Characterizations of Anti-Alpha-Fetoprotein-Conjugated Magnetic Nanoparticles Associated with Alpha-Fetoprotein for Biomedical Applications

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Abstract: In this work, we report characterizations of biofunctionalized magnetic nanoparticles (BMNPs) associated with alpha-fetoprotein (AFP) for biomedical applications. The example BMNP in this study is anti-alpha-fetoprotein (anti-AFP) conjugated onto dextran-coated Fe3O4 labeled as Fe3O4-anti-AFP, and the target is AFP. We characterize magnetic properties, such as increments of magnetization ∆MH and effective relaxation time ∆τeff in the reaction process. It is found that both ∆MH and ∆τeff are enhanced when the concentration of AFP, ΦAFP, increases. The enhancements are due to magnetic interactions among BMNPs in magnetic clusters, which contribute extra MH after the association with MH and in turn enhance τeff. The screening of patients carrying hepatocellular carcinoma (HCC) is verified via ∆MH/MH. The proposed method can be applied to detect a wide variety of analytes. The scaling characteristics of ∆MH/MH show the potential to develop a vibrating sample magnetometer system with low field strength for clinic applications.

Keywords: magnetic immunoassay; biofunctionalized magnetic nanoparticles; biomarker; alpha-fetoprotein; hepatocellular carcinoma; magnetization enhancement

1. Introduction

Immunoassays are biochemical tests used to detect or quantify a specific substance, such as analytes in samples of blood or bodily fluid, using immunological reactions. Immunoassay methods include the enzyme-linked immunosorbent assay (ELISA) [1], radioimmunoassay (RIA) [2], real-time polymerase chain reaction (real-time PCR) [3], immunonephelometry [4], etc. Some immunoassays, such as ELISA, require two antigens and separation of the unbound antigens, which can be tedious and time-consuming. On the other hand, magnetic immunoassay (MIA) is a novel type of diagnostic technology using magnetic nanoparticles (MNPs) as labels to replace conventional ELISA, RIA, real-time PCR, etc. MNPs are coated with dextran so that they are encapsulated or glued together with polymers in sizes of nanometers or even micrometers. In immunomagnetic tests, MNPs are first biofunctionalized against antibodies to target antigens. Reagents consisting of biofunctionalized magnetic nanoparticles (BMNPs) are then mixed with samples. Due to the molecular interactions among BMNPs and biomarkers, magnetic clusters are conjugated in the reaction process and their magnetic properties change after the association. The magnetic signal due to the changes of
magnetic properties is analyzed in order to determine the unknown amount of biomarkers. Magnetic properties (magnetic relaxation [5,6], remanent magnetization [7], Brownian relaxation [8], saturation magnetization [9], spin-spin relaxation of NMR [10], and alternative-current (AC) susceptibility reduction [11–15], etc.) have been developed recently. Magnetic immunoassays can be carried out simply by mixing reagents and tested samples together and taking physical measurements. Additionally, the background noise of magnetic detection is negligible; hence, high detection sensitivity can be achieved.

Based on the increment of saturation magnetization, \( \Delta M_S \), Chieh et al. [16] recently reported another assay method that used a vibrating sample magnetometer (VSM) to label tumor biomarkers of alpha-fetoprotein (AFP) in clinical studies via the \( \Delta M_S / M_S \)-versus-\( \Phi \) curve at the saturation field \( H_S \), where \( \Phi \) was the concentration of AFP. The authors demonstrated that VSM can be used to screen patients carrying hepatocellular carcinoma (HCC) with sensitivity better than the criterion set in clinics (0.02 \( \mu \)g/mL). It would be interesting to see whether we can screen HCC patients with high detection sensitivity at low magnetic fields (H). Therefore, in this work, we propose a detection method based on the scaling characteristic of the normalized increment of magnetization at low magnetic fields. It is found that \( M_{AFP} \) and \( \tau_{eff} \) are enhanced when \( \Phi \) increases, where \( M_{AFP} \) is the magnetization of the reagent and \( \tau_{eff} \) is the effective relaxation time. We attribute those results to the molecular interactions among BMNPs in the associated magnetic clusters, which contribute extra magnetization and in turn enhance \( \tau_{eff} \). The scaling characteristic of \( \left( \Delta M_{AFP}/M_{AFP} \right) \)-versus-\( \Phi \) curves at low magnetic fields is demonstrated, and the screening of HCC patients via the scaling characteristic is verified in clinical studies.

