Chapter 3

Molecular Detection of Biomarkers and Cells Using Magnetic Nanoparticles and Diagnostic Magnetic Resonance

Jered B. Haun, Tae-Jong Yoon, Hakho Lee, and Ralph Weissleder

Abstract

The rapid and sensitive detection of molecular targets such as proteins, cells, and pathogens in biological specimens is a major focus of ongoing medical research, as it could promote early disease diagnoses and the development of tailored therapeutic strategies. Magnetic nanoparticles (MNP) are attractive candidates for molecular biosensing applications because most biological samples exhibit negligible magnetic susceptibility, and thus the background against which measurements are made is extremely low. Numerous magnetic detection methods exist, but sensing based on magnetic resonance effects has successfully been developed into a general detection platform termed diagnostic magnetic resonance (DMR). DMR technology encompasses numerous assay configurations and sensing principles, and to date magnetic nanoparticle biosensors have been designed to detect a wide range of targets including DNA/mRNA, proteins, enzymes, drugs, pathogens, and tumor cells with exquisite sensitivity. The core principle behind DMR is the use of MNP as proximity sensors that modulate the transverse relaxation time of neighboring water molecules. This signal can be quantified using MR imagers or NMR relaxometers, including miniaturized NMR detector chips that are capable of performing highly sensitive measurements on microliter sample volumes and in a multiplexed format. The speed, sensitivity, and simplicity of the DMR principle, coupled with further advances in NMR biosensor technology should provide a high-throughput, low-cost, and portable platform for large-scale parallel sensing in clinical and point-of-care settings.

Key words: Magnetic nanoparticles, Molecular biosensing, Diagnostic magnetic resonance, Magnetic relaxation switches

1. Introduction

Magnetic nanoparticles (MNP) offer unique advantages for molecular detection of biological targets compared to other platforms. For example, MNP are inexpensive to produce, physically and chemically stable, biocompatible, and environmentally safe.

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In addition, biological samples exhibit virtually no magnetic background, and thus measurements can be performed in turbid or otherwise visually obscured samples. To date, numerous methods have been developed to sense biomolecules using magnetic labels (1), but one that has achieved considerable success in biomedicine is based on magnetic resonance. In this case, the MNP serve as proximity sensors that accelerate the relaxation rate of neighboring water molecules following exposure to a magnetic field. This phenomenon has been exploited for magnetic resonance imaging to obtain detailed anatomical information.

When magnetic resonance is used for molecular detection of biomolecules and cells outside of the body, it is referred to as diagnostic magnetic resonance (DMR) (2, 3). DMR assays employ affinity molecule-conjugated MNP to bind molecular targets and effect a change in proton relaxation rate by one of two methods (see Fig. 1). The first makes use of the phenomenon of magnetic relaxation switching (MRSw), in which molecular targets are used to self-assemble MNP into clusters and thereby cause a corresponding change in the bulk relaxation rate (4, 5). The second mode involves tagging large structures such as whole cells (2, 3, 6). In both cases, the binding interactions are performed homogeneously in solution and make use of the built-in amplification that magnetic resonance offers through its effect on billions of neighboring water molecules. These factors make DMR faster than techniques that require solid-phase immobilization, diffusion of nanoparticles to sensing elements, or discrete amplification steps. The magnetic resonance signal can be quantified by NMR or MRI as a decrease in longitudinal (spin–lattice, T_1) or transverse (spin–spin, T_2) relaxation times. Typically T_2 is used because the transverse relaxivity (r_2) is significantly greater than the longitudinal relaxivity (r_1) for most MNP.

To date, DMR has successfully been used to detect a wide variety of biomolecular targets with high sensitivity and specificity, including DNA, mRNA, proteins, enzyme activity, metabolites, drugs, pathogens, and tumor cells (see Table 1). Notably, detection thresholds in the femtomolar biomarker concentration and near single-cell range have been documented in complex media such as cell lysates, sputum, and whole blood (3, 4, 6). The magnetic resonance signal can be read-out using MRI scanners or benchtop NMR systems. Recently, however, a miniaturized, chip-based NMR (μNMR) detector system was developed that houses all components for DMR detection in a handheld, portable device (2, 3, 6). The μNMR chip device is capable of performing measurements on microliter sample volumes and contains multiple detection coils to enable parallel sensing of numerous biomarkers. The μNMR chip thus represents a critical advance in DMR technology that could facilitate detection of disease markers in sample-limited clinical specimen.

