Assaying Carcinoembryonic Antigens by Normalized Saturation Magnetization

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Abstract
Biofunctionalized magnetic nanoparticles (BMNs) that provide unique advantages have been extensively used to develop immunoassay methods. However, these developed magnetic methods have been used only for specific immunoassays and not in studies of magnetic characteristics of materials. In this study, a common vibration sample magnetometer (VSM) was used for the measurement of the hysteresis loop for different carcinoembryonic antigens (CEA) concentrations (ΦCEA) based on the synthesized BMNs with anti-CEA coating. Additionally, magnetic parameters such as magnetization (M), remanent magnetization (MR), saturation magnetization (MS), and normalized parameters (ΔMR/MS and ΔMS/MS) were studied. Here, ΔMR and ΔMS were defined as the difference between any ΦCEA and zero ΦCEA. The parameters M, ΔMR, and ΔMS increased with ΦCEA, and ΔMS showed the largest increase. Magnetic clusters produced by the conjugation of the BMNs to CEAs showed a ΔMS greater than that of BMNs. Furthermore, the relationship between ΔMS/MS and ΦCEA could be described by a characteristic logistic function, which was appropriate for assaying the amount of CEAs. This analytic ΔMS/MS and the BMNs used in general magnetic immunoassays can be used for upgrading the functions of the VSM and for studying the magnetic characteristics of materials.

Keywords: Magnetic immunoassays; Saturation magnetization; Magnetic clusters; Carcinoembryonic antigen; Biofunctionalized magnetic nanoparticles

Background
Magnetic nanoparticles interest researchers because of their potential applications in biomedicine, such as protein purification [1], magnetofection [2], tomographic imaging [3], magnetic resonance imaging [4–6], magnetic immunoassays [7, 8], tumor diagnosis [9], and hyperthermia therapy [10]. In magnetic immunoassays, magnetic nanoparticles are first biofunctionalized with antibodies to obtain biofunctionalized magnetic nanoparticles (BMNs), which are then dissolved in solutions to form magnetic reagents. To assay a biotarget, a magnetic reagent is mixed with a sample solution containing the biotarget. The conjugation of BMNs with the biotarget produces magnetic clusters because of molecular interaction (Fig. 1), and the magnetic properties of the reagent changes. Biological samples, unconjugated BMNs, and magnetic clusters of conjugated biotargets show a negligible magnetic background individually and differ in their magnetic characteristics. Hence, it is possible to develop magnetic immunoassays on the basis of several parameters and phenomena such as magnetic relaxation [11, 12], remanent magnetization (MR) [13, 14], saturation magnetization (MS) [15], magnetic resonance [16, 17], and alternating current (ac) susceptibility (χac) [8, 18–21].

In addition, because signal changes associated with the magnetic characteristics of BMNs are always small, a high-sensitivity high-critical-temperature superconducting quantum interference device (SQUID) sensor is usually used to enhance the signal-to-noise ratio and mu-metal shielding is provided to reduce environmental noise. A cryogenic biodetection system involving SQUIDs is difficult to construct.

Washing processes are sometimes required to separate magnetic clusters from reagents for measuring magnetic characteristics; however, they are time-consuming. Therefore, developing a biodetection system featuring an alternative detection mechanism and high detection sensitivity is crucial. A wash-free immunomagnetic reduction (IMR) method based on ac magnetic susceptibility reduction has been proposed [19], and various studies have
demonstrated the sensitive detection of biomolecules, such as nucleic acids [20], biomarkers (for diagnosing Alzheimer’s disease) [6], alpha-fetoprotein (for detecting liver tumors) [7], and human C-reactive protein (for diagnosing inflammation) [15].

In this study, we proposed a magnetic immunoassay method based on the BMNs used in magnetic immunoassay methods, like IMR; the proposed method does not require a SQUID sensor or washing process. The method involves the use of a vibration sample magnetometer (VSM) for measuring the hysteresis loop, from which the major magnetic characteristics can be inferred, and does not require a specific magnetic instrument for magnetic immunoassays. The magnetic parameters of the hysteresis loop were studied to determine the analytic method of magnetic immunoassay. When the method is applied to magnetic immunoassays, the magnetic parameters of the analytics are determined from the hysteresis loop.

