Noncovalent Spin Labeling

DOI: 10.1002/ange.201002637

Noncovalent and Site-Directed Spin Labeling of Nucleic Acids**

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Electron paramagnetic resonance (EPR) spectroscopy is widely used to study free radicals or paramagnetic centers associated with biopolymers.[1] With the advent of pulsed EPR methods, which allow accurate distance measurements between 20 and 80 Å, structures of biopolymers have increasingly been interrogated by this technique.[2] Some of the advantages of EPR spectroscopy over other structural techniques are its sensitivity, that it is not restricted by molecular size, and that measurements can be performed under biological conditions.[1b] However, stable free radicals, such as nitroxide spin labels, must be incorporated into the biopolymers prior to EPR studies.

In site-directed spin labeling (SDSL), spin labels are covalently attached to the biopolymers at a specific site of interest.[3] For nucleic acids there have been two main strategies for SDSL. First, spin labels have been incorporated during automated oligonucleotide synthesis by employing spin-labeled phosphoramidite building blocks.[3b,c,e] This approach has the advantage that very sophisticated and structurally complex labels can be incorporated at specific sites. However, the synthetic challenges of spin-labeled phosphoramidites can be considerable.[3e] Furthermore, spin labels can be partially reduced upon exposure to the reagents used in the automated synthesis of oligonucleotides.[4] The second SDSL approach is post-synthetic modification of the biopolymer.[3b,c,e] Here, a spin-labeling reagent is incubated with an oligonucleotide that contains a reactive functional group at a specific site. Post-synthetic labeling is in general less labor intensive than the phosphoramidite strategy, but drawbacks include incomplete labeling and side reactions of the spin label with inherent functional groups of the nucleic acids, such as the exocyclic amino groups of the nucleobases. Both strategies usually require purification of the spin-labeled material, which can be nontrivial. Here we report a new and straightforward SDSL protocol for nucleic acids that is based on noncovalent labeling.

The new approach utilizes a nitroxide that is structurally related to the rigid spin label **Ç**.[5] The spin label **Ç** is an analogue of cytidine (C), with a nitroxide-bearing isoindol moiety fused to cytosine by an oxazine linkage, and forms a stable Watson–Crick base pair with guanine (Figure 1). The rigidity of **Ç** enabled precise distance measurements by EPR, determination of the relative angular orientation between two spin labels,[6] and has been used to study DNA dynamics and folding.[4c,5,7] The strategy for noncovalent labeling was to disconnect the glycosidic bond of **Ç** to give an abasic site (F) and the free spin-labeled base **ç** (Figure 1). The spin label would bind in the abasic site through receptor–ligand interactions involving hydrogen bonding and π-stacking interactions.[8]

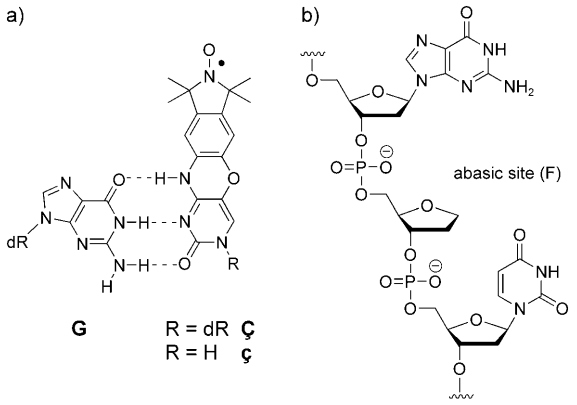

Figure 1. a) Base-pairing scheme of spin labels **Ç** and **ç** with G. dR is 2′-deoxyribose. b) Structure of an abasic site in DNA.

The synthesis of spin label **ç** started with regioselective alkylation of 5-bromouracil at N1 by a one-pot, two-step procedure using HMDS and benzyl bromide in the presence of a catalytic amount of iodine to obtain compound **2** (Scheme 1).[9] Activation of **2** by conversion to the O^4-sulfonylated derivative,[10] followed by coupling with isoindol amino phenol derivative **4** yielded conjugate **5**.[5] Subsequent ring closure, facilitated by cesium fluoride, yielded phenoxazine derivative **6**.[11] Removal of the N1-protecting benzyl group by boron tribromide[12] and oxidation of the amine to a nitroxide[13] with *m*CPBA gave spin label **ç**.

