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# Site directed nitroxide spin labeling of oligonucleotides for NMR and EPR studies

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ABSTRACT

Nitroxide labels are useful probes of biomolecule structure that can be detected by NMR and EPR spectroscopy. Although many methods exist for labeling oligonucleotides with nitroxides, most require reagents that are expensive or laborious to prepare. A simpler approach is described herein using commercially available phosphorothioate oligonucleotides and 2-(3-iodoacetamidomethyl)-PROXYL. We describe semi-optimized conditions for labeling DNA and RNA oligonucleotides and methodology for purifying and identifying these reagents by MALDI-MS. The nitroxide label showed some propensity to hydrolyze at high temperature and over prolonged periods at room temperature. Nitroxide-labeled DNA oligonucleotides gave characteristic EPR spectra and caused the disappearance of bound protein signals in $^{15}$N HSQC spectra consistent with the paramagnetic relaxation enhancement (PRE) effect.



## 1. Introduction

Site directed spin labeling (SDSL) is a useful tool for studying biomolecules by NMR and EPR spectroscopy.[1–4] In NMR spectroscopy, the dipolar interaction between unpaired electrons, normally nitroxide radicals or paramagnetic metal ions, and nearby nuclei gives rise to the paramagnetic relaxation enhancement (PRE) effect[5] resulting in increased nuclear relaxation rates. The PRE effect has a long range (>25 Å), a strong distance-dependence, and can be quantified making it well suited to providing additional accurate distance restraints for the structure determination of protein–-protein and protein–oligonucleotide complexes.

We are interested in using the PRE effect to study the interaction of proteins with oligonucleotides by NMR spectroscopy. The introduction of a paramagnetic probe to an oligonucleotide results in the distance-dependent broadening of protein resonances in the NMR spectra of a bound protein. Signals far from the probe are unaffected while signals close to the probe appear weaker or disappear completely.[6] The paramagnetic probe can then be replaced with a diamagnetic version by destroying the nitroxide radical (e.g., by reduction) or replacing a paramagnetic metal ion with a diamagnetic metal ion. Under these diamagnetic (but otherwise identical) conditions no line broadening is observed and the extent of line broadening under paramagnetic conditions and, hence, the distance of individual protein resonances from the label can be determined.

Multiple methods exist for SDSL of oligonucleotides with nitroxide or metal chelating moieties, although there are fewer reports for oligonucleotide labeling compared to protein labeling. The most well characterized approach involves incorporating a thymidine base modified with an EDTA metal-chelating moiety[7–10] during oligonucleotide synthesis. After synthesis, paramagnetic manganese(II) or diamagnetic calcium(II) is added to the purified oligonucleotides. Other labeling strategies involve incorporating modified nucleotides such as iodouridine[11] and thiouridine[12] during oligonucleotide synthesis and post-synthetic attachment of a nitroxide label using Sonogashira coupling,[13] thioalkylation, or azide–alkyne click chemistry.[14] All of these strategies utilize expensive, unnatural DNA bases and nitroxide reagents that must be prepared via multi-step syntheses.

A simpler strategy has been developed using oligonucleotides bearing the phosphorothioate modification. These oligonucleotides are commercially available, the phosphorothioate can be positioned anywhere within the sequence and is highly reactive toward electrophilic reagents such as 3-(iodomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (iodomethyl-PROXYL). This approach has been used extensively by a few laboratories to study the dynamics of DNA and RNA and their interaction with proteins.[15,16] However, the required iodomethyl-PROXYL must be synthesized in two steps from

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a carboxy-PROXYL and this might impede its use by structural biology laboratories that lack chemistry capabilities. An alternative strategy involves alkylation of thiouridine containing DNA with the commercially available 2-(3-iodoacetamidomethyl)-PROXYL (IAM-PROXYL).[17] We have combined these strategies to create a method that requires only commercially available phosphorothioate oligonucleotides and nitroxide reagents. We report our simplified methodology and findings with the aim of encouraging and enabling other laboratories to use SDSL for NMR and EPR studies involving oligonucleotides.

## 2. Results and discussion

We focused on two phosphorothioate-modified single-stranded DNA oligonucleotides G*GTTTTTT (**1**) and G*TGAACTTTCGGT (**2**) (*indicates position of the phosphorothioate). Our progress in reacting **1** or **2** with IAM-PROXYL was initially hampered by an inability to detect either of these oligonucleotides or their reaction products by MALDI-MS. Trace amounts of alkali metal ions can bind strongly to the oligonucleotide phosphate backbone to give adducts that ionize poorly.[18] We found pre-treating samples with ammonium-loaded cation-exchange resin[10] and loading these samples directly onto 2-hydroxypicolinic acid (HPA) matrix gave significantly improved signals in MALDI-MS (Fig. 1). We note that in our hands this procedure only worked for single-stranded DNA (ssDNA) oligonucleotides; annealed double stranded DNA (dsDNA) oligonucleotides gave no signal even after cation-exchange resin pre-treatment.