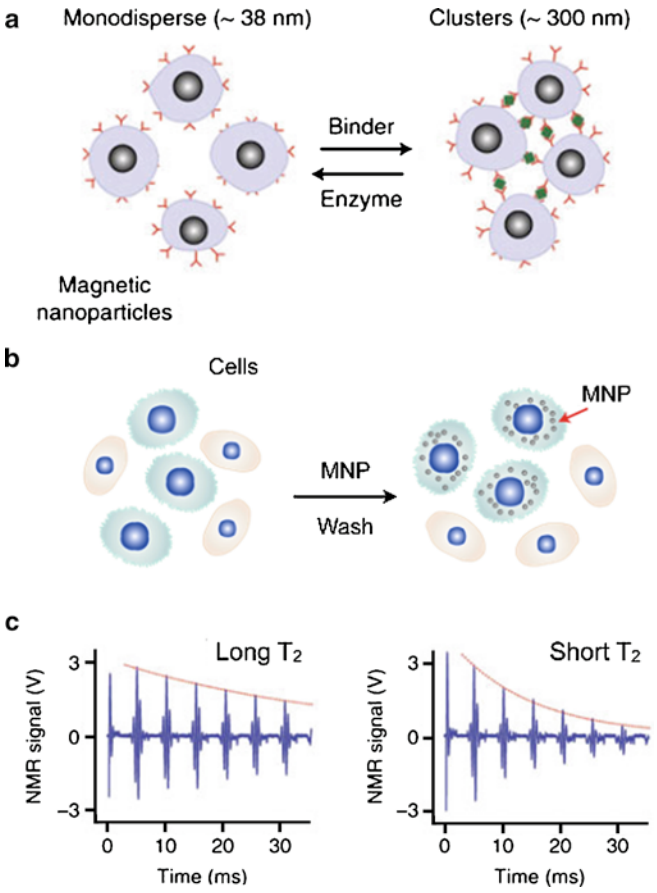

Fig. 1. DMR sensing principles. (**a**) Magnetic relaxation switching (MRSw) involves the assembly of MNP into clusters or disassembly of preformed clusters by the action of a target biomolecule. Clustered MNP dephase the nuclear spins of neighboring water molecules more efficiently than evenly dispersed MNP, shortening the bulk transverse relaxation time (T_2). (**b**) Tagging cells with MNP imparts a magnetic moment that is proportional to the number of nanoparticles bound. Following washing procedures to remove unbound MNP, the magnetic moment can be measured as a decrease in T_2 relaxation time. (**c**) Representative NMR output depicting the shortening of T_2 relaxation time that accompanies MNP clustering (MRSw) or cellular tagging. Modified with permission from ref. 2, copyright (2008) Nature Publishing Group.

2. Materials

2.1. Preparation for Bioconjugation of Affinity Molecules to MNP

1. Superparamagnetic nanoparticles: Synthesized (7, 8) or purchased commercially (Miltenyi Biotec, Auburn, CA; Ocean NanoTech, Springdale, AK) with primary amine functional groups or conjugated with proteins such as avidin or protein A.
2. Affinity molecule: Lyophilized or dissolved in buffer. This sample should be free of contaminants that would interfere with coupling interactions (see Note 1).

Table 1
DMR biosensors/applications to date

Class	Target	Mode	Magnetic nanoparticle sensor	References
DNA	Telomeres	MRSw (A)	CLIO-$(CCCTAA)_3$	(24)
RNA	GFP	MRSw (A)	CLIO-ATTTGCCGGTGT and CLIO-TCAAGTCGCACA	(4)
Protein	GFP	MRSw (A)	CLIO-antibody (anti-GFP)	(4)
	Avidin	MRSw (A)	CLIO-biotin	(4)
	β-HCG	MRSw (A)	CLIO-antibody (anti-β-HCG)	(25)
	Telomerase	MRSw (A)	CLIO-antibody (anti-telomerase)	(26)
	CA-125	MRSw (A)	CLIO-antibody (anti-CA-125)	(2)
	VEGF	MRSw (A)	CLIO-antibody (anti-VEGF)	(2)
	α-Fetoprotein	MRSw (A)	CLIO-antibody (anti-α-fetoprotein)	(2)
Enzyme activity	Caspase 3	MRSw (D)	CLIO-GDEVDG-biotin; CLIO-avidin	(4)
	*Bam*H1	MRSw (D)	CLIO-TTA-CGC-CTAGG-ATC-CTC and CLIO-AAT-GCG-GGATCC-TAC-GAG	(27)
	DNA methylase	MRSw (D)	Methylated *Bam*H1 sensor	(27)
	Renin	MRSw (D)	Biotin-IHPFHLVIHTK-biotin; CLIO-avidin	(28)
	Trypsin	MRSw (D)	Biotin-$(G)_4$RRRR$(G)_3$K-biotin or biotin-GPARLAIK-biotin; CLIO-avidin	(28)
	MMP-2	MRSw (D)	Biotin-GGPLGVRGK-biotin; CLIO-avidin	(28)
	Telomerase	MRSw (A)	CLIO-AATCCCAATCCC and CLIO-AATCCCAATCCC	(26)
	Peroxidases	MRSw (D)	CLIO-phenol or CLIO-tyrosine	(29)
Small molecule	Drug	MRSw (D)	CLIO-antibody (anti-D-phenylalanine)	(30)
	Folate	MRSw (D)	CLIO-antibody (anti-folate)	(31)
	Glucose	MRSw (D)	CLIO-Concavalin	(31)
	HA peptide	MRSw (D)	CLIO-antibody (anti-HA)	(31)
	Calcium	MRSw (A)	CLIO-Calmodulin and CLIO-M13 or CLIO-chelator	(32, 33)
Organism	Herpes virus	MRSw (A)	CLIO-antibody (anti-HSV1)	(34)
	Adenovirus-5	MRSw (A)	CLIO-antibody (anti-adenovirus-5)	(34)
	MAP	MRSw (A)	CLIO-antibody (anti-MAP)	(35)
	Staphylococcus aureus	MRSw (A)	CLIO-Vancomycin	(2)
	MTB/BCG	Tagging	CLIO-antibody or CB-antibody (anti-BCG)	(6)
Human cell	Tumor cell lines	Tagging	CLIO-antibody (anti-Her2/*neu*, EGFR, EpCAM)	(2)
	Murine tumor biopsies	Tagging	Mn-MNP-antibody (anti-Her2/*neu*, EGFR, EpCAM)	(3)