2. Experiments

The MNPs in this study were dextran-coated \( \text{Fe}_3\text{O}_4 \) (MF-DEX-0060, MagQu Co., Ltd., New Taipei City, Taiwan) with a mean core diameter of ~35 nm, as detected by x-ray diffraction (D-500, Siemens). The BMNPs were \( \text{Fe}_3\text{O}_4 \)-anti-AFP (MF-AFP-0060, MagQu Co. Ltd., New Taipei City, Taiwan), and the biotarget was AFP, which is a biomarker for diagnosing HCC. When the AFP level is abnormally high before surgery or other therapy, it is expected to fall to normal levels following the successful removal of all cancer cells.

In performing the AFP tests, the BMNPs consisting of \( \text{Fe}_3\text{O}_4 \)-anti-AFP were first mixed with AFP. The changes of magnetic properties after the reaction process were then characterized using a VSM (Model Hystermag, MagQu Co., Taiwan) and AC susceptometer. The data of the normalized increments of magnetization \( \Delta M/M \) were analyzed for a magnetic immunoassay. The AC susceptibility was measured by a highly balanced AC susceptometer in order to monitor the real-time reaction process. The AC susceptibility \( \chi_{ac}(\omega) \) can be expressed as follows:

\[
\chi_{ac} = \chi' + i\chi''
\]

where \( i = (-1)^{1/2} \), \( \chi''/\chi' = \tan\theta = \omega\tau_{eff}(t) \), and \( \theta \) is the phase lag of the time-varying magnetization \( M(t) \) with respect to the applied AC magnetic field \( H(t) \).

Figure 1a shows the detection schematic of the VSM used for characterizing \( M \) after the BMNPs had conjugated with AFP. In the measurement of \( M \), the sample vibrated with a frequency of ~30 Hz. The magnetic signal was detected with a second-order gradient coil. An electromagnet provided a magnetic field of up to 1.0 Tesla, so that the \( M-H \) curves of reagents were characterized. In assaying AFP, a reagent composed of 40 \( \mu \)L \( \text{Fe}_3\text{O}_4 \)-anti-AFP was mixed with 60 \( \mu \)L AFP. We measured the \( M-H \) curves and analyzed the magnetization enhancement \( \Delta M \) at low external fields (H) to establish the relationship between \( \Delta M/M \) and the concentrations of AFP (\( \Phi_{AFP} \)). Figure 1b shows the high-\( T_C \) SQUID-based AC susceptometer for characterizing the AC magnetic susceptibility. The excitation frequency is ~16 kHz. The magnetic signal of BMNPs is picked up by a gradient coil that is coupled to a high-\( T_C \) SQUID via a flux transformer. The detailed design of the pickup coil, gradient coil, and
compensation coil in a homemade AC susceptometer that did not use a high-Tc SQUID was reported in [17,18].

Figure 1. Detection scheme of (a) vibrating sample magnetometer; (b) high-Tc SQUID-based AC susceptometer.

The reagent was composed of anti-AFP-conjugated Fe$_3$O$_4$ labeled as Fe$_3$O$_4$-anti-AFP. The bio-target was AFP. Figure 2 depicts Fe$_3$O$_4$-anti-AFP, AFP, and a magnetic cluster composed of Fe$_3$O$_4$-anti-AFP-AFP.

