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Optimization of ^{13}C direct detection NMR methods

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Abstract

^{13}C-detected experiments are still limited by their inherently lower sensitivity, as compared to the equivalent ^{1}H-detected experiments. Improving the sensitivity of ^{13}C detection methods remains a significant area of NMR research that may provide better means for studying large macromolecular systems by NMR. In this communication, we show that ^{13}C-detected experiments are less sensitive to the salt concentration of the sample solution than ^{1}H-detected experiments. In addition, acquisition can be started with anti-phase coherence, resulting in higher sensitivity due to the elimination of the final INEPT transfer step.

Cryogenic probes are now widely used in NMR studies of protein structure, dynamics, and intermolecular interactions. Recently, several experiments utilizing direct ^{13}C detection, such as the HCACO (Serber et al., 2000, 2001), MQ-HACACO (Pervushin and Eletsky, 2003), ^{13}C-^{13}C TOCSY (Eletsky et al., 2003), COCAMQ (Bermel et al., 2003), and ^{13}C-^{13}C NOESY (Bertini et al., 2004), have been developed to overcome the fast relaxation characteristic of many traditional NMR experiments relying upon ^{1}H detection when studying high molecular weight proteins or proteins with paramagnetic centers (Bermel et al., 2003; Serber et al., 2000). These experiments are feasible because cryogenic probes have brought ^{13}C detected experiments into an experimentally useful sensitivity range even for biomolecular applications. Presently, however, these ^{13}C detected experiments are still limited by their inherently lower sensitivity, as compared to the equivalent ^{1}H detected experiments. Therefore improving the sensitivity of ^{13}C detection methods remains a significant area of NMR research that may provide better means for studying large macromolecular systems by NMR. Here, we investigate sample conditions and pulse sequences that improve the sensitivity of ^{13}C detected experiments relative to their ^{1}H-detected counterparts. In this communication we demonstrate the advantage of ^{13}C detection over ^{1}H detection at high salt concentrations and show that the sensitivity can be significantly improved by removing the final INEPT step in the H(CC)CACO experiment. In this experiment the carbonyl signals are detected as anti-phase doublets, caused by the ^{13}C′-^{13}Cα coupling. Deconvolution using maximum entropy (MaxEnt) reconstruction (Hoch and Stern, 1996, 2001; Shimba et al., 2003) to eliminate the splitting in the spectra can result in greater signal-to-noise ratios than the corresponding in-phase detected experiments.

The signal-to-noise ratio of NMR experiments depends on several factors. For cryogenic probes, the

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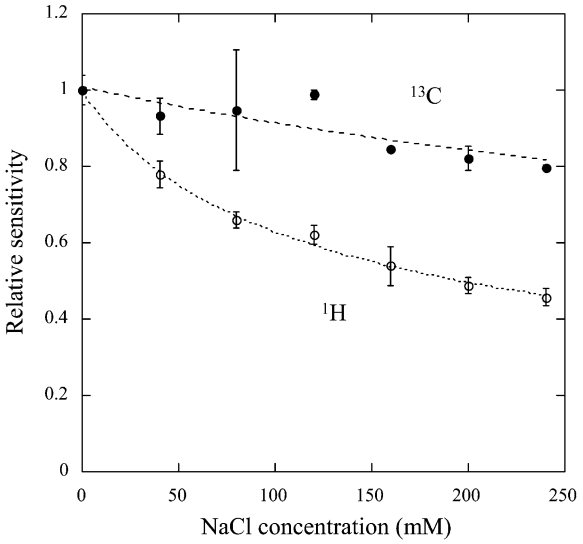

Figure 1. NaCl concentration dependence of the signal-to-noise ratio obtained with 2 mM ^{13}C-labeled leucine dissolved in a 500 µl solution of 90% H_2O/10% D_2O. Experimental values for ^{1}H (open circles) and ^{13}C (filled circles) are plotted as intensity ratios of samples with and of samples without salt. The best fit curves shown by the dashed lines were generated from Equation 1.