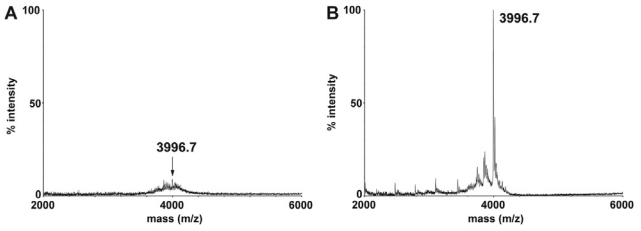


**Fig. 1.** The effect of $NH_4^+$-loaded cation-exchange resin on the quality of MALDI spectra of ssDNA oligonucleotides. (A) Compound **2** without pre-treatment. (B) Compound **2** with pre-treatment.

A variety of reaction conditions have been reported for labeling phosphorothioate oligonucleotides. We sought to develop a set of standard reaction conditions that could be applied to any oligonucleotide, and at the very least would provide a convenient starting point for further optimization. In developing the reaction conditions we did not attempt to separate the phosphorothioate diastereomers even though this is reported to be possible.[19] Phosphorothioate alkylation conditions developed by McLaughlin[20] used phosphate buffer at pH 6 in 30% dimethylformamide (DMF) and at 50 °C. Given our initial difficulties with MALDI signal suppression from alkali metal adducts, we opted for an organic buffer system, triethylammonium acetate (TEAA) at pH 6.5. Alkylation of **1** and **2** was attempted with 10 equiv of IAM-PROXYL in 50 mM TEAA/DMF (2:1) at 50 °C. The progress of the reaction was followed directly by HPLC and MALDI-MS as shown in Fig. 2. The appearance of a large +198 amu peak in the MALDI spectrum indicated successful alkylation of the phosphorothioate. HPLC analysis of the reaction revealed that the starting material was completely consumed after 1 h. For **1**, later eluting products were observed that corresponded to multi PROXYL-alkylated **1**, however, a longer reaction time reduced the amount of these side products. This result suggests that phosphorothioate alkylation is irreversible, whereas alkylation at other sites within the DNA (e.g., guanine N7) is reversible.[21]

Next, we undertook screening of the reaction conditions including temperature (room temperature, 50, 75 °C), equivalents of IAM-PROXYL (5, 10, 30 equiv), concentration of DMF (0%, 33% 66%, 100%), organic solvent (THF, acetone, ethanol, DMSO, acetonitrile), pH (5, 6.5, 8), and concentration (3, 0.3, and 0.03 mM). In general, the reaction proceeded best at room temperature or 50 °C. Reaction at 75 °C gave rapid alkylation but also led to the hydrolysis of the labeled product back to an unmodified oligonucleotide that lacked sulfur. 5-10 equivalents of IAM-PROXYL gave the best results; a higher number of equivalents resulted in significant over-alkylation. In terms of solvent, DMF gave the best results. Other organic solvents could be used for up to 8 h but gave variable results after this time. The reaction performed best in 33% DMF in 50 mM TEAA. Reaction proceeded with both no DMF and 66% DMF, but gave the hydrolysis product after longer reaction times. The reaction was incomplete in 100% DMF after 24 h. The reaction

