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Site-Directed Spin Labelling of Nucleic Acids

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The study of the structure and dynamics of the nucleic acids and their complexes with other biomolecules is the basis for understanding their functions. Electron paramagnetic resonance (EPR) spectroscopy is a biophysical technique that in recent years has been increasingly used to investigate nucleic acids. EPR studies require paramagnetic centre(s), usually nitroxide spin-label(s) that are incorporated at specific sites in the nucleic acid by site-directed spin labelling (SDSL). In the last few years, spin labels with improved spectroscopic properties have emerged and new SDSL techniques have been developed. This microreview describes SDSL of nucleic acids in the context of the three spin labelling strategies: post-synthetic spin labelling, labelling during oligonucleotide synthesis and noncovalent labelling.

Introduction

Nucleic acids are biopolymers that are essential to life. DNA contains the genetic blueprint whereas RNA has a wide range of functions, such as carrying genetic information and being a component of functional RNA–protein complexes such as the ribosome and the spliceosome.[1] RNA is also capable of catalysing reactions[1] and is involved in regulation of gene expression.[2] Understanding these functions of nucleic acids not only satisfies scientific curiosity but also provides a basis for influencing their function, as a strategy for, for example, combating disease. Such an understanding is based on knowledge of both structure and motion of the nucleic acid.

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Several biochemical and biophysical techniques have been utilized in the area of nucleic acid structural biology. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy yield information about the three-dimensional arrangements of atoms or groups in space.

However, to obtain good-quality crystals of nucleic acids is a challenging task and the crystal structures that are obtained are not necessarily of the biologically active conformations.[3] NMR spectroscopy is valuable for obtaining high-resolution solution structures under biologically relevant conditions and to give information about dynamics, but it becomes more difficult as the sizes of the nucleic acids exceed ca. 50 kDa.[4] Fluorescence resonance energy transfer (FRET)[5] and electron paramagnetic resonance (EPR) spectroscopy[6] have also been used to study nucleic acids and other biopolymers under biologically relevant conditions. Advantages of these two techniques include determination of long-range distances that can be used to probe

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large-scale conformational changes in folding and function, as well as the fact that substantially smaller amounts of material are required than for NMR studies.

EPR spectroscopy detects unpaired electrons and can be used to obtain information about the environment of a paramagnetic centre and its interactions with other unpaired electrons or nuclear spins. EPR provides structural information through measurement of distances; continuous wave (CW) methods can be used to measure distances up to 25 Å,[7] whereas pulsed electron-electron double resonance (PELDOR), also called double electron–electron resonance (DEER), yield distances between 15–80 Å.[6c,6e,6f,8] In addition to distance measurements, EPR can also provide information about the orientations of paramagnetic centres and thereby yield information beyond distance measurements.[9] EPR spectroscopy is also very useful for determining motion (dynamics) in the pico- to microsecond range.[6f,9c,10] EPR spectroscopy thus provides a view of both structure and dynamics that can give mechanistic insights into the biopolymer function.

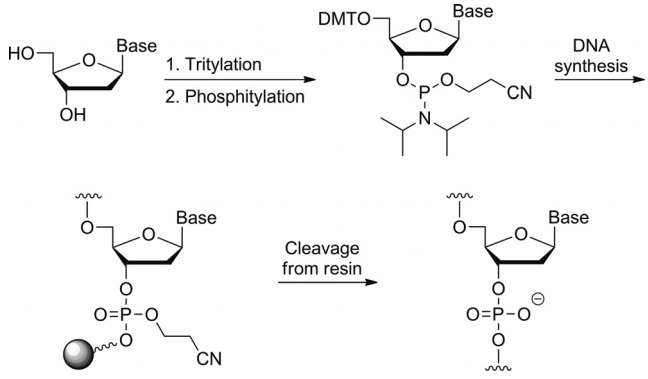
Nucleic acids are diamagnetic and therefore require the incorporation of paramagnetic spin centres (spin labels) for EPR studies. Some nucleic acids contain metal-ion-binding sites that can be used to bind paramagnetic metal ions. Mn^{II}, for example, has been used to study important metal-ion-binding sites in both the hammerhead[11] and the Diels–Alder ribozymes.[12] However, the most common strategy for incorporation of spin labels is the covalent attachment of aminoxyl radicals, commonly referred to as nitroxides. Nitroxides are persistent radicals that are readily accessible and can be easily manipulated with the tools of organic synthesis. Nitroxides are stable under biologically relevant conditions, with the exception of their ease of reduction by mild reducing agents, such as thiols.

When spin labels are conjugated to nucleic acids, it is essential that they only be linked to chosen sites. This is known as site-directed spin labelling (SDSL) or site-specific spin labelling. Several spin-labelling methods have been developed for nucleic acids, enabling linking of nitroxides to specific nucleotides, either to the base, the sugar or the phosphate. This article provides a fairly comprehensive overview of the known nucleic acid labelling methods, with the main focus on the most recent work. The description is in the context of the three labelling strategies: spin labelling during chemical synthesis of nucleic acids, post-synthetic labelling and noncovalent labelling. The most recent advances in the field are highlighted.

Spin Labelling During Chemical Synthesis of Nucleic Acids – The Phosphoramidite Approach

The advent of automated chemical synthesis of nucleic acids on a solid support revolutionized the use of modified nucleic acids in the life sciences, in particular molecular and structural biology, as well as biotechnology. The stepwise synthesis of polynucleotides allows incorporation of modified nucleotides into predetermined positions in the nucleic acid. Although a few different approaches for the chemical synthesis of nucleic acids were developed, the phosphoramidite method, developed by Caruthers, has become the mainstay of oligonucleotide synthesis.[13]

Scheme 1 shows the synthesis of DNA by the phosphoramidite approach.[14] For preparation of the phosphoramidite building block, the 5′-hydroxy group is protected as a dimethoxytrityl ether and the exocyclic amino groups of the bases as amides. The 3′-hydroxy group is subsequently phosphitylated and the resulting phosphoramidite is a stable solid that can be stored for extended periods of time. The oligonucleotide is synthesized on the solid support in the 3′- to 5′-direction to take advantage of the higher reactivity of the 5′-hydroxy group during the coupling step. After coupling of the first phosphoramidite, the phosphorus atom is oxidized and the 5′-hydroxy group is deprotected, setting the stage for the next coupling reaction. After the synthesis of the oligomer, it is deprotected and cleaved from the resin by heating in aqueous ammonia. The phosphoramidite approach has also been used for RNA synthesis, with use of an appropriate protecting group for the 2′-hydroxy group.[15] When the phosphoramidite method is used for spin labelling, a phosphoramidite containing a nitroxide is prepared and used in the chemical synthesis.



