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Miniaturized Nuclear Magnetic Resonance Platform for Detection and Profiling of Circulating Tumor Cells

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Abstract

Accurate detection and profiling of circulating tumor cells (CTCs) is a highly sought after technology to improve cancer management. Such “liquid biopsies” could offer a non-invasive, repeatable window into each patient’s tumor, facilitating early cancer diagnosis and treatment monitoring. The rarity of CTCs, approximated at 1 CTC for every billion peripheral blood cells, however, poses significant challenges to sensitive and reliable detection. We have recently developed a new biosensor platform, namely a micro-nuclear magnetic resonance (μ NMR). Through the synergistic integration of microfabrication, nanosensors, and novel chemistries, the μ NMR platform offers high detection sensitivity and point-of-care operation, overcoming technical barriers in CTC research. We herein review the μ NMR technology with emphasis on its application on CTC detection. Recent advances in the sensing technology will be summarized, followed by the description on the dynamic interplay between preclinical and clinical CTC studies.

I. INTRODUCTION

Mounting evidence supports the view that molecular profiling of cancer is a preferred method of classifying tumors, stratifying patients for targeted therapies, assessing treatment efficacy, and achieving clinical benefit.^{1–4} Failure to detect molecular differences of otherwise histologically identical tumors can also lead to underpowered clinical trials,⁵ thus creating missed opportunities for identifying effective therapies in specific patient subsets. Furthermore, molecular profiling is helpful in assessing treatment efficacy over time but serial surgical biopsies raise morbidity, compliance, safety and cost concerns. For these reasons, there has been a desire to shift to more clinically available samples, notably peripheral blood where circulating tumor cells (CTCs) can be analyzed.

Attempts to identify CTC have gained traction in solid tumors,^{6–9} but their reliable detection has been challenging using currently accepted enumeration techniques. The most commonly used cytometric method, Cell Search, is FDA-approved and based on enumeration of epithelial cells using anti-epithelial cell adhesion molecule (EpCAM) antibodies and

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subsequent staining for visualization.¹⁰ Its comparatively lengthy isolation and staining steps, however, are accompanied by considerable cell loss (~20–40%).^{11,12} It is generally accepted that EpCAM-based detection also has a low sensitivity in EpCAM-negative cancers, which may explain why a considerable fraction (up to 70% in some studies) of patients with metastatic epithelial malignancies fail to exhibit detectable CTCs using such methods. This is especially the case for aggressive tumor cells, which often downregulate EpCAM during epithelial-mesenchymal transition (EMT).¹³ Novel and rapid detection strategies extending beyond EpCAM are needed to promote rare cancer cell studies. Given that some trials are starting to stratify and tailor patient therapy based on CTC changes (clinicaltrials.gov ID: NCT00382018), this is becoming increasingly important and relevant today.

We previously developed a novel sensing technology, termed micro-nuclear magnetic resonance (μ NMR), which enables rapid and highly sensitive biomarker detection.¹⁴ μ NMR exploits magnetic resonance technology (similar to a contrast enhanced MRI scan) to detect cells labeled with immunospecific magnetic nanoparticles (MNPs). These nanoparticles are typically much smaller (tens of nm) compared to larger beads used for immunoseparation and are superparamagnetic, rather than ferromagnetic. Samples containing MNP-labeled cells display faster relaxation of NMR signals due to local magnetic fields created by MNPs.¹⁵ Since signal detection is based on magnetic interactions, μ NMR can be performed with minimal sample purification steps, which reduces cell loss and simplifies assay procedures.¹⁴ Through systematic optimization of nanoagents,^{16–20} conjugation chemistry,^{21,22} and NMR detectors,^{14,23,24} the μ NMR platform has been considerably advanced, enabling sensitive and robust detection on a wide range of targets, including nucleic acids,^{25,26} proteins,¹⁴ exosomes,²⁷ bacteria,^{28–30} and tumor cells³¹. Most recently, the platform has been adopted to detect and profile CTCs for point-of-care read outs. By leveraging the synergies between the preclinical and clinical spaces (Fig. 1), μ NMR technology has enabled robust detection and molecular profiling of CTC.^{32,33} This article will comprehensively review μ NMR technology, detailing recent technical developments and translational work.

II. μ NMR TECHNOLOGY

Detection of MNP-labeled cells can be facilitated by exploiting the “ T_2 -shortening” effect of MNPs in NMR measurements.¹⁵ When placed in static, polarizing magnetic fields for NMR detection, MNPs produce local dipole fields with strong spatial dependence, which efficiently destroys 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 (Fig. 2).

The capability of MNPs to induce T_2 changes is defined as transverse relaxivity (r_2).³⁴ With MNPs in solution, the relaxation rate ($R_2 = 1/T_2$) can be expressed as¹⁶

$$R_2 = R_w + r_2 \cdot \frac{N_P}{V}, \quad (9)$$

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 ($\Delta R_2 = R_2 - R_w$) due to MNPs is given as

$$\Delta R_2 = r_2 \frac{N_P}{V} = \frac{n \cdot r_2}{V} N_C = r_2^{cell} \cdot \frac{N_C}{V}, \quad (10)$$

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. μ NMR can thus be used effectively for molecular profiling of target cells.¹⁶ Eq. 2 highlights three important ways to enhance μ NMR sensitivity.

