Chapter 8
Site-Directed Spin Labeling for EPR Studies of Nucleic Acids

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Abstract Electron paramagnetic resonance (EPR) spectroscopy has emerged as a valuable technique to study the structure and dynamics of nucleic acids and their complexes with other biomolecules. EPR studies require incorporation of stable free radicals (spin labels), usually aminoxyl radicals (nitroxides), at specific sites in the nucleic acids using site-directed spin labeling (SDSL). In addition to the advancement of EPR instrumentation and pulsed EPR techniques, new strategies for SDSL have emerged, in particular, use of click chemistry, biopolymer catalysis, and noncovalent labeling. Furthermore, tailor-made spin labels with improved stability and spectroscopic properties have evolved, such as rigid spin labels that allow determination of accurate distances in addition to orientations between two spin labels. This chapter gives an overview of nucleic acids spin labeling using the three main strategies of SDSL, namely spin labeling during oligonucleotide synthesis, post-synthetic-, and noncovalent labeling. The spin-labeling methods have been categorized according to the labeling site.

8.1 Introduction

Nucleic acids are the reservoir of genetic information for all living organisms. DNA carries the genetic blueprint, which is transmitted to the ribosome through RNAs via transcription and translation. A subtle structural difference between DNA and RNA, namely the presence of a 2′-hydroxyl group on the sugar moieties of the latter, leads to much more diverse structural and chemical properties of RNA. RNA is not only a carrier of genetic information, it also carries out a wide range of other cellular functions central to the life, such as catalysis of chemical reactions by

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ribozymes [1–3] that are found in the catalytic core of both ribosomes [4] and spliceosomes [5], regulation of gene expression by small interfering RNAs [6], metabolite-responsive regulatory control by riboswitches [7, 8], protein recognition, and cellular signaling [9]. These wide ranges of functions are attributed to their flexibility and ability to fold into complex three-dimensional structures. During the last few decades, a variety of biochemical and biophysical methods has been utilized to investigate the structural basis of the functions of these biomolecules.

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are high-resolution techniques that give precise information about three-dimensional arrangements of atoms in space. Although NMR spectroscopy is useful for studying structure and dynamics of biomolecules under biologically relevant conditions, it is still limited by the molecular weight of biopolymers, of up to approximately 50 kDa [10, 11]. Moreover, the range of distances between atoms that can be deduced by NMR, and are used to generate a three-dimensional structure, are limited to only a ca. 20 Å, although residual dipolar coupling (RDC) has been used to get information about the orientation of helical domains [12]. X-ray crystallography also suffers from technical challenges. For example, it remains difficult to obtain crystals of biomolecules that diffract well. This is especially true for RNAs due to their tendency to misfold, oligomerize and, in general, accommodate conformational heterogeneity. Additionally, a crystallized form may not represent a biologically active conformation.

Lower-resolution spectroscopic techniques, such as Förster resonance energy transfer (FRET) [13–15] and electron paramagnetic resonance (EPR) [16–24], have proven to be valuable for the study of structure and dynamics of biopolymers and are complementary to NMR and X-ray crystallography. Both FRET and EPR are highly sensitive and thus require small amounts of material. They can be used to map distances of up to 100 Å, enabling observation of long-range conformational changes that are triggered by a change in conditions or upon binding to other biomolecules [25, 26]. Both techniques require incorporation of reporter groups for distance measurements. In the case of FRET, two different fluorophores are required; a donor and an acceptor that are usually connected with a flexible tether, that yield moderate-to-large distance distributions. Another potential complication for FRET is that the efficiency of the fluorescence transfer depends on the relative orientation of the two fluorophores. An important feature of FRET is that it can be used for single-molecule studies [27, 28]. There are also a few other low-resolution techniques, such as small angle X-ray scattering (SAXS) [29] and circular dichroism (CD) spectroscopy [30], that have been used for probing the global shape and conformational folding of nucleic acids.

EPR spectroscopy, also called electron spin resonance (ESR), was first reported by Zavoisky in 1945 [31]. EPR is a highly sensitive and useful technique to probe the local environment of paramagnetic centers. As such, it can probe polarity and solvent accessibility. In addition, the reporter groups (spin labels) that are commonly used for EPR studies are relatively small, compared to other exogenous tags (such as fluorophores), which makes them less perturbing to the native structure of

biopolymers. Like NMR, EPR is based on the principles of magnetic resonance, interrogating spins of unpaired electrons, such as those present in free radicals. EPR detects transitions of electron spins from a lower to a higher energy level, induced by absorption of electromagnetic (microwave) radiation in the presence of an applied external magnetic field. The magnetic moment of an unpaired electron can also interact with neighboring nuclei, usually referred to as hyperfine coupling, and split each electronic spin state into $2I + 1$ levels, where I is the spin quantum number of the nuclei. The energy levels of an electron can also be affected by the presence of other electron spins through both exchange- and dipolar coupling.

The mobility of a radical is reflected in the shape of its continuous wave (CW) EPR spectrum. Figure 8.1 shows the EPR spectra of an aminoxyl radical (usually called a nitroxide, Fig. 8.1) in the fast, intermediate, and slow motion regime. The EPR spectrum of a nitroxide has three lines, due to the hyperfine coupling to the nitrogen atom ($I = 1$). As the motion of the nitroxide slows down, the spectrum becomes broader. The mobility of a spin label attached to a biopolymer is a combination of motions of the linker used to attach the spin label as well as the local and global motions of the biopolymer itself. This feature can be used to indirectly extract structural information about the dynamics of the biopolymer, sometimes referred to as structure-dependent dynamics [32–34].

Most structural studies of biopolymers with EPR are based on distance measurements between spin labels, made possible through dipolar coupling. CW-EPR can be used for measuring intermediate distances, lower than 25 Å [35, 36]. However, when the distances between the spin labels are larger, the dipolar couplings become smaller than the inhomogeneous broadening of the EPR spectrum, caused by unresolved surrounding hyperfine couplings. In order to resolve these long-range dipolar couplings from the inhomogeneous line broadening, pulsed EPR techniques are required. The most widely used pulsed EPR technique, pulsed electron–electron double resonance (PELDOR), also called double electron–electron resonance (DEER), can yield accurate distances in the range of 15–100 Å [20, 21, 37–39]. In this technique, short (5–30 ns long) but intense microwave pulses (in kW) are used, usually applying the four-pulse method [40, 41]. EPR can also yield information about relative orientations between two interacting spin centers, which can provide additional constrains to build more accurate structural models [42–

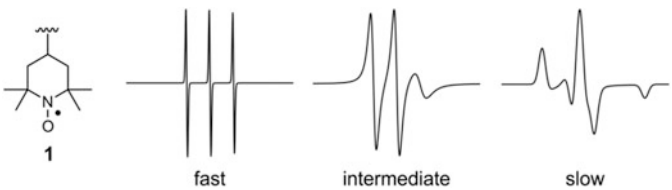


Fig. 8.1 Structure of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) radical (**1**) and its CW-EPR spectra in the fast, intermediate, and slow motion regime, respectively, showing the effect of its mobility on the spectral line shapes

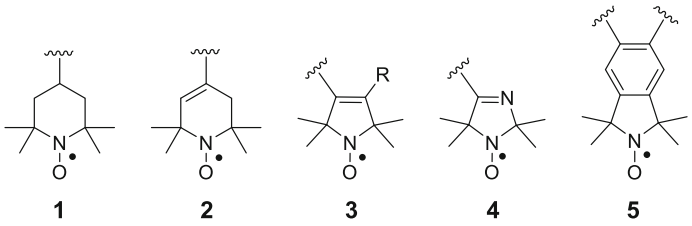


Fig. 8.2 Structures of the most commonly used nitroxide radicals for spin labeling

45]. This requires the use of rigid spin labels [46, 47], which have also enabled determination of the internal dynamics of nucleic acids [25, 48].

