Magnetic Nanoparticle Relaxation Dynamics-based Magnetic Particle Spectroscopy (MPS) for Rapid and Wash-free Molecular Sensing: A Strategy for Future Point-of-Care, Sensitive, and Versatile Bioassays

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Abstract
Magnetic nanoparticles (MNPs) have been extensively used as contrasts and tracers for bioimaging, heating sources for tumor therapy, carriers for controlled drug delivery, and labels for magnetic immunoassays. Here, we describe a MNP relaxation dynamics-based magnetic particle spectroscopy (MPS) method for the quantitative detection of molecular biomarkers. In MPS measurements, the harmonics of oscillating MNPs are recorded and used as a metric for the freedom of rotational motion, which indicates the bound states of the MNPs. These harmonics can be collected from microgram quantities of iron oxide nanoparticles within 10 seconds. Using a streptavidin-biotin binding system, we demonstrate the feasibility of using MPS to sense these molecular interactions, showing this method is able to achieve rapid, wash-free bioassays, and is suitable for future point-of-care (POC), sensitive, and versatile diagnosis.

Keywords: magnetic nanoparticle, magnetic particle spectroscopy, Brownian relaxation, wash-free, point-of-care, bioassay

1. Introduction
In recent years, magnetic nanoparticles (MNPs) have been successfully applied as nano-heaters for hyperthermia therapy 1–5, nano-carriers for drug delivery 6–8, nano-tracers for magnetic particle imaging (MPI) 9–13, nano-
contrast agents for magnetic resonance imaging (MRI)\textsuperscript{14–21}, and nano-labels for magnetic bioassays\textsuperscript{22–27}. MNPs, with physical size comparable to biologically important substances, have many unique physicochemical properties such as high surface to volume ratio and size-dependent magnetic properties, making them ideal for a host of many novel applications. Nowadays, MNPs with a properly functionalized surface can be physically and chemically stable, biocompatible, and environmentally safe. Furthermore, biological samples exhibit virtually no magnetic background, thus high sensitivity measurements can be performed on minimally processed samples in MNP-based biomedical applications. In addition, the ease of synthesis and facile surface chemistry have generated much eagerness in applying MNPs to clinical diagnostics and therapy.

Magnetic particle spectroscopy (MPS) is a novel measurement method that closely relates to MPI, which has been widely explored by many groups in recent years\textsuperscript{28–37}. In MPS, sinusoidal magnetic fields periodically drive MNPs into magnetically saturated regions which exhibit magnetic responses that contain not only the driving field frequencies but also a series of harmonic frequencies. These harmonic components can be easily extracted by means of filtering and fast Fourier transform (FFT). The harmonics are very useful metrics of the MNP ferrofluids such as the viscosity\textsuperscript{29,38–40} and temperature\textsuperscript{41,42} of MNP solution as well as the conjugation of any ligands/chemicals onto MNPs\textsuperscript{43–46}.

In this work, we are reporting a MNP relaxation dynamics-based MPS method for the rapid and wash-free molecular sensing. This type of measurement relies on the fact that the magnetic moments of MNPs tend to align with the external magnetic field, but this tendency is countered by the Brownian relaxation that randomizes the MNPs’ alignment. The extent of disorder caused by Brownian relaxation is most sensitive to the bound state of MNPs, namely, the hydrodynamic size. The higher harmonics (the 3\textsuperscript{rd} and 5\textsuperscript{th} harmonics) are extracted from the MPS for analyzing the bound states of MNPs. Herein, we verified the feasibility of MNP Brownian relaxation-based MPS method for molecular sensing: the MNP’s restricted rotation due to the analyte binding process. The well characterized streptavidin (SAV) and biotin system with ultra-high binding affinity is chosen as the model system, demonstrating the validity of this technique for future point-of-care (POC), sensitive, and versatile immunoassays. The results show that MNP Brownian relaxation-based MPS method is capable of detecting analytes directly from biological samples with minimum sample preparation and in a wash-free way.