- **MNPs engineered for high transverse relaxivity.** Pronounced changes in the relaxation rate (R_2) will occur when biological targets are labeled with MNPs of high transverse relaxivity (r_2).³⁵ Making magnetically stronger MNPs will benefit measurements, as r_2 concomitantly increases with the magnetic moment of particles.^{36,37}
- **Efficient MNP labeling on cells.** Detection sensitivity will improve by increasing the number (N_c) of MNPs per cell, which calls for the optimization of MNP-labeling strategy.
- **Miniaturized NMR probes.** Higher sensitivity can be achieved by decreasing the NMR detection volume (V), as it can effectively increase the analyte concentration (N_c/V) and lead to large R_2 . This is the most important rationale for implementing a miniaturized NMR system.

The following sections describe how each of these aspects were addressed during μ NMR development, particularly as they relate to CTC detection.

II.1. New magnetic nanoparticles

The transverse relaxivity (r_2) of MNPs is known to scale as $r_2 \sim d_p^2 \cdot M_p^2$, where d_p and M_p denote particle size and magnetization, respectively.^{36,37} Efforts to improve r_2 have thus focused on synthesizing magnetically stronger and larger particles.

Ferrite MNPs—Ferrite-based MNPs have gained favor in biomedical applications due to their excellent stability, biocompatibility, and ease of synthesis. Among the various ferrite types, Manganese (Mn)-doped magnetite (MnFe_2O_4) assumes the highest magnetization due to the high spin quantum number (5/2) of Mn^{2+} . We thus selected MnFe_2O_4 as a core material, and applied the sizing strategy to synthesize large MnFe_2O_4 MNPs.¹⁶ These particles were produced by reacting iron (III) acetylacetonate and manganese (II) acetylacetonate at high temperature (300 °C). The initial reaction led to the creation of 10-nm MnFe_2O_4 particles. Subsequently, the particle size was incrementally grown to 12, 16 and 22 nm through seed-mediated growth (Fig. 3a). MnFe_2O_4 nanoparticles with diameter 16 nm were found to be highly monodisperse and superparamagnetic at 300 K. Prepared particles were rendered water-soluble by coating their surface with small molecules (meso-2,3-dimercaptosuccinic acid; DMSA).³⁸

Figure 3c compares the r_2 values of different types of magnetite MNPs. For a given particle composition, r_2 values increase with particle size (d_p). Interestingly, M_p also increases with d_p , possibly due to reduced surface effect (e.g., spin-canting) in larger particles. Enlarging particle size, therefore, improved r_2 values much faster than expected. For a given particle size, MnFe_2O_4 MNPs assumed the highest r_2 ($= 6 \times 10^{-14} \text{ L} \cdot \text{s}^{-1}$ per particle) on account of their high magnetization. The maximum r_2 of ferrite MNPs, however, was limited by the bulk magnetization of MnFe_2O_4 .

Fe-core MNPs: It has been suggested that elemental iron (Fe), rather than iron oxides, may serve as a superior constituent material for MNPs.^{39,40} Among ferromagnetic crystals, Fe assumes the highest saturation magnetization and relatively low magnetocrystalline anisotropy. It is thus possible to make larger Fe MNPs that possess high magnetic moments but still remain superparamagnetic. Fe MNPs, however, undergo rapid oxidation that

necessities protective shells.^{40,41} To date, most core/shell approaches have yielded less optimal Fe-MNPs, as the shell was formed either by artificially oxidizing the core^{17,42} or by coating it with non-magnetic material^{43,44}.

We recently developed a new type of Fe-MNP construct that maximizes particle magnetization.¹⁸ The particle has a hybrid structure (Fe@ferrite), consisting of an elemental Fe-core and a protective ferrite shell. The synthetic method has been optimized to make large Fe-cores and preserve them in subsequent processes. The Fe-core particles were first formed through the thermal decomposition of iron (0) pentacarbonyl. The particle size increased in proportion to the reaction temperature, which could be attributed to the higher reactivity of Fe ions at elevated temperatures.⁴⁵ Applying this approach, we could prepare Fe MNPs with diameters up to 16 nm while maintaining relative size variations at < 5%. We next coated the Fe-core with a ferrite shell by carrying out ferrite-synthesis in the presence of as-prepared Fe MNPs. As in ferrite MNP synthesis, the shell could be metal-doped (Fe@MFe₂O₄, M = Mn, Co, Fe; Fig. 3c) to further improve particle magnetization. The resulting particles showed high stability against oxidation with negligible changes in particle shape and magnetic properties. Importantly, the M_p values of Fe@MFe₂O₄ particles were higher than those of magnetite-based MNPs, which led to >400% increase in r_2 (Fig. 3b).

II.2. Bio-orthogonal strategy for efficient MNP labeling

Besides improving the physical properties of MNPs, our group has also developed more efficient methods for MNP-labeling. A conventional approach uses MNPs pre-conjugated with target-specific affinity ligands,¹⁴ which often requires extensive optimization of both the affinity ligands and the conjugation methods for each new target. This becomes cumbersome when larger numbers of markers are being investigated or when routine clinical use is contemplated since immune-conjugates typically have a shorter half-life compared to unlabeled antibodies.

