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Synthesis and characterization of a magnetically active ^{19}F molecular beacon

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Abstract

Gene expression is used extensively to describe cellular characteristics and behaviors; however, most methods of assessing gene expression are unsuitable for living samples, requiring destructive processes such as fixation or lysis. Recently, molecular beacons have become a viable tool for live-cell imaging of mRNA molecules in situ. Historically, beacon-mediated imaging has been limited to fluorescence-based approaches. We propose the design and synthesis of a novel molecular beacon for magnetic resonance detection of any desired target nucleotide sequence. The biologically compatible synthesis incorporates commonly used bioconjugation reactions in aqueous conditions and is accessible for labs without extensive synthesis capabilities. The resulting beacon uses fluorine (^{19}F) as a reporter, which is broadened, or turned “off,” via paramagnetic relaxation enhancement from a stabilized nitroxide radical spin label when the beacon is not bound to its nucleic acid target. Therefore, the ^{19}F NMR signal of the beacon is quenched in its hairpin conformation when the spin label and the ^{19}F substituent are held in proximity, but the signal is recovered upon beacon hybridization to its specific complementary nucleotide sequence by physical separation of the radical from the ^{19}F reporter. This study establishes a path for magnetic resonance-based assessment of specific mRNA expression, providing new possibilities for applying molecular beacon technology in living systems.

Graphical Abstract

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Notes

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Supporting Information

PA gel method, theoretical mRNA concentration calculations, Supplementary figures

INTRODUCTION

Chemical biologists have made many recent strides in the imaging and detection of gene expression. The development of molecular beacons, or short, hairpin-shaped nucleic acid hybridization probes, has contributed to the growth in this area.¹ Molecular beacons typically contain a modified fluorescence resonance energy transfer (FRET) pair featuring a “donor” fluorophore on one end of the oligonucleotide and an “acceptor” quencher on the other. In its native hairpin state, the stem region of the beacon is bound, which holds the fluorophore and quencher in close proximity, resulting in a drop in fluorescence at the donor wavelength. Once the stem is denatured, either by hybridization of the beacon loop region to a target sequence or the application of heat, the beacon linearizes and physically separates the fluorophore and quencher, resulting in donor fluorescence.

Molecular beacons have long been used to improve the signal-to-noise ratio for qPCR and fluorescence in situ hybridization experiments, but more recently, beacons have also been applied to the imaging of live cell gene expression.^{2,3} Molecular beacon sequences can be designed to target a specific messenger RNA (mRNA) molecule of interest. Once bound to the intended mRNA, the intensity of the fluorescence emitted by the linearized beacon serves as a reporter for the expression of that particular gene. This technique has been used to monitor viral infection of mammalian cells, to follow expression of oncogenes in cell lines, and, in our own studies and others, to track differentiation of primary mesenchymal stem cells.^{4–8}

While fluorescence-based imaging is useful in many contexts, it is difficult to implement for in vivo imaging due to the limits of light penetration into tissues.^{9,10} A need therefore exists for a molecular beacon that is compatible with a deeper imaging modality. Magnetic resonance (MR) has been a critical technology with uses including deep macroscale imaging of tissues and organs via magnetic resonance imaging (MRI), visualization of metabolites in specific physiological and pathological sites by magnetic resonance spectroscopy (MRS), and subnanoscale investigations of molecular structures via nuclear magnetic resonance (NMR). Thus, an opportunity exists to combine the utility of molecular beacon technology to the broad applicability of magnetic resonance spectroscopy. Fluorine (¹⁹F) has been successfully used as a reporter in paramagnetic molecular probes for the detection of proteins, enzyme activity, and nucleic acids.^{11–17} The ¹⁹F isotope is widely used in these probes due to its 100% natural abundance and high gyromagnetic ratio, facilitating sensitivity in MR studies. Additionally, ¹⁹F is rare in biological systems, so the background

signal is low, improving signal-to-noise ratio in cells or tissues compared to ^1H NMR. We propose herein a novel, magnetically active ^{19}F molecular beacon for specific nucleotide sequence detection. Beacon NMR signal is “off,” or quenched, in its hairpin conformation and “on,” or visible, in its open conformation. The behavior of the magnetically active ^{19}F beacon was assessed by 1D NMR as a proof of concept for future use in magnetic resonance imaging modalities.

