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## Wash-free detection of C-reactive protein based on third-harmonic signal measurement of magnetic markers

Keiji Enpuku<sup>1\*</sup>, Misako Shibakura<sup>2</sup>, Yujiro Arai<sup>2</sup>, Takako Mizoguchi<sup>3</sup>, Akihiko Kandori<sup>3</sup>, Misato Hara<sup>4</sup>, and Keiji Tsukada<sup>5</sup>

<sup>1</sup>Department of Electrical and Electronic Engineering, Kyushu University, Fukuoka 819-0395, Japan

<sup>2</sup>Graduate School of Health Sciences, Okayama University, Okayama 700-8558, Japan

<sup>3</sup>Research and Development Group, Hitachi Ltd., Kokubunji, Tokyo 185-8601, Japan

<sup>4</sup>Biotronics Laboratory, Tamagawa Seiki Co., Ltd., Iida, Nagano 395-8515, Japan

<sup>5</sup>Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan

\*E-mail: enpuku@sc.kyushu-u.ac.jp

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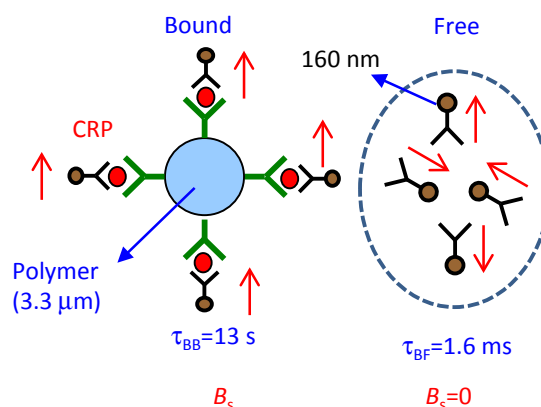
We demonstrate wash-free detection of C-reactive proteins (CRPs) based on third-harmonic signal measurement of magnetic markers. In the method presented here, the CRP concentration can be detected from the decrease in the third-harmonic signal from the sample solution. The relationship between the detected signal and the CRP concentration can be modeled quantitatively using a logistic function. The quantities of CRP that were detected using the proposed method showed good correlation with those obtained using the conventional optical method with a washing process. We also demonstrate CRP detection in a hemolysis sample solution that is not optically transparent.

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The detection of biological targets using bio-functionalized magnetic nanoparticles (or magnetic markers) has been studied widely.<sup>1–4</sup> In this magnetic method, the targets are detected by measuring the magnetic fields generated by the markers. Measurement methods and systems have been developed for this purpose using the various magnetic properties of the markers, including magnetization,<sup>5–8</sup> magnetic relaxation,<sup>9–12</sup> and their linear and non-linear AC susceptibilities.<sup>13–18</sup>

The magnetic markers that are bound to their targets are called bound markers, while the markers that are not bound to their targets are called free markers. The characteristic feature of the magnetic method is that these bound and free markers can be differentiated magnetically using the Brownian relaxation properties of the markers in solution.<sup>2,3</sup> Therefore, the time-consuming washing process that is required in the conventional optical method to separate the bound and free markers, i.e., so-called bound/free (B/F) separation, can be eliminated using the magnetic method. This wash-free detection property is one of the major advantages of the magnetic method and enables rapid and simple detection of the targets. Another advantage of the magnetic method is that it enables detection of targets in sample solutions that are not optically transparent. This is in contrast to the optical method, where sample solution transparency is required.

In this study, we present wash-free detection of C-reactive proteins (CRPs) using magnetic markers. First, we demonstrate the principle of wash-free detection based on third-harmonic signal measurement of magnetic markers in solution. We then measure the relationship between the detected signal and the CRP concentration. It is shown that the measured relationship can be modeled quantitatively using a logistic function. We also compare the presented results with those obtained using the conventional optical method that requires the washing process for B/F separation, and demonstrate good correlation between the results obtained using the two methods. Finally, we demonstrate that the proposed method enables CRP detection even when the sample solution is not optically transparent.