MRSw (A): MRSw assay using assembly of MNP into clusters. MRSw (D): MRSw assay based on disassembly of pre-clustered MNP

3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 in de-ionized water, adjust pH to 7.4 with NaOH. PBS can also be purchased, but be sure that divalent cations are not present (see Note 2).
4. Ethylenediamine tetraacetic acid (EDTA) buffer: 10× Solution with 100 mM EDTA in the PBS buffer listed in item 3, Subheading 2.1. EDTA is necessary for thiol-based chemistries to prevent formation of disulfide bonds between free thiol groups.
5. Bicarbonate buffer: 0.1 M $NaHCO_3$, adjust pH to 10 with NaOH.
6. Dimethylformamide (DMF) [if desired, dimethylsulfoxide (DMSO) can be used interchangeably].
7. Thiol chemistry bioconjugations will require one or more of the following reagents:
   (a) Sulfosuccinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC, available in convenient no-weigh format from Thermo Fisher, Waltham, MA).
   (b) *N*-Succinimidyl-*S*-acetylthioacetate (SATA, Thermo Fisher).
   (c) β-Mercaptoethylamine (MEA, Sigma Aldrich, St. Louis, MO).
8. Deacetylation buffer: 0.5 M Hydroxylamine·HCl, 25 mM EDTA in the PBS buffer listed in item 3, Subheading 2.1, adjust pH to 7.4 with NaOH. This reagent is required for point (b) of item 7, Subheading 2.1 only.
9. Click chemistry bioconjugations can be performed using commercially available reagents including succinimidyl ester or iodoacetamide conjugated azide and alkyne moieties (Invitrogen, Carlsbad, CA).
10. Amicon Ultra centrifugal filtration device, 15 mL volume, 100,000 MWCO (Millipore, Billerica, MA).
11. Sephadex G-50 (GE Healthcare, Piscataway, NJ) or similar gel filtration media.
12. PD-10 (GE Healthcare) or Zeba (5 mL, Thermo Fisher) desalting columns.
13. Coomassie protein stain (Thermo Fisher).

2.2. Coupling, Processing, and Characterization

1. Copper catalyst buffer: 10× Solution with 50 mM sodium ascorbate, 10 mM $Cu^{(II)}SO_4$, 50 mM of a polytriazole ligand (see Note 3).
2. Sephadex G-100 Superfine (GE Healthcare) or similar gel filtration media.

3. Fe digestion buffer: 6 M HCl, 0.3% H_2O_2 in de-ionized water.
4. Micro BCA™ Protein Assay Kit (Thermo Fisher).
5. 96-Well plate (Becton Dickinson, Franklin Lakes, NJ).

2.3. MRSw Assay for Soluble Analyte Sensing

1. Analyte samples can be obtained from numerous sources, including unpurified biological samples such as cell culture supernatants, cell lysates, milk, sputum, and whole blood. For disassembly assays, the MNP are typically pre-clustered using purified cross-linkers such as oligonucleotides or proteins prior to addition of analyte.

2.4. Magnetic Tagging of Cells

1. PBS with bovine serum albumin (BSA) (PBS+): 1 g/L BSA added to the PBS buffer listed in item 3, Subheading 2.1, sterile filter.
2. Cell samples can be obtained from any source of interest, such as in vitro cultures or in vivo specimen, but should be suspended as single cells. This can be accomplished using various enzymes (trypsin, collagenase, etc.) and/or EDTA (see Note 4). See Note 5 for total cell requirements.

2.5. Detection Using NMR

1. 5 or 10 mm NMR tubes (Thermo Fisher).
2. Benchtop NMR relaxometer (i.e., Minispec, Bruker Optics, Billerica, MA). Alternatively, custom devices such as miniaturized NMR (μNMR) detectors have been used (2, 3, 6). The indicated options both operate at approximately 20 MHz and 0.5 T.

2.6. Detection Using MRI

1. 384-Well plate (Becton Dickinson).
2. Clinical or experimental MRI scanner operating at 1–11 T [i.e., ClinScan (7 T) or PharmScan (4.5 T), Bruker BioSpin, Billerica, MA].
3. Software to analyze T_2 images (i.e., OsiriX Viewer, available at http://www.osirix-viewer.com).