The theory for quenching of the beacon magnetic resonance signals is based on paramagnetic relaxation enhancement (PRE) where the ^{19}F MR signal is turned “off” by PRE. Due to dipolar interaction between an unpaired electron and a nuclear spin, an NMR-active nucleus experiences a strongly increased rate of relaxation when in close proximity to a paramagnetic moiety, causing its resonance to be broadened beyond detection in an NMR spectrum. PRE is distance-dependent, exhibiting analogous behavior to fluorescent quenching, with a maximum effective distance of 35 Å.^{18,19} Several of the “on-off” ^{19}F probes previously mentioned use gadolinium, a paramagnetic lanthanide widely used as an MRI contrast agent, to generate this PRE effect.^{13,15,20,21} However, in this work, PRE-mediated on-off signaling is achieved with a stabilized pyrroline-based nitroxide radical. This compound was chosen for its stability in buffered systems, simple attachment chemistry to the oligonucleotide beacon, and the unique configuration of its single unpaired electron, which has a nearly isotropic g tensor that eliminates other paramagnetic effects like pseudocontact shifts.^{18,22} With the oligonucleotide in its hairpin conformation, the radical is held within 6–12 Å of the fluorine compound, which enables PRE broadening of the ^{19}F resonance. However, in the linearized DNA conformation, the moieties have ~100 Å separation, causing the effects of PRE to become negligible. This work establishes a proof-of-concept, MR-based, on-off beacon that is easily synthesized in aqueous conditions using only commercially available materials and without specialized equipment. Due to its facile synthesis, we are optimistic that research groups without organic synthesis facilities will be able to adopt this approach and subsequently use the molecule to investigate complex biological problems involving oligonucleotide recognition both in vitro and in vivo.

RESULTS AND DISCUSSION

Synthesis of ^{19}F Modified Beacon

The objective of this study was to synthesize a magnetically active molecular beacon that displays predictable on-off signaling behavior in response to target hybridization. The synthesis used commercially available components and took advantage of high-yield, aqueous chemistry (Scheme 1). Once the beacon was fully synthesized, hybridization assays with matched and mismatched targets were performed to verify the binding specificity of the beacon. ^{19}F signal intensity was also assessed at varying beacon concentrations. These experiments served to demonstrate functionality of the novel, magnetic resonance beacon.

The synthesis of this beacon relies on methods adapted from those used for post-translational modification of proteins. This relatively facile synthesis relies on the modification of functional groups that are either naturally present on biological macromolecules or that can be added with relative ease during synthesis. Further, these modifications can be done in aqueous buffers.^{23,24} While these bioconjugation reactions

proceed according to conventional conditions with proteins and peptides, the conditions had to be adjusted to allow for compatibility with DNA. The presented approach for beacon synthesis can be accomplished in any laboratory, independent of synthetic skill or expertise.

Fluorinated beacons were generated using a meta-disubstituted trifluorobromoacetyl (TFB) molecule, 2-bromo-1-[3,5-di(trifluoromethyl)phenyl]ethan-1-one. This compound was chosen because of its reactivity with thiol groups, its commercial availability, and its six magnetically identical fluorine atoms (two trifluoromethyl groups), which proportionately increase the signal-to-noise ratio in NMR.²⁵ However, TFB is largely insoluble in aqueous buffers while the DNA hairpin is largely insoluble in organic solvents. To alleviate solubility issues, a co-solvent system was developed consisting of 35% v/v 1-propanol in 20 mM, pH 7.4 sodium phosphate buffer. This co-solvent kept all components in solution and allowed for complete reaction of the reduced thiol groups on the oligonucleotide with TFB. Despite the aqueous insolubility of TFB, the full beacon was found to be highly soluble in the NMR buffer at the concentrations used in these experiments.