Scheme 1. Chemical synthesis of DNA by the phosphoramidite approach. A spin-labelled phosphoramidite is employed when this method is used for spin labelling.

When modified nucleotides are incorporated into oligonucleotides by chemical synthesis, it is important to determine whether or not the modification is compatible with the reagents used in the oligonucleotide synthesis. Because radicals are inherently unstable, it is not surprising that nitroxides have been shown to decompose partially under some of the reaction conditions. In particular, a considerable amount of nitroxides are reduced in the iodine/water solution that has traditionally been used for oxidation of the phosphorus atom from P^{III} to P^{V}.[16] This problem has been circumvented by the use of *tert*-butyl hydroperoxide as an oxidant.[16] Some disproportionation of nitroxides has also been observed upon treatment with the trichloroacetic acid used for removal of the trityl group, which can be minimized by the use of dichloroacetic acid.[16b] Thus, the majority (>90%) of the spin-labelled oligomers prepared by

this method contain an intact nitroxide, which can be purified by denaturing polyacrylamide gel electrophoresis or HPLC.

This section contains a description of the nitroxide spin labels that have been incorporated by the phosphoramidite method. There are also early examples of spin-labelled nucleosides that were incorporated into oligonucleotides by the related phosphotriester method. Spin labelling methods that have been used to incorporate nitroxides into oligonucleotides by conjugation of the nitroxide to a nucleotide during oligomer synthesis by, for example, the Sonogashira coupling reaction[16a,17] are included here because the nitroxide is exposed to the reagents of the oligonucleotide synthesis to the same extent as a phosphoramidite would be.

Single-Bond Base Labelling by Oligonucleotide Synthesis – Labelling Through C–C Bond Formation

Before the phosphoramidite approach became the method of choice for incorporation of spin labels into nucleic acids during chemical synthesis of the oligonucleotide, other methods were used for spin labelling. Bobst and co-workers, for example, incorporated several spin-labelled pyrimidine nucleotides enzymatically through the use of spin-labelled nucleotide triphosphates.[18] However, this method leads to multiple spin labelling with lack of site selectivity,[18–19] unless a single nucleotide extension is used.[18f] The phosphotriester approach was subsequently used by this group for site-directed incorporation of nitroxide-labelled pyrimidines, such as **1** and **2** (Figure 1), through chemical synthesis of the oligonucleotide.[20]

Hopkins and co-workers were the first to report SDSL of nucleic acids by automated chemical synthesis of oligonucleotides by phosphoramidite chemistry.[21] The spin-labelled nucleoside **3** was prepared by coupling 5-iodouridine to (3-ethynyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yl)oxidanyl ("2,2,5,5-tetramethylpyrrolin-1-yloxy-3-acetylene", TPA) by means of a palladium-catalysed Sonogashira cross-coupling reaction. The spin label was designed to project into the major groove of the DNA duplex and was found to be tolerated well. The 2′-deoxycytidine analogue of this spin label was also prepared and incorporated into DNA.[22]

Engels and co-workers reported a slightly different strategy for incorporation of TPA-containing pyrimidine nucleosides into both DNA (**3**)[17,23] and RNA (**6**),[16a] as well as incorporation of adenosine derivative **5** into RNA.[16a] On-column derivatization through a Sonogashira cross-coupling reaction was used during the solid-phase chemical synthesis. Although this approach does not utilize spin-labelled phosphoramidites, it is listed here because the nitroxide spin label is exposed to the chemicals used in oligonucleotide synthesis after its incorporation. Although this method circumvents the preparation of a spin-labelled phosphoramidite, its disadvantage is that the spin-label coupling is not quantitative. Therefore, this approach requires separation of the spin-labelled oligomers from the corresponding iodo-modified oligonucleotide used in the Sonogashira coupling.

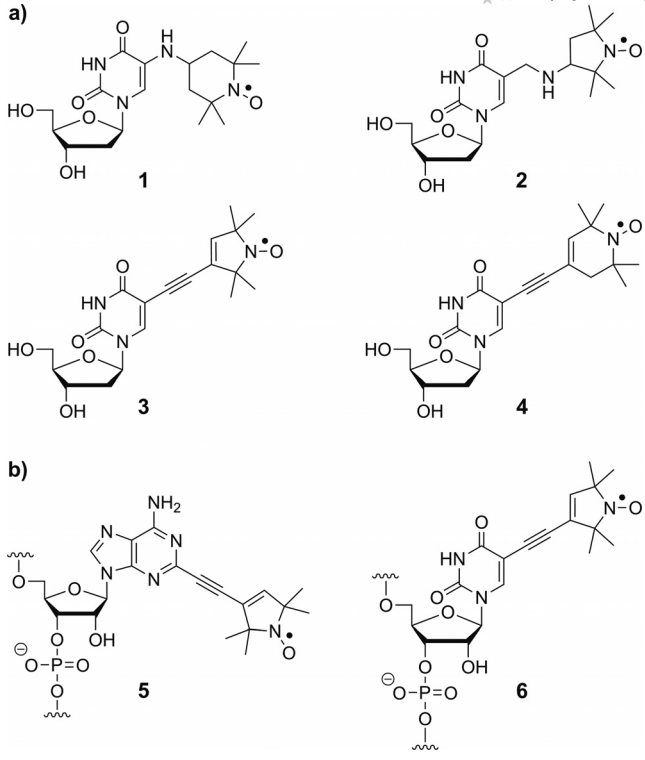


Figure 1. a) Pyrimidine nucleosides that contain spin labels at their 5-positions and have been incorporated into oligonucleotides by chemical synthesis by the phosphoramidite approach. b) Spin-labelled nucleotides that have been incorporated into RNA oligomers through on-column Sonogashira reactions during solid-phase synthesis.

