Review Paper:

A novel approach of ^{19}F NMR based sensor

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Abstract

Reliable and precise methods are to be applied ranging from biological field to diagnosis and quality control to identify target analytes in samples. Nuclear magnetic resonance (NMR) is a strongly recommended process that is suitable for collecting data multidimensionally at molecular and atomic levels. Information obtained from the NMR technique provides molecular structures, molecular interactions, dynamic and diffusional properties of molecules. Overlapping of NMR signals of each component makes it difficult to interpret data and low sensitivity makes it unfitting for many samples with a low concentration of analytes.

These limitations can be rectified by adding heteronuclear specially designed sensors, inventive transduction mechanism and strong pulse sequences. ^{19}F NMR tool is extensively accepted method to detect the interactions among a variety of biological substrates with ^{19}F-isotopic species. This review includes the detection of biomolecules, mainly enzymes and enzymatic reactions based on sensor applicability.

Keywords: Analytes, dynamic, transduction, sensitivity.

Introduction

Nuclear magnetic resonance is one of the strong analytic tools to provide multidimensional structural information at the atomic level.[11,36] Chemical shifts, signal intensities, relaxation and chemical exchange provide information on molecular structures as well as various dynamic processes including molecular interactions. Because of these exceptional capabilities, NMR is a must-have tool for a variety of difficult characterizations. Due to the high penetrability of radiofrequency radiations via deep tissues, NMR is a very powerful tool to investigate large and non-transparent tissues *in vivo*. These studies are generally very difficult with other optical-based methods like mass spectrometry and electron spin resonance.[9,23,35,38]

Besides its wholeness, NMR has some limitations including interference of signals in complex mixtures and low sensitivity of NMR spectroscopy due to low population difference even in an ultra-strong magnetic field,[32] making it unsuitable for relative spectroscopic changes in reactions and bindings. Only a few NMR active nuclei with sufficient natural abundance and high gyromagnetic ratio are detectable with commonly available NMR spectrometers. To simplify the interpretation of the NMR spectrum, ^{19}F containing substrates are frequently used in biomolecule assays.[4,29] To detect miscellaneous metal ions[43] and to rectify the sensitivity of NMR-based detection, ^{19}F-labeled chelators and hyperpolarised sensing systems were recommended.[26]

This review focuses on molecular and nanosized sensors that aid in NMR-based detection in a variety of ways including a generalization of the NMR spectrum, simplification of data elucidation, visualization of low or inactive NMR analytes and increment of sensitivity and selectivity. Changes in chemical shifts and shortening of relaxation time are two ways to indicate sensing feedback. Some substrates showing NMR relaxation properties are also reviewed in short.[6,20] NMR has been employed in a wide range of applications from process monitoring to structural determination to mechanistic research. The most often employed nuclei for the creation of sensing systems based on the disruption of NMR chemical shifts are ^{19}F, ^{31}P and ^{129}Xe.

Because of these nuclei's high chemical shift dispersion, small changes in the immediate environment around these heteronuclear labels may be easily seen using different NMR signals. As there is no endogenous xenon or organofluorine compounds in the body, background signals are removed and data interpretation is simplified. Using a 400 MHz NMR spectrometer, a chemical shift difference of 0.03 ppm is generally enough to accomplish baseline separation of two singlet ^{19}F resonances. For qualitative analysis, chemical shift perturbations as tiny as 0.01 ppm can still be useful. These benefits enable the identification of many labelled species in complicated mixtures at the same time.

When it comes to nuclei having low natural abundance (e.g. ^{13}C), isotope labelling is a good approach to improve NMR sensitivity. For nuclei having 100 percent natural abundance (e.g. ^{19}F), multiple magnetically equivalent ^{19}F atoms are integrated into the sensor to improve the detection of NMR signals. To observe more meaningful signals, a variety of hyperpolarization techniques are taken into effect.[13,19] The use of these sensitivity augmentation technologies in sensing devices significantly expands the detection range of NMR.

Detection of enzyme and enzymatic activity: The measurement of enzymatic activity is crucial for biological research and diagnostics. An abnormal quantity of enzyme activity is typically associated with underlying diseases and has a wide variety of biological and clinical effects. Enzyme activity is normally assessed by observing an enzymatic transformation in which the rates of substrate consumption and the synthesis of the enzymatic products are used to

calculate the enzyme's activity. Although optical-based sensing strategies for measuring enzyme activity are well established, these technologies are typically impeded by numerous forms of interference found in biological materials. Due to the restricted depth of light penetration, imaging big opaque organs is also problematic using optical-based sensors.

NMR-based detection systems with strategically placed sensors offer alternative solutions to these problems by providing precise and unambiguous sensing results. Because of ^{19}F NMR spectroscopy's high sensitivity and minimal background signals, ^{19}F-containing substrates have been frequently used to aid in the monitoring of enzyme operations.[2,4] Using well-established synthetic processes, fluorine atoms and fluorinated moieties may be selectively integrated into diverse target substrates, making the required fluorinated substrates easily accessible. Fluorinated substances usually have greater biological and pharmacological capabilities than nonfluorinated equivalents due to their unique electrical characteristics.[14,28]

As a result, it is not surprising that many pharmaceutical substances include one or more fluorine atoms. The fluorinated analogues of biomolecules are great candidates for investigating the metabolism of bioactive substances since fluorine is isosteric to hydrogen. Monitoring newly produced ^{19}F-containing products enable the assessment of enzymatic activity when metabolism is stimulated or catalysed by an enzyme. The microbial gene enzyme cytosine deaminase (CD) was employed in gene-directed enzyme prodrug delivery and ^{19}F NMR was used to evaluate biologically-based therapies employing cytosine deaminase.

The goal of the tests was to see how well the prodrug 5-fluorocytosine (Figure 1, a) is converted to 5-fluorouracil (Figure 1, b) after being treated with cytosine deaminase from an attenuated *Salmonella typhimurium* strain recombinant. These findings show that magnetic resonance spectroscopy may be used to noninvasively monitor therapeutic transgene expression in malignancies.[10,15,31]

Gene therapy has enormous promise for the treatment of a variety of disorders, but its application is impeded by lack of exact methods for determining transfection success. Phosphocreatine (Figure1, d) is the metabolic product of the creatine kinase process that is frequently employed as a gene transfection indicator. To show that the CK_{BB} identified in extracts was active *in vivo*, ^{31}P NMR spectra were obtained *in situ* from the liver of a mouse. The Bruker integration technique was used to the targeted expression of a transgene in a measurable NMR peak showing the use of NMR as a non-invasive gene expression measurement.[22] Despite the simplicity of this label-free method, the limited sensitivity of ^{31}P NMR and the existence of other endogenous ^{31}P-containing compounds curtail its widespread adoption.