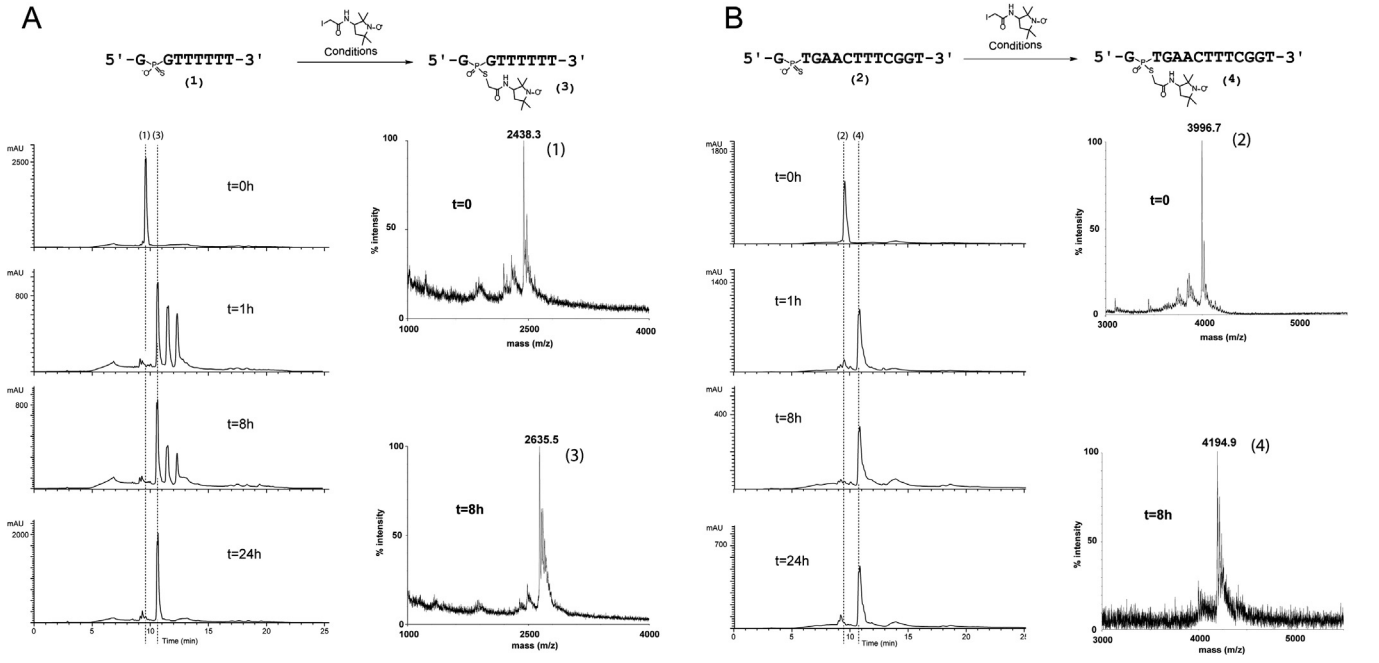


**Fig. 2.** Monitoring the reaction of oligonucleotides **1** (A) and **2** (B) with IAM-PROXYL by rpHPLC and MALDI-MS.

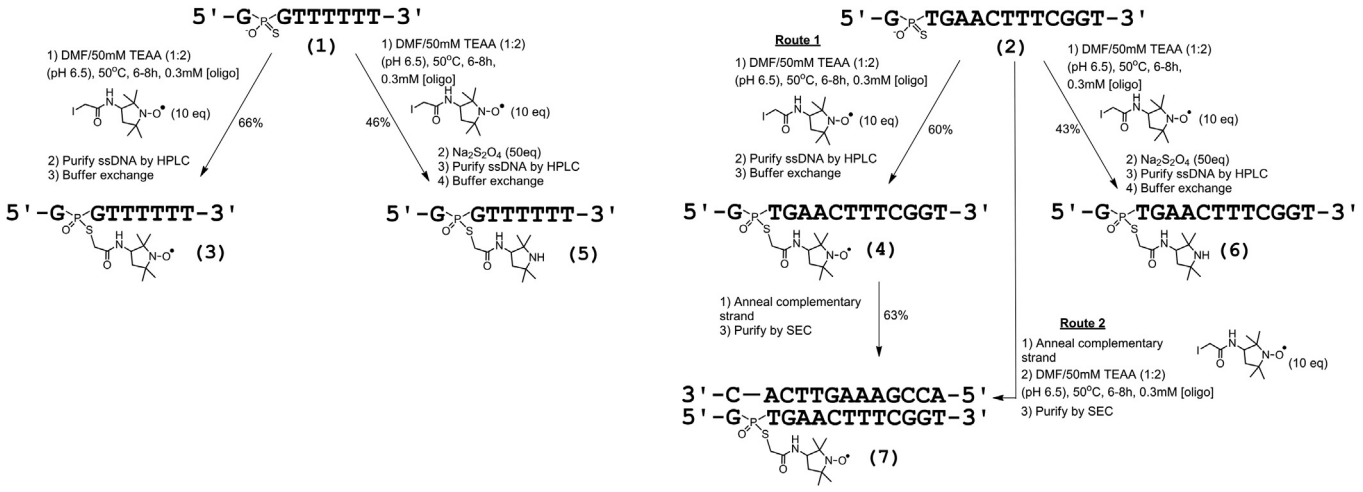


**Fig. 3.** Reaction sequences used to prepare PROXYL- and reduced PROXYL-labeled single and double stranded oligonucleotides.

proceeded well over the pH range 5–8. The optimal oligonucleotide concentration was 0.3 mM with oligonucleotide-dependent hydrolysis occurring at both higher (3 mM) and lower (0.03 mM) concentrations. Based on these data, we recommend a starting point for alkylation reactions that uses the following semi-optimized conditions: 50 °C for 1–24 h, 10 equiv of IAM-PROXYL, TEAA pH 6.5/DMF (2:1), an oligonucleotide concentration of 0.3 mM and monitoring the progress of the reaction by rpHPLC and MALDI-MS. The temperature, length of reaction, and number of equivalents of IAM-PROXYL are variables that can be tuned for specific oligonucleotides.

Larger scale (300 nmol) labeling reactions were next conducted on both **1** and **2** using the semi-optimized conditions to give 197 nmol of **3** (66% yield) and 180 nmol of **4** (60% yield) after HPLC purification. As a diamagnetic control, an oligonucleotide sample was also prepared that had been labeled but where the nitroxide radical had been reduced. This was achieved by conducting the alkylation reaction and then immediately reducing the samples using excess sodium dithionite for 10 min at 50 °C to give 137 nmol of **5** (46% yield) and 128 nmol of **6** (43% yield) after HPLC purification (Fig. 3). A loss of 15 mass units was observed by MALDI-MS for each of these reduced samples consistent with the reduction of the nitroxide to the secondary amine.