Although some biopolymers contain intrinsic paramagnetic centers, such as metal ions [23], most are diamagnetic and require incorporation of spin labels for EPR studies. Nitroxides are the most commonly used spin labels due to their persistent nature and relatively small size [49, 50]. Three factors contribute to the stability of nitroxide spin labels, delocalization of the unpaired electron between the oxygen and nitrogen atoms, electron donating effects of the alkyl groups on the carbon atoms adjacent to the nitroxide, and the steric shielding by the alkyl groups. Figure 8.2 shows the structures of the nitroxide moieties most commonly used for spin labeling, classified into three groups according to the size of the nitroxide-bearing ring: six-membered piperidines (**1**, **2**), five-membered pyrrolines (**3**, **4**), and isoindolines (**5**). The size of the nitroxide-bearing ring and the nature of its substituents affect the stability of the nitroxides, especially under reducing conditions [51–56]. Nitroxides are almost exclusively the spin labels of choice for EPR studies, although carbon-centered trityl radicals have recently been reported [57–59] as well as paramagnetic metal ions, such as Gd^{3+} [60].

This chapter gives an overview of site-directed spin labeling (SDSL), starting with strategies used for nucleic acids, followed by a fairly comprehensive description of nucleic acid spin labeling. The structures of the spin-labeled nucleotides will be shown, and the spin-labeling methods will be narrated. The organization of the material describing covalent labeling is by the labeling sites and will begin with nucleobase labeling, followed by labeling of the sugar moiety and the phosphate backbone. After that, noncovalent labeling will be covered, and the chapter concludes with a short perspective.

8.2 Nucleic Acid Spin Labeling

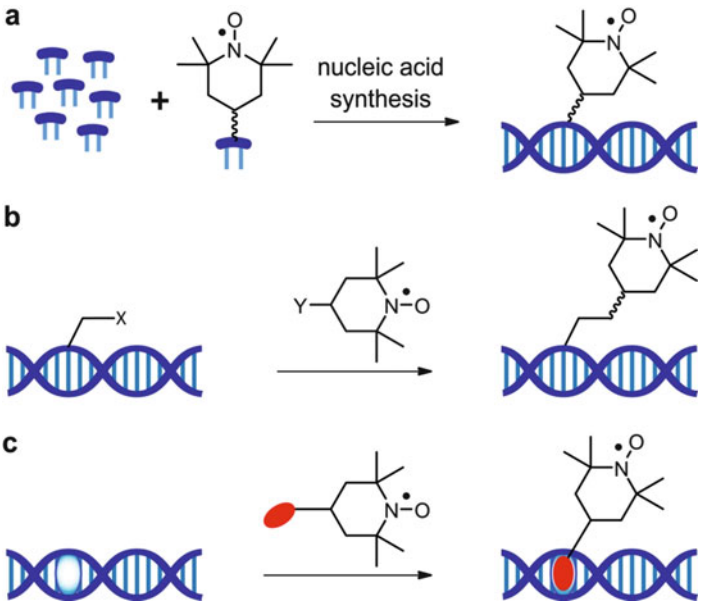
In 1965, McConnell introduced the concept of biopolymer spin labeling using nitroxide radicals [61, 62], which was initially applied to proteins [16, 18, 63] and subsequently to nucleic acids [22, 64]. During the early days of spin labeling, when automated chemical synthesis of nucleic acids was not available, spin labeling was performed by alkylation reviewed in [65]. However, due to the presence of

several reactive functional groups on the nucleic acid, this approach lacked specificity. This chapter will focus on spin labeling at selected sites.

While choosing the spin labels, a few criteria must be considered. First, the spin label has to be compatible with the synthetic methods used to prepare the spin-labeled biopolymers [47, 66]. Second, the label should be stable enough to allow EPR measurements under biologically relevant conditions [51, 67–69]. Third, the spin label must be non-perturbing to the native structure of the nucleic acid and its function, which is usually evaluated by thermal denaturation of nucleic acid helices and functional assays with and without the spin labels. Fourth, the structure of the linker for attachment of the spin labels to the nucleic acid must be carefully chosen. As mentioned earlier, the shape of an EPR spectrum is sensitive to the motion of the spin label. If the spin label is attached to the nucleic acid with a long and flexible linker, the EPR spectrum will be dominated by the motion of the spin label and has, therefore, limited use for studies of dynamics. Also, such labels will give a larger distance distribution, which reduces the accuracy of the distance measurements. In contrast, spin labels that are attached with a rigid linker that does not move independently of the biopolymer will report the actual dynamics of the site to which they are attached [70] and also yield accurate distances between two such labels [45, 48]. In some instances, a semi-flexible linker can be advantageous for studying conformational changes [71, 72] and binding interactions with other molecules [69, 73]. Although these labels yield less accurate distances, compared to the rigid spin labels, they show less orientational effects in PELDOR measurements, which can simplify the EPR measurements and data processing [74].

Another key aspect to be considered for SDSL is the spin-labeling method. There are three main strategies used for incorporation of spin labels at chosen sites (Scheme 8.1). The first two approaches rely on covalent attachment of the spin label, while the third approach takes an advantage of noncovalent interactions. The first approach utilizes incorporation of the spin labels during the chemical synthesis of nucleic acids by employing spin-labeled phosphoramidite building blocks (Scheme 8.1a). Due to advancement of automated chemical synthesis of nucleic acids, tailor-made and structurally complex labels can be incorporated at specific sites using this approach [24, 64, 75]. However, synthesis of the spin-labeled phosphoramidite building blocks is sometimes laborious and challenging. Furthermore, spin labels can be partially reduced upon exposure to the chemicals involved in oligonucleotide synthesis [47, 66].

The second SDSL approach is incorporation of spin labels into the nucleic acid after synthesis of the oligonucleotide, referred to as post-synthetic spin labeling (Scheme 8.1b). In this method, modified nucleotide(s) containing a uniquely reactive functional group, such as 4-thio-uridine [76, 77], a 2′-amino nucleoside [32, 35, 78], a phosphorothioate [79], an alkyne [80, 81], or a 2′-azido nucleoside [82], are incorporated at specific site(s) using either chemical or enzymatic synthesis. The modified oligonucleotides are subsequently reacted with a spin-labeling reagent containing the appropriate functional group; examples of functionalized spin labels for post-synthetic labeling are shown in Fig. 8.3. Post-synthetic labeling has the advantage of minimizing possible decomposition of the nitroxide during the



Scheme 8.1 A general scheme of the three main strategies used for site-directed spin labeling of nucleic acids, using TEMPO as a representative nitroxide. (**a**) Labeling during nucleic acid synthesis. (**b**) Post-synthetic labeling, where X and Y represent functional groups that undergo reaction to form a covalent bond between the spin label and oligonucleotide. (**c**) Noncovalent spin labeling

