CHAPTER FIFTEEN

Site-Directed Spin Labeling of RNA by Postsynthetic Modification of 2′-Amino Groups

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Abstract

To elucidate mechanisms that govern functions of nucleic acids, it is essential to understand their structure and dynamics. Electron paramagnetic resonance (EPR) spectroscopy is a valuable technique that is routinely used to study those aspects of nucleic acids. A prerequisite for most EPR studies of nucleic acids is incorporation of spin labels at specific sites, known as site-directed spin labeling (SDSL). There are two main strategies for SDSL through formation of covalent bonds, i.e., the phosphoramidite approach and postsynthetic spin-labeling. After describing briefly the advantages and disadvantages of these two strategies, postsynthetic labeling of 2′-amino groups in RNA is delineated. Postsynthetic labeling of 2′-amino groups in RNA using 4-isocyanato-TEMPO has long been established as a useful approach. However, this method has some drawbacks, both with regard to the spin-labeling protocol and the flexibility of the spin label itself. Recently reported isothiocyanate-substituted aromatic isoindoline-derived nitroxides can be used to quantitatively and selectively modify 2′-amino groups in RNA and do not have the drawbacks associated with 4-isocyanato-TEMPO. This chapter provides a detailed description of the postsynthetic spin-labeling methods of 2′-amino groups in RNA with a special focus on using the aromatic isothiocyanate spin labels.

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1. INTRODUCTION

Nucleic acids are essential molecules for sustaining life. DNA and RNA are responsible for storage, expression, and transmission of genetic information—DNA carries the genetic information, whereas RNA has varied functions, such as transferring genetic information and acting as a chief constituent of ribonucleoprotein complexes involved with mRNA processing and translation. RNA can also catalyze reactions; a prominent example is formation of peptide bonds by the ribosome (Nissen, Hansen, Ban, Moore, & Steitz, 2000). RNA has also been implied in the catalytic function of the spliceosome (Fica et al., 2013). Recently discovered siRNAs play a notable role in RNA interference, where they inhibit particular gene expressions (Brummelkamp, Bernards, & Agami, 2002). Moreover, riboswitches have an important role in regulating gene expression (Mandal & Breaker, 2004).

It is of interest to know the structure and dynamics of nucleic acids, because these properties govern their functions. There are several biochemical and biophysical techniques that have been applied for the study of the structure and function of nucleic acids. The most powerful technique is undoubtedly X-ray crystallography, which is capable of providing a "photographic" representation of the three-dimensional molecular structure. However, this highly informative technique requires a sufficiently large and regular single crystal, which can be a daunting task to obtain for nucleic acids. In addition, a crystal structure might not represent a biologically active conformation. Moreover, an X-ray structure provides a static view, whereas conformational changes are usually required to carry out specific functions. Another high-resolution technique to study nucleic acid structure is nuclear magnetic resonance (NMR) spectroscopy, which provides structural information of the nucleic acid in solution, thus revealing their conformation under biologically relevant conditions. However, NMR of nucleic acids often requires relatively large amounts of isotopically labeled samples. Furthermore, NMR studies are usually restricted to nucleic acids that are smaller than 50 kDa (Xu & Matthews, 2013), because the increased anisotropy associated with slower tumbling of large molecules in solution causes peak broadening. Another common technique for studying nucleic acids is Förster resonance energy transfer, which is capable of measuring distances in the nanometer range. This technique can also be used to study nucleic acids under biologically relevant conditions, in addition to enabling

single-molecule studies (Roy, Hohng, & Ha, 2008; Sisamakis, Valeri, Kalinin, Rothwell, & Seidel, 2010). However, since natural nucleic acids do not possess any fluorescent chromophores, a prerequisite for this technique is the incorporation of a pair of rather bulky fluorophores.

