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Nitroxides and nucleic acids: Chemistry and electron paramagnetic resonance (EPR) spectroscopy*

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Abstract: Electron paramagnetic resonance (EPR) spectroscopy has increasingly been applied for the study of nucleic acid structure and dynamics. Such studies require incorporation of free radicals (spin labels) into the biopolymer. The labels can be incorporated during chemical synthesis of the oligomer (phosphoramidite approach) or postsynthetically, by reaction of a spin-labeling reagent with a reactive functional group on the oligonucleotide. Incorporation of the rigid nitroxide spin label **Ç** is an example of the phosphoramidite method, and reaction of a spin-labeled azide with an alkyne-modified oligomer to yield a triazole-derived, spin-labeled nucleotide illustrates the postsynthetic spin-labeling strategy. Characterization and application of these labels to study structural features of DNA by EPR spectroscopy is discussed. Finally, a new spin-labeling strategy is described for nucleic acids that relies on noncovalent interactions between a spin-labeled nucleobase and an abasic site in duplex DNA.

Keywords: EPR spectroscopy; fluorescence; site-directed spin labeling (SDSL); spin labels; structural biology.

INTRODUCTION

Nucleic acids play a central role in biology. DNA contains the genetic blueprint, and RNA has a multitude of functions, for example, transferring the genetic information to protein structure (mRNA, tRNA, ribosome), processing and modification of RNA (spliceosome, RNAse P), housekeeping functions (signal recognition particle, telomers), and regulation of gene expression (riboswitches). RNA can also act as a catalyst and has, therefore, been identified as a key biopolymer in the evolution of life, since it can both carry genetic information and catalyze reactions [1]. Thus, RNA may have served as the primary catalytically active molecule before the emergence of proteins.

Information about the structure and motion of biopolymers, such as nucleic acids, provides insights into how they are able to carry out their biological function, i.e., their mechanism of action. In addition to satisfying academic curiosity about life itself, this mechanistic understanding can also be used to develop therapeutic methods to interfere with the biological function of biopolymers involved in the pathology of diseases.

There are several techniques that have been applied for the study of nucleic acids structure. X-ray crystallography [2] and NMR spectroscopy [3] provide a high-resolution view, showing positions of atoms in three-dimensional space. Lower-resolution techniques include cryo-electron microscopy [4],

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small-angle scattering [5], fluorescence resonance energy transfer (FRET) [6], solid-state NMR (ssNMR) [7,8], and electron paramagnetic resonance (EPR) spectroscopy [9–11]. FRET, ssNMR, and EPR have been used to obtain structural information through distance measurements between atoms or reporter molecules. These lower-resolution methods complement the high-resolution methods as they can be used to study motion/rearrangements of the biopolymer under biologically similar conditions.

EPR has become a particularly attractive spectroscopic technique for the study of biopolymers with recent advances in instrumentation and pulsed methods [9]. EPR studies of nucleic acids require incorporation of stable radicals, referred to as spin labels. In recent years, several different methods of spin labeling have been developed [10], giving ready access to spin-labeled oligonucleotides. The spin labels differ in their structures, points of attachment to the nucleic acid, and the chemistry used for their incorporation. Before providing specific examples of EPR studies of nucleic acids, a brief introduction of spin labeling and EPR spectroscopy will be given.

Nitroxides and their site-directed incorporation into nucleic acids

Site-directed spin labeling (SDSL) refers to incorporation of spin labels into specific sites of a biopolymer [10], sometimes referred to as site-specific spin labeling. A spin label contains an unpaired electron, and the most common functional group of spin labels for biopolymers is a nitroxide (Fig. 1). Nitroxides that are flanked by tertiary carbon atoms are stable under biologically relevant conditions in vitro and can be synthetically manipulated. Thus, synthesis and covalent attachment of the spin label to the biopolymer can be readily performed using the tools of organic synthesis.