3. Methods

The MNP used for magnetic resonance detection must be superparamagnetic, meaning that they become magnetized when placed in an external magnetic field but lose their magnetic moment when the field is removed (no magnetic remanence). The bulk of DMR assays have been performed using iron oxide nanoparticles, in particular cross-linked iron oxide (CLIO) (7, 9). CLIO has a magnetic core composed of a monocrystalline iron oxide nanoparticle (MION), and the MION core is caged in

cross-linked dextran. Such iron oxide nanoparticles can be obtained in various forms from commercial sources. Aside from standard iron oxide options, it has been demonstrated that doping iron oxide with metals such as manganese, cobalt, and nickel can increase magnetization, and thus detection sensitivity (10). Likewise, a core–shell nanoparticle composed of an elemental iron core and protective iron oxide shell has also been shown to possess greater magnetic susceptibility than iron oxide (11). Manganese-doped iron oxide nanoparticles (Mn-MNP) and elemental Fe core nanoparticles (cannonballs, CB) have been validated for DMR applications and significantly improve detection sensitivity (3, 6). Micron-sized particles composed of many iron oxide cores embedded in a polymer matrix have been used for DMR assays as well (12, 13), but their extremely high magnetization is offset by rapid settling out of solution, limiting utility.

Regardless of the type of superparamagnetic core used, a polymer coating is required to render the core water soluble, prevent aggregation, and provide chemical functional groups for bioconjugation. Most often the functional groups are primary amines, but carboxylic acids or thiols are also used. The protocols listed below detail the attachment of affinity molecules to amine-terminated MNP; treatments of MNP with other surface-based functional groups can be found elsewhere (14). Subheading 3.1 or 3.2 can also be followed to first attach avidin, proteins A or G, secondary antibodies, or fluorescent molecules (see Note 6) to the MNP or alternatively MNP can be purchased with these moieties already conjugated to their surface. In these cases, skip to Subheading 3.3 for attachment of affinity molecules modified with biotin or antibody Fc domains. The choice of affinity molecule is dependent on the target and application of interest. To date, strategies have included the attachment of oligonucleotides, antibodies, peptides, small molecules, metal chelators, and natural biological binding partners, as listed in Table 1.

3.1. Bioconjugation Using Thiol Chemistry

1. These instructions are intended for the attachment of affinity molecules that contain a free thiol group, such as peptides with a terminal cysteine residue and proteins that are appropriately modified to bear a thiol group (mild reduction of disulfide bonds or conversion of a primary amine to a thiol group).
2. Dilute amino-MNP with PBS to approximately 0.5 mg Fe/mL concentration and adjust the pH to 8.0 using the bicarbonate buffer. Lower Fe concentrations can be used but a different purification process is required (see Note 7).
3. Add at least 0.1 mg sulfo-SMCC per mg Fe (approximately 30-fold molar excess for CLIO containing 30 amines/MNP, see Note 8). The addition of more sulfo-SMCC will not

negatively affect the reaction. Incubate the reaction mixture while shaking at room temperature for 1–2 h (see Note 9).

4. Concentrate the MNP by adding to a centrifugal filtration device and centrifuging for 10 min at 2,000 × *g*. The final volume is usually about 0.2 mL.
5. Remove excess sulfo-SMCC reagent using a desalting column packed with Sephadex G-50. The total column volume should be at least 50 times greater than the sample volume to ensure effective separation. The resulting maleimide-MNP should be used as soon as possible, but can be stored at 4°C for up to 24 h if necessary.
6. Prepare the affinity molecule by dissolving the sample in or diluting it with the 10× EDTA buffer, and additional PBS if necessary, to obtain a final sample volume of 1 mL containing EDTA at 1× concentration. If the affinity molecule contains a free thiol functional group, it can be used directly (skip to Subheading 3.3).
7. For proteins that contain a disulfide bond that can be reduced without affecting binding function (such as most antibodies), add 1 mg of MEA (26 mM final concentration) and incubate for 2 h at 37°C. Alternatively, the primary amine groups of certain proteins can be converted to protected sulfhydryls using SATA; these groups can then be deprotected to yield free sulfhydryls. In this case, dissolve the SATA in DMF and add it to the protein sample at two- to fivefold molar excess (see Note 10). Incubate the mixture for 1–2 h at room temperature (see Note 9), then add 0.1 mL deacetylation buffer, and incubate for another 1.5 h.
8. Purify these protein affinity molecules by desalting with a gravity (PD-10) or centrifugal column (Zeba). First, equilibrate the column with PBS containing 1× EDTA buffer before applying the sample. For PD-10 columns, collect ~1 mL fractions and check for protein by mixing 50 µL sample with 50 µL Coomassie reagent (a color change will be observed if protein is present). Pool positive fractions. For the Zeba columns, use the flow-through directly (skip to Subheading 3.3).

