



Deoxynucleotide Triphosphates and Oligonucleotides by ^{31}P DNP NMR

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■ The highly enhanced signal to noise ratio obtained by DNP NMR makes it possible to directly detect certain heteronuclei (for example, ^{31}P) in only one scan and also in very low concentrations. Such methods allow us to obtain important structural information of bio-molecules at very low concentrations. In this application note, we report observation of ^{31}P nuclei in microgram amounts of deoxynucleotide triphosphates. In addition the ^{31}P NMR spectrum was observed for less than 200nmol of 12-mer oligonucleotide.

Introduction

The low signal to noise ratio of certain heteronuclei is caused by the low natural abundance of the respective NMR active isotopes (e.g. 1% for ^{13}C , 0.037% for ^{15}N) and also by the low polarisation of the sample. The extent of polarisation is linked to the relative populations of spin states, which is typically in the region of 5-20ppm and reflects very small amount of sample that actually contributes to the NMR signal.

One way of increasing the sensitivity of NMR is to create

an enhanced, non-Boltzmann spin state population, which yields enhanced NMR signals. The approaches used to achieve this are generally referred to as "hyperpolarisation techniques", and perhaps the most generally applicable is Dynamic Nuclear Polarisation (DNP). DNP has been shown to yield signals for ^{13}C and ^{15}N nuclei in solution-state NMR that are enhanced by over 10,000 times in comparison with conventional NMR spectra¹.

The technique involves cooling a sample to <4K in a strong magnetic field ($B_0 = 3.35\text{T}$) in the presence of a trityl radical². Under such conditions, the unpaired electrons on the trityl radical become strongly polarised, and this polarisation can be transferred to nearby atomic nuclei using microwave irradiation ($\nu \cong 94\text{GHz}$). Once the polarisation has built up to a sufficient level, the sample is dissolved by the injection of an aliquot (typically 3–5mL) of hot solvent and rapidly (~1s) transferred to a conventional NMR spectrometer for measurement.

^1H NMR spectra of bio-molecules such as deoxynucleotide triphosphates (dNTP), nucleotides, oligonucleotides, and DNA/RNA are usually complicated by poor chemical shift dispersion resulting in signal overlap between the aromatic and sugar proton signals. In addition, several protons of the DNA (such as amino and/or imino protons) undergo rapid exchange with solvent thereby impeding quality of structural elucidation. In this regard, ^{31}P NMR spectral data for oligonucleotides and dNTPs

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often provide valuable information regarding the structural orientation of the molecules (such as the structural information of the phosphodiester backbone of DNA and/or RNA). However, ^{31}P NMR of biomolecules (e.g. DNA, RNA, proteins, etc.) is hampered by a relatively low intrinsic sensitivity coupled with large line widths and requires high sample concentration or long data collection time. In this article, we report single scan acquisition of ^{31}P DNP NMR of dNTPs and a 12-mer oligonucleotide (Mol Wt $\sim 3.5\text{kDa}$) in microgram and nanomole concentrations, respectively. In addition, we show that monitoring ^{31}P nuclei by DNP NMR can allow identification of dNTPs in a mixture of two or more different dNTPs.

Experimental considerations

For DNP measurements, solid samples were dissolved in a mixture of glycerol:H₂O (2:3 v/v). A trityl radical (OX63)³ was added at a concentration of 15mM to act as the source of free electrons. The sample was then immersed in supercooled liquid helium ($T = 1.4\text{K}$) in a 3.35T magnetic field and irradiated with microwaves ($\nu \cong 94\text{GHz}$) for two to four hours (see below). After polarisation, the samples were dissolved in 4mL of hot aqueous EDTA solution and rapidly transferred to a conventional 400MHz NMR spectrometer. Single scan ^{31}P NMR spectra were recorded by using a single 90° tip-angle pulse.

Results and Discussion:

Deoxynucleotide Triphosphates (dNTP) and Identification of dNTPs from a Mixture

Significant gain in SNR allows single scan DNP NMR to efficiently probe ^{31}P nuclei that helps in structural elucidation of phosphorus containing molecules at very low concentration. Such results are difficult to obtain using conventional NMR spectroscopy. The gain in ^{31}P SNR makes it possible to observe all the three phosphate signals for 800 μg of dATP, dGTP, dTTP, and dCTP (Figure 1) in a single scan.

The observation of ^{31}P nuclei by DNP NMR can be further extended in determining and distinguishing between mixtures of phosphorus containing molecules. This is illustrated for a mixture of dATP and dTTP (Figure 2A) and a mixture of dATP, dTTP, and dGTP (Figure 2B).