The technique that will be addressed here is electron paramagnetic resonance (EPR) spectroscopy, which is applicable for the study of paramagnetic centers. EPR can provide structural information for biomolecules through measurement of distances between paramagnetic centers, using continuous wave (CW)- or pulsed EPR. CW EPR can be used to measure distances up to 25 Å through analysis of peak broadening (Kim, Murali, & DeRose, 2004; Macosko, Pio, Tinoco, & Shin, 1999). Pulsed EPR, such as pulsed electron–electron double resonance, also called double electron–electron resonance, can yield distances of 15–100 Å (Duss, Yulikov, Jeschke, & Allain, 2014; Jeschke, 2012; Milov, Salikhov, & Shirov, 1981; Reginsson & Schiemann, 2011; Schiemann & Prisner, 2007). EPR is also capable of probing the orientation of paramagnetic centers, which can provide information about both structure and dynamics (Denysenkov, Prisner, Stubbe, & Bennati, 2006; Marko et al., 2011; Schiemann, Cekan, Margraf, Prisner, & Sigurdsson, 2009). EPR is valuable for studying dynamics on a range of timescales (Marko et al., 2011; Nguyen & Qin, 2012; Sowa & Qin, 2008). Thus, EPR is a multifaceted tool that can provide valuable insights into both structure and dynamics of nucleic acids.

Nucleic acids are not inherently paramagnetic and, therefore, it is necessary to modify them with paramagnetic atoms or groups, referred to as spin labels. Although there are some examples of paramagnetic metal ions that have been used as spin probes (Goldfarb, 2014; Hunsicker-Wang, Vogt, & DeRose, 2009; Schiemann, Fritscher, Kisseleva, Sigurdsson, & Prisner, 2003), the most commonly used spin labels are aminoxyl radicals, usually called nitroxides. Many of these nitroxide radicals are commercially available or can be readily synthesized using standard techniques of organic synthesis. Therefore, nitroxides have found extensive use as spin labels. Although there are examples of noncovalent spin labeling of nucleic acids with nitroxides (Belmont et al., 1998; Chalmers et al., 2014; Maekawa et al., 2010; Shelke, Sandholt, & Sigurdsson, 2014; Shelke & Sigurdsson, 2010), the most common spin-labeling approach for nucleic acids is attachment of spin labels through covalent bonds.

There are several methods available for incorporation of spin labels at the end of nucleic acids (Shelke & Sigurdsson, 2012, 2013), but end-labeling has limited applicability for EPR studies. Therefore, this text focuses on

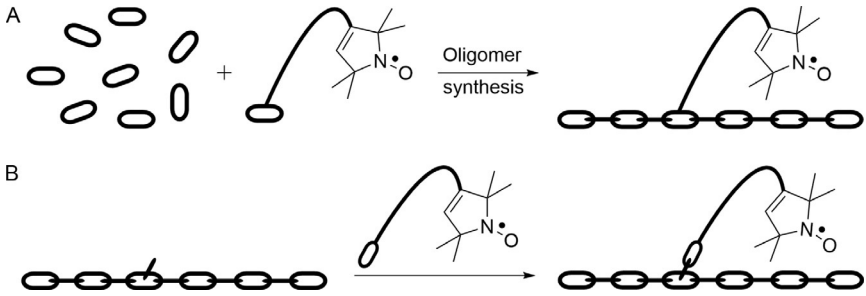


Figure 1 Strategies for site-directed spin labeling through covalent bonding. (A) The phosphoramidite approach. (B) Postsynthetic spin-labeling. A pyrrolidine-based spin label is used as a representative nitroxide spin label. Nucleotides are represented by links that form oligonucleotide chains.

methods for incorporation of spin labels at internal sites. Moreover, it will address how spin labels can be incorporated at specific sites of choice, referred to as site-directed spin labeling (SDSL). There are two main strategies that have been applied for covalent SDSL (Fig. 1). The first one utilizes spin-labeled phosphoramidites that are incorporated at specific positions during automated chemical synthesis of the nucleic acid (Shelke & Sigurdsson, 2012), shown schematically in Fig. 1A, and sometimes referred to as the phosphoramidite method. The second SDSL strategy is postsynthetic spin labeling, where spin labels are incorporated after the synthesis of the oligonucleotide, by either chemical or enzymatic methods (Fig. 1B).