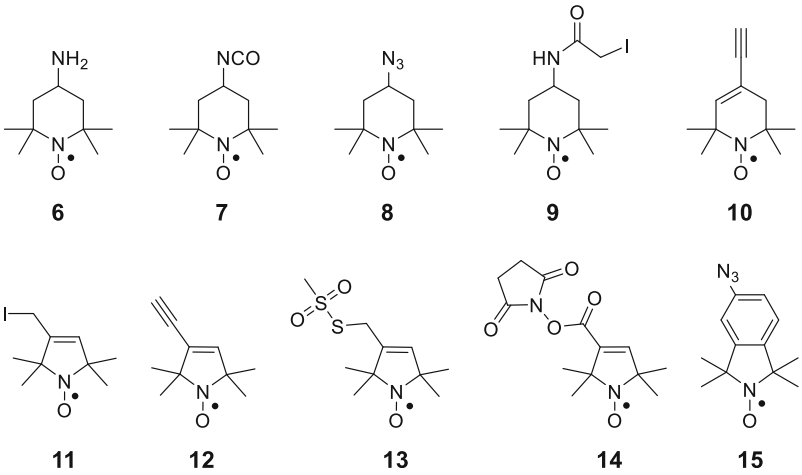


Fig. 8.3 Structures of nitroxide reagents commonly used in post-synthetic spin labeling of nucleic acids

oligonucleotide synthesis. It is also less labor intensive and often utilizes modified oligonucleotides and reagents obtained from commercial sources. Disadvantages of the post-synthetic method include incomplete labeling and possible side reactions of the spin-labeling reagent with innate functional groups of nucleic acids, such as the exocyclic amino groups of the nucleobases.

The third SDSL approach is based on ligand–receptor interactions, where a spin-labeled ligand binds through noncovalent interactions such as hydrogen bonding, ionic-, and Van der Waals interactions (Scheme 8.1c). Although there are only a few examples of using this approach for nucleic acids, there are several examples in protein spin labeling, where an active site of an enzyme or cofactor binding site has been utilized for site-specific binding of spin-labeled derivatives of their natural ligands (reviewed in [65]). The spin label ligand binding can be easily monitored by EPR spectroscopy as bound and free ligands have very different rotational correlation times [83]. Although this noncovalent strategy circumvents challenges associated with both of the aforementioned SDSL approaches that utilize covalent bonding, it requires binding sites that have a relatively high affinity for their spin-labeled ligands in order to get enough labeling for EPR studies.

8.2.1 Base Labeling

Nucleobases are the most common sites for incorporation of spin labels into nucleic acids, due to the availability of various attachment sites and functional groups that can be readily modified using a variety of different organic synthetic methods. Another advantage of nucleobase labeling is that the attached spin labels can readily be accommodated in one of the grooves, in particularly the major groove and thereby cause minimal structural perturbation. In addition to modification of the exocyclic amino groups, spin labels have been incorporated into the *C2*, *C*4, and *C*5 positions of pyrimidines. In particular, the *C*5 position is the most frequently used because of the availability of relatively simple conjugation methods, such as transition-metal-catalyzed coupling to *C*5-halogenated nucleobases and copper-catalyzed cycloaddition reactions between *C*5-alkynes and azido-nitroxides. Purine nucleobases are spin-labeled at the *C*2, *C*6, and *C*7 positions, where *C*7 is labeled by using 7-deaza nucleobase analogues. The following section contains a brief description of nucleobase spin labeling, starting with labeling of exocyclic amino groups, followed by pyrimidine and purine spin labeling through C–C bond formation. The last section describes rigid spin labels.

8.2.1.1 Exocyclic Amino Groups of Pyrimidines and Purines

The exocyclic amino groups of cytosine (*N*4), guanine (*N*2), and adenine (*N*6) nucleobases have all been modified with a spin label. Although amino groups of nucleobases are involved in base pairing and structural integrity of nucleic acid

helices, a single substitution on an amino group still allows one proton to participate in hydrogen bonding to its complementary base. Bannwarth and Schmidt demonstrated the synthesis of spin-labeled phosphoramidites of the *N*4-TEMPO-modified 2′-deoxycytidine (**16**) (Fig. 8.4a) and 5-methyl-2′-deoxycytidine (**17**) and their incorporation into DNA oligonucleotides [84]. Subsequently, Giordano and

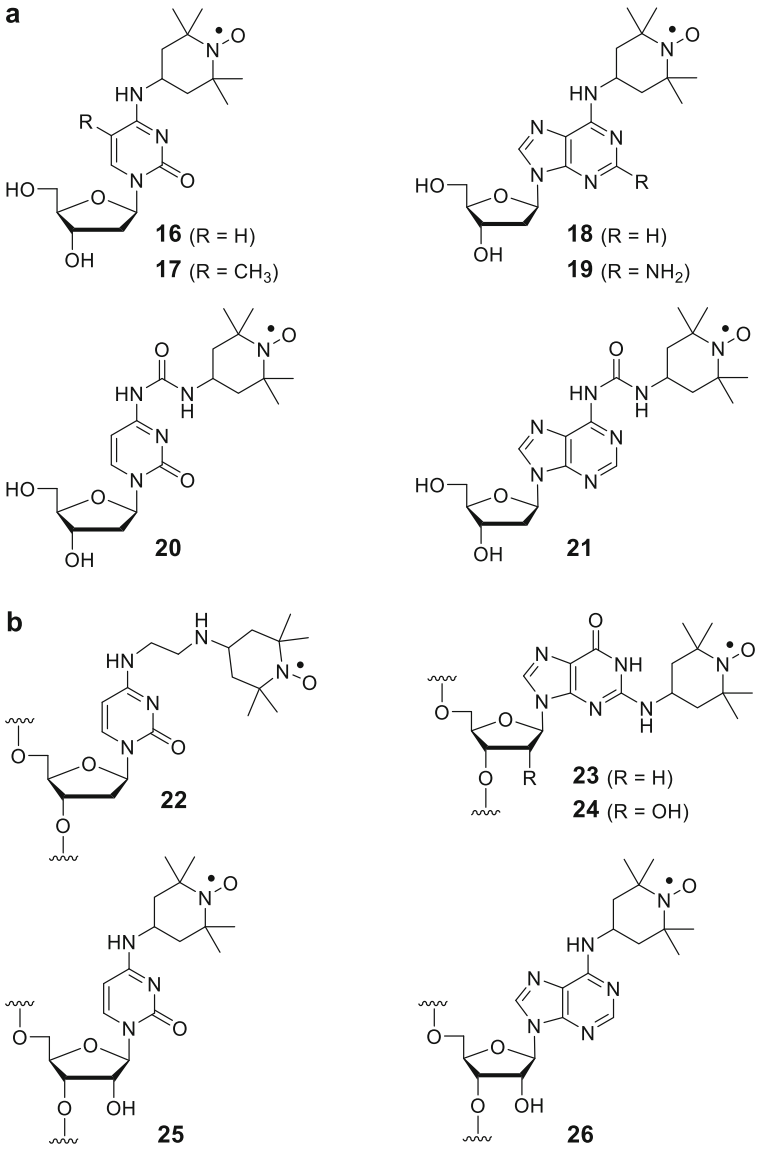


Fig. 8.4 TEMPO-labeled exocyclic amino groups of nucleosides/nucleotides using the phosphoramidite approach (**a**) and the post-synthetic convertible nucleoside approach (**b**)

coworkers reported improved yield of these phosphoramidites, along with the synthesis and incorporation of spin-labeled 2′-deoxyadenosine (**18**) and 2-amino-2′-deoxyadenosine (**19**) into DNA [85]. Since the nitroxides in these labels are directly connected to the nucleobases, their motion is sensitive to the microenvironment of the amino group, in particular, hydrogen bonding. This feature has been utilized to demonstrate that spin label **16** can not only detect mismatches but also identify its base-pairing partner in duplex DNA [86]. The spin-labeled nucleosides **20** and **21** (Fig. 8.4a), where TEMPO is connected to the exocyclic amino groups of C and A through a semi-flexible urea linkage, have also been incorporated into DNA oligomers using the phosphoramidite approach [87].