3.2. Bioconjugation Using "Click" Chemistry

1. These instructions are intended as a general method to attach affinity molecules to amine-modified MNP using bioorthogonal "click" chemistries (15). The most widely used "click" reaction is the copper-catalyzed azide-alkyne cycloaddition (16, 17), which has been used to conjugate small molecules and antibodies to MNP (18, 19), along with many other applications. Recently, a new catalyst-free cycloaddition between a 1,2,4,5-tetrazine and *trans*-cyclooctene dienophile

(20) was successfully used in the same capacity (21). Regardless of the reaction pair employed, they can be used interchangeably on the affinity molecule of choice and MNP, and therefore will generically be referred to as Click Reactants 1 and 2. They can be attached using amine- or thiol-reactive moieties, such as a succinimidyl ester or iodoacetamide/maleimide, respectively.

2. Dilute amino-MNP with PBS containing 5% DMF to a concentration of 0.5 mg Fe/mL. Adjust the pH to 8.0 using the bicarbonate buffer.
3. Dissolve amine-reactive Click Reactant 1 with DMF and add a tenfold molar excess relative to the total amine content per MNP. For example, 2 mg of CLIO with 30 amines/particle would require a minimum 1.4 μmol amine-reactive Click Reagent 1 (see Note 8). Incubate the reaction mixture while shaking at room temperature for 2 h.
4. Concentrate and desalt as indicated in steps 4 and 5, Subheading 3.1.
5. Prepare the affinity molecule by dissolving the sample in or diluting it with PBS to 0.8 mL. Add 0.1 mL sodium bicarbonate buffer. Dissolve Click Reactant 2 (amine- or thiol-reactive) in DMF at 10 mg/mL and add to the reaction solution at two- to fivefold molar excess (see Note 10). Include additional DMF to achieve a total concentration of 10% if necessary. Incubate at room temperature for 1–2 h (see Note 9).
6. Purify the affinity molecule as described in step 8, Subheading 3.1, but omit EDTA from the wash solution because it is only necessary if free thiol groups are present in the sample. For small molecules or peptides, HPLC purification may be required.

3.3. Coupling, Processing, and Characterization

1. Combine the appropriate MNP (conjugated with maleimide, Click Reactant 1, avidin, protein A/G, or secondary antibody) with the appropriate affinity molecule (conjugated with a free thiol group, Click Reactant 2, biotin, or antibody Fc domain) samples. If necessary, adjust the reaction volume with PBS such that the final MNP concentration is less than 0.5 mg Fe/mL to prevent cross-linking of nanoparticles (see Note 10). The affinity molecule should be added in excess to increase MNP valency, and thus binding activity, and prevent cross-linking of MNP for cases in which the affinity molecule contains multiple coupling domains. In general, a fivefold molar excess is sufficient, but a tenfold excess is preferred. For the copper-catalyzed azide-alkyne cycloaddition, the copper catalyst buffer must also be added to the reaction mixture at 1× concentration.
2. Incubate for 4 h at room temperature or overnight at 4°C.

3. For thiol conjugations, add ammonium chloride or cysteine at 1 μM final concentration to cap unreacted maleimide groups and prevent nonspecific binding to biological samples (see Note 11). Incubate 30 min at room temperature.
4. Concentrate by adding to a centrifugal filtration device and centrifuging for at least 10 min at 2,000 × *g*.
5. Remove excess affinity molecules using a size-exclusion column packed with Sephadex G-100. Again, the total column volume should be at least 50 times greater than the sample volume to ensure effective separation.
6. Quantify the MNP concentration based on Fe content by digesting with hydrochloric acid to yield $FeCl_3$ and determining the Fe absorbance at 410 nm. Dilute the MNP solution with at least nine volumes of Fe digestion buffer and incubate at 50–60°C for 1 h. Determine the absorbance at 410 nm and convert to Fe concentration using an extinction coefficient of 1,370 M^{-1} cm^{-1} and the appropriate dilution factor. Alternatively, a quick estimate of the MNP concentration can be obtained by measuring the absorbance at 410 nm and calibrating with a known standard (i.e., the original MNP stock solution).
7. For protein and some peptide affinity molecules, successful conjugation can readily be determined using the Micro BCA™ Protein Assay. Combine equal volumes (minimum 50 μL) of the MNP sample solution and the Micro BCA working reagent in a 96-well plate. Incubate for 2 h at 37°C, cool to room temperature, and measure the absorbance of the reacted BCA reagent at 562 nm. The absorbance can be converted to a concentration using a calibration curve prepared from a stock sample of the affinity molecule with known concentration, or BSA protein as an estimate. The number of molecules per MNP can then be calculated using the molecular weight, the MNP concentration from step 6, Subheading 3.3, and the molecular weight of the MNP (see Note 8).
8. Use dynamic light scattering, if desired, to ensure that the MNP did not aggregate during conjugation procedures.

3.4. MRSw Assay for Soluble Analyte Sensing

1. This section describes the detection of soluble molecules based on the principle of MRSw, in which the molecular target induces assembly or disassembly of MNP into clusters and causes a change in the bulk T_2 relaxation time (see Fig. 2). Disassembly MRSw assays are the preferred method for the detection of enzymes (cleavage of specific sites in the cross-bridges) and small molecules (competitive binding). In this case, MNP must first be clustered, which has been accomplished using several different strategies (see Table 1).

