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Diastereomer characterizations of nitroxide-labeled nucleic acids

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Abstract

Site-directed spin labeling (SDSL) obtains structural and dynamic information of a macromolecule using a site-specifically attached stable nitroxide radical. SDSL studies of arbitrary DNA and RNA sequences can be achieved using an efficient phosphorothioate labeling scheme, where a nitroxide is attached to a phosphorothioate substituted at a target site during chemical synthesis. The chemically introduced phosphorothioate contains two diastereomers (Rp and Sp), and nitroxides attached to each diastereomer may experience different local environments. Here, we report work on using anion-exchange HPLC to separate and characterize diastereomers in three DNA oligonucleotides and one RNA oligonucleotide. In all oligonucleotides studied, the Rp diastereomer was found to elute earlier than the Sp in the unlabeled oligonucleotides, while a reversal in the elution order (Sp earlier than Rp) was observed for nitroxide-labeled oligonucleotides. The results enable a one-step purification procedure for preparing diastereomerically pure nitroxide-labeled oligonucleotides. This expands the scope of nucleic acids SDSL.

Keywords

Site-directed spin labeling; DNA; RNA; Phosphorothioate; Diastereomer

In the method of site-directed spin labeling (SDSL), a nitroxide (spin label) is attached at a specific site of a target biomolecule, and electron paramagnetic resonance (EPR) spectroscopy is utilized to obtain information at the labeling site [1–3]. SDSL is capable of studying high-molecular weight systems in solution, and provides unique structural and dynamic information about DNA and RNA at the level of individual nucleotides [2,3]. Specifically, in nucleic acid studies, SDSL has been used to measure long-range distances [4–10], to probe site-specific dynamic features [11–15], and to monitor conformational changes [16–18].

Recently, a phosphorothioate labeling scheme has been reported for SDSL studies of DNA and RNA (Fig. 1A) [7,8,19,20]. In this scheme, a phosphorothioate is introduced at the desired labeling site during chemical synthesis of the oligonucleotide. This is followed by a reaction of the phosphorothioate-containing oligonucleotide to a thiol-reactive nitroxide derivative, producing a spin label that is linked via a thioester bond to the phosphorous of the oligonucleotide. Because the phosphorothioate substitution is a simple and efficient modification, the phosphorothioate labeling scheme is an efficient means for introducing a nitroxide to a target nucleotide within arbitrary DNA or RNA sequences. Nitroxides labeled using this method have been utilized to measure nanometer distances [7,8], to probe molecular interactions [19], and to reveal site-specific structural and dynamic features at the level of individual nucleotides (AP and PZQ, manuscript in preparation).

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In the phosphorothioate modification, one of the non-bridging oxygen atoms of the phosphodiester is replaced with sulfur. This creates a chiral center at the phosphorous atom and produces two chemical configurations known as the Rp and Sp diastereomers (Fig. 1B). With the labeling procedure used [20], the nitroxide reacts equally to the two diastereomers that are created in a ~50/50 ratio during the chemical synthesis. It is possible to conduct SDSL studies without separating the phosphorothioate diastereomers. For example, nitroxide attached to mixed diastereomers have been used to measure nanometer distances in DNA and RNA [7,8,20], and computational approaches have been developed to address the diastereomer issue [7,8,20,21]. On the other hand, because a nitroxide attached at the Rp diastereomer (Rp-nitroxide) may experience a different local environment compared to that of the Sp-nitroxide, SDSL measurements on diastereomerically pure samples may offer additional information. For example, molecular dynamic simulations predicted a Rp-specific DNA–nitroxide interaction at one site of a DNA duplex [21], and this interaction has been confirmed experimentally (AP and PZQ, manuscript in preparation).

Previously, phosphorothioate diastereomers in the context of oligonucleotides were separated using reverse phase HPLC, and their configurations were determined using nuclease digestions [22–24]. We recently reported that phosphorothioate diastereomers in nitroxide-labeled oligonucleotides can be separated with anion-exchange HPLC using a DNAPac column [20]. However, the exact diastereomer configurations of the nitroxide-labeled oligonucleotides have not been determined. Although the phosphorothioate configuration remains the same before and after labeling (Fig. 1B and C), a covalently bound nitroxide neutralizes a negative charge and changes the polarity at the labeling site. Therefore, one cannot assume that the phosphorothioate diastereomers elute in the same order between the labeled and unlabeled oligonucleotides.

