

Site-Directed Spin-Labeling of Nucleic Acids by Click Chemistry: Detection of Abasic Sites in Duplex DNA by EPR Spectroscopy

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Abstract: This paper describes a spin label that can detect and identify local structural deformations in duplex DNA, in particular abasic sites. The spin label was incorporated into DNA by a new postsynthetic approach using click-chemistry on a solid support, which simplified both the synthesis and purification of the spin-labeled oligonucleotides. A nitroxide-functionalized azide, prepared by a short synthetic route, was reacted with an oligomer containing 5-ethynyl-2'-dU. The conjugation proceeded in quantitative yield and resulted in a fairly rigid linker between the modified nucleotide and the nitroxide spin label. The spin label was used to detect, for the first time, abasic sites in duplex DNA by X-band CW-EPR spectroscopy and give information about other structural deformations as well as local conformational changes in DNA. For example, reduced mobility of the spin label in a mismatched pair with T was consistent with the spin label displacing the T from the duplex. Addition of mercury(II) to this mismatch resulted in a substantial increase in the motion of the spin label, consistent with formation of a metallopair between the T and the spin-labeled base that results in movement of the spin label out of the duplex and toward the solution. Thus, reposition of the spin label, when acting as a mercury(II)-controlled mechanical lever, can be readily detected by EPR spectroscopy. The ease of incorporation and properties of the new spin label make it attractive for EPR studies of nucleic acids and other macromolecules.

Introduction

EPR spectroscopy is a valuable technique to study the structural properties and dynamics of macromolecules, including oligonucleotides.¹ However, these studies require the incorporation of a spin label (a stable free radical, e.g., a nitroxide) into the oligonucleotide. This can be accomplished either by direct incorporation of the corresponding spin label phosphoramidite during automated synthesis of the oligonucleotide,² by enzymatic incorporation of spin-labeled nucleoside triphosphates,³ or by incorporating a modified nucleotide or another building block which can selectively react with a spin-labeling reagent after

the oligonucleotide synthesis (postsynthetic functionalization).⁴ The postsynthetic strategy is often advantageous, as synthesis of spin-labeled phosphoramidites is often tedious and the spin label may decompose under the conditions employed in automated DNA synthesis.^{2a,d} Examples of postsynthetic labeling include reaction at the amino group in 2'-amino-modified RNA,^{4a-c} at modified internucleotide linkages,^{4d,e} and at 4-thiouridine.^{4f,g}

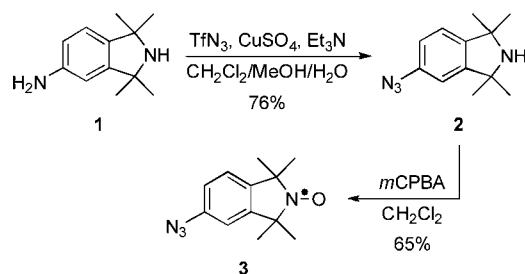
The Cu^I-catalyzed Huisgen–Meldal–Sharpless [3 + 2] cycloaddition⁵ (click chemistry)⁶ between an alkyne and an azide has been used for modification of nucleobases⁷ as well as for

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Scheme 1. Synthesis of Azide-Containing Nitroxide **3**

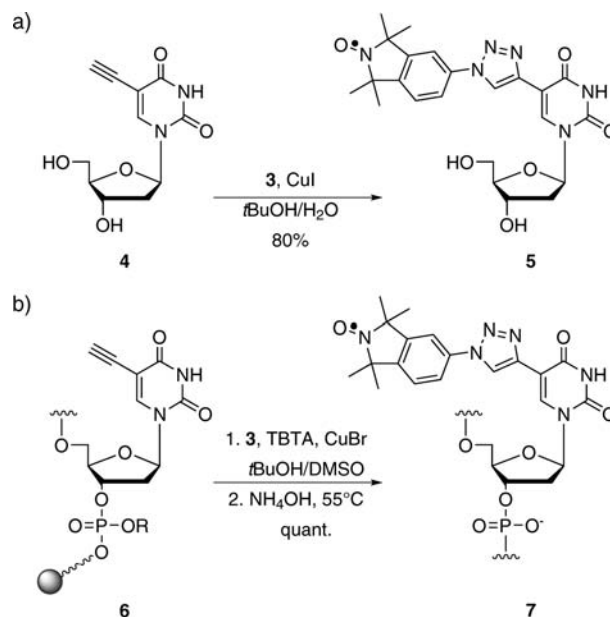
the labeling of DNA in solution^{7b,8} and on solid support.^{7b} The reaction is attractive, since it can be performed under mild conditions, tolerates a broad range of functional groups and reaction conditions, usually gives high yields, and is orthogonal to most organic reactions and consequently chemoselective. Here, we report site-directed spin-labeling of DNA by a click reaction and the use of the new label to detect local structural deformations in duplex DNA, in particular abasic sites.