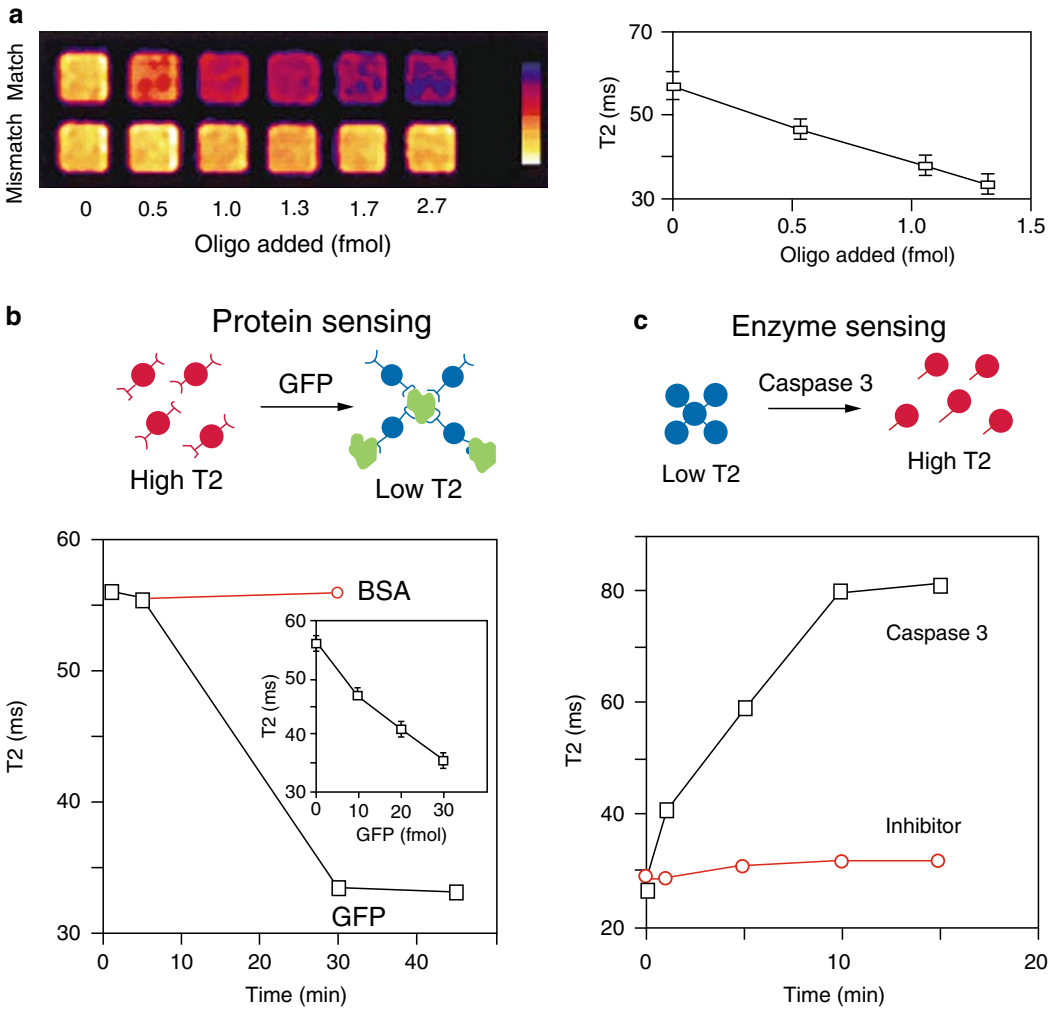

Fig. 2. DMR detection of biomolecules using magnetic relaxation switching (MRSw). (**a**) Detection of an oligonucleotide target using MNP conjugated with complementary oligonucleotide sequences. The left panel displays a T_2-weighted MR image of a 384-well plate containing varying amounts of target or mismatched oligonucleotide and constant levels of MNP. Hybridization to the target causes the MNP to cluster, resulting in a corresponding decrease in T_2 relaxation time. (**b**) Detection of GFP protein using MNP conjugated with a polyclonal antibody specific for GFP. T_2 relaxation time, which was determined using a benchtop NMR relaxometer, decreased linearly with GFP concentration, but was not affected by the concentration of a control protein (BSA). (**c**) Detection of caspase 3 enzymatic activity using MNP that were clustered using a linker containing the peptide sequence DEVD. Introduction of caspase 3 resulted in rapid cleavage of the peptide sequence and an increase in T_2 relaxation time, which was abrogated by the addition of a caspase 3 inhibitor. Reproduced with permission from ref. 4, copyright (2002) Nature Publishing Group.

2. Dilute the affinity molecule-MNP sample with PBS to 10–20 μg/mL total Fe concentration (see Note 12).
3. For preclustering MNP prior to a disassembly assay, add the cross-linking agent at the optimal concentration to induce aggregation as determined by experimentation (see Note 13). Incubate for at least 1 h at room temperature. Longer incubations

may produce more pronounced aggregation depending on the rate of cluster formation. Confirmation of MNP aggregation can be obtained by dynamic light scattering at this point. Then, combine equal volumes of the aggregated MNP solution and analyte sample.