sensitivity factor, L, can be expressed as (Kelly et al., 2002):

$$L = \left\{ 1 + 7.45 \frac{\frac{\pi\omega^2\mu^2\sigma n^2 b^4 \ell}{32[a^2+(\ell/2)^2]}}{Rc} \right\}^{-0.5}, \quad (1)$$

$$\sigma = \sum ciqi\lambda i, \quad (2)$$

where ω is the angular frequency of operation, σ is the conductivity of the sample and c is the ionic concentration. The other parameters, R_c, a and n, µ, b and ℓ, q, and λ, are the resistance of the coil, its radius, the permeability of free space, the radius and the length of the sample, the respective charge of the ions, and their mobility, respectively (Kelly et al., 2002). These equations show that the sensitivity is related to the frequency of the detected nucleus and the conductivity of the sample. Since the ^{13}C frequency is approximately four times lower than the ^{1}H frequency, sensitivity loss in samples of high ionic conductivity is expected to be less in ^{13}C detected experiments than in ^{1}H detected ones.

To test this prediction we have performed signal-to-noise measurements on a 2 mM ^{13}C labeled leucine sample at different NaCl concentrations. The salt dependency of the sensitivity of ^{1}H and ^{13}C detected experiments is shown in Figure 1. With increasing NaCl concentration, the ^{1}H sensitivity is reduced significantly. In contrast, the ^{13}C sensitivity is far less affected even at high salt concentrations. Previously it has been shown that the sensitivity of ^{13}C-detected experiments can become comparable to the sensitivity of ^{1}H-detected experiments for example for large or paramagnetic proteins (Bermel et al., 2003; Serber et al., 2000). The results shown above demonstrate that, the inherent loss of sensitivity of ^{13}C detected experiments relative to ^{1}H detection becomes partially compensated at high salt concentrations and therefore significantly extend the range of sample conditions under which ^{13}C detection becomes competitive.

The sensitivity of ^{13}C-detected experiments can further be increased by optimizing the pulse sequences. In ^{13}C detected experiments, acquisition can be started with anti-phase coherence, resulting in a shorter pulse sequence through elimination of the last INEPT step (see supporting information). Anti-phase detection was originally introduced in the COSY pulse sequence (Piantini et al., 1982), and recently also used in ^{13}C detected experiments (Pervushin and Eletsky, 2003). In ^{1}H detected pulse sequences such as ^{1}H-^{13}C HSQC, the final INEPT step has a delay of $1/2J_{CH}$, where J_{CH} is the coupling constant between the ^{13}C and ^{1}H spins and in ^{13}C detected pulse sequences, for example in the HCACO experiment (Serber et al., 2000), the final magnetization transfer delay is $1/2J_{C\alpha-C'}$, where $J_{C\alpha-C'}$ is the coupling constant between ^{13}Cα and ^{13}C′. Compared to coupling constants involving protons, which have values between 120 and 220 Hz, ^{13}C-^{13}C coupling constants such as $J_{C\alpha-C'}$ (∼55 Hz) are smaller, necessitating a longer transfer delay. Consequently, in ^{13}C detected experiments, anti-phase detection is expected to result in higher sensitivity because elimination of the final magnetization transfer delay significantly shortens the overall length of the experiment.

As an example of the improvement in sensitivity achieved by anti-phase detection as compared to in-phase detection, H(CC)CACO experiments were recorded on a 0.5 mM ^{13}C labeled sample of the 25-kDa Kaposi's sarcoma-associated herpesvirus protease (KSHV Pr) M197D variant (Pray et al., 2002). The experiments were performed on a Bruker Avance 500 MHz spectrometer equipped with a cryogenic Z-gradient DUAL ^{13}C/^{1}H probe. The Rowland NMR Toolkit (RNMRTK) was used for data processing. (RNMRTK is available via the Internet at http://www.rowland.org/rnmrtk)