Figure 2. Pictures showing (a) biofunctionalized Fe$_3$O$_4$-antiAFP; (b) AFPs; (c) magnetic cluster composed of Fe$_3$O$_4$-anti-AFP-AFP.
3. Results and Discussion

This section addresses and discusses the results from the characterization of magnetic properties when biofunctionalized Fe₃O₄-anti-AFPs are associated with AFP. Additionally, we present the results from the real-time association of Fe₃O₄-anti-AFP with AFP via the time-dependency studies of τ_eff(t) in the reaction process using the technique of AC susceptibility. We also briefly summarize the findings. Finally, we present the clinical research on screening HCC patients via normalized increments of magnetization and address and discuss advances in sensitive bio-sensing.

Figure 3 shows ∆M_H as a function of ϕ AFP at μ₀H = 0.02 T, 0.06 T, and 0.16 T and ∆M_H = M_H(ϕ AFP) − M_H(ϕ AFP = 0). For a fixed magnetic field at μ₀H = 0.02 T, ∆M_H = 0.015 emu/g when ϕ AFP = 0.01 μg/mL, and ∆M_H increases to ∆M_H = 0.02 T = 0.13 emu/g when ϕ AFP = 10 μg/mL. For μ₀H = 0.16 T, ∆M_H = 0.16 T = 0.03 emu/g when ϕ AFP = 0.01 μg/mL, and ∆M_H increases to ∆M_H = 0.16 T = 0.23 emu/g when ϕ AFP = 10 μg/mL. Hence, we have demonstrated an enhancement of ∆M_H when ϕ AFP increases at a fixed magnetic field. We attribute those enhancements to the fact that more magnetic clusters are associated and stronger magnetic interactions among BMNPs are present.

![Figure 3. The increments of magnetization ∆M_H as a function of ϕ AFP at low magnetic fields at μ₀H = 0.02 T, 0.06 T, 0.16 T.](image)

Figure 4 shows the normalized increment of magnetization, ∆M AFP/M AFP,0, as a function of ϕ AFP at μ₀H = 0.02 T, 0.06 T, and 0.16 T, where ∆M AFP = M(ϕ AFP) − M(ϕ AFP = 0), M AFP,0 = M_H(ϕ AFP = 0). It is found that ∆M AFP/M AFP,0 as a function of ϕ AFP in external magnetic fields can be scaled to a universal logistic function described by the following formula [15]:

$$\Delta M_{\text{AFP}}/M_{\text{AFP},0} = (A - B)/(1 + [(\Delta M_{\text{AFP}})/(\Phi_{\text{AFP}})]^\gamma) + B$$

(2)

where A and B are dimensionless quantities and Φ₀ is dimensionless. The fitting parameters are as follows: A = 0.173, B = 34.2, Φ₀ = 3410 μg/mL, and γ = 0.5. We have established a relationship between ∆M AFP/M AFP,0 and ϕ AFP with ϕ AFP varied from 0.01 μg/mL to 10 μg/mL. Therefore, the unknown amounts of AFP can be determined via a scaling characteristic of the (∆M AFP/M AFP,0)-versus-ϕ AFP curve, which is versatile and can be applied to assay other biomarkers. In assaying other biomarkers, the relationship between ∆M biomarker/M biomarker,0 and ϕ biomarker is first established and then ∆M biomarker/M biomarker,0 and the ϕ biomarker curve are applied to determine the unknown amount of biomarkers quantitatively.
\[ \tau_B = 3 \nu H \kappa \]  

In the reaction process, we assume that the viscosity and temperature are constant. The Brownian relaxation time is proportional to the hydrodynamic volume of the magnetic particle. The ratio of the increase in \( \tau_{\text{eff}} \) after the reaction process is 1.35 with an \( \Phi_{\text{AFP}} \) value of 1 \( \mu \text{g} / \text{mL} \). The effective diameter of the magnetic cluster is 2.4 times larger than a single magnetic particle when \( \Phi_{\text{AFP}} = 1 \mu \text{g} / \text{mL} \). The reagent shows \( \tau_{\text{eff}} = 1.3 \mu \text{s} \), and \( \tau_{\text{eff}} \) is stable to \( \tau_{\text{eff}} = 1.3 \mu \text{s} \) at \( t = 7200 \text{s} \). It takes approximately 6000 s for the reagent to complete the association and \( \tau_{\text{eff}} \) is increased to \( \tau_{\text{eff}} = 1.75 \mu \text{s} \) with \( \Phi_{\text{AFP}} = 1 \mu \text{g} / \text{mL} \). Therefore, a detection time of 7200 s is suggested. The real-time association of Fe\(_3\)O\(_4\)-anti-AFP with AFP is verified.