2. Materials and Methods

2.1 Material
The MNPs used in this work are iron oxide nanoparticles (IONPs) with average core size of 30 nm and coated with a layer of biotin, purchased from Ocean NanoTech, San Diego, California (catalog no. SHB-30). The streptavidin from streptomyces avidinii is a salt-free, lyophilized powder with biotin binding capacity of 13 units/mg protein, purchased from Sigma-Aldrich inc., Atlanta, Georgia (product no. S4762). Phosphate buffered saline (PBS) is purchased from Sigma-Aldrich inc., Atlanta, Georgia (product no. 79378).
2.2 Sample Preparation
The lyophilized streptavidin powder is reconstituted in PBS and prepared with different concentrations vary from 75 nM to 15 µM. As shown in Scheme 1, six samples (vial I - VI) are prepared and each sample contains approximately 100 µL MNPs. Vials I - V are active groups and each vial is added with 100 µL streptavidin of different concentrations. Vial VI serves as control group and it is added with 100 µL PBS. All the samples are mixed well and incubated at room temperature for 30 minutes to allow the binding of streptavidin to biotins from MNPs.

| Vial # | I   | II  | III | IV  | V   | VI  |
|-------|-----|-----|-----|-----|-----|-----|
| Streptavidin Concentration | 15 µM | 7.5 µM | 3.8 µM | 1.5 µM | 75 nM | 0 (PBS) |

Scheme 1. Composition of 6 MNP samples.

2.3 Conjugation of Streptavidin to MNPs
Streptavidin is a crystalline tetrameric protein, with a molecular weight of 4 × 15 kDa, it binds four molecules of biotin and has a high binding affinity to biotin ligands (dissociation constant $K_d = 10^{-14} M$). The binding between biotin and streptavidin is very fast, and once formed, it is independent of temperature, solvents, pH, and other denaturing agents. Each streptavidin hosts 4 biotin binding sites, which allows the interaction with multiple biotin moieties from different MNPs and, as a result, forms MNP clusters.

2.4 Experimental Setups
The MPS measurement system setups and signal chain are shown in Figure 1(a) & (b). A personal computer (PC) controls the data acquisition card (DAQ, NI USB-6289) to generate two sinusoidal signals, which are amplified by two instrument amplifiers (IA, HP 6824A), followed by two band pass filters (BPFs) to suppress higher harmonics that might be introduced by IAs. These amplified and filtered sinusoidal signals drive the outer and inner coils (see Figure 1(c) & (d)) to generate oscillating magnetic fields: one with frequency $f_L = 10\, Hz$ and amplitude $A_L = 170\, Oe$, the other with frequency $f_H$ varies from 500 Hz to 20 kHz and amplitude $A_H = 17\, Oe$. 
One pair of differentially wound pick-up coils (600 windings in clock-wise and 600 windings in counter-clock-wise) collect the induced voltage and phase signals from MNPs and send back to a BPF before digitalized on the DAQ. The response signals at combinatorial frequencies $f_H \pm 2f_L$ (3rd harmonic) and $f_H \pm 4f_L$ (5th harmonic) are analyzed.

![Image](image_url)

**Figure 1.** MPS measurement system setups. (a) Photograph of system setups. (b) Signal chain. The system used in this work records nonlinear magnetic response of MNPs under two oscillating magnetic fields with frequencies of $f_H$ and $f_L$. (c) Photograph of coils: (i) low frequency drive coil (outer coil); (ii) high frequency drive coil (inner coil); (iii) a pair of pick-up coils; (iv) plastic vial with a capacity of 300 $\mu$L. (d) Photograph of assembled coils.

### 2.5 Molecular Sensing Via MPS

When the single domain MNPs are suspended in solution and subjected to an external magnetic field, there are two mechanisms by which the magnetic moments rotate in response to the magnetic field (see Scheme 2(a) & (b)): the intrinsic Néel motion (rotating magnetic moment inside a stationary particle) and the extrinsic Brownian
motion (rotating the entire particle along with its magnetic moment). In principle, both the Néel and Brownian mechanism play a role in determining the magnetization of MNP ferrofluids subjected to external oscillating magnetic fields. In this work, we use MNPs with core size of 30 nm, where the Brownian mechanism is dominant and the Néel mechanism is minimized. For these Brownian-relaxation-dominated MNPs, their MPS responses are most sensitive to the hydrodynamic sizes of MNPs. As shown in Scheme 2(c) & (d), when the target biomarker (i.e., streptavidin) has multiple binding sites, ligands (i.e., biotins) from more than one MNP can bind to the same biomarker, resulting in the clustering of MNPs. Such an interaction greatly increases the hydrodynamic sizes of MNPs as well as the Brownian relaxation time. As a result, noticeable changes could be found from the MPS responses due to this specific binding process. Mathematical models of the relaxation mechanisms can be found in Notes S1 & S2 in the Supporting Information.