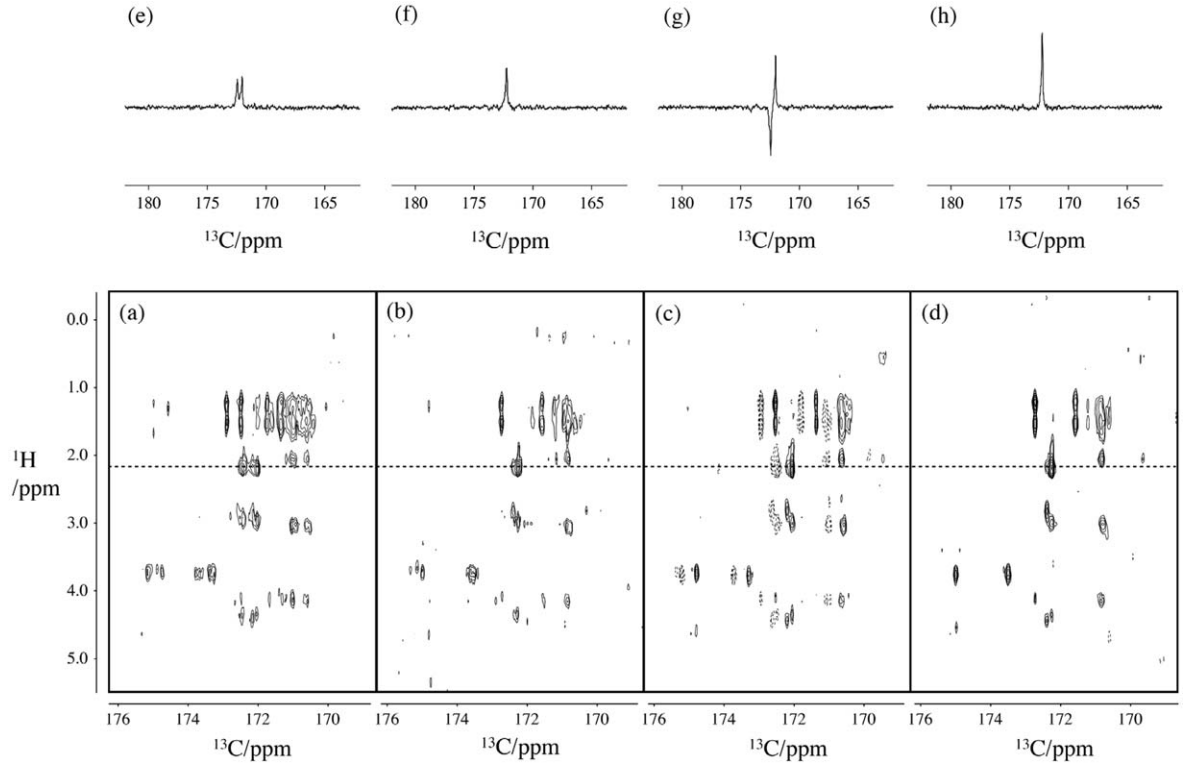

Figure 2. ^{1}H-^{13}C′ planes from 3D H(CC)CACO experiments with a 0.5 mM sample of a 25-kDa protein of the ^{13}C-labeled KSHV Pr M197D variant. Contour levels are shown just above the noise levels. For the H(CC)CACO experiments, data sets of 33 (^{1}H) × 20 (^{13}Cα) × 1024 (^{13}C′) complex points were recorded with spectral widths of 3000 (^{1}H), 5000 (^{13}Cα), and 2520 Hz (^{13}C′). The 1D cross-sections on top of each spectrum are taken along the ^{13}C′ dimension at the position indicated by the dashed lines. The spectral width of the 1D cross-section is larger than that of the 2D plane, to allow a better representation of the noise level. (a) A ^{1}H-^{13}C′ plane, taken from the H(CC)CACO experiment with ^{13}C′ in-phase detection. The ^{13}C′ dimension was processed using Fourier transformation, showing the ∼55 Hz coupling between ^{13}C′ and ^{13}Cα. (b) The ^{13}C′ dimension was transformed and the coupling was deconvolved with MaxEnt reconstruction. The deconvolution kernel was based on a modulation in the ^{13}C′ dimension by $\cos(\pi J t)$, where J is an approximation to the ^{13}C′-^{13}Cα coupling constant. (c) A ^{1}H-^{13}C′ plane, taken from the H(CC)CACO experiment with ^{13}C′ anti-phase detection. (d) The anti-phase^{13}C′-^{13}Cα coupling was deconvolved with MaxEnt reconstruction, assuming a modulation in the ^{13}C′ dimension by $\sin(\pi J t)$.