The Brownian relaxation time, \( \tau_B \), is a function of the hydrodynamic volume of a magnetic particle, \( V_H \), the viscosity of the medium, \( \eta \), the Boltzmann’s constant, \( k \), and the absolute temperature, \( T \), which is expressed as follows [19]:

\[ \tau_B = 3 \nu H \kappa / kT \]  

In the reaction process, we assume that the viscosity and temperature are constant. The Brownian relaxation time is proportional to the hydrodynamic volume of the magnetic particle. The ratio of the increase in \( \tau_{\text{eff}} \) after the reaction process is 1.35 with an \( \Phi_{\text{AFP}} \) value of 1 \( \mu \text{g} / \text{mL} \). The effective diameter of the magnetic cluster is 2.4 times larger than a single magnetic particle when \( \Phi_{\text{AFP}} = 1 \mu \text{g} / \text{mL} \). The reagent shows \( \tau_{\text{eff}} = 1.3 \mu \text{s} \), and \( \tau_{\text{eff}} \) is enhanced to \( \tau_{\text{eff}} = 1.75 \mu \text{s} \) when \( \Phi_{\text{AFP}} = 1 \mu \text{g} / \text{mL} \). The enhancement of \( \tau_{\text{eff}} \) is due to the presence of magnetic clusters in the reaction process. The magnetic interaction among BMNPs enhances \( M \), which in turn increases \( \tau_{\text{eff}} \). The \( (\Delta \tau_{\text{eff}} / \tau_{\text{eff},0}) \)-versus-\( \Phi_{\text{AFP}} \) curve follows the characteristic curve [15]:

\[ \Delta \tau_{\text{eff}} / \tau_{\text{eff},0} = (A_1 - B_1) / \{1 + [\Phi_{\text{AFP}} / (\Phi_0)]^\gamma \} + B_1, \]  

where \( \Delta \tau_{\text{eff}} = \tau_{\text{eff}}(7200 \text{s}) - \tau_{\text{eff}}(t = 0) \) and \( \tau_{\text{eff},0} = \tau_{\text{eff}}(t = 0) \). The curve is fitted to the following parameters: \( A_1 = -0.013 \mu \text{s}, B_1 = 0.56 \mu \text{s}, \Phi_0 = 0.15 \mu \text{g} / \text{mL} \), and \( \gamma = 0.52 \). Equation (4) reveals the concentration dependency of the characteristic of \( \Delta \tau_{\text{eff}} / \tau_{\text{eff},0} \) after the BMNPs have completed the association with AFP. The \( (\Delta \tau_{\text{eff}} / \tau_{\text{eff},0}) \)-versus-\( \Phi_{\text{AFP}} \) curve shown in Figure 5b can be applied to screening patients carrying HCC. Normalized \( \Delta \tau_{\text{eff}} / \tau_{\text{eff},0} \) is analyzed instead of \( \Delta \tau_{\text{eff}} \) for a magnetic immunoassay, because this enables us to eliminate minor differences in magnetic signals due to minor differences in sample amounts used from run to run, which will enhance the detection sensitivity.