4. For aggregation assays, add an equal volume of analyte sample to the MNP solution.
5. Incubate for at least 1 h at room temperature and proceed to analysis (Subheading 3.6 or 3.7).

3.5. Magnetic Tagging of Cells

1. This section describes the detection of biomarkers on the surface of intact, suspended cells by tagging with MNP to impart magnetic susceptibility (see Fig. 3). Similar methods can be used to label adherent cells, followed by disruption and suspension. In addition, intracellular markers can be tagged if the cells are first permeabilized (see Note 14).
2. Wash the cell sample by centrifuging at 300 × *g* for 5 min, aspirating the supernatant, and resuspending in 0.5 mL PBS+.
3. Add the affinity molecule-MNP sample to the cell suspension. When using antibodies as the affinity molecule, a final MNP concentration of 100 nM (~45 μg/mL total Fe concentration for CLIO, see Note 8) should be sufficient (see Note 15).
4. Incubate for 30 min at room temperature on an orbital shaker.
5. Remove unbound MNP using two rounds of centrifugation as described in step 2, Subheading 3.5, but use 1 mL of ice-cold PBS+ each time.

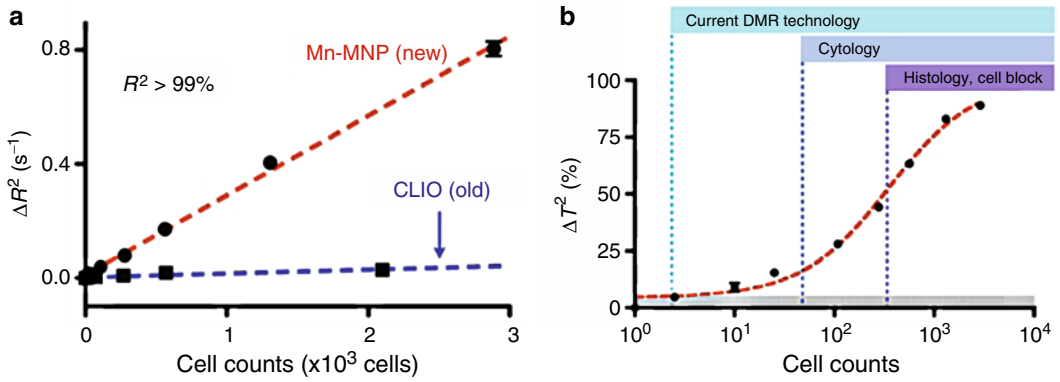

Fig. 3. DMR detection of tumor cells using the tagging method. (**a**) Her2/*neu* was detected on breast cancer cells (BT474) using an anti-Her2/*neu* antibody that was conjugated to CLIO and Mn-MNP nanoparticles. Transverse relaxation rate ($r_2 = 1/T_2$) was measured using a miniaturized NMR (μNMR) detector, and varied proportionally with cell number and the magnetic susceptibility of the nanoparticle employed. (**b**) As few as two cells could be detected using the Mn-MNP nanoparticle, well above the detection threshold of established clinical methods (cytology and histology). Reproduced with permission from ref. 3, copyright (2009) National Academy of Sciences, USA.

6. Resuspend in the minimum volume PBS+ necessary for the detection method of choice (see Subheadings 3.6 and 3.7).

3.6. Detection Using NMR

1. Magnetic resonance signal from MRSw or tagged cell samples can be detected using NMR relaxometers operating at low frequency and magnetic field strength. Benchtop systems such as the Bruker Minispec (20 MHz, <1 T) measure T_1 and T_2 relaxation times of samples in NMR tubes (see Fig. 2b, c). Alternatively, μNMR devices can be used to detect T_1 or T_2 within microfluidic channels (see Fig. 3).
2. For benchtop NMR systems, load sample into 5 (>0.3 mL) or 10 mm (>0.5 mL) NMR tubes. For μNMR devices, sample volumes as low as 1 μL have been achieved using microfluidic elements (3).
3. Measure T_2 relaxation time (and T_1 if desired). For both benchtop and miniaturized systems, T_1 relaxation time is measured using inversion recovery pulse sequences. For T_2 measurement, Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequences are employed to compensate for the spatial inhomogeneity of the external magnetic field. The typical echo time is 2–5 ms and the repetition time is $\approx 5 \times T_1$ to ensure full recovery of nuclear spins (2).

3.7. Detection Using MRI

1. Clinical or experimental MRI scanners can be used to detect magnetic resonance signals from samples loaded into multi-well plates (see Fig. 2a). MRI scanners employ strong magnetic fields (1–11 T) generated by superconducting magnets, and use sophisticated data acquisition schemes.
2. Load 50 μL sample into a 384-well plate.
3. Obtain a T_2 map using T_2 spin-echo sequences with variable echo times. Typically, echo time is varied between 25 and 1,000 ms, and the repetition time ranges between 2,000 and 3,000 ms.
4. Analyze the T_2 image using the appropriate software.