During our reaction screening we had observed hydrolysis of the PROXYL label at higher temperatures. We therefore next wanted to confirm that **4** could be successfully annealed with its complementary strand to make double stranded oligonucleotides under standard conditions. Aliquots of purified **4** were subjected to different temperatures and analyzed by HPLC. Fig. 4 shows that the PROXYL label is gradually lost with prolonged heating at 95 °C. However, there was minimal hydrolysis with 1 min of heat

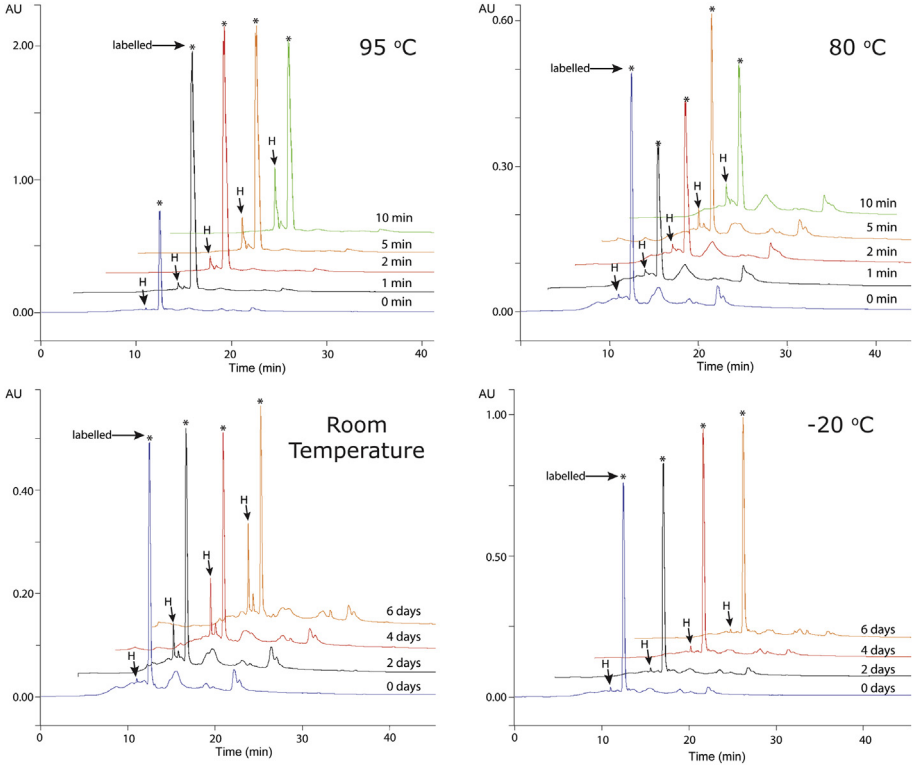


**Fig. 4.** HPLC traces of **4** after treatment at 95, 80 °C, room temperature, and at −20 °C for the indicated time. *=Labeled product. H=hydrolysis product.

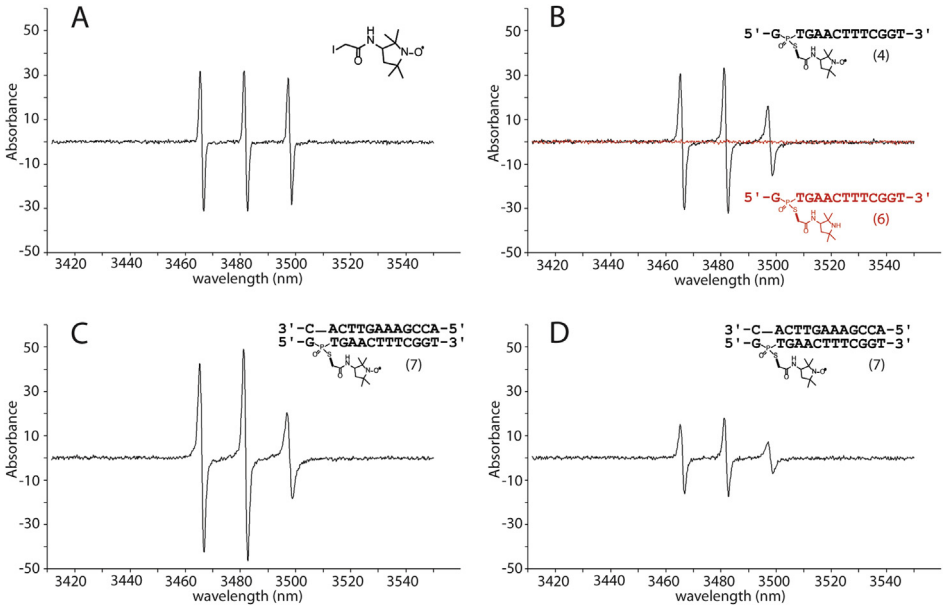


**Fig. 5.** EPR spectra of oligonucleotides in PBS. (A) IAM-PROXYL reagent (100 μM). (B) Compounds **4** (black) and **6** (red) (both 100 μM). (C) Compound **7** produced by post-alkylation annealing of dsDNA (route 1 in Fig. 3, 100 μM). (D) Compound **7** produced by pre-alkylation annealing of dsDNA (route 2 in Fig. 3, 100 μM).

