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A Site-Specific Low-Enrichment ^{15}N,^{13}C Isotope-Labeling Approach to Unambiguous NMR Spectral Assignments in Nucleic Acids

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Resonance assignment is the first decisive step in NMR studies of nucleic acid structure and dynamics. Traditionally, the assignment techniques are based on NOE and through-bond correlations.[1] The latter are usually obtained following usage of uniformly ^{15}N,^{13}C-labeled nucleic acids,[1] but several through-bond correlations can also be obtained at natural abundance.[2] On the other hand, base resonances can be identified by isotopic or chemical substitutions,[3,4] provided that the latter do not cause structural alterations. Site-specific 100% isotope enrichment provides instantaneous identification of the labeled spin without structural alterations,[3] but its general applicability could be limited on the basis of cost. Recently, it has been proposed that a nucleotide, whose ^{15}N or ^{13}C level increases by a factor of 2 or more compared to natural abundance, could be distinguished from its unlabeled counterparts.[4,5] This communication demonstrates that different resonances within DNA oligomers can be unambiguously assigned by site-specific ^{15}N,^{13}C-enrichment[6] at a level as low as 1%.[7] Using isotopically labeled phosphoramidites, with enrichment as low as this, assignment strategies by isotope enrichment become not only straightforward but also affordable. We demonstrate the methods on the DNA sequence d(GGGTTCAGG) which forms a dimeric G-quadruplex containing G·G·G·G tetrads sandwiched between G·(C-A) triads.[8]

For assignment procedures, protons are correlated to ^{15}N or ^{13}C, with one-bond correlations being usually the most sensitive. Then, on the basis of intensity differences between correlation peaks in labeled and unlabeled samples, one can identify the resonances associated with the site-specifically labeled nucleotide. Depending on resolution, measurements can be performed using 1D or 2D NMR experiments. One can also exploit (see below) the fact that a labeled nucleotide contains ^{15}N (or ^{15}N,^{13}C) at every position, in contrast to random distribution of ^{15}N and ^{13}C at natural abundance.

In studies of nucleic acids, exchangeable protons are very important because they can participate as hydrogen-bond donors and define base-pairing alignments. Figure 1 shows assignment of imino and amino protons for the d(GGGTTCAGG) fold and confirms the previous assignments for this dimeric quadruplex architecture containing triads and G-tetrads (Supporting Information Figure S1). Imino protons can be assigned in ^{15}N-filtered spectra (Figure 1A) following enrichment at a level as low as 0.5% (i.e., 0.87% of total ^{15}N). Amino protons can also be similarly assigned[9] (Figure 1B) despite their broader line widths.

Nonexchangeable aromatic base protons can be correlated by either one-bond couplings to attached ^{13}C nuclei in a ^{13}C-labeled sample, or alternately by long-range couplings to ^{15}N nuclei in a ^{15}N-labeled sample. There are advantages associated with ^{15}N-labeling relative to ^{13}C-labeling approaches based both on cost and the fact that ^{15}N exhibits a larger difference relative to natural abundance when compared to ^{13}C (factor of 4 vs factor of 2) for 1% labeled samples. Examples of guanosine H8 and cytidine H6 resonance assignments based on 1% ^{13}C-labeling at positions G2 and C6 in the dimeric d(GGGTTCAGG) quadruplex are shown in ^{13}C-filtered spectra Figure 2A. An example of guanosine H8 assignment based on 1% ^{15}N-labeling at position G3 follows from comparison of boxed peaks in the labeled (Figure 2C) and natural abundance (Figure 2B) $^{15}N-^{1}H$ correlation spectra.[10]