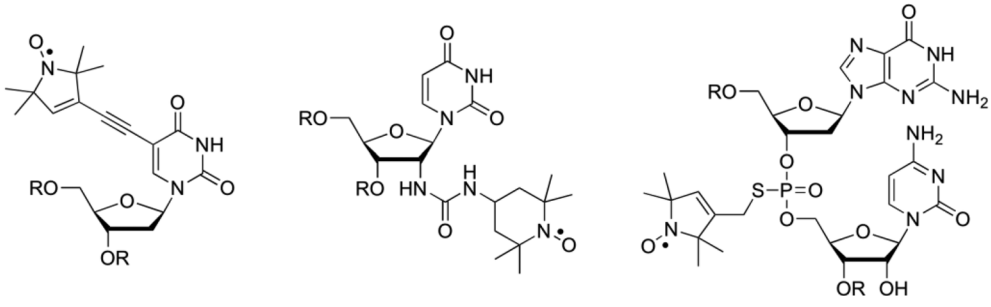
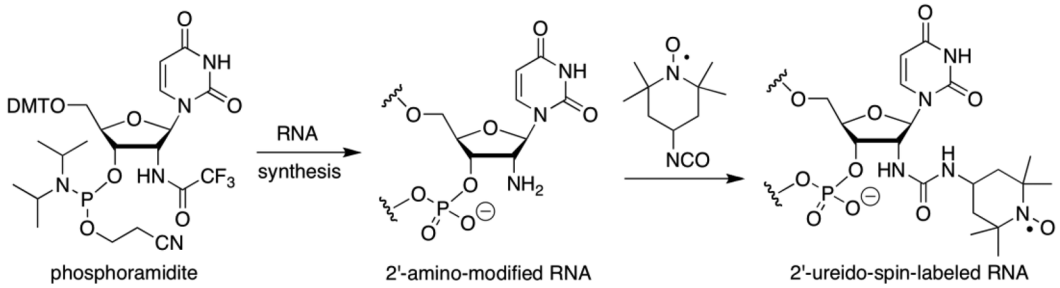


Fig. 1 Examples of nitroxides that have been conjugated to the nucleobase (left), the sugar (middle), and the phosphate backbone of nucleic acids (right).

Figure 1 shows examples of nucleic acid spin labels that have been attached to the nucleobase [12], the sugar [13], and the phosphate backbone [14]. Two general strategies have been used for incorporation of spin labels into nucleic acids: labeling during chemical synthesis of the nucleic acid (the phosphoramidite approach) and postsynthetic labeling. The phosphoramidite approach requires preparation of a spin-labeled phosphoramidite. Phosphoramidites are the building blocks for the automated chemical synthesis of nucleic acids. An example of a phosphoramidite, used for the site-directed incorporation of a 2'-amino group into RNA, is shown in Scheme 1, and the synthesis of a spin-labeled phosphoramidite is shown in one of the case studies below. The main advantage of the phosphoramidite approach is that it enables incorporation of labels that are structurally sophisticated. A potential drawback of the phosphoramidite method is that preparation of a labeled phosphoramidite may require a considerable synthetic effort.

In postsynthetic labeling, a spin label is reacted with a nucleic acid that contains a reactive functional group at a specific location, for example, spin labeling of 2'-amino-modified RNA with an aliphatic isocyanate [15] (Scheme 1). Postsynthetic labeling often uses readily available materials, but



Scheme 1 Incorporation of 2'-amino-modification into RNA using the phosphoramidite approach (left) and postsynthetic spin labeling of 2'-amino-modified RNA with a spin-labeled isocyanate (right). DMT = 4,4'-dimethoxytrityl.

this strategy is limited to certain sites of attachment as it requires chemically modified oligomers, and the spin labels are linked by a flexible tether. Furthermore, incomplete labeling may require purification of the spin-labeled material, which may be nontrivial.

EPR spectroscopy

EPR spectroscopy is a magnetic resonance technique that enables the study of unpaired electrons. As described above, nitroxides are stable free radicals that are commonly used as spin labels for nucleic acids. Examples of nitroxide EPR spectra are shown in Fig. 2. The spectrum on the left is typical for a small nitroxide in solution and shows clearly three lines, which result from coupling of the electron with the nuclear spins of the ^{14}N nitroxide atom, which has three spin states ($I = 1$).