Recently, we developed a novel targeting strategy, BOND (*BioOrthogonal Nanoparticle Detection*), that is modular, broadly applicable, and amplifies MNP-binding to biological targets.²¹ BOND is based on {4+2} Diels-Alder cycloaddition, notably the reaction between tetrazine (Tz) and *trans*-cyclooctene (TCO; Fig. 4a).⁴⁶ The reaction is fast, irreversible (covalent) and can be performed at room temperature without catalyst (historically copper). BOND chemistry has been adapted to cell-labeling with MNP (Fig. 4b), wherein cells are pre-targeted with TCO-modified antibodies and subsequently incubated with Tz-loaded MNPs (Tz-MNPs). Multiple TCO tags (usually ~20) can be incorporated onto an antibody without loss of affinity. Consequently, the antibodies function as scaffolds to promote multiple attachment of Tz-MNPs. Furthermore, compared to using direct antibody-MNP conjugates, the two-step approach (i.e., sequential application of antibody-TCO and Tz-MNPs) allows for easier access of antibodies to cell surface. More target markers thus can be occupied by separate antibody scaffolds, which leads to denser MNP-packing. Indeed, flow cytometry measurements showed that BOND yielded ~15-fold improvements in MNP-loading on cells, compared to labeling with antibody-MNP direct conjugates (Fig. 4c). The trend was further confirmed in μ NMR-based cell detection (Fig. 4d); the BOND method yielded more pronounced R_2 changes and improved the cellular detection limit.

To further improve BOND-mediated labeling, we focused on TCO modification of secondary IgG (and not primary) antibodies.³³ Specifically, cells are: 1) first labeled with primary antibodies (typically requiring lesser amounts); 2) targeted with secondary IgG antibodies previously modified with TCO and 3) then coated with MNPs. This approach spares excessive consumption of valuable primary antibodies, amplifies signal, and increases sensitivity given that multiple secondary antibodies bind to one primary antibody; these are key ingredients for successful CTC detection.

II.3. Miniaturized NMR system

We have developed different versions of miniaturized NMR devices for point of care measurements. System miniaturization affords the following advantages: 1) lowering detection limits by reducing sample volumes and effectively increasing analyte concentrations (Eq. 2); 2) increasing sensitivity of NMR probes (coils) since smaller coils generate stronger radio-frequency (RF) magnetic fields per unit current;⁴⁷ and 3) enabling the use of small, portable magnets to generate NMR magnetic fields as NMR probes become smaller.

μNMR prototypes—Figure 5a shows the first μNMR prototype (μNMR-1) developed to test the feasibility of system miniaturization. The μNMR-1 has four main components: microcoils, microfluidic networks, custom-designed NMR electronic and a portable permanent magnet ($B_0 = 0.5$ T). To enable parallel detection, eight planar microcoils were arranged into a 2×4 array format. On top of the coils, microfluidic networks were implemented for sample handling and distribution. The sample volume per microcoil was ~ 10 μL. The NMR electronics was designed to allow for spin-echo measurements. Such a detection scheme was needed to compensate for the magnetic field inhomogeneity from the small permanent magnet. We designed a new circuit that enabled versatile pulsed-NMR detection while using minimal electronic parts. This schematic has served as a blueprint for subsequent NMR systems.

In the second prototype (μNMR-2), we focused on improving the detection sensitivity by optimizing the NMR probe (Fig. 5b).^{16,17} We opted for solenoidal coils as the probe format, as they could produce more homogeneous RF magnetic fields and has less electrical resistance than planar coils. Unlike the conventional design wherein the coils were wound around capillary tubes, we completely embedded the solenoidal coils into the fluidic system. In this approach, the coils were first prepared by winding wires around a polyethylene tube. The coil-tube assembly was then cast-molded by pouring polydimethylsiloxane (PDMS). After PDMS curing, the tube was extracted to open up fluidic channels. With this new design, the entire coil bore can be filled with samples to maximize NMR signal level. Combined with lower electrical noise of the coils, the new probe showed 10-fold higher mass detection sensitivity than μNMR-1 and enabled highly sensitive detection. Furthermore, the PDMS-coil block could be seamlessly and easily integrated with other fluidic components (e.g., pumps, pneumatic valves, filters), enabling on-chip sample processing and NMR detection (Fig. 5c).

We also sought to further miniaturize our NMR system for portable operations. Specifically, we custom-designed a CMOS (complementary-metal-oxide semiconductor) chip (1.4×1.4 mm²) that integrates all necessary NMR electronics (Fig. 5d).²³ The whole NMR setup was packaged as a hand-held device, making μNMR-2 the world's smallest NMR system.

μNMR-3 system for clinical applications—Despite the successful demonstration of μNMR prototypes, it had still been challenging to adopt the platform for routine clinical applications. The major limitation arose from temperature-dependent fluctuation of the NMR frequency (f_0). The f_0 changes as the magnetic field (B_0) by a permanent magnet drifts with temperature. For example, with 1 °C increase in temperature, B_0 field by an NdFeB magnet will drop $\sim 0.1\%$ from its initial value, and f_0 will proportionally decrease by 0.1%. Such changes can place down-converted NMR signals near or beyond the low frequency cutoff (f_c) in the amplification stage (see the circuit diagram in Fig. 5a), distorting the measured signal. In a laboratory setting, the problem can be addressed by controlling the environmental and system temperatures. This solution, however, significantly increases the cost and size of the μNMR system, undermining its use for point-of-care applications.