4. Notes

1. If the MNP or affinity molecule storage buffer contains contaminants that will interfere with downstream processing (i.e., Tris or carrier protein for amine reactions, sodium azide for click chemistry), they must first be removed by desalting or chromatography.
2. It is important to avoid divalent cations when working with nanoparticles because they can crosslink the nanoparticles, leading to aggregation. Care should also be taken that chemical modifications to the nanoparticles, including conjugation

of proteins or peptides, do not alter the surface chemistry such that aggregation results.

3. A polytriazole ligand such as bathophenanthroline disulfonic acid should be added to the copper catalyst buffer to stabilize the reduced form of copper [$Cu^{(I)}$] that is required for catalytic activity (22).
4. When using an enzyme to disrupt tissues, caution should be taken that the biomarker of interest is not affected by the activity of the enzyme.
5. The total cell requirement for tagging assays is dependent on the target expression level and the detection sensitivity of the magnetic resonance sensor platform. For NMR measurements, detection thresholds using CLIO are in the range of 10,000 cells using a benchtop relaxometer (Minispec) and 1,000 cells using the miniaturized NMR (μNMR) for a high expression level marker (millions of copies per cell, see Fig. 3). Use of higher magnetization MNP can decrease the detection threshold to near single-cell for the μNMR.
6. Fluorophores can aid significantly in optimizing the binding of the MNP conjugates to cells using fluorescence microscopy or flow cytometry and in tracking the MNP concentration during processing steps using a fluorometer. Fluorophores should be attached prior to affinity molecule conjugation. Since excess reagents are not required for this conjugation, it is easier to control the degree of modification and MNP cross-linking is not an issue. Care should be taken that functional groups remain for affinity molecule conjugation, however. After dye conjugation, the free dye can be removed by desalting and the amount of dye attached can be determined by absorbance (known extinction coefficient) or fluorescence (compared to a standard) measurement. Following dye conjugation, the nanoparticles are typically referred to as magneto-fluorescent nanoparticles (MFNP).
7. Iron oxide MNP are brown and visible to the naked eye at concentrations above 100 μg Fe/mL. Therefore, when working with samples above this concentration, column purifications can be performed manually. For lower concentration samples, purifications should be performed using an automated system (i.e., AKTA fPLC from GE Healthcare) so that absorbance (410 nm) can be monitored.
8. The molecular weight of the MNP can be calculated based on the number of Fe atoms per core and the molecular mass of Fe (55.85 g/mol). For example, CLIO have approximately 8,000 Fe atoms/core (23), thus the estimated average molecular weight is 447,000 g/mol.
9. For modifications of MNP and affinity molecules using amine-reactive *N*-hydroxy-succinimidyl (NHS) esters, reaction times

of 1–2 h are common since the NHS groups hydrolyze in water. However, longer incubations may increase the reaction yield somewhat and should not adversely affect the final product provided that stability is not an issue.

10. MNP aggregation can result if the affinity molecule contains multiple coupling sites (e.g., such as multiple thiol, click reagent, or biotin molecules on a single macromolecular protein). For this reason, the affinity molecule modifications call for a minimal excess of amine-reactive reagent (i.e., two- to fivefold). These recommended values may need to be adjusted to yield approximately 1–2 coupling moieties per affinity molecule. Also of note, excess thiols can self-react to form disulfide bonds, and therefore should be capped with a reagent such as iodoacetamide.
11. Unreacted maleimide and thiol groups remaining after thiol couplings can potentially react with biomolecules or cells, increasing background adhesion and thus decreasing detection specificity. In some cases, capping of free thiol groups using iodoacetamide can help improve binding specificity. However, this should be performed following the purification steps described in steps 4 and 5, Subheading 3.3. Capping is not required when using a bioorthogonal click coupling chemistry.
12. For CLIO, the 10–20 μg/mL Fe recommended for MRSw assays equates to approximately 20–40 nM and typically registers a T_2 relaxation time of 50–100 ms. Due to the extra dilution factor associated with disassembly MRSw assays, the initial MNP concentration used is usually 20 μg/mL.
13. MNP clustering is governed by the equivalence principle, which dictates that clustering is greatest when a multivalent binder (i.e., MNP) and cross-linking agent are present at equimolar concentrations (13). In this case, the pertinent molar concentration is the number of affinity molecules per MNP, not the number of MNP.
14. Intracellular markers can be detected by adding 0.1% saponin to the PBS+ buffer. In this case, longer incubations are recommended (1 h) to increase MNP penetration into the cell, as well as extended washes at room temperature to allow unbound MNP to diffuse out of the cells.
15. The concentration of affinity molecule-MNP used for cell tagging can be lowered below 100 nM if the binding kinetics of the affinity molecule is sufficiently high. For example, a concentration of 10 nM would be sufficient if the equilibrium dissociation constant (K_D) of the interaction is subnanomolar. Likewise, the concentration should be increased if the binding kinetics is poor (i.e., micromolar K_D). The ideal concentration should be determined experimentally for each affinity molecule-MNP of interest.

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