**Fig. 1.** (Color online) Schematic of wash-free detection of biological targets using Brownian relaxation of magnetic markers. Bound and free markers are distinguished magnetically without use of a washing process to separate them.

Figure 1 shows a schematic of the presented method for wash-free biological target detection. In this study, we used CRPs as targets. Polystyrene beads with nominal diameters of 3.3  $\mu\text{m}$  (Spherotech PP-30) were used to fix the CRPs. Fixing antibodies (HyTest C2-anti-CRP antibody) were immobilized on the surfaces of the polymer beads: the fixed amount of C2 antibodies was 4.9  $\mu\text{g}/\text{mg}$ -polymer beads. The magnetic markers were made from magnetic nanoparticles (Tamagawa Seiki FG beads) that were conjugated with detecting antibodies (HyTest C6cc-anti-CRP): the fixed amount of C6 antibodies was 8.6  $\mu\text{g}/\text{mg}$ -FG beads. The hydrodynamic diameter of the marker, which was obtained from dynamic light scattering (DLS) measurements, was 160 nm.

In the experiment, the CRPs, fixing polystyrene beads and magnetic markers were placed in a reaction well and incubated. After the binding reaction finished, magnetic markers were bound to CRPs that were fixed to the polymer bead, as shown in Fig. 1: these markers are called the bound markers. There were also unbound markers in solution: these markers are called the free markers. The bound and free markers were



then differentiated magnetically using the difference between their Brownian relaxation times, as shown below.

The Brownian relaxation time of particles in solution,  $\tau_B$ , is given by<sup>2,3)</sup>

$$\tau_B = \frac{\pi\eta}{2k_B T} d_H^3, \quad (1)$$

where  $d_H$  is the hydrodynamic diameter of the particle,  $\eta$  is the viscosity of the solution,  $k_B$  is the Boltzmann constant and  $T$  is the absolute temperature.

Because the  $d_H$  value of the magnetic marker used here was  $d_H = 160$  nm, the Brownian relaxation time of the free markers can be calculated using Eq. (1) as  $\tau_{BF} = 1.6$  ms for  $\eta = 10^{-3}$  Pa·s and  $T = 300$  K. In addition, the  $d_H$  value of the bound markers is given by the diameter of the polystyrene beads, i.e.,  $d_H = 3.3$   $\mu$ m. As a result, the Brownian relaxation time of the bound markers is  $\tau_{BB} = 13$  s, which is much longer than  $\tau_{BF} = 1.6$  ms.

Using the difference between  $\tau_{BB}$  and  $\tau_{BF}$ , we could then magnetically differentiate the bound and free markers. For this purpose, we measured the nonlinear magnetization of a sample solution when an AC excitation field of  $H_{ac}(t) = H_0 \sin(2\pi ft)$  was applied. Specifically, we measured the third-harmonic magnetization of the sample, which was caused by the nonlinear magnetization of the sample. The third-harmonic measurement method has several advantages when compared with the fundamental-frequency measurement method, as previously noted in Ref. 19. For example, we can reduce the interference from the excitation field dramatically by measuring the third-harmonic signal. The effect of the solution, i.e., the diamagnetic signal from the water, can be also eliminated through use of the third-harmonic measurement method.

Here, we consider the case where bound markers with number  $N_B$  and free markers with number  $N_F$  coexist in a sample solution.  $N_F$  is given by  $N_F = N_T - N_B$ , where  $N_T$  is the total number of markers added to the solution. In this case, the third-harmonic signal of the sample,  $M_3(N_B)$ , can be expressed as

$$M_3(N_B) = A_F N_F + A_B N_B = A_F N_T - (A_F - A_B) N_B, \quad (2)$$

where  $A_F$  and  $A_B$  are the third-harmonic signals per unit number of bound and free markers, respectively.