treatment. Less hydrolysis occurs at 80 °C. The label was also gradually lost by standing at room temperature for several days, however, it is completely stable when frozen at −20 °C. Compound **4** was annealed with its complementary strand by heating for 30 s at 95 °C, allowed to cool to room temperature over 1 h and immediately purified by size exclusion chromatography (SEC) at 4 °C.

To confirm the presence of the nitroxide label, EPR spectra were obtained for paramagnetic **4**, its reduced, diamagnetic version **6**, and for the paramagnetic double stranded **7**. As can be seen in Fig. 5, both **4** and **7** gave a characteristic EPR signal whilst **6** gave no signal. A labeling reaction was also attempted on **2** pre-annealed with its complementary strand in an effort to generate **7** by a second route (Fig. 3, route 2). Due to the difficulties we experienced detecting double stranded DNA oligonucleotides by MALDI-MS we compared the extent of labeling (after size exclusion chromatography purification) using EPR spectroscopy. Much lower incorporation of the nitroxide label was observed when the labeling reaction was performed on the pre-annealed double stranded phosphorothioate oligonucleotide, and we therefore recommend performing labeling on the single-stranded oligonucleotides and annealing post-alkylation (Fig. 3, route 1). This route also enables definitive purification and identification of the labeled reaction products.

To determine whether the nitroxide-labeled **4** gave rise to significant PRE effect in protein NMR spectra we acquired HSQC spectra of $^{15}$N-labeled *Sulfolobus solfataricus* single-stranded DNA binding protein (*Sso*SSB) in isolation and in the presence of **3** or **5**. *Sso*SSB is known to bind to ssDNA with the sequence TTTTTT.[22] In the presence of diamagnetic **5** several *Sso*SSB signals shifted, indicative of binding to DNA (Fig. 6A). However, when HSQC spectra of *Sso*SSB were acquired under identical conditions but in the presence of paramagnetic **3** several protein signals were no longer visible[6] or had reduced intensity. This was presumably because of their close proximity to the nitroxide label which caused increased relaxation of their nuclei and broadening of their signals (Fig. 6B). Several of the non-visible protein signals corresponded to those that shifted upon DNA binding.[23]

Finally, we examined whether this approach was applicable to RNA using the phosphorothioate-modified ssRNA strand 5′-

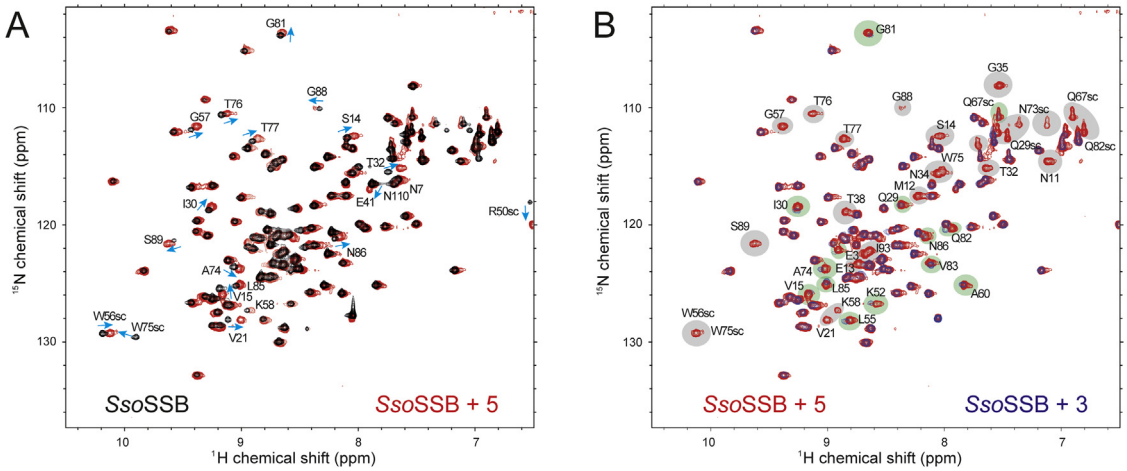


**Fig. 6.** (A) Overlay of $^{15}$N HSQC spectra of *Sso*SSB alone (black) and in the presence of diamagnetic **5** (red). Blue arrows indicate protein resonances that shift upon binding to the DNA. (B) Overlay of HSQC spectra of *Sso*SSB in the presence of paramagnetic **3** (blue) or diamagnetic **5** (red). Shading indicates protein resonances that disappear (gray), or have reduced intensity (green) in the presence of paramagnetic **3**. sc=side chain.

