PAPER Special Section on Current Situation of Ultra-High Sensitivity Magnetic Sensors and Measurement Techniques: Present Location of SQUID Sensors

Development of Liquid-Phase Bioassay Using AC Susceptibility Measurement of Magnetic Nanoparticles

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SUMMARY Simple and quick tests at medical clinics have become increasingly important. Magnetic sensing techniques have been developed to detect biomarkers using magnetic nanoparticles in liquid-phase assays. We developed a biomarker assay that involves using an alternating current (AC) susceptibility measurement system that uses functional magnetic particles and magnetic sensing technology. We also developed compact biomarker measuring equipment to enable quick testing. Our assay is a one-step homogeneous assay that involves simply mixing a sample with a reagent, shortening testing time and simplifying processing. Using our compact measuring equipment, which includes anisotropic magneto resistance (AMR) sensors, we conducted high-sensitivity measurements of extremely small amounts of two biomarkers (C-reactive protein, CRP and α-Fetoprotein, AFP) used for diagnosing arteriosclerosis and malignant tumors. The results indicate that an extremely small amount of CRP and AFP could be detected within 15 min, which demonstrated the possibility of a simple and quick high-sensitivity immunoassay that involves using an AC-susceptibility measurement system.

key words: *magnetic nanoparticles (MNPs), anisotropic magneto resistance sensor (AMR sensor), biomarker, homogeneous assay*

1. Introduction

Rapid detection of biomarkers using magnetic particles is required for early diagnosis of acute disease. Functional magnetic particles are extensively used in medical-care and biotechnology fields. A functional magnetic particle is a type of magnetic particle that can be bonded to a variety of substances by adding a function group, antibody, ligand, etc. to its surface. Experiments have been conducted on detecting protein or cells bonded to such particles by quantifying the concentration of magnetic particles using sensing technology [1]–[7]. Along with the progress in magnetic particles, many magnetic-sensing techniques have been developed to detect biomarkers using magnetic nanoparticles (MNPs) in liquid-phase assays, magnetic permeability measurements [8]–[15], magnetic particle spectroscopy measurement [16], and magnetorelaxation measurements [17]–[24]. The remanence of bound MNPs has been measured to detect biomarker concentration by applying direct current (DC) magnetic fields. In such measurement, the problem is that the residual magnetism of unbound MNPs causes non-specific aggregation due to the application of strong DC magnetic fields.

We are developing an application of measuring the AC susceptibility of MNPs for detecting biomarkers [25], [26]. The frequency of the excitation AC magnetic field is used as a reference signal, and the signal from the magnetic marker is lock-in detected during AC-susceptibility measurement. Therefore, it is less affected by the DC magnetic field of geomagnetism and electronic devices and eliminates the need for an expensive magnetic shield. We also developed a biomarker assay that involves using AC-susceptibility measurement system that uses HTS-SQUID sensor [25] and anisotropic magneto-resistance (AMR) sensors [26]. An AC-susceptibility measurement system bonds cells or proteins in the liquid phase to magnetic particles and applies Brownian relaxation to achieve a one-step homogeneous assay that involves simply mixing a sample with a reagent. Homogeneous assays enable simple and quick testing, so their application to on-site emergency medical care, e.g., diagnosis of myocardial infarction, is anticipated. In this regard, C-reactive protein (CRP, MW 110,000) is a typical inflammatory biomarker that can be used to diagnose a variety of infectious diseases, including COVID-19, or myocardial infarction [27]–[29]. It has been reported that the state of arteriosclerosis can be determined by testing for minute amounts of CRP (~0.1 µg/mL). We investigated the detection of α-Fetoprotein (AFP, MW 72,000), a type of tumor marker. Tumor markers are used for diagnosing malignant tumors and make rapid testing in hospitals possible [30]–[33]. AFP is a fetal-specific serum protein and increases in the blood of patients diagnosed with liver cancer. Liver cancer can be diagnosed when the AFP concentration in the blood exceeds 400 ng/mL (5.0×10^{-12} mol/mL).

Against the above background, we developed a biomarker assay that involves using an AC-susceptibility measurement system and developed compact measuring equipment that includes AMR sensors for quick testing. We argue that this equipment can be used to conduct one-step homogeneous biomarker assays that are highly sensitive to extremely small amounts of CRP and AFP.

