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^{19}F NMR indicator displacement assay using a synthetic receptor with appended paramagnetic relaxation agent[†]

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Abstract

An admixture of zinc(II)-bis(dipicolylamine) receptor with covalently attached paramagnetic relaxation agent and fluorine-labeled phosphate indicator enables ^{19}F NMR detection of phosphorylated analytes with amplified switched-on signal intensity.

One of the major practical goals of supramolecular chemistry is improved analytical methods for detecting molecular and ionic analytes in aqueous solution.¹ A common strategy is to design reporter molecules with ability to associate with a specific analyte and produce an observable signal change.² An alternative approach is the Indicator Displacement Assay (IDA) which creates a binding competition between the analyte (**A**) and indicator molecule (**I**) for a suitably designed receptor (**R**).³ In the most common assay configuration, the signal for **I** is quenched when it associates with **R** and the signal is turned on when **I** is displaced by **A**.⁴ To date, the vast majority of IDA studies utilize optical indicators that produce a change in absorbance or luminescence.^{5, 6} In principle, IDAs based on other spectroscopic methods have specific performance advantages. For example, NMR spectroscopy has greater signal dispersion, which favors the creation of multiplex sensing assays.⁷ In addition, the ability to manipulate NMR spin states using multipulse sequences allows selective signal detection and spectral editing. Furthermore, the ability of MRI to visualize signals that are spatially deep within physical objects, including living subjects, raises the intriguing idea of buried IDAs that are detected remotely. The largest technical drawback with an NMR-based IDA is the relatively low detection sensitivity. Ongoing improvements in NMR signal enhancement are expected to ameliorate this concern,⁸ as well as the invention of generalizable methods that magnify the theoretical maximum signal from **I**. A potential solution to the latter problem is to devise IDAs with catalytic cycles that produce amplified signals.⁹ Although inherently appealing, the catalysis design challenges are considerable, especially with low molecular weight analytes.

Here, we describe an alternative design concept that is well suited to NMR spectroscopy - a rapidly exchanging association system that allows **R** to quench the excited state of multiple

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I. This strategy is not feasible with an optically active **I** because electronic excited state lifetimes (10^{-10} – 10^{-6} s) are much shorter than the time-scale for intermolecular exchange of a typical **R:I** pair. In contrast, the excited state lifetime of a nuclear magnetic moment is typically on the order of seconds and usually sensitive to chemical exchange. There are several ways that a rapidly exchanging association system can be exploited to modulate NMR relaxation time or chemical shift. One established approach takes advantage of the large change in correlation time or chemical shift anisotropy for **I** upon association with a high molecular weight **R** such as a protein.¹⁰ But this strategy is typically not applicable with an IDA that employs a low molecular weight **R**. A potential solution is to equip **R** with a paramagnetic relaxation agent. Precedence for this idea is the SLAPSTIC screening method that identifies transient association of small molecules with a protein that has an appended relaxation agent.¹¹ Paramagnetic relaxation efficiency has an r^{-6} dependency on the distance between the paramagnetic center and the nucleus under observation. The highly sensitive distance dependency is the basis of the paramagnetic relaxation effect (PRE) which has been exploited as an NMR method to measure intramolecular distances¹² and identify intermolecular binding partners.¹³ With this literature knowledge in mind, we decided to develop a ^{19}F NMR-based IDA. ^{19}F NMR spectroscopy has many useful attributes for screening and imaging including high signal sensitivity, wide chemical shift range, and minimal biological and environmental background signal.¹⁴ Recent studies have developed ^{19}F NMR imaging probes,¹⁵ analyte reporters,¹⁶ and ligand binding assays (notably the FAXS method)¹⁷ that produce a modulation of either ^{19}F chemical shift or relaxation properties. Of particular relevance to this present study are published examples of ^{19}F NMR molecular imaging probes that are comprised of a paramagnetic center connected by a cleavable linker to a proximal ^{19}F label.¹⁸ The ^{19}F NMR signal for the intact molecular probe is effectively ‘switched-off’ due to the PRE and then restored by an enzyme catalyzed bond cleavage event. The ^{19}F NMR IDA in Figure 1a is a non-covalent version of this PRE switching concept but with a new performance feature. Not only is the assay signal intensity ‘switched-on’ by displacement of a ^{19}F -labeled **I** from a suitably designed **R** with appended relaxation agent, there is potential for signal amplification since substantially more **I** molecules are ‘switched-on’ than one molar equivalent of **R** (see Figure S2c for supplementary schematic picture).