Methods

Figure 1 shows a schematic of the clustering process involving BMNs and dextran-coated Fe$_3$O$_4$ nanoparticles. The procedures used for synthesizing BMNs consisting of anticarcinoembryonic antigens (anti-CEAs) coated on dextran-coated Fe$_3$O$_4$ nanoparticles (MF-DEX-0060, MagQu Corp., Taiwan) were similar to those used in a previous study for synthesizing dextran-coated Fe$_3$O$_4$ nanoparticles coated with anti-goat C-reactive protein [22]. Dextran-coated Fe$_3$O$_4$ nanoparticles was oxidized using NaI$_2$O to create aldehyde groups (−CHO), and dextran reacted with the antibodies of anti-CEAs (10C-CR2014M5, Fitzgerald, MA, USA) through −CH = N- to covalently conjugate the antibodies of anti-CEAs. After magnetic separation, the unbound antibodies were separated from conjugated BMNs consisting of dextran-coated Fe$_3$O$_4$ nanoparticles coated with anticarcinoembryonic antigens (Fe$_3$O$_4$-anti-CEAs). Subsequently, a reagent was synthesized by dissolving the BMNs in phosphate-buffered saline. The biotargets were carcinoembryonic antigens (CEAs; 30-AC30, Fitzgerald, MA, USA). These antigens are typically used as a tumor marker for colorectal cancers, which are caused by uncontrolled cell growth in the colon or rectum [23] and are the second leading cause of cancer death in adults worldwide [24].

The mean value of the hydrodynamic diameter of the BMNs was 40.8 nm, as detected through dynamic laser scattering (Nanotrac 150, Microtrac, PA, USA). The conjugation capability of BMNs was verified by tissue staining. The colon tumors induced on the backs of mice were sampled to form paraffin-embedded sections. Figure 2a shows the process of staining the colon tumor tissue with BMNs. First, the sections of the colon tumors were immersed in the Fe$_3$O$_4$-anti-CEA reagent. Consequently, a secondary antibody conjugated to a fluorescent indicator (goat anti-rabbit IgG antibody, Millipore, USA) was added. Here, the binding occurred because the fluorescent indicator with an isothiocyanate reactive group was reactive toward nucleophiles containing amine and sulfhydryl groups on the protein [25]. Because of conjugation between the secondary antibodies and anti-CEA antibodies, the fluorescent indicators were bound to the BMNs on the tissue. Both the tissue and fluorescent indicators of the BMNs were obtained through fluorescence microscopy (IX70, Olympus, Japan).

In assaysing the CEAs, 40 μL of the Fe$_3$O$_4$-anti-CEA reagent with a saturation magnetization of 0.07 emu/g was mixed with 60 μL of a CEA solution with a CEA
concentration ($\Phi_{CEA}$) in the range from 0 to 10 ppm. To verify the formation of magnetic clusters during the assay, the effective relaxation time $\tau_{\text{eff}}(t)$ was monitored. This was because the presence of magnetic clusters would increase $\tau_{\text{eff}}$. Furthermore, $\chi_{ac}(t)$ can be expressed as follows [26, 27]:

$$
\chi_{ac}(t) = \chi_{ac,0} \left\{ \frac{1}{1 + \left( \omega \tau_{\text{eff}}(t) \right)^2} \right\}^{1/2}
$$

(1)

Here, $\chi_{ac,0}$ is $\chi_{ac}$ of the Fe$_3$O$_4$-anti-CEA reagent initially mixing with the CEA solution, and $\omega$ is the angular frequency. Therefore, $\tau_{\text{eff}}$ can be obtained by substituting $\Delta\chi_{ac}$ defined as $\chi_{ac,0} - \chi_{ac}$ in Eq. (1). The test materials were the Fe$_3$O$_4$-anti-CEA reagent and a CEA solution with a $\Phi_{CEA}$ of 10 ppm. The complete experiment process first involved the measurement of the hysteresis loop for only the Fe$_3$O$_4$-anti-CEA reagent by using the VSM (Model Hystermag, MagQu Corp., Taiwan). Subsequently, $\chi_{ac}$ for the mixture of the reagent and the CEA solution was measured continuously during the entire assay period by using an analyzer (\chi_{ac}Pro-E101, MagQu Corp., Taiwan). After the assay, the mixture was again measured using the VSM.

For a $\Phi_{CEA}$ of 10 ppm, the formation of magnetic clusters in the assay of the CEA solutions was verified by measuring $\chi_{ac}$ along with the hysteresis loop during the assay period. For all the other CEA concentrations (0, 0.01, 0.5, 1, 2.5, and 5 ppm), only the hysteresis loop was measured. Figure 3 shows a schematic of the measurement of the hysteresis loop, which expresses the magnetization $M$ as a function of the applied field $H$. An electromagnet that provided a maximum $H$ of 1.0 T was used to determine $M$, $M_R$, and $M_S$. The sample was vibrated with a frequency of approximately 30 Hz by using an oscillating device. The magnetic signal was then detected using a second-order gradient pickup coil. In addition to characterizing the variation of $\Delta M_R$ or $\Delta M_S$ with $\Phi_{CEA}$, the relationship between $\Delta M_R/M_R$ or $\Delta M_S/M_S$ and $\Phi_{CEA}$, which represented the merit function of the CEA amount, was determined.