Detection sensitivity can be defined by the noise level with standard deviations for the detected signal at low concentrations [20]. In this study, the detection sensitivity levels are 0.0024 \( \mu \text{g} / \text{mL} \) and
0.0177 µg/mL, as determined by measuring $\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$ and $\Delta M_{\text{AFP}}/M_{\text{AFP,0}}$ respectively. The reference criterion of the AFP serum level for HCC is 0.02 µg/mL. The sensitivity of both methods reaches the criteria for a clinical AFP assay. The feasibility of AFP is demonstrated by measuring $\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$ and $\Delta M_{\text{AFP}}/M_{\text{AFP,0}}$.

![Figure 5](image.png)

**Figure 5.** (a) $\tau_{\text{eff}}$ as a function of time, (b) $\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$ as a function of $\Phi_{\text{AFP}}$ with $\Phi_{\text{AFP}}$ from $\Phi_{\text{AFP}} = 0.001$ µg/mL to $\Phi_{\text{AFP}} = 1$ µg/mL.

In this study, we characterized magnetic properties when BMNPs are associated with AFPs for biomedical applications. The findings in the characterization of magnetic properties are briefly summarized as follows. First, $M$ and $\tau_{\text{eff}}$ are enhanced when reagents composed of BMNPs are conjugated with AFP in the reaction process. The magnetic interactions among BMNPs in magnetic clusters enhance $M$, which in turn increases $\tau_{\text{eff}}$. Second, the real-time association of BMNPs with AFP was demonstrated in the time-dependent $\tau_{\text{eff}}$. Third, bio-detection based on the ($\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$)-versus-$\Phi_{\text{biomarkers}}$ curve provided a sensitive methodology for assaying unknown amounts of AFP, and BMNPs could be applied to assay large molecules such as AFP as well as small molecules such as C-reactive protein (CRP) [21]. Finally, the proposed detection methodology based on the ($\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$)-versus-$\Phi_{\text{biomarkers}}$ curve was versatile, and the ($\Delta M_{\text{AFP}}/M_{\text{AFP,0}}$)-versus-$\Phi_{\text{AFP}}$ curves shown in Figure 4 were scaled to a characteristic function described by Equation (2). The results confirm that both changes in $\Delta M_{\text{AFP}}/M_{\text{AFP,0}}$ and $\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$ are caused by the formation of magnetic clusters and can be applied to sense a wide variety of biomarkers.

