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Sub-second 2D NMR Spectroscopy at Sub-millimolar Concentrations

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Progress in NMR rests on two areas that have traditionally defined the capability of a spectroscopic characterization: the method's sensitivity and its spectral resolution. Foremost among the technical advances that enhanced NMR's sensitivity was the introduction of pulsed NMR and the time-domain signal-averaging of data.[1] A similar impact but in the site-resolution front came with the advent of multidimensional spectroscopy.[2] Notwithstanding the maturity achieved by these principles, the quest for enhancing sensitivity and resolution in NMR continues. Important developments that have influenced these areas include the introduction of faster modes for acquiring multidimensional spectra,[3] and methods that increase NMR's sensitivity by creating macroscopic nuclear magnetizations that greatly exceed the natural thermal values.[4] Dramatic gains have been reported on both accounts: among the new multidimensional NMR methodologies counts an "ultrafast" approach capable of completing *n*D NMR acquisitions within a single sub-second transient;[5] among the sensitivity strategies, enhancements factors reaching up to 10^4 have been achieved.[4d] Yet despite their unquestionable potential, these strategies exhibit certain handicaps that may limit their applicability. Signal-enhancement procedures, for instance, usually require relatively long times to prepare their very peculiar polarization states, and do so with a degree of reproducibility that is less than perfect. These features make them impractical starting points for traditional multidimensional NMR techniques demanding the acquisition of several consistent scans. Ultrafast 2D acquisition schemes, on the other hand, suffer from sensitivity limitations, which compromise their full potential when involving studies on dilute analytes. It is noteworthy that both kinds of complications could be simultaneously lifted if pre-polarization and ultrafast 2D NMR schemes were combined. The present study explores this potential using what is arguably the simplest of these combinations: chemically induced dynamic nuclear polarization (CIDNP) for enhancing NMR's sensitivity, and ^{1}H TOCSY[6] as the 2D experiment tested.

A CIDNP experiment begins with light irradiating a suitable photoexcitable molecule.[7] The fate of the radical pairs created by this irradiation will depend on hyperfine interactions between the unpaired electrons and their surrounding nuclei. This, in turn, endows these transient electronic species with the capability of affecting the steady-state polarization reached by the surrounding nuclear spins. When the irradiation is carried out on a peptide or protein sample placed in the presence of a suitable photosensitizer, the radicals generated during CIDNP will enhance the NMR signals of certain aromatic residues (tryptophan, tyrosine, histidine) that have sufficient physical access to the photosensitizer. Despite its apparent limitations, the CIDNP approach has been shown to serve as an excellent marker for quantifying solvent accessibility to an aromatic residue, and thus for measuring protein folding at a particular aromatic site.[7c−f] As usual when implementing NMR studies on peptides or proteins, it would be desirable to carry out this sensitivity-enhancement procedure while spreading the affected resonances throughout a 2D frequency spectrum. Here, however, CIDNP shares with most other nuclear hyperpolarization schemes a limited compatibility with 2D NMR, stemming from significant photobleaching effects that set on after the first few light irradiation cycles. These, in turn, reflect in a significant decrease in the nuclear signal following the initial scans, leading to broad peaks and increased t_1 noise along the indirect spectral domain. A number of solutions have been proposed and exploited over the years to alleviate such complication, including the use of light-stable photosensitizers, the addition of tiny aliquots of photosensitizers or oxidants between scans, as well as evacuating/replenishing the sample following each scan.[7d−f] On the other hand, as was mentioned earlier, ultrafast 2D NMR could complete the photo-CIDNP acquisition within a single scan and thus avoid such complications altogether. To assess this possibility, a CIDNP setup was built around a Bruker DMX500 NMR spectrometer, capable of implementing ultrafast 2D NMR based on isotropic homonuclear mixings (Figure 1). A Spectra-Physics CW argon laser operating at 2 W and 488 nm (single mode) provided the light source for these experiments; its output was pulsed into the NMR sample by a mechanical shutter, operating under control from the spectrometer's pulse programmer. The laser's light was led through an optical fiber into the top of the superconducting magnet, and on to a sample tube inserted into the triple-resonance Nalorac probehead used. To maximize the CIDNP effect, the end of the fiber was maintained within an inner coaxial tube, which dipped into the solution studied. Two samples were analyzed in this fashion: a 0.5 mM solution of the cyclic octapeptide C-17, synthesized around the TyrTyrGluGlu motif,[8] and bovine crystalline zinc insulin (Sigma) dissolved at 1 mM. Both compounds were measured at 27 °C in D_2O solutions containing 0.125 mM flavin mononucleotide as photosensitizer. To control insulin's association, the pH of its solution was held at 2.75 with acetic acid-d_3, conditions where the dimeric form predominates.[9]

