Miniaturized Electromagnetic Biosensors for Circulating Cancer Biomarker Detection

by

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Submitted to the Harvard-MIT Program in Health Sciences and Technology on August 28, 2017 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Engineering

Abstract

Cancer is one of leading cause of deaths, and responsible for 8.2 million deaths worldwide. Especially, 70% of deaths from cancer occur in low or mid income countries. In order to deliver affordable and accessible cancer care to low income developing countries, it is critical to develop rapid, low cost, and highly accurate tools for cancer detection and treatment. Recently, liquid biopsy and circulating cancer biomarkers such as circulating tumor cells (CTC), extracellular vesicles (EV), and cell free DNA (cfDNA) have gained great attentions for early diagnosis, prognosis, and treatment monitoring of cancer patients because they can be accessed in less invasive approaches through body fluids while providing quantitative information about original tumors at low cost. To facilitate detection of circulating cancer biomarkers, we developed electromagnetic biosensing systems for rapid and quantitative molecular analysis. First, we report portable nuclear magnetic resonance (NMR) system that detects cancer cells or proteins labelled with magnetic nanoparticles (MNPs). The developed NMR system could detect as low as 20 cancer cells in 5 uL samples. Second, we describe micro-Hall magnetometer that molecularly profiles single cancer cell with magnetic multiplexing. The micro-Hall magnetometer, which consisted of an array of 7 um x 7 um Hall sensors, showed its capability to differentiate magnetic particles with distinct magnetic moments. We applied this technology to molecular profiling of single ovarian cancer cell. Last, we introduce wirelessly powered electrochemical system that detect cancer specific EV and DNA. Using immuno-magnetic sandwich assay, we could enrich almost 100% of EVs from clinical specimens without ultracentrifugation and profile cancer specific transmembrane proteins from as low as $10^5$ EVs. Also, we demonstrated PCR-free detection of single stranded DNA with in-vitro protein synthesis assay. These electromagnetic biosensors will be powerful tools to deliver more accessible and affordable cancer care to resource limited areas in developing countries.

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I would like to dedicate this thesis to my beloved parents, family, and lovely girl friend who always support my journey toward Ph.D.
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Chapter 1. Introduction

1.1 Motivation

Cancer is the second leading cause of deaths, and responsible for 8.2 million deaths worldwide\textsuperscript{1,2}. Many research institutes forecasts newly diagnosed case will increase by about 70% over next two decades\textsuperscript{3}. Furthermore, 70% of deaths from cancer have occurred in low-middle income countries including Africa, Latin America, and Asia\textsuperscript{3-5}. Cancer burden in these areas is increasing significantly due to lack of medical resources such as hospitals, equipments, and well trained healthcare professionals and relatively high cost of health care service compared to their gross annual income\textsuperscript{1,3}. My research question started from here. How can we provide affordable and accessible health care, especially cancer care, to people who live in medically underserved area? I have tackled this problem by developing rapid, low cost, and highly accurate tools for cancer diagnosis, prognosis, and treatment monitoring.

1.2 Liquid Biopsy and Circulating Cancer Biomarkers\textsuperscript{6}

In a current cancer care, imaging and tissue biopsy are the standard care for cancer diagnosis and prognosis. However, imaging methods are costly because the equipments such as MRI, CT, and PET are expensive and required well-trained radiologists. Tissue biopsy is invasive, painful and
time intensive procedures that require well-trained medical doctors. Recently, liquid biopsy and circulating cancer biomarkers such as circulating tumor cells (CTC)\textsuperscript{7–16}, extracellular vesicles (EV)\textsuperscript{17–25}, and cell free DNA (cfDNA)\textsuperscript{26–31} have gained great attentions for early diagnosis, prognosis, and treatment monitoring of cancer patients because liquid biopsy can access those circulating cancer biomarkers in less invasive approaches through body fluids while providing quantitative information about original tumors at low cost. Thus robust, sensitive and easy-to-use biosensors for the detection and quantification of circulating cancer biomarkers will have significant applications in cancer research and clinical practices. If made available, these platforms could aid in understanding of fundamental biology; in accurately detecting diseases at their early stage; and in evaluating and monitoring the efficacy of therapy\textsuperscript{32–34}. To realize such sensors, the underlying detection technology should ideally 1) enable high sensitivity and specificity, 2) support short assay time with minimal sample processing, and 3) allow for multiplexed detection in a single sample\textsuperscript{35}. Different types of sensing platforms, fulfilling some of these requirements, have been developed based on optical\textsuperscript{36,37}, electrical\textsuperscript{38–40} or magnetic detection\textsuperscript{6,16,35,41–50}. These systems, however, often require expensive equipments, lengthy sample purification, large sample volumes or long assay time, which can potentially limit their clinical utility and adaption.

1.3 Point of Care Technology for Cancer Detection
1.3.1 Magnetic Approaches

Biosensors based on magnetic detection have recently emerged as a promising diagnostic platform. Due to the intrinsically negligible magnetic susceptibilities of biological entities, magnetic detection experience little interference from native biological samples; even optically turbid samples will often appear transparent to magnetic fields. Biomarkers of interests, when magnetically labeled, however, can attain a high contrast against the biological background. Recent progresses in the synthesis of magnetic nanoparticles (MNPs) have further advanced the magnetic detection technology. With their size scale similar to that of biological molecules, MNPs can efficiently and abundantly bind to biological targets, amplifying analytical signals. Various detection technologies have been developed based on this magnetic-tagging concept. These include techniques that use magnetometers, such as superconducting quantum interference device (SQUID)\textsuperscript{51–53}, magnetoresistive sensors\textsuperscript{35,41,42,48,49}, all of which directly measure the magnetic fields arising from the magnetically-labeled targets.

1.3.2 Optical Approaches

Optical observations of tumor signatures are the most common approaches for cancer detection in research labs and clinics. Pathologists normally identify cancer cells from biopsied tissue using H&E staining or immunohistochemistry under bright field microscope. However, these
approaches require well trained pathologists and have limited throughput due to small field of view under conventional microscope. More quantitative and automated methods are fluorescent microscopy and flow cytometry (FACS). They both are based on fluorescent labeling of biological targets, enabling multiplexed detections using fluorescent dyes with different wavelength. However, these approaches cannot be easily adapted for point of care settings because of its bulky and expensive optics. Enzyme linked immunosorbent assay (ELISA) is another conventional laboratory tools to measure quantity of target proteins. ELISA is relatively easier to be miniaturized, but vulnerable to auto-fluorescence or background noise. Recently, digital inline holography was introduced to cancer cell detection\textsuperscript{54}. Advantage of this technology are wide field of view, thus enabling high throughput analysis.

1.3.3 Electrical Approaches

Electrical biosensors are another common approaches for cancer cell detection. There are two categories of electrical biosensors. One is electrochemical sensing\textsuperscript{55} and another is impedance sensing\textsuperscript{56–58}. Originating from analytical chemistry, electrochemistry has been widely used in biochemical sensing, which is based on chemical reactions producing electric currents. Electrochemical sensors were developed for wide range of target applications including pH, gas, ions, metals, glucose, proteins, cells, DNAs, and so on. In contrast, impedance sensor measures changes of resistance or capacitance at interface between samples and detectors, which are
mostly metal electrodes. The biggest advantage of these sensors is that they require simple
electronic circuits, thus achieving the highest portability at the lowest cost.

1.4 Thesis Overview

This thesis introduces rapid, low cost, and highly sensitive platforms for circulating cancer
biomarkers using electromagnetic methods. In the first section, we report portable nuclear
magnetic resonance (NMR) system that detects cancer cells or proteins labelled with MNPs. We
show a variety of MNP assaying schemes to detect cancer cells and proteins, and techniques to
achieve robust NMR measurement with mobile device in point of care settings. Also, we
demonstrate multichannel digital NMR system for high throughput and multi-frequency NMR
measurement. In the second section, we describe micro-Hall magnetometer that molecularly
profiles single cancer cell with magnetic multiplexing. The micro-Hall magnetometer shows its
capability to differentiate magnetic particles with distinct magnetic moments. We apply this
technology to molecular profiling of single ovarian cancer cell. In the third section, we introduce
wirelessly powered electrochemical system that detects cancer specific EV and DNA. To
minimize sample purification steps, we show immuno-magnetic sandwich assaying scheme for
the enrichment of EVs from clinical specimens without ultracentrifugation. Also we profile
cancer specific transmembrane proteins from EV of bladder cancer cell line and demonstrate
PCR-free detection of single stranded DNA with in-vitro protein synthesis assay. In the last section, we summarize the work and conclude with future perspectives.
Chapter 2. Miniaturized Nuclear Magnetic Resonance System for Molecular Analysis

2.1 Introduction and Background\textsuperscript{59}

We have recently developed a new magnetic sensing platform, portable nuclear magnetic resonance (NMR) system\textsuperscript{6,45,46}. Contrary to directly measuring the magnetic moments of the labeled targets, the developed NMR uses water proton magnetic resonance as the detection mechanism. When placed in NMR magnetic fields, MNPs create local magnetic fields and change the relaxation rate of surrounding water molecules\textsuperscript{60}. The detection offers an intrinsic signal amplification mechanism, as more than millions of water molecules can be affected by a single MNP. Moreover, since the signal is generated from the entire sample volume, the assay procedure is significantly simpler than the direct magnetic detection in which MNP-labeled targets have to be closely positioned to the sensing elements.

By optimizing MNPs and miniaturizing NMR detectors, the detection sensitivities for various target types have been considerably improved over the last few years. These developments enable rapid and multiplexed detection on a wide range of targets in microliter sample volumes, including nucleic acids\textsuperscript{61}, proteins\textsuperscript{62}, drugs\textsuperscript{63}, bacteria\textsuperscript{64,67}, and tumor cells\textsuperscript{43,44,46}. With the recent integration of bioorthogonal targeting strategies\textsuperscript{43,68} as well as accurate and real-time control of
device temperature$^{69}$, the developed portable NMR platform has become more robust and sensitive, allowing operation in clinical settings. This chapter demonstrated the the latest development of the portable NMR technology, focusing on its four major components: single channel temperature independent miniature NMR systems, multichannel digital NMR system, MNP based assaying schemes and their biomedical applications.

### 2.1.1 Nuclear Magnetic Resonance$^{59}$

The NMR detection of MNP-labeled cells is realized by exploiting the "$T_2$-shortening" effect of MNPs in NMR measurements$^{70}$. When placed in static, polarizing magnetic fields for NMR detection, MNPs produce local dipole fields with strong spatial dependence, which efficiently destroy the coherence in the spin-spin relaxation of water protons. MNP-labeled objects consequently cause faster decay of NMR signal, or shorter transverse relaxation time $T_2$, than non-targeted ones.

The capability of MNPs to induce $T_2$ changes is defined as transverse relaxivity ($r_2$)$^{71}$. With MNPs in solution, the relaxation rate ($R_2 = 1/T_2$) can be expressed as$^{47}$

\[
R_2 = R_w + r_2 \cdot \frac{N_L}{V},
\]
where $R_w$ is the relaxation rate of the background (usually water), $V$ is the NMR detection volume, and $N_p$ is the total number of MNPs in $V$. If each biological cell has $n$ MNPs and the total number of cells is $N_c (= N_p/n)$, the net change of $R_2$ ($\Delta R_2 = R_2 - R_w$) is given as

\[
\Delta R_2 = R_2 - R_w \cdot \frac{N_c}{V} = \frac{n \cdot r_2}{V} \cdot \frac{N_c}{V} = r_2 \cdot \frac{N_c}{V},
\]

where $r_2^{cell} (= n \cdot r_2)$ is defined as the cellular relaxivity (transverse relaxivity per given cell concentration). Note that $r_2^{cell}$ is indicative of the abundance of relevant surface biomarkers. NMR thus can be used effectively for molecular profiling of target cells.\textsuperscript{43,47} Eq. 2 provides valuable insights into how to increase the sensitivity and specificity of NMR-based sensors.

\textbf{MNPs with high $r_2$ relaxivity.} Pronounced $R_2$ changes will occur when cells are labeled with MNPs of high $r_2$ relaxivity.\textsuperscript{60} Because $r_2$ is proportional to the magnetic moment ($\mu_p$) of particles,\textsuperscript{72,73} making magnetically stronger MNPs will benefit the measurements.

\textbf{Maximal MNP-labeling on cell.} The $R_2$ changes are also directly proportional to the number of MNPs loaded onto cells. In addition to increasing the $r_2$-potency of individual MNPs, it is equivalently important to establish a labeling protocol to maximize and/or amplify MNP loading on cells.
Miniaturized NMR probes. Higher sensitivity can be achieved on the device level by decreasing the NMR detection volume \( (V) \). This approach can effectively increase the analyte concentration \( (N_c/V) \), leading to large \( \Delta R_2 \). Furthermore, smaller NMR probes assume higher SNR (signal-to-noise) ratio due to the increased sample-filling factor. It can be shown that the sensitivity of NMR coils (with a typical dimension of \( d \)) scales as \( d^{-1/2} \).

These recognitions motivated us to explore three major activities in portable NMR development: synthesis of new MNPs, optimization of such MNPs for cellular labeling, and miniaturization of NMR systems. The following sections will describe these accomplishments.

2.2 Results

2.2.1 System Overview and Implementation

2.2.1.1 Single Channel NMR system

Single channel NMR system is designed for practical applications in a clinical environment (Fig. 2.1). Its small size, easy accessibility and high robustness provide end-users with fast and stable measurements of biological samples. The single channel NMR
Figure 2.1. Single Channel NMR system. Single channel NMR system consists of three major components. 1) NMR probe 2) NMR electronics 3) NMR control software. System is composed of three core parts: a newly designed probe for clinical samples, NMR electronics, and a user-friendly software.
The system design is based on that of conventional NMR, and consists of a small portable magnet ($B_0 = 0.5$ T) and a solenoidal coil for higher SNR. A custom-made PMMA (polymethylmethacrylate) housing cages the magnet, the microcoil and RF matching circuit. With its major improvement focused on clinical translatability, this new system uses disposable thin-walled polyimide tubes to load biological samples for measurement, thereby eliminating potential contamination of the NMR probe. The disposable tubes are filled with samples (~5 µL) and are inserted into the coil bore for NMR detection. Modular coils made in a variety of size can be plugged into the system to optimally accommodate available sample volumes (1-100 µL).