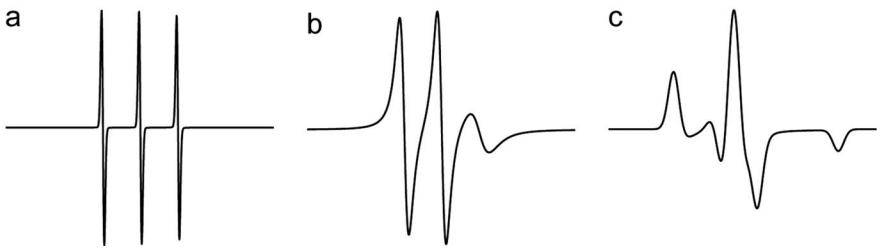


Fig. 2 Continuous wave (CW) EPR nitroxide spectra in the fast (a), intermediate (b), and slow (c) motion regime.

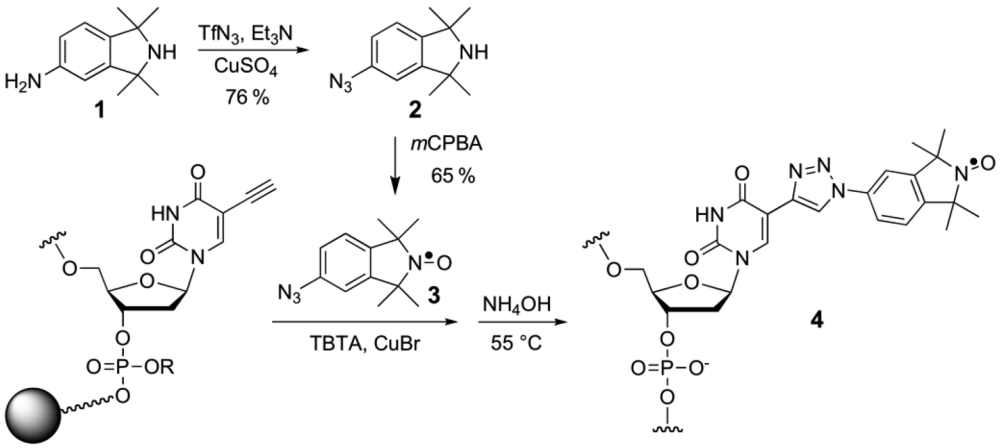
EPR spectroscopy is a valuable technique for studying dynamics, because the line width is sensitive to the mobility of the label. A nitroxide that is rapidly tumbling shows sharp lines (Fig. 2a), while the spectrum becomes more anisotropic with concomitant line-broadening when the motion slows down (Figs. 2b,c). Simulation of EPR spectra can be used to quantify the motion, i.e., to provide the rotational correlation time [16].

Changes in dynamics of a spin-labeled nucleic acid upon binding to another molecule can, for example, provide structural insights into ligand–receptor interactions [17,18] and hydrogen bonding [19]. The spectral changes can be quantified, either by measuring the width of the central peak (for spectra in the fast-motion regime) or the total spectral width, from the crest of the low-field peak to the trough of the high-field peak [11]. In the examples below, spectral width is used to probe local structural deformation in duplex DNA to study noncovalent binding of a ligand to DNA.

SITE-DIRECTED SPIN LABELING: CASE STUDIES

Postsynthetic spin labeling of nucleobases by click chemistry: Detection of abasic sites

The success of postsynthetic modification of nucleic acids depends on the chemoselectivity and yield of the reaction. The Huisgen–Meldal–Sharpless 1,3 dipolar cycloaddition between an azide and an alkyne to produce a disubstituted triazole ("click chemistry") is such a reaction and has been used for the modification of oligonucleotides [20]. To use this method for spin labeling, we prepared nitroxide-containing azide **3** (Scheme 2) [21]. The azide will only react with an alkyne and thereby ensure a site-specific modification. Unfortunately, the yields of the reaction between the azide and an alkyne-modified oligonucleotide in solution were low; increasing the reaction time or the reaction temperature led to decomposition of the oligomer. However, quantitative yields were obtained by perfoming the reaction before cleaving the oligomer from the solid support, on which it was synthesized, and deprotecting with aqueous ammonia (Scheme 2). The synthesis of **3** is simple, and the phosphoramidite for the alkyne-modified 2'-deoxyuridine is commercially available, giving ready access to site-specifically spin-labeled oligonucleotides.