The main features of these two spin-labeling strategies, the phosphoramidite method and postsynthetic labeling, will be described briefly below. Both of these SDSL routes are useful and complement each other. A facile approach for postsynthetic labeling of 2′-amino groups in RNA will subsequently be described in detail.

1.1 The Phosphoramidite Method for SDSL

Nucleoside phosphoramidites are derivatives of natural nucleosides and serve as building blocks in solid-phase synthesis of nucleic acids. A generic structure of a phosphoramidite is shown in Fig. 2A, where the 5′-hydroxyl group of a ribonucleoside is protected as a 5′-dimethoxytrityl (DMT) ether, while the phosphoramidite group is at the 3′-position. The 2′-position also needs to be protected when synthesizing RNA. The main advantage of the phosphoramidite method is that spin labels with specific and desired structural features can be inserted at chosen sites, which might not be possible using postsynthetic labeling.

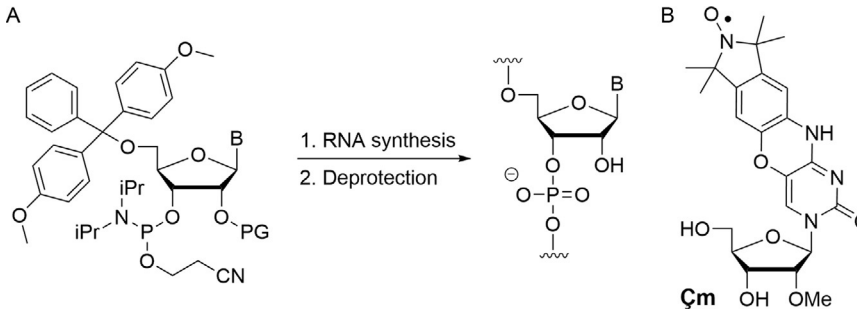


Figure 2 (A) Phosphoramidite monomer building block. PG is a protecting group for the 2′-hydroxy group. B is a nucleobase. (B) The rigid spin label **Çm** that has been incorporated into RNA by the phosphoramidite method.

There are several examples of spin labels that have been incorporated into DNA by the phosphoramidite method (Shelke & Sigurdsson, 2012). However, there is only one example of a spin-labeled nucleoside that has been incorporated into RNA by this method, the nucleoside **Çm** (Fig. 2B). **Çm** is a rigid spin label containing a nitroxide that has been fused to a nucleobase (Höbartner, Sicoli, Wachowius, Gophane, & Sigurdsson, 2012). Synthesis of spin-labeled phosphoramidites usually requires a substantial effort and involves a high degree of expertise in synthetic organic chemistry. Another drawback is the exposure of the spin labels to the reagents used during the oligonucleotide synthesis, which may result in partial reduction of the nitroxide radical. For example, iodine/water, which has traditionally been used to oxidize the phosphorous atoms from P(III) to P(V), needs to be replaced by *tert*-butyl hydroperoxide to avoid degradation of the radical (Cekan, Smith, Barhate, Robinson, & Sigurdsson, 2008; Piton et al., 2007). Moreover, the acid treatment, which removes the DMT groups from the 5′-end of the growing chain during elongation, can also result in decomposition of nitroxide spin labels, depending on their stability.

1.2 Postsynthetic Spin-Labeling

Postsynthetic spin labeling is the other main method of choice for incorporation of spin labels at specific sites (Fig. 1B). This strategy requires oligonucleotides that have uniquely reactive groups at specific sites where the spin label is to be incorporated. Such oligomers are normally prepared by the phosphoramidite method, often using commercially available reagents. This is a useful feature of this method, because both the modified oligonucleotide and a suitable spin label can often be either purchased or readily prepared. The other merit of this method is that the spin label does not get exposed

to the reagents used in the chemical synthesis of oligonucleotides. However, a drawback of this method is the possibility of nonspecific labeling due to the nucleophilic groups present in the nucleic acids, such as the exocyclic amino groups of the nucleobases, the N7 of purines, and nonbridging oxygen atoms of the phosphodiesters. In addition, incomplete spin labeling is also a well-known drawback of this method.