With nine comparable fluorenes and no scalar coupling to other nuclei, perfluoro-*t*-butyl hydroxyprolines outperform other fluorinated amino acids in terms of sensitivity. To monitor the activity of the protein kinases PKA and Akt in real-time, ^{19}F NMR was used to integrate 4R- and 4S-perfluoro-*t*-butyl hydroxyproline into substrate peptides (Figure 2). Each protein kinase rapidly phosphorylated peptides containing each diastereomeric proline, causing ^{19}F chemical shift changes ($\Delta\delta$ trans-cis = 0.12 ppm) as a result of phosphorylation.[34]

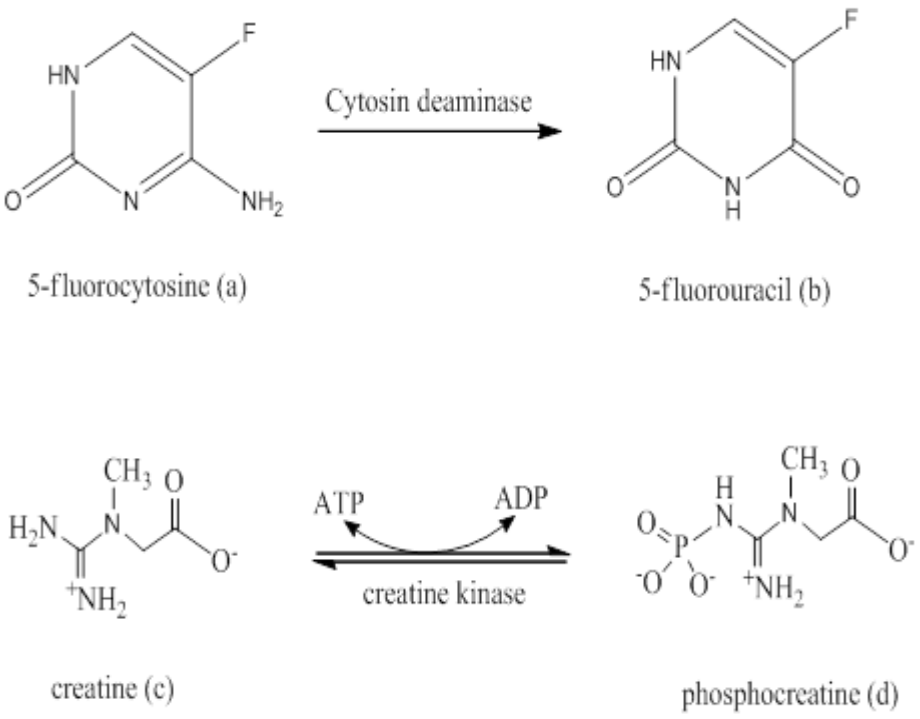


Figure 1: Detection of creatine kinase enzyme activities by metabolism processes.

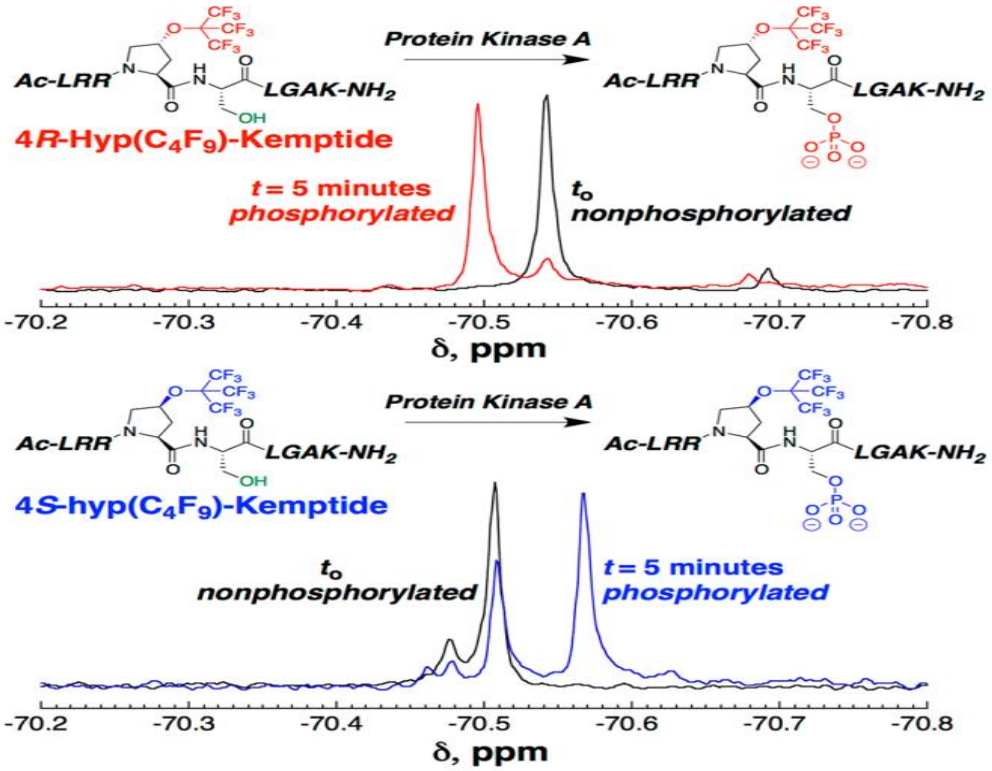


Figure 2: ^{19}F NMR-based analysis of PKA activity via phosphorylation of Kemptide-based substrate peptides with perfluoro-*t*-butyl hydroxyprolines. Minor peaks are due to the presence of a small population of species with cis-proline amide bonds. The Figure is reprinted with permission from a reference.[34]