Here, we report results on diastereomer separation and characterization in nitroxide labeling studies of three DNA oligonucleotides and one RNA oligonucleotide using the DNAPac column. We demonstrated separations of diastereomers in both the phosphorothioate-containing oligonucleotides and the nitroxide-labeled oligonucleotides. Furthermore, in all oligonucleotides studied, the Rp diastereomer was found to elute earlier than the Sp in the unlabeled oligonucleotides, while a reversal in the elution order (Sp earlier than Rp) was observed for nitroxide-labeled oligonucleotides. These studies enable the use of diastereomerically pure nitroxide-labeled oligonucleotide for probing structure and dynamic features in nucleic acids.

Materials and methods

Sample preparations

Table 1 lists the name and sequence of phosphorothioate-containing oligonucleotides used in this study. All oligonucleotides were chemically synthesized using the phosphoramidite chemistry by Integrated DNA Technologies, Inc. (Coralville, IA). The oligonucleotides were deprotected by the vendor, and used directly without purification.

Nitroxides were attached to oligonucleotides that contain a phosphorothioate modification following procedures reported previously [7,8,20]. The labeled nitroxide was designated as R5 (Fig. 1).

Anion-exchange HPLC

Anion-exchange HPLC was carried out on an ÄKTA basic system (GE Healthcare, Inc.) using a DNAPac PA-100 column (4 × 250 mm, Dionex Inc., Sunnyvale, CA). The low salt buffer

(buffer A) is 25 mM Tris-HCl (pH 6.8). The high salt buffer (buffer B) is 25 mM Tris-HCl (pH 6.8) and 400 mM NaCl.

Characterizations of diastereomer configurations using enzymatic digestion

The configurations of phosphorothioate diastereomers in unlabeled oligonucleotides were determined following a prior reported procedure [22,25,26]. Individual anion-exchange HPLC fractions of oligonucleotides were directly subjected to nuclease digestion without desalting. A typical P1 nuclease reaction mixture contained 750 μ l of nucleic acids samples and 7.5 μ l P1 nuclease (10 U/ μ l, United States Biological, Swampscott, MA). A typical snake venom phosphodiesterase I (SVP) reaction contained 750 μ l of nucleic acid samples, 75 μ l 10 $\times$ buffer (0.5 M Tris-HCl, pH 7.5 and 0.15 M MgCl₂), 0.3 μ l of 0.3 M ZnCl₂, and 7.0 μ l SVP (10 U/ μ l, United States Biological, Swampscott, MA). All digestions were carried out at 37 °C with an overnight incubation. To convert the digested mononucleotides to mononucleosides, 1.0 μ l of calf intestinal alkaline phosphatase (CIAP, 10 U/ μ l) and 90 μ l of the corresponding 10 $\times$ buffer (0.5 M Tris-HCl, pH 7.5, 0.01 M MgCl₂, and 1 mM ZnCl₂) were added to each digestion mixture, and incubation was continued at 37 °C for 2 h.

After nuclease and CIAP treatments, the mixture was analyzed using reverse phase HPLC. Reverse phase HPLC was carried out on a C18 column (Ultrasphere ODS, 4.6 $\times$ 250 mm, Beckman Coulter, Inc., Fullerton, CA), using 0.1 M triethylammonium acetate and 5% acetonitrile as buffer A, and 50% acetonitrile as buffer B. The elution gradient used was 100% buffer B in 20 min. with a flow rate of 1 ml/min.

To determine the elution profile of mononucleosides, the corresponding deoxyribonucleotide triphosphate was treated with CIAP, and then analyzed using reverse phase HPLC.

Results and discussion

Separation and identification of phosphorothioate diastereomers in an unlabeled DNA

Although reverse phase HPLC was the method of choice in previous work on separating phosphorothioate diastereomers [22–24], we decided to explore anion-exchange HPLC methods, which are efficient in purifying nitroxide-labeled oligonucleotides [7,8,19,20]. It has been shown that diastereomer separations can be achieved with anion-exchange HPLC using the DNAPac column [20] (D. Herschlag, personal communications), which is capable of discriminating samples based on both charge and polarity. An example of this is shown using the CS7 DNA, which contains one phosphorothioate modification at the center of the oligonucleotide (see Table 1 for name and sequence). When the CS7 DNA was analyzed using the DNAPac column (see Materials and methods), the major populations eluted as two separate peaks (Fig. 2A). Each peak corresponds to ~50% of the DNA, and represents one of the phosphorothioate diastereomers.