There are a number of sites on a nucleotide in RNA that can in principle be spin labeled postsynthetically, namely the nucleobase, the sugar, and the phosphodiester backbone. Postsynthetic spin labeling of a nucleobase can, for example, be performed by the reaction of 4-thiouridine with a suitable spin-labeling reagent. Figure 3A shows such examples, where thiol-specific methane-thiosulfonate spin-labeling reagents have been reacted with 4-thiouridine in RNA to yield a variety of spin-labeled oligomers (Qin, Hideg, Feigon, & Hubbell, 2003; Qin, Iseri, & Oki, 2006). 4-Thiouridine can also be spin labeled through alkylation (Ramos & Varani, 1998). Another facile postsynthetic method is the reaction of phosphorothioates, in which one of the nonbridging oxygen atoms has been replaced with sulfur by oxidation with a sulfurizing agent during oligonucleotide synthesis, with alkylating agents (Fig. 3B; Grant, Boyd, Herschlag, & Qin, 2009; Qin, Butcher, Feigon, & Hubbell, 2001). This method requires the use of a deoxynucleotide at the phosphorothioate site to prevent cleavage of the RNA strand. Exocyclic amino groups in RNA have also been modified with a spin label (Sicoli, Wachowius, Bennati, & Höbartner, 2010) using the "convertible nucleoside" approach (Macmillan & Verdine, 1990). In this method, a derivative of a nucleoside possessing a leaving group on its nucleobase (the convertible nucleoside) is

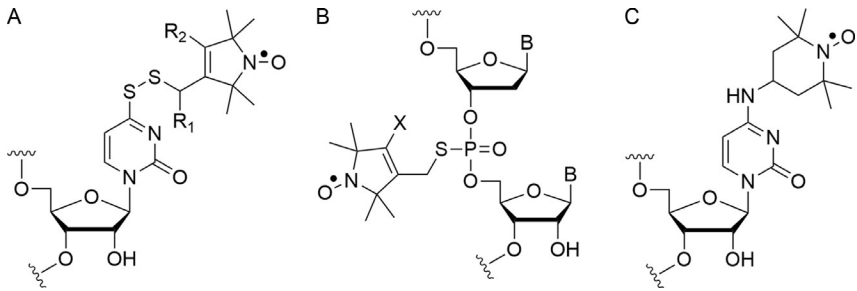


Figure 3 Representative examples of postsynthetic spin-labeling of nucleobases and phosphodiesters. (A) Attachment of spin labels at 4-thiouridine. (B) Spin-labeling at phosphate backbone. (C) Labeling of exocyclic amino groups of cytosine through the convertible nucleoside approach. R_1 and R_2 = H or CH_3, X = H or Br, and B is a nucleobase.

incorporated into RNA through solid-phase synthesis. After the synthesis of the full-length oligomer, it is treated with an amine-based nucleophile, which substitutes the leaving group on the nucleobase, and becomes covalently attached. Figure 3C shows an example, where TEMPO was utilized as the nucleophile (Sicoli et al., 2010).

Spin labels have also been incorporated at the 2′-position of sugars in oligonucleotides using postsynthetic methods (Fig. 4). The 2′-position is the only site that is readily available for labeling of sugars at internal positions of nucleic acids. Moreover, a spin label attached at the 2′-position gets projected out of the minor groove, causing minimal structural perturbation of the labeled RNA. Spin labels have been incorporated into 2′-positions of RNA using the Cu(I)-catalyzed Huisgen–Meldal–Sharpless [3+2] cycloaddition reaction (click chemistry), yielding triazole-linked spin labels (Büttner, Javadi-Zarnaghi, & Höbartner, 2014; Flaender et al., 2008; Fig. 4A).