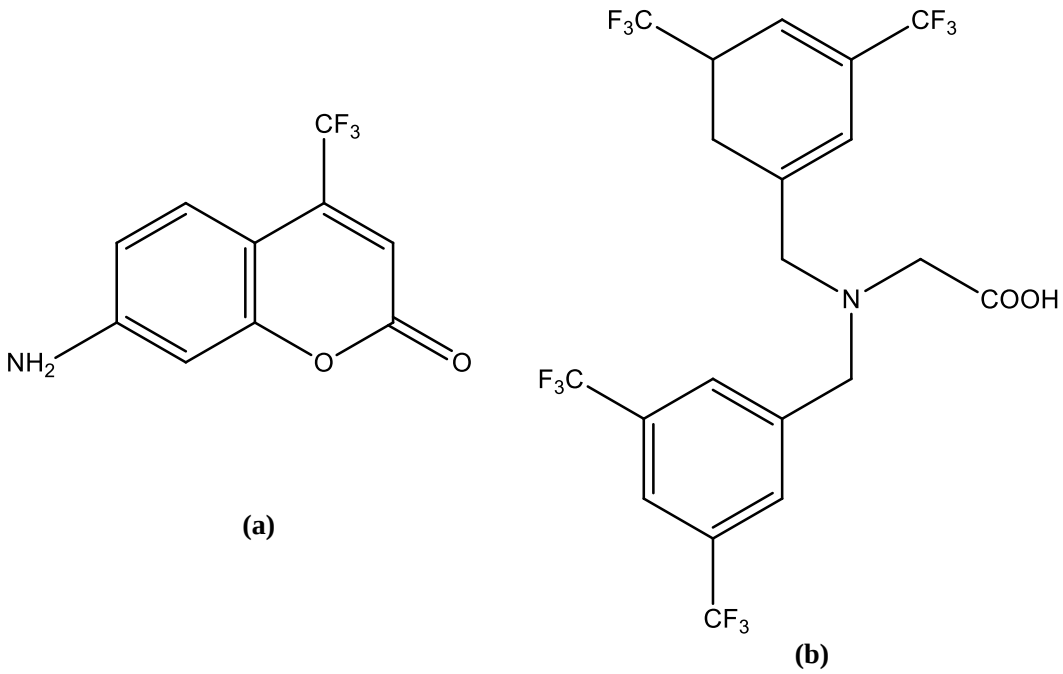


Figure 3: 7-amino-4-trifuoromethylcoumarin (Figure 5, a) is inserted at the C-terminus and Bis-(3,5-bis-trifluoromethyl benzyl)-amino]-acetic acid (Figure 5, b) is inserted at the N-terminus.

The n-FABS (n-fluorine atoms for biochemical screening, n being the number of magnetically equivalent fluorine atoms in the moiety) may be used to screen complicated chemical and biological systems for genuine inhibitors or boosters of a given enzymatic process. Dalvit et al[8] used a ligand-based strategy to screen a library of fluorinated molecules in a direct or competitive format using an appropriate fluorinated reporter molecule. The protein-based technique necessitates

the use of fluorinated amino acids to mark the protein. These approaches are presently in use at many academic institutions and in the pharmaceutical industries due to their effective application in drug development projects.

Another approach for performing a functional test based on ^{19}F NMR spectroscopy is the substrate or cofactor-based fluorine NMR screening. The substrate or cofactor of an enzyme must be labelled with a fluorinated moiety in this direct test. The n-FABS peptidic substrates provide excellent fluorine tagging flexibility. A fluorinated moiety can be added to a peptide's N- or C-terminus or fluorinated amino acid (Figure 3) introduced within the peptide sequence.

In addition to the drugs/prodrugs and endogenous biological molecules discussed above, substrates with strategically placed fluorine probes are designed to detect the presence of enzymes and measure their activity. Another valuable signal for determining the effectiveness of gene transfection is β - galactosidase (β - gal) generated by the lacZ gene. It was confirmed that 4-fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG, Figure 4, a) may be employed as a sensor to detect β - gal. Enzymatic processes rapidly yield 4-fluoro-2-nitrophenol (Figure 4, b) whose ^{19}F NMR resonance is 510 ppm different from that of PFONPG. The enzyme activity and pH value of the sample may be evaluated concurrently because the magnitude of the chemical-shift change[7,18,21] is dependent on the ionic state of the 4-fluoro-2-nitrophenol.

Various techniques were used to modify the sensor's cytotoxicity and substrate effectiveness toward β-gal including substituent modification on the phenyl group (Figure 5)[42] conjugation with heterocyclic moieties,[41] and inclusion of multiple glycosyl groups.[17,39] The detection of β-gal in live *Escherichia coli* proved the feasibility of *in vivo* experiments using MR imaging with this sensing method.[24,40]

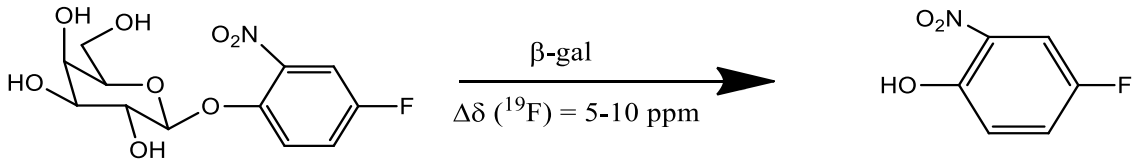


Figure 4: Detection of β-gal with PFONPG.

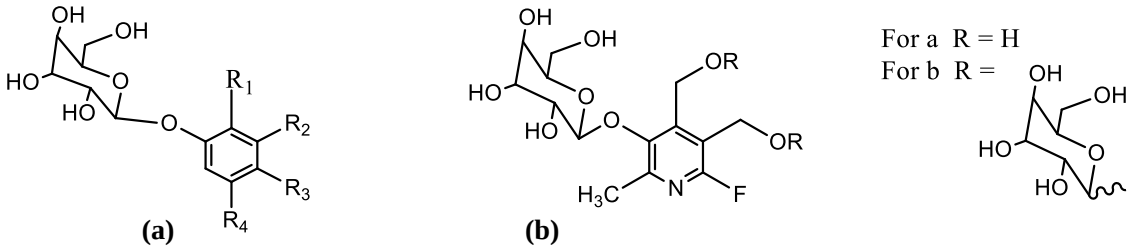
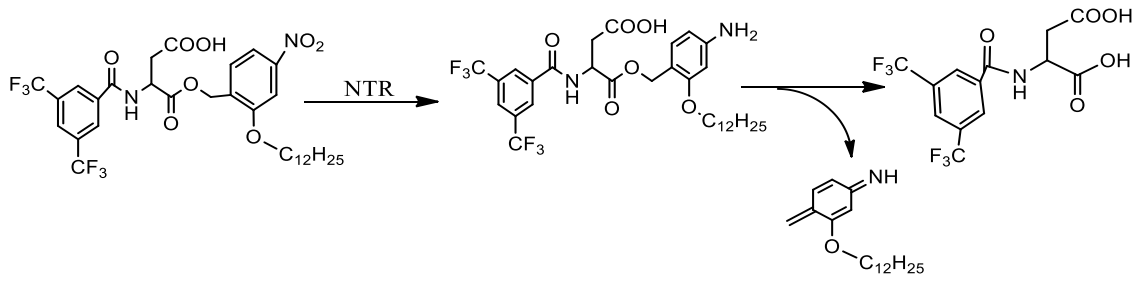


Figure 5: Sensors with structural modifications for detecting β-gal.