For $\mu\text{NMR-3}$, we adopted an alternative, software-based approach: the f_0 was monitored before each measurement and the measurement settings were accordingly adjusted.²⁴ Figure 6a summarizes the tracking algorithm. When a measurement starts, the system sweeps the excitation frequency f to find the resonance frequency f_0 . For each f , the system measures the free induction decay of the NMR signal and transforms the signal into the frequency domain via fast Fourier transformation. A peak-finding routine then determines the frequency offset $\Delta f (= f - f_0)$. The whole process iterates until Δf reaches a target value, and a standard NMR measurement is then performed. To reduce processing time, the tracking routine is further divided into two parts: a coarse and a fine tracker. For the first measurement, the system will conduct both tracking methods. In the subsequent operations, however, the fine tracker will find the resonance frequency, based on the f_0 in the immediate previous measurement. The developed method guarantees reliable and robust μNMR measurements. For example, when the tracking routine was turned off, R_2 values fluctuated up to 200% of its initial value in a typical laboratory setting. With tracking on, the fluctuation was $< 3\%$ (Fig. 6b).

The $\mu\text{NMR-3}$ has other important aspects for practical use. The electronics were assembled using off-the-shelf integrated circuit (IC) parts (e.g., microcontrollers, RF synthesizers), which reduced system costs ($< \$200$) while improving its programmability. Moreover, the electronics interfaced with a mobile terminal (e.g., smartphones, tablet computers) for system control (Fig. 6c). A customized application program was implemented to allow for system operation and data-logging with minimal input from end-users. For translational testing, $\mu\text{NMR-3}$ has anchored our recent preclinical and clinical work as detailed in the following section.

III. CLINICAL INVESTIGATION

III.1 Determining a unique tumor protein panel for detection

Selection of appropriate protein biomarkers for ubiquitous cancer detection has been challenging for a number of reasons including i) absence of unique cancer specific proteins, ii) low abundance levels of certain proteins, iii) the molecular heterogeneity of tumors,^{48,49} iv) temporal changes of molecular profiles among others. Since it appears unlikely that a single protein can identify all cancers, the focus has shifted towards multiplexed testing. With $\mu\text{NMR-3}$ in place to support our first-in-human studies, we profiled a wide array of solid epithelial tumors grounded in the typical clinical workflow of an interventional radiology department.³¹ We applied $\mu\text{NMR-3}$ to fine needle aspirates (FNA) from 50 patients presenting to the Massachusetts General Hospital with suspected intra-abdominal malignancies (Fig. 7). Notably, with one FNA pass we quantitated the protein expression levels of nine well-established cancer markers and one leukocyte marker. We identified a four marker panel set (MUC-1+EGFR+HER2+EpCAM), coined quad-marker, that conferred a 96% diagnostic accuracy compared to clinical pathology interpretation of their paired core biopsies. To eliminate potential sources of error and data over-fitting, we tested our quad marker in 20 additional patients. $\mu\text{NMR-3}$ correctly diagnosed all 20 patients in the validation set, supporting the quad-marker as a reliable signature of solid epithelial tumors. The rapid readouts (~ 1 hour) and user friendly operation of the smartphone interface aligned with our pre-clinical experiences.

III.2 Preclinical optimization of quad- μNMR for CTC testing

We integrated quad marker analyses into our platform (quad- μNMR) and explored its potential to identify CTCs.³² We hypothesized that applying a quad-marker cocktail for simultaneous cellular targeting and testing would prove superior to multiplexing. In the following sections, we discuss steps taken to optimize our quad- μNMR assay to detect CTC

directly in whole blood. This occurred in highly regulated laboratory conditions followed by using patient-derived samples from the clinic.

Assay protocol for CTC detection—We tested various whole blood preparation assays and measured quad- μ NMR detection for each preparation method.³² Detecting cells in untreated whole blood was desirable for practical reasons and to enhance yield. Among various methods tested, red blood cell (RBC)-lysis and fixation was shown to generate the highest μ NMR signal. This method allowed prompt disposal of RBCs and subsequent fixation of the remaining leukocytes and CTCs. We further tested if sample fixation compromised the integrity of cell labeling. Our previous clinical study demonstrated rapid degradation of protein markers unless cells were fixed promptly after harvesting.³¹ However, fixing cells before labeling could alter antibody-binding epitopes on proteins. We thus performed spiking experiments using SKBR3 (breast cancer) cell lines and healthy control blood. Following RBC-lysis, samples were either fixed first and labeled with MNPs, or vice versa. For all cell concentrations tested (140 /mL, 1,100/mL, 6,300/mL), best results were obtained with the fixation-labeling sequence.³²