PRE-mediated broadening of the fluorine resonance was achieved by a pyrroline-based nitroxide radical-NHS ester compound, 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate N-hydroxysuccinimide (NHS) ester. This compound was chosen based on the well-established, methyl-group-stabilized tetramethylpyrroline radical with an isotropic magnetic moment for optimal PRE.¹⁸ The NHS ester attached to the nitroxide radical allowed ready reaction with the 5' primary amine modification on the oligonucleotide, a common attachment mechanism used to modify oligonucleotides.^{25,26} Initially, we attempted to use amine-reactive gadolinium-ion chelates to generate PRE following a similar study by Sakamoto and colleagues.¹⁵ Unfortunately, the desired on-off behavior was not observed when using gadolinium. This was possibly due to the affinity of gadolinium ions for phosphate groups, which may have led to non-specific attachment of the metal to random sites on the oligonucleotide near the ¹⁹F moiety, resulting in a permanent "off" signal.²⁷ Inadequate synthetic or purification methods may have also prevented reproduction of this work in our lab, as the use of gadolinium quenchers in the presence of phosphates has been demonstrated by other groups.^{13,20} Comparatively, the nitroxide radical compound does not use any paramagnetic metals, eliminating the potential for metal ion interaction with the phosphodiester backbone.

Mass Spectrometry Confirmation of Modifications

Mass spectra were collected using electrospray ionization-mass spectrometry (ESI-MS) at each step of the synthesis to confirm reaction and successful moiety conjugation to the oligonucleotide. Results verified the expected molecular weights of the starting material oligonucleotide (MW: 9824 Da, Fig. 1A), oligonucleotide with deprotected thiol group (**1**, MW: 9734 Da, Fig. 1B), oligonucleotide conjugated to the TFB (**2**, MW: 9988 Da, Fig. 1C), and fully conjugated oligonucleotide with TFB and stabilized nitroxide radical (**3**, MW: 10,154 Da, Fig. 1D). Additionally, the presence of DNA in the reaction was verified by UV absorbance and nondenaturing polyacrylamide gel electrophoresis (Supp. Fig. S1). Based on integration and comparison of the extracted ion chromatogram (EIC) peaks, yield of deprotected oligonucleotide by thiol group reduction (**1**) was effectively 100%. The average

percentage of TFB-only oligonucleotide (**2**) from the next reaction step was $90 \pm 14\%$ ($n=7$ syntheses) with some residual deprotected oligonucleotide (**1**) left unmodified. Following the remainder of the synthesis, average percentage of fully modified oligonucleotide (**3**) was $84 \pm 6\%$ ($n=7$ syntheses) with the minor, secondary products corresponding to $3 \pm 2\%$ nitroxide radical-only oligonucleotide and $12 \pm 7\%$ TFB-only oligonucleotide (**2**). Final yield of fully modified oligonucleotide synthesized from the starting material was $\sim 50\%$ due to DNA loss in the concentration and buffer exchange steps. Though several synthesis batches had slight background signal in NMR from residual, TFB-only beacons, they could generally be converted to fully modified beacons by repeating the nitroxide radical reaction step of the synthesis (step 3) as the NHS ester hydrolysis takes place relatively quickly and may not allow enough time for complete reaction with all of the oligonucleotides.

Characterization of On-off ^{19}F Beacon Behavior

After conjugating TFB to the oligonucleotide, baseline ^{19}F NMR spectra of the beacon containing only the fluorine compound (**2**) were established. Since PRE requires a paramagnetic moiety and its activity is a distance-dependent phenomenon, the ^{19}F resonance when attached to one half of the beacon stem should be visible both when the nitroxide radical is totally absent from the other side of the stem as well as when the radical compound is present but not attached. The NHS ester is rapidly hydrolyzed, so addition of stoichiometric amounts at NMR conditions does not result in labeling, which was confirmed by mass spectrometry. As expected, both conditions resulted in a strong peak from the conjugated ^{19}F TFB at approximately -63.1 ppm, indicating that no nonspecific PREs arose from incidental interactions with unconjugated nitroxide radical (Fig. 2, top and middle). However, when the radical is bound to the beacon stem and held in close proximity to the ^{19}F reporter, PRE broadens the resonance into the baseline, resulting in the absence of a resonance peak (Fig. 2, bottom). It is notable that the chemical shift position of TFB-only beacon (-63.1 ppm) (**2**) differs slightly from free TFB in solution and the fully modified beacon when linearized, which both appear slightly downfield at -62.9 ppm (Supp. Fig. S2).