2. AC Susceptibility Measurement System

2.1 Measurement Principle

A biomarker assay involves using an AC-susceptibility measurement system. Such a system uses two types of particles, polystyrene beads and MNPs, with different particle sizes.

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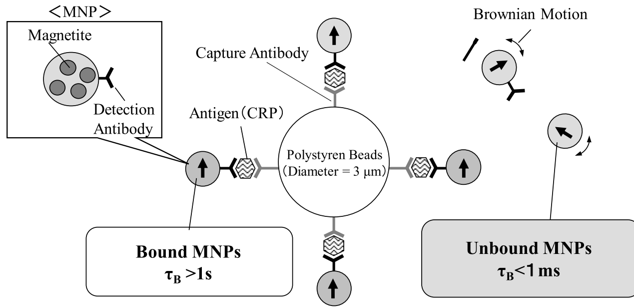

Fig. 1 Principle of AC-susceptibility measurement system for biomarker detection using polystyrene beads and MNPs.

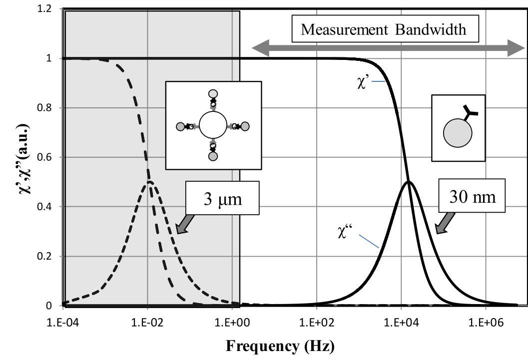

Fig. 2 Signal bandwidth for different diameter beads.

These particles differ greatly in size to detect the difference in Brownian relaxation times originating from the difference between those particle diameters. The principle of this measurement system is shown in Fig. 1. The system targets a liquid-phase sample using two types of particles, as shown in the figure. One type is a polystyrene bead having a diameter on micrometer scale that immobilizes "capture" antibodies on its surface. The other type is a magnetic particle having a diameter on nanometer scale that immobilizes a "detection" antibody on its surface. These two types of antibody particles are used to bind an antigen (CRP), the target of detection, by an antigen-antibody reaction (sandwich method). Since the capture and detection antibodies each recognize different antigenic epitopes, a highly specific biomarker detection system needs to be constructed. Which would result in a state in which magnetic particles bound to polystyrene beads via CRP antigens coexist in the liquid phase with surplus magnetic particles not bound to the polystyrene beads.

An AC magnetic field having a specific frequency is applied to a sample, the overall diameter of MNPs bound to beads (bound MNPs) is large, lengthening the Brownian relaxation time τ_B (> 1 s). The diameter of unbound MNPs, however, is small, shortening τ_B (< 1 ms). The behavior of these MNPs is expressed as their AC magnetic susceptibility in terms of χ' following the magnetic field (real component) and phase delayed χ'' (imaginary component) respectively given as

$$\chi'(\omega) = \frac{\chi_0}{1 + (\omega\tau_B)^2} + \chi_\infty \tag{1}$$

$$\chi''(\omega) = \frac{\omega\tau_B\chi_0}{1 + (\omega\tau_B)^2} \tag{2}$$

The amount of MNPs with immobilized antibodies can be determined by measuring the frequency-response characteristics of AC susceptibility in a frequency band appropriate for MNPs of a specific size. Figure 2 shows the frequency dependence of the magnetic particles for the real and imaginary components of AC susceptibility. The figure shows ideal monodisperse particles with a particle size of 3 μm for polystyrene beads (antigen and MNP-bound complexes) and 30 nm for MNPs. MNP-bound complexes have large particles with a frequency band of 1 Hz or less, but MNPs alone have a small particle size of 30 nm, so the AC-susceptibility frequency band is 100 Hz or more. Therefore, it is possible to measure single MNPs of several tens of nanometers by carrying out excitation and detection with an alternating magnetic field at 100 Hz or more.