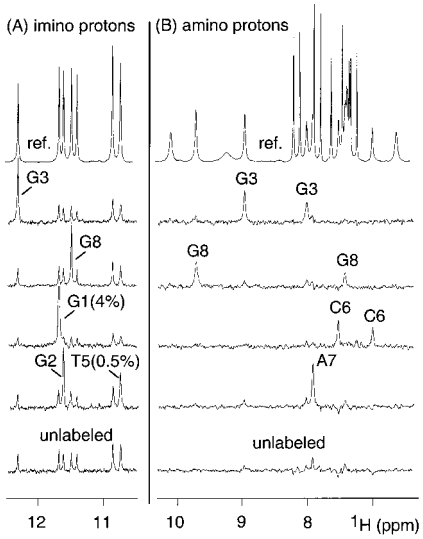

Figure 1. (A) Assignments of imino and (B) amino protons for site-specific low-enrichment (0.5−4.0%) ^{15}N-labeled samples of the dimeric d(GGGTTCAGG) quadruplex. Strand concentration was 4 mM, except for the G1-labeled sample, for which the strand concentration was 2 mM. Experimental conditions: 100 mM NaCl, 2 mM phosphate, pH 6.6, 0 °C, in 95% H_2O, 5% D_2O. Unless otherwise indicated in the Figure, the enrichment level of site-specific ^{15}N-labeled samples is 1%. From the top, the first reference spectrum, designated "ref.", was obtained by the jump-and-return sequence (JR).[13] Other spectra were ^{15}N-filtered in an HMQC experiment with JR water suppression[2d]. The last spectrum, from unlabeled sample, is used as a reference. The remaining spectra are from oligonucleotides ^{15}N-labeled at the positions indicated in the Figure.

Sugar protons are usually broader and more crowded than base protons, making it sometimes difficult to identify site-specifically 1% ^{13}C-labeled peaks in 1D spectra. Thus, not all sugar protons can be readily identifiable in a ^{13}C-filtered spectrum of a sample containing 1% ^{13}C-labeling at position G2 in the dimeric d(GGGTTCAGG) quadruplex (Figure 3B), relative to its reference counterpart (Figure 3A). Such ambiguities can be overcome following recording of 2D $^{13}C-^{1}H$ correlation spectra (Supporting Information Figure S3). Thus, the H1′−C1′ cross-peak assigned to G2 is stronger in the 1% labeled sample (Figure 3C′) compared to the reference spectrum of unlabeled sample (Figure 3C). An alternate approach, based on a $^{13}C-^{1}H$ constant-time HSQC experiment[11] (constant

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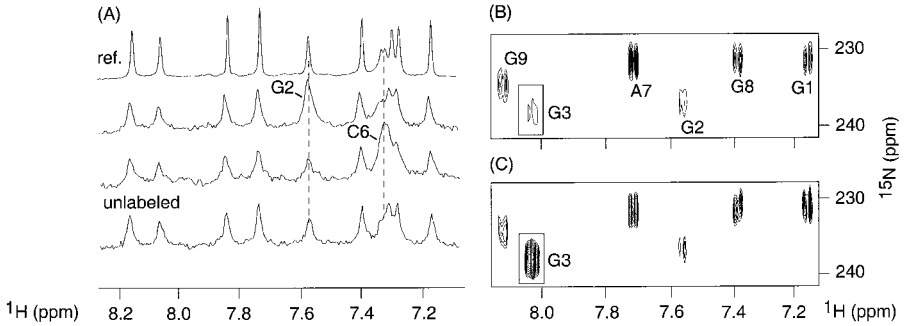

Figure 2. Assignments of aromatic protons for site-specific 1% ^{15}N (and ^{15}N,^{13}C)-labeled samples of the dimeric d(GGGTTCAGG) quadruplex (same buffer as in Figure 1, except in 99.9% D_2O). (A) Top spectrum is a 1D reference spectrum. Other spectra are ^{13}C-filtered by the HMQC pulse sequence.[14] Note that resonances in these spectra are broader than in the 1D reference because of one-bond coupling to ^{13}C. Most broadened peaks are from labeled nucleotides (^{13}C,^{15}N-labeled at the position indicated in the Figure) due to supplementary couplings to all ^{13}C and ^{15}N. (B, C) Long-range H8−N7 correlation for purine bases in a 2D selective HMBC experiment of unlabeled (B) and G3-^{15}N-labeled (C) samples obtained using the pulse sequence in Supporting Information Figure S2. The correlation peak for G3 in (C) is stronger than in (B). The line-shape of this peak is also different due to simultaneous couplings of H8 to N7 and N9 during acquisition.