Once the radical was conjugated to create the fully assembled beacon (**3**), the functionality of the molecule as an “on-off,” beacon-type probe was verified. To demonstrate that the PRE arising from the nitroxide radical is responsible for this behavior, sodium ascorbate was added to the beacon to reduce the unpaired electron of the nitroxide radical as previously demonstrated with other nitroxide spin label probes in NMR.²⁸ NMR spectra following incubation of the beacon with ascorbate showed a ^{19}F NMR signal, which was previously broadened beyond detection by the active nitroxide radical (Supp. Fig. S3). The peak was notably not as intense as the TFB-only-modified beacon, which suggests that the ascorbate did not completely reduce all of the radicals on the full beacons and consequently did not eliminate all PRE effects.

Denaturation of the hairpin stem and linearization of the oligonucleotide through elevated temperature also demonstrated the on-off behavior of the beacon. ^{19}F NMR spectra were collected in 10 degree increments from 20 to 80°C , which represent temperatures below and above the theoretical melting temperature of the beacon, 64°C (Supp. Fig. S4). As predicted, no NMR peak was observed below 50°C due to the dominance of the hairpin structure,

which brings the nitroxide radical close to the fluorinated TFB and eliminates MR signal. Above 50°C, a peak began to be visible at -63.0 ppm, indicating denaturation or melting of the hairpin structure. These results were direct, thermodynamic indicators of the presence of the desired molecular beacon construct (**3**). No peak was observed at physiological and room temperatures. Kieger and colleagues produced a similar, temperature-dependent signal increase by thermally denaturing their gold nanoparticle-conjugated, ^{19}F -oligonucleotide probe, though the resulting signal was unable to be turned “off” once after observation of the signal since the molecules were physically released from the nanoparticle probe.¹⁶ These observations further support the notion that the PRE arising from the nitroxide radical broadens the signals of the conjugated ^{19}F TFB in a distance-dependent manner.

^{19}F Beacon Hybridization Specificity

In fluorescence studies, molecular beacons are sufficiently specific to distinguish between a perfectly complementary target sequence and varying mismatched sequences.¹ To test the specificity of the magnetically active molecular beacon, a hybridization assay was performed using NMR (Fig. 3). This was done by first acquiring a baseline NMR spectrum of a 13 μM sample of fully modified molecular beacon (**3**). Mismatched target was then introduced to this sample at a 12:1 molar ratio to the beacon, and an NMR spectrum was acquired. The mismatched target was one nucleotide shorter than the beacon loop with 78% mismatched nucleotides in a 5' to 3' beacon orientation. Subsequently, the complementary matched target was added to the same sample at a 12:1 ratio to the beacon, and the spectrum acquired once more. The ^{19}F signal of the beacon was not observed when alone or with the mismatched target but became detectable once in the presence of its matched target (Fig. 3). These results indicated that the beacon was able to hybridize specifically to its target sequence while otherwise remaining in its unbound, hairpin configuration. Similar results were observed in analogous hybridization assays using a fluorescent version of the same molecular beacon sequence (Supp. Fig. S5). The match-mismatch assay provided indirect, yet compelling, evidence of the hybridization ability of the molecular beacon to its intended target, and, in principle, this same thermodynamic behavior should be replicated when using this beacon in living systems.

^{19}F Beacon Concentration Dependence of Signal Intensity

In all of the previously described experiments, ^{19}F NMR spectra were collected at the same beacon concentration, but it remained to be seen whether NMR could be used to determine otherwise unknown concentrations based on signal intensity below this concentration. This capability would allow for relative quantification of target mRNA concentrations in biological samples using only MR signal intensity. Kanazawa and colleagues demonstrated this approach by quantitating the ^{19}F signals from samples extracted from 5-fluorouracil-treated mice.²⁹ Here, NMR spectra of (**3**) were obtained in vitro at 4, 5, 7, 10, and 13 μM of full beacon in the presence of excess matched target DNA to investigate the feasibility of using NMR to determine concentration of signaling beacons, as well as defining an estimated lower limit of detection for the probe (Fig. 4A). As before, beacons displayed on-off behavior in response to exposure only to complementary DNA and, notably, the intensity of the NMR peak increased with increasing concentration. Using the signal-to-noise ratio for each concentration spectrum, a practical lower limit of detection of “on” beacons due to a