The exocyclic amino groups have also been modified by a post-synthetic modification through the convertible nucleoside approach, developed by Macmillan and Verdine [88]. In this method, a leaving group is displaced by an amine at the end of the chemical synthesis, which also deprotects the oligonucleotide and cleaves it from resin. The flexible spin label **22** (Fig. 8.4b) has been incorporated into DNA using this strategy and was used for studying dynamics as well as DNA–protein interactions by high-field EPR [89, 90]. A similar approach has been used to label the exocyclic amino group of guanine by treating 2-fluorohypoxanthine-containing oligonucleotides with 4-amino TEMPO to afford nucleotide **23**, used for studying DNA hybridization and folding of G-rich DNA sequences into G-quadruplex [91]. This spin label (**23**) has also been used to probe conformational transitions between duplex DNA [92] and structural changes induced by lesions in DNA duplexes using pulsed EPR spectroscopy [93]. Spin labels have also been installed on the exocyclic amino groups of RNA nucleobases guanine (**24**), cytosine (**25**), and adenine (**26**) (Fig. 8.4b) with good yields by Höbartner and coworkers and used for mapping secondary structures of RNAs by pulsed EPR spectroscopy [94]. More recently, the same group reported a strategy for SDSL of long RNAs that are beyond the limit of solid-phase oligonucleotide synthesis, which entailed a ligation of short spin-labeled RNA oligonucleotides to an in vitro transcribed RNA, catalyzed by a deoxyribozyme. This method was used to synthesize the *S*-adenosylmethionine-I (SAM-I) riboswitch containing **24** [95].

8.2.1.2 C5 of Pyrimidines

Conjugation of spin labels through amino groups has been reported for the 5-position of uridines, such as nucleosides **27** and **28**, which were incorporated into oligonucleotides using phosphotriester-based synthesis (Fig. 8.5a) [96, 97]. However, the first spin-labeled phosphoramidite for the incorporation of spin labels into DNA by automated chemical synthesis was reported by Hopkins and coworkers [98]. A spin-labeled uridine (**29**) and a cytosine (**30**) were prepared by a palladium-catalyzed Sonogashira cross-coupling reaction between their corresponding 5-iodo analogues and the nitroxide 2,2,5,5-tetramethylpyrrolin-1-yloxy-3-acetylene (TPA) [98, 99]. Later, Prisner and coworkers developed an on-column version of this method, where the coupling was performed during the

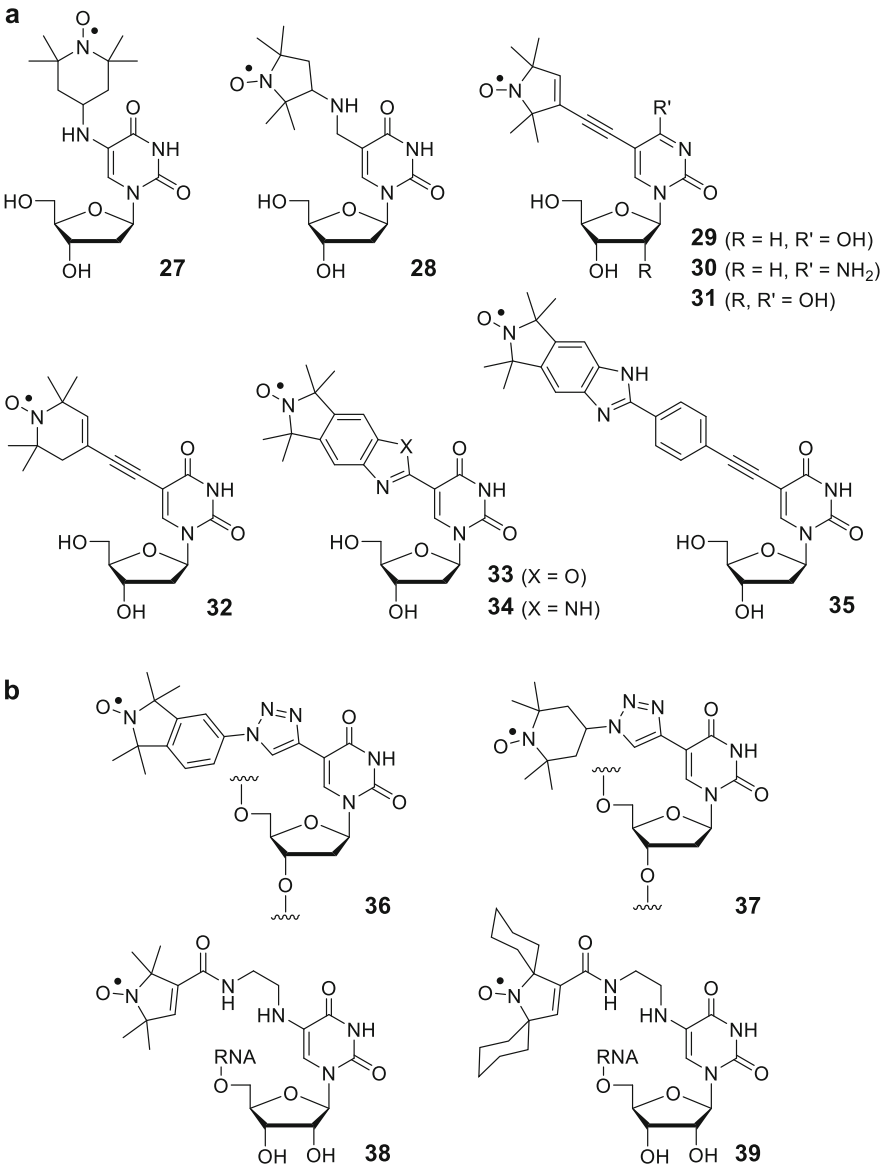


Fig. 8.5 *C*5-labeled pyrimidines. (**a**) Spin labels incorporated by the phosphoramidite method. (**b**) Spin labels incorporated post-synthetically

solid-phase synthesis of both DNA (**29**) [100, 101] and RNA (**31**) [66]. The TPA label is connected to the nucleobase by a short linker that has only rotation around the single bonds flanking the acetylene and has been a useful probe for measuring accurate long-range distances in nucleic acids by PELDOR [66, 69, 73, 100,

101]. Spin label **32** is of similar design as the TPA label and has relatively short synthesis compared to the TPA [102] and has been used to investigate G-quadruplex formation in human telomeric DNA by DEER [103].

Recently, we have reported the synthesis and incorporation of the isoindoline-derived spin labels **33** and **34** (Fig. 8.5a) into DNA oligonucleotides. These labels showed limited mobility in duplex DNA, especially **34**, where an intramolecular hydrogen bond between the *N*–H of the imidazole and *O*4 of the uracil restricted rotation around the bond connecting the spin label to the base [104]. A structurally similar but more flexible spin label (**35**) was also incorporated into DNA by the phosphoramidite approach [74]. Spin labels **32**–**35** are advantageous for distance measurements as the *N*–O bond of the nitroxide lies on the same axis as the rotatable single bonds linking the label to the nucleobase, thereby causing limited displacement of the nitroxide, relative to the nucleobase, upon bond rotation [74].