Scheme 2. (a) Néel relaxation is the rotation of magnetic moment inside a stationary MNP. (b) Brownian relaxation is the rotation of entire MNP along with the magnetic moment. (c) The streptavidin has a high binding affinity to the biotin ligands on MNP surface. (d) As the quantity of streptavidin increases in the MNP suspension, the MNPs are likely to form clusters. The dashed lines represent the hydrodynamic sizes of MNPs due to the clustering induced by streptavidin. As the MNP clustering level increases, the hydrodynamic size increases and the harmonic amplitude decreases.
3. Results and Discussions

3.1 MPS Characterization of Magnetic Relaxation Dynamics

The MPS response upon increasing streptavidin concentrations from 75 nM to 15 µM is investigated, and one control group is added in this experiment to verify this detection strategy: biotin coated MNPs with the addition of PBS. The frequency of high frequency drive field $f_H$ is varied at 500 Hz, 1 kHz, 2 kHz, 4 kHz, 6 kHz, 8 kHz, 10 kHz, 12 kHz, 14 kHz, 16 kHz, 18 kHz, and 20 kHz, and the frequency of the low frequency drive field $f_L$ is set at 10 Hz. The amplitudes of both high and low frequency drive fields are identical in each test. During each test, the background noise is monitored for 10 s, then the plastic vial containing 200 µL sample is inserted into the pick-up coils and followed by another 10 s of data collection on the total signal. Some examples of the real-time magnetic responses sensed by pick-up coils are shown in Note S8 in the Supporting Information. The MPS response of MNPs is extracted by subtracting the background noise from the total signal using the phasor theory we reported before $^{36,47,48}$,

$$A_{MNP}e^{j\phi_{MNP}}|_m = A_{TOT}e^{j\phi_{TOT}}|_m - A_{Noise}e^{j\phi_{Noise}}|_m \quad (1),$$

Where $A_{MNP}$, $A_{TOT}$, $A_{Noise}$ are the amplitudes from MNPs, total signal, and the background noise, respectively, and $\phi_{MNP}$, $\phi_{TOT}$, and $\phi_{Noise}$ are the phase angles from MNPs, total signal, and the background noise, respectively, $m$ represents the $m^{th}$ measured harmonic. Phasor model can be found in Note S4 in the Supporting Information.
Figure 2. (a, c) MPS measurements of the 3rd and 5th harmonics from 6 MNP samples in different bound states. Error bar represents standard deviation. (b, d) MPS measurements of the normalized 3rd and 5th harmonics from 6 MNP samples in different bound states. Dotted line represents the position of peak harmonic signal for each sample as the drive field frequency varies.

The 3rd and 5th harmonic amplitudes of MNPs from six samples are reconstructed from the total signals and background noise, they are summarized in Figure 2(a) & (c). According to the Debye model and Faraday’s law of induction (mathematical models of the MPS responses can be found in Notes S3 - S5 in the Supporting Information), the harmonic amplitude is dependent on the drive field frequency $f_H$, the cosine of phase lag $\varphi$, and the quantity of MNPs in the testing sample. The increased streptavidin concentration/quantity in the MNP sample causes larger MNP clusters, thus, the hydrodynamic size of MNPs increases and the phase lag increases, which, as a result, causes noticeable drop in harmonic amplitudes (see Scheme 2(d)). Since the harmonic amplitude is largely dependent on the quantity of the MNPs from the sample, the testing results could be biased by the deviations of MNP quantities in each sample, especially for the very low concentration biomarker detection scenarios. We report here the normalized 3rd and 5th harmonics as a MNP quantity-independent metric for biomarker detection, which are plotted in Figure 2(b) & (d). The dotted lines represent the frequencies $f_H$ where the harmonic amplitudes reach to peaks for 6 samples. There is a clear trend that the peaks move to lower $f_H$ for
samples with higher concentrations/quantities of streptavidin. Furthermore, the full width at half maximum (FWHM) decrease as the concentrations/quantities of streptavidin increases in the sample. Which, as far as we know, no group has reported these two indicators as metrics for characterizing the biomarker concentrations/quantities, and are summarized in Table I.