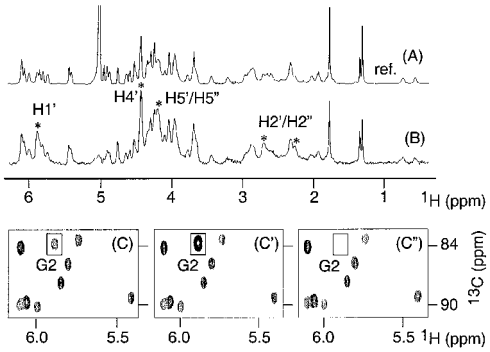

Figure 3. Assignments of sugar protons of G2 following site-specific 1% ^{15}N,^{13}C-labeling at this position in the dimeric d(GGGTTCAGG) quadruplex (same buffer as in Figure 1, except in 99.9% D_2O). (A) 1D reference spectrum and (B) ^{13}C-filtered 1% G2-labeled spectrum. Asterisks indicate sugar protons assigned to G2. The H1′-C1′ correlation spectra for (C) unlabeled sample and (C′, C″) 1% G2-labeled sample. The spectra of (C) and (C′) were obtained with a HSQC sequence,[15] while the spectrum of (C″) was obtained using CT-HSQC sequence.[11] Compared to (C), the correlation peak for G2 is stronger in (C′) and cancels out in (C″).

delay $T = 25$ ms), capitalizes on the observation (for uniformly labeled samples) of negative cross-peaks for sugar protons (such as H1′), whose attached ^{13}C have an odd number of ^{13}C neighbors. In the sample containing 1% ^{13}C-labeled at G2, cancellation occurs between the uniformly labeled and natural abundance contributions because of opposite sign, resulting in the disappearance of the H1′−C1′ cross-peak assigned to G2 (Figure 3C″). The corresponding experimental data for several other sugar protons are outlined in Supporting Information Figure S3.

The current studies were undertaken with 0.5−4.0% site-specific labeling within DNA sequences whose concentration ranged from 2 to 4 mM in strands. Such strand concentrations are necessary for studies of multimeric DNA structures. This approach is also applicable to more diluted samples, in which case one may have to appropriately increase the extent of site-specific label incorporation. Practically, sample handling is much easier, when one dilutes labeled material with its unlabeled counterpart, as outlined in this contribution. In conclusion, the proposed site-specific labeling approach is a direct and affordable method for unambiguous resonance assignment of base and sugar protons in DNA oligomers. Other heteronuclei can also be assigned, following correlation to assigned base and sugar protons. This approach opens opportunities for NMR studies of higher-molecular weight DNAs, and eventually RNAs[12] through site-specific labeling and assignment of structurally and catalytically important residues. The approach would be also applicable to peptides and proteins.

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Supporting Information Available: Three figures (schematic structure of the dimeric d(GGGTTCAGG) quadruplex, selective HMBC pulse sequence, and assignments of sugar protons). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) DNA oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method. 100%-^{15}N-labeled and 100%-^{15}N,^{13}C-double labeled phosphoramidites, purchased from Martek, were diluted with unlabeled phosphoramidites to obtain desired enrichment levels. One or a small number of nucleotides are labeled in the DNA oligomer, and percentage (%) refers to added enriched products.
(7) A 1% added label (i.e., 100-fold dilution of 100%-labeled phosphoramidites) translates to 1.37% for ^{15}N and 2.1% for ^{13}C (i.e., these isotopes increase by a factor of 4 and 2, respectively, compared to natural abundance values of 0.37% for ^{15}N and 1.1% for ^{13}C).
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(9) It is worth noting that adenine amino protons, which are distant from other protons within the base, can also be assigned as shown in Figure 1B.
(10) The purine H8 protons of site-specifically labeled nucleotides exhibit not only intensity differences but also line-shape differences due to simultaneous couplings to both N7 and N9 during detection. The pulse sequence outlined in Supporting Information Figure S2 allows suppression of the H8−N9 correlation pathway during transfer delays. In this long-range correlation experiment, the intensity of different correlation peaks (from different aromatic base protons) can be very different (Figure 2, B and C), depending on relaxation rate, coupling values, and ^{15}N frequency. Note that, under favorable conditions aromatic protons of pyrimidines and H1′ protons can also be correlated to a base nitrogen.
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