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Editor's Choice

Turn-on Detection of Targeted Biochemical Reactions by Triple Resonance NMR Analysis Using Isotope-labeled Probe

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We report on a strategy for the "turn-on" detection of target biochemical (metabolic) reactions using a triple resonance NMR technique with an isotope-labeled probe. Our NMR study clearly reveals that otherwise NMR-nonactive-$^{13}C/^{2}H$-labeled glucose actually turns "on" its ^{1}H NMR signal by conversion to an important biomarker lactate as the end product of anaerobic glycolysis in cells and in injected mice with high selectivity.

There has been a long-term effort into the analysis of specific chemical events in complex biological systems, and metabolic profiling is one of the most intriguing targets among a variety of events. Metabolites are chemical products resulting from essential biological activities and, therefore, can be good biomarkers to reveal the physiological or chemical status of cells, tissues, and organs.

NMR/MRI-based technology is one of the most promising techniques for the analysis of such biochemical (metabolic) reactions. However, the detection of target metabolites or biochemical reactions of interest is often difficult because of spectral overlaps with a large number of biological components. To overcome this, attempts to trace metabolic pathways by the administration of stable isotope-labeled compounds as probes has been reported.[1] In principle, these isotope-labeled compounds are highly potential probes since these are NMR-sensitivite and constitutively NMR-active. However, this advantage can also be a disadvantage since, for the same reason, these probes and derived various intermediates/metabolites all serve as possible origins of background noise signals, and thus target metabolite should be carefully discriminated from others by the precise analysis of the chemical shifts, typically by measuring the 2D NMR spectra.[1,2] Especially, such spectral overlaps are problem for detection of ^{1}H, the most sensitive and thus attractive nucleus, due to its small chemical shift range.

In this context, ideal chemical probes are those with a signal on/off device, that are otherwise NMR-silent and is rendered NMR-active only when accumulated at target sites and subjected to specific biochemical events.[3–14] In this work, we have used a triple resonance NMR technique for the detection of isotopically labeled metabolites generated in cells and in injected mice with high selectivity. In addition, for the first time, we report on a signal-activatable glucose-based probe, which turns "on" its ^{1}H NMR signals as a result of target anaerobic glycolytic reaction.

The triple resonance technique is a method to correlate three NMR-active nuclei with different Lamor frequencies. For example, when the pulse scheme allows the magnetic coherence of ^{1}H to transfer to two successive ^{13}C nuclei with different Lamor frequencies through scalar couplings, only the proton in the particular sequence ^{1}H–^{13}C–^{13}C is detectable. Due to the low natural abundance of ^{13}C (1.1%), the probability of a naturally occurring ^{1}H–^{13}C–^{13}C sequence is as low as 0.01%, suggesting that the triple resonance technique can markedly improve the selectivity of detection of a target molecule having ^{1}H–^{13}C–^{13}C by lowering the background signal.[15]

As a model metabolic reaction for demonstrating the proof-of-concept of our idea, we chose the glucose-to-lactate production. Among the stable isotopomers for metabolic analysis by NMR, ^{13}C-labeled glucose is one of the most intensively studied. Glucose is a common source of energy in cells and is converted to a variety of metabolites during glycolysis. Among these, lactate H_3C–CH(OH)–CO_2H is a key end metabolite. In particular, in tumor cells, glucose is actively converted to lactate through an anaerobic glycolytic pathway.[16,17] We challenged to develop a method to detect generation of lactate from glucose using isotope-enriched glucose as a probe that turns from "off" to "on" its own ^{1}H NMR signals in synchronization with a target anaerobic glycolytic reaction (Figure 1a).