Figure 2a shows a typical ^{1}H-^{13}C′ plane from the H(CC)CACO spectrum using standard Fourier transform processing. Each peak is split into a doublet by the ^{13}C′-^{13}Cα coupling in the acquisition dimension. Figure 2b shows the same spectrum with the ^{13}C′-^{13}Cα splitting removed by deconvolution using MaxEnt reconstruction. The one dimensional (1D) cross-sections on top of each spectrum show that the signal-to-noise ratio is increased by the elimination of the ^{13}C′-^{13}Cα splitting.

Figures 2c and 2d show the corresponding ^{1}H-^{13}C′ plane of the H(CC)CACO spectrum using ^{13}C anti-phase detection, measured under the same conditions with the same sample. The ^{13}C′ dimension of the spectrum shown in Figure 2d was deconvolved using MaxEnt reconstruction. The anti-phase detection experiment clearly exhibits a higher signal-to-noise ratio compared to the H(CC)CACO spectrum using in-phase detection. In the case of the KSHV protease an approximately two-fold improvement in sensitivity is achieved based on comparison of the one-dimensional cross sections shown on top of each spectrum.

This example also demonstrates the power of MaxEnt reconstruction in removing the splitting of peaks observed in ^{13}C detection experiments. In earlier publications we had already demonstrated its application for the deconvolution of in-phase splittings (Serber et al., 2001) which even worked reliably in regions with overlapping peaks (Shimba et al., 2003). In the case of anti-phase detection, potential problems could arise from the partial cancellation of positive and negative peaks for proteins with large line width. To investigate the question whether sensitivity gains are expected also in these cases we simulated FIDs of

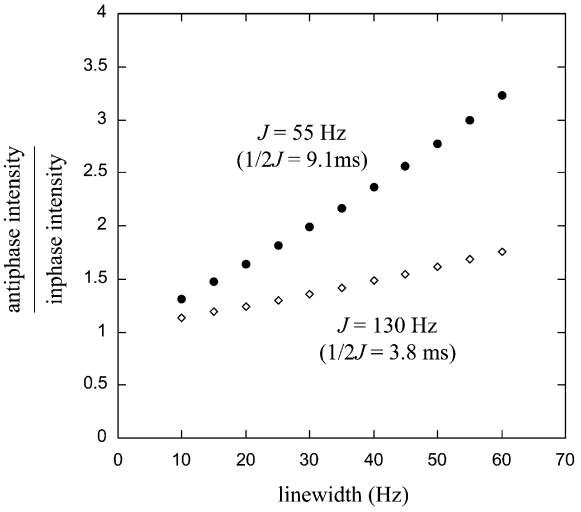

Figure 3. Improvement in sensitivity through anti-phase detection with varying line widths and coupling constants. Magnetization transfer delays, $1/2J$, were set to 9.1 ms ($J = 55$ Hz, filled circles) and 3.8 ms ($J = 130$ Hz, open diamonds), respectively. With increasing line width, the intensity gain of anti-phase over in-phase signals is increased due to the shorter overall length of the experiment. FIDs for anti-phase doublet signals were created using the Rowland NMR toolkit (RNMRTK) with 1024 complex points and a spectral width of 5000 Hz. The line width was varied as shown in the plot to simulate proteins of increasing molecular weight. In-phase signals were generated by eliminating points comprising the initial 9.1 ms and 3.8 ms of the FIDs, respectively. Each anti-phase signal and in-phase signal was calculated separately and their intensities were plotted as a ratio. For peaks showing large line widths, overlap of their positive and negative components led to partial cancellation of the signal. However, deconvolution with MaxEnt still resulted in an increase in sensitivity relative to in-phase detection.

in-phase as well as anti-phase coherences assuming a coupling constant of 55 Hz and compared the relative intensities of the resulting peaks after deconvolution with MaxEnt. As can be seen from Figure 3 the ratio of the intensities of peaks corresponding to anti-phase coherence versus in-phase coherence increases with increasing line width. This demonstrates that MaxEnt is able to deconvolve also anti-phase signals with a large line width and that significant sensitivity gains can be obtained particularly for fast relaxing proteins. Furthermore, comparison of the spectra of the KSHV protease processed with MaxEnt showed that also in crowded regions in which the positive and negative components of different peaks lead to partial cancellation of the anti-phase signals a significant sensitivity gain of the anti-phase detection over the in-phase detection method is achieved. In a second simulation we have assumed a larger coupling constant of 130 Hz. As expected – and in agreement with experimental results – a significantly smaller sensitivity gain is achieved since the duration of the eliminated INEPT step is shorter.