An advantage of the TPA label is that the nitroxide is connected to the nucleobase through a short, semi-rigid linker, which restricts the motion of the nitroxide relative to the nucleic acid to which it is attached. This feature of TPA-labelled nucleotides has enabled their use in study of site-specific dynamics, and thereby in distinguishing between different structural features of the DNA,[24] as well as to measure accurate long-range distances in RNA and DNA by PELDOR.[16a,17,23,25]

Another example of a spin label that has restricted motion relative to the nucleic acid to which it is attached is nucleoside **4** (Figure 1), prepared by Gannett and co-workers.[26] The synthesis of spin-labelled nucleoside **4** requires less effort than required for synthesis of **3**. Nucleoside **4** also has the advantage that rotation of the single bonds flanking the acetylene bond does not displace the nitroxide functional group relative to the nucleobase because these bonds lie on the same axis as the N–O bond. This allows the determination of more accurate distances in **4**-labelled nucleic acids by EPR spectroscopy, which has been used to probe the structural changes during DNA duplex and triplex formation by CW-EPR[27] as well as for studying the G-quadruplex conformations in human telomeric DNA by PELDOR.[28]

Single-Bond Base Labelling through Oligonucleotide Synthesis – Labelling of Exocyclic Amino Groups

Nucleosides containing spin-labelled exocyclic amino groups have also been incorporated site-specifically into nucleic acids by phosphoramidite chemistry. A single modification of an amino group leaves one proton that can participate in hydrogen bonding with its complementary base. Spin labelling of the exocyclic amino groups of 2′-deoxycytidine (to give product **7**, Figure 2) and 5-methyl-2′-deoxycytidine (product **8**) with (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (2,2,6,6-tetramethylpiperidine-1-oxyl, TEMPO) and their incorporation into DNA were demonstrated by Bannwarth and Schmidt.[29] However, the spin-labelled phosphoramidites were obtained in relatively low yields, which were later improved by Giordano and co-workers,[30] who also incorporated spin-labelled 2′-deoxyadenosine (**9**) and 2-amino-2′-deoxyadenosine (**10**) into oligonucleotides.[30] Amino-spin-labelled oligonucleotides have also been prepared by post-synthetic modification of convertible nucleosides, as described below.[31]

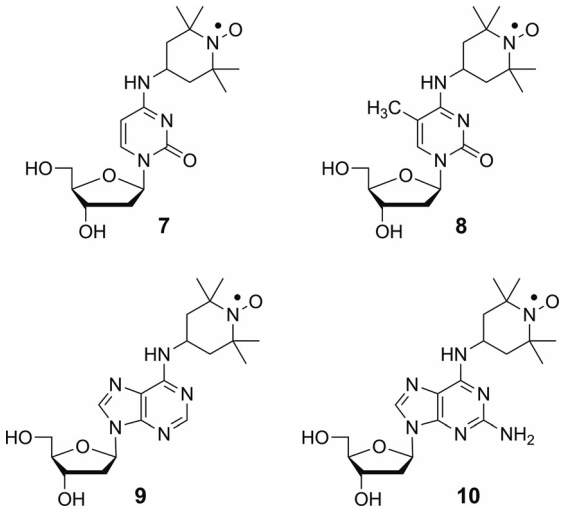


Figure 2. Nucleosides containing spin labels on exocyclic amino groups that have been incorporated into oligonucleotides by the phosphoramidite method.

In addition to being used for distance measurements,[31c,32] spin labels on amino groups are also useful reporter groups for dynamics. In particular, the motion of the amino group has a large effect on the dynamics of the spin label. We have used this feature to probe hydrogen bonding and have shown by CW-EPR spectroscopy that spin label **7** can detect mismatches and also identify its base-pairing partner in duplex DNA.[33]

Ring-Fused Base Labelling through Oligonucleotide Synthesis – Rigid Spin Labels

Most spin labels have been attached to nucleic acids through flexible or semi-flexible linkers and therefore the labels can move independently of the nucleic acid to which they are attached. As a result, the labels cannot directly report the actual dynamics of the nucleic acid. Furthermore, distance measurements by EPR spectroscopy through the use of such labels results in a wider distribution of distances (i.e., less accurate distance measurements). The ideal probe for studies of dynamics and structure would therefore not have movement independent of its site of attachment.

The spin-labelled nucleoside **Q** (Figure 3), the first rigid label for nucleic acids, was synthesized and incorporated into oligodeoxynucleotides by solid-phase chemical synthesis by Hopkins and co-workers.[34] The spin label **Q** is a C-nucleoside, in which the nitroxide moiety is fused to a quinolonyl ring system. The rigidity that results from the nitroxide being incorporated into the ring system of the base has been utilized to study the sequence-dependent dynamics of DNA.[35] The drawbacks of **Q** are that it is a C-nucleoside that requires a lengthy synthesis and that the non-natural 2-aminopurine is its base-pairing partner.

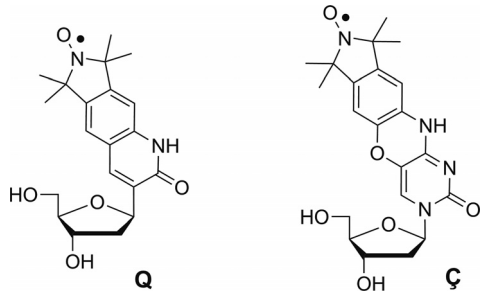


Figure 3. Rigid spin labels incorporated by the phosphoramidite approach.