Postsynthetic labeling of 2′-amino groups is another particularly facile and selective approach for labeling the 2′-position; the aliphatic 2′-amino group is more nucleophilic than the aromatic amines on the nucleobases or the hydroxyl groups on the phosphodiester and can be converted to ureas and esters (Fig. 4B). Moreover, RNA oligonucleotides having 2′-amino modification(s) are commercially available or can be synthesized in-house on an automated synthesizer using commercially available 2′-amino-modified phosphoramidites. Thus, easy availability of 2′-amino-modified RNAs makes this approach attractive. The 2′-amino group has been spin labeled through reaction with a succinimidyl ester of a pyrrolidine-derived

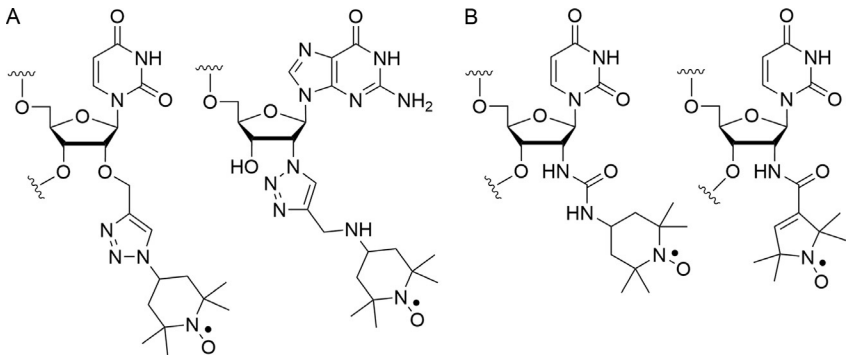


Figure 4 (A) Postsynthetic spin labeling of RNA at the 2′-position by using cycloaddition reaction between an azide and an alkyne. (B) Postsynthetic spin labeling at 2′-amino position through formation of urea or amide linkage.

nitroxide spin label to yield amide-modified spin label (Fig. 4B); however, this modification was found to cause considerable destabilization of RNA duplexes (Kim et al., 2004). Spin labeling of 2′-amino groups through reactions with aliphatic isocyanates and aromatic isothiocyanates is a more useful route than amide formation and is described in detail below.

2. 2′-AMINO SPIN-LABELING WITH ALIPHATIC ISOCYANATES AND AROMATIC ISOTHIOCYANATES

The first example of spin labeling of the 2′-position in RNA was the reaction of 4-isocyanato-TEMPO (**1**) with 2′-amino groups in RNA, forming a urea linkage (Fig. 5; Edwards, Okonogi, Robinson, & Sigurdsson, 2001). Spin-labeled oligonucleotides, prepared by this method, were used to study the structure-dependent dynamics of the transactivation response RNA (Edwards, Okonogi, & Sigurdsson, 2002; Edwards, Robinson, & Sigurdsson, 2005; Edwards & Sigurdsson, 2002, 2003) and the hammerhead ribozyme by EPR spectroscopy (Edwards & Sigurdsson, 2005). This spin-labeling method has been used by several other research groups and has the advantage that the starting materials are commercially available. However, it also has a few drawbacks. First, the isocyanate functional group is highly reactive and can lead to incomplete labeling in RNA due to a competing hydrolysis reaction, requiring a careful control of the reaction conditions. Second, at the low temperatures under which the spin-labeling reaction is performed, long RNAs sometimes form secondary structures that reduce

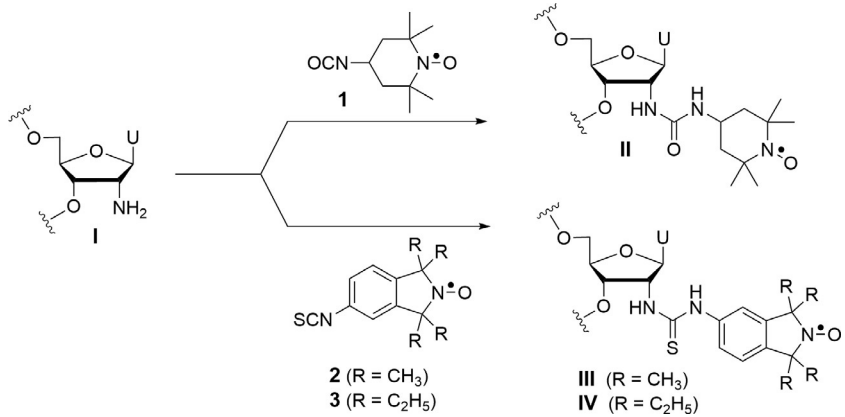


Figure 5 Spin labeling at the 2′-amino position of the oligonucleotide **I** by isocyanate **1** and isothiocyanate spin-labeling reagents **2** and **3**. U, uracil.

the reactivity of 2′-amino groups and result in low yields. In addition, TEMPO is not the optimal spin label for EPR studies due to its inherent flexibility.