| Vial # | I (15 µM) | II (7.5 µM) | III (3.8 µM) | IV (1.5 µM) | V (75 nM) | VI (0 nM) |
|--------|-----------|-------------|--------------|-------------|-----------|-----------|
| $f_H$ at peak harmonic amplitude (Hz) | 2000 | 2500 | 2700 | 2800 | 2850 | 2900 |
| $\frac{1}{2}$ FWHM* (Hz) | 11,300 | 11,800 | 12,300 | 12,700 | 12,900 | 13,100 |

*$\frac{1}{2}$ FWHM is the difference of $f_H$ at peak harmonic amplitude and 50% of the peak value.

As is shown in Table I, the $f_H$ at peak harmonic amplitude and the $\frac{1}{2}$ FWHM increases as the biomarker concentrations/quantities increase.

On the other hand, the ratios of the 3rd to the 5th harmonics (R35) at different drive field frequencies have also been used as a MNP quantity-independent metric for characterizing the biomarker concentrations/quantities from samples (the harmonic ratio model can be found in Note S6 in the Supporting Information). Figure 3 summarizes the harmonic ratios, R35, from 6 samples as we vary the $f_H$ from 500 Hz to 20 kHz. The harmonic ratios R35 increases as the concentrations/quantities of streptavidin increases. The inset figure on the right lists the harmonic ratios R35 from six samples at a drive field frequency of $f_H = 10$ kHz. The R35 drops from 1.7978 for sample I (streptavidin concentrations: 15 µM) to 1.4332 for sample VI (streptavidin concentrations: 0 nM). Besides the peak shift, FWHM, and harmonic ratio methods, the harmonic angle is also a MNP concentration/quantity independent metric for characterizing the bound states of MNPs in MPS. The 3rd and the 5th harmonic angles are reconstructed from the total signals and background noise, they are summarized in Note S7 in the Supporting Information.
3.2 Morphological Characterization of MNPs in Different Bound States

Transmission electron microscopy (TEM) images are taken to investigate the MNP clusters and bound states in six samples after the MPS measurements. A droplet of the MNP solution (~ 10 µL) is dipped onto a TEM grid (copper mesh with amorphous carbon film) with filter paper underneath. The MNP droplet forms a thin liquid layer on the TEM grid and will be ready for TEM characterization when the solution evaporates. Then the TEM grids are characterized in TEM (FEI Tecnai T12, 120 kV). Well-dispersed MNPs are observed from the control sample without streptavidin (vial VI, 0 nM) as seen in Figure 4 (i) & (k) while more MNP clusters could be found with the increase of streptavidin concentrations. As shown in Figure 4 (e) - (i), increased streptavidin concentration produces larger MNP clusters as observed in TEM images. Figure 4 (a) shows different bound states of MNPs in the presence of streptavidin. Due to the fact that there are multiple biotins on each MNP and the tetrameric structure of streptavidin hosting 4 biotin bindings sites, MNPs could form clusters, chains, tetramers, trimers, dimers, etc. Figure 4 (b) - (d) show the corresponding models of MNP bound states from Figure 4 (a). Figure 4 (j) & (k) are the zoomed in views of one “כ-shape” MNP cluster from vial I and the well-dispersed MNPs from vial VI.
3.3 Hydrodynamic Size Analysis of MNPs in Different Bound States

Dynamic light scattering (DLS) measurements confirmed the streptavidin-specific clustering and the hydrodynamic size increased with streptavidin concentration. Statistic results from Figure 5 give us the mean hydrodynamic sizes of vials I - VI: 706, 102.8, 63.2, 58.8, 58.4, and 58.3 nm, respectively. The results from DLS are in good agreement with our MPS and TEM measurements.
4. Conclusions and Future Perspectives