The sensitivity levels of $\Delta \tau_{\text{eff}}/\tau_{\text{eff,0}}$ and $\Delta M_{\text{AFP}}/M_{\text{AFP,0}}$ reach the criteria for a clinical AFP assay. The cost of a high-$T_C$ SQUID-based AC susceptometer is much higher than that of a VSM with a low-strength magnet. The low-strength VSM has high potential for commercial and clinical applications. Therefore, the screening of HCC patients can be addressed by measuring $\Delta M_{H}/M_{H,0}$. Since the data shown in Figure 4 are scaled to a characteristic function described by Equation (2), it would be interesting to verify whether we can also obtain high detection sensitivity at low
magnetic fields via Equation (2). Hence, we can apply Equation (2) at a low magnetic field, say $\mu_0 H = 0.065 \, \text{T}$, to analyze AFP levels in clinical studies. To verify this, we show in Figure 6a ($\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$)-versus-$\Phi_{\text{AFP}}$ with data analyzed at $\mu_0 H = 0.065 \, \text{T}$, where $\Delta M_{\text{AFP}} = M_H(\Phi_{\text{AFP}}) - M_H(\Phi_{\text{AFP}} = 0)$ and $M_{\text{AFP,0}} = M_H(\Phi_{\text{AFP}} = 0)$. The background magnetic signal of serum from healthy persons in $\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$ is deducted in the data analysis. To screen patients carrying HCC and healthy persons, we mixed 40 $\mu$L of serum with 60 $\mu$L of AFP solution. The data for establishing the standard curve are marked with a solid dot ($\bullet$). AFP levels in serum for HCC patients are marked with an open triangle ($\Delta$), while AFP levels for healthy persons are marked with an open square ($\square$). The reference magnetization, $M(\Phi_{\text{AFP}} = 0)$ and $M_{\text{AFP,0}} = M_H(\Phi_{\text{AFP}} = 0)$. The background magnetic signal of serum from healthy persons is deducted in the data analysis. To screen patients carrying HCC and healthy persons, we mixed 40 $\mu$L of serum with 60 $\mu$L of serum. The data for establishing the standard curve are marked with a solid dot ($\bullet$). AFP levels in serum for HCC patients were higher than $\sim 0.2 \, \mu\text{g/mL}$, which is significantly higher than the criterion set in clinics ($0.02 \, \mu\text{g/mL}$). The average AFP levels for healthy persons were below $\sim 0.02 \, \mu\text{g/mL}$, except for one healthy person who showed a false positive (AFP level = $\sim 0.03 \, \mu\text{g/mL}$). Figure 6b shows $\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$ as a function of $\Phi_{\text{AFP}}$ with data analyzed at $0.16 \, \text{T}$. HCC patients showed AFP levels higher than the clinical criterion. Healthy persons showed AFP levels of $0.001 \, \mu\text{g/mL}$, except for one healthy person who showed a false positive (AFP level = $\sim 0.03 \, \mu\text{g/mL}$). Figure 6b shows $\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$ as a function of $\Phi_{\text{AFP}}$ with data analyzed at $0.16 \, \text{T}$. HCC patients showed AFP levels higher than the clinical criterion. Healthy persons showed AFP levels of $0.001 \, \mu\text{g/mL}$, except for one healthy person who showed a false positive (AFP level = $\sim 0.03 \, \mu\text{g/mL}$). The estimated values of $\Phi_{\text{AFP}}$ were different between $\mu_0 H = 0.065 \, \text{T}$ and $0.065 \, \text{T}$. It was probably due to the magnetic clustering effect that induces background magnetic noises. Besides, $\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$ of serum tested at $0.16 \, \text{T}$ is higher than that at $0.065 \, \text{T}$. It leads that the estimated AFP concentration at $0.16 \, \text{T}$ is higher than that at $0.065 \, \text{T}$. The reason may be due to the larger background magnetization of serum than that of the AFP solution. The reference magnetization, $M(\Phi_{\text{AFP}} = 0)$, in the clinical test may be considered by using the averaging magnetization of healthy persons to reduce the effect in the clinical test. Thus, the feasibility of screening HCC patients by assaying AFP levels in serum was verified.

**Figure 6.** The normalized increment of magnetization $\Delta M_{\text{AFP}} / M_{\text{AFP,0}}$ as a function of $\Phi_{\text{AFP}}$ with data analyzed at $\mu_0 H = 0.065 \, \text{T}$. On the standard curve, AFP levels for healthy persons and HCC patients are shown.
The AFP level in serum was recently determined via the $\Delta M_S$-versus-$\Phi_{\text{AFP}}$ curve at the saturation field $\mu_0 H_S \approx 0.4$ T [16], where $\Delta M_S$ is the increment of the saturated magnetization. A clear demarcation between the normal group and the HCC group was verified in the test results, which indicates the feasibility of using $\Delta M_S$-versus-$\Phi_{\text{AFP}}$ at the saturation field as the primary analysis factor for identifying the AFP risk level in patients. In this work, the screening of HCC patients was fulfilled at low magnetic fields, which makes the detection platform simple for biomedical application users.

4. Conclusions

In summary, we performed measurements of magnetization (M–H curves) and AC susceptibility when reagents consisting of Fe$_3$O$_4$-anti-AFP were conjugated with AFP. The scaling characteristic of $(\Delta M_{\text{AFP}}/M_{\text{AFP0}})$-versus-$\Phi_{\text{AFP}}$ curves at low magnetic fields was demonstrated, and bio-sensing using BMNPs via increments of magnetization was proposed. We showed that BMNPs can be applied to assay large as well as small molecules. The screening of HCC patients via the scaling characteristic was verified in clinical studies. The detection mechanism based on the scaling characteristic showed potential to develop a compact VSM with a low magnetic field for biomedical applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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