binding event for these experimental conditions and acquisition parameters was determined to be $\sim 1.3 \mu\text{M}$, denoting where the signal was twice as intense as the noise (Fig. 4B). Due to the tight binding of beacon to target, it is assumed in this dilution experiment that all beacons present in the sample are hybridized to the matched target due to its large molar excess. However, the ability to detect a signal above the noise in a system of lower matched target concentrations is more relevant to physiological applications and was therefore assessed (Fig. 4C). The linear portion of the concentration curve suggests a similar theoretical limit of detection, with a signal-to-noise ratio of 2 corresponding to $2.7 \mu\text{M}$ of added matched target if extrapolated. However, when attempting to detect a signal in NMR with these lower concentrations of matched target, the signal was indistinguishable from the noise of the spectrum below two signal-to-noise ratios. The absence of any detectable background signal from the unbound beacon (alone and with mismatched DNA, Fig. 3) at the -62.9 ppm hybridized beacon resonance position does suggest high sensitivity for detection of binding. Therefore, this result may be due to slowed hybridization kinetics which cannot be observed over the lengthy spectrum acquisition time necessary for these NMR-based experiments and may differ from the kinetics of the fluorescent version of this beacon (Supp. Fig. S5). It is important to note that the practical limit of detection will depend on experimental parameters in addition to concentration, such as number of scans, probe configuration, and magnetic field strength.

Target mRNA levels vary widely in the body depending on gene expression, turnover, and tissue density, with one study of 5000 mammalian genes showing a range of 10 to 10,000 mRNA copies per cell.³⁰ The cell density of tumors ranges from 10^7 to 10^9 cells/cm^3 , resulting in a possible mRNA concentration range of 0.17 pM to 17 nM .³¹ If the ^{19}F beacons were administered and bound to these mRNA targets with 100% hybridization efficiency, this is currently two to seven orders of magnitude away from being detectable using our existing experimental setup (calculation in Supporting Information). Therefore, future efforts to improve sensitivity to within physiological mRNA concentration ranges will include design of beacons that incorporate additional fluorine attachment positions and application of dynamic nuclear polarization. As our magnetically active molecular beacons develop, it is also desirable for magnetic resonance technologies to improve in strength and sensitivity to enhance the potential of these beacons as biological probes.

While the current study provides a synthetic pathway for the development of magnetically active molecular beacons and provides evidence of on-off functionality by binding targets in solution, use of this molecule in complex cellular environments is yet to be demonstrated. However, this beacon was intentionally developed with many advantages for eventual studies in biological systems. ^{19}F is a favorable nucleus for NMR due to its large gyromagnetic ratio, 100% isotopic abundance, and low biological background signal. Additionally, while stabilized nitroxide radicals are still relatively unstable, especially in an uncontrolled cellular environment where they are subject to reduction by radical scavengers, multiple studies have demonstrated use of nitroxide radicals as contrast agents for in vivo imaging.^{22,32,33} These studies showed that pyrroline-based nitroxide radicals can remain stable for at least 20–30 minutes in vivo, which is sufficient time for injection, target hybridization, and imaging, though longer stability may be achieved by gadolinium chelates.²¹ It has also been shown previously that molecular beacons can be modified for greater

stability and resistance to nuclease degradation in cellular environments using 2'-o-methyl bases, peptide nucleic acids, locked nucleic acids, or phosphorothioate.^{34–37} Since our magnetically resonant beacon is a standard, unmodified oligonucleotide, there is potential to incorporate any of these modifications for improvement in biostability in future experiments. The current study establishes a framework from which potential future applications can be developed, with the ultimate goal being the facilitation of gene expression-based MR imaging in large organisms.