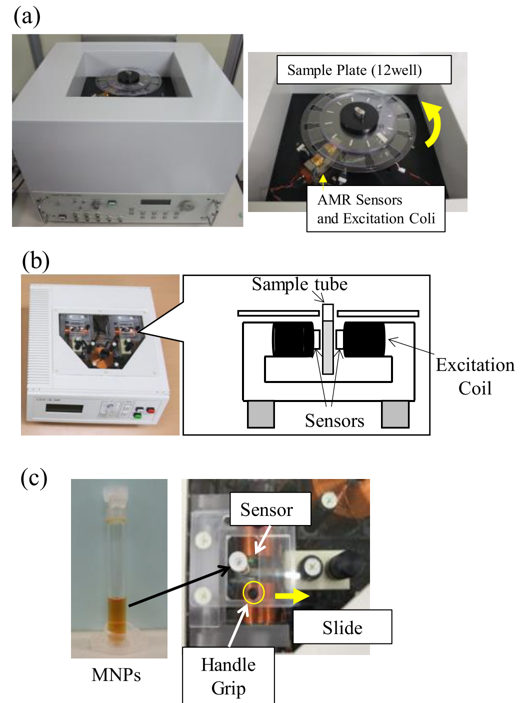

Fig. 3 Biomarker-detection equipment: (a) benchtop type, (b) compact type, (c) sample transfer of compact equipment.

2.2 Assay Equipment

We previously developed benchtop biomarker-detection equipment that involves using an AC-susceptibility measurement system (shown in Fig. 3 (a)) [26]. To further reduce weight and size, we developed compact measuring equipment to more easily conduct biomarker assays that involves using an AC-susceptibility measurement system. We downsized the equipment to A4 paper size (W290×D310×H130) and installed an excitation coil and AMR magnetic sensors (HMC1001, Honeywell) together with a circuit board inside

the equipment (shown in Figs. 3 (b)(c)). We chose the AMR sensor not only because of its high sensitivity but also because its size and functionality were suitable for the present measurement.

This equipment enables a biomarker assay to be conducted that involves using an AC-susceptibility measurement system by applying an AC magnetic field of a specific frequency to a magnetic-particle sample while moving the sample container and obtaining a fluctuating magnetic field in the form of signals as the sample passes the AMR sensors.

2.3 Reagent-Preparation Method

We used 3-μm-diameter polystyrene beads (hereafter, beads) coated with carboxyl groups and 30-nm-diameter MNPs (Ocean NanoTech, LLC). The MNPs were water soluble and coated with a polymer layer (oleic acid and amphiphilic polymer) approximately 4 nm thick. Antibodies were immobilized on the beads and MNPs using water-soluble carbodiimide (WSC). The beads were coupled with anti-human CRP monoclonal antibodies (capture antibodies), and the MNPs were coupled with anti-human CRP monoclonal antibodies (detection antibodies). The amount of antibodies immobilized on each type of particle was confirmed to be approximately 1.8 μg/cm^2 of the bead surface area and approximately 80 μg/mg of the MNP unit weight (Fe converted). Given that the MNPs bound with antibodies tend to condense, a blocking treatment was conducted and a 0.45-μm filter was used to remove condensed MNPs. We used human CRP (HyTest Co., Ltd.) as the CRP antigen targeted for detection and diluted it to final concentrations of 0.1–5 μg/mL (9.1×10^{-13}–4.6×10^{-11} mol/mL).

For detecting AFP, we also used 3-μm-diameter polystyrene beads coated with carboxyl groups, and 250-nm-diameter MNPs (FG beads, Tamagawa Seiki Co., Ltd.). Anti-human AFP antibodies (capture antibodies) were immobilized on the polystyrene beads using WSC. MNPs, in which the carboxyl groups on the particle surface were activated with N-hydroxysuccinimide, were used to conjugate the detection antibody. The amount of antibody immobilized on each particle was 12 μg/mg of polystyrene beads and 31 μg/mg of MNPs (Fe converted). To prevent aggregation, The MNPs were dispersed using an ultrasonic device. We used human AFP (BBI Solutions Co., Ltd.) as the AFP antigen targeted for detection and diluted it to final concentrations of 3.1–1250 ng/mL (4.3×10^{-14}–1.7×10^{-11} mol/mL).