A small form-factor (20 cm × 12 cm × 5 cm) NMR electronics is implemented using off-the-shelf integrated circuit (IC) chips, to achieve cost-effective (< $200) and highly-programmable NMR platform. The NMR electronics generates the NMR pulse sequences, acquires the NMR signal, and communicates with external terminals (computer, mobile devices). It has three main parts: a microcontroller unit (MCU), an RF transmitter, and a signal receiver. The MCU (TMS320F28235; Texas Instruments) controls overall RF transceiver operations as well as data communication with external terminals. As an RF transmitter, a direct digital synthesis chip (AD9954; Analog Devices) is employed to generate two RF signals with 90° phase difference, that are modulated by voltage controlled switches (ADG1419BRMZ; Analog Devices). The RF heterodyne system is implemented to process the NMR signal. First, the signal is amplified by low noise amplifier (AD604; Analog Devices), and down-converted to baseband (1-10kHz) by a mixer (ADE-6; Mini Circuits). Baseband signals subsequently pass a low pass filter, and are digitized by an analog-to-digital converter (AD7625; Analog Devices). Mobile devices (e.g.,
iPhone™, iPad™), which are connected to the MCU via a standard communication channel (e.g., USB, Bluetooth), receive digital data and show graphical outputs.

The NMR software incorporates graphical user interface (GUI), data acquisition/process, data logging/sharing and a temperature compensation engine, which deliver a user-friendly interface and contribute to the robustness of the developed system. The software is programmed with Objective-C using Cocoa and Cocoa Touch frameworks and operates on iOS™ and OS X™. In order to implement a graphical representation of R₂ relaxation curve, open source plotting framework (Core Plot) is cross-linked with Cocoa and Cocoa Touch frameworks. Time domain NMR data are acquired at the negative edge of spin echoes and processed real-time. Negative edge-triggered data acquisition reduces the size of the data to be transferred, and consequently decreases the data acquisition time via USB/Bluetooth connections by more than 10-fold. Transferred NMR data are then processed to obtain T₂ relaxation time. The overall signal pattern is displayed and the data is stored in the terminal device. In addition, wireless communication module is embedded in the software for data logging/sharing over an encrypted wireless network, promoting usability in a remote clinical site. To ensure stable and reliable T₂ relaxation time measurements, a temperature compensation engine has been implemented to run independently from the main thread. This temperature compensation engine transforms NMR data from time domain to frequency domain using Fast Fourier Transformation (FFT), and detects any changes in the NMR frequency due to environmental temperature fluctuation.
The NMR frequency $f_0$ is the parameter that requires most frequent adjustments and affects the measured NMR signals most significantly. The frequency $f_0$ changes as the magnetic field ($B_0$) from the permanent magnet drifts with temperature. For example, with a 1 °C increase in temperature, $B_0$ field from a NdFeB magnet will drop ~ 0.1% from its initial value, and $f_0$ will proportionally decrease by 0.1%; when the initial $f_0$ is 20 MHz ($B = 0.47$ T), the frequency change is then ~ 20 kHz. Such changes can place down-converted NMR signals near or beyond the low frequency cutoff in the amplification stage, distorting the measured signal. Commercial benchtop NMR systems address the problem by housing the entire magnet block inside a heated container. This solution, however, significantly undermine the portability of the system due to the use of bulky and power-consuming parts. In the developed NMR system, we employed a dynamic control approach. Namely, programmable hardware in the NMR electronics and temperature compensation engine in the NMR software are designed to track and compensate for temperature dependency of the system. These implementations ensure optimal measurement settings for reliable and robust performance.

Figure 2.2 shows the algorithm for temperature compensation. The feedback loop tracks the Larmor frequency $f_0$ and reconfigures the frequency $f$ of NMR excitation. Coarse tuning mode starts with initial NMR excitation frequency $f_i$ and increases $f$ by $\Delta f$. When the spectral power ($P$) of NMR spin echo reaches a predefined threshold $P_{th}$, a fine tuning mode takes over to measure the frequency offset $f_d (= |f-f_0|)$. The fine tuning iterates until $f_d$ reaches a target value. The target $f_d$ value is carefully selected to keep down-converted NMR signal within the
Figure 2.2. Temperature Compensation. (a) System diagram for magnetic resonance frequency search. Tracking routine searches Lamor frequency for every NMR measurement to compensate temperature dependency. (b) Tracking routine. To find exact Lamor frequency in real time, tracking routine measures single spin echo, and converts time domain signal to frequency domain via FFT. (c) Drift of Lamor frequency. The effect of temperature compensation was compared when the tracking routine is turned on and off. (d) The robustness of NMR measurements across a broad range of temperature (4 – 50 °C) was demonstrated. The dotted line indicates a theoretical prediction.
passband of the low pass filter. Once the new NMR excitation frequency \( f \) has been established, CPMG pulse sequence is used to measure the \( T_2 \) relaxation time of the sample.

**Figure 2.2(c)** demonstrates the effectiveness of the developed temperature compensation method. When \( f_0 \) was allowed to drift but the RF frequency \( f \) for sample excitation was fixed, \( T_2 \) values varied up to 200% relative to its starting value with typical fluctuation of room temperature \( (\Delta T \sim 2 \, ^\circ C) \). When the temperature compensation engine was activated, however, \( T_2 \) variations were significantly reduced to \(< 1\%\). We further tested the system in environmental settings with a wide range of temperature differences \( (4 - 50 \, ^\circ C) \). To determine the measurement accuracy, the linear dependence of \( T_2 \) on temperature was utilized. **Figure 2.2(d)** shows the \( T_2 \) values of a MNP-solution monitored at different temperature. For a given environment setting, the single channel NMR system was operated with the temperature tracking activated, to compensate for minute temperature variations \( (~1 \, ^\circ C) \). The results show a linear relationship \( (R^2 > 98\%) \) as theoretically predicted, demonstrating the capacity for reliable \( T_2 \) measurements in various settings.

### 2.2.1.2 Multi Channel NMR system

The system overview and architecture are shown in **Figure 2.3**. The multichannel NMR is designed to measure \( T1/T2 \) relaxation time and NMR spectrum in four to six channels concurrently. Time interleaved NMR sequence is developed to send NMR excitation pulse to
each channel within one echo time (TE) for concurrent NMR measurement in multiple NMR probes (Fig. 2.3(a)). To enhance digital programmability and multichannel capability, the transceiver architecture of the multichannel NMR system is based on digital transceiver of software defined radio, which is composed three parts; 1) digital processing unit 2) analog transceiver 3) multi channel NMR probes (Fig. 2.3(c)).

The digital processing unit is implemented on FPGA (Virtex5, Xilinx) and operates signal RF modulation/demodulation, baseband signal processing, microcontroller (picoblaze, Xilinx). For demodulation in a receiver pathway, digital synthesizer generates programmable radio frequency signal, and then the generated signal is down-converted to baseband using digital multiplier. In a transmitter pathway, digital switch modulates the generated radio frequency signal for appropriate NMR sequence. Four copies of identical signal modulation/demodulation unit is implemented in a parallel structure to measure multichannel NMR signal concurrently. Baseband signal processing block executes low pass filtering and fast fourier transform of the down converted signal. The microprocessor unit orchestrates all functional blocks in FPGA and communicates with control software (Matlab). The analog transceiver parts consist of pre-amplifiers, analog filters, and a duplexer. Pre-amplifiers and analog filters are tuned to be operated from 11 MHz to 48 MHz, thus enabling the system perform wide band proton NMR with 0.25 T – 1 T magnet. Multichannel NMR probes incorporate four or six solenoidal coils and pin diodes for channel selection (Fig. 2.3(b) and (c)). When pin diode is turned on, NMR coil is
detuned in high impedance state. While, NMR coil is matched to NMR frequency when pin
diode is turned off.
Figure 2.3. Multi Channel NMR. (a) Interleaved NMR measurement scheme for multi channel detection (b) Hexa channel NMR probes (c) Block diagram of multi channel NMR system.
2.2.2 Applications

2.2.2.1 Cellular Analysis

To validate clinical utility of the multichannel NMR system, we demonstrated its applications to cellular analysis (Fig 2.4). We first explored the detection limit of cancer cell detection using ovarian cancer cell (OvCAR3) as a model cell line. We cultured and prepared OvCAR3 cells with different concentrations via serial dilution. The samples were then labeled with MNPs against epithelial cell adhesion molecule (EpCAM), and 5 uL of each sample was loaded in four NMR coils. We measured T2 from multichannel NMR coils concurrently using the interleaved CPMG sequence and converted it to R2 changes. As expected, ΔR2 was linearly proportional to the number of cancer cells in the sample. Also, we were able to detect as low as 25 cancer cells in 5 uL of sample (Fig 2.4(a)).

Next, we profiled multiple biomarkers of OvCAR3 cells. Four aliquots of sample (5 uL) were prepared in the same concentration (5–6 x 10^2 cells/uL), and then labeled with MNPs targeting three different cancer biomarkers (EpCAM, EGFR, and HER2), which are known to be overexpressed in OvCAR3 cells. We assigned four samples including one isotype control (IgG1), one per each NMR coil, and measured T2 concurrently. The converted R2 relaxativity of each sample was plotted in Figure 2.4(b). Higher R2 indicates elevated expression of target molecules on cancer cell surface. By using time interleaved scheme, we could test expression levels of multiple cancer biomarkers in the same sample concurrently, thus reducing T2 measurement time by four times.
Figure 2.4. Cancer Cell Detection. (a) Titration curve for cancer cell. OvCAR3 cells are labelled with anti-EpCAM antibody-MNP conjugates. $R_2$ changes are linearly proportional to concentration of MNP labelled cells. Limit of detection was 25 cancer cells in 5 $\mu$L. (b) Multichannel profiling of OvCAR3 cells. OvCAR3 cells are labelled with four different markers (EpCAM, HER2, EGFR, and IgG1). $R_2$ changes of the labelled samples are measured in different NMR coils concurrently.
2.2.2.2 Protein analysis

2.2.2.2.1 Magnetic Relaxation Switch Assay

We first synthesized a panel of MNPs with different size and composition (Fig. 2.5(a); see Methods for details). Small MNPs (CLIO; cross-linked iron oxide) were synthesized through chemical coprecipitation of ferric (Fe$^{3+}$) and ferrous (Fe$^{2+}$) chloride with the addition of a base solution (NaOH). The magnetic core measured ~8 nm in diameter and was covered with a thick layer of 10 kDa dextran, crossed-linked with epichlorohydrin. The resulting particles had a hydrodynamic diameter of ~30 nm. Additional ferrite MNPs (Fe$_3$O$_4$) were synthesized via thermal decomposition of metal-complexes (iron (III) acetylacetonate [Fe(acac)$_3$]) at high temperature (300 °C). The core size of these particles was increased from 12 to 16 and then 22 nm, through a seed-mediated growth approach. In a similar manner, Mn-doped ferrite (MnFe$_2$O$_4$) particles, which have higher magnetization than Fe$_3$O$_4$, were also prepared by thermally decomposing Fe(acac)$_3$ in the presence of manganese complexes (Mn(acac)$_2$). To further improve the magnetization, elemental iron (Fe) was selected as a core material of new nanoparticles. Initially, Fe-MNPs were synthesized by thermally decomposing iron (0) pentacarbonyl [Fe(CO)$_5$]. To prevent its oxidation, Fe-MNPs were then encased with an artificially grown ferrite shell (Fe@MnFe$_2$O$_4$). All prepared MNPs were rendered...
Figure 2.5. Panel of MNPs with different transverse relaxivities. (a) To study the effect of particle relaxivity ($r_2$) on MRSw assays, different types of MNPs with varying size and magnetization were synthesized. The transmission electron micrographs confirmed the narrow size distribution of the prepared MNPs. Clockwise from the top left are: 16 nm Fe$_3$O$_4$, 22 nm Fe$_3$O$_4$, 16 nm Fe-core and MnFe$_2$O$_4$ shell (Fe@MnFe$_2$O$_4$), 16 nm MnFe$_3$O$_4$ MNPs. (b) The measured transverse relaxivity ($r_2$) showed good agreement with those predicted by an outer-sphere model (dashed lines). These MNPs thus were in the motional averaging regime in their non-clustered state. $M_s$, saturation magnetization; CLIO, cross-linked iron oxide nanoparticle.
water-soluble by coating the particle surface with small molecules (2,3-dimercaptosuccinic acid) with the exception for CLIO which had a hydrophilic dextran coating.

For each type of MNPs, we measured its transverse relaxivity \( r_2 \), the capacity of the particles to accelerate the \( R_2 \) relaxation of water protons (Methods). With different diameter \( (d; 8 - 22 \text{ nm}) \) and magnetization \( (M) \), the prepared MNPs assumed a wide range of \( r_2 \) values (Fig. 2.5(b)). All MNPs, however, were in the motional averaging regime of \( R_2 \) relaxation, where the diffusional motion of water protons was fast enough to average out the effects of MNPs. Consequently, the observed \( r_2 \) values could be fitted to \( r_2 \sim d^2 \cdot M^2 \), as predicted by the outer-sphere model (dotted lines, Fig. 2.5(b))\(^{73}\).

Prepared particles were used to characterize the effect of different MNP types on MRSw assay. As a model mechanism for particle-clustering, we used avidin-biotin interaction. MNPs were biotinylated by forming amide bonds between carboxylic acids in MNPs and amine groups in biotin (Methods). On average, 40 biotin molecules were found to be immobilized per particle. For MRSw assay, a varying amount of avidin was introduced to the biotinylated-MNP solution; control samples were prepared in the same way, but with the addition of PBS (phosphate buffered saline) solution (Methods). Following a 15-min incubation at \( T = 300 \text{ K} \), the samples were subjected to \( R_2 \)-measurements using a miniaturized NMR system previously reported\(^{46,69}\). The corresponding size of MNP clusters was measured \textit{via} dynamic light scattering (DLS).