In addition to MaxEnt two other methods have been proposed to eliminate the splitting of resonances in ^{13}C-detected experiments. Band-selective homodecoupling (Bermel et al,. 2003) can be used if the resonance positions of the coupled spins are sufficiently separated such as the $^{13}C'$ and $^{13}C\alpha$ spins. However, this method can only be used in experiments detecting in-phase coherences but not in anti-phase detecting experiments like the one described here. The second method was introduced by Pervushin and Eletsky (2003) and is based on adding and subtracting an anti-phase signal to and from its absolute-value spectrum and then adding the two individual peaks. While this method would also work for the anti-phase detected H(CC)CACO experiment it cannot be used for the deconvolution of in-phase signals.

In summary, we have shown that ^{13}C-detected experiments are less sensitive to the salt concentration of the sample solution than ^{1}H-detected experiments. In addition, acquisition can be started at the point of anti-phase magnetization, resulting in higher sensitivity, due to the elimination of the final INEPT transfer step. The anti-phase doublet caused by the anti-phase detection can be easily deconvolved using for example maximum entropy (MaxEnt) reconstruction. Although ^{13}C detection is inherently less sensitive than ^{1}H detection, ^{13}C detected experiments have certain advantages which make them useful tools to complement traditional ^{1}H-detected pulse sequences. In particular NMR experiments on high molecular weight proteins in high salt concentrations and at high pH values, where amide protons exchange rapidly with bulk water protons, may benefit from ^{13}C detection.

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Supporting Information Available: One figure at: http://kluweronline.com/issn/0925-2738.

References

Baselgia, L., Laukien, F., Stern, A.S., Hoch, J.C. and Dötsch, V. (2000) *J. Am. Chem. Soc.*, **122**, 3554–3555.

Bermel, W., Bertini, I., Felli, I.C., Kummerle, R. and Pierattelli, R. (2003) *J. Am. Chem. Soc.*, **125**, 16423–16429.

Bertini, I., Felli, I. C., Kummerle, R., Moskau, D. and Pierattelli, R. (2004) *J. Am. Chem. Soc.*, **126**, 464–465.

Eletsky, A., Moreira, O., Kovacs, H. and Pervushin, K. (2003) *J. Biomol. NMR*, **26**, 167–179.

Hoch, J.C. and Stern, A.S. (1996) *NMR Data Processing*, Wiley-Liss, New York.

Hoch, J.C. and Stern, A.S. (2001) *Meth. Enzymol.*, **338**, 159–178.

Kelly, A.E., Ou, H.D., Withers, R. and Dötsch, V. (2002) *J. Am. Chem. Soc.*, **124**, 12013–12019.

Pervushin, K. and Eletsky, A. (2003) *J. Biomol. NMR*, **25**, 147–152.

Piantini, U., Sørensen, O.W. and Ernst, R.R. (1982) *J. Am. Chem. Soc.*, **104**, 6800–6801.

Pray, T.R., Reiling, K.K., Demirjian, B.G. and Craik, C.S. (2002) *Biochemistry*, **41**, 1474–1482.

Serber, Z., Böhlen, J.M., Gerfin, T., Marek, D., Häberli, M., Baselgia, L., Laukien, F., Stern, A., Hoch, J.C., Dötsch, V. (2000). *J. Am. Chem. Soc.*, **122**, 3554–3555.

Serber, Z., Richter, C., Dötsch, V. (2001) *ChemBioChem*, **2**, 247–251.

Shimba, N., Stern, A.S., Craik, C.S., Hoch, J.C. and Dötsch, V. (2003) *J. Am. Chem. Soc.*, **125**, 2382–2383.