2.4 Detection Method

To calculate the amount of change in consumed magnetic particles due to antigen-antibody reactions, it is necessary to measure two antigen (CRP or AFP) samples and a reference sample that includes no antigen. The reaction procedure of CRP detection is shown in Figs. 4 (a)(b). To prepare the CRP sample, we first added human CRP to the capture-antibody beads and then mixed the diluted detection-antibody MNPs to a total volume of 100 μL (Fig. 4 (a)).

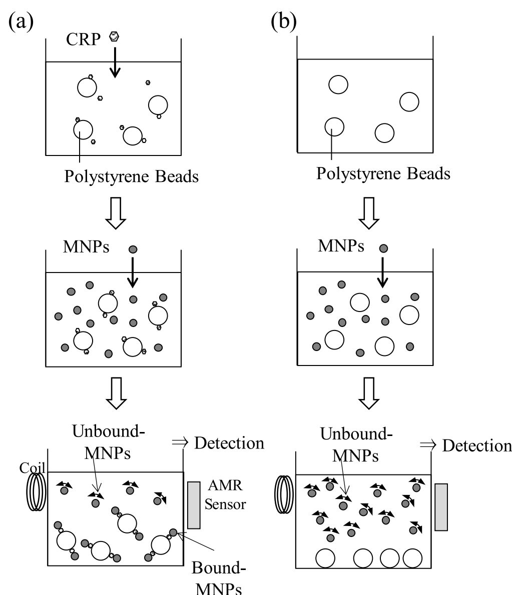

Fig. 4 Reaction procedure of biomarker detection: (a) CRP sample (b) reference sample.

On the other hand, for preparation of the reference sample, we mixed capture-antibody beads with no added antigens with detection-antibody MNPs to a total volume of 100 μL (Fig. 4 (b)). We measured the CRP reactions at room-temperature for 5, 15, and 30 min after mixing in the MNPs. To detect magnetic signals, we used our assay equipment to apply a magnetic field to a sample at an excitation frequency of 230 Hz and magnetic field intensity of 1 mT and detected magnetic signals input to the magnetic sensor from the sample.

We first added human AFP antigen to the capture-antibody beads and then mixed with diluted detection antibody MNPs to prepare AFP samples in a total volume of 100 μL. In contrast, for preparation of the reference sample, we mixed capture-antibody beads with no added antigens with detection-antibody MNPs to a total volume of 100 μL. After sample preparation, we measured the AFP reactions at room temperature for 15 and 30 min after mixing in the MNPs. To detect magnetic signals, we used our assay equipment to apply a magnetic field to a sample at an excitation frequency of 80 Hz and magnetic field intensity of 1 mT and detected magnetic signals input to the magnetic sensor from the sample. In the detection of AFP, we used 250-nm-diameter MNPs, so the excitation frequency was set to a low frequency of 80 Hz in this study. This is because the Brownian relaxation time τB becomes longer for larger particle and we have to decrease the frequency with increasing the particle size in order to use the Brownian-relaxation property.

The reaction ratio of antigen-antibody reactions was calculated using Eq. (3) on the basis of the magnetic signals obtained from the CRP sample and reference sample.

In the equation, the reaction ratio (%) expresses the ratio of magnetic particles bound to the beads.

$$\text{Reaction ratio (\%)} = \{1 - (\text{CRP sample}/\text{reference sample})\} \times 100 \quad (3)$$

3. Measurement Results

3.1 CRP Detection Results

Figures 5(a)(b) show the results of CRP detection. The results of the signal intensity change at 5, 15, and 30 min after the reactions began are shown in Fig. 5 (a). The horizontal axis represents the elapsed time from reaction initiation and the vertical axis represents the signal intensity of the reference and CRP samples (n = 5). The variation in the reference signal over the elapsed time was small (coefficient of variation = 2–3%). This indicates that the MNPs (30 nm in diameter, Ocean NanoTech, LLC) were well dispersed and stable in the liquid phase. It also suggests that the MNPs had little non-specific binding reaction with the beads. The reaction ratios of the CRP sample at 5, 15, and 30 min after the reactions began are shown in Fig. 5 (b). The horizontal axis represents the CRP concentration and the vertical axis represents the reaction ratio calculated from Eq. (3). At 5 min after the reaction began, the reaction ratio of 40% could be detected for a CRP concentration of 4.6×10^{-11} mol/mL (5 μg/mL) while a reaction ratio of 14% could be detected for a low CRP concentration of 9.1×10^{-13} mol/mL (0.1 μg/mL, 910 fmol/mL). These results indicate that a sample with a low CRP concentration of 0.1 μg/mL could be detected in a short time (within 5 min) with a biomarker assay that involves using an AC-susceptibility measurement system.