(a)



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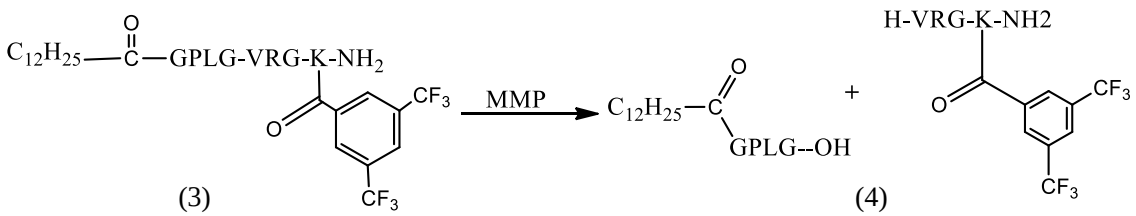


Figure 6: Detection of NTR by Probe 1 (Figure a) and Detection of MMP by Probe 3 (Figure b).

The ability to identify specific turn-ons of enzyme activity is crucial in drug discovery research and medical diagnostics. Even though magnetic resonance imaging (MRI) is one of the most powerful methods for noninvasively observing enzyme activity *in vivo* and *ex vivo*. Feasible methodologies for imaging individual enzymes with high contrast have been exceedingly limited thus far. Matsuo et al[25] developed innovative signal amplifiable self-assembling ^{19}F MRI/MRS probes for detecting and visualizing nitroreductase (NTR) and matrix metalloproteinase (MMP) enzyme activity. While aggregated in NMR spectroscopy, these probes are "quiet," but show a disassembly-driven turn-on signal shift once the catalytic enzyme cleaves the substrate part.

Using ^{19}F NMR, they were able to visualize the activity of MMP generated under tumor cell development settings. When both probes were dissolved in a buffer solution, no NMR signals were seen, but a strong signal developed at δF -62.9 ppm with the addition of NTR with probe 1(Figure 6, a) and MMP with probe 3 (Figure 6, b). PRE (paramagnetic relaxation enhancement) is an interaction of two magnetic moments between paramagnetic nuclei and observed nuclei that result in an effective shortening of longitudinal relaxation time T_1 and transverse relaxation time T_2 of the observed nuclei. The NMR signal of the ^{19}F will be hidden as a consequence of the lower T_2 generated by the PRE effect if a paramagnetic metal and a ^{19}F label are together by an enzymatically cleavable linkage of adequate length.

Caspase-3[27,33] was chosen as a target enzyme for the development of a ^{19}F MR imaging probe. Caspase-3 activity is increased during apoptosis and Caspase-3 hydrolyzes peptides such as DEVD sequences. Gd-DOTA-DEVD-Tfb (Figure 7), an MRS probe, was created by connecting the Gd^{3+} complex with the ^{19}F moiety through DEVD. The NMR peak of Gd-DOTA-DEVD-Tfb at ^{19}F was greatly widened and was not seen. After the addition of Caspase-3, this peak was regained in a time- dependent manner. Then, for the measurement of ^{19}F MRI, a phantom MR image was measured in a glass capillary. As a result, it was demonstrated that enzyme detection MRS probes may be created using a molecular design based on PRE.

Detection of other biomolecules: A ^{19}F NMR-based sensor can be used to diagnose diabetes mellitus without requiring a complicated sample. Gao et al[12] produced a ^{19}F NMR-based fluorinated boronic acid sensor (Figure 8, b) for selective glucose detection. The fluorinated di boronic acid sensor may attach to two pairs of vicinal diols of glucose to produce a unique ^{19}F NMR signal at δF -114.93 ppm that is distinct from that of other saccharides. The sensor was able to preferentially detect glucose (Figure 8, c) in a mixture of up to 10 saccharides (Figure 8, a). Furthermore, the sensor was utilized to detect glucose in real human urine samples without any pre-treatment and the results were good with a detection limit of 0.41 mm.

Axthelm et al[1] used ^{19}F NMR spectroscopy to successfully discriminate a variety of diols containing saccharides (and other bio analytes). From 4,4′-bipy, 3,3′-bipy and 3,4′-bipy, three water-soluble fluorinated bisboronic acid bipyridinium salts with distinct substitution patterns were synthesized (Figure 9). The binding of each receptor to each analyte was then monitored using ^{19}F-NMR spectroscopy, which revealed unique spectrum changes as the sp^2 boronic acids were converted to the appropriate sp^3 boronate esters. On the NMR time scale, the equilibrium of the production of the boron diol complexes is gradual, ensuring that the peaks are well separated.

The discrimination of analytes using a single receptor was not always conclusive. Compounds 1 and 2 displayed a single peak in DMSO-d6 at δF -110.45 and δF -110.42 ppm respectively. Due to its asymmetry, receptor 3 exhibited two signals at δF -110.49 and -110.54 ppm. Receptor 1 exhibited a single wide signal at δF -111.76 ppm in a buffered aqueous solution. In contrast, 2 and 3 had weaker signals at δF -111.86 and -111.89 ppm respectively. The free boronic acid form can be provisionally ascribed to this downfield shifted minor group of signals for 2 and 3.

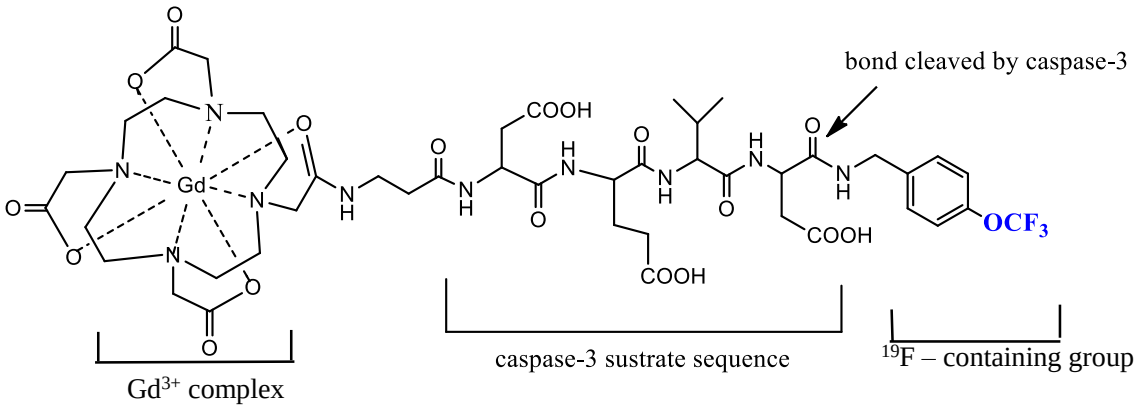


Figure 7: Structure Gd-DOTA-DEVD-Tfb Probe for detection of Capsase-3.