C5-labeled pyrimidines have also been incorporated into nucleic acids using post-synthetic labeling. The Cu(I)-catalyzed Huisgen–Meldal–Sharpless [3 + 2] cycloaddition reaction (click reaction) has been used to incorporate an isoindoline-derived spin label by an on-column reaction of an azido-nitroxide (**15**, Fig. 8.3) with a DNA oligomer containing 5-ethynyl-2′-deoxyuridine to produce **36** (Fig. 8.5b), which was used for probing local structural lesions in duplex DNA, such as abasic sites and mismatches [81]. Subsequently, Seela and coworkers used a similar click chemistry approach for incorporation of spin label **37,** which was used for distance measurements in DNA oligonucleotides and for studying DNA structure and DNA–protein interactions [105]. Spin labels **38** and **39** have also been incorporated into RNA oligonucleotides using post-synthetic labeling. Spin label **39** with the improved stability toward nitroxide reduction and longer relaxation time was used for distance measurements using Q-band DEER [106].

8.2.1.3 Other Pyrimidine Modifications

During early 1970s, thio-modified nucleotides, which are generally found in tRNAs, were used for SDSL of nucleic acids. For example, 4-thiouridine found in tRNAs of *E. coli* was selectively spin-labeled by alkylation under mild reaction conditions to afford spin-labeled tRNA (**40**) (Fig. 8.6) without affecting their activity [107]. Similarly, 2-thiocytidine has been used for spin labeling of tRNA, which can be enzymatically incorporated into tRNAs using tRNA nucleotidyl transferase and alkylated to yield spin-labeled nucleotide **41** [108]. Dugas and coworkers reported site-specific spin labeling of *E. coli* tRNA using the rare base 2-thio-5-(*N*-methylaminomethyl)-uridine, present in the anticodon region of Glu-tRNA, by acylation to yield nucleotide **42** [109].

After the development of phosphoramidite chemistry and solid-phase synthesis of oligonucleotides, 4-thiouridine has been site-specifically incorporated into RNA oligonucleotides and used for SDSL of RNAs by reacting it with thio-specific spin-labeling reagents to yield spin-labeled nucleotides, such as **40** [77, 110] and **43**–**45** (Fig. 8.6) [76, 110]. Spin-labeled nucleotides **44** and **45** were shown to have

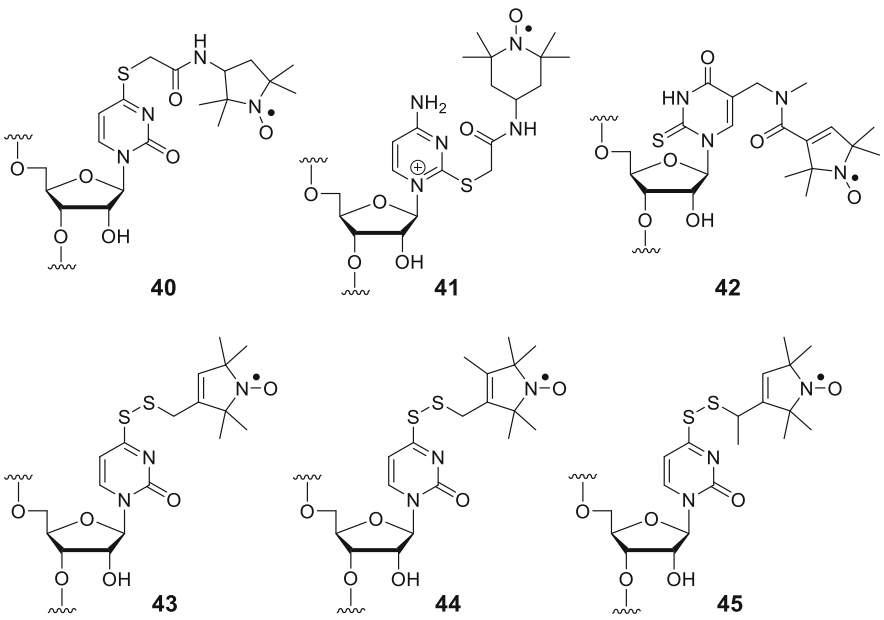


Fig. 8.6 Spin-labeled thiouridines and cytosine in RNA, from post-synthetic labeling

restricted internal motion, relative to the nitroxide **43**, due to the presence of an extra methyl group. These spin labels have been used for studying conformational changes and dynamics of RNA duplexes [76, 111, 112] as well as a synthetic tetracycline RNA aptamer upon ligand binding using pulsed EPR spectroscopy [113].

8.2.1.4 Other Purine Modifications

As described above, purines have mostly been spin-labeled at their exocyclic amino groups (**18**, **19**, **21**, **23**, **24**, and **26**, Fig. 8.4) as shown in the aforementioned section; however, there are a few examples where purines have been spin-labeled by carbon–carbon bond formation. Spin-labeled nucleotide **46** (Fig. 8.7) was prepared by a post-synthetic Diels–Alder [4 + 2] cycloaddition reaction of a nitroxide-functionalized maleimide with 7-vinyl-7-deaza-2′-deoxyguanosine [114]. Spin label **47** was incorporated into RNA using an on-column Sonogashira cross-coupling reaction between 2-iodo adenine and an alkyne-functionalized nitroxide (**12**, Fig. 8.3), during the chemical synthesis of the oligomers [66]. The spin-labeled 7-deazaadenosine analogue **48** was prepared by a post-synthetic click reaction with an alkyne-modified DNA for distance measurements by PELDOR [80]. Spin label **49** has recently been incorporated post-synthetically into RNA oligonucleotides by using a previously reported strategy [115], in which a linker containing an aliphatic amino group was delivered to a chosen RNA nucleobase (guanosine in this case)

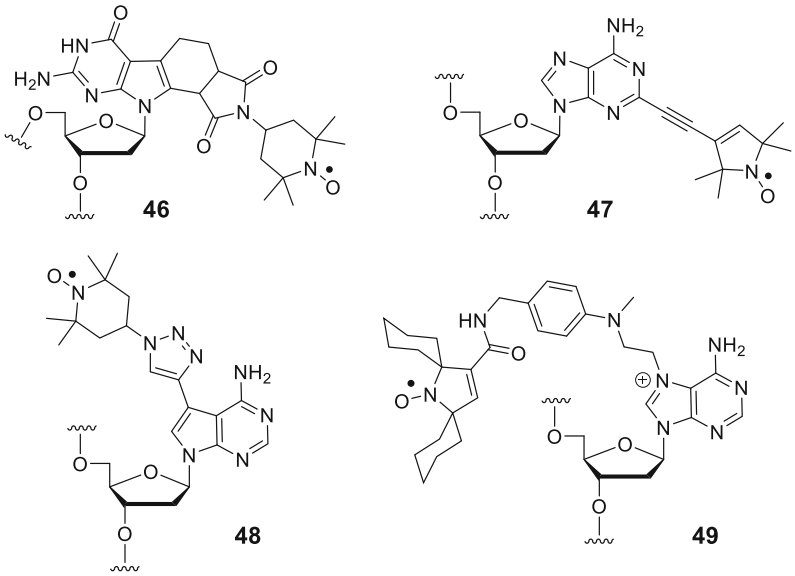


Fig. 8.7 Miscellaneous spin-labeled purine nucleotides

using a complementary DNA reagent and subsequently acylated with a spin-labeling reagent [106]. Kieffer and coworkers have used a DNA-splint-mediated ligation strategy to incorporate 6-thioguanosine at internal sites of long RNA followed by alkylation with a nitroxide to yield spin-labeled RNAs [116].

8.2.1.5 Rigid Labels

The spin labels described thus far have been attached through either flexible or semi-flexible linkers. As mentioned earlier, such labels cannot accurately report the actual dynamics of the nucleic acids, and the distance measurements by EPR using these labels result in wider distance distributions. Therefore, the ideal spin label does not move independently of the nucleic acid to which it is attached. Such spin labels are here referred to as rigid spin labels, although there are examples in the literature where spin labels that have some mobility have been called rigid, when they should more appropriately have been called semi-rigid.