Two distinct MRSw modes were observed (Fig. 2.6). With small particles (CLIO), the \( R_2 \) values initially rose and then decreased with increasing avidin concentration (\([Av]\); Fig. 2.6(a)). In
contrast, larger MNPs showed an initial decrease and plateau of $R_2$ values with increasing [Av] (Figs. 2.6(b)-(d)). For all types of MNPs, a strong correlation between $R_2$ and cluster size ($d_c$) was observed, which led to the development of a new physical model of MRSw phenomena. (1) For small MNPs, the clustered particles remain in the motional averaging (MA) mode of relaxation; these clusters are still small enough that the effect of their magnetic fields is averaged out by the diffusional motion of water molecules. In the MA mode, the $R_2$ values concomitantly increase with the particle size. With a core size of 8 nm and water-permeable coating, the clustered CLIO falls into this regime, showing a close match between $R_2$ and $d_c$. Further addition of avidin, however, leads to a decrease of both $R_2$ and $d_c$, as excess avidin coats MNPs (prozone effect) to hinder inter-particle clustering. (2) Larger MNPs assume a different relaxation mode upon clustering, namely the static dephasing (SD), as their cluster size exceeds the traveling distance of diffusing water molecules. These clusters appear as randomly-distributed, stationary objects. In the conventional SD model, where MNPs are assumed to be a solid sphere with a constant magnetization ($M$), the $R_2$ values are independent of the particle size but only proportional to $M$. For the MRSw, however, the observed $R_2$ values declined with the cluster size $d_c$, indicating that the effective $M$ decreased in the corresponding clusters.
Figure 2.6 Different MRSh behaviors for a panel of MNPs. Two distinct relationships between $R_2$ and cluster size ($d_c$) were observed. With small MNPs (e.g., 8 nm core MNP; CLIO), the $R_2$ values were commensurate with the cluster size (a), which indicated that the clusters remained in the motional averaging (MA) regime. For all other MNP types, $R_2$ values decreased with the cluster size (b – d), as the clusters entered a different relaxation mode (static dephasing; SD).
The observed new $R_2$-dependence on the cluster size was further analyzed in the framework of the diffusion-limited aggregation model\textsuperscript{82-84}. Clusters of nanoparticles are known to have a fractal structure; the number of particles ($n$) per cluster is given as $n \sim \left(d_c/d_s\right)^f$, where $f$ is the fractal dimension. Accordingly, we hypothesize that the magnetization $M_c$ of the MNP clusters scales as $M_c = M_s (d_c/d_s)^f$, where $M_s$ and $d_c$ are the magnetization and the diameter of a single MNP, respectively (see Appendix A for details). By denoting $R_{2c}$ and $R_{2s}$ as the relaxation rates for clusters and individual MNPs respectively, we then obtain the following power law for the normalized relaxation rate and particle size. For the MA mode, $R_2$ is proportional to $d^2 \cdot M^2$, hence 

$$(R_{2c}/R_{2s})_{\text{MA}} \sim (d_c/M_c)^2/(d_s/M_s)^2 \sim (d_c/d_s)^{2f-4} \text{(Fig. 2.7(a)).}$$

Likewise $(R_{2c}/R_{2s})_{\text{SD}} \sim (d_c/d_s)^{-3}$ for the SD mode, since $R_2$ is proportional to $M$ (Fig. 2.7(b)). When the observed MRSw data was replotted in these normalized units, it indeed showed the power law behavior. The dimension ($f$) determined for each MNP type had a universal value ($f \sim 2.4$), revealing the generic fractal nature of MNP clusters. Similar results were also observed when MRSw assays were performed using DNA molecules as a crosslinker. Clusters of small MNPs (8 nm Fe$_3$O$_4$) were found to be in the MA mode, whereas larger MNPs (16 nm MnFe$_2$O$_4$) fell into the SD mode upon clustering; the fractal dimension of MNP clusters for both modes was around 2.4. This value is in good agreement with those ($f = 2.1 - 2.5$) measured by other methods for nanoscale clusters.
Figure 2.7. Characterization of MNP clustering. The normalized $R_2$ and $d$ showed different power law behaviors than that expected from a conventional MA or SD model, which could be attributed to the fractal nature of MNP clusters. From the observed data, the dimension constant ($f$) was obtained. Clusters, both in MA (a) and SD (b) mode of relaxation, assumed a universal $f$ value (= 2.4), which is close to the theoretical maximum (2.7). Gray areas indicate 95% prediction level from the fit.
To evaluate the utility of MRSw for molecular sensing, we next investigated the detection limit and dynamic range for each MNP type. For a given MNP concentration, an avidin-dose response curve was obtained (Figs. 2.8(a), (b)). The lower and upper bounds of [Av] for detection were defined from 5 – 95% of the overall $R_2$ responses. The detection limit was found to improve with decreasing MNP concentrations, presumably due to a favorable stoichiometric ratio between MNPs and avidin molecules; the $R_2$ changes are maximized since all MNPs could be transformed into clusters under these conditions. Lowering MNP concentrations, on the other hand, reduced the dynamic ranges, as the $R_2$ of MNP solutions became closer to that of the background (e.g., water). These opposing behaviors led to the following consequences: 1) MNPs with higher $r_2$ achieve lower detection limit by producing larger $R_2$ changes even at low particle concentrations; 2) each MNP type has an absolute lower detection limit, set by the diminishing dynamic ranges.

The experimental data further confirmed this hypothesis. The detection limit scaled inversely with particle $r_2$; the lowest detection limit was $\sim 1$ pM with 22 nm Fe$_3$O$_4$ ($r_2 = 1.2 \times 10^{-15} \text{ L} \cdot \text{s}^{-1}$), whereas 8 nm ferrite ($r_2 = 7.0 \times 10^{-16} \text{ L} \cdot \text{s}^{-1}$) had the limit of $\sim 2$ nM.

The observed detection limits and dynamic ranges were further formulated into a general analytical model, based on the developed MRSw modes (MA, SD) for different MNPs (see Appendix A for details). The model correlated well with experimental observation (Fig. 2.8(c)); the detection limit was found to scale as $1/r_2$, and the dynamic range was proportional to $k/a$, where $k$ and $a$ are the average numbers of individual MNPs and avidin molecules per cluster, respectively. For the case of DNA molecules, their lower binding affinity led to the formation of smaller MNPs clusters, resulting in smaller $k$. The overall detection
Figure 2.8. Analytical modeling of MRSw assays. (a, b) The detection threshold and dynamic range of each MNP type were determined. For both MA and SD modes, the detection sensitivity improved with decreasing MNP concentrations. With lower MNP numbers, however, the detection dynamic range became narrower with the $R_2$ of MNP solution approaching that of background. These effects set the detection limit for each MNP type. (c) An analytical MRSw model (for MA and SD modes) was constructed, that can estimate the detection limit and the dynamic range for a given MNP type and concentration. The model showed good correlation with the observed data (dotted lines with triangles). (d) The $R_2$ relaxivity of MNPs determines the relaxation mode of clusters, with the transition from MA to SD occurring around $R_2 \sim 10^{-15} \text{ } \text{L} \cdot \text{s}^{-1}$. The MRSw model also revealed that the detection limit is proportional to $1/R_2$ (solid lines), which agreed well with the experiment data (filled circles). Notably, the sensitivity enhancement becomes progressively slower with $R_2$ increases, which places practical limits on further sensitivity improvement. The practical detection limit ($\sim 100 \text{ fM}$) was calculated assuming the use of hypothetical, highly magnetic Fe-MNPs ($d_s = 22 \text{ nm}$). Nevertheless, these limits could be overcome by designing new assays employing target amplification strategies and magnetic microspheres.
sensitivity and dynamic ranges were thus reduced. The developed model can further estimate effective MRSw responses for a given MNP type and concentration (Fig. 2.8(c)), which can facilitate assay determination and optimization for intended detection targets. Importantly, we could accurately predict the MRSw mode and the absolute detection limit as a function of particle relaxivity $r_2$ (Fig. 2.8(d)). The transition from MA to SD for MNP clusters happened at $r_2 \sim 10^{-15} \text{L.s}^{-1}$ (Appendix A), and higher $r_2$ lowered the absolute detection limit, all of which agreed well with experimental data.

2.2.2.2 Polystyrene Bead Sandwich Assay

We also applied multichannel NMR system to viral protein detection. As a model disease system, we chose dengue infection that is caused by any one of four dengue virus serotypes, called DENV-1, -2, -3, and -4. Current ELISA testings target three proteins; 1) non-structural protein 1 (NS1) DENV antigen, 2) IgM, and 3) IgG antibodies produced by immunological response against DENV viral envelope proteins. Generally, diagnosis of dengue infection depends on the phase of infection. NS1 protein has been used for acute dengue infections because it is detectable at earlier stage of the infection from 1 to 18 day post onset of symptoms (DPO), while IgM antibodies appear at later stage of the infection upto 90 DPO. Moreover, primary or secondary dengue infection can be determined by measuring fold changes of IgG antibody between acute phase and convalescent phase of the infection.
Figure 2.9. Dengue Virus Detection. (a) Titration curve for DENV NS1 protein (b) Detection of DENV reactive IgG and IgM
We prepared sandwich assays for dengue viral protein and antibody detection using polystyrene beads and MNPs. Recombinant NS1 proteins were first captured by polystyrene beads conjugated with capturing antibodies, and then labeled with detection antibody-MNP conjugates as listed in Table x. By varying concentrations of the NS1 proteins, we were able to detect as low as 2 pg/μL of NS1 protein, which is ten fold higher sensitivity than conventional ELISA method (Fig. 2.9(a)). Also, we could detect presence of dengue virus reactive IgM and IgG. The target antibodies were captured by polystyrene beads conjugated with DENV virus envelop proteins and labeled with anti-IgM or anti-IgG antibody-MNP conjugates. The prepared samples and controls were loaded, and T2 relaxation time was measured in multichannel NMR coils concurrently (Fig. 2.9(b)).

2.3. Summary and Conclusion

As a novel biosensing platform, the portable NMR offers many synergistic advantages over traditional approaches, such as high detection sensitivity, multiplexed capability, rapid measurement, small sample volume requirement with minimal sample processing. Indeed, the developed system thrives through the complement of several cutting-edge technologies, namely magnetic nanomaterials, bioconjugation chemistry and microfabrication. With new developments such as multi channel NMR system, optimized magnetic nanoparticles and advanced labeling techniques, the portable NMR technology has proven itself as a robust and sensitive approach for quantitative and molecular analyses for biomedical research. Its remarkable performance and
potential impact on clinical disease management would no doubt accelerate the advance of personalized treatment, by providing valuable information on molecular signature of individual patients.

We further envision broader application of the portable NMR in global healthcare. The developed NMR technology does not require extensive sample purification, and can be packaged as a portable device. The system thus is well-suited for rapid and point-of-care (POC) testing, especially in resource-limited primary clinics where majority of diagnoses are made based on physical symptoms only.

2.4 Materials and Methods

2.4.1 Synthesis and Characterization of Magnetic Nanoparticles

All MNPs were prepared as previously reported. Briefly, amine-terminated cross-linked iron oxide (CLIO) nanoparticles were generated by aqueous co-precipitation and coated with dextran\textsuperscript{77}.

Ferrite nanoparticles (Fe\textsubscript{3}O\textsubscript{4} and MnFe\textsubscript{2}O\textsubscript{4}) were synthesized via thermal decomposition and enlarged through a seed-mediated growth process\textsuperscript{47}. Iron (III) acetylacetonate [99.9\%, Fe(acac)\textsubscript{3}], manganese (II) acetylacetonate [Mn(acac)\textsubscript{3}], oleylamine (70\%), 1-octadecene (95\%), 1,2-hexadecanediol (90\%), chloroform (99\%), sulfosuccinimidyl-(4-N-maleimidomethyl)cyclohexane-1-carboxylate (99\%, sulfo-SMCC), 2,3-dimercaptosuccinic acid (98\%,
DMSA) and dimethyl sulfoxide (99.9%, DMSO) were purchased (Sigma–Aldrich) and used without further modification. Isopropanol (99.5%), hexane (98.5%), ethanol (99.5%), and NaHCO₃ were purchased (Fisher Scientific) and used as received.

We first synthesized 10-nm MnFe₂O₄ MNPs. Fe(acac)₃ (4 mmol, 1.4 g), Mn(acac)₂ (2 mmol, 0.5 g), 1,2-hexadecanediol (10 mmol, 2.9 g), oleic acid (6 mmol, 1.9 mL), oleyamine (6 mmol, 2.8 mL), and 1-octadecene (20 mL) were mixed by stirring under N₂ flow (1 h). The mixture was then heated and kept at 200 °C for 2 h. Subsequently, the temperature was ramped to 280 °C to initiate particle formation. After reflux, the mixture was cooled to room temperature, and isopropanol (80 mL) was added. Particles were collected via centrifugation (1,811 × g, 15 min) and then dispersed in hexane. To make 12-nm particles via the seed-mediated growth, 10-nm MnFe₂O₄ MNPs (100 mg) were dispersed in hexane (10 mL) along with the same amount of metal acetylacetonates, 1,2-hexadecanediol, oleic acid, oleylamine, and 1-octadecene as described above. The mixture was heated and kept at 100 °C for 1 h under N₂ flow. The mixture was then heated and kept at 200 °C for 2 h. Finally, the temperature was increased to 300 °C, and the mixture was refluxed for 2 h. After cooling down to room temperature, the particles were collected by the same washing and isolation procedure. 16-nm MnFe₂O₄ MNPs were synthesized in a similar manner using 12-nm particles as a seed.

Fe@MnFe₂O₄ were prepared through annealing of manganese and iron-oleate complexes on Fe nanoparticles⁷⁹. Fe-only MNPs was first synthesized. 20 mL ODE and 0.3 mL oleylamine (0.64 mmol) were mixed and the mixture was heated (60 °C) under vacuum (1 h) and recharged with N₂ gas. The mixture was then heated to 260 °C. When the temperature became stable,
Fe(CO)$_5$ (1.4 mL, 10 mmol) was injected. The solution was kept at 260 °C and under N$_2$ flow for 1 h, after which it was cooled to room temperature. While the Fe MNPs were formed, manganese and iron-oleate complex was separately prepared. Mn$_2$(CO)$_{10}$ (156 mg, 0.8 mmol), OA (2.3 mL, 7.26 mmol) and 10 mL ODE were mixed and the mixture was heated to 60 °C under vacuum (1 h) and recharged with N$_2$. The mixture was heated to 120 °C and Fe(CO)$_5$ (0.21 mL, 1.61 mmol) was subsequently injected. The solution, containing metal-oleate complexes, was cooled to room temperature and transferred to the Fe MNP solution using double-ended needles. The mixture of Fe MNPs and metal-oleate complexes was stirred (0.5 h) at room temperature. The reactor temperature was then ramped (5 °C/min) to the optimal annealing temperature (300 °C) for the ferrite-shell formation. When the temperature stabilized, the mixture was stirred for 1 h. The solution was then cooled to room temperature and 150 mL isopropanol solution (ODE/isopropanol = 0.2 v/v) was added. MNPs were collected via centrifugation (1,811 × g, 15 min) and dispersed in 10 mL hexane.

The shape, structure, and composition were further characterized using a transmission electron microscope (TEM; JEOL 2100, JOEL USA), an X-ray powder diffractometer (XRD; RU300, Rigaku), and an inductively-coupled plasma atomic emission spectrometer (ICP-AES; Activa-S, HORIBA Jobin Yvon), respectively. The magnetic properties were analyzed using a vibrating sample magnetometer (EV-5, ADE Magnetics). The $r_2$ relaxivity of MNPs was obtained by measuring $R_2$ of samples with varying MNP concentrations using a commercial relaxometer (0.47 T; Minispec mq20, Bruker). After the magnetic measurements, samples were dissolved in acid (HCl 10%), and the amounts of metals (Fe, Mn) were quantified by ICP-AES.
2.4.2 Surface Modification and Biotinylation

Amine-terminated CLIO nanoparticles were biotinylated in the presence of 20-fold molar excess of sulfo-NHS-biotin (Pierce Biotechnology), in phosphate-buffered saline containing 0.1M sodium bicarbonate for 3 h at room temperature. Following conjugation, unbound biotin molecules were removed using Sephadex G50 columns (GE Healthcare).