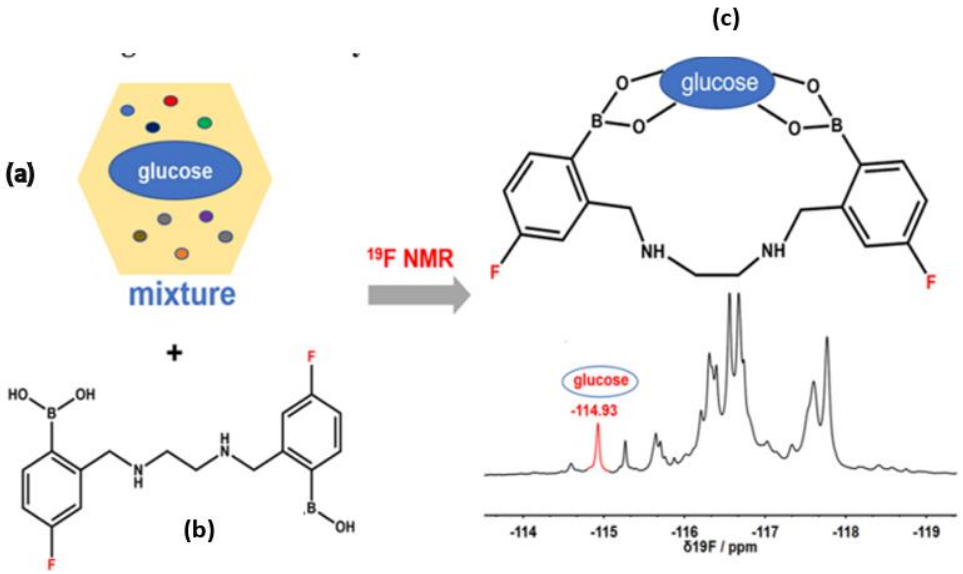


Figure 8: Schematic illustration of Selective Glucose Sensing and ^{19}F NMR Spectrum of Sensor. The figure is reprinted with permission from reference.[12]

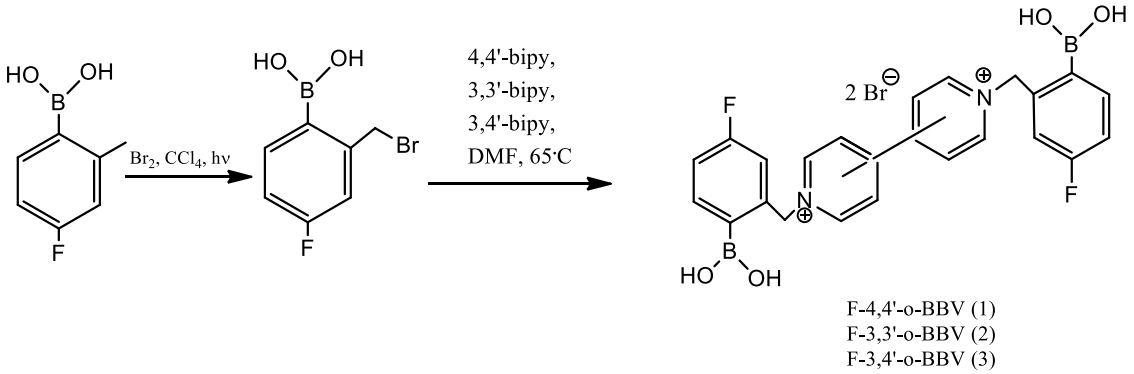


Figure 9: Synthesis path and molecular structures of receptors 1-3.

When catechol was added as an aromatic 1,2-diol, all peaks of unbound 1 vanished, leaving just one clear signal at δF -116.05 ppm, suggesting strong 1:1 boronic acid catechol esters (Figure 10). The symmetric isomer 2 generated one strong signal at δF -115.81 ppm whereas 3 with two different ^{19}F nuclei, produced two sharp signals at δF -115.92 and -116.09 ppm. Interestingly, the related neurotransmitter dopamine may be distinguished from catechol by shifting the ^{19}F peaks to 1 (δF -115.93 ppm), 2 (δF -115.60 ppm) and 3 (δF -115.76 and -115.95 ppm). D-fructose, which has a high affinity for boronic acids 1,2 produced a single sharp receptor analyte peak at δF -116.10 ppm with 1 and -115.97 ppm with 2 (δF -116.00 and -116.11 ppm with 3).

D-glucose, which is normally difficult to distinguish from D-fructose, produced two peaks with 1 at δ -115.72 and -117.04 ppm. There were also two binding peaks for 2 (δF -116.04 and -116.65 ppm) and 3 (δF -116.68 and -117.07 ppm). Acetylation of histone tails and non-histone proteins was discovered to be an important component of the 'chromatin code' which controls transcription by recruiting transcription factors, co-regulators and DNA-binding proteins. Acetylation can alter protein-protein interactions, protein function, localization and stability. Using nuclear magnetic resonance Nocca et al[30] developed a simple method for detecting acetyl moieties at the amino function of lysine residues using peptides generated from the amino-terminal regions of histone H4 and TDG. Acetylated lysine residues were created by combining the commercially available N-Fmoc-N-acetyl-lysine synthon (Novabiochem, Figure 11).

Even in the presence of multiple acetylated substrates, scalar interactions between lysine side chain protons and the distinctive NH_2 acetamide signal at 8.01 ppm allow for the recognition of acetylation sites.

Cysteine (Cys), a thiol-containing biomolecule, is involved in a variety of biological activities. Furthermore, as a common antioxidant, vitamin C (ascorbic acid, AA) plays an important function in a variety of biological events. The use of a fluorogenic probe to identify these physiologically relevant small compounds *in vivo* provides a critical tool for unraveling real-time biomolecular dynamics. The production of cell-permeable, water-soluble, non-toxic fluorogenic compounds is always favorable in terms of spatiotemporal sensitivity and accuracy.