Scheme 2 Synthesis of nitroxide-containing azide **3** and its use for postsynthetic modification of an alkyne-bearing oligonucleotide by the Huisgen–Meldal–Sharpless 1,3 dipolar cycloaddition. TBTA = *tris*-(benzyltriazolylmethyl)amine.

The three aromatic rings of the spin-labeled nucleotide have the potential to stack within a DNA helix. This structural feature has been used to detect abasic sites in DNA [21], which are biologically relevant nucleic acid lesions [22]. Two 14-mer DNA duplexes containing the spin label **4** were synthesized, where **4** was paired with either A or an abasic site (F) (Figs. 3a,b). The EPR spectra of the two duplexes were strikingly different (Fig. 3c); the mobility of the spin label opposite the abasic site was much lower, consistent with the spin label stacking in the pocket created by the absence of a base at the abasic site. Subsequent modeling showed that all three aromatic rings could participate in aromatic stacking interactions within the duplex [21].

To investigate if **4** could specifically identify abasic sites, i.e., if the EPR signal for an abasic site would be different from that of other local structural lesions, we recorded the EPR spectra of spin-labeled duplexes, where **4** was mispaired with either C, G, or T. Only the **4**•**T** pair showed a significant slow component in the EPR spectrum, which was slightly more mobile than for the abasic site, indicating that the T was flipping out of the helix and allowing the spin label to stack within the duplex.

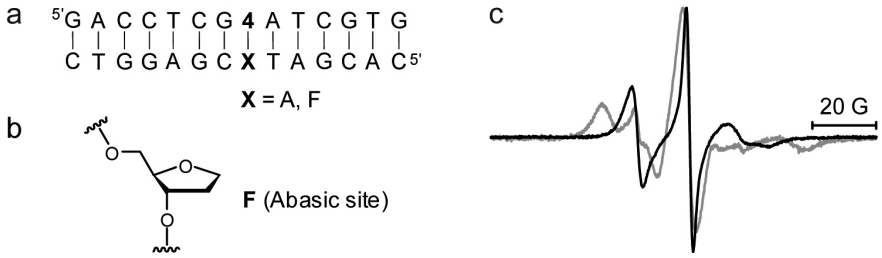


Fig. 3 (a) DNA duplexes containing spin label **4**, paired with either A or an abasic site (F). (b) The abasic site 3-hydroxy-2-(hydroxymethyl)tetrahydrofuran. (c) EPR spectra of the duplexes; the gray and black lines are the spectra of the duplex containing the **4**·**F** and **4**·**T** pairs, respectively.

Mercuric ions have been used to stabilize T-T mismatches by forming a T-Hg-T metallopair through bridging the *N*3 atoms of the nucleobases [23]. We hypothesized that formation of a metallopair between **4** and T would place the nitroxide ring in the major groove and thereby increase the mobility of the spin label. Indeed, addition of mercuric ions to the **4**·**T** duplex gave a spectrum that was nearly superimposable with that of the **4**·**A** duplex [21].

These results show that nucleic acids can be site-specifically spin labeled in quantitative yield with click chemistry using azide **3** and that the resulting spin label **4** is useful for detection of local structural deformations in DNA duplexes, in particular, abasic sites and T-mismatches.