The prototype IDA used in this preliminary study exploits the phosphate anion recognition properties of synthetic zinc(II)-bis(dicolylamine) (ZnBDPA) receptors.¹⁹ Prior work has shown that ZnBDPA coordination complexes have high affinity for highly charged polyphosphates like pyrophosphate (PPi) in water ($K_a \sim 10^{-7} \text{ M}^{-1}$).²⁰ Two homologous receptors were prepared by covalently linking a ZnBDPA unit to a paramagnetic relaxation agent - a proxyl spin label in the case of receptor **1**^{11b} and a Gd^{3+} -DOTA in the case of **2** (Figure 1b).²¹ The ^{19}F -labeled phosphate indicator, **3**, was designed to associate rapidly and relatively weakly with the ZnBDPA unit in **1** or **2** ($K_a \sim 10^{-4} \text{ M}^{-1}$).²⁰ As expected, ^{19}F NMR titration experiments showed that the peak for **3** broadened substantially upon addition of sub-stoichiometric amounts of receptor **1** or **2** with a concomitant reduction in peak height (Figure 2 and Figure S1). Specifically, addition of either **1** or **2** (0.25 mM) reduced the relative peak height for **3** (1 mM) by 82%. A subsequent titration of the **1:3** admixture with PPi showed that only 0.25 mM of PPi was needed to fully restore the peak height to a

value corresponding to 1 mM of **3** (Figure 2 and Figure S2). Essentially the same amount of signal restoration was observed when PPI was added to the homologous IDA admixture of **2:3** (compare Figures S3 and S4).

Further characterization of the PRE was gained by measuring ^{19}F NMR relaxation times for indicator **3** in the presence and absence of the other assay additives. As listed in Table 1, T_2 for the ^{19}F signal of free **3** (1 mM) was 1.13 ± 0.15 s, and it decreased to 0.03 ± 0.01 s after addition of proxyl-appended receptor **1** (0.25 mM). A subsequent addition of excess PPI restored T_2 to its original value (1.08 ± 0.09 s). These results confirm the rapidly exchanging association of **1** and **3** leading to a substantial PRE on **3**. The PRE is lost when the added PPI associates strongly with **1** and prevents **1** from making long-lived intermolecular contact with **3**. A similar decrease in T_2 relaxation was observed when **3** (1 mM) was mixed with Gd^{3+} -DOTA-appended receptor **2** (0.25 mM). But the subsequent addition of excess PPI only returned the T_2 value for **3** to 50% of its original value (Table 1). We attribute this incomplete recovery of T_2 to the known propensity of small oxyanions like **3** to directly coordinate with the metal center in the Gd^{3+} -DOTA unit of **2**.²² This provides a second, non-competitive relaxation pathway for indicator **3** that is not blocked by association of PPI to the ZnBDPA unit in **2**. Evidence supporting this rationalization is the observation that the addition of **apo-2** (a version of **2** without zinc cations) also decreases the T_2 for **3** in the presence or absence of added PPI (Table 1, Figure S5). So while receptors **1** and **2** are equally effective in a spectroscopic IDA like Figure 2, they are not expected to exhibit the same performance if the IDA is detected as a T_2 -weighted image using MRI. In this latter case, a smaller image voxel dynamic range is expected with **2** due to the incomplete restoration of T_2 upon titration with PPI.