The samples [dATP and dTTP (1:2 by w/w, Figure 2A) or dATP, dTTP, and dGTP (1:2:3 by w/w, Figure 2B)] were polarised for 90 minutes using glycerol-d₈ and D₂O as glassing agents, followed by dissolution using hot aqueous EDTA solution. The samples after dissolution were transferred into a 5mm NMR tube inside a 400MHz JEOL NMR spectrometer.

Figure 1: ^{31}P NMR spectra of 800 μg of dATP(A), dGTP(B), dTTP(C), dCTP(D). The assignments of α , β , and γ phosphates are indicated and the chemical structures of dNTPs are shown along with their respective ^{31}P DNP NMR spectrum.

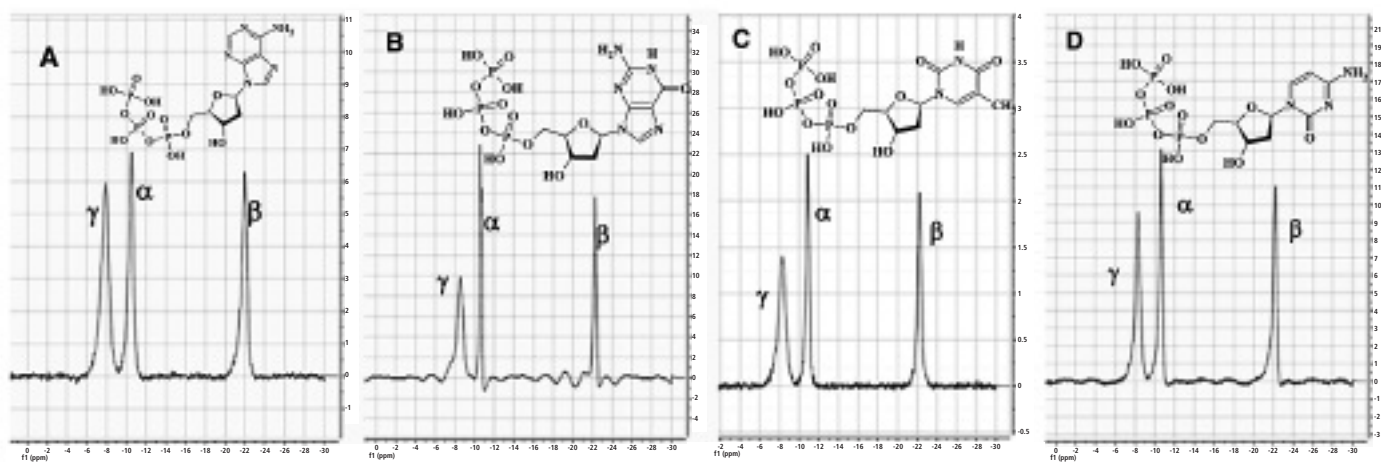


Figure 1

Figure 2: (A) ^{31}P DNP NMR of mixture of dATP and dTTP in 1:2 ratio. The difference in chemical shifts observed for the α -phosphate signals are shown (inset). (B) ^{31}P DNP NMR of mixture of dATP, dTTP, and dGTP in 1:2:3 ratio. The difference in chemical shifts observed for the α -phosphate signals are shown (inset).

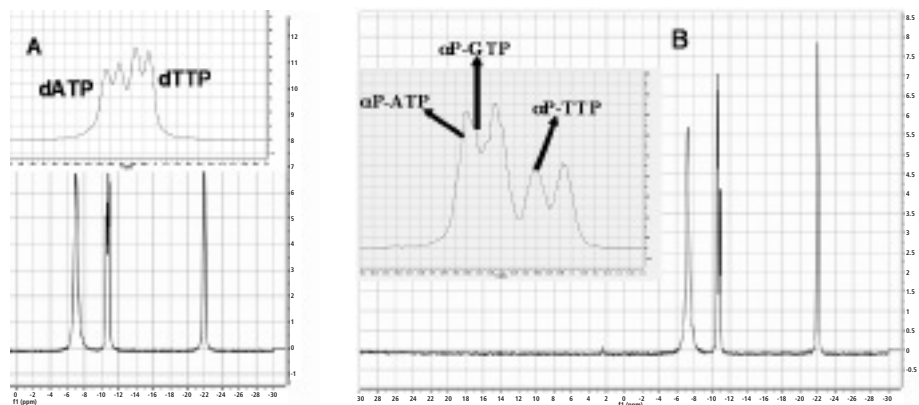


Figure 2

The dNTPs in the mixture were identified based on the chemical shifts of the respective dNTPs observed from the DNP NMR experiments conducted before (Figure 1). The main differences in chemical shifts were observed for the α -phosphate signals only, as shown in Figure 2A and Figure 2B. In addition, the dNTPs that were mixed in a definite w/w ratio were identified based on their relative signal intensities (signal integration values not shown). Identification of isomers and/or impurities in microgram amount of samples is a long-standing challenge faced by the chemists. In this article, we exhibit use of ^{31}P DNP NMR in identifying respective entities in a mixture of dNTPs. This technique can also be extended in distinguishing and structural elucidation of other phosphorus containing compounds.