Results and Discussion

Synthesis of Spin Label and Postsynthetic DNA Labeling by Click Chemistry. Since azides are sensitive to the conditions used in automated DNA synthesis,⁹ the nitroxide was functionalized with an azide, rather than the DNA. Thus, the azide-containing nitroxide **3** was synthesized from the known aminoindoline **1**¹⁰ by reaction with triflyl azide¹¹ to give azide **2**, which was oxidized with $m\text{CPBA}$ (Scheme 1).

The alkyne 5-ethynyl-2'-dU **4** was chosen for reaction with azide **3** as it has previously been used in Cu^{I} -catalyzed Huisgen cycloadditions^{7a,c} and results in a relatively short and rigid linker between the spin-label and the oligonucleotide. The reaction is usually performed using a copper(II) salt and sodium ascorbate as a reducing agent,^{5a} but since these conditions cause degradation of DNA¹² and reduction of nitroxides,¹³ a copper(I) salt was used directly. Thus, the commercially available 5-ethynyl-2'-deoxyuridine **4** was reacted with the azide functionalized nitroxide **3** in a Cu^{I} -catalyzed Huisgen–Meldal–Sharpless [3 + 2] cycloaddition to yield the spin-labeled uridine derivative **5** (Scheme 2a). The nitroxide functional group was stable under these reaction conditions.

The 5'-DMT protected phosphoramidite of 5-ethynyl-2'-deoxyuridine is commercially available, and thus, the alkyne can easily be incorporated into an oligonucleotide using standard conditions for automated DNA synthesis. However, due to low coupling yields obtained with the standard activator 1*H*-tetrazole, 1.8 M pyridinium hydrochloride was used as an activator and the coupling time increased to 15 min for the modified amidite. The optimized coupling conditions resulted in nearly quantitative coupling yields (DNA sequence shown in Figure 2a).

Scheme 2. Synthesis of Nitroxide-Labeled Nucleoside **5** (a) and Oligonucleotide **7** (b) by Click Chemistry^a

^a The modification of the oligomer takes place before deprotection and cleavage from the resin. TBTA is the Cu^{I} -stabilizing ligand *tris*-(benzyltriazolylmethyl)amine.

Copper(I)-catalyzed [3 + 2] cycloadditions on oligonucleotides containing the 5-ethynyl-2'-deoxyuridine derivative have been reported in solution with a number of different azides.^{8a} However, this method^{8a} proved unsuccessful in our hands, and analysis of the reaction mixture by MALDI-TOF showed only a minor trace of the spin-labeled oligonucleotide. Prolonged reaction times resulted in decomposition of the oligonucleotide, even in the presence of the Cu^{I} -stabilizing ligand *tris*-(benzyltriazolylmethyl)amine (TBTA)¹⁴ which has been shown to protect the metal from oxidation and to protect DNA from degradation by reactions mediated by Cu^{I} .^{8a} The oligonucleotide was consequently reacted with an excess of compound **3**, CuBr , and TBTA while still attached to the solid support (Scheme 2b). After the reaction, the excess reagents were removed by washing and the oligonucleotide was cleaved from the solid support using standard conditions (conc. aqueous NH_3 for 12 h, 55 °C). Analysis of the reaction mixture by MALDI-TOF showed a nearly quantitative conversion to the spin-labeled oligonucleotide (MW 4494.9 g mol^{-1}) (Figure 1). The effect of the spin label on the stability of duplex DNA was determined by thermal denaturation experiments. The modification decreased the stability of the corresponding DNA duplex by 3.5 °C, when compared to an oligonucleotide containing T instead of the modified dU derivative (Supporting Information).

