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DETECTION OF SPECIFIC BASE SEQUENCES IN SOLUTION USING DNA PROBES LABELED WITH D- AND/OR ^{15}N-SUBSTITUTED SPIN-LABELS

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In order to enhance the sensitivity and the accuracy of the detection by the DNA probe method in which spin-labeled oligonucleotides are used as probes, 4-amino-2,2,6,6-tetramethylpiperidine-^{15}N-oxyl-d_{16} (4-amino-TEMPO-^{15}N-d_{16}) and 4-amino-2,2,6,6-tetramethylpiperidine-^{14}N-oxyl-d_{16} (4-amino-TEMPO-^{14}N-d_{16}) were incorporated into the phosphate linkage of oligonucleotides by the hydrogen phosphonate method and these DNA probes were used for the detection of target DNAs in solution. The intensities of the electron paramagnetic resonance (EPR) signals of the oligonucleotides labeled with 4-amino-TEMPO-^{14}N-d_{16} and 4-amino-TEMPO-^{15}N-d_{16} were 3-fold and 4-fold larger than that of the oligonucleotide labeled with 4-amino-TEMPO-^{14}N, respectively. Also, the EPR lines of these labeled oligonucleotides do not overlap each other, allowing the detection of two different regions in the same target DNA by the single EPR measurement.

KEY WORDS: DNA-probe, spin-label, spin-labeled oligonucleotide, deuterated TEMPO, ^{15}N-substitution, EPR.

INTRODUCTION

The DNA probe method detecting specific base sequences of target DNA is one of the most useful tools for genetic diagnosis. We have studied the DNA probe method (spin-labeled DNA probe method) which uses site-specifically spin-labeled oligonucleotides and can detect the target DNAs in solution without separation of the bound and free probes (B/F separation).

In our previous papers, we have reported synthetic procedures involving the hydrogen phosphonate method by which oligonucleotides are labeled on the internucleotide phosphoramidate linkage with 4-amino-2,2,6,6-tetramethylpiperidine-^{14}N-oxyl (4-amino-TEMPO-^{14}N), and successful detection of the target oligonucleotides using this probe in solution[1,2]. When such spin-labeled oligonucleotides formed hybrids with their complementary sequences (oligonucleotides), marked line broadening was observed in the EPR spectra obtained. Since this method does not require B/F separation, the spin-labeled DNA probe method proposed in the present study is characteristic of high detection speed, simple operation, and easiness of complete automation.

Compared to other proposed DNA probe techniques all of which require B/F separation, however, the sensitivity of this DNA probe method is rather low: the minimum amount of the target required for the diagnosis is 160 pmol. Although

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enhancement of the sensitivity can be achieved by use of the polymerase chain reaction (PCR) technique which amplifies target DNAs or RNAs nearly 1 million times, it may also be helpful to enhance the sensitivity by use of deuterated spin-labels. Deuterium(D)-substitution on the piperidine ring will result in the sharpening of the EPR lines: the 5-fold enhancement of the signal for 4-maleimide-2,2,6,6-tetramethypiperidine-^{14}N-oxyl-d_{17} has been reported previously[3]. In the present study, all the piperidine ring protons were substituted with deuterium.

Furthermore, for enhancing the detection accuracy, double detection of a single target DNA was studied here. Substitution of the ^{14}N with ^{15}N will lead to dramatic change in the EPR signal structure since the number of nuclear spin states decrease from three ($m = +1, 0, -1$) to two ($m = +1/2, -1/2$)[4,5]. Thus the EPR signals from ^{15}N nucleus of the spin-labeled probe will not overlap those from ^{14}N. Therefore, if two oligonucleotides whose base sequences are complementary to the two different regions of the target DNA are labeled with ^{14}N- and ^{15}N-nitroxide, it is feasible to detect these two regions simultaneously with a single EPR measurement. In the present study, oligonucleotides labeled with deuterated ^{14}N- or ^{15}N-aminoxyl spin-labels were prepared and used for the double detection of the target DNA in solution.