To determine the diastereomer configuration of each of the peaks from anion-exchange HPLC, enzymatic digestions using nuclease P1 (P1) and snake venom phosphodiesterase I (SVP) were conducted (see Materials and methods). P1 hydrolyzes the phosphodiester bonds in DNA and RNA, but cleaves only the Sp phosphorothioate diastereomer and leaves the Rp diastereomer intact [26]. On the other hand, SVP hydrolyzes the phosphodiester bonds, and cleaves only the Rp phosphorothioate diastereomer and leaves the Sp diastereomer intact [25,27].

The reverse phase HPLC trace of SVP digestion of CS7 fraction 1 (the early eluting fraction from the DNAPac column) showed four peaks (Fig. 2B). Based on comparisons with mononucleoside standards (data not shown), these four peaks were identified as dC, dG, dT, and dA, respectively (Fig. 2B). The identities and elution order of the mononucleosides are consistent with previous reports [28]. SVP digestions of fraction 2, however, gave five peaks

(Fig. 2B). The first four peaks were mononucleosides, and the fifth peak was assigned as the phosphorothioate-containing dinucleotide (GpsC). For the dinucleotide fragment to survive SVP digestion, it must contain a Sp phosphorothioate. This identified fraction 2 from the anion-exchange HPLC as the Sp diastereomer. Consequently, fraction 1 was assigned as the Rp diastereomer. This is consistent with the absence of a dinucleotide fragment in the SVP digestion of fraction 1 (Fig. 2B).

P1 digestions were carried out to confirm the assignment of Rp diastereomer to fraction 1 and Sp diastereomer to fraction 2 (Fig. 2C). P1 cleaves the Rp diastereomers, which is opposite to that of SVP. A reversal in the digestion patterns is expected, and was indeed observed (Fig. 2C). P1 digestion of CS7 fraction 1 (Rp diastereomer) gave five peaks (Fig. 2C). The fifth peak corresponded to the GpsC dinucleotide, and its identity was further confirmed by mass spectrometry measurements (Fig. 2C, inset). In addition, the dinucleotide was absent in P1 digestions of fraction 2 (Sp diastereomer) (Fig. 2C).

The data above unambiguously show that without a nitroxide attached, the Rp diastereomer (fraction 1) elutes earlier than the Sp diastereomer (fraction 2) on the DNAPac column.

Reversal of elution order on the DNAPac column observed for nitroxide-labeled CS7 DNAs

We have reported a one-step purification procedure in the phosphorothioate labeling scheme [7,8,20]. In this procedure, a crude phosphorothioate-containing oligonucleotide is reacted with the nitroxide. After nitroxide coupling, the DNAPac column is used to achieve, in one step, separation of failure sequences from synthesis, removal of excess nitroxide precursors, and, in some cases, separation of phosphorothioate diastereomers [19,20] (see below). It will greatly simplify the preparation of diastereomerically pure nitroxide-labeled sample if one can assign diastereomer configurations directly after DNAPac column purification. However, nuclease digestions of nitroxide-labeled DNA showed a complex HPLC pattern (data not shown), preventing assignment of diastereomer configurations.

To examine the behavior of nitroxide-labeled DNA on the DNA-pac column, the CS7 DNA was first eluted on the DNAPac column to separate the Rp diastereomer (RpCS7, the early eluting fraction) from the Sp diastereomer (SpCS7, the late eluting fraction) (Fig. 2A). Each DNA fraction was separately labeled with the R5 nitroxide, and the products were analyzed again using the DNAPac column. As shown in Fig. 3, the R5-labeled DNAs eluted earlier than the unlabeled DNAs in either RpCS7 or SpCS7, consistent with a reduction of the negative charge upon nitroxide labeling (Fig. 1A) [20]. Surprisingly, the nitroxide-labeled Sp diastereomer (R5-SpCS7) clearly eluted earlier than that of R5-RpCS7 (Fig. 3). This indicates that attachment of R5 nitroxide reverses the elution order of phosphorothioate diastereomers on the DNAPac column.

In both traces in Fig. 3, one can observe a population of unlabeled DNA, which is the result of incomplete nitroxide labeling. The unlabeled fragment serves as an internal control to confirm the DNA diastereomer identity in each trace. As expected, in the R5-RpCS7 trace, the unlabeled DNA eluted earlier than that in the R5-SpCS7 trace (Fig. 3). This excludes any possible mis-assignment of diastereomer configuration in the nitroxide-labeled DNA.