Engels and co-workers have also reported an on-column spin-labeling method using a Sonogashira coupling.^{15a} However, after spin-labeling, the oligomer synthesis is continued, which exposes the spin label to the nucleic acid synthesis reagents. This causes a partial reduction of the nitroxides.^{15b} In contrast, the on-column method described here is a postsynthetic approach, that is, the

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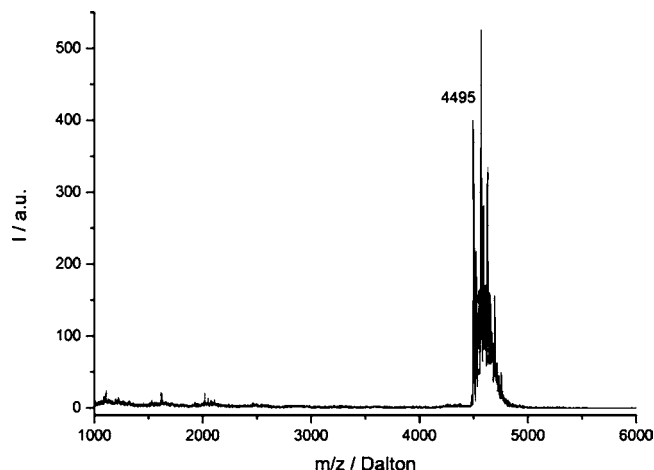


Figure 1. MALDI-TOF spectrum of the crude click reaction mixture between azide **3** and 5-ethynyl-2'-dU modified oligonucleotide **6** (MW = 4263.7 g mol⁻¹) to yield spin-labeled oligonucleotide **7** (MW = 4494.9 g mol⁻¹). The response of the alkyne-containing oligomer in the MALDI-TOF was shown in a separate experiment to be within a maximal factor of 2 of the spin-labeled oligomer.

oligomer has already been synthesized when the spin labeling is carried out, and therefore, the spin label is not exposed to harmful reagents. Another advantage of this on-column protocol is that it is not moisture sensitive.

EPR Studies. Incorporation of the nitroxide into DNA was verified by continuous-wave (CW) EPR spectroscopy, which showed the expected three-line pattern for a nitroxide (Figure 2). EPR spectra of nitroxide radicals also give information about their mobility; as the motion slows down, the spectrum becomes wider. Thus, the EPR spectrum of the spin-labeled nucleoside **5**, which can tumble freely in solution, has narrower lines than the spin-labeled single stranded DNA (Figure 2b). As expected, the spectral width increased even more after annealing the spin-labeled single strand to the complementary oligomer (see Figure S1 in Supporting Information for comparison of ssDNA and duplex DNA at the same temperature). However, a slow-moving component of the single strand spectrum indicates a minor foldamer where the spin label has lower mobility than in the duplex.

However, the EPR spectrum of the spin-labeled duplex DNA is considerably narrower than what has been observed for the rigid nitroxide \dot{C} in duplex DNA.^{2d} Thus, there is some motion in the linker connecting the nitroxide to the nucleoside. The motion of the spin label, independent of the duplex, results from rotation of the two single bonds that connect the triazole to the base and the nitroxide moiety. Since all the atoms in the tether are sp²-hybridized, the three aromatic rings can lie in the same plane, as supported by modeling studies of a triazole ring connected at the 5-position of a pyrimidine nucleobase.^{7a} Furthermore, if rotation around the single bonds connecting the rings could be restricted, the motion of the nitroxide would be reduced and result in a much broader EPR spectrum. Thus, EPR spectroscopy could be used to detect sites to which the extended aromatic system of the label could bind and thereby substantially slow the motion of the nitroxide. We have successfully used this promising feature of the spin label to detect an abasic site in DNA by EPR spectroscopy.

Abasic sites are common lesions that result from spontaneous hydrolytic cleavage of the N-glycosidic bond in DNA or from

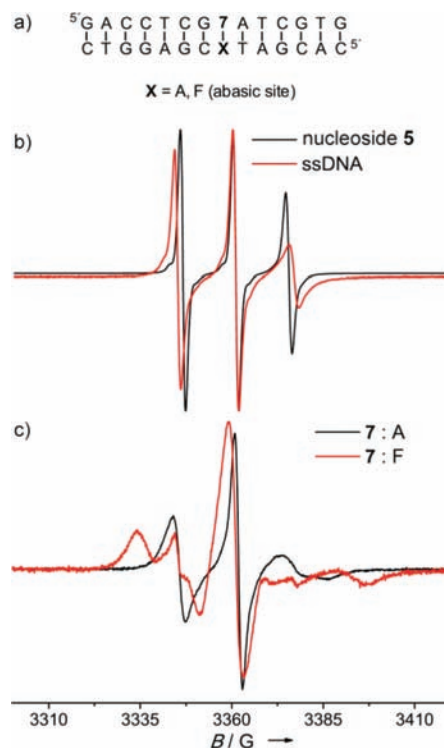


Figure 2. EPR spectra of the spin-labeled nucleoside (**5**), ssDNA, and DNA duplexes opposite an A or an abasic site (**F**). (a) Sequences of the spin-labeled duplexes. The identity of each spectrum is shown in insets. (b) EPR spectra of spin-labeled DNAs and the free spin label **5** at 30 °C. **5** is only soluble in DMSO and was measured at 30 °C because of the relatively high freezing point of DMSO. (c) EPR spectra of the fully matched duplex and a duplex containing the abasic site opposite the spin label measured at 10 °C.