Our group developed the rigid spin label **Ç** (“C-spin”), an analogue of cytidine (C) that can base-pair with guanine (G).[36] In **Ç**, a nitroxide-bearing isoindoline ring has been fused to C through an oxazine linkage. Thermal denaturation studies showed that **Ç** had a very minor effect on the stability of duplex DNA, and more recently, a crystal structure of **Ç**-labelled duplex DNA confirmed that **Ç** forms a stable and structurally non-perturbing base-pair with G (Figure 13, A).[37]

In addition to allowing accurate distance measurements in duplex DNA, the rigidity of **Ç** has for the first time enabled the determination of relative orientations of two such labels by PELDOR at X-band frequencies.[9b,38] Moreover, PELDOR studies of **Ç**-labelled DNA duplexes at low and high magnetic fields have given insights into the major modes of motion that contribute to the conformational flexibility of duplex DNA.[9c] It has therefore become clear that in addition to yielding more accurate distance measurements by EPR, the rigid probes give access to information that it is not possible to obtain with use of flexible labels.

An interesting feature of **Ç** is that upon reduction of the nitroxide functional group with a mild reducing agent, the nucleoside becomes fluorescent. The resulting fluorescent probe can both detect single-base mismatches in duplex DNA and identify its base-pairing partner.[39] **Ç** can therefore be used as a bifunctional probe (i.e., **Ç**-labelled oligonucleotides can be studied by EPR spectroscopy and sub-

sequently by fluorescence spectroscopy after reduction of the nitroxide). The bifunctional nature of the probe has been used to study conformational dynamics of DNA hairpin loops[16b] and folding of the cocaine aptamer.[40]

Sugar-Phosphate Labelling through Oligonucleotide Synthesis

Spin labels have also been attached to the nucleic acid sugar-phosphate backbone. However, most of the spin labels reported in this category are attached by post-synthetic spin labelling methods that are discussed later, where a more detailed description of the advantages and disadvantages of phosphodiester labelling is also presented. The spin labels that have been incorporated into the phosphodiester backbone through oligonucleotide synthesis were installed at the end of the synthesis, but prior to removal from the solid support. One method is to connect amines to the phosphorus atoms of phosphodiesters through oxidation of H-phosphonates in the presence of the amine.[41] Makino et al. used this strategy to incorporate 4-amino-TEMPO at specific sites in short oligodeoxynucleotides as the spin label **11** (Figure 4) in good yields.[42] As with any method that replaces one of the non-bridging oxygen atoms with a substituent, this resulted in a pair of diastereomers that could be separated by HPLC.[42b] Murakami and co-workers also used the same strategy to link TEMPO to the phosphodiester with different tethers and used the spin-labelled oligomers for detecting DNA hybridization.[43] They also spin-labelled the 5′-terminus of DNA oligonucleotide through a carbamate linker by sequential treatment of the 5′-hydroxy group with *N*,*N*′-carbonyldiimidazole (CDI)[44] and 4-amino-TEMPO to afford spin label **12**.[43]

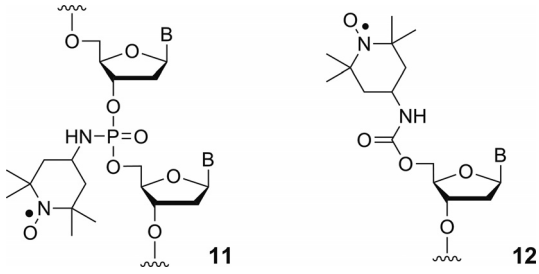


Figure 4. Sugar-phosphate backbone labelling through chemical synthesis of nucleic acids.

Post-Synthetic Spin Labelling

Incorporation of spin labels into a nucleic acid after the synthesis of the oligomer, either by chemical or enzymatic methods, is referred to as post-synthetic labelling (Figure 5). This method relies on having a reactive group at a specific site (or sites) on the nucleic acid, which reacts with a spin-labelling reagent. For site-specific labelling, the functional group to be modified needs to be more reactive towards the spin-labelling reagent than the innate groups on the nucleic acid, such as N7 in purines, the exocyclic amino groups of the nucleobases and the non-bridging oxygen atoms of the phosphodiesters.

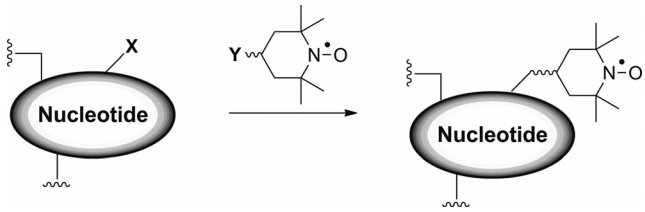


Figure 5. Post-synthetic spin labelling of nucleic acids. X and Y represent functional groups that react in the process of conjugation. TEMPO is used as a representative nitroxide spin label.

Early on, alkylating or acylating agents, such as compounds **13**[45] and **14**[46] (Figure 6, A), were used for spin labelling,[47] but for the aforementioned reasons had very limited sequence selectivity. In an attempt to increase the selectivity, bifunctional cross-linking agents, such as the hydrazine mustard spin label **16**,[48] the psoralene derivative **17**[49] and the spin-labelled cisplatin **18**,[50] were developed. Although these cross-linking agents have a sequence selectivity that spans a few nucleotides, such as intra-strand cross-linking of the sequence GG for **18**, they still lack enough specificity to be generally applicable for SDSL.

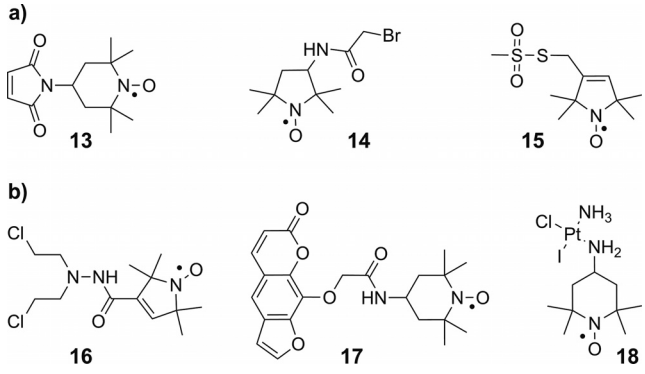


Figure 6. a) Spin-labelled electrophiles and b) cross-linking agents used for post-synthetic spin labelling of nucleic acids.