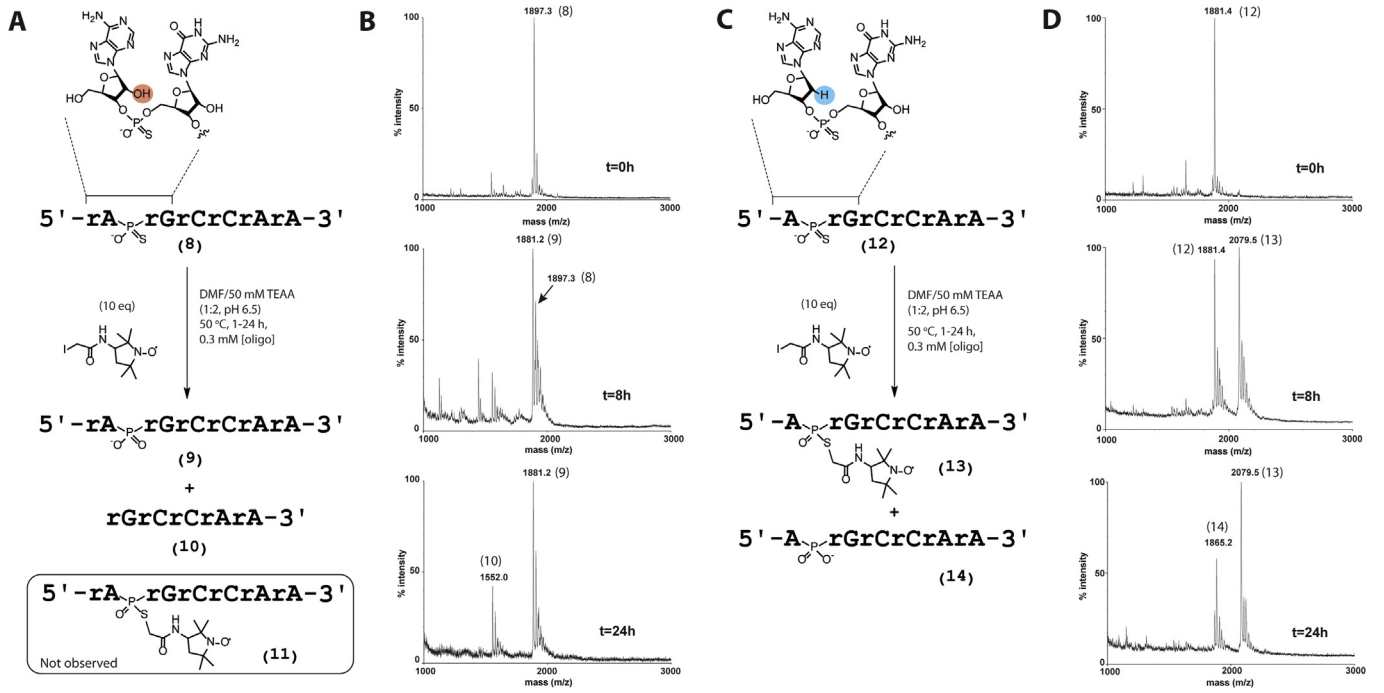


**Fig. 7.** (A) Attempted labeling reaction on ssRNA oligonucleotide **8**. (B) Monitoring the reaction on **8** by MALDI-MS. (C) Attempted labeling reaction on ssRNA oligonucleotide **12**. (D) Monitoring the reaction on **12** by MALDI-MS.

rA*rGrCrCrArA-3′ (**8**). Using the optimized conditions, and following the reaction progress by MALDI-MS we observed only hydrolysis products **9** and **10** and none of the desired **11**. We had observed similar hydrolysis reactions for **3** and **4** at extreme conditions and suspected that the adenosine 2′-OH, being in close proximity to the phosphorothioate linkage, might accelerate these hydrolysis reactions. When the reaction was reattempted using 5′-A*rGrCrCrArA-3′ (**12**), which has a deoxyribonucleotide on the 5′ side of the phosphorothioate,[24] the reaction proceeded to give the desired **13**. We noted that the reaction of RNA phosphorothioates with IAM-PROXYL was slower than that of DNA, being only 50% complete after 8 h as judged by MALDI-MS (Fig. 7). After 24 h the starting material was completely consumed and **13** was the major product along with a small amount of hydrolysis product **14**.