First, we measured a triple resonance NMR spectrum of chemically synthesized ^{13}C-labeled lactate. We used a pulse sequence for the detection of a ^{1}H bound to an aliphatic ^{13}C, which in turn was connected to a carbonyl ^{13}C (^{1}H–{^{13}C–$^{13}C'$}).[18] The conventional ^{1}H NMR spectrum of 1,2,3-^{13}C-labeled racemic lactate, prepared by chemical reduction of 1,2,3-^{13}C-labeled pyruvate using $NaBH_4$,[19] in D_2O shows two double multiplets attributable to methine protons (2-C, 4.21 ppm, $J_{C-H} = 147$ Hz) and methyl protons (3-C, 1.30 ppm, $J_{C-H} = 129$ Hz) (Figure S1a[21]). In marked contrast, only methine protons (2-C) were detected in the triple resonance experiment (Figure S1b[21]) with a detection limit of 77 μM under our experimental conditions (data not shown).

Once the triple resonance technique was optimized to detect targeted lactate, we moved on to sensing of the biomarker lactate using isotope-labeled glucose as a precursor probe. Initially, we used the glucose probe **1** (Figure 1a), where all the carbon atoms were enriched with ^{13}C. Again, our purpose was to develop an OFF-to-ON-type metabolic sensing probe that would ideally be undetected until subjected to the metabolic reaction of concern.

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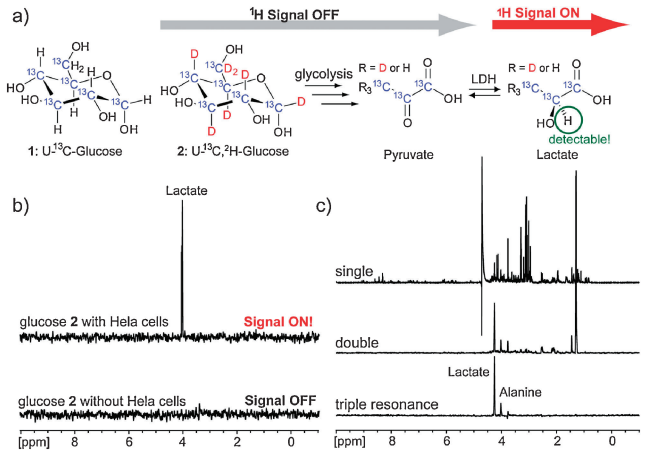

Figure 1. NMR spectroscopic observations on the conversion of glucose to lactate in cells or in mice. a) Chemical structure of the glucose-based probes **1** and **2**, and a schematic drawing showing how the glucose probe **2** works as an OFF-to-ON-type NMR probe for the production of a lactate biomarker. b) Triple resonance ^{1}H NMR spectra (^{1}H–{^{13}C–^{13}C′}) of the DMEM containing glucose probe **2** incubated with (upper) or without (lower) HeLa cells for a period of 24 h. c) Single (^{1}H), double (^{1}H–{^{13}C}), and triple (^{1}H–{^{13}C–^{13}C′}) resonance NMR spectra of the extracts from a tumor (murine colon adenocarcinoma C-26) in a mouse, which was infused with glucose probe **2** via the tail vein. Detection of lactate in tumor by triple resonance NMR was successfully repeated in at least five separate experiments.

The ^{13}C-labeled glucose probe **1** is not ideal in this respect, since probe **1** and its glycolysis intermediates have many ^{1}H–^{13}C–^{13}C sequences, while not of the ^{1}H–^{13}C–^{13}C′-type, which could possibly give rise to undesired ^{1}H signals, even through the triple resonance selection scheme. This was indeed the case. The glucose probe **1** afforded undesired signals even without the metabolic reactions (Figure S2[21]). Thus, we redesigned the probe and used the fully ^{13}C- and ^{2}H-labeled glucose **2**. Since the number of ^{13}C-bound ^{1}H in probe **2** and its metabolites up to pyruvate in the first nine enzymatic reactions are highly suppressed (Figure S3[21]), they should be undetectable under the triple resonance conditions. In the final step of glycolysis, an NMR-active (detectable) species is generated, where the pyruvate is converted (reduced) to lactate by lactate dehydrogenase (LDH) with concomitant incorporation of ^{1}H from the coenzyme NADH to give a detectable ^{1}H–{^{13}C–^{13}C′} sequence. Therefore, glucose probe **2** can be a turn-on sensing probe to selectively monitor an anaerobic production of lactate (Figure 1a).

