Published in final edited form as:

Adv Healthc Mater. 2014 July ; 3(7): 1015–1019. doi:10.1002/adhm.201300672.

Magnetic Ligation Method for Quantitative Detection of MicroRNAs

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Abstract

A magnetic ligation method is utilized for the detection of microRNAs amongst a complex biological background without polymerase chain reaction or nucleotide modification. The sandwich probes assay can be adapted to analyze a panel of microRNAs associated with cardiovascular diseases in heart tissue samples.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Keywords

microRNA; enzymatic ligation; magnetic labeling; diagnostics; magnetic resonance

Mature microRNAs (miRNAs) are small (typically ~22 nucleotides) molecules of noncoding RNA that regulate gene expression.^[1] These molecules form part of the multi-protein RNA-induced silencing complex, which binds to target messenger RNA and inhibits protein translation. Given that deregulated expression levels of distinct miRNAs in tissue or blood samples have been shown to correlate strongly with the status of several human diseases, miRNAs have been highlighted as potential novel diagnostic markers.^[2] However, the clinical application of miRNA analyses has been hindered by their need for extensive and time-consuming sample preparation steps. Namely, due to the inherently low concentration of miRNAs in biological samples, they need to undergo multiple enrichment processes, including total RNA extraction, as well as amplification by polymerase chain reaction (PCR). Furthermore to improve target specificity, miRNAs need to be elongated prior to PCR (e.g., via poly-A tail elongation or primer ligations).^[3] To exploit the potential of miRNAs in clinical settings, sensitive platforms that can detect miRNAs without complementary DNA (cDNA) synthesis and enzymatic reactions will need to be developed.^[4]

We herein describe a new magnetic method for fast miRNA detection directly in clinical samples. The approach is based on the self-amplifying effect of magnetic nanoprobles (MNPs) during nuclear magnetic resonance (NMR) measurements. Target nucleotides are first labeled with sequence-specific MNPs, which subsequently increases the transverse relaxation rate (R_2) of surrounding water protons. Since a single MNP can affect more than 10^6 surrounding water protons, there is built-in signal amplification, rendering the assay highly sensitive even with small sample volumes. The detection is highly robust against biological backgrounds with minimal need for sample purification.^[5] In previous work, we were successful in applying this method to the detection of PCR-amplified, long-strand DNAs.^[6] The detection of much shorter miRNAs without PCR, however, proved challenging since the method did not have sufficient target specificity and binding affinity. To overcome this problem, we thus combined the use of multivalent labeling with DNA ligation. Target miRNAs in solution were first captured on microbeads and labeled with DNA-modified MNPs. The capture and labeling strands were then enzymatically ligated. By adopting this strategy, we were able to perform robust and selective miRNA detection without nucleotide modification or PCR amplification. Importantly, the assay design was capable of detecting target nucleotides with high specificity, and could even discriminate closely related miRNAs. To demonstrate the clinical utility of the developed assay, we profiled a series of miRNAs implicated in cardiovascular diseases. With its capacity for point-of-care miRNA detection, this magnetic ligation assay has the potential to have significant clinical applications.

Figure 1a shows the scheme of the magnetic ligation assay. The method has two main components: i) polymeric beads modified with single-stranded capture probes (either covalently bound or attached via avidin/biotin), and ii) MNPs conjugated to single-stranded

labeling probes via a noncleavable heterobifunctional linker. The assay begins by capturing target miRNA strands on microbeads. The unbound portion of the bead-immobilized miRNAs is then labeled with sequence-specific MNPs, to create a sandwich nucleotide complex. This is subsequently subjected to ligation and denaturation to improve detection specificity; namely samples are treated with *Taq* DNA Ligase to link the 5' phosphate terminus of the labeling DNA with the 3' hydroxyl terminus of the capture DNA, before being incubated under low-salt conditions at 45°C for denaturation. The level of miRNA expression is then quantified by measuring the R_2 values of samples by NMR.

Initially, we focused on optimizing the assay conditions in order to obtain reliable measurements. The miR-208 family was chosen as the target miRNA, based on its importance in cardiovascular diseases.^[7,8] Microbeads with a diameter of 1 μm were chosen (see Supporting Information), as they yielded the highest analytical signals whilst still maintaining colloidal stability (no sedimentation). MNPs used in the assay consisted of a superparamagnetic iron-oxide core (7 nm) and a cross-linked dextran coating, capable of withstanding exposure to variable temperatures, high salt conditions, and reducing agents during the labeling process.^[9] The capture and labeling DNAs were designed to contain a poly(C) spacer, which improved the binding efficiency by extending the DNA probes from the particle surface. The estimated number of DNA probes per microbead and MNP were 10^5 and 50, respectively. Cross-section electron microscopy confirmed the presence of dense MNP-packing on the bead surface after hybridization (Figure 1b); control beads without the ligation process showed negligible MNP binding (Figure S1).