**Results and Discussion**

Figure 2b shows BMPs conjugated to the CEA reagents on the tumor tissue. The blue and green colors represent the nucleus of a colon tumor cell and the fluorescent indicator, respectively. Here, the excitation/emission wavelengths of the observed green and blue colors were 495 nm/519 nm and 358 nm/461 nm, respectively. Superposing these two images shows that the blue and green spots are located in close proximity, indicating that the BMPs were bound to colon tumor cells. The proximity of the blue and green spots also confirms the bioconjugation capability of the BMNs.

Figure 4a shows that $\chi_{ac}$ was initially constant and that it subsequently decreased with time and reached a steady value. These stages corresponded to the preconjugation, conjugation, and postconjugation period, in which the reference is to the conjugation between BMNs and CEA reagents. In the immunoconjugation reduction (IMR) assay [8, 18–21], the normalized parameter $\Delta\chi_{ac}/\chi_{ac}$ (the
was enhanced to 0.23 emu/g after the conjugation.

In addition to the $\chi_{ac}$ measurement, typical hysteresis loops of the Fe$_3$O$_4$-anti-CEA reagent before the assays and the mixture of the same reagent and the CEA solutions after assaying 10 ppm of CEA were separately shown in Fig. 4b. The parameter $M_s$ for the reagent was equal to 0.07 emu/g at 0.15 T and near the saturation field, and $M_s$ was enhanced to 0.23 emu/g after the conjugation.

One part of the hysteresis loops for various $\Phi_{CEA}$ values is shown in Fig. 5a. For all $\Phi_{CEA}$ values, $M$ rapidly increased with an increase in $H$ from 0 to 1000 Oe, and then gradually reached $M_s$. Furthermore, for each $H$, $M$ (including $M_s$) increased with $\Phi_{CEA}$. From the hysteresis loops, both $\Delta M_R$ at zero $H$ and $\Delta M_S$ at the maximum $H$, defined as the difference between $\Delta M_R$ and $\Delta M_S$ between any $\Phi_{CEA}$ and zero $\Phi_{CEA}$, also increased with $\Phi_{CEA}$, as depicted in Fig. 5b, c. Each of the parameters $\Delta M_R$ and $\Delta M_S$ increased to 0.009 and 0.17 emu/g for a $\Phi_{CEA}$ of 10 ppm.

To quantify the detected $\Phi_{CEA}$ amount and to improve the capability of distinguishing the small measured values of $M$, the parameters $\Delta M_R/M_R$ and $\Delta M_S/M_S$ were used. In addition to the increase in the variation of $\Delta M_R$ or $\Delta M_S$ with $\Phi_{CEA}$, both $\Delta M_R/M_R$ and $\Delta M_S/M_S$, represented as $\Delta M/M$, can be expressed by a characteristic logistic function $\Phi_{CEA}$, as shown in Fig. 6a, b [28, 29, 19]:

$$\Delta M/M = (A - B)/(1 + [(\Phi_{CEA})/\Phi_0]^y) + B \quad (2)$$

where $A$, $B$, and $y$ are dimensionless quantities, and $\Phi_0$ is the dimensional concentration. The parameters $A$, $B$, $y$, and $\Phi_0$ for the fitting curve were $-0.2$, 30.1, 0.5, and $3222.7$ ppm for $x = R$ and 0.018, 83.3, 0.63, and 2874 ppm, respectively, for $x = S$.

A comparison of Fig. 4a, b, and c shows that $\chi_{ac}$ decreased, and $M$, which was related to the dc magnetic susceptibility, increased after the assaying of the CEA solutions. The opposite variations of the ac and dc magnetic susceptibilities are attributed to the presence of magnetic clusters. The verification performed in this study was for the increase in $\tau_{eff}$ during conjugation, consistent with similar assays of C-reactive proteins [30]. Yang et al. [31] conducted a study on temperature-dependent immunoreaction kinetics of the BMN assay for biomarkers of colorectal cancer. They observed a gradual increase in the mean diameter of the magnetic nanoparticles from 41.53 to 45.13 nm after the reagent and CEA solution were mixed. Their results suggested the presence of magnetic clusters in the reagents. Here, the diameter of the magnetic cluster might be considerably greater than 45.13 nm, as indicated in Fig. 1. However, the magnetic clusters were confined to a limited part of the entire Fe$_3$O$_4$-anti-CEA reagent. Therefore, the observed increase in the mean diameter of the mixture, consisting of the Fe$_3$O$_4$-anti-CEA reagent and CEA solution, was small, even though individual magnetic clusters showed a considerably larger increase.