The post-synthetic methods listed below are commonly used for SDSL. They each involve a uniquely reactive functional group that has been installed during the synthesis of the oligonucleotides to direct the spin label to a specific site. Thus, for chemical synthesis of the nucleic acid that contains the reactive functional group, a modified phosphoramidite containing the appropriate functional group must be incorporated. However, most of these modified phosphoramidites are commercially available.

Post-Synthetic Base Labelling

4-Thiouridine nucleotides are found in several tRNAs and can be selectively spin-labelled by alkylation. Treatment with bromoacetamide spin label **14**, for example, afforded nucleoside **19** (Figure 7) under mild reaction conditions while retaining the biological activity of the tRNAs.[51] Var-

ani and co-workers incorporated 4-thiouridine into RNA by chemical synthesis, and subsequent treatment with the iodo derivative of spin label **14** yielded the spin-labelled nucleoside **19**,[52] which has been used for NMR studies of RNA–protein complexes[52–53] and for determination of long-range distances in RNA by double-quantum coherence (DQC) EPR spectroscopy.[54]

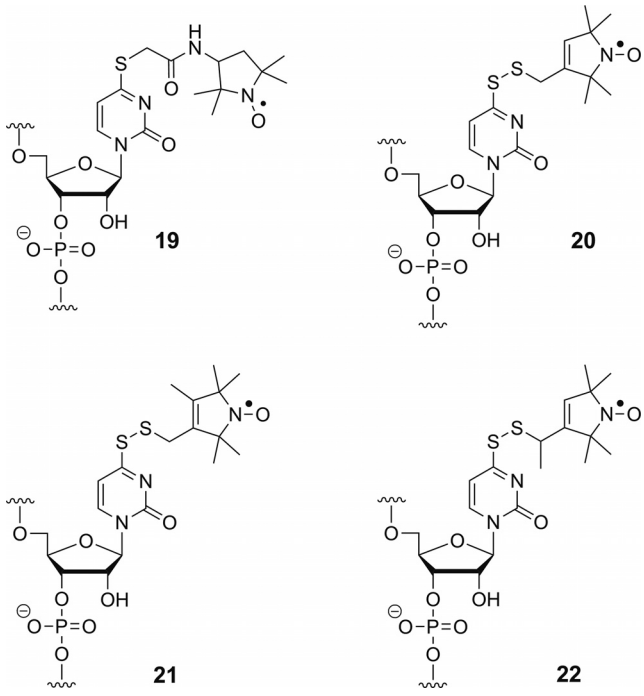


Figure 7. Post-synthetic spin labelling through reactions of nucleotides containing 4-thiouridine units.

Although alkylating agents react much more rapidly with sulfur atoms than with other functional groups in nucleic acids, they are not as selective as the thiol-specific methanethiosulfonate spin label reagent **15**.[55] Qin and co-workers treated **15** with 4-thiouridine in RNA to yield nucleotide **20**, containing a disulfide bridge between the spin label and the nucleotide (Figure 7); this was used to study nucleotide mobility in different structural contexts in RNA.[56] Spin-labelled nucleosides **21** and **22**, both of which have restricted internal motions relative to **20**, have also been prepared by the same strategy and used to investigate RNA dynamics and conformations.[56–57]

Spin labels have recently been incorporated into nucleic acids through the use of the high-yielding and bio-orthogonal Cu^{I}-catalysed Huisgen–Meldal–Sharpless [3+2] cycloaddition reaction (click chemistry).[58] Our group used this approach to incorporate spin-labelled nucleotide **23** (Figure 8) through a reaction between an isoindoline-derived azide and an oligonucleotide containing 5-ethynyl-2′-deoxyuridine.[58b]

The spin-labelling reaction was performed on a solid support, prior to removal of the oligonucleotide from the resin, to give the spin-labelled oligomer in quantitative yield. Nucleoside **23** has an unusual structural feature in relation to other spin labels, which is the three aromatic

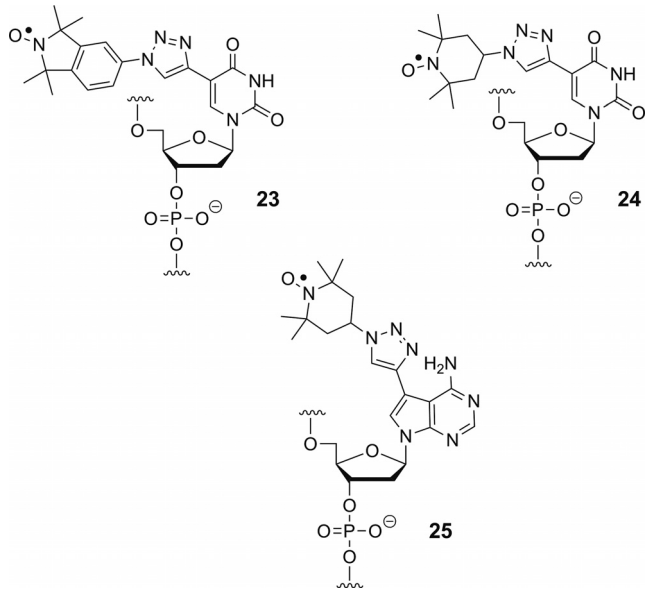


Figure 8. Spin-labelled nucleotides prepared by post-synthetic click chemistry.

rings that are connected by single bonds. Intercalation or aromatic stacking of this π-system should therefore be possible, which should dramatically reduce the mobility of the nitroxide. In fact, this feature has allowed the detection of local structural lesions, such as abasic sites and mismatches, in duplex DNA, as well as a mercury(II)-mediated metallobase pair formation.[58b] Incorporation of nucleosides **24** and **25** by click chemistry has been reported by Steinhoff and co-workers, who used those labels for distance measurements in DNA by CW-EPR and PELDOR.[58a]