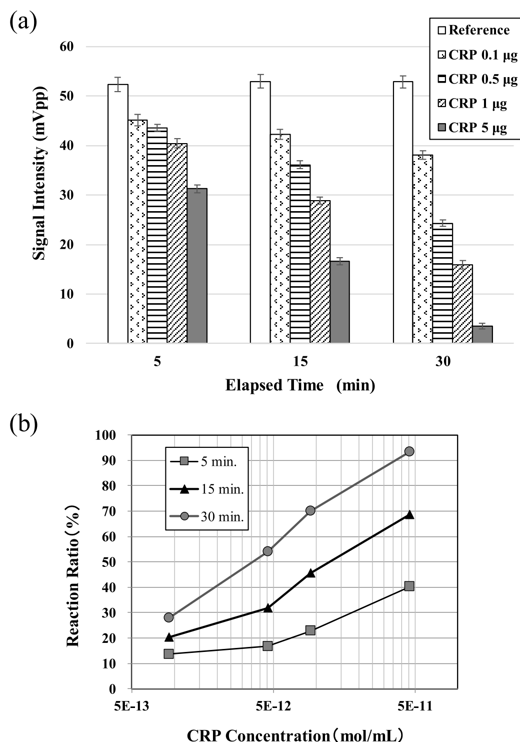

Fig. 5 Detection results of human CRP: (a) signal intensity change per elapsed time, (b) Reaction ratio of reference and CRP samples.

To confirm the surface state of the beads after the reaction, we observed them with a scanning electron microscope (SEM, S5000, Hitachi Co., Ltd.). The SEM image of a 3-μm-diameter bead and MNPs bound to the bead surface is shown in Fig. 6. The MNPs have a uniform diameter of approximately 30 nm and exhibit good dispersibility. From this SEM image, many 30-nm magnetic particles are bound to the surface of the bead. This is a state in which the bead and magnetic particles form a complex through an antigen-antibody reaction with CRP. There is also no agglomeration of the magnetic particles, and they are bound to the bead surface in a monolayer state. However, the magnetic particles are unevenly distributed on the bead surface. A possible reason for this phenomenon is that the antibody binding to the surface of the bead is not uniform. The non-uniform binding distribution of antibodies is caused by the surface state of the functional beads. The second reason is that the bound magnetic particles may have fallen off during the pretreatment stage when acquiring the SEM image. Acquisition of SEM images requires processing steps such as washing with organic solvents and drying. It is suggested that these steps may destabilize the bond between beads and MNPs.

3.2 AFP Detection Results

The results of detecting AFP at 15 and 30 min after the reactions began are shown in Figs. 7 (a)(b). The results of the signal intensity change in the AFP sample at 15 and 30 min after the reactions began are shown in Fig. 7 (a). The horizontal axis represents the elapsed time from reaction initiation and the vertical axis represents the signal intensity of the reference and AFP samples (n = 5). The variation in the reference signal over the elapsed time was small (coefficient of variation = 2%). The 250-nm-diameter MNPs with a tended to aggregate over time. The reason the time change in the magnetic signal of the reference sample was small is due to the appropriate pretreatment of the MNPs. The reaction ratio of the AFP sample at 15 and 30 min after

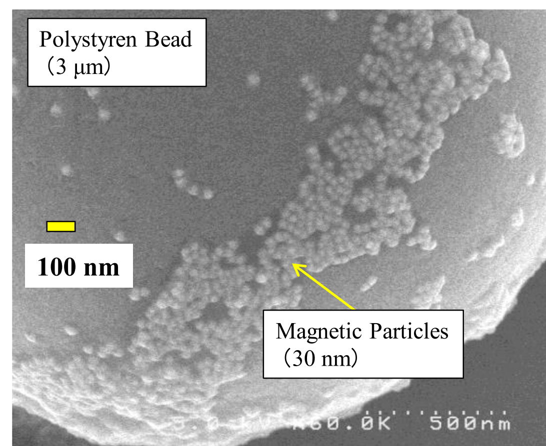

Fig. 6 SEM image of MNPs bound on the polystyrene bead surface.