All other MNPs prepared in the organic phase were transferred into the aqueous phase prior to biotinylation. Briefly, the prepared MNPs were suspended in 10 mL chloroform, and treated with 50 μL triethylamine and dimercaptosuccinic acid (DMSA; 50 mg in 10 mL DMSO). The mixture was incubated for 6 h at 40 °C until it gradually turned heterogeneous, and precipitated down by centrifugation (3,000 rpm, 10 mins). The precipitate was washed with ethanol to remove excess DMSA, and dispersed in 10 mL ethanol. DMSA treatment was then repeated to improve nanoparticle aqueous stability. The precipitated MNPs were finally dispersed in 10 mL water and have terminal sulfhydryl and carboxylic acid groups. The number of sulfhydryl group per nanoparticle was ~50 as determined by Ellman's reagent (Pierce Biotechnology). To conjugate DMSA-treated MNPs with (+)biotin-hydrazide (Aldrich), amide bonds were formed using carboxylic acids in MNPs and amine groups in biotin through NHS/EDC chemical reactions. The DMSA-treated MNPs (25 mg) were dispersed in 10 mL water, followed by the addition of NHS (3.5 mg), EDC (5 mg), and biotin (1 mg). The mixture was shaken for 3 h at room temperature. The conjugated MNPs was precipitated down (12,000 rpm, 20 min) and washed three times with
water. The number of biotins per particle, quantified using the EZ Biotin Quantitation Kit (Pierce Biotechnology), was \(~40\).

2.4.3 Magnetic Relaxation Switch Assays

Avidin (ImmunoPure Avidin #21121; Pierce Biotechnology) was first dissolved in PBS, and serially diluted. MRSw samples were prepared by adding 100 \(\mu\)L of avidin solution, containing varying avidin doses, into biotinylated MNP solutions (100 \(\mu\)L). After 15 minutes of incubation at 37 \(^\circ\)C, \(T_2\) values of all samples were measured from 1 \(\mu\)L aliquots using a miniaturized nuclear magnetic resonance (NMR) system. Independently, the size of MNP clusters was measured via dynamic light scattering (Zetasizer Nano-ZS, Malvern). These experiments were then repeated using samples with different MNP concentrations. For the relaxation measurements, we used Carr-Purcell-Meiboom-Gill pulse sequences with the following parameters: echo time (TE), 4 msec; repetition time (TR), 6 sec; the number of 180\(^\circ\) pulses per scan, 500; the number of scans, 8.

2.4.4 DNA conjugation to MNPs

The oligonucleotides were purchased from Integrated DNA Technologies. The list of oligonucleotides used in the experiments are summarized below:

DNA-1: 5'-thiol modifier-CGCATTCCAGGAT-3'
DNA-2: 5'-TCTCAACTCGTA-thiol modifier-3'

Target DNA: 5'-TACGAGTTGAGAATCCTGAATGCG-3' for DNA-1 and DNA-2

MNPs (0.25 mg) were first mixed with sulfo-SMCC (90 μg) in 0.625 mL PBS solution (pH 7.2) for 3 hours. 20 μL thiol-modified oligonucleotides (40 nmol) were mixed with 80 μL dithiothreitol (0.1 M solution in 0.1 mL PBS, pH 8, containing 1 mM EDTA) for 2 hours. The reduced oligonucleotides were then purified using a NAP-5 column (GE Healthcare) and deionized water as the eluent solution. Absorbance measurements were used to determine the fractions containing the pooled oligonucleotides and the bicinchoninic (BCA) protein assay solution were used to determine the fractions containing the DTT. MNP-SMCCs were subsequently purified using membrane filtration (Millipore Amicon, MWCO 30,000) and Sephadex G-50 with PBS as the eluent buffer. The purified maleimide-activated MNPs were eventually mixed with the reduced oligonucleotides in 2 mL PBS solution (1 mM EDTA, pH 7.2) and the reaction proceeded overnight at 4°C. After this incubation, the conjugates were purified using membrane filtration and Sephadex G-100 with PBS as the eluent buffer. Conjugation with FAM-modified oligonucleotides was used to confirm that approximately 50 oligonucleotides were conjugated to each fluorescent MNP-DNA probe.

2.4.5 DNA detection

MRSw samples were prepared by adding 100 μL of target DNA (Integrated DNA technologies) solutions, serially diluted in PBS, into MNP solutions (150 μL), containing MNP-DNA-1 and
MNP-DNA-2. After 24 hours of incubation at room temperature, $T_2$ values of all samples were measured from 1 μL aliquots using a miniaturized nuclear magnetic resonance (NMR) system. Independently, the size of MNP clusters was measured via dynamic light scattering (Zetasizer Nano-ZS, Malvern). These experiments were then repeated using samples with different MNP concentrations. For the relaxation measurements, we used Carr-Purcell-Meiboom-Gill pulse sequences with the following parameters: echo time (TE), 4 msec; repetition time (TR), 6 sec; the number of 180° pulses per scan, 500; the number of scans, 8.

2.4.6 Biotinylation of antibodies

20 μg of antibody of interest was resuspended in 100 μL of PBS using 7K MWCO desalting columns (89883, Thermofisher). The antibodies in PBS was reacted with 2.66 μL of 10 mM Sulfo-NHS Biotin (21326, Thermofisher) suspended in ultrapure water for 2 hours in room temperature. The excessive Sulfo-NHS biotins were removed by 7K MWCO desalting columns and stored at 4°C.

2.4.7 Preparation of carboxylated polystyrene beads

3 mg of polystyrene beads with carboxyl groups (3 μm, Polysciences) were washed three times and suspended in 100 μL of 0.1 M MES (2-(N-morpholino) ethanesulfonic acid) buffer (28390, Thermofisher). The washed beads were first reacted with 2 mg of EDC (22980, Thermofisher)
and Sulfo-NHS (24520, Thermofisher) in 50 μL of 0.1 M MES buffer, and incubated for 20 minutes at room temperature. After incubation, the NHS-EDC coupled beads were washed with PBS three times using 0.45 um centrifugal filter for 1 min at 3000 rpm (UFC30HV00, EMD Millipore) and resuspended in 100 μL of PBS. 100 ug of ligands against target proteins were added and mixed thoroughly, and then the whole mixture was incubated overnight at 4°C with slow tilt rotation. The beads were washed four times with PBS solution containing 0.05 % TWEEN and resuspended in 200 μL of 1 % BSA PBS solution.

2.4.8 Polystyrene beads and magnetic nanoparticle sandwich assay

50 μL of target protein was mixed with 2 μL of the ligand coupled polystyrene beads and 48 μL of 1 % BSA 0.05 % TWEEN PBS solution, and the mixture was incubated for 30 mins at room temperature, washed with 0.05 % TWEEN PBS three times using 0.45 um centrifugal filter for 1 min at 3000 rpm (UFC30HV00, EMD Millipore), and resuspended in 1 % BSA 0.05 % TWEEN PBS solution. The target captured beads were then mixed with 10 μL of biotinylated antibodies of interest (10 ug/mL) and incubated for 30 mins at room temperature. After incubation, the antibody bound beads were washed 3 times and resuspended in 100 μL of 1 % BSA 0.05 % TWEEN PBS solution. The washed beads were mixed with 2 μL of streptavidin modified magnetic nanoparticles (1.0 mg[Fe]/mL, 30 nm, SHS-30-01, OceanNanoTech) and incubated for 20 mins at room temperature. The MNP coated beads were washed 3 times with 0.05 % TWEEN PBS and reconstituted in 10 μL of PBS.
2.4.9. Cancer cell labeling

OvCAR3 ovarian cancer cell line was maintained in RPMI 1640 medium with 10% fetal bovine serum (Atlas Biologicals, S12450), supplemented with L-glutamine 1× (Corning Mediatech, 25-005-CI). Cells were cultured in a standard humidified incubator at 37°C in a 5% CO₂ atmosphere. Cells were detached from culture dish by trypsin with 0.05% EDTA (Corning Mediatech, 25-052-CI) before labeling with MNPs. Cancer cells were first washed with PBS, and then fixed with a 3:1 mixture of PBS with paraformaldehyde for 20 mins at room temperature. The fixed cells were washed and blocked with a blocking buffer (1% BSA with PBS) for 30 mins at room temperature. The washed cells were incubated with antibody-biotin conjugates (5 μg/mL, 1% BSA with PBS) for 30 min, and washed with centrifugation (350 x g, 5 min). Finally, cells were mixed and incubated with magnetic nanoparticles (1.0 mg[Fe]/mL, 30 nm, SHS-30-01, OceanNanoTech) for 30 mins at room temperature, and washed 3 times with centrifugation (350 x g, 5 min). All labeling experiments and measurements were performed at least three times.

2.4.10 Enzyme-linked immunosorbent assay (ELISA)

Ligands for capturing target proteins were diluted to 5 μg/mL in PBS, and then 100 μL of the diluted ligands was added to Maxisorb 96 well ELISA plate and incubated overnight at 4°C. The
plate was washed and blocked 3 times with 2 % BSA 0.1 % TWEEN in 1X TBS (2 % BSA-TBST). 100 uL of Target proteins were added to the plate and incubated at room temperature for an hour. After washing the plate with 2 % BSA-TBST 3 times, biotynolated antibodies against target proteins were added with adequate concentration (1 ug/mL) and incubated at room temperature for an hour. 100 uL of 1: 500 streptavidin HRP (Thermofisher) was added and incubated at room temperature for an hour. Unbound HRP was removed with 2 % BSA-TBST. TMB solution was added to the sample plate, and, after 20 mins, 1M HCl was used to stop the TMB-HRP reaction. Chemiluminescence signal was read with microplate reader (Spark, TECAN).
Chapter 3. MicroHall Magnetometer for Molecular Profiling of Single Cancer Cells

3.1 Introduction and Background

The biological and clinical use of magnetic nanoparticles (MNPs) continues to increase in concert with advances in medical imaging, DNA sequencing, cell sorting, and therapeutics. MNPs generate excellent contrast within inherently non-magnetic biological systems, which enables highly selective, interference-free actuation or detection. Overall, the utility of MNPs is commensurate with their magnetic moment, which needs frequent monitoring for process optimization during particle synthesis and for quality-control thereafter. Conventional magnetometers (e.g., superconducting quantum interference device, vibrating sample magnetometer) are generally used for such measurements, but their availability is often limited to specialized laboratories. Furthermore, such equipment often needs large amounts of MNPs (e.g., milligram amounts in a powder form); producing these amounts is often impractical in the particle developmental phase.

We reasoned that Hall-effect magnetometers can be adopted for point-of-use MNP characterization. Hall magnetometry offers many practical advantages. It assumes a linear response across many orders of magnetic field strength. Large polarizing magnetic fields that fully magnetize MNPs, can therefore be applied without saturating the sensors. Sensor
fabrication is also compatible with standard semiconductor technology. Sensor arrays as well as signal processing electronics can be integrated into the same chip to achieve high signal-to-noise ratio (SNR) and throughput. The entire detection system can thus be realized as a self-contained, cost-effective magnetometer platform.

We herein report a microHall magnetometer developed for micro-magnetometry. The microHall magnetometer consists of an array of Hall sensors and auxiliary circuits. Each Hall sensor attains micrometer-length scale and is connected to an external amplifier. We operated the chip in alternating current (AC) mode, thereby selectively measuring signals from MNPs while suppressing background. Importantly, we devised a new detection scheme that allowed us to: i) estimate MNPs' average saturation magnetic moment ($\mu_p$), and ii) differentiate MNP types within a mixture. We applied the microHall magnetometer to measure the magnetic moment of various types of MNPs; the measurement only required $<0.1$ pL of MNP samples to produce $\mu_p$ values. We further applied the system to profile single cancer cells labeled with MNPs. The developed microHall magnetometer could thus be positioned as an affordable and accessible biosensing platform or characterization tool for MNP development.
3.2 Results

3.2.1 System Overview and Implementation

We implemented an integrated microHall chip (Fig. 3.3) containing $2 \times 4$ array of Hall elements. For each column in the array, two Hall elements shared a current bias line. To guide sample loading above each Hall element, we made an array of fluidic wells and placed them on top of the Hall chip. Each well had a sample volume of $8 \times 8 \times 25 \, \mu m^3$. The assembled sensor was mounted on a custom-designed carrier. AC magnetic field was generated by a coil attached underneath the sensor, and an adjustable direct-current (DC) magnetic field was applied by an electromagnet.
Figure 3.1. microHall magnetometer chip. (a) Microscope image of the sensor chip. Eight μHall elements were fabricated into $2 \times 4$ array (b) SEM image of microHall magnetometer chip (c) SEM image of cross section of μHall element (d) Current related sensitivity of μHall element
3.2.1.1 Fabrication

Hall sensor device was fabricated on a epitaxially grown pHEMT GaAs wafer (IntelliEPi). Cross-shaped active Hall sensor area was first defined on the GaAs wafer with photolithography and wet chemical etching (H2SO4/H2O2/H2O, 1:3:50) for 90s. Electrodes to make a ohmic contact with two-dimensional electron gas was patterned with photolithography, and multi metal layers (Au/Ge/Ni/Au, 270Å/200Å/120Å/1500Å) were deposited with a thermal evaporator. Subsequently, the metal deposited device is stored in RemoverPG solution overnight to be lifted off. To form a eutectic alloy, the device was annealed at 450 °C for 90 s with a rapid thermal processor. A SiO2 layer (200 nm) was deposited by atomic layer deposition (ALD) to protect Hall sensor device from the MNP solution. Metal contact areas were opened by wet etching with BOE7:1 for 90s. Al wires were bonded to connect Hall sensor contacts with PCB landings (Figs. 3.2 and 3.3).

The microfluidic multi wells were fabricated with standard soft lithography. A SU-8 negative resist (SU-8 2025, Microchem) was spin-coated on a Si wafer at 3,500 r.p.m. for 30 s. The resist was then baked at 65 °C and 95 °C for 1 and 6 min, respectively. After being exposed under UV light with the multi well patterned mask, the resist was baked once again at 65 °C and 95 °C for 1 min and 6 min, respectively. Then the wafer was developed for 5 min with SU-8 developer.
Figure 3.2. Mask Design of microHall magnetometer chip. Eight μHall elements were designed into $2 \times 4$ array.
1. Define mesa (Wet etch)

2. Metal deposition (Thermal Evaporation)

3. Define electrodes (Lift off)

4. Rapid thermal annealing

5. SiO₂ deposition (Atomic Layer Deposition)

6. Bonding pad opening (Wet etch)

Figure 3.3. Fabrication Process. Hall sensor device was fabricated on an epitaxially grown pHEMT GaAs wafer (IntelliEPi). Cross-shaped active Hall sensor area was first defined on the GaAs wafer with photolithography and wet chemical etching (H₂SO₄/H₂O₂/H₂O, 1:3:50) for 90s. Electrodes to make an ohmic contact with two-dimensional electron gas were patterned with photolithography, and multi metal layers (Au/Ge/Ni/Au, 270Å/200Å/120Å/1500Å) were deposited with a thermal evaporator. Subsequently, the metal deposited device was stored in RemoverPG solution overnight to be lifted off. To form a eutectic alloy, the device was annealed at 450 °C for 90 s with a rapid thermal processor. A SiO₂ layer (200 nm) was deposited by atomic layer deposition (ALD). Metal contact areas were opened by wet etching with BOE7:1 for 90s. Al wires were bonded to connect Hall sensor contacts with PCB landings.
The developed wafer was then rinsed by isopropyl alcohol (IPA) and dried by nitrogen. The SU-8 mold was chemically treated by trichlorosilane vapor inside a desiccator for 30 min. Polydimethylsiloxane (PDMS), mixed with a curing agent with a 10:1 weight ratio and degassed, was cast on the SU-8 mold and cured on a hotplate at 60 °C overnight.