As mentioned in the section on spin-labelling by phosphoramidite chemistry, several exocyclic amino groups have been spin-labelled by the post-synthetic convertible nucleoside approach, developed by Verdine and co-workers.[59] In this instance, an amino-modified nucleobase is prepared by treatment of an electrophilic nucleobase with an amine at the end of the chemical synthesis of the oligonucleotide, which also serves to deprotect the oligomer and to cleave it from the solid support. Budil and co-workers used this strategy for incorporation of spin-labelled nucleoside **26**, containing a flexible tether (Figure 9), which was used for study of dynamics and DNA–protein interaction by high-field EPR spectroscopy.[31a] A post-synthetic modification of 2-fluorohypoxanthine with 4-amino-TEMPO to afford spin-labelled guanosine nucleoside **27** was reported by Saito and co-workers, who used it to probe the hybridization and structural folding of DNA.[31b] Nucleotide **27** has also been used for study of conformational equilibria of DNA duplexes[32a] and influence of lesions on nucleic acid structure by pulsed EPR spectroscopy.[32b] Spin label modifications of the exocyclic amino groups in RNA by the convertible nucleoside approach have more recently been reported, affording good yields of RNA oligonucleotides containing spin-labelled nucleosides **28**–**30** (Figure 9), which have been used to study conformations and secondary structures of RNA duplexes by PELDOR.[31c]

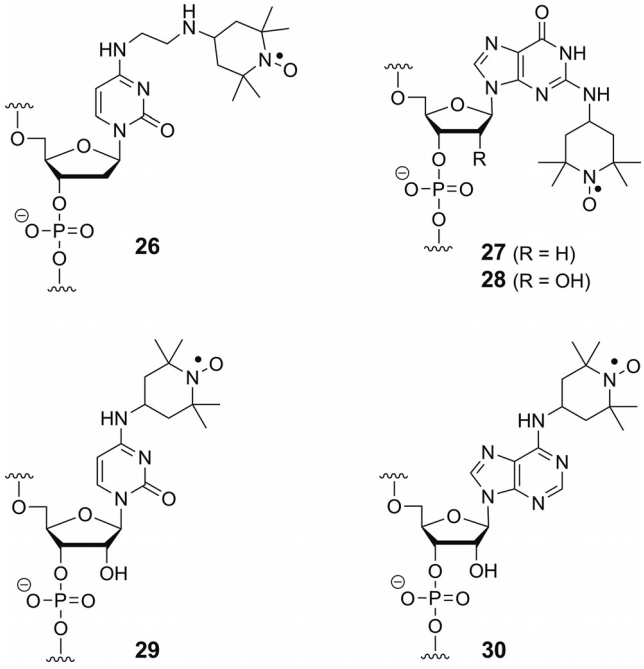


Figure 9. Nucleotides containing spin labels that have been incorporated into oligonucleotides by the post-synthetic convertible nucleoside approach.

Post-Synthetic Sugar Labelling

Spin labels have been incorporated into sugar moieties of nucleotides in oligonucleotides, both at terminal and at internal positions. The first example of sugar labelling was reported by Caron and Dugas, who labelled tRNA at the 3′-end by utilizing the *cis*-geminal diol functional group of the 3′-sugar. Incubation of the RNA with periodate gave the corresponding dialdehyde, which could be reductively aminated with 4-amino-TEMPO and sodium borohydride to afford the morpholino spin label **31** in good yield (Figure 10).[60] Reductive amination with cyanoborohydride yields a different spin label tether that has more motional freedom.[61] This approach has the advantage that a native (unmodified) RNA can be labelled but is restricted to 3′-end labelling.

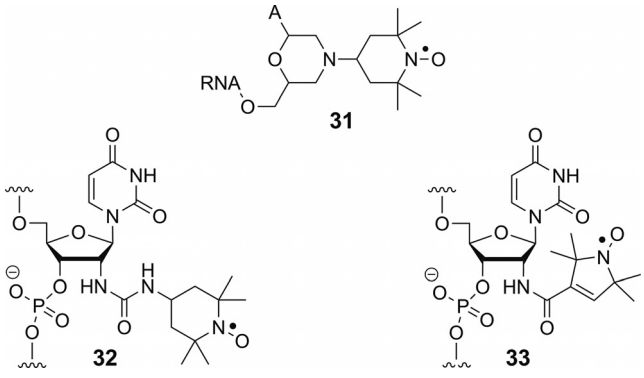


Figure 10. Spin labels incorporated into nucleotide sugar moieties through post-synthetic modification.

For internal labelling of sugars, the 2′-position has been the location of choice. We developed a high-yielding, post-synthetic spin-labelling strategy for RNA based on 2′-amino modified RNA oligonucleotides and 4-isocyanato-TEMPO,[62] both of which are commercially available, to yield spin-labelled RNAs containing nucleotide **32**. This strategy is based on the higher reactivity of the 2′-amino group in relation to the exocyclic amino groups of the nucleobases and has been used for labelling both DNA[63] and RNA.[62,64] The ureido-linked nitroxide-containing nucleotide **32** (Figure 10) has been used for studying the dynamics of the transactivation responsive (TAR) RNA upon binding of small organic molecules,[65] metal ions[66] and peptides,[66a,67] as well as site-specific dynamics of the hammerhead ribozyme upon metal-ion-induced folding by EPR spectroscopy.[64b,66b] It has also been used for accurate distance measurements in nucleic acids,[63–64,64c] as well as for study of a conformation transition of the tetracycline aptamer upon ligand binding by pulsed EPR.[68] A spin label has also been conjugated to a 2′-amino group in RNA through an amide linkage (**33**), but the short amide linkage was found to have a destabilizing effect on duplex RNA.[7b]

Post-Synthetic Labelling of the Phosphodiester Backbone

Spin labels have been conjugated to phosphorus both in terminal phosphate groups and in the phosphodiesters, by replacement of one of the non-bridging oxygen atoms. This approach has the advantage that the phosphodiesters can be labelled at any position in the oligonucleotide sequence without having to prepare specifically modified nucleosides or nucleotides as in the cases of sugar labelling and base labelling. Moreover, because the phosphorus atoms are positioned at the edges of nucleic acid helices, the labels should not interfere with duplex formation and should have minimal effects on the overall structure of the nucleic acid. However, the loss of a negative charge associated with the modification can affect the electrostatic interactions within the labelled nucleic acid, as well as those with other molecules. As mentioned earlier, another potential complication is that phosphodiester labelling leads to the formation of two diastereomers, S_p and R_p, although in some instances they can be separated by HPLC.[69] Furthermore, when an RNA phosphodiester is modified, the 2′-OH group adjacent to the modified phosphodiester needs to be replaced with a hydrogen atom or a 2′-*O*-methyl group, because the presence of a 2′-OH group readily results in strand cleavage or loss of the label through transesterification.[70]

