origin of the spurious signal will be the degradation of dispersion of the free markers in the solution, i.e., occurrence of aggregation and precipitation. When aggregation of the free markers occurs, a cluster of free markers is formed. Due to a large volume V of the cluster, Brownian relaxation time τ_B of the cluster becomes very long since τ_B is proportional to V. Precipitation of the cluster will also occur. Since these effects prevent the Brownian rotation, they will increase the spurious signal. Although it is difficult to make a quantitative discussion at present, we experienced a strong relationship between the spurious signal and the dispersion of the maker. For example, the spurious signal was much decreased when potential clusters of free markers were removed using a 400 nm diameter filter. Although quantity of the cluster could not be measured, it was estimated to be very few since the remanence signal of the dried sample is unchanged between the filtered and unfiltered ones. Therefore, it is very important to avoid aggregation and improve the dispersion of the marker in order to reduce the spurious signal.

Another origin will be residual dc magnetic field H_{res} existing in the SQUID system. When the residual field H_{res} exists, the free markers in the solution are tending to be aligned in the direction of the magnetic field. This prevents the Brownian rotation and causes the spurious signal. In other words, the residual field H_{res} causes susceptibility signal from the free markers. 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. 4, the relationship between the spurious signal of the free markers and the dc field is shown. The horizontal axis represents the sum of the residual field $\mu_0 H_{res}=40$ nT in the present system and the external field $\mu_0 H_{ex}$. As shown, linear relation was obtained between the spurious signal and the dc field. In the present case, the sensitivity of the spurious signal to the dc field was roughly given by 62.5 mΦ_0/μT. Therefore, it is also necessary to decrease the residual field in order to reduce the spurious signal from the free markers.

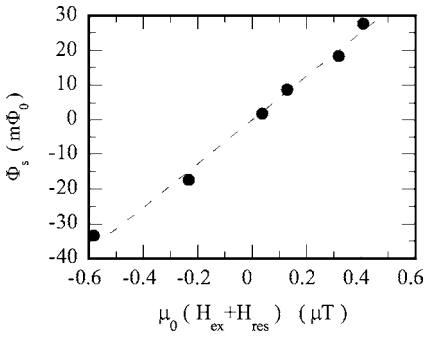

FIG. 4. Relationship between the spurious signal from the free markers and the magnetic filed existing in the SQUID system. The horizontal axis represents the sum of the residual field H_{res} and the applied field H_{ex}.

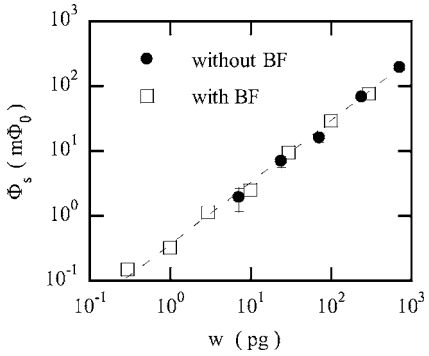

FIG. 6. Relationship between the signal flux Φ_s and the weight w of IgE. Circles show the result obtained *without* BF separation, while rectangles show the results obtained *with* BF separation.

IV. IMMUNOASSAY EXPERIMENT

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.[11–13] 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 h. 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, some of them were bound to the antibodies, but others remained unbound, i.e., bound and free markers coexisted in the solution as shown schematically in Fig. 1. The signal from the bound markers was detected without BF separation.

In Fig. 5, change of the detected signal is shown after the marker was added. The horizontal axis represents the time after the marker was added. The vertical axis shows the detected signal Φ_s at each time. Here, the sample was magnetized by the field of 0.1 T outside the SQUID system in each measurement, and the remanence field from the sample was measured after 2 min waiting time. As shown in Fig. 5, the detected signal increased with time, and then began to saturate. Since the marker begins to react to the antibody after the marker is added, this time dependence represents the binding process between them. In the present case, long reaction time was needed since the reaction speed of the homemade marker was slow. The reaction speed may be hampered by the chemical property of the marker or the clumsy particles. The reaction time will become much shorter when these properties are improved.

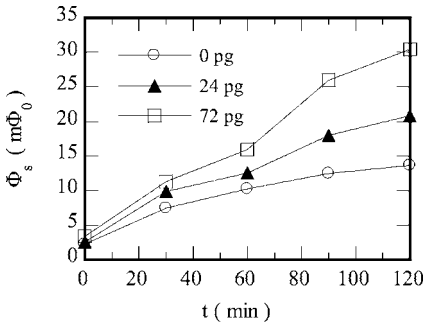

FIG. 5. Change of the detected signal after the marker is added. The horizontal axis shows the time after the marker is added. The vertical axis shows the detected signal Φ_s at each time. Weights of IgE are w=0, 24 pg, and 72 pg.