To increase the number of MNPs on each bead and thereby enhance detection sensitivity, we applied the layer-by-layer assembly approach.^[10] Namely, we designed pairs of MNP conjugates with oligonucleotide sequences that are mutually complementary (see Supplementary Table S1 for the list of oligonucleotides), and added these probes in a sequential, alternating order (Figure 2a). The complementary sets of MNPs were also each modified with different fluorochromes (VivoTag 680/VT680 and fluorescein isothiocyanate/FITC) for comparative optical measurements. Figure 2b shows the fluorescence analyses confirming the layer-by-layer probe assembly. After initial labeling with VT680-modified miR-208a-specific MNPs, the capture beads displayed far-red fluorescence. With the formation of a second layer of FITC-modified MNPs, we subsequently observed both far-red and green fluorescence with flow cytometry; complementary fluorescence micrographs also showed colocalization of both colors on the capture beads. Importantly, such successive layering led to higher R_2 values, and improved overall detection sensitivity (Figure 2c). We subsequently performed titration experiments to establish the limit of detection (LOD) for the assay. Samples were prepared by spiking different amounts of miRNAs into a background of total *E. coli* RNA. We observed a LOD of ~ 1 pM miRNAs in 10 μL sample volumes (Figure 2d); the R_2 value of 1 pM miRNA samples was significantly different from that of negative control ($P = 0.0213$; two-sided t -test). The high detection sensitivity could be attributed to: 1) the higher binding affinity of multivalent MNPs ($K_D \sim 56$ pM) compared to individual oligonucleotide strands ($K_D \sim 1.4$ nM);^[11] 2) the amplifying effect of MNPs in NMR detection; or 3) the multilayering strategy for maximal MNP loading. The assay assumed a dynamic range of $\sim 10^3$, and the signal was linear at low miRNA

concentrations (1–100 pM; $R^2 = 93\%$). The assay was also highly reliable. For example, three separate measurements on the same sample yielded statistically identical R_2 values ($P = 0.4643$; one-way analysis of variance/ANOVA).

We next assessed the capacity of the magnetic ligation assay to distinguish target miRNA from related nucleic acid strands. This is essential for accurate diagnosis since several miRNAs in a related family can differ by only a few nucleotide bases. To achieve such capability, two key components were incorporated into the developed assay: 1) shorter nucleotide probes that have higher specificity for their complementary target; and 2) ligation and denaturation processes that minimize off-target binding.^[12] The shorter nucleotide probes can increase the specificity for the target strand by decreasing the thermodynamic stability for single-nucleotide mismatch binding.^[6] For this experiment, we designed pairs of probes specific for miR-208b (Figure 3a), and then measured the NMR signals after their hybridization with either miR-208a or miR-208b. Upon hybridization with miR-208b, enzymatic ligation resulted in connections between capture strands and adjacent labeling strands; the beads also remained magnetically labeled after the denaturing step. Upon hybridization with miR-208a, however, because of the gap between adjacent nucleotides, the base mismatches between the probes and miRNA inhibited enzymatic ligation. Moreover, denaturation of the nucleic acid complex resulted in beads that were no longer magnetically labeled and which displayed R_2 values close to those of buffer solution. We subsequently further expanded the assay to analyze a panel of miRNAs related to cardiovascular diseases.^[8,13] Accordingly, for a given pair of nucleotide probes, we observed consistent high signals with target miRNA strands (Figure 3b). In contrast, with nonspecific miRNAs, nucleotide mismatches significantly reduced the hybridization efficiency, resulting in minimal background signals.

We next applied the magnetic ligation assay to the analysis of miRNA in heart tissues, and directly compared our NMR measurements with real-time PCR quantification via the gold standard SYBR Green assay. Accordingly, we found that our NMR measurements showed high expression of miR-208a and miR-1 similar to real-time PCR experiments (Figure 3c). In previous studies, it had been reported that both miRNA types are highly expressed in the heart, and that they may thus serve as useful diagnostic biomarkers for cardiovascular diseases.^[8,14] However, it is important to consider that the current analysis of miRNA levels with NMR measurements are primarily qualitative, and our work is currently focused on stringent quantitative assessment for miRNA detection in order to discern differences in expression levels between wild-type and disease phenotypes. Since the current method does not distinguish between precursor, primary, and mature miRNAs, additional work will be conducted to differentiate these strands. Nevertheless, the magnetic ligation assay can be done without reverse transcription and PCR amplification, is faster (60 minutes) and requires smaller sample volumes (10 μ L) than standard techniques.