MATERIALS AND METHODS

Materials and General Procedure

4-Oxo-2,2,6,6-tetramethylpiperidine-^{14}N-d_{16} (4-oxo-TEMPO-^{14}N-d_{16}) and 4-oxo-2,2,6,6-tetramethylpiperidine-^{15}N-d_{16} (4-oxo-TEMPO-^{15}N-d_{16}) were synthesized according to the previous procedures[6]. $(CD_3)_2CO$, NaOD, D_2O and $NaBH_3CN$ were obtained from Aldrich Chemical Company (St. Louis, MO, USA) and used without further purification. $^{15}NH_3$ was purchased from Sigma Chemical Company (St. Louis, Mo, USA). Other chemicals of guaranteed grade were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CH_3OH was refluxed over CaH_2 for 8 hr and distilled over Molecular Sieves 3A. Ammonium acetate was dried over phosphoric anhydride. Oligonucleotides used as models of the target DNAs were synthesized by the solid-phase phosphoramidite protocol on a Milligen Biosearch Cycron Plus DNA synthesizer and purified by reversed-phase liquid chromatography (RPLC). Isotope substituted spin-labels, 4-amino-2,2,6,6-tetramethylpiperidine-^{14}N-oxyl-d_{16} (4-amino-TEMPO-^{14}N-d_{16}) and 4-amino-2,2,6,6-tetramethylpiperidine-^{15}N-oxyl-d_{16} (4-amino-TEMPO-^{15}N-d_{16}) were synthesized from 4-oxo-TEMPO-^{14}N-d_{16} and 4-oxo-TEMPO-^{15}N-d_{16} according to the procedure reported previously[6], respectively.

Synthesis of 4-Amino-TEMPO-^{15}N-d_{16}

To a solution of 0.77 g (10 mmol) of ammonium acetate dissolved in 10 ml of CH_3OH added was 0.092 g (0.5 mmol) of 4-oxo-TEMPO-^{15}N-d_{16} and 0.025 g (0.4 mmol) of $NaBH_3CN$. Molecular Sieves 3A was added to the solution to absorb water. The reaction mixture was allowed to stir for 24 hr at room temperature. After filtering, solvent was removed under vacuum and the red oil taken up into a 1N HCl solution (30 ml). The solution was extracted with $CHCl_3$ three times. The organic layer was washed with H_2O. After drying over $MgSO_4$, solvent was removed under

vacuum. The resulting residue was dissolved in H_2O. After filtering, solvent was removed under vacuum to leave a red oil (0.063 g). RPLC of the red oil indicated about 100% purity (68% yield). EPR studies showed that the ^{15}N composition was about 100%.

Synthesis of 4-Amino-TEMPO-^{14}N-d_{16}

4-Amino-TEMPO-^{14}N-d_{16} was synthesized from 4-oxo-TEMPO-^{14}N-oxyl-d_{16} by the similar procedure described for 4-amino-TEMPO-^{15}N-d_{16}.

Synthesis of Spin-Labeled Oligonucleotide

Oligonucleotides labeled with isotope-substituted 4-amino-TEMPO were synthesized by a combination of automated solid-phase phosphoramidite protocol[7] and the hydrogen phosphonate method[8,9] according to our previous procedure[1,2].

Purification of Spin-Labeled Oligonucleotides

Synthesized spin-labeled oligonucleotides were purified by RPLC. An high-performance liquid chromatography system used was composed of an HLC-803D pump unit (Tosoh, Tokyo) equipped with a gradient system (GE-4, Tosoh) and a UV-flow monitor (UV-8, Tosoh). The chromatographic conditions were as follows: column, Wakosil-DNA (4 × 150 mm); eluent, (low) 5% CH_3CN in 0.1 M triethylammonium acetate (TEAA, pH 6.8) and (high) 50% CH_3CN in 0.1 M TEAA; gradient, linear; flow rate, 1.0 mL/min; detection, 260 nm.