Optimizing μ NMR sensitivity for CTC detection—Appreciating the rarity of CTCs and high potential for cell loss during processing, our experimental testing supported use of a quad marker “cocktail” for optimal signal and detection³². Rather than aliquoting the samples for parallel testing, CTCs were simultaneously labeled with *trans*-cyclooctene (TCO) modified MUC-1, EGFR, HER2, and EpCAM antibodies and subsequently coupled with Tz-MNPs. Receiver operating characteristic analyses defined the optimal cutoff values for malignancy. Another challenge in CTC detection is the potential epithelial-to-mesenchymal (EMT) transition of cancer cells. During EMT, CTCs may express little or no EpCAM.⁵⁰ To experimentally test the influence of EpCAM expression on the performance of quad- μ NMR, we compared EpCAM-only versus quad-marker labeling, using 12 different cell lines with variable levels of EpCAM expression: low, intermediate and high. μ NMR signal was highest for quad- μ NMR across all cell lines irrespective of the EpCAM expression levels (Fig. 8a).³² This was likely due to more efficient recruitment of MNPs to cancer cells. In other words, the presence of four different TCO-modified antibodies led to more MNPs targeted per cell, thus generating higher μ NMR signal. Given the heterogeneous nature of biomarker expression in cancer, the application of quad- μ NMR is particularly helpful for detecting cancer cells with variable EpCAM expression levels, including those with aggressive physiology (i.e. EMT).

We also compared quad- μ NMR to CellSearch and noted an average recovery rate of 9.1% across all concentrations accessed (i.e. 200, 100, 50 and 25 spiked cells) for the latter (Fig. 8b). However, quad- μ NMR demonstrated an average recovery rate of 38% across the various cell concentrations, leading to higher CTC detection sensitivity ($p < 0.05$) in all cases (Fig. 8c).³² In our preclinical studies, the detection sensitivity of quad- μ NMR was 400% fold higher than CellSearch. Quad- μ NMR was even more sensitive in the low EpCAM expressing breast cell line, MDA-MB-436 with known EMT behavior.

III.3 Utility of quad- μ NMR in clinical studies

To examine the clinical performance of quad- μ NMR, we pursued a pilot study using freshly collected peripheral blood from 15 patients with advanced ovarian cancer.³² Quad- μ NMR's broader dynamic range of CTC enumeration correlated well with clinical metrics. For example, average CTC counts were higher in more advanced cases such as stage IV, platinum resistant and progressive disease, and in patients not pursuing active therapy. Quad- μ NMR also performed better than EpCAM-based CellSearch, reporting higher CTC

counts. Studies are under way to validate these findings in ovarian cancer and to explore the utility of quad- μ NMR for minimally invasive monitoring of response or early recurrence.

III.4. Comparing CTC with matched distal metastases

A key aspiration of many cancer investigators is understanding how well CTC protein profiles correlate with their metastatic deposits. Strong associations could justify leveraging CTCs for diagnostic and potentially pharmacodynamic monitoring rather than pursuing more invasive biopsies. Using μ NMR, we recently demonstrated that comparing the protein profiles of CTCs with their respective FNA was feasible.³³ First, quad- μ NMR detected CTCs in all 21 enrolled subjects with a range of ~3 to 27 CTC/mL.³³ More CTCs were detected 1) in patients with metastatic disease than with locally advanced cancers, and 2) in patients with progressive disease (~10 CTC/mL) than clinical responders (~4 CTC/mL). Beyond enumeration, we used quad- μ NMR to compare the molecular profiles of CTCs with their matched distal metastasis. Cellular expression levels of EpCAM, EGFR, HER2 and vimentin were positive in 67%, 62%, 76%, and 76% of CTC samples, respectively (Fig. 9). The expression of vimentin in patients with worsening clinical trajectories was 50% lower compared to patients with stable or improving trajectories, which supports associations between EMT and aggressive clinical course. Overall, our data suggests that, across a wide spectrum of intra-abdominal solid epithelial tumors, the molecular profiles of CTCs are not similar to their matched distal metastases (Fig. 9). As such, “benign” results in one specimen type would still demand testing the other (i.e. CTC may not be adequate diagnostic surrogates to obviate additional testing). We are currently examining whether such discrepancies hold true by testing larger numbers of subjects and focusing on specific cancer types (e.g., lung and melanoma).

CONCLUSION

The clinical application of the μ NMR technology would fulfill many (if not all) of the key requirements desirable for biodiagnostic technologies, namely: assay sensitivity, selectivity, versatility, low cost and portability.⁵¹ Manufacturing costs from reusable NMR components (< \$200) and the disposable microfluidic chip (< \$1) also render μ NMR technology a practical and scalable option. These contributions will be important because they are all predicated on the use and analyses of human specimens attained through minimally invasive means – an unmet need for serial studies. Opportunities from our CTC work and those of others include better powered trials, improved tumor monitoring, and rational selection of therapies at the relevant time with higher potential for clinical benefit. We anticipate μ NMR technology, with its ability for molecular profiling of CTCs at point-of-care settings, would provide an important step forward beyond enumeration to the clinical deployment of personalized and highly specific cancer therapies.