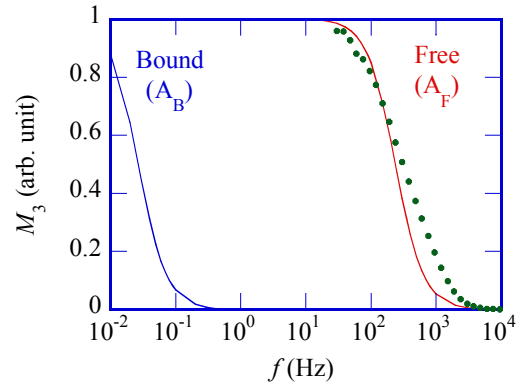
Because  $M_3(N_B = 0) = A_F N_T$ , Eq. (2) can be rewritten as

$$\Delta M_3 = M_3(N_B = 0) - M_3(N_B) = (A_F - A_B) N_B. \quad (3)$$

Note that  $\Delta M_3$  represents the reduction of the third-harmonic signal that is caused by the bound markers. We also note that the values of  $A_F$  and  $A_B$  are strongly dependent on the excitation field frequency  $f$ . Figure 2 shows the frequency dependences of  $A_F$  and  $A_B$  when the excitation field amplitude is chosen to be  $\mu_0 H_0 = 4$  mT. The solid lines were calculated using the equation given in Ref. 20. In the calculations, we assumed that the magnetic moment of the marker was

$$m = \frac{\pi}{6} M_s d_c^3 = 1.6 \times 10^{-17} \text{ Am}^2,$$

where  $d_c = 40$  nm is the magnetic core diameter and  $M_s = 4.8 \times 10^5$  A/m is the saturation magnetization of the  $\text{Fe}_3\text{O}_4$  nanoparticles that form the marker. As shown in Fig. 2, the  $A_B$  value of the bound marker becomes almost zero when



**Fig. 2.** (Color online) Frequency dependence of third-harmonic signals generated by bound and free markers.

$f > 1$  Hz. This is because the bound marker cannot respond to excitation fields at  $f > 1$  Hz because of its long relaxation time of  $\tau_{BB} = 13$  s. However, we obtain a finite  $A_F$  value for the free marker at frequencies up to a few kHz because of its short relaxation time of  $\tau_{BF} = 1.6$  ms. The circles in Fig. 2 represent the experimental results for the frequency dependence of the third-harmonic signals of the free markers. As the figure shows, reasonable agreement was obtained between the experiments and calculations, and better agreement will be obtained if we take the distribution of  $d_H$  in the magnetic markers into account.<sup>20)</sup>

In the proposed method, we select the excitation field frequency  $f$  to satisfy the condition  $1/\tau_{BB} \ll f \approx 1/\tau_{BF}$ . In this case, we can approximate  $A_B = 0$ , as indicated by Fig. 2. As a result, Eq. (3) can be rewritten as

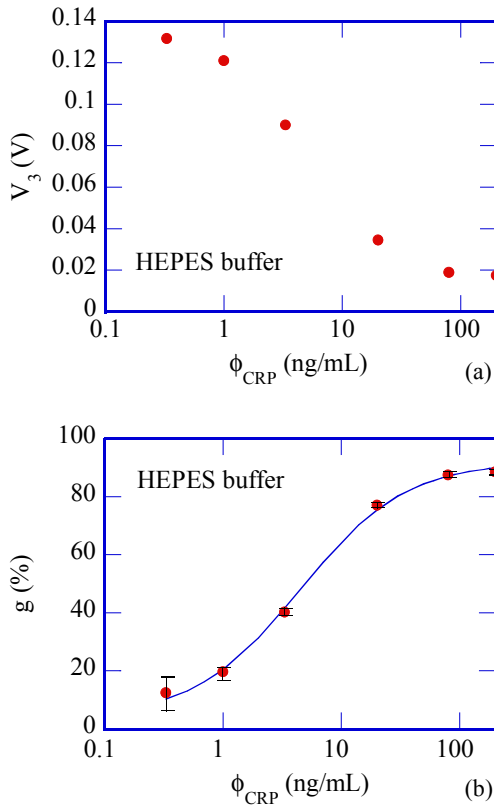
$$g = \frac{\Delta M_3}{M_3(N_B = 0)} = \frac{N_B}{N_T}, \quad (4)$$

where  $g$  is the normalized reduction of the third-harmonic signal. Therefore, we can determine the number of bound markers from the measured  $g$  value.