Rigid nitroxide spin label Ç: A fluorophore in disguise

Most spin labels have a degree of motion, independent of the biopolymer itself, due to conformational flexibility of the tether used to attach the spin label. Sometimes conformational flexibility is advantagous, for example, when changes in the structure of the biopolymer changes the mobility of the spin label, as described above for the "click" spin label. We have also utilized changes in mobility of nucleotides linked to a semi-flexible urea-linked TEMPO spin label to study ligand binding to RNA [24] and TEMPO-modified nucleobases in duplex DNA to investigate hydrogen bonding. However, accurate distance measurements between two spin labels with EPR spectroscopy require a rigid spin label. Following the work of Matteucci and co-workers, who prepared the C-analog phenoxazine (**tC^{O}**, Fig. 4) that forms a base pair with G [25], we designed the rigid spin label **Ç** (Fig. 4) [26].

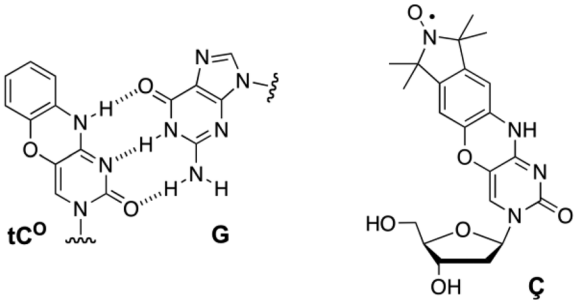
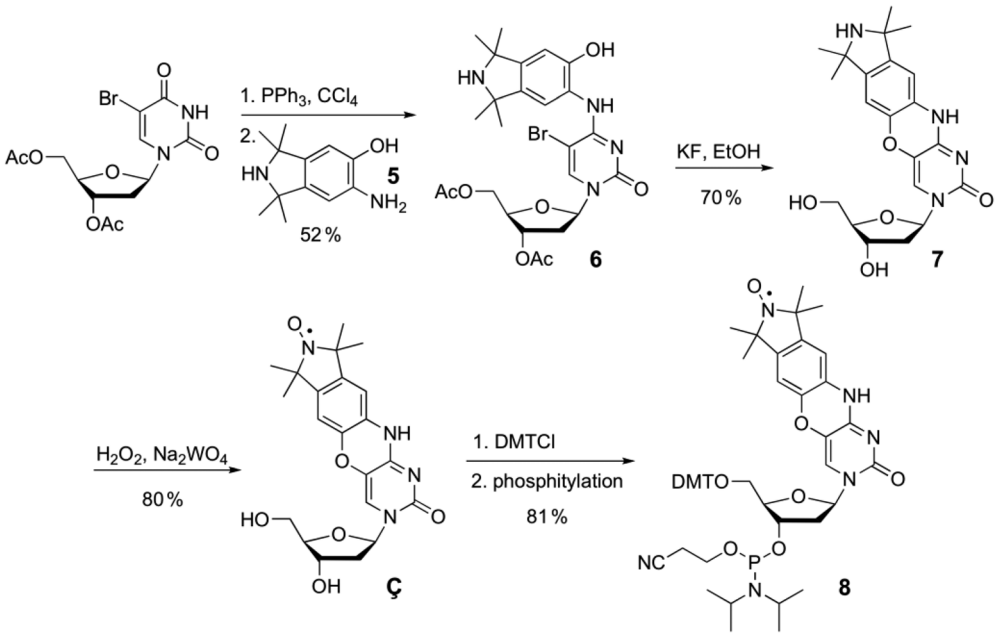


Fig. 4 Phenoxazine nucleoside **tC^{O}**, shown base-paired with G, and the rigid spin label **Ç**.

The synthesis of **Ç** started with reaction of acetyl-protected 2'-deoxy-5-bromouridine with PPh_3 and CCl_4 and subsequent addition of amino phenol **5** [26], followed by ring closure in the presence of KF to make tetracycle **7** (Scheme 3). Oxidation of **7** yielded nucleoside **Ç**, which was converted to the corresponding phosphoramidite (**8**).



Scheme 3 Synthesis of the rigid spin label **Ç** and its phosphoramidite (**8**).