We were first challenged to monitor the glucose-to-lactate conversion in cultured HeLa cells. The fully ^{13}C/^{2}H-labeled glucose probe **2** (6.5 mM) was added to HeLa cells (2.4×10^6 cells) in a DMEM medium. After incubation at 37 °C for 24 h, the medium was collected, lyophilized, resuspended in D_2O, and subjected to NMR analysis. As shown in Figure 1b, a single signal (4.02 ppm) was observed in the triple resonance ^{1}H–{^{13}C–^{13}C′} NMR analysis, which was assigned and confirmed by 2D ^{1}H–^{13}C HSQC analysis (Figure S4 vs. S1c[21]) to the methine protons of the lactate metabolically produced in the HeLa cells. Importantly, completely no signal was detected from glucose probe **2** incubated in the absence of HeLa cells (Figure 1b). These results clearly indicate that the fully ^{13}C/^{2}H-labeled glucose **2** works as an OFF-to-ON switching NMR probe to detect lactate production from glucose in cells selectively.

Then, we applied our turn-on lactate-sensing approach to metabolic reaction analysis in mice. Glucose probe **2** (3 g kg^{-1} body weight) was infused via the tail vein into a mouse bearing subcutaneously inoculated murine colon adenocarcinoma C-26. After a period of 1 h, the tumor was collected, lysed, redissolved in D_2O, and subjected to NMR analysis. Figure 1c shows the ^{1}H NMR spectra of the tumor in single (^{1}H, upper spectrum), double (^{1}H–{^{13}C}, middle spectrum), and triple resonance (^{1}H–{^{13}C–^{13}C′}, lower spectrum) experiments. As can be clearly seen in Figure 1c, the conventional ^{1}H (upper) spectrum is useless due to the presence of a variety of signals. The double resonance spectrum (^{13}C-selected ^{1}H, middle spectrum) still displays more than 10 signals. In marked contrast, only two signals, a major signal at 4.26 ppm and a minor signal at 4.02 ppm, were observed in the triple resonance spectrum (lower spectrum), which were assigned using 2D ^{1}H–^{13}C HSQC analysis to the methine protons of lactate (^{1}H–^{13}C(OH)–^{13}C=O) and alanine (^{1}H–^{13}C(NH_2)–^{13}C=O), respectively, the latter compound being produced from pyruvate by a transaminase reaction (Figure S5).[20,21] Thus, the present signal turn-on strategy using a combination of the triple resonance technique and a dual ^{13}C/^{2}H-labeled probe is applicable to ex vivo analysis.

In conclusion, this work reveals the potential utility of triple resonance NMR technique for monitoring of particular biochemical events in situ. Suppression of the background noise is not perfect in double resonance ^{1}H–{^{13}C} experiments but becomes satisfactory using a triple resonance technique for selective detection of lactate-producing reaction. Importantly, the present ^{1}H probe is a novel "switch-on" type, which is otherwise NMR-silent and is rendered active only when transformed into the target metabolite by the metabolic reaction in concern. The method thus allows simple and unambiguous detection of specific metabolic event without requiring laborious and possibly misleading chemical shift analysis of the probe and its (various) metabolites. The present strategy is also applicable to other NMR-active ($I = 1/2$) nuclei, such as ^{15}N. Since sequences such as H–C–C and H–C–N are common in biorelevant molecules, there are plenty of potential candidate molecules as biomarkers that can be detected using the present technique. Conceptually, this approach can be applied for in vivo MRI/MRS and further challenge is now underway along these lines.

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References and Notes

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