removal of damaged nucleic bases by DNA glycosylases.¹⁶ These lesions have been detected by a number of different ways, including fluorescent probes, either incorporated into DNA¹⁷ or bound to specific sites in DNA,¹⁸ mass spectrometry,¹⁹ acoustic wave,²⁰ and electrochemical methods.^{20b,21} Pulsed EPR spectroscopy of doubly spin-labeled duplex DNA in frozen solutions has been used to measure a shortening of DNA helices containing an abasic site,²² but until now, CW EPR spectroscopy has not been used to directly detect an abasic site.

The single-stranded, spin-labeled oligomer was annealed to a DNA strand containing a stable analogue of an abasic site, 3-hydroxy-2-(hydroxymethyl)-tetrahydrofuran. Introduction of an abasic site creates a cleft into which the spin-label can

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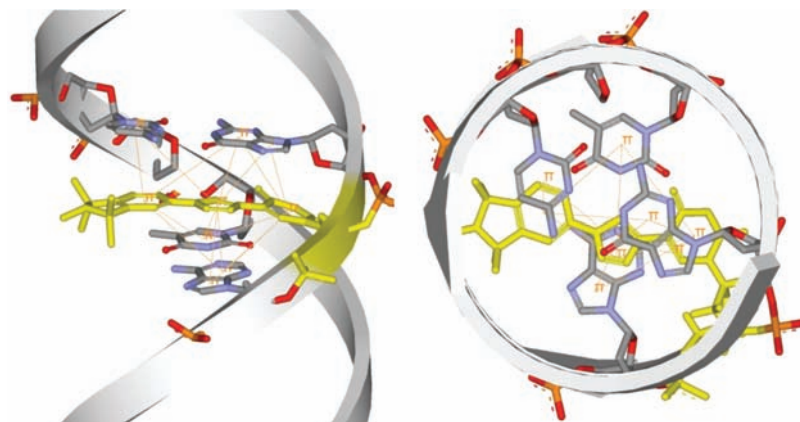


Figure 3. Putative model for the location of the spin label (yellow) when intercalated in the duplex cleft formed by an abasic site. Side view (left) and top view (right) with π - π interactions indicated by orange lines. H-atoms have been omitted for clarity.

intercalate, driven by favorable stacking interactions with neighboring bases as well as hydrophobicity of the label. Indeed, the EPR spectrum of the DNA duplex containing a spin label directly across from the abasic site shows significant broadening (Figure 2c). This is consistent with the spin label flipping into the abasic site pocket with concomitant reduction of its motion, in contrast to the fully base-paired duplex where the spin label has more degrees of freedom as it extends into the solution. A model of the spin label (Figure 3) shows that the spin label fits well in the abasic site, where it forms a network of favorable π - π interactions while placing the methyl groups of the tetramethylindoline nitroxide moiety into the major groove. The spin-labeled base is also stacked within the duplex, which is consistent with NMR studies that have shown that a T in a similar sequence context placed opposite an abasic site stacks within the helix.²³ In addition to the slow motion component in the EPR spectrum, there are also narrower lines that indicate a faster moving component with mobility similar to that obtained for the single strand/duplex. Double integration of the EPR spectrum shows that ca. 80% of the sample contains the spin label in the abasic site (Supporting Information).

How specific is the detection of an abasic site by **7**? Do other structural deformations in the vicinity of **7** show a similar EPR spectrum? To determine if the new spin label could be used to differentiate abasic sites from other structural lesions by EPR spectroscopy, DNA duplexes with different mismatches opposite the spin label were prepared (Figure 4a) and their EPR spectra recorded (Figure 4b). The spectra of the individual mismatches are different from each other, although the difference is not as clear as when a TEMPO-spin-labeled dC was used to detect and distinguish between different bases.²⁴ For example, the EPR spectra of **7**:A and **7**:C are quite similar. On the other hand, both **7**:T and **7**:G show a slow-moving component that can be used to distinguish each pair from all others. The only pair that shows a somewhat similar spectrum to the abasic site is **7**:T; however, the two spectra are clearly distinguishable (Figure 4c).