EPR Measurements

EPR measurements of spin-lables and spin-labeled oligonucleotides were performed on a JEOL PE-3X EPR spectrometer (JEOL, Tokyo) with X-band cavity resonator at room temperature. An aqueous quartz flat cell (outer dimensions being 45 × 10 × 2 mm, LABOTEC, Tokyo) was used. To remove molecular oxygen, samples were degassed by bubbling argon gas for 3 min immediately prior to the measurements. For the detection of hybrid formation, to make stable hybrids, a solution containing both a spin-labeled oligonucleotide (final concentration, 5 μM) and its target oligonucleotide (final concentration, 5 μM) was maintained at 85°C and then gradually cooled down to the room temperature. EPR spectra were recorded at 9.56 GHz resonant frequencies and with 100 kHz field modulation of 0.02 mT amplitude, 1.0 min of response time, 36 min of sweep time, and non-saturating microwave power of 10 mW. When D- and/or ^{15}N-4-amino-TEMPOs were measured, EPR spectra were recorded using the same EPR settings except that the response time and the sweep time were 0.3 sec and 8 min, respectively.

RESULTS AND DISCUSSION

Before the incorporation of the spin-labels into oligonucleotides, EPR measurements were performed for the labels. EPR spectra obtained from aqueous solutions of 4-amino-TEMPO-^{14}N, 4-amino-TEMPO-^{14}N-d_{16} and 4-amino-TEMPO-^{15}N-d_{16} are shown together with their chemical structures in Figures 1a, b, and c, respectively:

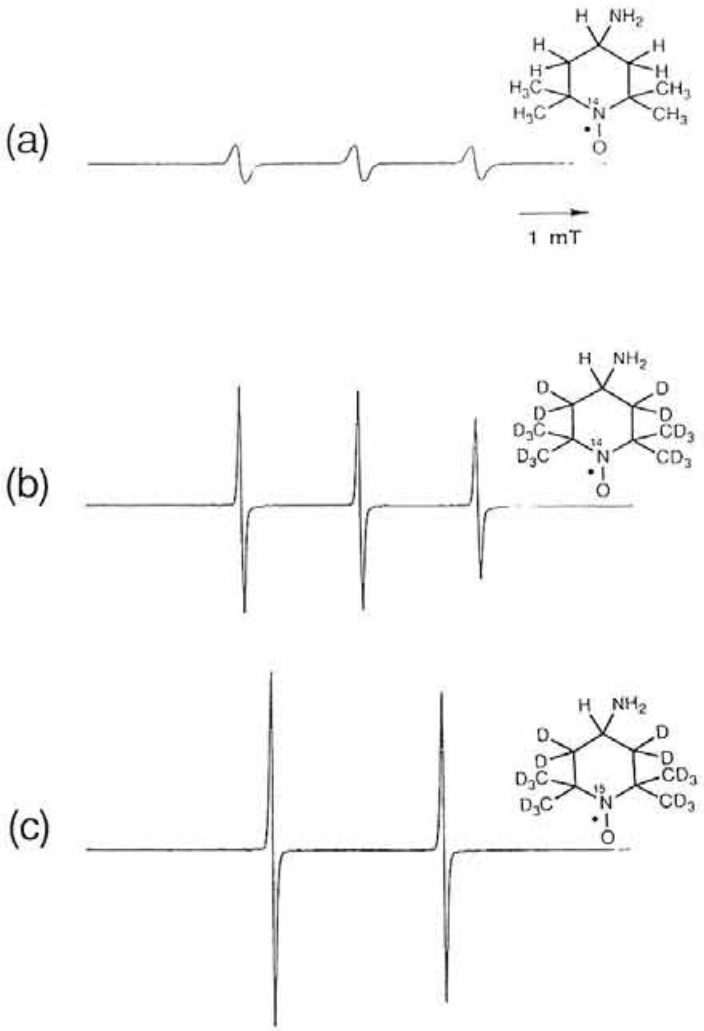

FIGURE 1 Structures and EPR spectra obtained from (a) 4-amino-TEMPO, (b) 4-amino-TEMPO-^{14}N-d_{16}, and (c) 4-amino-TEMPO-^{15}N-d_{16}. *Conditions*: concentration of the spin-label, 30 μM; solvent, water; temperature, room temperature. *EPR settings*: modulation, 0.02 mT; gain, 3.2 × 100; responce time, 0.3 sec; sweep time, 8 min.