With the finding that R5-SpCS7 elutes earlier than R5-RpCS7 on the DNAPac column, one can now directly assign the diastereomer configuration without going through multiple HPLC runs. Fig. 4A shows the result of nitroxide labeling carried out directly on a crude CS7 DNA that has not been subjected to any prior purification. In the HPLC trace, it is clear that the nitroxide-labeled DNA separated into two peaks. Based on the elution order discussed above, the early eluting peak was assigned as the Sp diastereomer (R5-SpCS7), while the late eluting peak was assigned as the Rp diastereomer (R5-RpCS7) (Fig. 4A).

Fig. 4B shows the EPR spectra of DNA duplexes that contain either the R5-RpCS7 or the R5-SpCS7 oligonucleotide. The spectra show reproducible small differences, which report the different local environment experienced by the nitroxide attached to the different diastereomer [21]. The spectrum of R5-RpCS7 (Fig. 4B, red trace) is broader than that of the R5-SpCS7 (Fig. 4B, black trace), indicating a slight reduction in the mobility of the nitroxide attached to the Rp diastereomer. This is consistent with modeling studies showing that in the Rp configuration the nitroxide is positioned towards the DNA major groove, while in the Sp configuration the nitroxide is directed towards the solvent [21].

The generality of the nitroxide-related elution order reversal

Following procedures described above, diastereomers were separated and characterized in two additional DNA oligonucleotides (CS4 and CS21) and in an RNA oligonucleotide (GpI) (Table 1). In all cases, the two diastereomers were nicely separated on the DNA-pac column, and the Rp diastereomer eluted earlier than the Sp diastereomer (Table 2).

Furthermore, R5 was attached to diastereomerically pure oligonucleotides, and the products were analyzed using the DNAPac column. In all cases of nitroxide-labeled oligonucleotides, the Sp diastereomer eluted earlier than the corresponding Rp diastereomer (Table 2). Therefore, the reversal of elution order was observed for all DNA and RNA oligonucleotides analyzed, and seems to be a general feature in nitroxide-labeled oligonucleotides.

Summary

Data reported here support two conclusions. First, phosphorothioate diastereomers in nitroxide-labeled short oligonucleotides (11–12 nts) can be separated using anion-exchange HPLC with the DNAPac column, and the procedure was effective for phosphorothioates inserted at variable positions within DNA and RNA. Second, in all nitroxide-labeled oligonucleotides studied, the Sp diastereomer elutes earlier than the Rp on the DNAPac column. This allows direct diastereomer configuration assignments, and enables a one-step purification procedure for preparing diastereomerically pure nitroxide-labeled oligonucleotides. The results have a direct impact in the development and application of nucleic acid SDSL, and are being used in the investigation of diastereomer-specific DNA/nitroxide interaction and in the analysis of RNA dynamics in large ribozymes.

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Fig. 1. Schematics of the phosphorothioate labeling method. (A) A schematic showing the attachment of the R5 nitroxide. The stars indicate the phosphorous chiral centers. (B) Chemical configurations of phosphorothioate-substituted oligonucleotides. (C) Chemical configurations of nitroxide-labeled oligonucleotides.

Fig. 2. CS7 DNA diastereomer separations. (A) Diastereomer separation on the D-NApac column. The elution gradient was 0–61% of buffer B in 2 min., followed by 61–75% of buffer B in 47 min. The flow rate was 2 ml/min. Based on enzymatic digestion patterns (see (B) and (C)), the early eluting peak (fraction 1) was identified as the Rp diastereomer, and the late eluting peak (fraction 2) as the Sp diastereomer. (B) RP-HPLC analyses of SVP digestions. Fractions 1 and 2 corresponded to the two fractions shown in (A). RP-HPLC was carried out on a C18 column as described in Materials and methods. It should be noted that the ratio among the four mononucleoside peaks deviated from the expected values calculated based on the sequence. This is likely due to incomplete digestion. (C) RP-HPLC analyses of P1 digestions. The SVP

and P1 digestions were carried out on different dates, accounting for the slight offset in the mononucleoside elution positions. The inset shows a MALDI-TOF mass spectrum of the peak identified as the GpsC dinucleotide. The measured mass was 571.6 Da, which matches the expected mass of GpsC (571.5-Da). Spectra were acquired on a Voyager-DE STR system (Applied Biosystems, Foster City, CA) in the range of 500–1200 Da following procedures provided by the vendor. To prepare samples for mass spectrometry, reverse phase HPLC fractions eluted from 8.9 to 9.5 ml were combined and lyophilized. The resulting powder was dissolved in 5 mM sodium phosphate (pH 7), and the sample concentration was adjusted to approximately 2–3 μ M. The sample was then mixed with a matrix solution (8:1 ratio of 3-hydroxypicolinic acid/diammonium citrate) in a 1:2 ratio. Droplets of the mixture (1.50 μ l) were spotted onto the sample plate and air-dried.