3.2.1.2 Experimental Setup

We used an electromagnet (3470, GMW) to generate an adjustable DC magnetic field (-0.3 T to 0.3 T). For the AC field generation, we made a coil (35 turns; diameter, 17 mm) and placed it under the iMH chip. The coil was excited with a sinusoidal input (1 kHz, 2.425 V peak-to-peak). The bias current (500 µA) to the Hall sensor was supplied by a DC current source (2400, Keithley), and the sensor output was fed to a voltage amplifier (5113, Signal Recovery), and then the amplified signal was demodulated by lock-in amplifier (1 kHz; integration time, 2 sec). All data were automatically recorded with MATLAB (MathWorks).
3.2.2 Applications

3.2.2.1 Hall Magnetometry

We set up the microHall magnetometer system to measure volume magnetic susceptibility ($\chi_v$) of MNPs (Fig. 3.4(a)). Specifically, MNPs placed on top of the microHall magnetometer, were magnetized by a static DC magnetic field ($H_{DC}$). Directly measuring MNP magnetization, however, was technically challenging, because the Hall generated much larger signal in response to $H_{DC}$. To single out signal from MNPs, we thus applied lock-in detection\textsuperscript{109,110}; a probing alternating current (AC) field was superimposed to $H_{DC}$, and Hall voltage ($V_H$) was measured at the AC field frequency. The resulting $V_H$ is proportional to the slope of MNP magnetization ($M$) curve at the given $H_{DC}$ ($V_H \sim dM/dH \sim \chi_v$; Fig. 3.4(b)). By sweeping $H_{DC}$, the overall magnetic susceptibility could be measured to construct the magnetization curve (see Appendix B for details).

We next applied the microHall magnetometer system for MNP characterization. Ferrite MNPs of different sizes and composition (e.g., doped ferrite) were synthesized to vary magnetic properties (Fig. 3.5(a), see Methods). We also used various commercial magnetic beads embedded with maghemite ($\gamma$-Fe$_2$O$_3$) MNPs. The particle solution was directly loaded over the microHall
magnetometer, and the Hall voltage was measured using the AC detection scheme. The DC magnetic field was swept from –200 to 200 (kA/m).

**Figure 3.5(b)** shows normalized $\chi_v$ curves for different magnetic particles. For superparamagnetic particles, the normalized $\chi_v$ curve can be approximated as (see Appendix B for details)
Figure 3.4. Working principle of microHall magnetometer. (a) Measurement setup. MNPs were magnetized by an excitation coil underneath the sensor chip. Differential output was amplified by pre-amplifier (differential input to single output) and demodulated by lock-in amplifier. (b) Magnetic susceptibility measurement scheme. Alternating current magnetic field ($H_{AC}$), superimposed to a static direct current magnetic field ($H_{DC}$), was applied perpendicular to the microHall magnetometer chip. The resulting magnetization, induced from MNPs at the same frequency of the applied $H_{AC}$, was proportional to the slope of MNP magnetization curve at the given $H_{DC}$. The overall magnetic susceptibility curve was constructed by sweeping $H_{DC}$. 
Figure 3.5. microHall magnetometer measurements. (a) Transmission electron microscopy images of MNPs (ZnFe$_2$O$_4$, left; CoFe$_2$O$_4$, middle) and magnetic beads (right). (b) Magnetic susceptibility ($\chi_v$) curves of different magnetic particles were measured by sweeping the external field ($H_{ext}$) from -200 to 200 kA/m. The measured $\chi_v$ curve was normalized to $\chi_v$ at $H_{ext} = 0$, and fitted to the first derivative of Langevin function. (c) Normalized magnetization curves were constructed from $\chi_v$ curves in (b). Magnetic beads with $\gamma$-Fe$_2$O$_3$ MNPs were saturated faster than other MNP types.
\[ \chi_v = 3 \cdot \chi_{v0} \left[ -\text{csch}^2 \left( \frac{\mu_0 \mu_p H_{DC}}{kT} \right) + \left( \frac{\mu_0 \mu_p H_{DC}}{kT} \right)^{-2} \right] \]

where \( \chi_{v0} \) is the susceptibility at \( H_{DC} = 0 \), \( \mu_0 \) is the vacuum permeability, \( k \) is Boltzmann constant, \( T \) is temperature, and \( \mu_p \) is the average magnetic moment of individual MNPs\(^{109,111}\). By fitting measured data to Eq. (1), we could construct a magnetization curve \( M \) for each particle type (Fig. 3.5(c)). Magnetic beads containing maghemite (\( \gamma \)-Fe\(_2\)O\(_3\)) MNPs were quickly saturated at lower \( H_{DC} \), presumably due to their low magnetic anisotropy\(^2\). Conversely, CoFe\(_2\)O\(_4\) MNPs, whose material has high magnetic anisotropy, showed slower magnetization response.

We further noted that the normalized response curve \( \chi/\chi_{v0} \) is a function of MNPs' magnetic moment \( \mu_p \) only. Particularly, the full-width at half maximum (FWHM) of \( \chi_v \) is inversely proportional to \( \mu_p \) with

\[ H_{\text{FWHM}} = 4.16 \cdot kT \cdot (\mu_0 \mu_p)^{-1}. \]

We reasoned that this metric can be used to estimate \( \mu_p \) of MNPs regardless of sample type or loading volume. To test the hypothesis, we measured two types of particles: free \( \gamma \)-Fe\(_2\)O\(_3\) MNPs and polystyrene microbeads labeled with MNPs. Indeed, the \( \chi_v \) curves from the \( \mu \)Hall detection were nearly identical (Fig. 3.6(a)), and extracted \( \mu_p \) values were comparable to each other: \( 0.41 \times 10^{-18} \text{ A} \cdot \text{m}^2 \) (MNPs only); \( 0.39 \times 10^{-18} \text{ A} \cdot \text{m}^2 \) (MNPs on microbeads). We extended the
Figure 3.6. Characterization of MNPs. (a) Two samples, each containing free γ-Fe$_2$O$_3$ MNPs and polystyrene bead (6.8 μm) labeled with γ-Fe$_2$O$_3$ MNPs, were measured by the microHall magnetometer system. The normalized $\chi'$ curves were nearly identical and independent of the sample volume or MNP configurations (i.e., bead-bound or free floating). (b) microHall magnetometer was used to measure magnetic moments ($\mu_p$) of free floating MNPs (ZnFe$_2$O$_4$ and CoFe$_2$O$_4$) and bead-bound γ-Fe$_2$O$_3$ MNPs. Due to their larger size, γ-Fe$_2$O$_3$ MNPs in magnetic beads showed higher $\mu_p$ than ZnFe$_2$O$_4$ and CoFe$_2$O$_4$. Bead type 1: Streptavidin Superparamagnetic Particles, SVM-025-5H, 100 ~ 390 nm, Spherotech 2: Mono Mag Streptavidin Beads, 500 nm, MV0502, Oceananotech 3: MyOne Streptavidin T1, 1μm, 65602, Dynabead 4: M-280 Streptavidin, 2.8 um, 11205D, Dynabead
measurements to other MNPs and commercial magnetic beads embedded with γ-Fe$_2$O$_3$ MNPs. Overall, γ-Fe$_2$O$_3$ MNPs in commercial magnetic beads showed higher $\mu_p$ values than ZnFe$_2$O$_4$ and CoFe$_2$O$_4$ MNPs, presumably due to their large crystal size (~22 nm).

We next extended the microHall magnetometer measurement for multiplexed MNP detection. When different MNP type ($i$) of known magnetization ($\mu_i$) are mixed, the total magnetization $M$ is written as

$$M = \sum_i f_i \mu_i L(\mu_i, H_{DC})$$  

where $f_i$ is the number fraction of the particle type $i$ ($\sum f_i = 1$) in a given sample volume, and $L$ is Langevin function. We approximated $M$ as a single functional form with an effective magnetic moment $\mu_{est}$,

$$M = \mu_{est} L(\mu_{est}, H_{DC}).$$

Note that $\mu_{est}$ value can be obtained from the width of $\chi_v$ peak measured by microHall magnetometer. With known $\mu_i$ and $\mu_{est}$, the fraction of each MNP type ($f_i$) can be estimated by solving a set of equations, $\mu_{est} L(\mu_{est}, H_{DC}) = \sum f_i \mu_i L(\mu_i, H_{DC})$ measured at different $H_{DC}$. For the mixture of two particle types ($i = 1, 2$), the process can be further simplified to solving $f_1$ from $\mu_{est} \approx f_1 \mu_1 + (1 - f_1) \mu_2$. 

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We applied the model to the mixture of two particle types: ZnFe$_2$O$_4$ ($i = 1$), $\gamma$-Fe$_2$O$_3$ ($i = 2$). We first checked the model for accuracy. We measured the $\chi$ curve for each particle type alone, and
Figure 3.7. Measurement of MNP mixtures. (a) Magnetic susceptibility ($\chi_s$) curves of single type MNPs and their mixture were measured. The average magnetic moment of a sample was estimated from full-width at half maximum ($H_{FWHM}$). (b) Samples containing ZnFe$_2$O$_4$ and $\gamma$-Fe$_2$O$_3$ MNPs at different ratios were prepared. From the susceptibility measurements, the average magnetic moment ($\mu_{avg}$) of each mixture was estimated. The obtained value (block dots) showed a good match with $\mu_{avg}$ (dotted line) which is a number-weighted average of comprising MNPs’ magnetic moments. (c) The number fractions of ZnFe$_2$O$_4$ and $\gamma$-Fe$_2$O$_3$ MNPs was estimated from the measured $\mu_{est}$ (block dots). The dotted line indicates the expected values.
obtained μ\text{r} values for ZnFe₂O₄ and γ-Fe₂O₃ MNPs. We then mixed the particles, and obtained μ\text{e} from the χ\text{v} curve (Fig. 3.7(a)). The measured μ\text{e} matched well with the expected magnetic moments (μ\text{ avg}) of two particle mixture, μ\text{ avg} = f_1·μ₁ + (1 - f_1)·μ₂, over varying f₁ ranges (Fig. 3.7(b)); this result supports the use of a single functional form (Eq. 4) to approximate the magnetization of a MNP mixture. Consequently, the number fraction of MNPs in a mixture could be estimated from the measured μ\text{e} (Fig. 3.7(c)).

3.2.2.2 Single Cancer Cell Profiling

As translational proof-of-concept, we applied the microHall magnetometer system to detect biological cells. We first calibrated the sensor output by measuring Hall signals (at H\text{DC} = 0 kA/m) at different MNP concentrations (Fig. 3.8(a)). The Hall voltage level was linearly proportional to MNP numbers, which enabled quantitative comparison on levels of targeted molecules. We next profiled single cancer cells by targeting the epidermal growth factor receptor (EGFR) and epithelial cell adhesion molecule (EpCAM). Ovarian cancer cells (OVCA420) were incubated with biotinylated antibodies and subsequently labeled with streptavidin-coated magnetic particles. After removing excess particles, we loaded cells to the microHall magnetometer device through the microfluidic well structure. Figure 3.8(b) shows single-cell magnetization curves for different markers. Higher slope at zero DC magnetic field and saturation magnetization indicated higher number of MNPs present in the cell. We also used
samples incubated with control IgG to account for non-specific binding. The expression levels of corresponding target molecules (EGFR, EpCAM and IgG)
Figure 3.8. Application of microHall magnetometer system. (a) The microHall magnetometer system was calibrated by measuring the Hall voltage outputs of serially diluted ZnFe$_2$O$_4$ MNP samples. The Hall signal was linearly proportional to MNPs concentrations. (b) Ovarian cancer cells were labeled with MNPs specific to EGFR or EpCAM. The $\chi_v$ curves of a single cell were measured and quantified by the microHall magnetometer system. The Hall voltage at $H_{ext} = 0$ is proportional to marker expressions. (c) Molecular profiling. The expression levels of target markers (EGFR, EpCAM) were compared for OvCA420 cells. The control sample was OvCA420 cells incubated with isotype-matched IgG antibody and MNPs. were quantified by subtracting baseline output hall voltage from output hall voltage at zero DC magnetic field (Fig. 3.8(c)).
3.3 Summary and Conclusion

We developed the microHall magnetometer system for rapid, multiplexed, small-volume magnetometry. The detection chip was implemented with standard semiconductor technology, integrating an array of microHall elements and microfluidic capturing wells. We operated the device in frequency lock-in mode with AC magnetic field superimposed to strong DC magnetizing field. This strategy enabled us to measure the average magnetic moments of individual MNPs directly from a small volume (0.01 pL) of MNP solution. By deconvoluting magnetic responses over a range of external magnetic fields, we could identify MNP types and their fractional ratios in a mixture. Moreover, by tagging MNPs with existing or emerging cancer markers, microHall magnetometer is poised for facile molecular profiling of single cancer cells to expand the breadth and depth of cancer diagnostics available for preclinical and clinical research.

3.4 Materials and Methods

3.4.1 Polystyrene Bead Labeling
The biotin coated polystyrene beads (TP-60-5, 6.8 um, Spherotech) were washed with PBS three times using 0.45 um centrifugal filter for 1 min at 3000 rpm (UFC30HV00, EMD Millipore). The washed polystyrene beads were diluted ten times (50 ul) and blocked with a blocking buffer (1% BSA with PBS) for 30 min at room temperature. After blocking, five microliter of streptavidin modified magnetic beads (Dynabeads M280, 1:10 dilution in 1% BSA PBS, ThermoFisher Scientific) were mixed with the BSA blocked polystyrene beads for 45 min at room temperature, and then washed three times with PBS using 5.0 um centrifugal filter for 1 min at 3000 rpm (UFC30HV00, EMD Millipore).

3.4.2 Biotinylation of labeling antibodies

Sulfo-NHS-biotin (10 mM, Pierce) solution in PBS was incubated with antibodies for 2 h at room temperature. Unreacted sulfo-NHS-biotin was removed using Zeba spin desalting column, 7K MWCO (Thermo Scientific). Antibodies were kept at 4 °C until use.