the concentration of the each solute was same (30 μM). Comparing the spectra in Figures 1a and b, the triplet due to 4-amino-TEMPO-^{14}N (a_N = 1.70 mT and the line width (peak to peak, δH_{pp}) being 0.1598 mT for the m = 0 component) is sharper by the D-substitution on the piperidine ring: the line width (δH_{pp}) of the m = 0 component of the EPR signals of 4-amino-TEMPO-^{14}N-d_{16} was 0.062 mT. Accordingly the signal intensity of the m = 0 component became 6.0-fold larger than that for 4-amino-TEMPO-^{14}N. A characteristic doublet with sharp lines (a_N = 2.39 mT and the line width being 0.064 mT for the m = + 1/2) was

obtained for 4-amino-TEMPO-^{15}N-d_{16}. Since the total integration area of the signal should be independent of the ^{15}N-substitution, the EPR signal intensity is further increased up to a total of 8.2-fold ($6 \times 3/2 = 9$ in theory).

Since the EPR peaks of the 4-amino-TEMPO-^{15}N-d_{16} do not overlap those of the 4-amino-TEMPO-^{14}N-d_{16}, combined use of these two spin-labels will lead to the simultaneous detection of the two different base sequences in the same target DNA with a single EPR measurement.

These three spin-lables were incorporated into the oligonucleotides whose base sequences appear in Table 1 together with their abbreviations. In the same table, oligonucleotides used as targets for these probes are also listed. Although each probe consists of a set of stereoisomers arising with respect to the phosphoramidate linkage bound with the labels, a mixture of the isomers was utilized without separation in the present study. In order to see how these spin-labeled oligonucleotides behave in solution in the absence or in the presence of the target molecules, following EPR measurements were performed as follows.

Shown in Figures 2a and 3a are EPR spectra obtained from oligonucleotides labeled with 4-amino-TEMPO-^{15}N-d_{16} (S1) and 4-amino-TEMPO-^{14}N-d_{16} (S2), respectively. Compared to the spectra obtained from the spin-labels (4-amino-TEMPO-^{15}N-d_{16} and 4-amino-TEMPO-^{14}N-d_{16}) (Figures 1c and b), all the EPR lines from S1 and S2 were broadened by the incorporation of the labels into oligonucleotides, respectively. This may come from the steric hindrance of the main chain preventing free rotation of the labels. Nevertheless, when each of S1 and S2 was mixed with their complementary oligonucleotide (C1), further broadening arose, as represented in Figures 2b and 3b, respectively, indicating the hybrid formation between S1 (or S2) and C1. These results imply that hybrid formation between the spin-labeled oligonucleotides and their complementary targets can be detected simply by monitoring the change in the EPR line shape.

The minimum quantity detectable by the spin-labeled probes was also roughly estimated by assuming that a probe makes 1:1 hybrid with the target and the quantity is equivalent to the minimum EPR-observable amount of the probe. The minimum EPR-observable quantities were as follows: 50 pmol for the probe bound with 4-amino-TEMPO-^{14}N-d_{16} and 40 pmol for that with 4-amino-TEMPO-^{15}N-d_{16} as well as 160 pmol for that with 4-amino-TEMPO. These values suggest the minimum quantities of the targets detectable by the present method. Several times enhancement of the detection limit was obtained.

Another improvement of the method was studied here by introducing the detection of two different base sequences in the same target. The EPR lines of S1 did

TABLE 1
Base sequences and abbreviations for spin-labeled oligonucleotides

Abbreviation	Base sequence	Spin-label
S1	5′-dG*GGAATTCGT	4-amino-TEMPO-^{15}N-d_{16}
S2	5′-dG*GGAATTCGT	4-amino-TEMPO-^{14}N-d_{16}
S3	5′-dA*ATCATGGTC	4-amino-TEMPO-^{14}N-d_{16}
C1	5′dACGAATTCCC	
C2	5′-dTGATTACGAATTCCCGGGGS	
C3	5′-dGACCATGATTACGAATTCCC	

*Site to which spin-label is incorporated.