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REFERENCES

1. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K, Chapman PB. *N. Engl. J. Med.* 2010; 363:809–819. [PubMed: 20818844]

2. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Janne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW, Iafrate AJ. *N. Engl. J. Med.* 2010; 363:1693–1703. [PubMed: 20979469]
3. Sequist LV, Martins RG, Spigel D, Grunberg SM, Spira A, Janne PA, Joshi VA, Mccollum D, Evans TL, Muzikansky A, Kuhlmann GL, Han M, Goldberg JS, Settleman J, Iafrate AJ, Engelman JA, Haber DA, Johnson BE, Lynch TJ. *J. Clin. Oncol.* 2008; 26:2442–2449. [PubMed: 18458038]
4. Von Hoff DD, Stephenson JJ Jr, Rosen P, Loesch DM, Borad MJ, Anthony S, Jameson G, Brown S, Cantafio N, Richards DA, Fitch TR, Wasserman E, Fernandez C, Green S, Sutherland W, Bittner M, Alarcon A, Mallery D, Penny R. *J. Clin. Oncol.* 2010; 28:4877–4883. [PubMed: 20921468]
5. Betensky RA, Louis DN, Cairncross JG. *J. Clin. Oncol.* 2002; 20:2495–2499. [PubMed: 12011127]
6. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ. *J. Clin. Oncol.* 2008; 26:3213–3221. [PubMed: 18591556]
7. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. *N. Engl. J. Med.* 2004; 351:781–791. [PubMed: 15317891]
8. Scher HI, Jia X, De Bono JS, Fleisher M, Pienta KJ, Raghavan D, Heller G. *Lancet Oncol.* 2009; 10:233–239. [PubMed: 19213602]
9. Stott SL, Lee RJ, Nagrath S, Yu M, Miyamoto DT, Ulkus L, Inserra EJ, Ullman M, Springer S, Nakamura Z, Moore AL, Tsukrov DI, Kempner ME, Dahl DM, Wu CL, Iafrate AJ, Smith MR, Tompkins RG, Sequist LV, Toner M, Haber DA, Maheswaran S. *Sci. Transl. Med.* 2010; 2:25ra23.
10. Sleijfer S, Gratama JW, Sieuwerts AM, Kraan J, Martens JW, Foekens JA. *Eur. J. Cancer.* 2007; 43:2645–2650. [PubMed: 17977713]
11. Punnoose EA, Atwal SK, Spoerke JM, Savage H, Pandita A, Yeh RF, Pirzkall A, Fine BM, Amler LC, Chen DS, Lackner MR. *PLoS One.* 2010; 5:e12517. [PubMed: 20838621]
12. Riethdorf S, Fritsche H, Muller V, Rau T, Schindlbeck C, Rack B, Janni W, Coith C, Beck K, Janicke F, Jackson S, Gornet T, Cristofanilli M, Pantel K. *Clin. Cancer Res.* 2007; 13:920–928. [PubMed: 17289886]
13. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, Herold CI, Marcom PK, George DJ, Garcia-Blanco MA. *Mol. Cancer Res.* 2011; 9:997–1007. [PubMed: 21665936]
14. Lee H, Sun E, Ham D, Weissleder R. *Nat. Med.* 2008; 14:869–874. [PubMed: 18607350]
15. Gillis P, Koenig S. *Magn. Reson. Med.* 1987; 5:323–345. [PubMed: 2824967]
16. Lee H, Yoon T, Figueiredo J, Swirski F, Weissleder R. *Proc. Natl. Acad. Sci. U S A.* 2009; 106:12459–12464. [PubMed: 19620715]
17. Lee H, Yoon T, Weissleder R. *Angew. Chem. Int. Ed. Engl.* 2009; 48:5657–5660. [PubMed: 19554581]
18. Yoon T, Lee H, Shao H, Weissleder R. *Angew. Chem. Int. Ed. Engl.* 2011; 50:4663–4666. [PubMed: 21495138]
19. Yoon T, Lee H, Shao H, Hilderbrand S, Weissleder R. *Adv. Mater.* 2011; 23:4793–4797. [PubMed: 21953810]
20. Liong M, Shao H, Haun J, Lee H, Weissleder R. *Adv. Mater.* 2010; 22:5168–5172. [PubMed: 20859943]
21. Haun J, Devaraj N, Hilderbrand S, Lee H, Weissleder R. *Nat. Nanotechnol.* 2010; 5:660–665. [PubMed: 20676091]
22. Agasti S, Liong M, Tassa C, Chung H, Shaw S, Lee H, Weissleder R. *Angew. Chem. Int. Ed. Engl.* 2012; 51:450–454. [PubMed: 22113923]
23. Sun N, Liu Y, Lee H, Weissleder R, Ham D. *IEEE J. Solid-State Circuits.* 2011; 46:342.
24. Issadore D, Min C, Liong M, Chung J, Weissleder R, Lee H. *Lab Chip.* 2011; 11:2282–2287. [PubMed: 21547317]
25. Chung H, Castro C, Im H, Lee H, Weissleder R. *Nat. Nanotechnol.* 2013; 8:369–375. [PubMed: 23644570]