The EPR spectrum of **ç** in an aqueous solution containing ethylene glycol (30%) shows three narrow lines that broaden on reducing the temperature from 0 to −30 °C (Figure 2, left), due to slower tumbling of **ç** in solution.[3c,e] On mixing a DNA duplex containing an abasic site with **ç**, a slow-moving component appears in the EPR spectrum (shown by arrows, Figure 2 middle), indicating binding of the spin label to the abasic site. On further cooling, the extent of spin-label binding increased, and at −30 °C the narrow lines (the fast-motion component of the spectrum) had completely disappeared, consistent with the spin label being fully bound. For comparison, EPR spectra of a covalently **Ç**-labeled 14-mer were recorded under identical conditions (Figure 2, right). The mobility of the spin label that is covalently linked to the dsDNA is the same as that of the slow-moving component in the sample containing **ç** and the abasic DNA (Figure 2,

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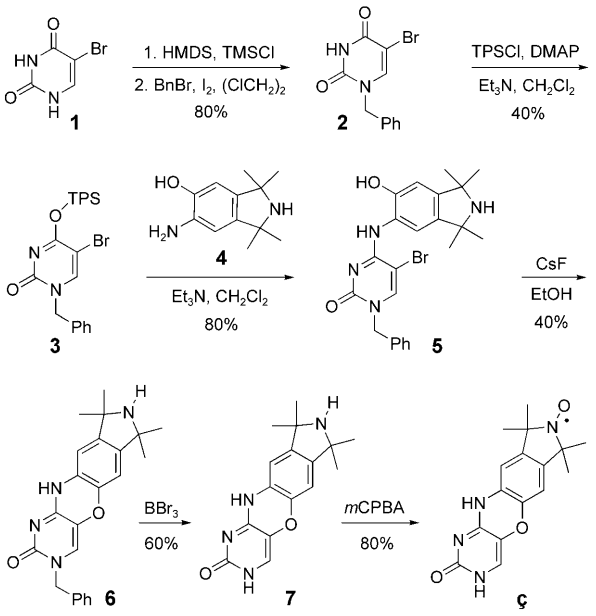
[**] This work was supported by the Icelandic Research Fund (080041022) and the University of Iceland Research Fund, including a doctoral fellowship to S.A.S.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201002637.



Scheme 1. Synthesis of spin label **ç**. Bn = benzyl, DMAP = 4-dimethylaminopyridine, HMDS = 1,1,1,3,3,3-hexamethyldisilazane, *m*CPBA = *meta*-chloroperbenzoic acid, TMS = trimethylsilyl, TPS = 2,4,6-triisopropylbenzenesulfonyl.

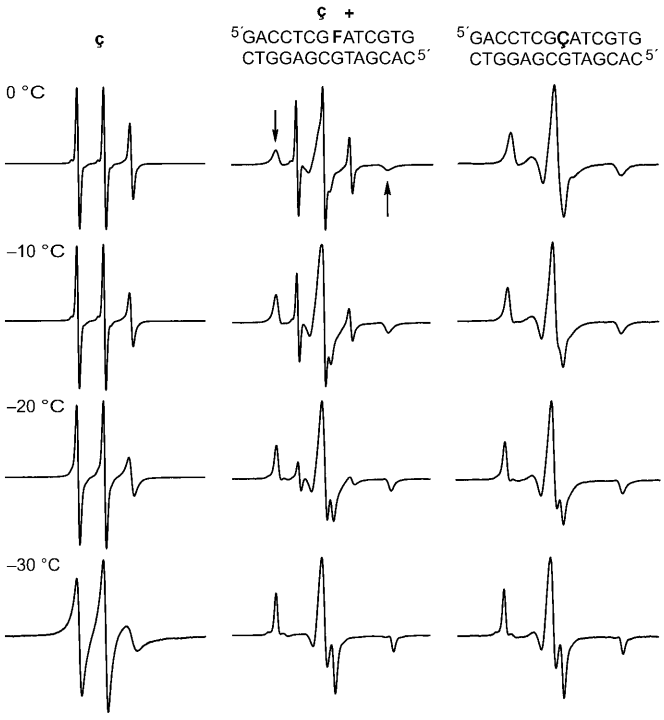


Figure 2. EPR spectra of spin label **ç** (left), of **ç** (200 μM) in the presence of abasic DNA (400 μM) (middle), and of a **Ç**-labeled 14-mer (right). The EPR spectra were recorded in phosphate buffer (pH 7.0) containing 30% ethylene glycol and 2% DMSO at different temperatures, with the same number of scans, phase corrected and aligned to the height of central peak. F denotes an abasic site.

middle) at all temperatures, and this provides further evidence for noncovalent binding of **ç** to the abasic DNA. A duplex DNA containing two abasic sites was also shown to bind nearly two equivalents of spin label (Supporting Information).

Experiments were carried out to verify that the spin label was binding directly to the abasic site, rather than nonspecifically to the DNA, for example, by intercalation or groove binding. First, spin label **ç** was titrated into a solution containing a fixed concentration of abasic DNA and EPR spectra were recorded at −30 °C (Figure 3a). At 0.5 and