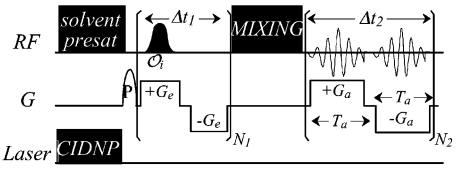

Figure 1. Ultrafast 2D TOCSY sequence used in this study, akin to that described elsewhere[5] except for the addition of the pre-acquisition laser irradiation period required by CIDNP.

Figure 2 presents single-scan 2D ^{1}H TOCSY NMR spectra obtained on C-17 in the absence and in the presence of the CIDNP enhancement—"dark" and "light" sets, respectively. Cross-sections placed inside the figure's panels correspond to 1D traces arising from conventional single-pulse ^{1}H experiments, and show the expected resonance enhancement for the two tyrosine residues in the oligomer. Particularly significant are the negative enhancements affecting the NMR peaks arising from the aromatic H3/H5 protons, which in the "light" spectrum appear with absolute intensities that are over 12 times larger than those in the "dark" counterpart. This, in turn, places the sensitivity of such resonances well above the limit of detection of single-scan 2D NMR, which on the basis of previous calculations we estimate at ca. 4 mM/scan for a TOCSY correlation run on a thermally polarized sample at 11.7 T.[5d] The consequences of this sensitivity enhancement are demonstrated by

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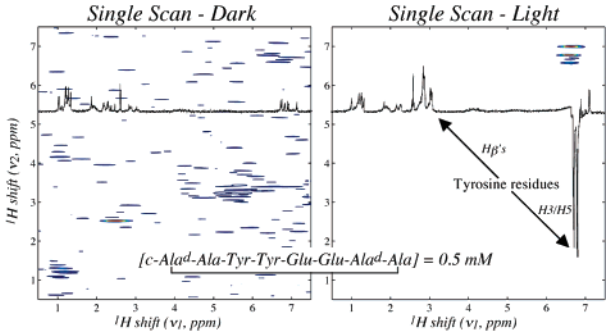

Figure 2. Single-scan ^{1}H 1D and TOCSY 2D NMR spectra of the C-17 octapeptide (inset), recorded in the absence ("dark") and in the presence ("light") of a 1 s CIDNP pre-acquisition enhancement. Ultrafast 2D acquisitions involved $N_1 = 22$ square excitation pulses applied at offset increments $\Delta O = 8$ kHz and spaced by $\Delta t_1 = 290$ μs while in the presence of a $\gamma_H G_e = 121$ kHz/cm, a 32 ms long WALTZ-based mixing period, and $N_2 = 192$ decoding gradient echoes with $\Delta t_2 = 270$ μs and $\gamma_H G_a = 139$ kHz/cm. Data were sampled throughout these decoding echoes at a 200 kHz rate, and processed into the displayed magnitude spectra (plotted at identical levels normal to their maximum peak intensity) as described elsewhere.[5]