Hopkins and coworkers reported the synthesis and incorporation of the first rigid spin label (**Q**, Fig. 8.8) into DNA oligonucleotides using solid-phase chemical synthesis [117, 118]. The rigid spin label **Q** is a C-nucleoside and has been used for studying sequence-dependent dynamics of duplex DNAs [119–121]. However, **Q** has a lengthy synthesis and also requires the nonnatural base-pairing partner 2-aminopurine (**2AP**), which hampered its further use for EPR studies of nucleic acids. We have synthesized the rigid spin label **Ç** ("C-spin") (Fig. 8.8) and incorporated it into DNA oligonucleotides using solid-phase synthesis [46]. In **Ç**,

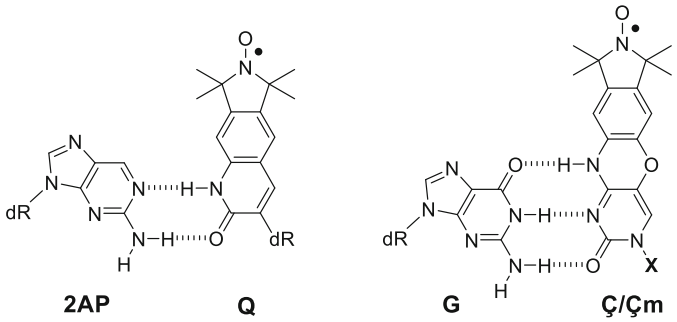


Fig. 8.8 Structures and base-pairing schemes of rigid spin label nucleosides **Q** and **Ç/Çm**, where X indicates either 2′-deoxyribose (in **Ç**) or 2′-methoxyribose (in **Çm**)

a nitroxide-bearing isoindoline ring has been fused to cytosine (C) through an oxazine linkage. The rigid spin label **Ç** can form a stable and structurally non-perturbing base pair with guanine (G), as observed in the crystal structure of a **Ç**-labeled DNA duplex [122].

Spin label **Ç** has enabled accurate distance measurements in DNA duplexes, as well as determination of the relative orientations between two such labels using pulsed EPR spectroscopy [45, 123]. Furthermore, **Ç** has been used to study the dynamics and conformations of DNA hairpin loops and bulges [47, 124] as well as motion associated with substrate recognition in a group I ribozyme [70] by CW-EPR. Spin label **Ç**, in conjunction with PELDOR, has also been used to obtain insights into internal mobility of duplex DNAs [48]. An interesting feature of **Ç** is that reduction of the nitroxide functional group yields a fluorescent probe, which has been used for both detecting single-base mismatches and to identify its base-pairing partner in duplex DNA [125–127]. The bifunctional nature of **Ç** also allowed for the study of the cocaine aptamer folding by both fluorescence and EPR spectroscopies [128]. A ribo-analogue of the rigid spin label **Ç** (**Çm**), which contains a methoxy group at 2′-position, has also been prepared and incorporated into different RNA oligonucleotides by solid-phase chemical synthesis [129] and used for distance determinations as well as orientation selections in RNA oligonucleotides by PELDOR [130].

8.2.2 Sugar Labeling

The sugar moieties of both DNA and RNA nucleotides have been used for conjugation of spin labels. However, the 2′-position is the only readily available site for labeling at internal positions of nucleic acids, which projects the label into the minor groove. In contrast, labeling of the 5′- and 3′-positions is restricted to the oligonucleotide termini. Post-synthetic modification is generally the method of choice to label the sugars. One such high-yielding spin-labeling method for RNA

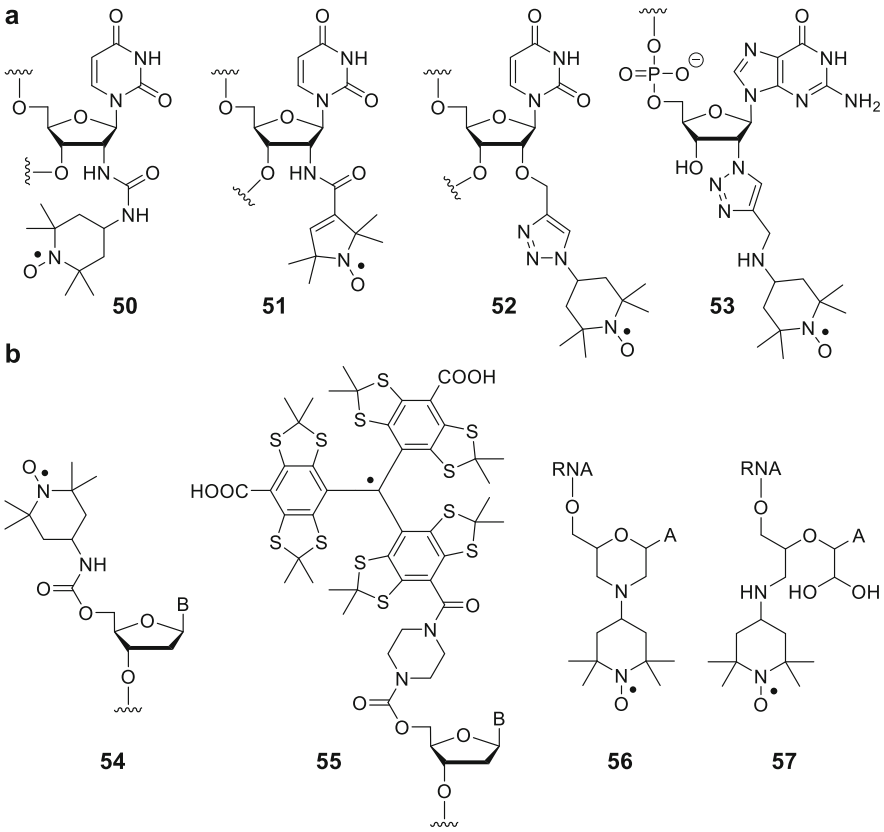


Fig. 8.9 Spin-labeled sugars. (**a**) Spin labels for incorporation at the internal positions through conjugation to the 2′-position of sugars. (**b**) Spin labels for incorporation at the 5′- and 3′-ends of nucleic acids. A stands for adenine and B for nucleobase

is the reaction of readily available 2′-amino-modified oligonucleotides with the commercially available 4-isocyanato-TEMPO (**7**, Fig. 8.3) to afford an urea-linked 2′-spin-labeled nucleotide (**50**, Fig. 8.9a) [32, 78]. Spin label **50** has been used for studying structure-dependent dynamics of the trans-activation-responsive (TAR) RNA [71, 119, 131] and metal-ion-induced folding of hammerhead ribozyme by EPR spectroscopy [132–134]. It has also been used for studying ligand-induced folding of the tetracycline aptamer [113] and for distance measurements in nucleic acids by pulsed EPR [133, 135, 136]. DeRose and coworkers have conjugated a nitroxide to the 2′-amino group through a short amide linker (**51**); however, it was found to have a destabilizing effect on RNA helices [35].

Spin labels have also been incorporated post-synthetically at 2′-positions of sugars using click-chemistry, such as the spin label nucleotide **52** [137]. This label has been used for distance measurements in DNA using DEER; however, large distance distributions were obtained due to the flexibility of the linker. Recently,

Höbartner and coworkers reported an elegant, deoxyribozyme-mediated approach for site-specific labeling of internal 2′-hydroxyls of in vitro transcribed long RNAs [82]. In this method, a 2′-labeled guanosine triphosphate (GTP) is used as a substrate for a Tb^{3+}-deoxyribozyme to install a spin label, such as **53**, on the 2′-hydroxyl group of any chosen internal adenine nucleotide through a 2′, 5′-phosphodiester linkage.