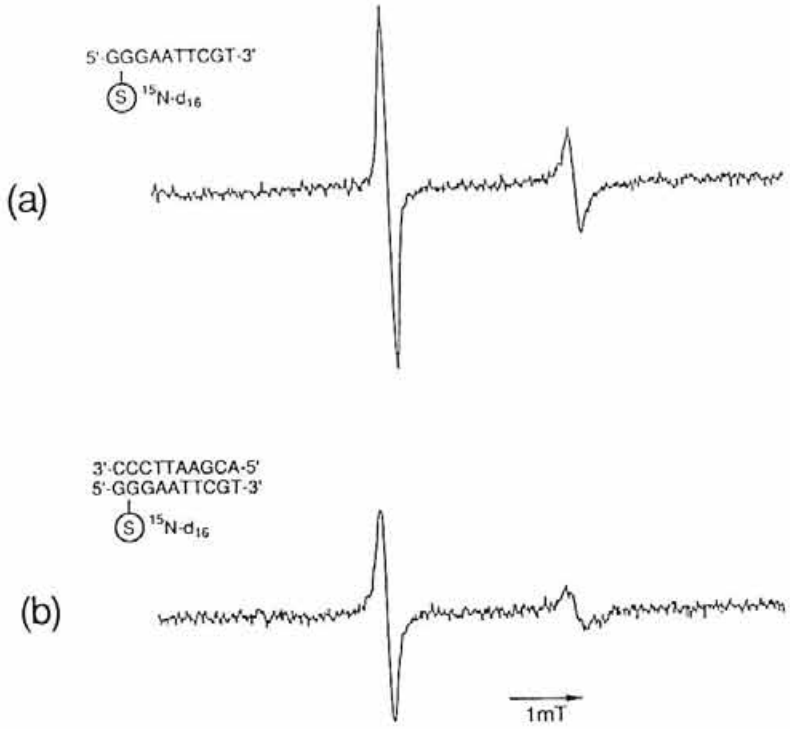

FIGURE 2 EPR spectra obtained from an oligonucleotide labeled with 4-amino-TEMPO-^{15}N-d$_{16}$ (S1) (a) in the absence and (b) in the presence of its complementary target (C1). Conditions and EPR settings same as in Figure 1 except: concentration of the oligonucleotide, 5 μM; gain, 1.8 × 1000: response time, 1.0 min; sweep time, 36 min. The base sequences appear in the figure and Table 1.

not overlap those of S2 whose base sequence is same as that of S1. For simulaneous detection with S1, an oligonucleotide with a base sequence different from that of S2 was also prepared using 4-amino-TEMPO-^{14}N-d$_{16}$ (S3 in Table 1). As shown in Figure 4a, an EPR spectrum (triplet) obtained from this probe was distinguishable from that from S1 (doublet). When an oligonucleotide (C2) a part of which is only partially complementary to S1 was added to this solution, only the doublet due to S1 was broadened while that of S3 (triplet) remained unchanged (Figure 4b). This result shows that only S1 formed hybrid with C2 in the solution: thus in the presence of two different oligonucleotides labeled with 4-amino-TEMPO-^{14}N-d$_{16}$ and 4-amino-TEMPO-^{15}N-d$_{16}$, selective detection was achieved. Furthermore, when an oligonucleotide (C3) which has a complementary base sequence to both S1 and S3 was added to the same system, both the EPR lines arising from S1 and S3 were broadened (Figure 4c). These results clearly indicate that the two different sites on the target DNA could be detected simultaneously using oligonucleotides labeled with deuterated 4-amino-TEMPO-^{14}N and -^{15}N. This double detection of the two different site on the target DNA enhances the precision of DNA probe method.