26. Liong M, Hoang A, Chung J, Gural N, Ford C, Min C, Shah R, Ahmad R, Fernandez-Suarez M, Fortune S, Toner M, Lee H, Weissleder R. *Nat. Commun.* 2013; 4:1752. [PubMed: 23612293]
27. Shao H, Chung J, Balaj L, Charest A, Bigner D, Carter B, Hochberg F, Breakefield X, Weissleder R, Lee H. *Nat. Med.* 2012; 18:1835–1840. [PubMed: 23142818]
28. Budin G, Chung H, Lee H, Weissleder R. *Angew. Chem. Int. Ed. Engl.* 2012; 51:7752–7755. [PubMed: 22744868]
29. Chung H, Reiner T, Budin G, Min C, Liong M, Issadore D, Lee H, Weissleder R. *ACS Nano.* 2011; 5:8834–8841. [PubMed: 21967150]
30. Liong M, Fernandez-Suarez M, Issadore D, Min C, Tassa C, Reiner T, Fortune S, Toner M, Lee H, Weissleder R. *Bioconjug. Chem.* 2011; 22:2390–2394. [PubMed: 22043803]
31. Haun JB, Castro CM, Wang R, Peterson VM, Marinelli BS, Lee H, Weissleder R. *Sci. Transl. Med.* 2011; 3:71ra16.
32. Ghazani A, Castro C, Gorbato R, Lee H, Weissleder R. *Neoplasia.* 2012; 14:388–395. [PubMed: 22745585]
33. Ghazani A, McDermott S, Pectasides M, Sebas M, Mino-Kenudson M, Lee H, Weissleder R, Castro C. *Nanomedicine.* 2013 in press.
34. Gossuin Y, Gillis P, Hocq A, Vuong Q, Roch A. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2009; 1:299–310. [PubMed: 20049798]
35. Gueron M. *J. Magn. Reson.* 1975; 19:58–66.
36. Brooks R, Moyny F, Gillis P. *Magn. Reson. Med.* 2001; 45:1014–1020. [PubMed: 11378879]
37. Gillis P, Moyny F, Brooks R. *Magn. Reson. Med.* 2002; 47:257–263. [PubMed: 11810668]
38. Lee J, Huh Y, Jun Y, Seo J, Jang J, Song H, Kim S, Cho E, Yoon H, Suh J, Cheon J. *Nat. Med.* 2007; 13:95–99. [PubMed: 17187073]
39. Huber D. *Small.* 2005; 1:482–501. [PubMed: 17193474]
40. Qiang Y, Antony J, Sharma A, Nutting J, Sikes D, Meyer D. *J. Nanopart. Res.* 2006; 8:489–496.
41. Seo WS, Lee JH, Sun X, Suzuki Y, Mann D, Liu Z, Terashima M, Yang PC, McConnell MV, Nishimura DG, Dai H. *Nat. Mater.* 2006; 5:971–976. [PubMed: 17115025]
42. Peng S, Wang C, Xie J, Sun S. *J. Am. Chem. Soc.* 2006; 128:10676–10677. [PubMed: 16910651]
43. Ban Z, Barnakov Y, Li F, Golub V, O'Connor CJ. *J. Mater. Chem.* 2005; 15:4660–4662.
44. Cheng J, Ni X, Zheng H, Li B, Zhang X, Zhang D. *Mater. Res. Bull.* 2006; 41:1424–1429.
45. Park J, An K, Hwang Y, Park J, Noh H, Kim J, Park J, Hwang N, Hyeon T. *Nat. Mater.* 2004; 3:891–895. [PubMed: 15568032]
46. Devaraj NK, Upadhyay R, Haun JB, Hilderbrand SA, Weissleder R. *Angew. Chem. Int. Ed. Engl.* 2009; 48:7013–7016. [PubMed: 19697389]
47. Webb AG. *Prog. Nucl. Magn. Reson. Spectrosc.* 1997; 31:1–42.
48. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C. *N. Engl. J. Med.* 2012; 366:883–892. [PubMed: 22397650]
49. Xu X, Hou Y, Yin X, Bao L, Tang A, Song L, Li F, Tsang S, Wu K, Wu H, He W, Zeng L, Xing M, Wu R, Jiang H, Liu X, Cao D, Guo G, Hu X, Gui Y, Li Z, Xie W, Sun X, Shi M, Cai Z, Wang B, Zhong M, Li J, Lu Z, Gu N, Zhang X, Goodman L, Bolund L, Wang J, Yang H, Kristiansen K, Dean M, Li Y, Wang J. *Cell.* 2012; 148:886–895. [PubMed: 22385958]
50. Konigsberg R, Obermayr E, Bises G, Pfeiler G, Gneist M, Wrba F, De Santis M, Zeillinger R, Hudec M, Ditttrich C. *Acta Oncol.* 2011; 50:700–710. [PubMed: 21261508]
51. Giljohann DA, Mirkin CA. *Nature.* 2009; 462:461–464. [PubMed: 19940916]

Figure 1. Translational loop behind μ NMR development and eventual CTC detection and profiling

The development of a μ NMR platform for exquisitely sensitive protein detection in scant human samples leveraged findings and advances between the preclinical and clinical spaces. The feedback loop has recently culminated in the ability to reliably detect and profile CTCs.

Figure 2. Principle of cellular detection with μ NMR

Cancer cells tagged with MNPs can accelerate the transverse relaxation of water protons. Compared to the non-tagged samples (left), the NMR signal will decay faster in time domain (right), providing a sensing mechanism. (Reproduced with permission from [14]. Copyright 2008 Nature Publishing Group.)