3.4.3 Cancer cell labeling

OV420 ovarian cancer cell line was maintained in RPMI 1640 medium with 10% fetal bovine serum (Atlas Biologicals, S12450), supplemented with L-glutamine 1× (Corning Mediatech, 25-005-CI). Cells were cultured in a standard humidified incubator at 37°C in a 5% CO₂ atmosphere. Cells were detached from culture dish by trypsin with 0.05% EDTA (Corning Mediatech, 25-052-CI) before labeling with MNPs. Cancer cells were first washed with PBS,
and then fixed with a 3:1 mixture of PBS with paraformaldehyde for 20 mins at room temperature. The fixed cells were washed and blocked with a blocking buffer (1% BSA with PBS) for 30 min at room temperature. The washed cells were incubated with antibody-biotin conjugates (5 μg/mL, 1% BSA with PBS) for 30 min, and excess antibodies were removed via centrifugation (350 × g, 5 min). Finally, cells were incubated with magnetic particles (30 min, 20 °C), and triple washed via centrifugation (350 × g, 5 min). All labeling experiments and measurements were performed at least three times.

### 3.4.4 Magnetic Nanoparticles

Tetramethylammonium (TMAOH), streptavidin (from Sigma-Aldrich); ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), hydroxysulfosuccinimide (NHS) (from Thermo Fisher Scientific); carboxymethyl (CM) dextran (from PK chemicals A/S); MACS column (from Miltenyi Biotec); amicon ultra centrifugal filters (from Merck Millipore) were used as received. All chemicals were of analytical grade and used without purification. Zinc-doped ferrite MNPs (ZnFe₂O₄) and cobalt-doped ferrite MNPs (CoFe₂O₄) were synthesized as previously reported methods. CM-dextran and streptavidin were used for the surface modification. For CM-dextran coating, 5 mg of MNPs (ZnFe₂O₄, CoFe₂O₄) and 0.72 g of TMAOH dissolved in 2 mL of n-butanol were mixed and sonicated for 1 h. Then, MNPs were collected by centrifugation (1500 × g, 5 min) and dispersed in 5 mL of deionized water. 375 mg of CM-dextran was added to the
MNP and was stirred overnight at 70 °C. Finally, unreacted reagents were purified by centrifugal filter. Streptavidin was used for further surface modification. 9.6 mg of EDC, 1.09 mg of NHS and 1 mg of streptavidin were added to 1 mg of CM-dextran coated MNPs dissolved in 1 mL of 10 mM phosphate buffer solution (pH 7.4) followed by shaking for 2 hrs. The final product was purified by MACS column.
Chapter 4. Wirelessly Powered Electrochemical Biosensor

4.1 Introduction and Background

4.1.1 Extracellular Vesicles

A promising new approach for timely cancer monitoring is to exploit circulating biomarkers ("liquid biopsy") that can be repeatedly and conveniently obtained with minimal complications. Exosomes (or extracellular vesicles; EVs) in particular could offer new breakthroughs for monitoring bladder cancer\(^{115-117}\). Exosomes are membrane-bound phospholipid vesicles (30 – 200 nm in diameter) actively secreted by most mammalian cells\(^{18,25}\). A growing number of studies show that i) EVs carry cellular constituents from their originating cells, including transmembrane and intracellular proteins\(^{118}\), mRNA\(^{24}\), DNA\(^{119}\), and miRNA\(^{120}\), and can thus serve as cellular surrogates\(^{121-123}\), and ii) with their abundance and structural stability, these vesicles can better reflect global tumor burden\(^{124}\), overcoming limitations of tumor heterogeneity and sampling bias\(^{18-24,125,126}\). Analyzing EVs for routine clinical diagnostics, however, remains challenging due to the lack of adequately sensitive and fast assay platforms. Conventional molecular assays (e.g., Western blot, ELISA) require large amounts of samples and are impractical in most clinical settings.
### 4.1.2 Bladder Cancer

Bladder cancer is the fifth most common cancer in the United States with approximately 74,000 new cases diagnosed and 16,000 deaths estimated in 2015\(^{127}\). Over 90% of bladder cancer is urothelial carcinoma, also known as transitional cell carcinoma (TCC); 75% of which are non-muscle invasive bladder cancer (NMIBC) and 25% are muscle invasive bladder cancer (MIBC) with 20~30% 5 year survival rate\(^{128}\). According to TNM stage system, 70% of NMIBC are stage Ta tumors confined to the urothelium, 20% are stage T1 tumors penetrate from the urothelium to lamina propria, and 10% are carcinoma in situ (CIS) confined to the urothelium with high grade but a flat non-papillary configuration\(^{129}\).

Current gold standard for bladder cancer diagnosis is cystoscopy, examining urethra and bladder wall using a slender tube with a lens and light that is inserted through urethra\(^{128,129}\). While this method is highly sensitive, the procedure is invasive and expensive, and it may fail to detect flat carcinoma in situ. Voided urine cytology, a microscopic examination of urothelial cells in urine, is a noninvasive procedure with high specificity (99%), but its main weaknesses are low sensitivity for low grade tumor, long test turn around time (2~5 days) and inter-observer variation. To develop a cost effective, non-invasive, and reliable testing method for bladder cancer detection, six molecular tests (NMP22 BladderChek, NMP22 BC test kit, BTA stat, BTA TRAK, uCyt+, and UroVysion Bladder Cancer Kit) were developed and approved by the Food
and Drug Administration (FDA). However, due to variable sensitivity and low specificity, they have not been widely accepted in clinical practice\textsuperscript{117,128,130}. The standard care guidelines for bladder cancer, by National Comprehensive Cancer Network (NCCN), recommend that NMIBC is treated with transurethral resection of bladder tumor (TURBT) and stage dependent intravesical instillation (mitomycin, BCG, and interferon), and MIBC is treated with radical cystectomy and bilateral pelvic lymph node dissection.

A major challenge in treating NMIBC patients is high recurrence rate after TURBT. Even with TURBT and intravesical instillation, 68\% of T1 stage patients experience non-invasive recurrence, and 30\% of them develop a progression to MIBC\textsuperscript{128,131}. Regular surveillance of the treated NMIBC patients is required to detect recurrence early in a timely manner and prevent its progression to MIBC. The current guideline for monitoring recurrence of high grade tumors after TURBT treatment is to perform a voided urine cytology an cystoscopy in every three month for first 2 years, every six month for the next 2-5 years and once a year thereafter\textsuperscript{128}. The frequent monitoring due to hight recurrence rate also burdens NMIBC patients with the most expensive life time treatment cost, ranging from $89,287 to $202,203, per patient among all cancer types\textsuperscript{117,132}.

There is an urgent need to develop a novel diagnostic and surveillance biomarker for bladder cancer in order to improve the quality of care and reduce the economic burden on patients. New biomarker should have a low false positive rate for better diagnosis and high sensitivity and
negative prediction value (NPV) for accurate recurrence monitoring. Extracellular vesicles (EVs) could be an ideal candidate satisfying the aforementioned criteria. EVs have gained attention as noninvasive diagnostic biomarkers and therapeutic agents in different types of cancer. Specifically, many research have found that circulating exosomes, endolysosomal pathway originated EVs, may function as surrogates of cancer cells and promote tumor progression, survival, angiogenesis, cell signaling, and immune response. However, only a few research have shown the clinical utility of urinary exosome in bladder cancer diagnosis and surveillance.

4.1.3 Electrochemistry

Electrochemistry has been used to detect a variety of analytes for biochemical sensing. There are many kinds of electrochemical methods to measure physical quantities (potential, current, and charge) in different types of electrochemical reactions such as bulk and interfacial methods. The interfacial methods are simply divided into static and dynamic methods depending on whether current in electrochemical cell is time-varying or not. The dynamic methods are further divided into controlled-current coulometry and controlled-potential voltammetry, which is one of the most common electrochemical detection method for biochemical sensing.
Electrochemical cell normally consists of two or three electrodes; 1) working electrode 2) reference electrode 3) counter electrode (optional for three electrode electochemical cell).

Voltammetry is a method that measures electric current flowing through working electrode when known potential is applied between working and reference (or counter) electrodes. Unlike other voltammetry methods, amperometry measures current flowing through working electrode over time when static potential is applied to electrochemical cell. The redox current is proportional to concentration of analytes in sample. The common applications of amperometry are $O_2$ sensor and glucose meter.

In this chapter, to provide rapid and quantitative urine analyses in point-of-care settings, we develop a smartwatch-enabled electrochemical sensing platform using NFC technology. This platform, which is wirelessly powered by and communicated with smartwatch, quantitatively measures the concentration of biological components in urine. The system was validated with several different assay schemes to detect a variety of biological molecules such as glucose, creatinine, proteins, and nucleic acids. We established a urine-NFC-powered Extracellular vesicle Test (NeXT) to detect bladder cancer extracellular vesicles (BCEVs) in urine (Fig. 4.1). In later part of this chapter, we profile bladder cancer cell lines and their EVs to identify transmembrane protein signatures. The assay protocol is optimized using urine spiked with bladder cancer-derived extracellular vesicles (BCEVs). We then compare enrichment efficiency of our immunomagnetic EV purification method with conventional ultracentrifugation.
Figure 4.1. Schematic of wirelessly powered electrochemical system. This system, which is wirelessly powered by smartwatch via NFC technology, quantitatively measures electronic signals from chemical reactions between biological samples and reagents. The measured signal is wirelessly transmitted to smartwatch and further processed for molecular analyses.
4.2 Results

4.2.1 System Overview and Implementation

4.2.1.1 System Architecture

The system comprised of three major parts (Figs. 4.2, 4.3, and 4.4); 1) disposable electrode strip for electrochemical sensing 2) chronoamperometry signal read-out circuits and 3) RF communication modules (RF430FRL152H, Texas Instrument). Once the sample is dropped on the surface of electrodes in a disposable strip, electric potential is applied to trigger oxidation or reduction of target analytes. Read-out circuits (LMP91000, Texas Instrument) amplify chronoamperometric signal from the disposable strip, and the amplified signal is converted from analog to digital signal. The converted signal is transmitted to a smartwatch (Sony smartwatch 3, android wear) or smartphone (Samsung galaxy 5) wirelessly via NFC protocol (ISO15693). The smartwatch or smartphone supplies power to the proposed system to be operated without battery. Application software sends commands to control the electrochemical chronoamperometry, and process the transmitted data.

Disposable electrode strip consists of three electrodes: reference, counter, and working electrodes. Counter and working electrodes are screen-printed with Au and reference electrode is
screen-printed with Ag/AgCl. Chronoamperometry signal read-out circuit is composed of a signal conditioning circuit, transimpedance amplifier, and analog-to-digital converter. Signal conditioning circuit set up an electrical potential between the reference and the working electrodes, and then electrons are transported in or out of working electrode upon oxidation or
Figure 4.2. Block Diagram and Device Photo. The system comprised of three major parts (Left): 1) disposable electrode strip for electrochemical sensing 2) chronoamperometry signal read-out circuits and 3) RF communication modules.
Figure 4.3. Schematic of Device Circuits.
Figure 4.4. Printed Circuit Board of Device.
Figure 4.5. Software flow chart. Control softwares divide into two parts; 1) Mobile application that is operated in smartphone or smart watch 2) Device firmware that runs in microprocessor of the electrochemical device. To initialize NFC and electrochemical measurement system, mobile application sends commands for identifying devices and setting various parameters. Then, device firmware executes the commands and send the status of device back to mobile application.
reduction of analytes. The transported electrons flow through the working electrode and be amplified and converted to voltage output by trans-impedance amplifier. 12 bit sigma-delta ADC filters and converts the amplified signal from analog to digital domain (Fig. 4.6). The converted signals are stored in the EEPROM of a microcontroller, and finally RF communication module transmits the stored date to smartwatch or smartphone. Power, up to 300 uW, is supplied from smartwatch or smartphone wirelessly, enabling the whole system to operate in a standalone mode for a long time without battery. Application software to control the whole system was developed using android wear and android for smartwatch and smartphone operation respectively.

Control softwares (Fig. 4.5) divide into two parts; 1) mobile application that is operated in smartphone or smart watch 2) device firmware that runs in microprocessor of the electrochemical device. To initialize NFC functionality and electrochemical measurement system, the mobile application sends initialization commands for transferring power, identifying devices and setting various system parameters. Then, device firmware executes the commands and send the system status back to mobile application. Once the system is set properly, the mobile application starts amperometry measurement and reads out ADC memory from the device. The retrieved ADC data, then, is processed and plotted on mobile device.
Figure 4.6. ADC calibration. ADC readout is linearly proportional to analog voltage input.
4.2.1.2 System Validation and Assay

4.2.1.2.1 Glucose

Glucose is a type of sugar that provides energy to most organisms through aerobic respiration. For human beings, blood glucose level is usually regulated by balancing insulin and glucagon. Failure in keeping glucose homeostasis is a major cause of metabolic disease such as diabetes. Normal range of blood glucose while fasting should be between 70 ~ 110 mg/dL. There are many portable glucose monitoring systems that are commercially available. Most of them measure glucose level with a type of electrochemistry, called amperometry. Once a drop of blood from finger tip is loaded in a sample chamber, glucose oxidase that is immobilized on electrode surface catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide. Then, hydrogen peroxide oxidized at a working electrode at given electric potential, thus generating current to be measured by read out circuits. To validate the NexT system, we prepared different concentration of glucose samples and measured oxidation currents from the working electrode using the NexT system. The result showed linear relationship between system read out and glucose concentration (Fig. 4.7).
Figure 4.7. System Validation - Glucose. Different concentrations of glucose samples were prepared, and oxidation currents were measured from the working electrode using the NexT system. The result showed linear relationship between system read out and glucose concentration.
4.2.1.2.2 Creatinine

Creatinine is a by-product of muscle metabolism that is filtered out at kidney and excreted in urine. Urine creatinine concentration is measured to normalize urine concentration and to test the validity of urine specimen. Urine creatinine is chosen to test because it is a normalizing factor for urine concentration. The NexT system quantified the level of creatinine by measuring the electric current from creatinine oxidation reaction. Concentration of urine creatinine normally ranges between 0 and 300 mg/dL. Creatinine concentration can be measured by electrochemical amperometry using a three enzymes method. First, creatinine is hydrolyzed to creatine by creatinine amidohydrolase, and creatine is then hydrolyzed to sarcosine by creatine amidinohydrolase. In turn, sarcosine is oxidized by sarcosine oxidase, and the reduced sarcosine oxidase is re-oxidized by the mediator, ferricyanide. By applying electric potential between reference and working electrodes, oxidation of ferrocyanide produces the electric current that is proportional to creatinine concentration in sample. The amperometry measurement of creatinine standard samples shows a linear relationship between the electric current and creatinine concentration (Fig. 4.8).
Figure 4.8. System Validation - Creatinine. Creatinine concentration was measured by electrochemical amperometry using a three enzymes method (Left). The amperometry measurement of creatinine standard samples showed a linear relationship between the output current and creatinine concentration (Right).
4.2.1.2.3 Protein