Oligonucleotides

Structural elucidation of oligonucleotides and DNA/RNA by ^1H NMR spectroscopy is complicated because of signal overlap and also because of protons that rapidly exchange with the solvent. These difficulties are avoided by recording NOESY spectra of the oligonucleotides/DNA at lower temperature and also by selective deuteration. However, the structural information obtained by such methods are often found to be inadequate. Moreover, the heteronuclear 2D experiments do not provide enough information because of the poor natural abundance of ^{13}C (~1%) and

^{15}N (~0.037%). The currently known procedures for synthesising ^{13}C , ^{15}N labelled oligonucleotides are difficult and expensive, unlike those of ^{13}C , ^{15}N labelled peptides and proteins. In this regard, ^{31}P NMR spectroscopy of oligonucleotides/DNA can provide valuable structural information about the phosphodiester backbone.

Figure 3: ^{31}P DNP NMR spectrum of 12-mer oligonucleotide (concentration = 175nmol of oligonucleotide; polarisation time = 3h; number of scans = 1)

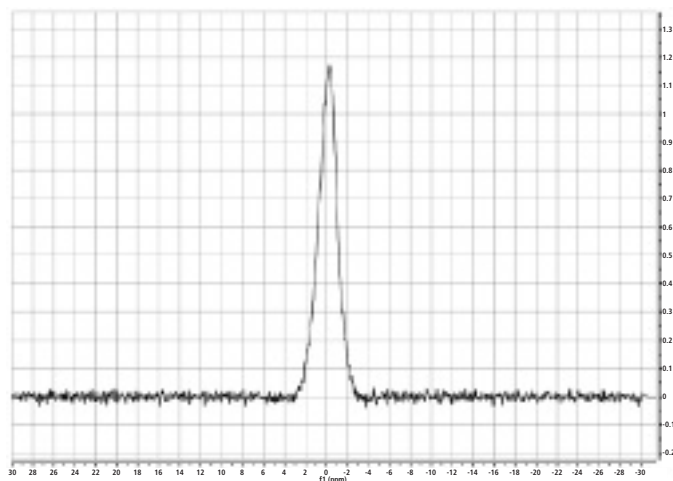


Figure 3

175nmol of a 12-mer oligonucleotide (5'-CCTCTGGTCTCC-3') was polarised for 3h using glycerol-d8 and D_2O as glassing agents. The polarisation was followed by dissolution using hot aqueous EDTA solution and the sample was transferred into a 5mm NMR tube inside a 400MHz JEOL NMR spectrometer for

data acquisition, Figure 3. The 11 possible phosphate signals could not be identified from this spectrum because of lack of spectral resolution. However, with the development of ultrafast single scan NMR pulse sequences for DNP⁴, several novel experiments (such as ³¹P-¹H HETCOR) could be performed to identify and assign the respective phosphate signals. The ³¹P DNP NMR can be extensively used in structural elucidation of DNA-drug adducts, DNA-protein complexes, DNA complexes of metal and non-metal based intercalators, and also in the field of RNA.

Conclusions

DNP can dramatically increase the signal to noise ratio of several heteronuclei. This feature of DNP allows us to use ³¹P NMR signals to monitor phosphorus containing compounds, identifying them from a mixture of several phosphorus containing compounds, and also in structural elucidation of several biomolecules.

References

1. Ardenkjaer-Larsen, J. H.; Fridlund, B.; Gram, A.; Hansson, G.; Hansson, L.; Lerche, M.H.; Servin, R.; Thaning, M.; Golman, K. *Proc. Nat. Acad. Sci.* **2003**, *100*, 10158.
2. Wolber, J.; Ellner, F.; Fridlund, B.; Gram, A.; Johannesson, H.; Hansson, G.; Hansson, L.H.; Lerche, M.H.; Mansson, S.; Servin, R.; Thaning, M.; Golman, K.; Ardenkjaer-Larsen, J. H. *Nucl. Inst. Meth. Phys. Res. A* **2004**, *526*, 173.
3. Blazina, D.; Reynolds, S.; Slade, R.; "Influence of Trityl Radical on the DNP process", Application note. **2006**, (OX63 available from OIMBL)
4. Frydman, L.; Blazina, D.; *Nat. Phys.*, **2007**, *3*(6), 415.

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