Conclusively, to achieve the DNA probe method which does not require B/F

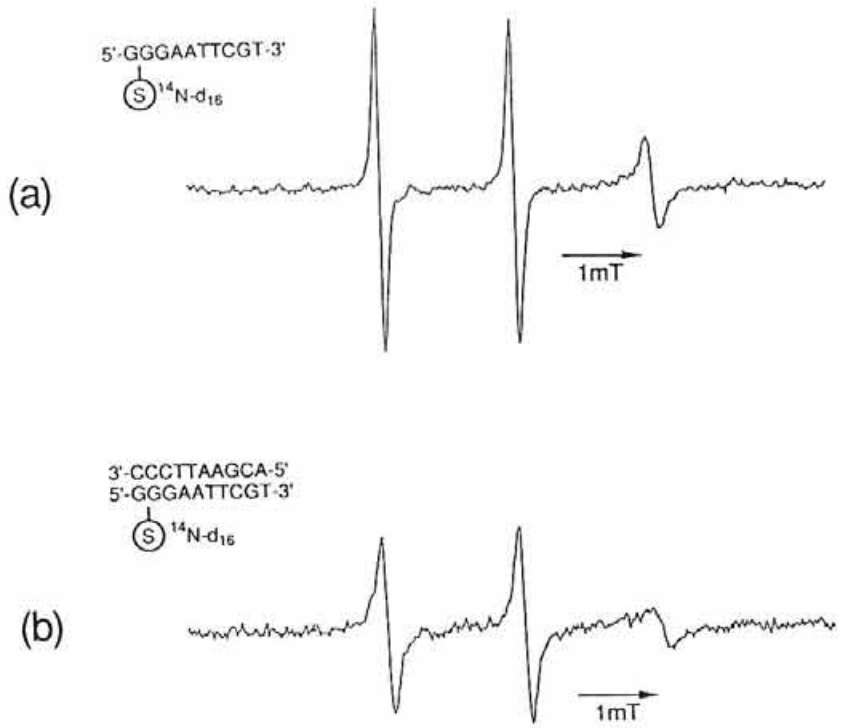

FIGURE 3 EPR spectra obtained from an oligonucleotide labeled with 4-amino-TEMPO-^{14}N-d$_{16}$ (S2) (a) in the absence and (b) in the presence of its complementary target (C1). Conditions and EPR settings same as in Figure 2. The base sequences appear in the figure and Table 1.

separation and can be used in solution, it is preferred to use oligonucleotide labeled with deuterated spin labels to enhance the detection sensitivity, and to utilize a combination of ^{14}N- and ^{15}N-aminoxyl spin-labels to provide the precision of the detection.

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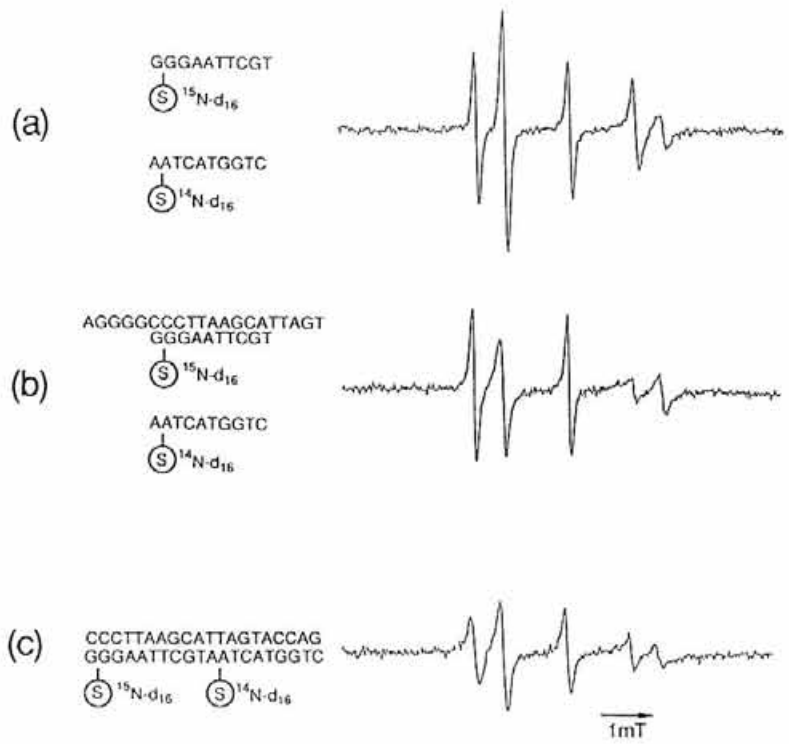

FIGURE 4 EPR spectra obtained from a mixture of oligonucleotides labeled with 4-amino-TEMPO-^{15}N-d_{16} (S1) and 4-amino-TEMPO-^{14}N-d_{16} (S3) (a) in the absence of the target, and in the presence of (b) a complementary target (C1) only for S1 and (c) C3 both for S1 and S3. Conditions and EPR settings same as in Figure 2.

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