## 3. Conclusion

In summary, we have described a simple and practical protocol for labeling oligonucleotides for NMR and EPR studies. Paramagnetic oligonucleotides were prepared in useful quantities using commercially available reagents and gave clear EPR spectra and the PRE effect on protein signals was evident in HSQC NMR spectra with $^{15}$N-labeled *Sso*SSB. We provide semi-optimized reaction conditions and highlight factors that must be considered especially if dsDNA or ssRNA are to be used. Studies utilizing these oligonucleotides are ongoing in our laboratories and will be reported in due course.

## 4. Experimental

### 4.1. General

Standard and phosphorothioate oligonucleotides were purchased from Integrated DNA technologies. 3-(2-Iodoacetamidomethyl)-PROXYL and triethylamine were obtained from Sigma Aldrich. Acetonitrile and DMF were obtained from Merck. Acetic acid and other solvents were obtained from Ajax chemicals. Oligonucleotide concentrations were determined by Nanodrop using extinction coefficients 70,600 L/($M^{-1}$ $cm^{-1}$) for **1**, **3**, and **5**, 126,200 L/($M^{-1}$ $cm^{-1}$) for **2**, **4**, and **6**.

### 4.2. Oligonucleotide detection by MALDI-MS

Oligonucleotide samples required pre-treatment with ammonium-loaded cation-exchange resin prepared according to Ref. 10. 3-Hydroxypicolinic acid (HPA) matrix (1 μL, 200 μM in 70% acetonitrile, 30% $H_2O$) was spotted onto the plate and allowed to dry. Oligonucleotide sample (1 μL) was mixed with 1 μL resin for 5 min and then 1 μL of this mixture was spotted on top of the dried matrix and allowed to dry. Samples were then analyzed on a Voyager DE-STR MALDI mass spectrometer in positive linear mode with laser intensities 2000–2500. Ion 'hot-spots' were normally found at the perimeter of the sample spots.

### 4.3. Reaction screening

Small-scale reaction screening was performed on 40 nmol of phosphorothioate oligonucleotide. The purity of crude oligonucleotides was checked by analytical HPLC and if >90% they were used in screening reactions without purification. A 1 mM stock solution of phosphorothioate oligonucleotide was dissolved in MilliQ water. 3-(2-Iodoacetamidomethyl)-PROXYL was dissolved in the required organic solvent (DMF, DMSO, ethanol, acetone, acetonitrile) to a final concentration of 10 mM. Triethylammonium acetate (TEAA) was prepared by adding equal molar quantities of triethylamine and acetic acid to MilliQ water to achieve a final concentration of 0.1 M and the pH was adjusted to 6.5 using either acetic acid or triethylamine. To 40 μL (40 nmol) of phosphorothioate oligonucleotide in a microfuge tube was added 40 μL of freshly prepared 0.1 M TEAA, followed by 40 μL of IAM-PROXYL solution. The microfuge tube was vortexed, centrifuged, and then heated in a heating block at the required temperature.

Aliquots (20 μL) were taken at the specified times (1, 4, 8, 24 h), lyophilized, and redissolved in 100 mM TEAA pH 6.5 and analyzed by HPLC and MALDI-MS.

For oligonucleotide concentration screening the solution was diluted appropriately with TEAA and DMF (e.g., for [oligonucleotide]=0.03 mM) or the reagents were mixed as normal and then immediately frozen and lyophilized and the dry mixture resuspended in the required volume (e.g., for [oligonucleotide]=3 mM). Similarly for % DMF screening all of the reagents were mixed as above and immediately lyophilized and resuspended in a solution containing the required amounts of TEAA buffer and DMF. For screening of the IAM-PROXYL, the concentration of the IAM-PROXYL–DMF solution was adjusted to 5 or 30 mM. For pH screening the pH of the 0.1 M TEAA solution was adjusted with acetic acid or triethylamine.

Reactions were analyzed on an Agilent HPLC system using a Phenomenex Luna C18 column (5 μm 300 Å, 150 mm×4.6 mm) using gradient mixtures of solvent A: 100 mM TEAA pH 6.5 and solvent B: 80% acetonitrile/20% 100 mM TEAA pH 6.5. A linear gradient of 100% solvent A to 100% solvent B over 20 or 30 min was used with UV detection at 260 nm.

### 4.4. NMR/EPR scale oligonucleotide labeling

To a solution of phosphorothioate oligonucleotide (300 μL, 300 nmol) in a 1.5 mL microfuge tube were added 300 μL of 0.1 M TEAA (pH 6.5) and 300 μL of 10 mM solution of IAM-PROXYL in DMF (3 μmol). The mixture was incubated at 50 °C with vortexing (1000 rpm) in an Eppendorf thermomixer for 8 h (a heating block worked equally well). After this time the mixture was lyophilized and redissolved in 2 mL 100 mM TEAA (pH 6.5) and purified by HPLC on a GBC HPLC system using a Phenomenex Jupiter C18 column (10 μm, 300 Å, 250 mm×15 mm) and a linear gradient of 0–40% B over 35 min at 3 mL/min (solvent A: 100 mM TEAA pH 6.5 and solvent B: 80% acetonitrile/20% 100 mM TEAA pH 6.5) with detection at 260 nm. Fractions were analyzed by MALDI-MS. Fractions containing the labeled oligonucleotides were pooled and lyophilized.