Because of their higher reactivities, terminal phosphates are easier to modify than phosphodiesters. Dzuba and coworkers spin-labelled both the 3′-ends and the 5′-ends of DNA oligonucleotides by treating the activated phosphate groups with 4-amino-TEMPO to yield phosphoramidates **34** and **35** (Figure 11), respectively. They subsequently used PELDOR to study the conformational changes induced in doubly spin-labelled single- and double-stranded DNA oligonucleotides by non-nucleotide inserts.[71]

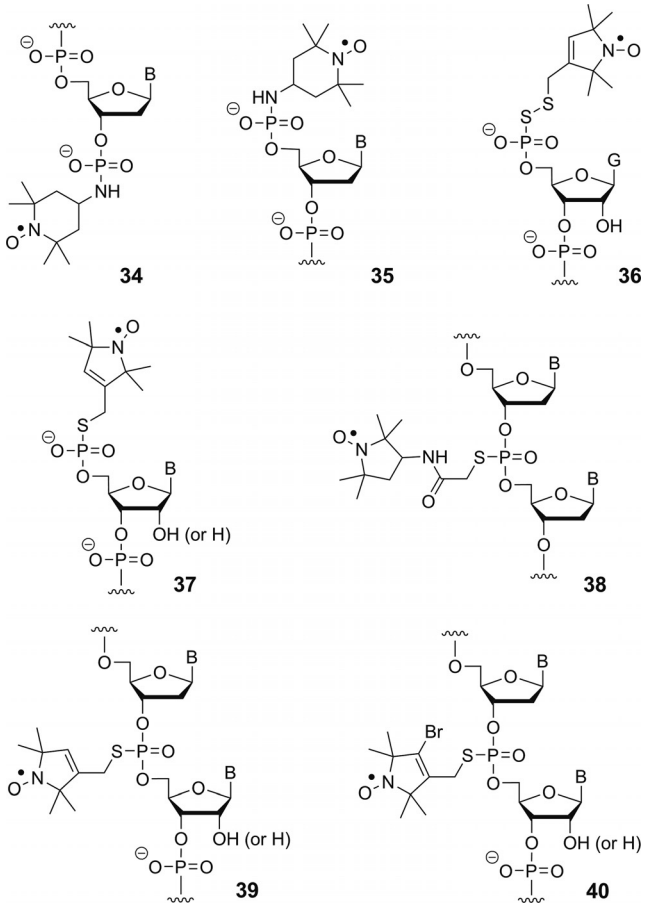


Figure 11. Nucleotides containing spin labels at their phosphate backbones, prepared by post-synthetic modification. B stands for a nucleobase.

Replacement of a non-bridging oxygen of a terminal phosphate or a phosphodiester with a sulfur atom dramatically increases the reactivity at that site, which can be selectively spin-labelled with thiol-specific spin-labelling reagents under mild reaction conditions. Shin and co-workers incorporated a guanosine monophosphorothioate (GMPS) unit at the 5′-end of RNA during in vitro transcription from a DNA template with use of T7 RNA polymerase. The phosphorothioate subsequently reacted with the thiol-specific methanethiosulfonate spin-labelling reagent **15** (Figure 6, A) to afford the spin-labelled RNA **36** (Figure 11).[7a] Similarly, Qin and co-workers reported a general approach for spin-labelling of terminal positions of both DNA and RNA through the incorporation of a phosphorothioate group at the 5′-end by use of T4 polynucleotide kinase.[72] Subsequent spin-labelling by alkylation yielded nucleotide **37**, which was used for study of nucleic acid hybridization and metal-ion-induced folding of RNA by EPR spectroscopy.

One of the non-bridging oxygen atoms of a specific internal nucleotide can be replaced with sulfur during the chemical synthesis of an oligomer by use of a sulfurizing agent instead of the normal oxidizing agent during the oxidation of the phosphorus atom.[73] The resulting phosphorothioate can be specifically alkylated by use of an alkylating spin label reagent such as **14** to yield a spin-labelled oligonucleotide **38**.[74] Similarly, RNA can be spin-labelled by treatment of a phosphorothioate-containing oligonucleotide with an alkylating agent to afford a spin-labelled nucleotide **39**.[75] This spin-labelling approach has been used to prepare site-specifically spin-labelled RNAs for study of metal-ion-dependent tetraloop–receptor interactions in RNA[75a] and measurements of dynamics[76] and distances in nucleic acids by PELDOR.[75b,77] Subsequently, Qin and co-workers prepared the 4-bromo-substituted analogue of **39** (**40**, Figure 11) and used it to study the motion of the substrate-recognition RNA element in the group I intron ribozyme by CW-EPR spectroscopy.[78] They also separated the individual spin-labelled diastereomers by HPLC[69a] and used them to study stereospecific dynamics in DNA.[69b]

Noncovalent Spin-Labelling

Noncovalent spin-labelling circumvents the challenges associated with formation of a covalent bond to the biopolymer at a specific atom. There are several examples of noncovalent labelling of proteins in which active sites of enzymes and cofactor binding sites have been utilized for specific binding to spin-labelled derivatives of their natural ligands.[79] In the context of proteins, the drawback of this approach is that it does not provide the option of labelling different sites because the binding sites cannot be moved around without changing the structure of the protein.