In this work, we have demonstrated the feasibility of using MNP relaxation dynamics-based MPS method for bioassay applications. The specific binding of target analytes onto MNP surface inhibits their rotational freedom, thus, changes the MPS pattern. The streptavidin and biotin system is applied in this study as a model system. Each streptavidin interconnect multiple MNPs and induces MNP clusters that tremendously increase the hydrodynamic sizes of MNPs, as a result, noticeable changes could be found in the harmonic phase lag, harmonic amplitude, $f_H$ at peak harmonic amplitude (Hz), ½ FWHM, and the ratios of the 3rd to the 5th harmonics (R35). The sensitivity of this method is 75 nM, which is 7.5 pmole streptavidin. There are several advantages in this MPS method for bioassays: wash-free and easy-to-use bioassay (measurements could be carried out on minimally processed biological samples by non-technicians with minimum training requirements), rapid (the testing time is within 10 seconds), cheap (the cost for each trial only requires microgram quantities of iron oxide nanoparticles), portable (the coils, amplifiers, filters, and DAQ could be assembled onto a single PCB board).

Future development in this MNP relaxation dynamics-based bioassay method is to improve the sensitivity of MPS measurements as well as increase the signal-to-noise ratio (SNR). In summary, this MNP relaxation dynamics-based MPS method for bioassay applications opens a door for future point-of-care, versatile, sensitive, rapid, and wash-free molecular sensing.

Figure 5. Statistical hydrodynamic size distribution collected using DLS. (a) - (f) are vials I - VI. Solid black lines are cumulative distribution curves, $\mu$ and $\sigma$ denote the mean and the standard deviation of hydrodynamic sizes.
ASSOCIATED CONTENT
Supporting Information
Note S1. Langevin Model of Magnetic Response
Note S2. Néel and Brownian Relaxation Models
Note S3. Phase Lag Model
Note S4. Phasor Theory
Note S5. Induced Signal Model
Note S6. Harmonic Ratio Model
Note S7. MPS Measurements of Harmonic Angles
Note S8. Recorded Real-time Magnetic Response from Pick-up Coils

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Notes
The authors declare no competing financial interest.

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Supporting Information

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Note S1. Langevin Model of Magnetic Response

In the presence of oscillating magnetic fields, MNPs are magnetized and their magnetic moments tend to align with the fields. For a ferrofluid system of monodispersed, noninteracting MNPs, the magnetic response obeys Langevin function:

\[ M_D(t) = m_s c L(\xi), \]

where,

\[ L(\xi) = \coth(\xi) - \frac{1}{\xi}, \xi = \frac{m_s H(t)}{k_B T} \]

The MNPs are characterized by magnetic core diameter \( D \), saturation magnetization \( M_s \) and concentration \( c \). Assume MNPs are spherical without mutual interactions. The magnetic moment of each particle is \( m_s = M_s V_c = M_s \pi D^3 / 6 \), \( V_c \) is volume of the magnetic core, \( \xi \) is the ratio of magnetic energy over thermal energy, \( k_B \) is Boltzmann constant, and \( T \) is the absolute temperature in Kelvin. \( H(t) = A_H \cos(2\pi f_H t) + A_L \cos(2\pi f_L t) \) are the external magnetic fields, where \( A_H, A_L, f_H, f_L \) are the amplitude and frequency of high and low frequency fields, respectively.
Note S2. Néel and Brownian Relaxation Models

The magnetization dynamics of MNPs are usually characterized by effective relaxation time $\tau_{eff}$, which is dependent on Brownian relaxation time $\tau_B$ and Néel relaxation time $\tau_N$. Both relaxation processes are dependent on the frequency and amplitude of applied magnetic fields. The $\tau_{eff}$ of a nanoparticle governs its ability to follow the external fields. The effective relaxation time $\tau_{eff}$ is related to the Brownian and Néel relaxation times as follows:

$$\frac{1}{\tau_{eff}} = \frac{1}{\tau_B} + \frac{1}{\tau_N}$$

$\tau_{eff}$ of a nanoparticle governs its ability to follow the alternating field. An analytical expression for Brownian relaxation time is 1:

$$\tau_B = \frac{\tau_{B0}}{1 + \frac{\xi^2 - \xi^2 \coth^2 \xi}{\xi \coth \xi - 1}}$$

$$\tau_{B0} = \frac{3\eta V_h}{k_B T}$$

where $\tau_{B0}$ is zero-field Brownian relaxation time, hydrodynamic volume $V_h = \pi(D + 2d)^3/6$, $\eta$ is viscosity, and $d$ is the thickness of nonmagnetic polymer coating layer 2-4.