In the following, we present the results of wash-free detection of CRPs. In the experiments, 5.0  $\mu$ L of the fixing polystyrene beads at a concentration of 10 mg/mL, 2.5  $\mu$ L of the magnetic markers at a concentration of 10 mg/mL, and 52.5  $\mu$ L of diluted CRPs were placed in a reaction well. The CRP concentration was varied here by diluting the original CRP (HyTest 8C72) with a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution. Next, 60  $\mu$ L of the sample solution was incubated for 20 min at room temperature. We then applied the excitation field ( $\mu_0 H_0 = 4$  mT,  $f = 1$  kHz) to the sample solution, and the third-harmonic signal at  $3f = 3$  kHz was measured using a previously developed measurement system that was presented in Ref. 19.

Figure 3(a) shows the measured third-harmonic signal when the CRP concentration,  $\phi_{\text{CRP}}$ , was changed from 0.3 to 200 ng/mL. The vertical axis of Fig. 3(a) represents the voltage signal  $V_3$  measured using the measurement system. Note that  $V_3(\phi_{\text{CRP}})$  is proportional to  $M_3(N_B)$  in Eq. (2). As shown in Fig. 3(a), the third-harmonic signal decreased with increasing CRP concentration. This reduction is caused by the increase in the number of bound markers  $N_B$ , as indicated by Eq. (3).

The third-harmonic signal of a reference sample without CRPs ( $\phi_{\text{CRP}} = 0$ ) was also measured to obtain the value of  $M_3(N_B = 0)$  in Eq. (3): we obtained  $V_3(\phi_{\text{CRP}} = 0) = 0.150$  V.



**Fig. 3.** (Color online) (a) Measured third-harmonic signals from sample solution when the CRP concentration,  $\phi_{\text{CRP}}$ , was changed. A HEPES buffer solution without serum was used in the experiments. (b) Relationship between percentage reduction of the third-harmonic signal,  $g$ , and  $\phi_{\text{CRP}}$ . The solid line was calculated using Eq. (5).

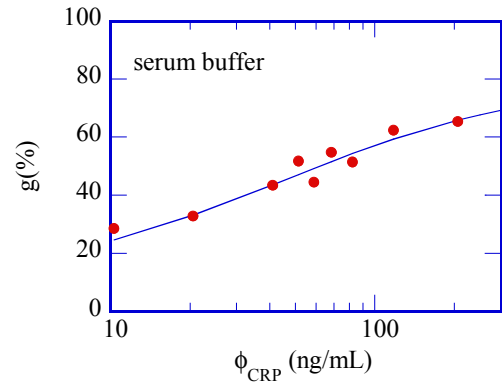
Note that the value of  $V_3(\phi_{\text{CRP}} = 0) - V_3(\phi_{\text{CRP}})$  is proportional to  $\Delta M_3(N_B)$ , which was given in Eq. (3). Using this value, we can obtain the  $g$  value from Eq. (4), which represents the normalized reduction of the third-harmonic signal. Figure 3(b) shows the relationship between  $g$  and  $\phi_{\text{CRP}}$  that was obtained from the data shown in Fig. 3(a), where  $g$  is represented by the percentage reduction of the third-harmonic signal. The circles represent the average of three measurements, while the error bars represent the variation among these three measurements. As shown, the  $g$  value increased with increasing  $\phi_{\text{CRP}}$ .