With comprehensive mechanistic inquiry, a fluorogenic probe, 4-azido-7-nitrobenzo-2-oxa-1,3-diazole (NBD-N_3), has been created to detect AA and Cys, among others a spectrum of physiologically relevant compounds. With the action of AA, the weakly fluorescent NBD-N_3 changes into the highly fluorescent NBD-NH_2. The direct production of NBD-NH_2 from NBD-N_3 is shown by ^{1}H NMR peaks at δH 6.5 ppm and δH 8.6 ppm. (Figure 12, a).

On the other hand, Cys uses aromatic nucleophilic substitution (SNAr) followed by S-N rearrangement to transform NBD-N_3 into highly fluorescent NBD-NHR (R = Cys). NMR spectroscopy has also been used to validate the fluorogenic response's chemical pathways. Detection of Cys by NBD-N_3 progressively develops additional doublets at δ 7.67 ppm and δ 8.62 ppm, probably due to the production of intermediate NBD-SR which eventually rearranges to product NBD-NHR[5] which exhibits peaks at δ 6.53 ppm and δ 8.59 ppm (Figure 12, b).

To distinguish Cys, Hcy and GSH mixes, a ^{19}F NMR probe (Figure 13) was created. The peak of δF -127.6 ppm vanished and three new signals at δF -128.0 ppm, -128.3 ppm and -135.4 ppm were recorded concurrently. The chemical shift increased by 7.8 ppm (from δF -127.6 ppm to -135.4 ppm), indicating that the probe was consumed within a few minutes and product 1 (Figure 13, a) was produced. The chemical shift of the probe at δF -127.6 ppm vanished fast after the addition of 5 equivalents Hcy and GSH. However, novel chemical changes were identified at δF -128.5 ppm, δF -128.3 ppm for Hcy and δF -128.4 ppm, δF -128.1 ppm for GSH {Due to the formation of products 2 (Figure 13, b) and 3 (Figure 13, c) respectively}. Importantly, the signal at δF -135.4 ppm was not detected by ^{19}F NMR[37] under the test circumstances even after 90 minutes, which is a substantial divergence from Cys.

Because of the remarkable sensitivity of the ^{19}F nucleus to changes in the chemical environment, fluorine-labeled molecular probes have been used to explore the structure and interactions of nucleic acids using ^{19}F NMR. So far, most attempts have concentrated on integrating the fluorine atom into nucleobase and ribose moieties via solid-phase synthesis using monomer building blocks or enzymatic synthesis with nucleoside triphosphates.

Baranowski et al[3] generated a variety of 5'-fluoro(mono)- and 5'-fluoro(di)phosphate-labeled oligonucleotides (FPONs and FPPONs) probes with FP (Mono fluoro phosphates) or FPP (Di fluoro phosphates) moieties at the 5' end (Figure14).

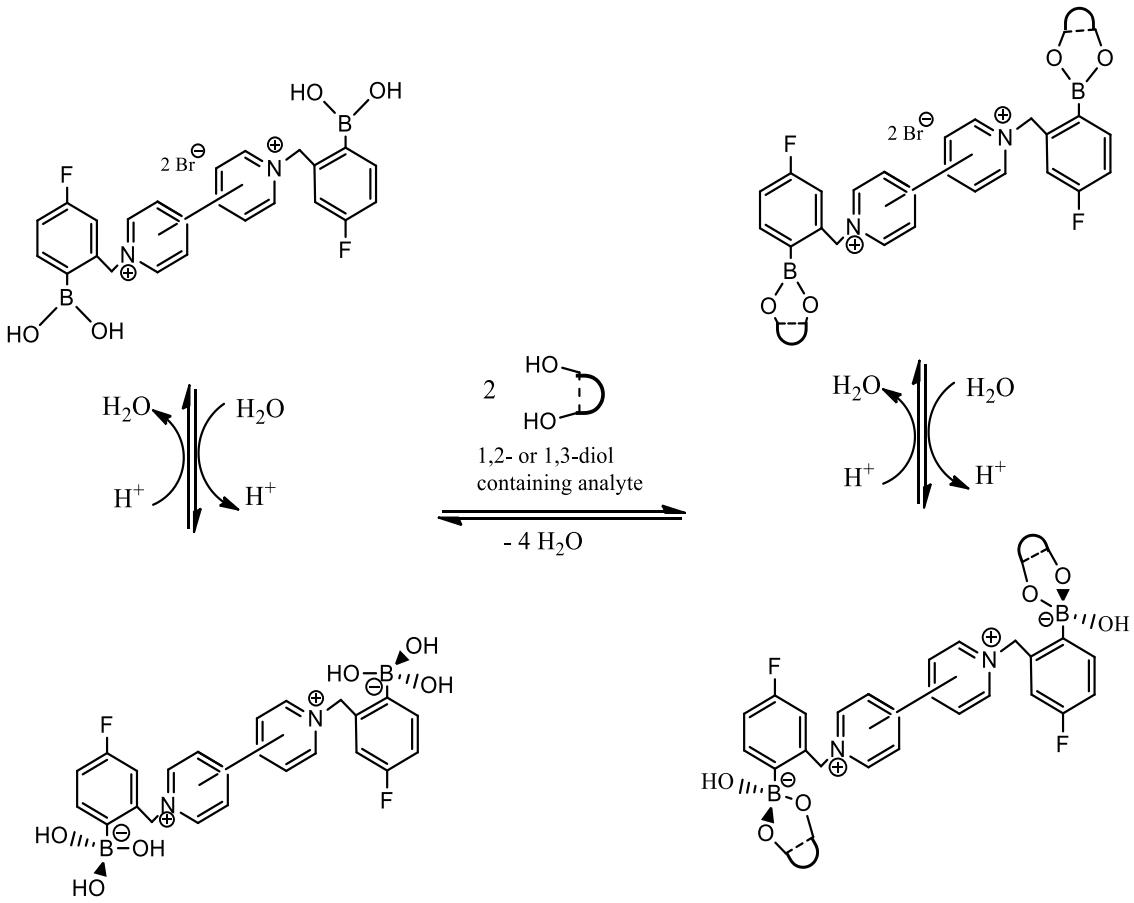


Figure 10: Illustration of Reversible Boronic Acid−Diol Interaction.