To overcome these shortcomings of 4-isocyanato-TEMPO, a new class of spin labels for 2′-amino labeling has recently been introduced: isoindoline-derived nitroxides **2** and **3** have an aromatic isothiocyanate functional group, which forms a stable thiourea linker upon reaction with 2′-amino groups (Fig. 5; Saha, Jagtap, & Sigurdsson, 2015). Aromatic isothiocyanates are less reactive than aliphatic isocyanates, which allows the reaction to be carried out at a higher temperature without any nonspecific labeling. Performing these reactions at higher temperature in the presence of an organic cosolvent reduces RNA secondary structure and thus avoids potential reduced reactivity of the 2′-amino group. The detailed protocols of the preparation of these spin-labeling reagents and their incorporation into 2′-amino sites in RNA will be described in the latter part of this chapter.

2.1 Spin-Labeling of 2′-Amino Groups in RNA with 4-Isocyanato-TEMPO

Isocyanate **1**, the spin-labeling reagent for this protocol, can be either purchased (Toronto Research Chemicals) or synthesized using a previously reported protocol (Edwards et al., 2001; Edwards & Sigurdsson, 2007). As previously mentioned, the 2′-amino-modified oligonucleotides are also commercially available. A representative 2′-amino spin-labeling protocol (Edwards et al., 2001; Edwards & Sigurdsson, 2007, 2014) using isocyanate **1** is as follows:

(1) To a solution of 2′-amino-modified RNA **I** (i.e., 5′-GACCUCG (2′-NH_2U)AUCGUG-3′) (30 nmol), previously precipitated to exchange ammonium ions with sodium ions, in boric acid buffer (15 μL, 70 m*M*, pH 8.6) was added formamide (9 μL). The resulting solution was cooled in a rock salt/ice water bath (−8 °C). It is recommended to perform this reaction in a cold room (4 °C), which helps keeping the temperature low during transfer of reagents. The low temperature minimizes the competing isocyanate hydrolysis reaction and ensures the specificity of the labeling reaction toward the 2′-amino groups.

(2) The solution was treated with freshly prepared **1** (9 μL) in anhydrous *N*,*N*-dimethylformamide (DMF) and incubated for 1 h at −8 °C. The solution of **1** was prepared by dissolving **1** (1 mg) in anhydrous DMF (67.6 μL) to a final concentration 75 m*M*. Isocyanates are electrophilic functional groups and as such they are reactive toward a variety of

nucleophiles, including amines and water. Therefore, anhydrous and amine-free DMF should be used.

(3) To ensure complete spin labeling, it is advisable to add a second aliquot of freshly prepared **1** in DMF (9 μL) after 1 h and a third aliquot after 2 h.

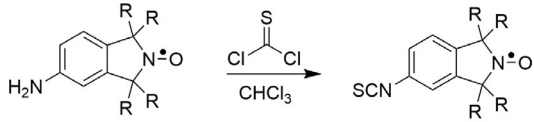
(4) The extent of the spin-labeling reaction can be determined by a denaturing polyacrylamide gel electrophoresis (DPAGE) analysis. An aliquot from the reaction mixture (1 μL) was run on 20% DPAGE gel along with the starting RNA **I**; one lane contained an equimolar mixture of the starting RNA and the RNA present in the reaction. The spin-labeled RNA displays reduced mobility on DPAGE (see Section 2.3 for an example of DPAGE analysis of 2′-amino spin labeling). DPAGE can be readily used to monitor the extent of spin labeling of oligonucleotides of up to ca. 20 nt long; for longer RNA sequences, it may be a challenge to gauge the difference in the mobilities of spin-labeled and unlabeled material. A quantitative conversion to spin-labeled RNA is usually observed. Non- or partial spin labeling indicates decomposition of isocyanate **1**. The purity of **1** can be examined by thin-layer chromatography (TLC) (silica gel, 5% MeOH:CH_2Cl_2, R_f (**1**) = 0.7) and IR spectroscopy (RNCO stretching at 2100–2270 cm^{-1}).