Next, we discuss the  $g$  vs  $\phi_{\text{CRP}}$  curve that is shown in Fig. 3(b). Note that, in general, the number of bound markers  $N_B$  does not increase linearly with increasing  $\phi_{\text{CRP}}$ . The relationship between  $N_B$  and  $\phi_{\text{CRP}}$  is dependent on the binding process between the CRPs and the magnetic markers. Therefore, the value of  $g$  given by Eq. (4) does not increase linearly with increasing  $\phi_{\text{CRP}}$ . To determine the relationship between  $g$  and  $\phi_{\text{CRP}}$ , we used the following logistic function from Ref. 14.

$$g = \frac{A - B}{1 + (\phi_{\text{CRP}}/\phi_0)^\gamma} + B, \quad (5)$$

where  $A$ ,  $B$ ,  $\phi_0$ , and  $\gamma$  are variable parameters.  $A$  and  $B$  are given by the value of  $g$  when  $\phi_{\text{CRP}} \ll \phi_0$  and when  $\phi_{\text{CRP}} \gg \phi_0$ , respectively. The value of  $\phi_0$  is given by the value of  $\phi_{\text{CRP}}$  when  $g = (A + B)/2$ .

The solid line shown in Fig. 3(b) was calculated using Eq. (5) with the parameters  $A = 4$ ,  $B = 92$ ,  $\phi_0 = 4.5$  ng/mL,



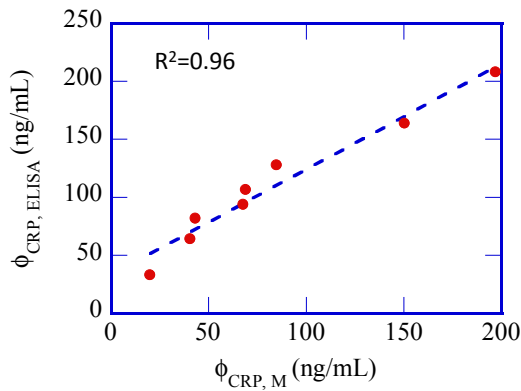
**Fig. 4.** (Color online)  $g$  vs  $\phi_{\text{CRP}}$  curve when CRP international standards was used. Note that normal human serum exists in the sample solution here. The solid line was calculated using Eq. (5).

and  $\gamma = 0.98$ . As the figure shows, good agreement was obtained between the experimental results and those obtained from Eq. (5). Therefore, Eq. (5) can be used to model the relationship between  $g$  and  $\phi_{\text{CRP}}$ .

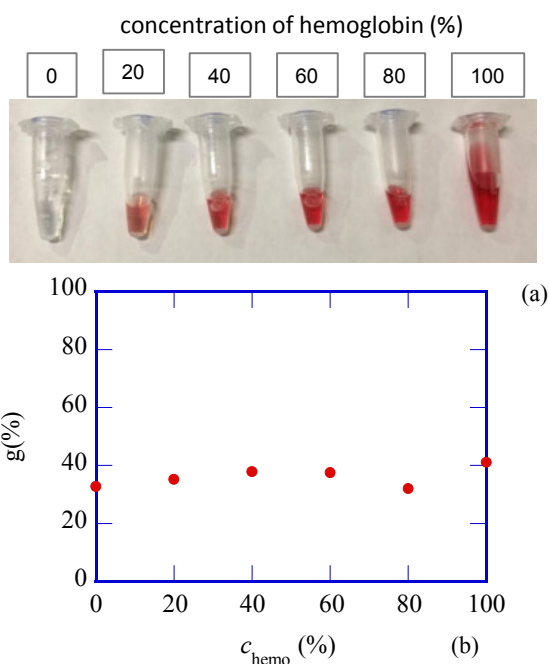
We note that the serum is usually contained in the sample solution in practical diagnostic applications. In this case, it is known that the serum causes the aggregation of magnetic markers to some extent.<sup>16)</sup> To study this case, we measured the  $g$  vs  $\phi_{\text{CRP}}$  curve when CRP international standards (ReCCS DA474) was used. Note that normal human serum exists in the sample solution here. In Fig. 4, circles represent the experimental results for the  $g$  vs  $\phi_{\text{CRP}}$  curve. The solid line was calculated using Eq. (5) with parameters  $A = 10$ ,  $B = 80$ ,  $\phi_0 = 45$  ng/mL, and  $\gamma = 0.9$ . As the figure shows, good agreement was also obtained between the experimental results and the results from Eq. (5) in this case. However, we should note here that the parameters obtained ( $A$ ,  $B$ ,  $\phi_0$ , and  $\gamma$ ) differ from those obtained in the case of the HEPES buffer shown in Fig. 3(b). This result indicates that the serum contained in the sample solution affects the parameters in Eq. (5).