Examples of 5′-labels include TEMPO-derived spin label **54** [138] and a carbon-centered triarylmethyl (trityl or TAM) spin label (**55**) (Fig. 8.9b). The trityl labels were incorporated into short DNA oligonucleotides by coupling a trityl acid chloride with 5′-piperazine-activated short DNA oligonucleotides and used for distance measurements at physiological temperature on immobilized duplex DNA [58]. Trityl radicals have emerged as a new class of spin labels for distance measurements [57, 59, 139] and offer certain advantages over nitroxide radicals, such as a narrow spectral width, stability in reducing environment [52, 140], and a long transverse relaxation time (T_M) in the liquid state at room temperature [141]. However, trityl radicals are considerably larger than nitroxides, which limits where they can be incorporated without causing structural perturbations.

Caron and Dugas developed a 3′-end labeling strategy for tRNA using periodate oxidation of the *cis*-geminal diol of the sugar moiety at the 3′-end to make the corresponding dialdehyde, which on reductive amination with 4-amino TEMPO and sodium borohydride afforded morpholino spin label **56** [142]. A milder reducing agent, sodium cyanoborohydride, yielded spin label **57**, which showed more motional freedom than spin label **56** [143]. These labels have been used for studying 3′-end conformations and aggregations of tRNAs [143, 144].

8.2.3 Phosphate Labeling

The phosphate group of the sugar-phosphate backbone of nucleic acids is another useful site for spin labeling. Spin labels have been conjugated to the phosphorous atoms at both terminal and internal positions by replacement of one of the non-bridging oxygen atoms with the label. Advantages of phosphate labeling include the availability of post-synthetic methods using commercially available materials and the fact that phosphodiesters can be labeled independent of the nucleotide sequence and without having to prepare specifically modified nucleosides or nucleotides. Furthermore, spin labels attached to phosphorous generally interfere less with the duplex formation since they are placed at the edges of the helices. However, labeling of the phosphodiester group yields a mixture of two diastereomers, which may lead to ambiguous structural information, although the isomers of short oligonucleotides can be separated by HPLC [145, 146]. In case of an RNA phosphate labeling, the 2′-OH group adjacent to the labeled phosphodiester needs to be either replaced with a hydrogen or a 2′-OMe group, because the 2′-OH group leads to strand cleavage through 2′,3′-transesterification [147].

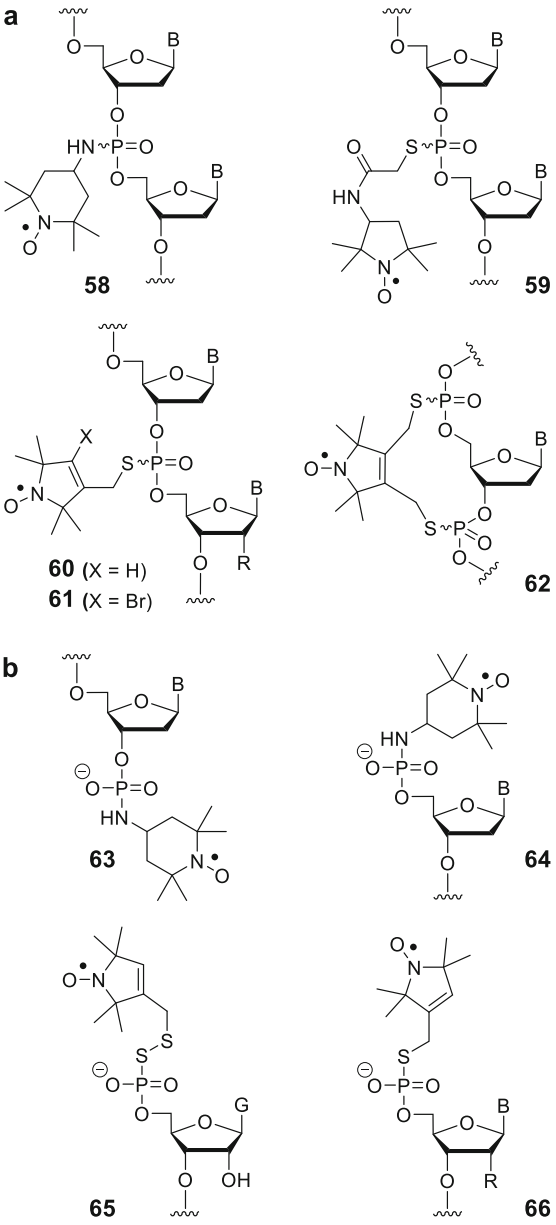


Fig. 8.10 Spin labels attached to internal phosphodiesters (**a**) and terminal phosphate groups (**b**). R stands for OH or H, and B represents a nucleobase

Phosphorous atoms of internal phosphodiesters have been spin labeled using H-phosphonate chemistry, where a hydrogen-phosphonate internucleotide linkage is introduced at a specific site during the oligonucleotide synthesis and oxidized in the presence of 4-amino TEMPO to yield phosphoramidate **58** (Fig. 8.10a) [148] or derivatives with different linkers [138]. One of the non-bridging oxygen atoms of a

phosphodiester can be replaced with sulfur by using a sulfurizing agent instead of an oxidizing agent during the chemical oligonucleotides synthesis [149]. The resulting phosphorothioate can be specifically alkylated to afford a spin-labeled nucleotide, such as **59** [150]. Similarly, RNA oligonucleotides have been spin-labeled to afford spin label **60** [79], designated as R5, which has been used for studying GNRA tetraloop–receptor interactions in RNAs [151], for dynamics [152, 153], for distance measurements [79, 154], and for studying protein–nucleic acid complexes using PELDOR [155].

Subsequently, Qin and coworkers reported a 4-bromo-substituted analogue of **60** (**61**), which has been used to study dynamics of the substrate-recognition RNA element in the group I intron ribozyme by CW-EPR spectroscopy [156], in addition to studying structure and dynamics of DNA [145, 146, 157]. Linking adjacent phosphorothioates with a nitroxide-containing bifunctional alkylating agent resulted in the conformationally restrained spin label **62** [158], similar to what has been reported for spin labeling of two cysteines in proteins [159, 160].

Due to their higher nucleophilicity, terminal phosphates are easier to modify than phosphodiesters. Dzuba and coworkers labeled both 3′- and 5′-terminal phosphates with 4-amino TEMPO to afford phosphoramidates **63** and **64** (Fig. 8.10b), respectively, and used the spin-labeled DNA to study conformational changes induced by non-nucleotide inserts in duplex DNAs by PELDOR [161]. A phosphoramidite derivative of 4-hydroxy TEMPO has been prepared and used to incorporate spin labels into the 5′-end of RNA hairpins [162]. Oligonucleotides containing terminal phosphorothioates have also been prepared by incorporation of 5′-guanosine monophosphorothioate (GMPS) during in vitro transcription of RNA using T7 RNA polymerase, which was subsequently spin labeled to afford **65** [36]. Similarly, a phosphorothioate group has been enzymatically incorporated at the 5′-position of either DNA or RNA using T4-polynucleotide kinase, followed by alkylation to yield spin-labeled nucleotide **66** [163].