Concentration of target proteins could be measured by our NexT system using the immuno-magnetic sandwich assay\(^{40}\) (Fig. 4.9). Capturing antibodies specific to target proteins are conjugated with epoxy magnetic bead (M-270; Dynabead). The conjugated magnetic beads capture and enrich target proteins. Then, biotinylated detection antibodies are incubated with target-captured magnetic beads, and then avidin-HRP (ThermoFisher Scientific) is conjugated together. Lastly, TMB substrate (ThermoFisher Scientific) was added to amplify signals from HRP by inducing redox recycling. The NexT system measures reduction current from oxidized TMB at the given electrical potential (-0.1 V) between working and reference electrodes. As a model system, WE chose to test ZIKA virus non structural protein, called ZIKA NS1. Recombinant ZIKA NS1 proteins were diluted in PBS, and the diluted samples were captured by anti-ZIKA NS1 antibody coated magnetic beads. Then, the ZIKA NS1 captured magnetic beads were labeled with biotinylated detection antibodies and HRP. The reduction current from HRP and TMB reaction showed good match with the result from ELISA measurement (Fig. 4.10).
Capturing antibodies specific to target proteins are conjugated with epoxy magnetic bead (M-270; Dynabead). The conjugated magnetic beads capture and enrich target proteins. Then, biotinylated detection antibodies are incubated with target-captured magnetic beads, and then avidin-HRP (ThermoFisher Scientific) is conjugated together. Lastly, TMB substrate (ThermoFisher Scientific) was added to amplify signals from HRP by inducing redox recycling.
Figure 4.10. System Validation - Protein. ZIKA viral NS1 protein was qualitatively analyzed using the NexT system (Right) and compared with ELISA measurement (Left).
4.2.1.2.4 Nucleic Acid

Nucleic acid could also be detected by our NexT system using in-vitro translation assay\textsuperscript{133} (Fig. 4.11). Template single stranded DNA, consisting of target binding site and LACZ reporter, was prepared. Once target ssDNA, which is complementary to binding site in template ssDNA, added to the template, β-galactosidase is produced from LACZ reporter with addition of T7 in-vitro translation mix (New England Biolabs). The produced β-galactosidase, in turn, hydrolyses 4-aminophenyl β-D-galactopyranoside (PAPG) into p-aminophenyl (PAP), and PAP generates oxidizing current on the working electrode at the given electric potential (0.3 V) between working and reference electrodes. As a model system, different concentrations of target RNA were prepared and assayed as described above. The resulting amperometric read outs showed good agreement with the result from western blot (Fig. 4.12).
Figure 4.11. System Validation - Nucleic Acid. ssDNA was detected using in-vitro protein synthesis assay. In the presence of target ssDNA, β-galactosidase is produced, and hydrolyses PAPG into PAP. Then, PAP generates oxidizing current on the working electrode.
Figure 4.12. Titration Measurement of ssDNA. Different concentrations of ssDNA were measured using the NexT system and compared with western blot result.
4.2.2 Bladder Cancer Extracellular Vesicle (BCEV) Profiling

We applied the NexT system to profile bladder cancer extracellular vesicles profiling (BCEV). For BCEV assaying, we adapted immuno-magnetic bead sandwich assay for isolation and detection of target proteins on the surface of BCEVs. The prepared EV solution will be mixed with anti-CD63/CD9/CD81 antibody coated magnetic bead solution for 15 min at room temperature. Magnetic beads with captured EV will be separated by a permanent magnet, and resuspended in PBS. The captured EV will then be labeled with biotinylated antibodies against target proteins. After another magnetic separation step to eliminate excessive biotinylated antibodies, streptavidin conjugated HRP will be added and washed. The prepared EV solution and TMB solution will be placed on disposable electrode strip for electrochemical detection using the NexT system. This approach has many practical advantages: i) cell-specific EVs can be isolated directly from complex media without the need for extensive filtration or centrifugation; ii) the assay can achieve high detection sensitivity, by combining the merits of both magnetic enrichment and enzymatic signal amplification; iii) based on the electrical detection scheme, sensors can be easily miniaturized and expanded for parallel measurements.

To test enrichment efficiency of EVs from clinical urine specimen, we spiked EVs from RT4 bladder cancer cell line into human urine. Then, EVs were isolated using both conventional ultracentrifugation and immuno-magnetic beads. The enrichment ratio was ten fold higher when using immuno-magnetic beads than when using conventional ultracentrifugation (Fig. 4.13).
Figure 4.13. Enrichment of Bladder Cancer Extracellular Vesicle.

UC: with ultracentrifuge
NC: NoUC, without ultracentrifuge
Ur: Urine
Ex: Extracellular Vesicles
We also performed CD63 positive EV titration experiment, and the limit of detection was $10^5$ EVs (Fig. 4.14).

A panel of bladder cancer cell lines (SV-HUC1, T24, RT4, VM-CUB-1) were cultured in vesicle depleted medium (containing 5% vesicle depleted FBS) for 48 hours. Conditioned medium from $\sim 10^7$ cells was collected and centrifuged at 300 g for 5 min. Supernatant was filtered through a 0.2 um filter (Millipore), and concentrated via ultra centrifugation (100 000 g for 1h). The exosome pellet was washed with PBS and centrifuged again at 100 000 g for 1 h, and resuspended in PBS. The final concentration was measured by nanoparticle tracking analysis (NanoSight).

Target surface markers are selected based on previous exosome screening from our group and prior studies. (EpCAM, EGFR, HER2, MUC1/4, FGFR1/3, PD-L1/2, SDC-1, TACSTD-2, NUMA-1, CD24, HSP8A, FAS, FASL, Vimentin, a6-integrin, b1-integrin). To investigate the cellular origin of the BCEV, the surface marker expression profile of EVs was compared with that of the cultured bladder cell lines. The surface marker expression of the cultured cell lines was observed by flow cytometry, and EV samples ($\sim 10^9$ vesicles/mL) were profiled for surface protein biomarkers by the NexT system as explained above (Fig. 4.15).
Figure 4.14. EV titration Experiment.
**Figure 4.15. Bladder Cancer Cell Line Profiling.** A panel of bladder cancer cell lines (SV-HUC1, T24, RT4, VM-CUB-1) were cultured and EVs from the culture cell lines were isolated using differential ultracentrifugation. Then, the surface marker expression profile of EVs was compared with that of the cultured bladder cell lines.
4.3 Summary and Conclusion

We have developed a portable platform, wirelessly powered electrochemical system, for fast, streamlined molecular analyses. The developed system was validated with various biological targets including glucose, creatinine, viral protein, and RNA. We also applied this technology to bladder cancer extracellular vesicle (BCEV) detection, BCEVs were isolated and molecularly profiled using immuno-magnetic bead assay. The enrichment ratio was ten fold higher when using immuno-magnetic beads than when using conventional ultracentrifugation.
Chapter 5. Conclusion and Perspectives

5.1 Summary

This thesis introduced the rapid, low cost, and highly sensitive and specific platforms for the detection of circulating cancer biomarkers using electromagnetic methods. First, we reported the single channel portable NMR system that could detect cancer cells or proteins labelled with MNPs. We showed diverse applications of target-MNP assaying schemes to detect cancer cells and proteins. Also we implemented adaptive temperature compensation techniques to achieve robust NMR measurement with mobile devices in point of care settings. The developed NMR system could detect as low as 20 cancer cells in 5 ul. samples. Also, we demonstrated the multichannel digital NMR system for high throughput and multi-frequency NMR measurement. The multichannel NMR system could measure T1, T2, and NMR spectrum of the biological or chemical samples with programmable resonance frequency. Furthermore, multichannel capability, together with time interleaving CPMG and inversion recovery techniques, could significantly reduce measurement time and energy. Second, we have developed micro-Hall magnetometer that could molecularly profile single cancer cell with magnetic multiplexing. The micro-Hall magnetometer showed its magnetic multiplexing capability to differentiate magnetic particles with distinct magnetic moments from their mixture. We applied this technology to measure molecular expressions of two surface protein markers from single ovarian cancer cell.
Third, we introduced wirelessly powered electrochemical system that could detect cancer specific EV and DNA. To minimize sample purification steps, we introduced immuno-magnetic sandwich assaying scheme for the enrichment of EVs from clinical specimens such as urine without ultracentrifugation, consequently reducing significant amount of time for sample preparation. Using the developed electrochemical system and immuno-magnetic sandwich assaying scheme, we profiled cancer specific transmembrane proteins from EV of bladder cancer cell lines and identified highly correlative cancer EV specific biomarkers for bladder cancer detection. We also demonstrated electrochemical detection of RNA using in-vitro protein synthesis assay. As a proof of a concept, we could detect as low as 0.1 uM of target RNA using β-galactosidase and PAPG electrochemical reaction.

5.2 Perspectives and Future Direction

We have developed three innovative biosensing platforms for circulating cancer marker detection in point of care settings. The portable NMR system was based on proton NMR and designed to detect water protons interacting with MNP-labeled biological targets. This system provides high throughput sample processing via bulky samples in multichannel NMR probes and the sample assay schemes are versatile for any types of biological targets including cells, proteins, and nucleic acids, but one minor disadvantage is that samples should be in a bulkily aqueous solution for proton NMR measurement, thus it is not easy to observe single particle or cell. To overcome this challenge, we designed micro-Hall magnetometer that can measure induced magnetic field
directly from magnetic particle itself or magnetic particles labeled single cell, enabling to observe both aqueous and solid forms of samples. Also, by fabricating micron size Hall sensor elements and integrating with microfluidics, the micro-Hall magnetometer can capture and measure micron size samples such that we could observe intrinsic magnetic properties of single magnetic particle or molecular expression of single cancer cell.

We expect several technical improvements to further advance micro Hall magnetometer technology. First, implementing a monolithically integrated chip, containing Hall elements, coils, amplifiers, and signal processing units, would improve detection sensitivity, SNR, and assay throughput. Since Hall sensor has a differential output, load balancing of analog amplifiers is an important factor to determine SNR. Reducing parasitic components from wire connections and electromagnetic interference could help achieve higher SNR of the system. Integrating a large number of Hall sensors in a single chip could increase assay throughput and efficiency of single cell analyses. Second, the capacity of magnetic multiplexing could be expanded. We note that this system can measure both magnetic susceptibility and saturation magnetic moment. Leveraging these properties through MNP size and composition control, it is conceivable to create a panel of MNPs with distinct magnetic signatures. These particles could be used, for example, to simultaneously profile different molecular markers in individual cells. With such improvements, the micro Hall magnetometer could serve as a convenient tool for both MNP characterization and point-of-care biosensing.

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We also developed wireless powered electrochemical system streamlines cancer testing processes including sample acquisition, purification, and measurement. Simplicity is the biggest advantage of this system compared to magnetic or optical detection methods. This system only needs simple amplifiers and printed electrodes unlike the portable NMR and micro Hall magnetometer require magnets, coils, sophisticated signal processing electronics. Furthermore, using immuno-magnetic sandwich assaying scheme, we could finally exclude time and energy intensive ultracentrifugation from sample purification steps such that we could directly purify our targets such as extracellular vesicles from liquid biopsied clinical specimens, thus saving time and cost of testings. Needless to say, wireless power supply feature enables the system work at any place without battery or wired electricity while carrying credit card size system in a wallet or pocket.

We will further evaluate clinical utility of the wirelessly powered electrochemical system. Urine or blood samples from bladder cancer patients can be analyzed for the identified surface protein markers in bladder cancer cell line profiling. The result will be further analyzed by supervised machine learning classification algorithms. Test sensitivity and specificity can be compared with urine cytology, tissue pathology, and FDA-approved rapid testing methods (NMP22 Bladder Chek, Alere; BTA-TRAK, Polymedco). To investigate mRNA expressions in BCEV, we can develop isothermal PCR assay for electrochemical sensing using DNA intercalating dyes.
Altogether, the portable NMR, micro Hall magnetometer, and wirelessly powered electrochemical system will be powerful tools for cancer diagnosis, prognosis, and treatment monitoring such as immunotherapy in both clinical laboratory and point-of-care settings. Each system has its own strength depending on types of clinical specimens and biological targets. The portable NMR and micro Hall magnetometer are more suitable for cellular analysis while the wirelessly powered electrochemical system performs well with smaller objects such as EVs, proteins, and nucleic acids. By deploying them against proper targets, we anticipate that the novel electromagnetic systems introduced in this thesis will bring us bright future to deliver affordable and accessible cancer care to people who live in medically underserved countries.
APPENDIX A

Symbols used in the text.