METHODS

Oligonucleotides

All oligonucleotides were purchased commercially. The loop region of the beacon is complimentary to a sequence specific to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (sequence: 5' Amino C3-GGTCCGGAGTCCCTTCCACGATACCACGGACC-Thiol C3 3', Eurofins MWG Operon, Huntsville, AL, USA).³⁸ Oligonucleotides were HPLC purified, verified by MALDI-TOF, lyophilized, and shipped dry. Upon arrival, oligonucleotides were resuspended at 100 μ M in 20 mM sodium phosphate buffer at pH 7.4 and stored at -20°C until use.

Conjugation of trifluorobromoacetyl (TFB) and stabilized nitroxide radical spin label

Reduction of protected 3' disulfide (1)—Oligonucleotide stock solution was added to dithiothreitol (DTT) (Acros Organics, Thermo Fisher Scientific, NJ, USA) in 20 mM sodium phosphate buffer at pH 7.4, bringing $[\text{DTT}]_{\text{final}}$ to 100 mM, and $[\text{oligo}]_{\text{final}}$ to 10 μ M. The reduction reaction was allowed to proceed at room temperature protected from light for 60 minutes. DTT was removed by buffer exchange using a 15 mL, 3 kD molecular weight cutoff spin concentrator according to manufacturer's instructions (EMD Millipore, Billerica, MA). In brief, the reaction solution was added to the reservoir of the spin concentrator and brought to 15 mL with 20 mM pH 7.4 sodium phosphate buffer. The concentrator was spun at $4000 \times g$ for 30 minutes until approximately 500 μ L was concentrated in the reservoir. The flow-through was then discarded, and the reservoir volume was brought back up to 15 mL with fresh 20 mM sodium phosphate buffer. The concentrator was spun for a total of four buffer exchange cycles. The resulting 500 μ L concentrate was collected for analysis by mass spectrometry before proceeding with the next step of the synthesis.

Conjugation of TFB to deprotected 3' thiol (2)—1-propanol was added to the deprotected oligonucleotide solution in 20 mM, pH 7.4 phosphate buffer to create a co-solvent system (35% 1-propanol/65% 20 mM sodium phosphate buffer, v/v). 100 mM TFB (2-bromo-1-[3,5-di(trifluoromethyl)phenyl]ethan-1-one, Maybridge Chemical, Trevillet, UK) stock solution was made fresh by dissolving TFB in 1-propanol. This was added to the co-solvent solution, bringing $[\text{TFB}]_{\text{final}}$ to 5 mM and $[\text{oligo}]_{\text{final}}$ to 10 μ M. The reaction solution was mixed well and placed at 4°C for 18–24 hours to yield (2).

Conjugation of nitroxide radical – NHS ester to 5' amine (3)—The TFB-only oligonucleotide was buffer exchanged four times by spin concentration, as described

previously, into 100 mM, pH 9.1 sodium borate buffer. 100 mM nitroxide radical-NHS ester (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate N-hydroxysuccinimidyl ester, Toronto Research Chemicals, Toronto, ON) stock solution, made fresh in DMSO, was added to the oligonucleotide concentrate, bringing [nitroxide radical – NHS ester]_{final} to 5 mM and [oligo]_{final} to 20 μ M. The reaction solution was mixed and left to react at 4°C for 18–24 hours. The pH and temperature of this reaction are critical to balance the deprotonation of the amine group on the hydrocarbon linker with preventing the rapid hydrolysis of the NHS ester at alkaline pH. The solution was buffer exchanged three times with nanopure water (DNase-, RNase-, protease-free, no divalent cations; Hyclone, Fisher Scientific) to yield the final molecular beacon (3).