Figure 3. Magnetic nanoparticles engineered for high transverse relaxivity (r_2)
 (a) Transmission electron micrograph (TEM) images of Mn-doped magnetite (MnFe₂O₄) particles. Smaller particles (left) were used as a seed to grow bigger particles (right). (b) TEM micrographs of Fe-MNPs. These particles have an elemental Fe-core passivated with a ferrite shell. The shell prevents the core from oxidation, and impart additional magnetic moments to the particle. Note that the shell can be metal-doped to further enhance magnetization. (c) The r_2 values of various types of MNPs were mapped as a function of both the particle size (d) and the saturation magnetization (M_s). As expected from the outer-sphere theory (solid line), the r_2 values of ferrite MNPs increased with the particle size (blue

squares). Among ferrite, MnFe_2O_4 MNPs have the highest r_2 due to their high M_s , but these values were upper limited by the bulk M_s of MnFe_2O_4 (dotted line). Fe-core MNPs, because of their stronger magnetization, passed the boundary and achieved higher r_2 relaxivities, with $\text{Fe@MnFe}_2\text{O}_4$ MNPs assuming the highest value. CLIO, cross-linked iron oxide nanoparticle; MION, monocrystalline iron oxide nanoparticle. (Adapted with permission from [16]. Copyright 2009 National Academy of Sciences, USA. Reproduced with permission from [18]. Copyright 2011 John Wiley and Sons, Inc.)

Figure 4. Bioorthogonal nanoparticle detection (BOND)

(a) The method is based on the Diels-Alder cycloaddition between *trans*-cyclooctene (TCO) and tetrazine (Tz). (b) Cells are pre-labeled with TCO-antibodies and targeted with Tz-MNPs. The antibody provides sites for multiple MNP binding. (c, d) Compared to the direct targeting with MNP-antibody conjugates, BOND method enabled higher MNP loading on target cells as confirmed by fluorescent (c) and μ NMR (d) measurements. (Adapted with permission from [21]. Copyright 2010 Nature Publishing Group.)

Figure 5. μ NMR system development

(a) μ NMR-1: The system consists of an array of microcoils for NMR detection, microfluidic channels for sample handling, embedded NMR electronics, and a permanent magnet. (b) μ NMR-2 (left): A solenoidal coil is embedded along with a microfluidic channel. The entire bore of the coil is available to samples, maximizing the filling factor (≈ 1). (right) The new coil offered $>350\%$ enhancement in NMR signal, compared to a similar coil wrapped around a tubing. (c) A NMR coil was integrated into a sophisticated microfluidic system. MNP-labeling, sample washing and NMR detection can be performed on-chip. (d) The entire NMR setup can be potentially packaged as a hand-held device, using a custom-designed IC

(integrate circuit) chip. (Reproduced with permission from [14, 27]. Copyright 2008, 2012 Nature Publishing Group. Adapted with permission from [16]. Copyright 2009 National Academy of Sciences, USA. Reproduced with permission from [23]. Copyright 2010 IEEE.)

Figure 6. μ NMR-3 designed for clinical applications

(a) Algorithm for robust NMR measurement. The routine keeps track of the NMR frequency and automatically adjust f (RF excitation frequency), maintaining a constant frequency offset (Δf). FID, free induction decay; FFT, fast Fourier transformation. (b) With the frequency tracking turned on, the measured T_2 values of the same sample showed $<1\%$ variation, whereas T_2 values fluctuated $>200\%$ without such scheme. (c) μ NMR-3 system for point-of-care operation, featuring automatic system configuration and a smartphone interface. The system was used in the recent clinical trial. (Reproduced with permission from [24]. Copyright 2011 RSC Publishing.)

Figure 7. Profiling of tumor cells using the μ NMR platform

Single fine-needle aspirates were obtained from patients, and were tagged via the BOND strategy for μ NMR detection. The profiling results indicate a high degree of heterogeneity in protein expression both across the different patient samples and even with the same tumor. (Reproduced with permission from [31]. Copyright 2011 American Association for the Advancement of Science AAAS.)

Figure 8. Performance of quad-μNMR and comparison with CTC gold standard

(a) Comparison of EpCAM-only and quad marker CTC detection using μNMR. Across all cell lines with different EpCAM-expression, quad-μNMR (red bars) showed higher NMR signals than EpCAM-only detection. (b) Using quad-μNMR, the average CTC recovery rate was 38% across the various cell concentrations assessed (i.e., 200, 100, 50 and 25 spiked cells). Identical experiments performed using the CellSearch detection system showed an average recovery rate of 9.1%. (c) Quad-μNMR indeed showed higher CTC detection sensitivity ($p < 0.05$) than CellSearch system. (Adapted with permission from [32]. Copyright 2012 Neoplasia Press.)

Sample	Marker	Patient No.																						
		1	2	3	5	6	7	8	9	10	11	13	14	15	16	17	18	23	29	32	33	34		
Blood	EpCAM	+	-	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-		
	EGFR	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	-	+	+	-		
	HER2	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	+	+		
	Vimentin	-	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+		
Biopsy	EpCAM	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	-	-	-		
	EGFR	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	-	-		
	HER2	+	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-		
	Vimentin	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-		

Figure 9. Comparison of molecular profiles between CTCs and biopsy from the site of metastasis
 Each column represents a patient sample. CTC results (top) are categorized as positive (+) or negative (–) for the expression of a given marker. Results from the biopsy from the site of metastasis (bottom) are similarly indicated as positive or negative. In each subject, concordant test result (i.e. both CTC and biopsy positive or both negative) is designated in green. Discordant results are shown in pink. (Reproduced with permission from [33]. Copyright 2013 Elsevier Inc.)