In summary, magnetic ligation is a promising new method for fast and accurate miRNA analyses. It has the capacity for quantitative miRNA detection without the need for PCR amplification or extensive sample purification steps. Because this novel ligation-denaturation strategy can detect down to single-nucleotide mismatch, it is possible to distinguish target miRNAs from closely related family members. The use of NMR for signal

detection enables the assay to be performed with minimal sample purification, minimizing sample loss and simplifying assay procedures. The assay also benefits from fast reaction kinetics, since the signal is detected on a bulk sample. Combined with the miniaturized NMR (μ NMR) platform,^[15] which has a small form factor and automatically maintains optimal detection settings, magnetic ligation is a versatile technology that could be potentially used for point-of-care diagnosis using miRNA biomarkers to detect a variety of human diseases. To increase the sensitivity further and to enable detection of circulating miRNAs in blood (femtomolar to attomolar ranges), we now intend to isolate individual capture beads on femtoliter-volume wells to enhance binding efficiency using lower copy numbers.^[16] In addition, by integrating magnetic ligation with single-bead magnetic detection technology, the sensitivity and sample throughput is expected to increase even further.^[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank P. Dutta, K. Lee, M. McKee, H. Sager, N. Sergeev, and H. Shao for assistance with the experiments; Y. Fisher-Jeffes for reviewing the manuscript. This work was supported in part by NIH grants R01-EB010011 (R.W.), R01-EB00462605A1 (R.W.), T32CA79443 (R.W.), R01-HL113156 (H.L.), and NHLBI contract HHSN268201000044C (R.W.).

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Figure 1.

Magnetic ligation for detection of miRNAs. (a) Schematic of the assay protocol. Target miRNAs are captured on DNA-modified capture beads (diameter 1 μm) via a complementary sequence. The MNPs coat the beads by hybridizing with the free, complementary portion of the bead-immobilized miRNAs. To increase specificity and minimize off-target binding, *Taq* DNA Ligase is added to catalyze bond formation between the 5' phosphate of the labeling DNA and the 3' hydroxyl of the capture DNA. After removing unbound MNPs, the magnetically-labeled beads are inserted into the microcoil for analysis by NMR. Because the R_2 value is proportional to the number of MNPs in the sample, the expression level of miRNAs can be quantified. (b) Electron microscopy analysis of the magnetically ligated beads shows dense coverage of MNPs on the bead surface.

Figure 2.

Assay performance and the amplification strategy. (a) Layer-by-layer assembly of the MNPs was used to enhance NMR signals for the miR-208a miRNAs. A pair of fluorochrome-modified MNPs with complementary sequences (see Supplementary Table S1 for full list of oligonucleotides) were designed for multilayer deposition and for increasing the number of MNPs deposited on the surface. (b) Scatter plots from flow cytometry measurements are shown for: i) unlabeled beads (left), ii) beads labeled with a single layer of MNPs (middle), and iii) beads labeled with two layers of MNPs (right). Fluorescence microscopy images (inset) are shown for the corresponding beads. (c) NMR signals from the beads were measured after each layer of labeling, and normalized with signals from the first layer of labeling. (d) The magnetic ligation assay (2 layers of labeling) could detect as low as 10^6 miRNAs in a 10 μ L sample containing background *E. coli* RNA. The μ NMR signal values were obtained by normalizing the relaxation rate of samples containing target miRNA with the relaxation rate of control samples. All experiments were performed in triplicate.

Figure 3.

Analysis of miRNAs associated with cardiovascular diseases. Enzymatic ligation between the capture and labeling probes was used to detect target miRNAs. a) Nucleotide probe pairs specific for miR-208b only ligate in the presence of a fully complementary target. Beads thus remain magnetically labeled even after denaturing the nucleic acid complex. In contrast, because nucleotides between miR-208b probes and miR-208a strands are mismatched, denaturing the nucleic acid complex results in MNP release from the bead surface. (b) Relaxation rates for detection of different miRNAs using the magnetic ligation probes. Positive signals only occurred when the capture and labeling probes matched the target miRNA. (c) Analysis of a panel of miRNAs related to cardiovascular diseases in human tissue samples. The NMR measurements using the magnetic ligation method were compared with real-time PCR quantification using SYBR Green. All experiments were performed in triplicate.