As shown in Fig. 5, the spurious signal from the free markers was 2.0 mΦ_0 just after the marker was added, i.e., for the case of IgE=0. The spurious signal, however, increased with time and began to saturate. The reason of this increase is not clear at present. We speculate that the precipitation of the marker with time or nonspecific binding of the marker to the substrate will cause the increase of the spurious signal.

In Fig. 6, the relationship between the detected signal Φ_s and the weight w of IgE is shown. In obtaining the signal flux, we regard the spurious signal from the free markers as an offset. The signal flux Φ_s was evaluated by subtracting the offset from the measured value in the presence of IgE. The value measured after the reaction time of 120 min was used. The circles show the results obtained without BF separation process. As shown, linear relationship was obtained between the detected signal and the weight of IgE. The minimum detectable weight was 7 pg, though the error was somewhat large for the case of 7 pg. Since the molecular weight of IgE is 180 000, 7 pg corresponds to 39 amol. We note that this sensitivity was limited by the spurious signal from the free markers.

For comparison, rectangles in Fig. 6 show the experimental results when we used the BF separation process, i.e., when the free markers were washed out.[12,13] 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. We note, however, that the minimum detectable weight of IgE was as small as 0.3 pg in the case with BF separation. In order to realize the same sensitivity without BF separation, it is necessary to decrease the spurious signal from the free markers.

V. CONCLUSION

We have developed the liquid phase immunoassay system utilizing the magnetic marker and the SQUID. The mag-

netic marker that shows the remanence was used, and the remanence field of the bound markers was detected with the SQUID. The Brownian rotation of the free markers was used to distinguish the bound markers from the free ones without using the BF separation process. Since imperfectness of the Brownian rotation caused the spurious signal from the free markers, the dispersion of the free markers in the solution should be improved as much as possible. Using the system, we showed the detection of the biological material called IgE without BF separation. At present, we could detect the IgE down to 7 pg. In order to improve the sensitivity, it is necessary to decrease the spurious signal from the free markers.

[1]R. Kotitz, H. Matz, L. Trahms, H. Koch, W. Weitschies, T. Rheinlander, W. Semmler, and T. Bunte, IEEE Trans. Appl. Supercond. **7**, 3678 (1997).
[2]K. Enpuku, T. Minotani, T. Gima, Y. Kuroki, Y. Itoh, M. Yamashita, Y. Katakura, and S. Kuhara, Jpn. J. Appl. Phys., Part 2 **38**, L1102 (1999).
[3]A. Haller, H. Matz, S. Hartwig, T. Kerberger, H. Atzpadin, and L. Trahms, IEEE Trans. Appl. Supercond. **11**, 1371 (2001).
[4]J. Lange, R. Kotitz, A. Haller, L. Trahms, W. Semmler, and W. Weitschies, J. Magn. Magn. Mater. **252**, 381 (2002).
[5]H. Grossman, W. Myers, V. Vreeland, R. Bruehl, M. Alper, C. Bertozzi, and J. Clarke, Proc. Natl. Acad. Sci. U.S.A. **101**, 129 (2004).
[6]D. Eberbeck, C. Bergemann, S. Hartwig, U. Steinhoff, and L. Trams, J. Magn. Magn. Mater. **289**, 435 (2005).
[7]G. Glockl, V. Brinkmeier, K. Aurich, E. Romanus, P. Weber, and W. Weitschies, J. Magn. Magn. Mater. **289**, 480 (2005).
[8]F. Ludwig, E. Heim, S. Mauselein, D. Eberbeck, and M. Schilling, J. Magn. Magn. Mater. **293**, 690 (2005).
[9]S. H. Chung, A. Offmann, K. Guslienko, S. D. Bader, C. Liu, B. Kay, L. Makowski, and L. Chen, J. Appl. Phys. **97**, 10R101 (2005).
[10]K. Enpuku, D. Kuroda, T. Q. Yang, and K. Yoshinaga, IEEE Trans. Appl. Supercond. **13**, 371 (2003).
[11]K. Enpuku IEICE Trans. Electron. *et al.*, **E88-C**, 158 (2005).
[12]K. Enpuku, D. Kuroda, A. Ohba, T. Q. Yang, K. Yoshinaga, T. Nakahara, H. Kuma, and N. Hamasaki, Jpn. J. Appl. Phys., Part 2 **42**, L1436 (2003).
[13]K. Enpuku, K. Inoue, K. Soejima, K. Yoshinaga, H. Kuma, and N. Hamasaki, IEEE Trans. Appl. Supercond. **15**, 660 (2005).
[14]A. Tsukamoto *et al.*, IEEE Trans. Appl. Supercond. **15**, 656 (2005).
[15]P. Debye, *Polar Molecules* (Chemical Catalog, New York 1929).