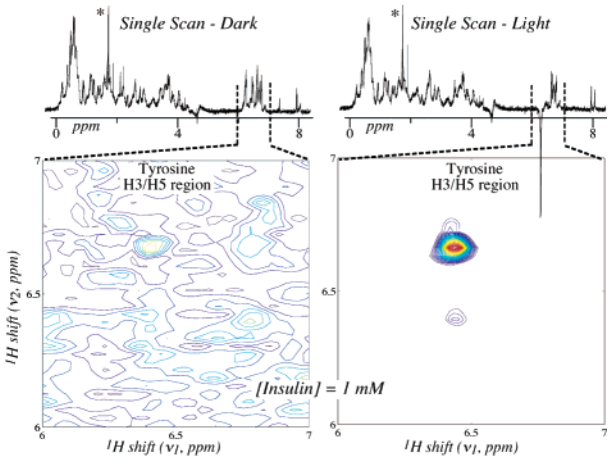

Figure 3. Top: Single-scan ^{1}H 1D NMR spectra recorded on 1 mM insulin in the absence and in the presence of CIDNP enhancement (asterisks mark an artifact arising from the acetic acid used to buffer the solution). Bottom: Single-scan 2D TOCSY ^{1}H spectra arising from the indicated portions of the 1D NMR traces. These zoomed regions were collected using the sequence in Figure 1 with a 32 ms long WALTZ mixing and $N_1 = 53$, $\Delta O = 4$ kHz, $\Delta t_1 = 560$ μs, $\gamma_H G_e = 142$ kHz/cm, $N_2 = 128$, $\Delta t_2 = 270$ μs, $\gamma_H G_a = 70$ kHz/cm.

the experimental 2D NMR results in Figure 2, the quality of which is marginal when "dark" single-scan experiments are recorded at a sub-millimolar level but become unambiguously good when intra-aromatic TOCSY connectivities are probed in the presence of CIDNP enhancement. Figure 3 extends these tests to the case of insulin. Shown on the top of this figure are single-pulse "dark" and "light" ^{1}H NMR spectra; in both cases, lines appear significantly broader than in the peptide spectrum, mainly due to distributions in chemical shifts arising from the protein's self-aggregation. Also inferior to the performance observed for the peptide is the CIDNP enhancement achievable for insulin; out of the four tyrosine residues in this protein, and in accordance with what has been previously reported, we observe most of the enhancement affecting solely Tyr14 at ca. 6.4 ppm.[10] This is apparently the only residue that is sufficiently exposed to the photosensitizer to undergo a significant CIDNP effect, leading in "light" experiments to a signal that is ca. 5 times more intense than in their "dark" counterparts. This degree of pre-polarization may appear modest, but it is sufficient for lowering the detection threshold of single-scan 2D TOCSY NMR below a 1 mM concentration, as illustrated by the dramatically different qualities of the "dark" and "light" 2D NMR data presented in Figure 3.

The main objective of the present study was to explore the benefits that could result from combining nuclear pre-polarization schemes with single-scan 2D NMR methods. The former provide sensitivity gains that would be hard to achieve by gradual improvements in the traditional NMR hardware, but do so at the expense of setups that are poorly suited to multiscan NMR acquisitions. The latter, on the other hand, are capable of providing the complete information being sought within a fraction of a second, but suffer from significant sensitivity limitations. The combination of both methodologies is therefore a natural avenue to exploit. For implementing an initial, test we chose to couple ultrafast 2D NMR with CIDNP, a sensitivity enhancement method of relatively wide applicability. As for the general merits of such a combination, it is worth noting that, in terms of sensitivity per unit acquisition time, the 2D TOCSY results in Figures 2 and 3 are very promising, particularly when recalling that CIDNP's enhancement is not nearly as dramatic as that achieved by other hyperpolarization methods discussed in the literature. The opportunities opened up by these additional combinations, as well as the ways by which these hybrid experiments could help expand the potential of biomolecular NMR, are currently being assessed.

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