Incorporation of **Ç** into DNA by automated chemical synthesis using phosphoramidite **8** was verified by mass spectroscopy and HPLC analysis of an enzymatic digest of a spin-labeled DNA, which returned **Ç** [26]. Moreover, EPR analysis confirmed the presence of the radical in the DNA and showed the successful design of the spin label: While the nucleoside gave sharp lines (Fig. 5a), the EPR spectrum of a single strand showed a reduction in mobility (Fig. 5b) and finally, the EPR spectrum of the DNA duplex containing **Ç** showed a significantly reduced motion (Fig. 5c). In fact, the rotational correlation time of the **Ç**-labeled duplex was similar to that of a cylinder with the dimensions of the duplex, indicating that the label was immobilized within the DNA helix [26].

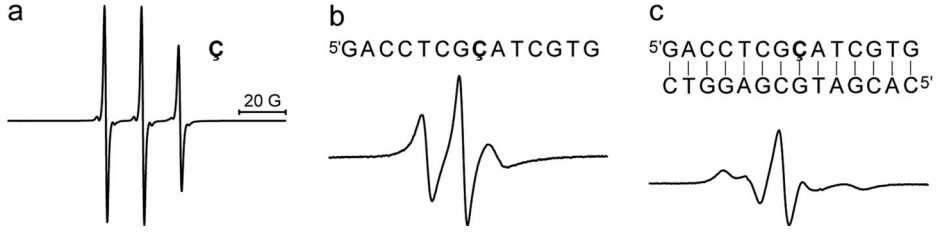
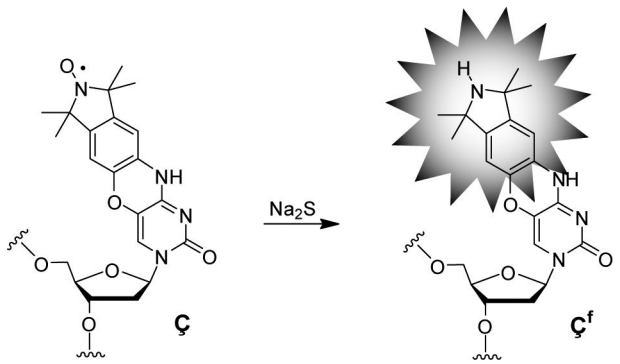


Fig. 5 EPR spectrum of **Ç** (a), a **Ç**-labeled single-stranded DNA oligomer (b), and a **Ç**-labeled duplex DNA (c).

Pairs of rigid spin label **Ç** have been incorporated into a series of DNAs for distance measurements between two radicals and shown to yield accurate long-range distances [27]. More importantly, the rigidity of **Ç** has also enabled determination of the relative orientations of two labels [27]. Thus, **Ç** has facilitated EPR measurements to go beyond distances in nucleic acids, since information about relative orientation gives additional structural insights and holds promise for investigation of complex tertiary structures of nucleic acids.

An interesting and useful feature of **Ç** is that it becomes fluorescent, with a relatively high quantum yield ($\Phi = 0.3$), upon reduction of the nitroxide functional group with a mild reducing agent [26]. This reduction can be carried out after incorporation into oligonucleotides (Scheme 4) [28]. The fluorescent nucleoside is sensitive to its microenvironment and is able not only to detect mismatches, but also to identify the mismatched base [28,29]. The bifunctionality of the spectroscopic probe allows, for the first time, the use of the same nucleic acid sample for both EPR and fluorescence spectroscopies and has been applied for the study of the conformation and dynamics of hairpin loops [30,31] and the folding of a DNA aptamer that binds cocaine [32].