The fact that **7**:T shows a slow-moving component indicates that the spin label is displacing the opposing T and stacking within the duplex. If that is the case, it should be possible to change the mobility of the spin label by adding Hg^{2+} , which can stabilize T:T²⁵ and U:U mismatches²⁶ by bridging the N3

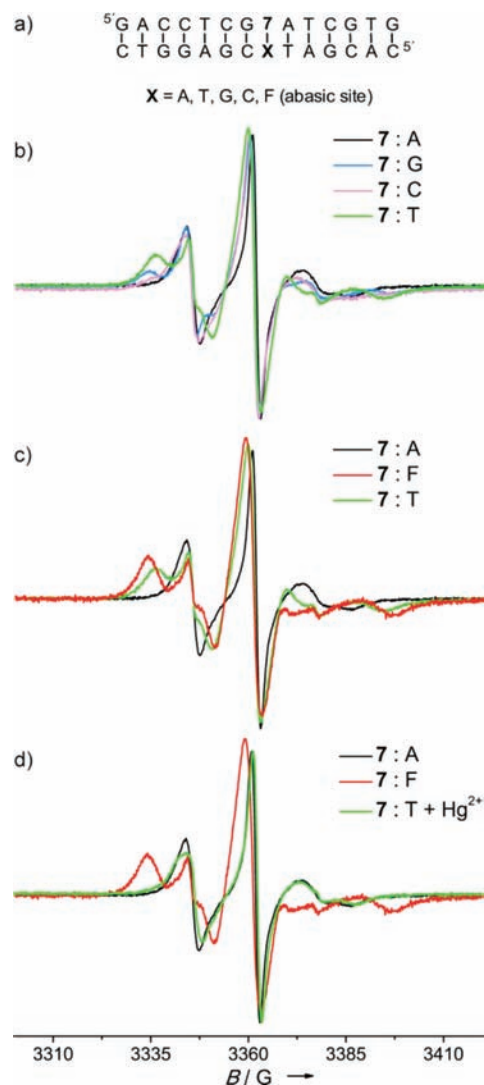


Figure 4. EPR spectra of DNA duplexes that contain spin label **7** opposite different bases (A, T, G, C) or an abasic site (**F**) at 10 °C. (a) Sequences of the spin-labeled duplexes. (b–d) EPR spectra of spin-labeled DNAs. The identity of each spectrum is shown in insets.

atoms of the nucleobases.²⁷ In fact, formation of a **7**-Hg-T base pair upon addition of Hg^{2+} should place the spin label in a similar position as in the fully matched duplex and thereby dramatically increase the mobility of the nitroxide. Indeed, the

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spectra of 7:A and 7:T in the presence of Hg^{2+} are nearly superimposable (Figure 4d). Thus, mercuric ions can effect displacement of the spin label out of the duplex and into the groove in a controlled fashion. Such a mercuric switch might find use for mechanical maneuvering of nucleic acid nanostructures.

Conclusion

In conclusion, we have incorporated a new spin label in a DNA strand by a new postsynthetic method using click-chemistry on a solid support, simplifying both the synthesis and purification of the spin-labeled oligonucleotides. The azide-functionalized nitroxide used in the reaction was synthesized by a short synthetic route and reacted with oligomers containing 5-ethynyl-2'-dU, the phosphoramidite of which is commercially available. The incorporation proceeds in excellent yield, avoids possible break down of the spin-label during oligonucleotide synthesis, and results in a fairly rigid linker between the modified nucleotide and the nitroxide spin label. This particular spin label

is an excellent probe for detecting abasic sites in duplex DNA by X-band CW-EPR spectroscopy. An advantage of using EPR for detection of abasic sites, compared to other methods, is that it is based on the mobility of the spin label. In contrast, fluorescence-based methods detect changes in fluorescence that can be influenced by factors other than the abasic site, such as identity of the flanking bases. Another advantage of EPR over fluorescence is the lack of background interference when doing measurements in a biological matrix. The spin label described here can also give information about local structural lesions, other than abasic sites. For example, reduced mobility of the spin label in a 7:T mismatch in a duplex DNA was consistent with the spin label displacing the T. Addition of mercury(II) resulted in an increased motion of the spin label, consistent with formation of a 7-Hg-T metallopair that flips the spin label out toward the solution. The azide-functionalized nitroxide **3** can also be used to spin-label other macromolecules using click chemistry.

Acknowledgment. This work was supported by the Danish National Research Foundation, the Icelandic Research Fund (080041022), and a doctoral fellowship to S.A.S from the University of Iceland.

Supporting Information Available: Experimental methods, including synthetic procedures, T_m studies, and EPR spectroscopy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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