Most of the above experiments used standard conditions of 0.25 mM of **R** and 1.0 mM of **I**. These relatively high concentrations enabled the spectra to be rapidly acquired in 8 scans. While detection sensitivity could be enhanced by simply increasing the number of scans or employing an indicator with a higher number of fluorine labels, the ^{19}F NMR IDA still may not be suitable for analyses that require highly sensitive analyte detection. We envision that a more likely application of the ^{19}F NMR IDA will be as a method that monitors abundant analytes and reports differences in analyte concentration or structure. For example, ZnBDPA receptors are able to recognize differences in membrane electrostatic charge caused by the presence of anionic phospholipids.¹⁹ Specifically, ZnBDPA receptors selectively associate with anionic membranes of dead and dying mammalian cells (produced by surface exposure of anionic phosphatidylserine) over the zwitterionic membranes of healthy cells. This raises the idea of a ^{19}F NMR IDA that can detect cell death. To demonstrate the basic concept we treated an admixture of **1:3** with two separate vesicle dispersions, zwitterionic vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC, mimic of healthy cells) and anionic vesicles containing 20 mol % of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPC: POPS 80:20, mimic of dead/dying cells). As shown in Figure 3, the ^{19}F NMR IDA can readily differentiate the two vesicle compositions. With further development this ^{19}F NMR IDA could become broadly useful in new types of biomedical assays that monitor changes in membrane surface charge or the number of anionic cells. An example of the former is a phospholipase D assay that monitors enzyme

catalyzed conversion of zwitterionic phosphatidylcholine to anionic phosphatidic acid, a biochemical process that is implicated in cancer.²³ An example of the latter is an antibiotic drug discovery assay that monitors changes in the number of anionic bacterial cells.²⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) ^{19}F NMR-based IDA assay using receptor **R** with appended relaxation agent (RA) and ^{19}F -labeled **I**. (B) Chemical structures of ZnBDPA receptors **1** and **2**, and ^{19}F -labeled indicator **3**.

Figure 2.

(left) ^{19}F NMR (376 MHz) spectra of indicator **3** (1.0 mM) upon addition of receptor **1**.

(right) ^{19}F NMR spectra of the admixture **1:3** (0.25: 1.0 mM) upon addition of PPI. N = 8 scans, 10 mM HEPES, pH 7.4, 25 °C, external trifluoroethanol as reference.

Figure 3.

^{19}F NMR (376 MHz) spectra of the admixture **1:3** (0.25: 1.0 mM) before and after addition of zwitterionic vesicles (POPC) or anionic vesicles (80:20 POPC:POPS). N = 8 scans, 10 mM HEPES, 145 mM NaCl, 3.2 mM KCl, pH 7.4, 25 °C, external trifluoroethanol as reference.

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Table 1

^{19}F relaxation times for **3** in IDA admixtures.^a

Admixture (mM)					
R	3	R	PPI	T ₁ (s)	T ₂ (s)
1	1.0	0.00	0.0	1.77 ± 0.02	1.13 ± 0.15
1	1.0	0.25	0.0	0.60 ± 0.04	0.03 ± 0.01
1	1.0	0.25	5.0	1.47 ± 0.03	1.08 ± 0.09
2	1.0	0.00	0.0	1.73 ± 0.01	1.08 ± 0.06
2	1.0	0.25	0.0	0.06 ± 0.04	0.03 ± 0.01
2	1.0	0.25	5.0	0.94 ± 0.02	0.50 ± 0.15
apo-2	1.0	0.00	0.0	1.73 ± 0.01	1.08 ± 0.06
apo-2	1.0	0.25	0.0	0.74 ± 0.01	0.33 ± 0.04
apo-2	1.0	0.25	5.0	0.91 ± 0.03	0.34 ± 0.04

^a 376 MHz, 10 mM HEPES, pH 7.4, 25 °C; external trifluoroethanol as reference.