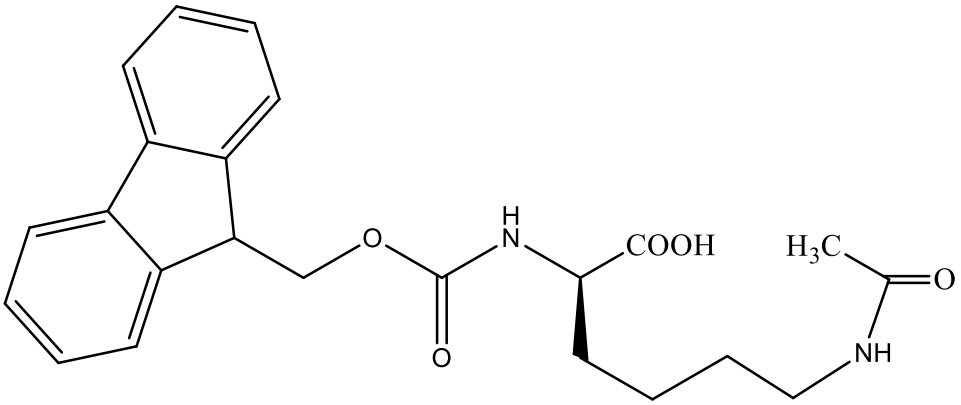
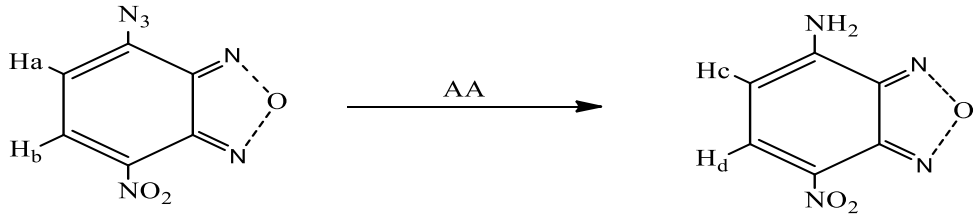


Figure 11: Structure of N-Fmoc-N-acetyl-lysine synthon (Novabiochem).

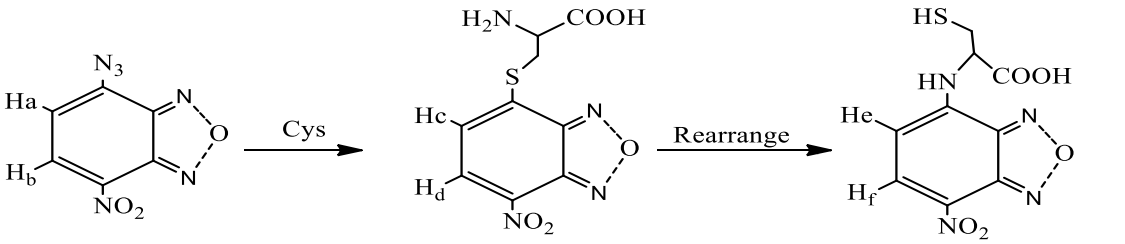
(a)



NBD-N_3 NBD-NH_2

(δ 6.5 ppm and δ 8.6 ppm)

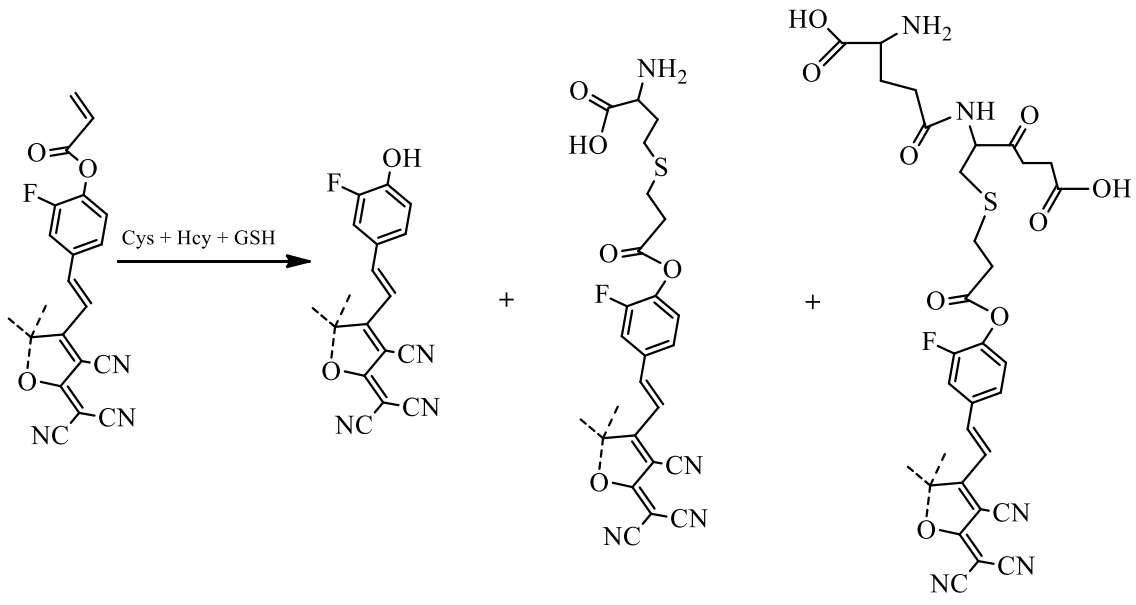
(b)



NBD-N_3 NBD-SR NBD-NHR

(δ 7.67 ppm and δ 8.62 ppm) (δ 6.53 ppm and δ 8.59 ppm)

Figure 12: Detection of (a) Vitamin-C and (b) Cysteine by NBD-N_3 sensor.



^{19}F Probe Product 1(a) Product 2(b) Product 3(c)

Figure 13: Discrimination of Cys, Hcy and GSH by ^{19}F Molecular probe.

Due to differences in chemical shift values, these fluorinated probes were able to discriminate between single-strand DNA (ssDNA) and double-strand DNA (dsDNA). FPPON14's (length - 16 nt) ^{19}F NMR spectra revealed a single doublet at δF -73.38 ppm. The production of duplexes with oligonucleotide 4 (ON4) and oligonucleotide 25 (ON25) led to the appearance of additional signals at δF - 73.40 ppm and -73.42 ppm respectively followed by the vanishing of the original peak of ssDNA. Analyzing changes in chemical shifts between ssDNA and dsDNA revealed a chemical shift of 0.02 ppm between duplexes (containing ON4 and ON25). This discovery showed that these probes may be utilized to detect 5'/3' terminal mismatches using ^{19}F NMR.