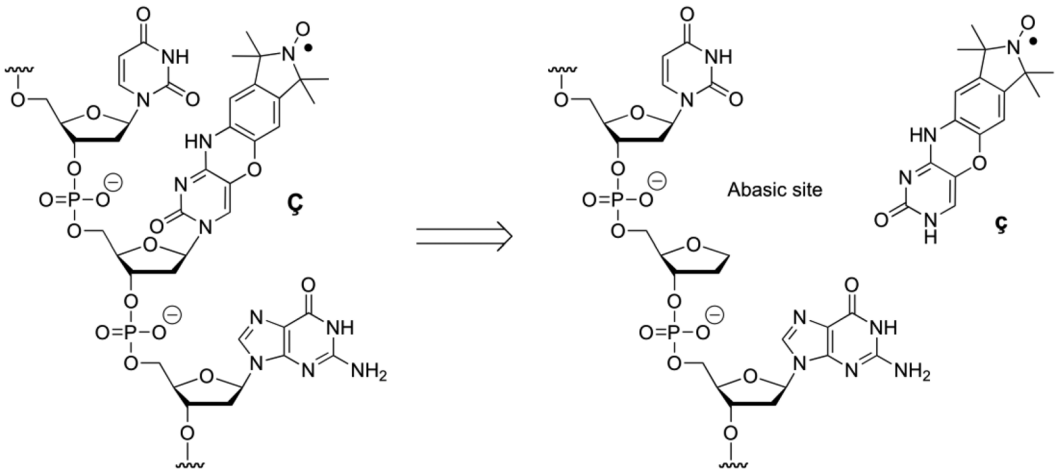


Scheme 4 Reduction of the nitroxide functional group of **Ç** in DNA. The halo placed on $\mathbf{Ç^f}$ indicates that the nucleoside is fluorescent.

Site-directed and noncovalent incorporation of spin labels into nucleic acids: A new strategy in spin labeling of nucleic acids

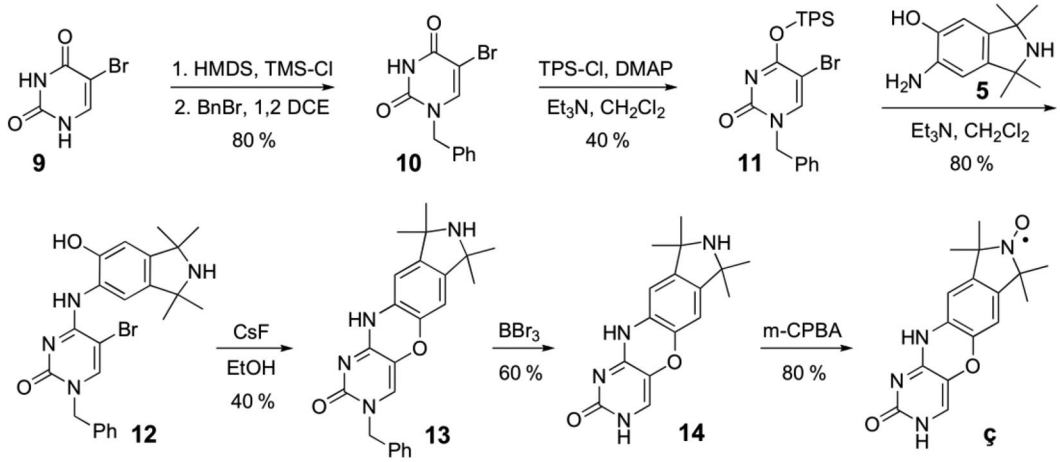
The traditional approaches for spin-labeling nucleic acids, both the postsynthetic method and direct labeling during chemical synthesis of the nucleic acid, require a degree of expertise in chemistry. Furthermore, the synthetic work is often labor intensive, as in the case of **Ç**, and the phosphoramidite approach requires a large excess of phosphoramidite during oligomer synthesis. Many research groups that would like to use EPR spectroscopy to study aspects of structure and dynamics of nucleic acids do not have the necessary synthesis expertise. For a biophysicist, being able to obtain a spin-labeled sample from a commercial source, ready for measurement would be desirable. As a step toward more readily accessible spin-labeled nucleic acids, we have developed a strategy that relies on noncovalent labeling. Thus, nucleic acids can be site-specifically labeled by mixing a chemically synthesized nucleic acid with the spin label.

The design of the new spin label was based on nucleoside **Ç**. We argued that by disconnection at the glycosydic bond, the spin-label base (**ç**) would be able to bind to the resultant pocket, created by the abasic site, through stacking and hydrogen bonding to the base on the opposing strand (Scheme 5). The phosphoramidite for an abasic site is commercially available, which makes preparation of nucleic acids containing the abasic site straightforward.