Characterization of oligonucleotides

HPLC and ESI-MS of oligonucleotides (1–3)—Oligonucleotide characterizations were carried out on an Agilent 6530 Accurate Mass Q-TOF mass spectrometry (MS) system (Agilent Technologies, Santa Clara, CA), with both UV and MS detection, coupled with the Agilent 1260 Infinity series liquid chromatography instrument. Both Agilent Zorbax (reverse phase C18, 2.1 $\times$ 50 mm, 3.5 μ m pore size) and Waters XTerra MS (RP-C18, 2.1 $\times$ 50 mm, 3.5 μ m pore size) columns were used at 25°C. Mobile phases used were A, 10 mM ammonium acetate in water and B, methanol. The time program of the LC gradient was as follows: 0 min 5% B, linear increase to 65% B at 8 min, increased to 95% B at 9 min, and back to 5% B at 11 min, followed by equilibration for 10 min. The Q-TOF MS instrument was operated with an electrospray ion (ESI) source in the negative ionization mode. Data analysis used Agilent MassHunter Qualitative Analysis (ver. B.06.00) with BioConfirm Software, and the average oligonucleotide mass was calculated from the raw data by maximum entropy deconvolution. For yield quantification, EIC peaks were manually integrated and summed to obtain the percentage of each product formed throughout the syntheses. The UV chromatograms were not used for yield estimation due to the strong absorbance of nitroxide radical and residual NHS at 260 nm which may have augmented some of the peaks. For the seven syntheses analyzed, the average percentage of each product with standard deviations are reported along with a total yield for the reaction based on DNA concentrations from starting material to final products quantified on a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA).

NMR of oligonucleotides (2) and (3)

All spectra were acquired on a Bruker AVANCE III HD Ascend 600 MHz NMR (Bruker Corp., Billerica, MA, USA) fitted with a 5 mm BBO probe tuned to 564 Hz to observe ¹⁹F directly. Beacon samples were run with 1024 scans plus 6 dummy scans with a 1.0 second acquisition time, 6.5 μ s pre-scan delay time, 8 μ s dwell time, 124,992 data points, 14 μ s pulse length, receiver gain set to 203, and 62,500 Hz spectral width. NMR spectra of beacons were acquired in thin-walled, precision glass tubes rated for 600 MHz with 500 μ L sample volumes (Wilmad LabGlass, Vineland, NJ). Data were apodized with 3 Hz exponential line broadening operation and analyzed in Topspin (Bruker).

Baseline NMR of TFB-only molecular beacon (2) with free nitroxide radical—A sample of (2), the partially assembled beacon lacking the nitroxide radical compound, was

prepared at 13 μM in testing solution, comprised of 50 mM pH 7.4 Tris-HCl buffer made with solvent grade deuterium oxide (D_2O) (Sigma-Aldrich, St. Louis, MO) and the spectrum was then acquired at 20°C. To test if nitroxide radical in free solution causes non-specific direct PREs, 15 μM nitroxide radical-NHS ester in DMSO was added and the NMR spectrum was again acquired.

Ascorbate and temperature effects on magnetically active molecular beacon

(3)—A sample of **(3)**, fully assembled beacon, was prepared at 13 μM in testing solution. The NMR spectrum was acquired as above. Subsequently, the sample was incubated with 100 mM sodium ascorbate solution for 3 hours at room temperature, protected from light. Spectra were then acquired on this sample in the presence of ascorbate. Separately, to determine the effect of temperature on the observation of ^{19}F signals from a fully assembled beacon, spectra were acquired at 20, 30, 40, 50, 60, 70, and 80°C on another 13 μM full beacon sample. Each spectrum was acquired using the standard NMR parameters listed previously.

MR molecular beacon (3) hybridization assay—A sample of **(3)** was prepared at 13 μM in testing solution. Spectra were acquired at 20°C. Mismatched target (5' CAAACCCCTATTCCATGC 3', Eurofins MWG Operon, 10 mM stock solution) was added to the sample at a final concentration of 150 μM and incubated for 10 minutes before spectra were again acquired at 20°C. Finally, matched target (5' TGGTATCGTGGGAAGGACTC 3', Eurofins MWG Operon, 10 mM stock solution) was added to the sample at a final concentration of 150 μM and incubated for 10 minutes before spectra were acquired at 20°C. Each spectrum was acquired using the standard NMR parameters listed above.

MR molecular beacon (3) concentration curve with excess matched target—To estimate the lower limit of detection for hybridized beacon, a spectrum of 13 μM beacon in testing solution in the presence of 480 μM mismatch and 480 μM match oligonucleotides was acquired in NMR. The sample was serially diluted to 10, 7, 5, and 4 μM , and spectra were acquired at each concentration. Signal-to-noise ratio was quantified using the signal-to-noise ratio function in the software.