For the reduced-label oligonucleotides the same procedure was followed except that after reacting the oligonucleotide with IAM-PROXYL reagent for 8 h, sodium dithionite was added (5 mg, 30 μmol) and left to react for 10 min at 50 °C, after which the mixture was lyophilized and purified by rpHPLC as above.

To generate **7** by route 1, 1:1 molar equivalents of **4** and the complementary strand (5′-ACCGAAAGTTCAC-3′, 130,000 L/($M^{-1}$ $cm^{-1}$)) were mixed in PBS and heated at 95 °C for 30 s and cooled to room temperature for 1 h. The annealed oligonucleotide was then immediately purified by SEC (Superdex-75) at 4 °C using PBS. dsDNA fractions were pooled and concentrated using centricons (1 KDa MWCO) at 4 °C. To generate **7** by route 2, a 1:1 molar ratio of **2** and the complementary strand were mixed in 0.1 M TEAA (pH 6.5), heated at 95 °C for 30 s, and cooled to room temperature for 30 min. IAM-PROXYL was added and the mixture was incubated at 50 °C with vortexing (1000 rpm) in an Eppendorf thermomixer for 8 h. After this time the mixture was lyophilized and redissolved in 2 mL PBS and purified by SEC (Superdex-75) at 4 °C in PBS. dsDNA fractions were pooled and concentrated using centrifugal concentrators (1 KDa MWCO) at 4 °C.

(**3**) 5′-G-(PROXYL)-GTTTTTT-3′: $t_R$: 7.97 min. MALDI-MS $[M+H]^+$=2635.5 (found), 2635.9 (calcd).

(**4**) 5′-G-(PROXYL)-TGAACTTTCGGT-3′: $t_R$: 7.17 min. MALDI-MS $[M+H]^+$=4194.9 (found), 4194.9 (calcd).

(**5**) 5′-G-(redPROXYL)-GTTTTTT-3′: $t_R$: 7.61 min. MALDI-MS $[M+H]^+$=2621.1 (found), 2620.9 (calcd).

(**6**) 5′-G-(redPROXYL)-TGAACTTTCGGT-3′: $t_R$: 7.79 min. MALDI-MS $[M+H]^+$=4180.8 (found), 4179.9 (calcd).

### 4.5. EPR spectroscopy

The spin labeling of IAM-PROXYL, **4**, **6**, and **7** (all 100 μM in PBS) was determined using a Bruker EMX X-band (9.5 GHz) EPR spectrometer with a standard rectangular TE cavity. First-derivative EPR absorption spectra were recorded at room temperature with a microwave power of 5.0 mW, a modulation amplitude of 1.0 G, and a sweep width of 140 G. EPR spectra were analyzed with the Spectrum Viewer program.

### 4.6. Preparation of $^{15}$N-labeled *Sulfolobus solfataricus* SSB

*Sso*SSB was prepared as described previously.[22] *Sso*SSB 1–117 was cloned into pET28C without any tags. Protein expression was induced by addition of 0.2 mM IPTG at 37 °C for 16 h. Cells were lysed by sonication in 20 mM Tris–HCl, pH 7.0, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.1% Triton X-100, and the supernatant was heated to 70 °C for 30 min in a water bath to denature the majority of proteins present in the bacterial cells. Following centrifugation, the supernatant was diluted two-fold with buffer A (20 mM Tris–HCl pH 7.0, 1 mM EDTA, 1 mM DTT) and applied to a HiTrap HP Heparin (295 mL tandem, GE) column equilibrated with buffer B (20 mM Tris–HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). A 500 mL linear gradient comprising 100–1000 mM NaCl was used to elute cationic proteins. Fractions corresponding to a distinct absorbance peak were analyzed by SDS-PAGE, pooled, concentrated, and loaded onto a Superdex-75 gel filtration column in NMR buffer.

### 4.7. NMR spectroscopy

NMR experiments were carried out using 0.8 mM SsoSSB in 50 mM NaCl, 10 mM sodium phosphate, pH 6.0 with 10% $D_2O$. Proton chemical shifts were referenced to 4,4-dimethyl-4-silapentanesulfonic acid (DSS) at 0 ppm. $^{15}$N chemical shifts were referenced indirectly to the same signal. NMR experiments were recorded at 298 K on either a Bruker 600 or 800 MHz spectrometer (Bruker Avance III) equipped with a 5 mm TCI cryoprobe. 1D $^1$H NMR and $^{15}$N HSQC spectra were recorded with 0, 0.5, 0.7, and 1 molar equiv of **3** and **5**. Data were processed using Topspin (Bruker Biospin) and assignments were made using Sparky (T.D. Goddard and D.G. Kneller, University of California at San Francisco).

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## Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2014.12.056.

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