Aro-Heinilä reported the discovery of a trifluoromethyl-containing organomercury nucleobase analog 2 (Figure 15) capable of forming stable metal-mediated base pairs with guanine and thymine.[16] The probe can distinguish between purine and pyrimidine bases based on melting temperature analysis in the middle of the sequence. T > G > C > A was the order of affinity. Analogue 2 was shown to be a sensitive ^{19}F NMR label for detecting local environmental changes in Hg (II)-mediated base pairs at micromolar concentrations.

The mercurated single strand {ON (2)} ^{19}F NMR shift reacted somewhat to temperature variations, which might imply intramolecular Hg (II)-mediated binding within the sequence. For 2-T, 2-C and 2-G base pairs, distinct and well-resolved ^{19}F NMR resonance signals were found in double helices, but the binding of 2 to adenine remained a mystery. The respective peak sizes of ^{19}F NMR signals might be used to calculate the melting temperatures of 2-T and 2-C base pairs.

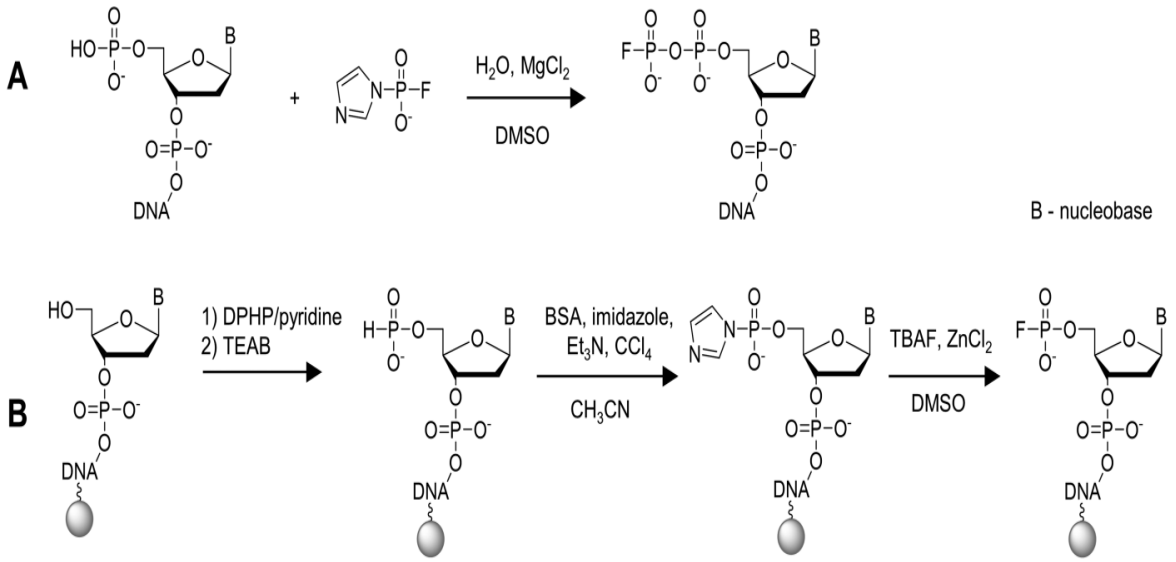
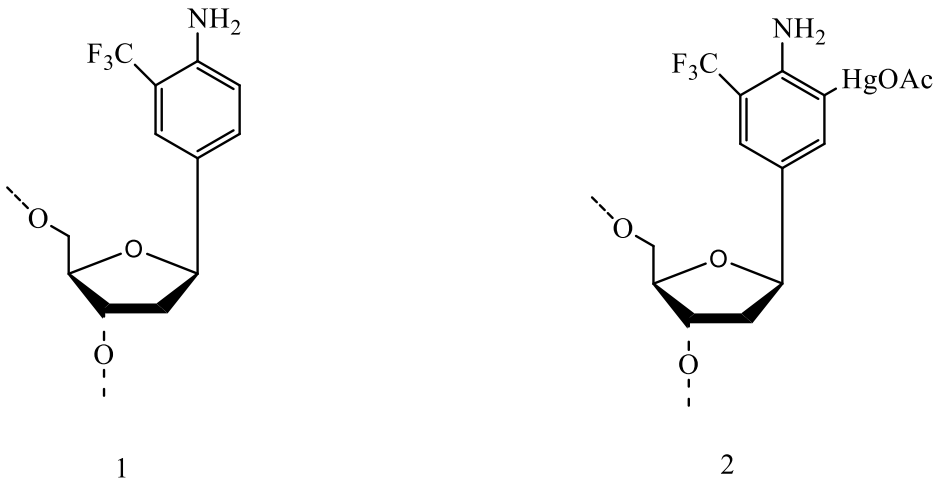


Figure 14: Synthesis of 5'-fluoro(mono)- and 5'-fluoro(di)phosphate-labelled oligonucleotides (FPONs and FPPONs). (A) FPPONs were obtained by reacting solid-phase synthesized oligonucleotide 5' -phosphates with an activated fluorophosphate. (B) FPONs obtained by activation on a solid support and reaction with TBAF. DPHP: Diphenyl H-phosphonate; BSA: N, O-Bis(trimethylsilyl)acetamide. The figure is reprinted with permission from reference.[3]



1 2

Figure 15: Structure of (1) Nucleobase and (2) its Mercurated analog.

Conclusion

NMR is one of the most powerful spectroscopic methods for determining molecule structures and interactions at the atomic level without a doubt but the overlap of NMR signals frequently complicates the interpretation of NMR data. Because of its intrinsic poor sensitivity, it is ineffective in many detections when the analytes of interest are present in low quantities. Some of the limitations of traditional NMR analysis can be overcome with the use of molecular sensors. Molecular MR imaging is an essential use of these NMR-based sensors, allowing *in vivo* viewing of bioactive chemical distribution, metabolic processes, molecular interactions and cells. Heteronuclear-based NMR sensors simply provide a "hot spot" image.

In vitro, the advancement of novel ^{19}F-sensors with a high number of magnetically comparable fluorine atoms and customized nanoparticle formulations will boost fluorine payloads and make ^{19}F-labeled sensors suitable for clinical applications. Hyperpolarization methods must be used to attain appropriate sensitivity for MR imaging in sensors with less sensitive hetero nuclei such as ^{13}C, ^{15}N and ^{129}Xe. With these encouraging developments, we anticipate that more powerful NMR-sensing schemes will be developed through the continuous development of robust synthetic sensors, novel transduction mechanisms and NMR hyperpolarization methods, significantly broadening the application of NMR techniques in detection.

References

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