Concentration curve of matched target with MR molecular beacon (3)—To determine the lower limit of detection of target DNA, spectra of 11 μM beacon in testing solution were acquired in the NMR in the presence of 0, 2.6, 5.1, 7.7, 10, 20, and 29 μM match oligonucleotides. Signal-to-noise ratio was quantified using the signal-to-noise ratio function in the Bruker TopSpin software for all spectra, including those spectra without a discernible peak for which the signal area specified was -62.89 to -62.92 ppm.

Fluorescent molecular beacon hybridization assay

A fluorescent version of the same molecular beacon sequence was purchased commercially (Eurofins MWG Operon, Huntsville, AL). The beacon was functionalized with a 5' 6-carboxyfluorescein molecule and a 3' Black Hole Quencher 1. Upon arrival, beacon was reconstituted in nanopure water to a final concentration of 100 μM . Four conditions were tested (final volume, 200 μL): NMR buffer alone (50 mM Tris-HCl in D_2O), NMR buffer

with 250 nM fluorescent *GAPDH* beacon, NMR buffer with 250 nM *GAPDH* beacon and 2.5 μ M mismatched target, and NMR buffer with 250 nM *GAPDH* beacon, 2.5 μ M mismatched target, and 2.5 μ M matched target. Samples were excited at 492 nm, and emission read at 520 nm at 25°C every 10 seconds for a total of 41 minutes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

¹⁹F	fluorine
FRET	fluorescence resonance energy transfer
mRNA	messenger ribonucleic acid
MR	magnetic resonance
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance
PRE	paramagnetic relaxation enhancement
TFB	trifluorobromoacetyl
ESI-MS	electrospray ionization-mass spectrometry
EIC	extracted ion chromatogram

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Figure 1.

ESI-MS analysis of oligonucleotides at each step of the reaction (products **1–3**). Predicted masses were verified for the starting oligonucleotide (A, MW: 9824 Da), the oligonucleotide with reduced thiol group (**1**) (B, MW: 9734 Da), the oligonucleotide modified with TFB (**2**) (C, MW: 9988 Da), and the fully modified oligonucleotide (**3**) (D, MW: 10,154 Da).

Figure 2.

^{19}F NMR spectra of TFB-only oligonucleotide (**2**) (green, top) in the presence of unconjugated nitroxide radical (red, middle) compared to conjugated nitroxide radical which forms the full beacon (blue, bottom). The ^{19}F resonance is unaffected by radicals in solution but is broadened in a distance-dependent manner once the radical is bound to the other end of the beacon stem. Exponential line broadening of 3 Hz applied.

Figure 3.

^{19}F NMR spectra of 13 μM fully modified beacon (**3**) alone (top, green), in the presence of 150 μM mismatched target (middle, red), and the addition of 150 μM matched target (bottom, blue). Spectra remained unchanged upon the addition of mismatched target, but matched target DNA hybridized to the beacon disrupting paramagnetic broadening of fluorine resonance by physical separation of the ^{19}F from the spin label. Exponential line broadening of 3 Hz applied. Data is representative of behavior observed in 3 additional replicate experiments.

Figure 4.

NMR spectra of ^{19}F molecular beacon (**3**) in the presence of excess matched target at full beacon concentrations of 4, 5, 7, 10, and 13 μM (A). Signal-to-noise ratio was linearly correlated with hybridized beacon concentration, allowing for prediction of the lower limit of detection for the beacon when signal is twice as high as noise under these same processing conditions if all of the available beacon is bound (B). Signal-to-noise ratio was also assessed for a different batch of ^{19}F molecular beacon (**3**) at 12 μM with increasing concentrations of matched target added (C). Data points above the red line correspond to the observance of a discernible signal while those concentrations below a signal-to-noise ratio of 2 were undetectable. Exponential line broadening of 3 Hz applied to each spectrum. Signal-to-noise ratios were determined using Bruker TopSpin NMR

Scheme 1.
Conjugation of Trifluorobromoacetyl and Nitroxide Radical to Oligonucleotide