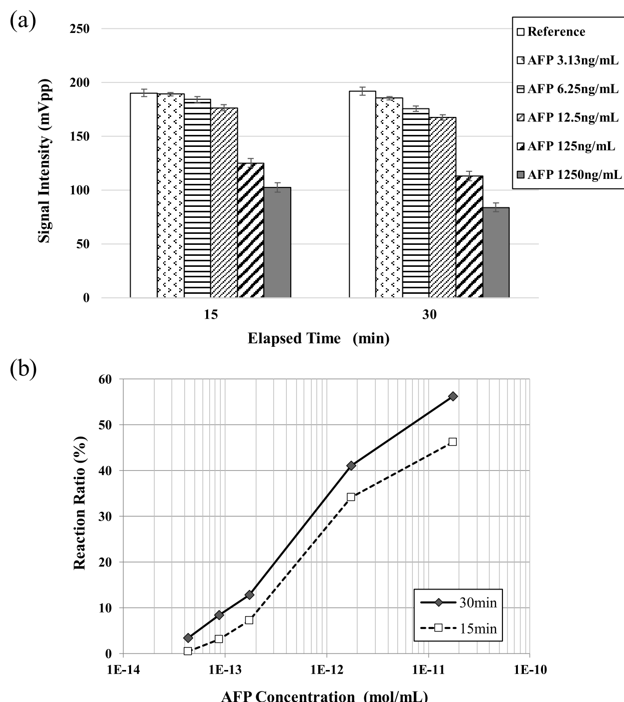

Fig. 7 Detection results of human AFP. (a) Signal intensity change per elapsed time. (b) Reaction ratio of reference and AFP samples.

the reactions began are shown in Fig. 7 (b). The horizontal axis represents the AFP concentration (mol/mL) and the vertical axis represents the reaction ratio calculated from Eq. (3). These results indicate that the AFP concentration has a linear relationship with the reaction rate over a wide range from 43 fmol/mL (4.3×10^{-14} mol/mL) to 17 pmol/mL (1.7×10^{-11} mol/mL). Furthermore, 15 min after the start of the reaction, a reaction rate of 3.1% was detected when AFP was 86 fmol/mL, and 30 min after the start of the reaction, a reaction rate of 3.3% was detected when the AFP concentration was 43 fmol/mL. The reaction rate increased approximately 2.7 times from 3.1 to 8.4% at a low concentration of 86 fmol/mL within 15 min to 30 min after the start of the reaction. Under the high concentration of 17 pmol/mL, the increase was only about 1.2 times from 46 to 56%. In other words, the change in response rate over time is greater in the low-antigen-concentration range than in the high range. This suggests that the reaction time has a large effect on low-concentration antigens, and longer reaction times are necessary to detect lower-concentration antigens. In this study, the AFP antigen was detected from AC-susceptibility measurement, and the limit of detection (LOD) was about 80 fmol/mL after 15 min of reaction time and about 40 fmol/mL after 30 min.