Scheme 5 Design strategy for noncovalent spin labeling.

The spin label **ç** was prepared using similar chemistry as applied for the preparation of **Ç** (Scheme 6) [33]. However, *N*1 of 5-bromouracil (**9**) required protection and, therefore, a benzyl group was regioselectively incorporated using a one-pot, two-step procedure with hexamethyldisilazane and benzyl bromide. *O*4-Sulfonylation, coupling with amino-phenol **5** and ring closure in refluxing ethanol in the presence of CsF yielded phenoxazine derivative **13**. After deprotection, oxidation of **14** with *m*CPBA gave spin label **ç**.



Scheme 6 Synthesis of the noncovalent spin label **ç**. TPS = 2,4,6-triisopropylbenzenesulfonyl. *m*-CPBA = *meta*-chloroperoxybenzoic acid.

EPR analysis of a solution of **ç** and a DNA duplex containing an abasic site at 0 °C showed that the major component of the spectrum corresponds to the unbound label, which shows sharp peaks (labeled with stars in Fig. 6b) [33]. However, there was also a slow-moving component (identified with arrows) from restricted motion of the spin label due to its binding to the abasic site. As the temperature of the sample was lowered, the amount of spin-label binding increased. At –30 °C, the label was com-

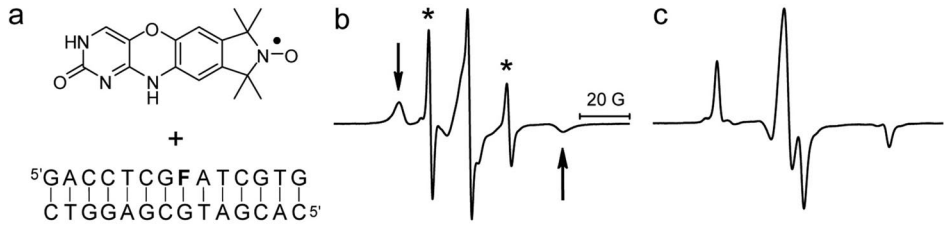


Fig. 6 Binding of the spin label **ç** to an abasic site (F) in duplex DNA (a), monitored by EPR spectroscopy at 0 °C (b) and –30 °C (c). The peaks associated with binding of the spin label to the abasic site are identified with arrows, and the peaks arising from unbound **ç** are labeled with a star.

pletely bound to the abasic site to yield a spectrum that is nearly superimposable with that of a sample covalently spin labeled with **Ç** (Fig. 6c) [33].

Several experiments were carried out to determine the specificity of binding, i.e., to verify that **ç** was binding to the abasic site, rather than in a groove or by intercalation. Incubation of **ç** with an unmodified DNA at –30 °C showed only minor binding (ca. 5 %). Changes in the abasic binding site also affected binding; the spin label bound tighter when G was opposite the abasic site than either A, T, or C, providing evidence that hydrogen bonding to G contributed to spin-label binding. Combined, our data show that nucleic acids can be spin labeled noncovalently and site-specifically through receptor–ligand interactions, where the receptor is placed in the nucleic acid helix by incorporation of an abasic site. Incorporation of two labels into DNA using this approach for distance measurements by pulsed EPR spectroscopy is currently in progress.

CONCLUSIONS

The application of EPR spectroscopy for the study of structure and dynamics of nucleic acids relies on spin labels with specific properties that can be readily incorporated at specific sites. The work described above has expanded the repertoire of spin labels and labeling methods. The postsynthetic labeling using click chemistry gives spin-labeled oligomers in high yield. Although the rigid spin label **Ç** requires extensive synthetic effort, its properties enable extraction of information about the structure and dynamics by EPR spectroscopy that have previously not been accessible. Finally, the noncovalent spin labeling of nucleic acids with **ç** is a new strategy that greatly simplifies spin labeling, while retaining the advantages of a rigid spin label.

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