Fig. 3. Characterizations of nitroxide-labeled CS7 DNAs that contain pure Rp or Sp phosphorothioate diastereomers. Elution patterns were obtained on a DNAPac column. The flow rate was 2.0 ml/min, and the gradient was 0–55% buffer B in 2 min, followed by 55–67% buffer B in 40 min. The bottom trace shows the Sp diastereomer, and the top trace shows the Rp diastereomer.

Fig. 4.

Nitroxide labeling and characterization of crude CS7 DNA. (A) Anion-exchange HPLC elution profile. Approximately 7 nonomoles of CS7 crude DNA was labeled with R5, then loaded on the DNAPac column (4 mm × 250 mm). Elution was carried out using as buffer A: 25 mM Tris-HCl (pH 6.8) and as buffer B: 500 mM NaCl and 25 mM Tris-HCl (pH 6.8). The gradient was 0–70% buffer B in 47 min. The flow rate was 2 ml/min. (B) EPR spectra of DNA duplexes containing R5-CS7. Shown are X-band continuous-wave spectra obtained with R5-SpCS7 (black) and R5-RpCS7 (red). Their differences are shown as the blue dotted line. Spectra were acquired on a Bruker EMX Spectrometer at 9.34 GHz, with the sample (10–15 μ l) placed in a glass capillary (1.0 mm ID × 1.2 mm OD, Vitrocom, Inc. Mountain Lakes, NJ). The incident microwave power was 2 mW and the 100 kHz field modulation amplitude was 1 G. Temperature was kept at 25 °C. All spectra were base-line corrected and normalized to the same number of spins. To prepare the EPR samples, 1 nono-mole purified R5-labeled DNA strand was combined with a 10% molar excess of unlabeled complementary strand in 10 μ l of buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5). After overnight incubation at room

temperature, excess un-annealed single-stranded DNA and detached nitroxides were removed by filtration using an Ultrafree centrifugal filter (Millipore, Inc.). The solution of the DNA duplex was then adjusted so that the final EPR sample contained 100 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 34% (w/w) sucrose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1

Oligonucleotides used in this study

Name	Sequence ^a
CS4 ^b	5'-dCdTdA*dCdTdGdCdTdTdTdAdG
CS7 ^b	5'-dCdTdAdCdTdG*dCdTdTdTdAdG
CS21 ^b	5'-dCdTdAdAdAdGdCdA*dGdTdAdG
GpI ^c	5'-rCrCrCmUrCrUrAdA*rArCrC ^d

^a Deoxyribonucleotides are marked by the letter “d”, and ribonucleotides are marked by the letter “r”. The position of the phosphorothioate is indicated by the “*.”.

^b Each of these oligonucleotides corresponds to one of the two complementary strands in a DNA dodecameric duplex [29]. They are named according to the PDB ID of the DNA dodecamer, which is 1CS2.

^c An RNA oligonucleotide corresponding to the substrate of the group I intron Tetrahymena ribozyme.

^d A deoxyribonucleotide is inserted 5' to the phosphorothioate in order to prevent strand scission upon nitroxide labeling [20]. The sequence also includes a 2'-O-methyl uridine (mU).

Table 2

Observed diastereomer elution order of phosphorothioate-substituted and nitroxide-labeled oligonucleotides on the DNAPac column

		Rp	Sp
Unlabeled	CS7	1st	2nd
	CS4	1st	2nd
	CS21	1st	2nd
	GpI	1st	2nd
Nitroxide-labeled	R5-CS7 ^a	2nd	1st
	R5-CS4 ^a	2nd	1st
	R5-CS21 ^a	2nd	1st
	R5a-GpI ^b	2nd	1st

^aR5 is 3-methylene-1-oxyl-2,2,5,5,-tetramethylpyrroline, and its chemical structure is shown in Fig. 1.

^bR5a = 3-methylene-4-bromo-1-oxyl-2,2,5,5,-tetramethylpyrroline. The procedure of R5a labeling is similar to that of R5, and the details of this procedure are reported elsewhere (AP and PZQ, manuscript in preparation).