Consequently, in Fig. 5, the higher the $\Phi_{CEA}$ value, the larger the $\Delta M_R$ and $\Delta M_S$ values. However, for small values of $\Delta M_R$ or $\Delta M_S$, it is difficult to determine the $\Phi_{CEA}$ amount because of the small difference between $\Delta M_R$ and $\Delta M_S$. The parameter $\Delta M_R$ was scattered and negative when $\Phi_{CEA}$ was smaller than 0.1 ppm. The reason is that the system noise intensity was greater than the intensity of the signal for the low $\Phi_{CEA}$. Consequently, $\Delta M_R/M_R$ or $\Delta M_S/M_S$ with larger values than $\Delta M_R$ or $\Delta M_S$ was used to obtain a characteristic logistic function of $\Phi_{CEA}$. These relationships were identified for assaying the amount of

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![Image of hysteresis loop measurement scheme using a VSM](image-url)
Fig. 4 The magnetic measurements of $\chi_{ac}$ and the hysteresis loop for mixing 40 μL of the Fe$_3$O$_4$-anti-CEA reagent with 60 μL and 10 ppm of a CEA solution. a The dynamic measurement of $\chi_{ac}$ with time. b Before and c after the measurement of $\chi_{ac}$, the measurement of the hysteresis loop for only the Fe$_3$O$_4$-anti-CEA reagent as well as the mixture of the same reagent and the CEAs.

Fig. 5 The dependence of magnetic characteristics of on $\Phi_{CEA}$ from 0.01 to 10 ppm. a One part of the hysteresis loop, the $M$ variation with $H$, under different $\Phi_{CEA}$. b $\Delta M_S$ and c $\Delta M_R$ as a function of $\Phi_{CEA}$. 
CEAs. In particular, because of having higher values than $\Delta M_R/M_R$, it is suggested that $\Delta M_S/M_S$ can be used to enhance the discrimination capability of $\Phi_{CEA}$ in magnetic immunoassays. In Fig. 5b, c, the detection limits of $\Delta M_R/M_R$ and $\Delta M_S/M_S$ are 0.1 and 0.01 ppm, respectively. For the mixture of the Fe$_3$O$_4$-anti-CEA reagent and CEAs, if the mixing conditions such as the concentration or volume of each material can be optimized instead of the IMR condition, the detection limit can be improved for a $\Phi$ value of 0.005 ppm. This study performed a more detailed investigation compared with a previous study [32]; the investigation included validating and comparing the analysis of $\Delta M_R/M_R$ and $\Delta M_S/M_S$, determining the immunoassay capability of the Fe$_3$O$_4$-anti-CEA reagent by tissue staining, and verifying the presence of magnetic clusters through an analysis of the effective relaxation time. Moreover, the biomarker studied here was also different from that studied previously [32].

The major clinical objectives of assaying CEAs are to screen a colorectal cancer, evaluate the effect of colorectal carcinoma treatment, identify recurrences after surgical resection, and control the spread of cancer. Although a variety of developed immunoassay methodologies exist, such as enzyme-linked immunoassays [33, 34], Western blot immunoassay [35, 36], fluorescence in situ hybridization [37], and polymerase chain reactions [38], washing processes are always required to avoid inaccuracies in the optical examination of sample interference colors. This results in the immunoassays being time-consuming and requiring large manpower. In this study, the magnetic detection platform using BMNs neither depends on the color of biological samples nor requires washing. The established relationship between $\Delta M_S/M_S$ and $\Phi_{CEA}$ followed a characteristic logistic function and was used for the determination of the CEA amount. The proposed method can be applied to the analysis of other biotargets once the relationship between $\Delta M_S/M_S$ and $\Phi_{biotargets}$ is established.

**Conclusions**

A detection mechanism was proposed to show that $M_S$ for BMNs consisting of Fe$_3$O$_4$-anti-CEAs increased after conjugation with CEAs. Hysteresis loops were measured and analyzed to determine $\Delta M_R/M_R$ and $\Delta M_S/M_S$. $\Delta M_S/M_S$ showed higher sensitivity and greater discrimination capability than $\Delta M_R/M_R$ for assaying CEAs. Consequently, the CEA amount could be determined using the relationship between $\Delta M_S/M_S$ and $\Phi_{CEA}$, expressed by a universal characteristic logistic function. This methodology has the potential to be used for other targets; for this purpose, magnetic reagents used in other magnetic immunoassays can be used with the VSM, and no specific instrument is required for applying the methodology to magnetic immunoassays.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

KWH designed the molecular study. JJC designed the measurement study and wrote the paper. JCS and MHC conducted the experiment. All authors read and approved the final manuscript.

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