4. Discussion

4.1 Detection Sensitivity of Biomarkers

We found that our homogeneous assay is easy to conduct, requiring only the mixing of a reagent with a sample; thus, it will be of great use in hospital settings as a quick and simple testing method. We discuss the superiority of the homogeneous biomarker assay that involves using an AC-susceptibility measurement system and associated issues. The homogeneous assay is superior because it simplifies the process and shortens testing time. The sandwich ELISA method, a typical immunoassay, requires bound/free (B/F) separation to wash out surplus labeled antibodies, thereby lengthening assay time and complicating the process. In contrast, our homogeneous biomarker assay negates the need for B/F separation since it only requires the mixing of beads and MNPs with an antigen sample. Using our compact measuring equipment, it was possible to detect CRP by using a low-concentration sample of 0.1 μg/mL (910 fmol/mL) within 5 min after mixing in the reagent. The LOD of AFP was about 40 fmol/mL after 30-min reaction time. This indicates that this biomarker assay not only enables simple and quick testing but also makes it possible to measure a low-concentration sample with high sensitivity. To achieve even more accurate testing, measures such as optimizing the amount of antibody sensitizing on MNPs or decreasing loss in MNPs due to condensation can be considered. It is thought that aggregates are easy to form since the surface charge of MNPs changes by immobilizing antibodies. In this study, non-specific agglutination was prevented by carrying out appropriate pretreatments such as blocking, filtering, and sonication. For future research, it will be important to optimize the amount of antibodies immobilized on particles and improve blocking and storage solutions to control non-specific reactions.

4.2 Sensitivity Comparison by Sensor Type

Since an AC-susceptibility measurement system does not require washing of reagents, processing can be shortened compared with current optical inspection methods, and faster inspection can be achieved. In a previous study, the detection limit of MNPs (Resovist, PDRadiopharma Inc.) was 0.2 μg/mL using a third harmonic measurement system with an HTS-SQUID sensor [25]. Assuming that the particle size of the magnetic particles is the same, the detection sensitivity of the tumor marker (AFP) with the HTS-SQUID sensor is approximately 1–4 fmol/mL (10^{-15} mol/mL). Figure 8 shows standard diagnostic levels of typical tumor markers and inflammatory markers. It also shows the lower LOD (calculated value) for two types of AC-susceptibility measurement systems, one using an AMR sensor and the other using an HTS-SQUID sensor. In the system using an AMR sensor, the LOD is lower than that of the HTS-SQUID sensor. Since the AMR sensor measures the fundamental wave, which has the same frequency as the excitation magnetic field, measurement sensitivity remained at about 40 fmol/mL (10^{-14} mol/mL). It can be estimated that the third harmonic AC-susceptibility measurement system using the HTS-SQUID sensor has a detection sensitivity of 10 times or more. Therefore, this system is superior for the detecting Pro-GRP (small cell lung cancer marker), which

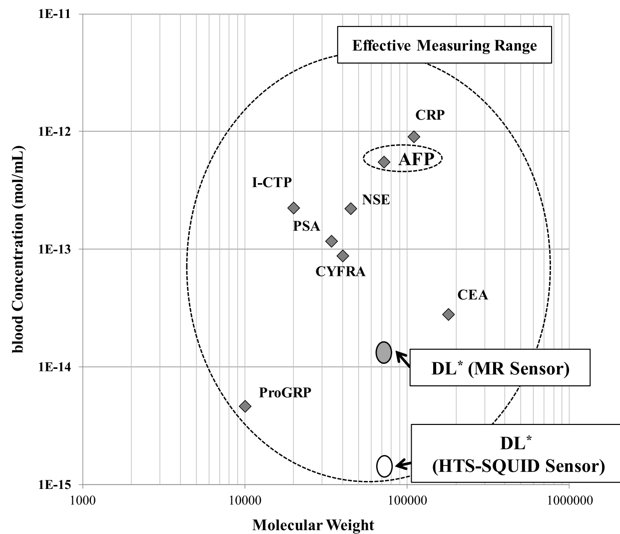

Fig. 8 Comparison of serum levels of biomarkers.

requires a measurement sensitivity of several fmol/mL. The system using an AMR sensor can be made smaller without a magnetic shield and is considered superior as a simple inspection system for hospitals. To advance biomarker-detection technology using a magnetic measurement method, it is necessary to develop devices that take advantage of the sensitivity and features of the sensor.

5. Conclusion

We developed a biomarker assay that involves using an AC-susceptibility measurement system and developed compact biomarker-detection equipment that includes AMR sensors that operate at room temperature. Magnetic sensors that operate at room temperature enable a device to be made smaller. In liquid-phase AC magnetometry, the Brownian relaxation of antibody-sensitized beads and magnetic particles is obtained as a magnetic signal, so biomarkers can be detected quickly and with high sensitivity. We demonstrated that the biomarkers CRP and AFP can be detected with high sensitivity using AC-susceptibility measurements.

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