The first noncovalent labelling example of nucleic acids was the intercalation of a chlorpromazine cation radical in a nucleic acid duplex.[80] A decade later, Piette and co-workers used a similar strategy by conjugating a nitroxide spin label to the known intercalator ethidium bromide (**41**, Figure 12) and polyaromatic carcinogens to study the thermal dissociation of DNA–carcinogen complexes by EPR spectroscopy.[81] Spin-labelled Ru^{II}-phenanthroline complexes[82] and acridine analogues (compound **42**,[83] for example) have been prepared and their binding to nucleic acids has been studied by EPR spectroscopy. However, these intercalating agents do not bind to specific sites and so have limited use for EPR studies. Although not for the purpose of nucleic acid spin-labelling, Lhomme and co-workers

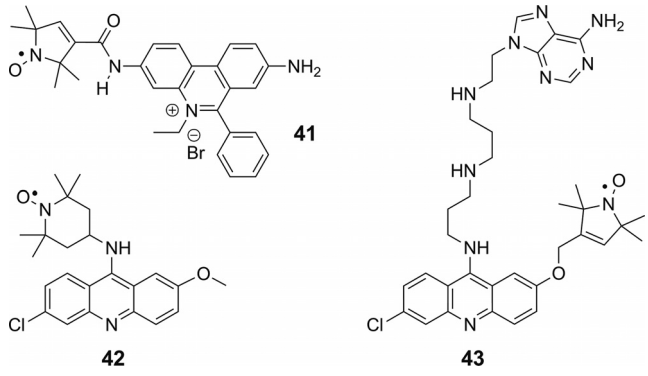


Figure 12. Spin-labelled intercalators used for noncovalent spin-labelling of nucleic acids.

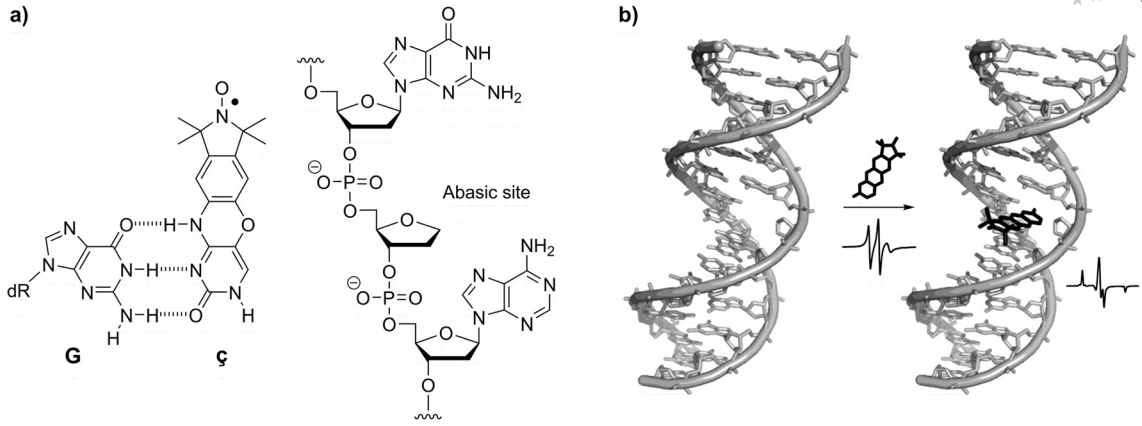


Figure 13. Noncovalent and site-directed spin-labelling. a) Structure of **ç** and its base-pairing scheme with G (left), together with the structure of an abasic site in DNA (right). b) A cartoon representation of the noncovalent spin-labelling approach based on the nitroxide **ç** (black) and a duplex DNA (grey) containing an abasic site. The EPR spectra shown are of the unbound spin label (left) and of the bound spin label (right) in duplex DNA containing an abasic site at –30 °C.

tethered a spin-labelled intercalator to adenine to produce compound **43**, which can bind to abasic sites in duplex DNAs and thereby direct the spin label to the vicinity of the abasic site.[84]

Recently, we reported a general strategy for noncovalent and site-directed spin labelling (NC-SDSL) of nucleic acids based on ligand–receptor interactions.[85] The receptor is an abasic site in duplex DNA, which can be readily installed at specific sites by automated chemical synthesis of the nucleic acid with use of commercially available phosphoramidites. An abasic site in a DNA duplex provides a binding site for the spin-labelled ligand **ç** (Figure 13, A), which binds through hydrogen bonding with a guanine on the complementary strand and through π-stacking interactions with the base pairs immediately adjacent to the abasic site (Figure 13, B). The binding of the spin label can be conveniently monitored by CW-EPR spectroscopy; after binding to the DNA duplex, the tumbling of **ç** slows down and its EPR spectrum becomes wider (Figure 13, B). At 25 °C the spin label **ç** showed only a small amount of binding, but the binding increased as the temperature was decreased. At –30 °C, **ç** was fully bound and control experiments showed that it bound specifically to the abasic site.[85]

This spin-labelling strategy has notable advantages over the other two spin labelling approaches – the phosphoramidite method and post-synthetic labelling. The synthesis of spin label **ç** is shorter than for similar spin-labelled phosphoramidites, for example, and it is more stable and can be stored for extended periods of time. Smaller quantities of spin label are required and the spin label is not exposed to the chemicals used for the oligonucleotide synthesis. Furthermore, sample preparation is very easy: the spin label is simply mixed with the nucleic acid containing the abasic site, prepared chemically from commercially available phosphoramidites, and no purification of the complex is required. Although complete binding of **ç** to an abasic site requires low temperatures, this strategy should be useful for distance measurements studies in nucleic acids by PELDOR because these are performed in frozen solutions.

Conclusions

EPR spectroscopy has evolved rapidly over the last years. Documented advances in instrumentation and method development have enabled routine investigations of the structure and dynamics of biopolymers in folding and function. There has thus been an increase in demand for nucleic acids containing tailor-made spin labels at specific sites; new labels as well as new methods for spin labelling have emerged. However, there is still a need for spin labels with improved spectroscopic properties and new labelling methods that provide easy access to nucleic acids. NC-SDSL, which permits spin labelling simply by mixing the spin label with the nucleic acid immediately prior to EPR measurements, provides a new strategy for delivering a variety of labels to different sites. Spin labelling through NC-SDSL and the two strategies that utilize covalent bonding will undoubtedly continue to be an active area of research in the near future.

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