<table>
<thead>
<tr>
<th>Description</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \omega_r$</td>
<td>$d_{c}$ diameter of a MNP</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>$d_{e}$ diameter of a MNP cluster</td>
</tr>
<tr>
<td>$D$</td>
<td>$M_i$ saturation magnetization for a MNP</td>
</tr>
<tr>
<td>$\nu$</td>
<td>$M_c$ saturation magnetization for a cluster</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$[C]$ concentration of target molecules</td>
</tr>
<tr>
<td>$\mu_0$</td>
<td>$R_{2i}$ relaxation rate before MNP clustering</td>
</tr>
<tr>
<td>$k$</td>
<td>$R_{2f}$ relaxation rate after MNP clustering</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$R_{2w}$ relaxation rate for water</td>
</tr>
<tr>
<td>$f$</td>
<td>$m_i$ initial MNP concentration</td>
</tr>
<tr>
<td>$R_{2c}$</td>
<td>$n_f$ MNP concentration after MRSw reaction</td>
</tr>
<tr>
<td>$R_{2e}$</td>
<td>$n_c$ MNP-cluster concentration</td>
</tr>
<tr>
<td>$r_{2c}$</td>
<td>$\zeta$ binding capacity of MNPs for target molecules</td>
</tr>
<tr>
<td>$r_{2e}$</td>
<td>$\theta$ number of binding sites per target molecule</td>
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</table>

1. Relaxation model for MNP clusters

The effective magnetization ($M_c$) of MNP clusters is lower than that of single MNPs due to the quasi-solid nature of the clusters. Inside a cluster of diameter $d_e$, the number of MNPs is given by

\[ k = \left( \frac{d_e}{d_i} \right)^f. \]

The cluster magnetization is then obtained as

\[ M_c = \frac{k \cdot M_i \cdot d_i^3}{d_e^3} = M_i \cdot (d_e/d_i)^f. \]

1.1. Motional averaging (MA) mode

From the outer-sphere relaxation model, the relaxation rate ($R_{2c} = 1/T_2$) with MNP clusters is given as

\[ R_{2c} = \frac{4}{9} \nu \tau_D (\Delta \omega_r)^2, \]

where

\[ \Delta \omega_r = \frac{1}{3} \mu_0 \gamma M_c. \]
Using Eq. (2), $R_2$ is expressed as

$$R_{2c} = \frac{4}{9} \nu \cdot \frac{d_s^2}{D} \left( \frac{1}{3} \mu_0 \gamma M_s \right)^2 \left( \frac{d_c}{d_s} \right)^{2j+q}$$

(6)

where $R_{2c}$ is the relaxation rate before MNP clustering. Note that the volume fraction $\nu$ of MNPs remains the same before and after MNP clustering. Thus the scaling law for relative $R_2$ changes is given by

$$\left( \frac{R_{2c}}{R_{2s}} \right) = \left( \frac{d_c}{d_s} \right)^{2j+q}$$

(7)

### 1.2. Static dephasing (SD) mode

In the static dephasing regime, the relaxation rate in the presence of MNP clusters is given by

$$R_{2c} = \sqrt{\frac{15\pi}{9}} \cdot \nu \cdot \Delta\omega_s$$

(8)

Substituting $\Delta\omega_s$ with Eq. (4) and using the expression for $M_s$, Eq. (8) is simplified as

$$R_{2c} = \frac{\sqrt{15\pi}}{9} \cdot \nu \left( \frac{1}{3} \mu_0 \gamma M_s \right) \left( \frac{d_c}{d_s} \right)^{j+q}$$

(9)

and the following scaling law is obtained,

$$\left( \frac{R_{2c}}{R_{2s}} \right)_{SD} = \left( \frac{d_c}{d_s} \right)^{j+q}$$

(10)
2. Analytical model for MRSw assays

For a given initial MNP concentration \(n_i\), the \(R_2\) of a sample is

\[
R_{2i} = R_2^s + r_2s \cdot n_i,
\]

where \(r_2s\) is the relaxivity of single MNPs. After clustering, the relaxation rate changes to

\[
R_{2f} = R_2^s + r_2s \cdot n_f + r_2c \cdot n_c,
\]

where \(r_2c\) is the relaxivity of MNP clusters, and \(n_c\) and \(n_f\) are final concentrations of single MNPs and clusters, respectively. The total number of MNPs is conserved before and after the reaction, leading to

\[
n_i = k \cdot n_c + n_f.
\]

Since all target molecules are consumed for clustering, their concentration \([C]\) will be proportional to \(n_c\),

\[
[C] = \alpha \cdot n_c,
\]

where \(\alpha\) is the average number of target molecules per MNP cluster. For the avidin-biotin system used in the report, we experimentally determined \(k\) and \(\alpha\) (CLIO: \(k \approx 13, \alpha \approx 11\); other type of MNPs: \(k \approx 17, \alpha \approx 9\)).

2.1. Lower detection limits for MA mode

In the MA mode, \(R_2\) values increase after MNP clustering. We apply a conservative 5\% cut-off for minimal, detectable \(R_2\) changes:

\[
\frac{R_{2f} - R_{2i}}{R_{2i}} \geq 0.05.
\]

Substituting Eqs. (11) & (12) into Eq. (15) leads to

\[
r_2s \cdot (n_f - 1.05n_i) + r_2c \cdot n_i \geq 0.05R_2^s.
\]

Using Eqs. (13) & (14), the lower bound for \([C]\) is given as

\[
[C] \geq \frac{0.05 \cdot \alpha (R_2^s + r_2s \cdot n_i)}{r_2c - k \cdot r_2s}.
\]

For a given MNP type, the absolute minimum value of \([C]\) that satisfies Eq. (15) is obtained when \(n_i = k \cdot n_c\) (i.e., all MNPs are consumed to make clusters, and \(n_f\) is minimized). Imposing such a condition, we find the theoretical lower limit of \([C]\),

\[
[C]_{\text{min}} = \frac{0.05 \cdot \alpha R_2^s}{r_2s - 1.05k \cdot r_2c}.
\]

From Eqs. (1) & (7), the relaxivity of MNP clusters \(r_2c\) is expressed as a function of the relaxivity of single MNPs \(r_2s\).
\[ r_{2c} = \frac{R_{2c}}{n_c} = \frac{R_{2c}^2 \cdot k \cdot k}{n_c} = r_{2c} \cdot k^{\frac{-4}{r}} \]

Substituting Eq. (19) into Eq. (18), the theoretical lower limit of \([C]\) is obtained as a function of \(r_{2n}\):

\[ [C]_{\text{min}} = \frac{1}{r_{2s}} \cdot \frac{0.05 \cdot \alpha R_2^*}{k \cdot k^{\frac{-4}{r}} - 1.05k} \]

2.2. Lower detection limits for SD mode

In the SD mode, \(R_2\) values decrease upon MNPs clustering. We thus apply the following criterion for reliable detection of \(R_2\) changes:

\[ \frac{R_{2i} - R_{2f}}{R_{2i}} \geq 0.05. \]

Following the same procedure as in the MA mode case, we obtain the lower bound for \([C]\):

\[ [C] \geq \frac{0.05 \cdot \alpha (R_{2s}^* + r_{2s} \cdot n_1)}{k \cdot r_{2s} - r_{2c}}. \]

Using the same condition \((n_1 = k \cdot n_c)\) as in the MA mode, the theoretical minimum for \([C]\) is given as

\[ [C]_{\text{min}} = \frac{0.05 \cdot \alpha R_2^*}{0.95 r_{2c} - k \cdot r_{2s}}. \]

From Eqs. (1) & (10), the relaxivity of MNP clusters, \(r_{2c}\), becomes

\[ r_{2c} = r_{2s} \cdot k^{\frac{2}{r-1}} \]

Substituting Eq. (24) into (23), the theoretical lower limit of \([C]\) is given as

\[ [C]_{\text{min}} = \frac{1}{r_{2s}} \cdot \frac{0.05 \cdot \alpha R_2^*}{0.95 k^{\frac{-2}{r-1}} - k} \]

2.3. Upper detection limits for MA and SD modes

The upper detection limit would be set by the binding capacity \((\xi)\) of MNPs for target molecules. If the amount of target molecules exceeds the total binding sites available, each MNP will be covered with target molecules and inter-particle clustering will be obstructed. The binding capacity \((\xi)\) can be estimated as follows. With \(\alpha\) and \(k\) respectively defined as the average number of target molecules and MNPs per
cluster, \( \alpha/k \) reflects the number of target molecules per MNP inside a cluster. The binding capacity \( \xi \) can be given as

\[
\xi = \theta \cdot \frac{\alpha}{k},
\tag{26}
\]

where \( \theta \) is the number of binding sites per target molecule. Each avidin molecule has four binding sites for biotin, and from the MRSw assay\textsuperscript{134}, \( \theta \) was found to be in the range of 3 - 4; for ELISA type MRSw assays, \( \theta \approx 2 \). For a given MNP concentration \( (n_i) \), we thus have the upper detection bound for \( [C] \),

\[
|C| \leq \theta \cdot \frac{\alpha}{k} \cdot n_i.
\tag{27}
\]
3. Transition from MA to SD for MNP clusters

All single MNPs start off in the MA mode, and their relaxivity is given as

\[ r_{21} = \frac{V_p}{1000} \cdot \frac{4}{9} \cdot \tau_p' \cdot \Delta \omega_s^2 \]

where

\[ \Delta \omega_s = \frac{2}{\sqrt{5}} \cdot \gamma \cdot \frac{4\pi}{3} \cdot M_s. \]

After clustering, the effective magnetization changes due to the fractal nature of the clusters [Eq. (2)] and \( \Delta \omega_c \) is accordingly written as

\[ \Delta \omega_c = \frac{2}{\sqrt{5}} \cdot \gamma \cdot \frac{4\pi}{3} \cdot M_s \cdot \left( \frac{d_i}{d_f} \right)^{1-s} = \Delta \omega_s \cdot k^{1-s}. \]

From Eq. (28), we obtain

\[ \Delta \omega_c = \left( \frac{1000 \cdot 9 \cdot r_{21}}{V_p \cdot 4 \cdot \tau_p'} \right)^{1/s}. \]

MNP clusters fall into the SD mode when

\[ \Delta \omega_c \cdot \tau_p' \geq 1. \]

Substituting Eqs. (30) & (31) into (32)

\[ \left( \frac{1000 \cdot 9 \cdot r_{21}}{V_p \cdot 4 \cdot \tau_p'} \right)^{1/s} \cdot \tau_p' \cdot k^{1-s} \geq 1. \]

The minimum relaxivity of single MNPs to have MNP clusters in the SD mode can then be expressed as

\[ r_{21} \geq \frac{8}{27} \cdot \frac{\pi}{1000} \cdot D \cdot d_s \cdot k^{1-s}. \]

For MNPs with \( d_s = 16 \) nm \( f = 2.4 \), \( k = 17 \) and diffusion constant of water \( D = 2 \times 10^{-5} \) cm\(^2\)/sec, the transition from MA to SD for MNP clustering happens when

\[ r_{21} \geq 1.1 \times 10^{-15} \text{ L/sec}. \]

4. Estimation of the lowest detection limit with MNPs

In this estimation, we assume the use of hypothetical, highly magnetic Fe-MNPs with a diameter of 22 nm. As compared to similarly-sized 22 nm FeO MNPs, these Fe-MNPs have a 3.4 (= 1750/510) times higher \( M_s \), and thus a 12-fold larger \( r_{21} \) [see Eq. (3)]. With the lower detection limit scaling as \( 1/r_{21} \) [Eq. (32)]...
(15), the Fe-MNPs are expected to have 12-times lower $[C]_{min}$ than that with the Fe$_3$O$_4$. Since the measured $[\text{avidin}]_{min}$ for the 22 nm Fe$_3$O$_4$ particles is ~1 pM, the Fe MNPs are expected to the detection limit of $[\text{avidin}]_{min}$ ~100 fM.
APPENDIX B

Symbols used in the text

<table>
<thead>
<tr>
<th>Description</th>
<th>Description</th>
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<tbody>
<tr>
<td>$V_H$ Hall voltage (Hall element)</td>
<td>$T$ Temperature</td>
</tr>
<tr>
<td>$V_{H, Lock}$ Hall voltage (from lock-in amplifier)</td>
<td>$\omega$ frequency of time varying magnetic field</td>
</tr>
<tr>
<td>$B$ total magnetic induction</td>
<td>$n$ number density</td>
</tr>
<tr>
<td>$H$ magnetic field strength</td>
<td>$V$ volume</td>
</tr>
<tr>
<td>$M$ magnetization of material</td>
<td>$G$ magneto-geometrical factor</td>
</tr>
<tr>
<td>$H_{DC}$ external magnetic field (static)</td>
<td>$R_H$ Hall coefficient</td>
</tr>
<tr>
<td>$H_{AC}$ external magnetic field (time varying)</td>
<td>$t$ thickness of device</td>
</tr>
<tr>
<td>$\mu_p$ magnetic moment</td>
<td>$I$ DC bias current</td>
</tr>
<tr>
<td>$\chi_v$ magnetic susceptibility</td>
<td>$\mu_0$ vacuum permeability</td>
</tr>
<tr>
<td>$\chi_0$ magnetic susceptibility at H=0</td>
<td>$k_B$ Boltzmann constant</td>
</tr>
</tbody>
</table>

1. Hall output is proportional to magnetic susceptibility at the given DC polarization field.

Hall voltage, $V_H$, is given by $^{105,110}$

\[
V_H = \frac{GR_H}{t} IB
\]  

If external magnetic fields, $H_{DC}$ and $H_{AC}$, are applied perpendicular to Hall sensor plane, then magnetic field induction, $B$, is sum of external magnetic field strength and induced magnetic field from magnetic materials.

\[
B = \mu_0 \left[ H_{DC} + H_{AC} e^{i\omega t} + M(H) \right]_{H=H_{DC}+H_{AC} e^{i\omega t}}
\]

where

\[
B = \mu_0 (H + M(H))
\]

\[
H = H_{DC} + H_{AC} e^{i\omega t}
\]

Using Eq. (2), $V_H$ is expressed as
The first order of Taylor approximation of Eq. (5) leads to

\[ V_H = \frac{\mu_0 G R_H}{t} I \left[ H_{DC} + H_{AC} e^{j \omega t} + M(H_{DC} + H_{AC} e^{j \omega t}) \right] \]

Frequency modulated Hall voltage is down-converted to DC signal by lock-in amplifier. Then Eq. (6) can be written as a function of magnetic susceptibility.

\[
V_{H,\text{lock}} = \frac{\mu_0 G R_H}{t} I \left( H_{AC} + \frac{dM}{dH} \bigg|_{H=H_{AC}} \cdot H_{AC} \right)
\]

\[= \frac{\mu_0 G R_H}{t} I H_{AC} \cdot \left( 1 + \frac{dM}{dH} \bigg|_{H=H_{AC}} \right)\]

\[= \frac{\mu_0 G R_H}{t} I H_{AC} \cdot \left[ 1 + \chi' \left( H_{DC} \right) \right] \]

2. Normalized magnetic susceptibility

Magnetization of superparamagnetic nanoparticles is given as\textsuperscript{109,111}

\[ M(H) = M_0 \cdot \left[ \coth \left( \frac{\mu_0 \mu_p H}{k_B T} \right) - \frac{k_B T}{\mu_0 \mu_p H} \right] \]

where

\[ M = \frac{m}{V} \]

\[ M_0 = n \mu_p \]

From Eq. (8), we can obtain magnetic susceptibility by differentiating magnetization with respect to magnetic field strength.
\[ \frac{dM}{dH} = M_0 \cdot \frac{\mu_0 \mu_p}{k_B T} \left[ -\csc h^2 \left( \frac{\mu_0 \mu_p H}{k_B T} \right) + \left( \frac{\mu_0 \mu_p H}{k_B T} \right)^{-2} \right] \]

\[(11)\]

\[ = M_0 \cdot \frac{\mu_0 \mu_p}{3k_B T} \cdot 3 \cdot \left[ -\csc h^2 \left( \frac{\mu_0 \mu_p H}{k_B T} \right) + \left( \frac{\mu_0 \mu_p H}{k_B T} \right)^{-2} \right] \]

\[ = \chi_0 \cdot 3 \cdot \left[ -\csc h^2 \left( \frac{\mu_0 \mu_p H}{k_B T} \right) + \left( \frac{\mu_0 \mu_p H}{k_B T} \right)^{-2} \right] \]

Therefore, normalized magnetic susceptibility becomes

\[(12)\]

\[ \therefore \frac{\chi}{\chi_0} = 3 \cdot \left[ -\csc h^2 \left( \frac{\mu_0 \mu_p H}{k_B T} \right) + \left( \frac{\mu_0 \mu_p H}{k_B T} \right)^{-2} \right] \]
REFERENCES