8.2.4 Noncovalent Labeling

Nucleic acids have been spin-labeled noncovalently by using intercalators (reviewed in [64]). However, spin-labeled intercalating agents have limited use, because they lack sequence specificity and since multiple ligands can bind to the same nucleic acid. Lhomme and coworkers reported the first example of a noncovalent SDSL (NC-SDSL) of nucleic acids, in which a spin-labeled acridine intercalator–adenine conjugate (**67,** Fig. 8.11) was bound to an abasic site in a duplex DNA [164, 165]. The abasic site can readily be incorporated at specific sites in DNA by nucleic acid synthesis using commercially available phosphoramidites. Nakatani and coworkers prepared naphthyridine carbamate dimer (NCD, **68**) that bound specifically to G–G mismatches and used it for site-specific programmable assembly of spin probes on one- and two-dimensional DNA tiles [166–169].

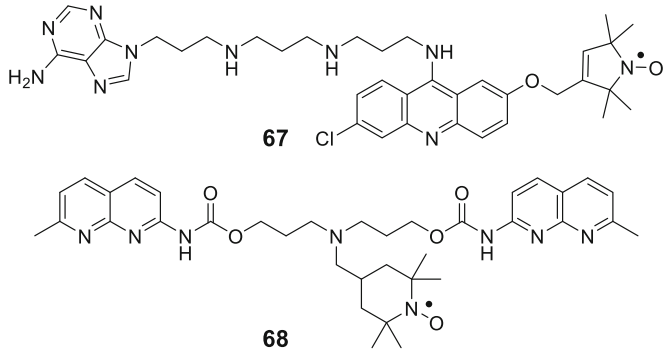


Fig. 8.11 Spin-labeled intercalator (**67**) and nitroxide-conjugated G–G mismatch-binder NCD (**68**) used for noncovalent spin labeling of nucleic acids

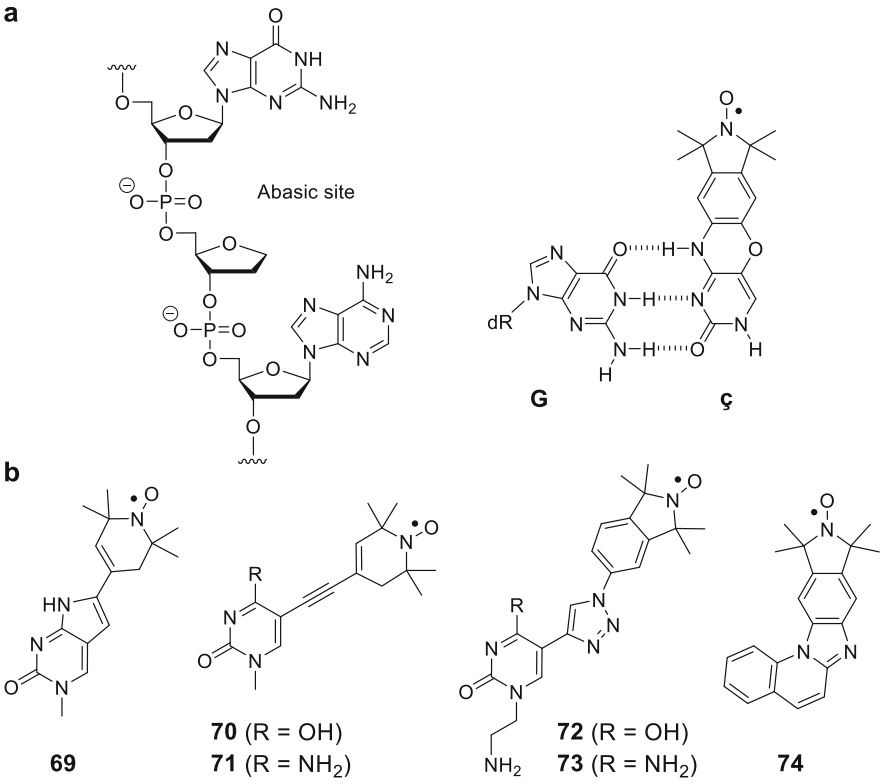


Fig. 8.12 Structure of an abasic site in DNA (**a**, *left*) and spin label **ç** base paired with G (**a**, *right*) used in NC-SDSL. (**b**) Structures of spin-labeled ligands for NC-SDSL

Our group has reported NC-SDSL of nucleic acids in which an abasic site in a duplex DNA served as a receptor for the spin-labeled ligand **ç** (Fig. 8.12a) [170]. The modified nucleobase **ç** is derived from the rigid spin label nucleoside

Ç, an analogue of the nucleobase cytosine (C). The spin label **ç** binds to the abasic site by forming hydrogen bonds with an orphan guanine base on the opposite strand and π-stacking interactions with base pairs immediately flanking the abasic site. The spin label **ç** has been used for distance measurements in duplex DNA and for studying protein-induced DNA bending using pulsed EPR [26]. A structure–function relationship study of **ç** showed that its binding is primarily governed by the identity of the base-pairing nucleotide (the orphan base) and flanking nucleotide sequence [171]. Several other derivatives of **ç** for NC-SDSL have also been reported [172].

More recently, several pyrimidine-derived spin-labeled ligands (**69–73**, Fig. 8.12b) have been screened for binding to abasic sites in nucleic acid duplexes. However, most of the labels showed lower binding affinity than **ç**, except **72**, which binds fully to abasic sites in both DNA and RNA [173]. Masters and coworkers recently reported a new class of profluorescent nitroxides, for example, spin label **74** for NC-SDSL of both DNA and RNA [174].

NC-SDSL provides a simple approach to direct spin labels to specific sites of nucleic acids and has several advantages over the other two spin-labeling strategies, the phosphoramidite- and the post-synthetic method. For example, syntheses of the spin label ligands are simpler than the spin-labeled phosphoramidites, they are relatively more stable and can be stored for extended periods of time. Furthermore, spin labeling can be performed simply by mixing the spin label ligand with the nucleic acid containing abasic sites. However, this strategy requires a binding site that has high enough affinity for the spin label ligand to ensure complete and specific binding. The NC-SDSL utilizing abasic sites is also restricted to base-pairing regions in nucleic acids.

8.3 Conclusions and Future Prospects

This chapter highlights advances in the development of site-specific spin-labeling strategies of nucleic acids. Coupled with recent advances in EPR techniques, such as pulsed EPR methods, SDSL strategies have enabled routine interrogations of the structure and dynamics of nucleic acids that give insights into their folding and functions. This includes recent examples of long RNAs, where spin labels were incorporated using either protein- or DNA catalysis. Several of the SDSL techniques are straightforward to carry out using readily available materials, which has given researchers an easy access to spin-labeled nucleic acids for EPR studies. This includes noncovalent labeling, where the label is simply mixed with a binding site for the spin label. Tailor-made spin labels with improved spectroscopic properties have evolved, for example, the rigid spin label **Ç**, which has allowed determination of relative orientations of two spin labels, in addition to accurate distance measurements in nucleic acids. The rigidity of **Ç** has also allowed the internal dynamics of DNA duplexes to be investigated by EPR spectroscopy. Carbon-centered trityl radicals have enabled distance measurements at physiological temperatures using

pulsed EPR. Trityl radicals have thus emerged as an exciting class of spin labels for EPR spectroscopy that are relatively stable under reducing conditions, which is a prerequisite for in-cell studies. While a number of spin labels have been described in the last few years, there is still a need for readily accessible spin labels with improved spectroscopic properties and stability. This will further the use of EPR spectroscopy to study nucleic acids, which has shown a great promise as a stand-alone technique or, more recently in combination with NMR spectroscopy [175] to obtain high-resolution solution structures of nucleic acids and their complexes with other biomolecules.

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