The analytical expression for Néel relaxation time is 5:

$$\tau_N = \frac{\tau_{N0}}{\sigma_{eff}(1 - h^2)} \left( \frac{\sqrt{\sigma_{eff}/\pi}}{1 + 1/\sigma_{eff}} + 2^{-\sigma - 1} \right)^{-1} \times \left( \frac{1 - h}{e^{\sigma_{eff}(1 - h)^2} - 1} + \frac{1 + h}{e^{\sigma_{eff}(1 + h)^2} - 1} \right)^{-1}$$

$$\tau_{N0} = \frac{\beta(1 + \alpha'^2)M_s}{2\gamma \alpha'}$$

$$h = \frac{\xi}{2\sigma_{eff}}$$

$$\beta = \frac{V_c}{k_B T}$$

where $\gamma = 2.8 \times 10^{10}$ Hz/T is electron gyromagnetic ratio, $\alpha' = 0.1$ is damping constant 6 for magnetite nanoparticles, and $\sigma_{eff} = K_{eff}V_c/k_B T$ is energy barrier.

Figure S1 shows that smaller MNPs relax via Néel process whereas larger MNPs relax via Brownian process. This cut off size is about 12 nm. In this paper, we are using MNPs with core diameter of 30 nm, thus Brownian process will dominate, which is most sensitive to the MNP’s hydrodynamic size.
Figure S1. Simulated Brownian, Néel, and effective relaxation time as function of MNP core diameters. The crystal asymmetry on the surface of nanoparticles (also called “magnetically dead layer”) yields a smaller saturation magnetization $M_s$ and a larger anisotropy constant $K_{eff}$ than the bulk materials $^7,^8$. Due to this surface spin-canting effect, $K_{eff}$ and $M_s$ are calculated for different sizes of MNPs, coating layer thickness $d = 4 \text{ nm}$, viscosity $\eta = 1 \text{ cp}$.
Note S3. Phase Lag Model

The phase lag is modulated by the low-frequency field and also can be monitored at the harmonic phase angles $^9$

\[ \varphi(t) = \arctan(\omega \tau_{\text{eff}}) \]

where $\omega$ is the angular frequency, $\tau_{\text{eff}}$ is modulated by the oscillating magnetic field.
Note S4. Phasor Theory

The voltage and phase generated from MNPs at specific frequencies are represented by a phasor: \( A \cdot e^{j(\omega t + \phi)} \) (or expressed as \( A \angle \phi \)), where \( \omega \) is the angular frequency of driving field, \( \phi \) is the phase lag, and \( j = \sqrt{-1} \).

In our experimental setup, two alternating currents (ACs) are applied to the driving coils. First, the background noise is collected with external fields on. The background noise can be expressed as \( A_{\text{Noise}} e^{j\phi_{\text{Noise}}} \). Second, a plastic vial containing MNP sample is inserted into the MPS system and the total signal is collected. The total signal is expressed as \( A_{\text{TOT}} e^{j\phi_{\text{TOT}}} \). This signal is the sum of two phasors: the background noise and the signal generated by MNPs (namely, \( A_{\text{MNP}} e^{j\phi_{\text{MNP}}} \)).