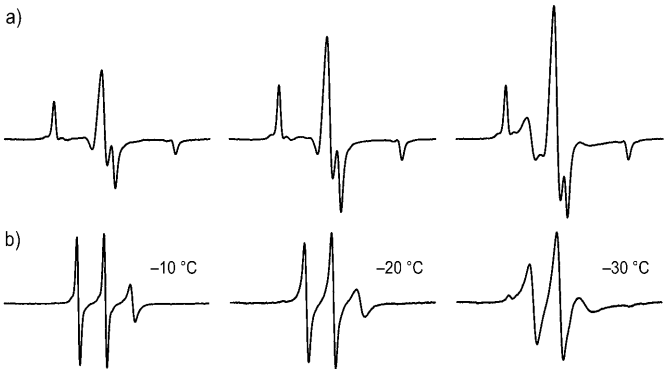


Figure 3. a) EPR spectra of 0.5 (left), 1 (middle), and 2 equiv (right) of spin label **ç** in the presence of a dsDNA (100 μm) containing an abasic site, recorded at −30 °C with the same number of scans and phase-corrected. The DNA sequence is the same as for the middle panel in Figure 2. b) EPR spectra of spin label **ç** (100 μM) in the presence of an unmodified dsDNA (100 μM) containing the same sequence as in a) except that the abasic site has been replaced by C. The EPR spectra were recorded in phosphate buffer (pH 7.0) containing 30% ethylene glycol and 2% DMSO.

1.0 equiv of **ç**, the spin label was fully bound, whereas both the bound and excess free spin label were observed for 2.0 equiv of **ç**. Thus, the spin label binds readily until the abasic binding site has been saturated. Second, the spin label was incubated with an unmodified DNA duplex of the same sequence, that is, a sequence that did not contain an abasic site. No binding was observed down to −20 °C, but at −30 °C a small amount (< 5%) of binding was observed (Figure 3b), and indicates minor nonspecific binding in the absence of an abasic site. Third, a duplex containing a G overhang did not show increased nonspecific binding (Supporting Information), although the spin label can, in principle, bind to the unpaired G through Watson–Crick pairing while stacking at the end of the duplex. This experiment clearly shows that, in addition to hydrogen bonding, efficient binding requires π stacking of both faces of the spin label, as provided by an abasic site in a duplex region.

The specificity of spin-label binding was also evaluated by determining the change in dissociation constant K_d for abasic DNA on addition of a large excess of unmodified DNA;[14] K_d was determined to be (6.95 ± 0.8) μM at −10 °C but (3.7 ± 0.3) μM (K_d') in the presence of a tenfold excess of unmodified DNA. High specificity of binding is reflected in the small change in the dissociation constant.

To probe specific interactions of the spin label within the abasic site, the structure of the abasic site was systematically varied. Specifically, the contribution of the base opposite the abasic site, which was expected to provide hydrogen-bonding sites for the label, to the binding affinity was evaluated. To

this end, binding of **ç** to four different DNA duplexes, containing either A, C, G, or T opposite to the abasic site, was determined by EPR spectroscopy at −10 °C. Visual inspection of the spectra showed significant differences in extent of binding; as expected, the spin label bound most efficiently when it was paired with G (Supporting Information). A plot of the dissociation constants of spin-label binding as a function of sequence shows a six- to eightfold higher affinity of the spin label with G than with A, C, or T (Figure 4). The

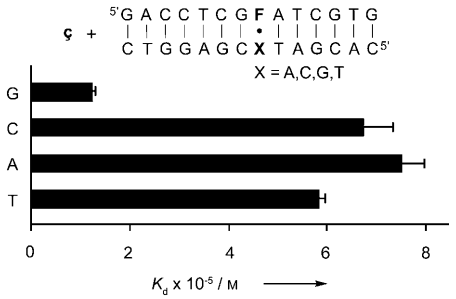


Figure 4. Dissociation constants of **ç** binding to dsDNA as a function of the nucleotide opposite to the abasic site (denoted F), determined at −10 °C (400 μM abasic dsDNA, 200 μM **ç** in a phosphate buffer, pH 7.0, containing 30% ethylene glycol and 2% DMSO). The DNA sequence is shown on top.

spin label binds with similar affinity to A and C and slightly higher affinity to T, in agreement with previously reported stability data for C mismatches.[15] This data is, therefore, supportive of a successful design: the spin label binds specifically in the abasic site, where it forms hydrogen bonds with G opposite to the abasic site.

In summary, we have developed a general and straightforward approach for site-directed spin labeling of nucleic acids using receptor–ligand interactions. Binding sites can simply be carved out at specific locations in the nucleic acid by incorporation of abasic sites, as long as the labeling site is in a duplex region. This strategy has clear advantages over the traditional spin-labeling methods using spin-labeled phosphoramidites or post-synthetic labeling, as it enables labeling by simply adding the spin label to a solution of commercially available nucleic acids that contain abasic sites. Since the design of **ç** is based on the rigid spin label **Ç**, it should have the same spectroscopic properties and facilitate extraction of both accurate distances and relative orientations by EPR spectroscopy. Although the noncovalent, site-directed spin-labeling (NC-SDSL) method requires cooling the sample to −30 °C to achieve complete binding, it can be readily used for structural studies by pulsed EPR spectroscopic techniques such as DEER or PELDOR, which are all carried out in frozen solutions. Easy access to site-specifically labeled nucleic acids will facilitate their structural studies by pulsed EPR spectroscopy, and applications along these lines will be reported in due course.

Received: May 2, 2010
Revised: July 7, 2010
Published online: September 15, 2010

Keywords: DNA · EPR spectroscopy · nucleic acids · receptor–ligand interactions · spin labels

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