We now compare the proposed wash-free method with the conventional optical method that requires the washing process for B/F separation. For the optical method, we used a detection kit based on enzyme-linked immunosorbent assay (ELISA). In the experiments, the international standard CRPs were detected using both the proposed method and ELISA. Figure 5 shows the results obtained when the CRP concentration was changed from 20.6 to 206 ng/mL. The horizontal axis of Fig. 5 represents the CRP concentration  $\phi_{\text{CRP,M}}$  that was measured using the proposed method, where the  $g$  vs  $\phi_{\text{CRP}}$  curve from Fig. 4 was used as a calibration curve. The vertical axis represents the concentration  $\phi_{\text{CRP,ELISA}}$  that was obtained using ELISA. The broken line in Fig. 5 is a linear fitting between  $\phi_{\text{CRP,M}}$  and  $\phi_{\text{CRP,ELISA}}$ . As the figure shows, good correlation was obtained between the two methods, with a correlation coefficient of  $R^2 = 0.96$ . This result indicates that the wash-free method presented here has the same quantitative performance as that of ELISA.

For practical applications, however, it will be necessary to compare the correlations between the two methods over a much wider range of concentrations of biological targets. It is also necessary to optimize the concentrations of polystyrene beads and magnetic markers in order to improve the sensitivity and reproducibility of target detection.



**Fig. 5.** (Color online) Comparison of the presented method with the conventional optical method, which required a washing process. International standard CRPs were detected using the presented method and ELISA.



**Fig. 6.** (Color online) CRP detection in hemolysis sample solutions. (a) Photographs of six hemolysis sample solutions with different hemoglobin concentrations. (b) Comparison of detected signals from six sample solutions.

Note here that the time required for sample preparation in the presented method was 20 min. In contrast, we required 90 min for sample preparation in ELISA because of the washing process. Therefore, the presented method can perform both simple and rapid detection of biological targets.

Finally, we demonstrate that the proposed method can be used on samples that are not optically transparent, e.g., hemolysis samples. Figure 6(a) shows photographs of six hemolysis sample solutions with different hemoglobin concentrations. The hemoglobin concentration was varied from 0 to 100%. We detected CRPs with a concentration of 60 ng/mL that were mixed into six sample solutions. The results in Fig. 6(b) show that we obtained almost the same signal from all six sample solutions: the  $g$  values were  $36.1 \pm 4.5\%$  among the six samples. This result indicates that the method presented here can be applied to hemolysis samples.

In summary, we have demonstrated wash-free detection of C-reactive proteins (CRPs) based on third-harmonic signal measurement of magnetic markers in solution. The CRP concentration was detected based on the decrease in the third-harmonic signal from the sample solution. The relationship between the detected signal and the CRP concentration can be modeled quantitatively using a logistic function, which can be used to provide a calibration curve in practical applications. The quantities of CRP that were detected using the presented method showed good correlation with those obtained using the conventional optical method with the washing process. We also demonstrated the detection of CRPs in hemolysis sample solutions that were not optically transparent.

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