So,
\[
A_{\text{Noise}} e^{j\phi_{\text{Noise}}} + A_{\text{MNP}} e^{j\phi_{\text{MNP}}} = A_{\text{TOT}} e^{j\phi_{\text{TOT}}},
\]
which reduces to an equation set:
\[
\begin{align*}
A_{\text{Noise}} \times \cos \phi_{\text{Noise}} + A_{\text{MNP}} \times \cos \phi_{\text{MNP}} &= A_{\text{TOT}} \times \cos \phi_{\text{TOT}} \\
A_{\text{Noise}} \times \sin \phi_{\text{Noise}} + A_{\text{MNP}} \times \sin \phi_{\text{MNP}} &= A_{\text{TOT}} \times \sin \phi_{\text{TOT}}
\end{align*}
\]

By solving the equation set above, we can get the harmonic amplitude \( A_{\text{MNP}} \) and phase lag \( \phi_{\text{MNP}} \) of each type of MNPs at different frequencies.
Note S5. Induced Signal Model

According to Faraday’s law, the induced voltage in a pair of pick-up coils is expressed as:

\[ u(t) = -S_0 V \frac{d}{dt} M_D(t) \]

where \( V \) is volume of MNP suspension. Pick-up coil sensitivity \( S_0 \) equals to the external magnetic field strength divided by current.
Note S6. Harmonic Ratio Model

Taylor expansion of $M_D(t)$ shows the major frequency mixing components:

$$
\frac{M_D(t)}{m_s c} = L \left( \frac{m_s H(t)}{k_B T} \right)
$$

$$
= \frac{1}{3} \left( \frac{m_s}{k_B T} \right) H(t) - \frac{1}{45} \left( \frac{m_s}{k_B T} \right)^3 H(t)^3 + \frac{2}{945} \left( \frac{m_s}{k_B T} \right)^5 H(t)^5 + \ldots
$$

$$
= \ldots + \left[ -\frac{1}{60} A_H A_L^2 \left( \frac{m_s}{k_B T} \right)^3 + \ldots \right] \times \cos \left[ 2\pi (f_H \pm 2f_L) t + \varphi_{f_H \pm 2f_L} \right]
$$

$$
+ \left[ \frac{1}{1512} A_H A_L^4 \left( \frac{m_s}{k_B T} \right)^5 + \ldots \right] \times \cos \left[ 2\pi (f_H \pm 4f_L) t + \varphi_{f_H \pm 4f_L} \right]
$$

The mixing frequency components are found at odd harmonics exclusively:

$$
M_D(t)|_{3rd} \approx -\frac{m_s c}{60} A_H A_L^2 \left( \frac{m_s}{k_B T} \right)^3 \times \cos \left[ 2\pi (f_H + 2f_L) t + \varphi_{f_H \pm 2f_L} \right]
$$

$$
M_D(t)|_{5th} \approx \frac{m_s c}{1512} A_H A_L^4 \left( \frac{m_s}{k_B T} \right)^5 \times \cos \left[ 2\pi (f_H + 4f_L) t + \varphi_{f_H \pm 4f_L} \right]
$$

Amplitudes of induced voltages at the 3rd and 5th harmonics are expressed as:

$$
u_{3rd} = -S_0 V \frac{d}{dt} M_D(t)|_{3rd}
$$

$$
u_{5th} = -S_0 V \frac{d}{dt} M_D(t)|_{5th}
$$

Harmonic ratio of the 3rd to the 5th harmonics is expressed as:

$$
R35 = \frac{u_{3rd}}{u_{5th}} = \frac{\frac{d}{dt} M_D(t)|_{3rd}}{\frac{d}{dt} M_D(t)|_{5th}}
$$

Hence, one advantage of using this harmonic ratio to characterize magnetic properties of MNPs is that this parameter is independent of concentration/quantity of MNPs in the testing sample.
Note S7. MPS Measurements of Harmonic Angles

The 3\textsuperscript{rd} and the 5\textsuperscript{th} harmonic angles are reconstructed from the total signals and background noise, they are summarized in Figure S2.

Figure S2. MPS measurements of (a) the 3\textsuperscript{rd} and (b) the 5\textsuperscript{th} harmonic phase angles from 6 MNP samples in different bound states.
Note S8. Recorded Real-time Magnetic Response from Pick-up Coils

The real-time magnetic responses sensed by pick-up coils are recorded from 6 MNP samples at different drive field frequencies: $f_H = 500 \text{ Hz}, 10 \text{ kHz}, 20 \text{ kHz}$.

![Figure S3](image)

Figure S3. The real-time magnetic responses recorded from 6 MNP samples at frequencies of (a) $f_H = 500 \text{ Hz}$; (b) $f_H = 10 \text{ kHz}$; (c) $f_H = 20 \text{ kHz}$.
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