(5) On completion of the reaction, H_2O (100 μL) was added to the reaction mixture, the solution was washed with $CHCl_3$ (4 × 300 μL), and the solvent was removed *in vacuo*.

(6) The spin-labeled RNA was precipitated in EtOH (NaOAc (5 μL, 3 *M*, pH 4.6) and EtOH (300 μL), −80 °C, 4 h) and purified by 20% DPAGE. The gel slices containing spin-labeled material were excised, extracted using the "crush and soak method" with Tris buffer (250 m*M* NaCl, 10 m*M* Tris, 1 m*M* Na_2EDTA, pH 7.5), and subsequently desalted using Sep-pak C18 cartridges following the manufacturer's instructions, to obtain the final product (28 nmol).

2.2 Synthesis of Isothiocyanate-Containing Spin Labels

Spin labeling with **2** and **3** is a newly published method at the time of this writing and thus, these reagents are not yet commercially available. Therefore, the protocol for their preparation has been included. In short, **2** and **3** were prepared by reaction of their corresponding amino derivatives **4** and **5** with thiophosgene (Fig. 6), according to the following representative protocol for the synthesis of **2**:



4 (R = CH_3)
5 (R = C_2H_5)

2 (R = CH_3)
3 (R = C_2H_5)

Figure 6 Synthesis of isothiocyanate spin-labeling reagents **2** and **3**.

(1) A solution of 1,1,3,3-tetramethylisoindoline-5-amine-2-oxyl (**4**) (Jagtap et al., 2015; Mileo et al., 2013) (100 mg, 0.49 mmol) in $CHCl_3$ (3.5 mL) was treated dropwise with a solution of thiophosgene (0.041 mL, 0.54 mmol) in $CHCl_3$ (1 mL) at 24 °C. (Note: Thiophosgene is a toxic reagent and it is strongly recommended to perform the reaction in an efficiently ventilated fume hood.) The progress of the reaction was monitored by TLC (20% EtOAc:pet. ether, R_f (**4**) = 0.2, R_f (**2**) = 0.8).

(2) After stirring for 2 h at 24 °C, the reaction mixture was washed successively with aq. NaOH (4 mL, 1 *M*), H_2O (2 × 5 mL) and brine (5 mL).

(3) The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography using a gradient elution (EtOAc:pet. ether from 0:100 to 5:95) to give **2** as a yellow solid (98 mg, 82%).

Spin-labeling reagent **3** was prepared from its corresponding amino derivative **5** (1,1,3,3-tetraethylisoindoline-5-amine-2-oxyl) (Jagtap et al., 2015) in the same manner. Isothiocyanates **2** and **3** are stable solids that have not shown any detectable decomposition after storing at −20 °C for several months.

2.3 Spin Labeling of 2′-Amino Groups in RNA with Isothiocyanates

The main difference between the protocols for spin labeling with isothiocyanates **2** and **3** and isocyanate **1** is that the spin-labeling reactions were performed at 37 °C for **2** and **3**, compared with −8 °C for **1**. A detailed representative protocol for this spin-labeling method is as follows:

(1) A solution of an isothiocyanate spin label (**2** or **3**) (2 μmol) in DMF (20 μL) was added to a solution of RNA oligonucleotide **I** (40 nmol) in borate buffer (20 μL, 100 m*M*, pH 8.6) and heated at 37 °C for 8 h. For isothiocyanate **2**, we observed a precipitate at the end of the reaction which was extracted into an organic solvent (see next step).

(2) Sterile water was added (200 μL) and the excess labeling reagent was removed by extracting the aqueous reaction mixture with EtOAc (6 × 500 μL). Each of the EtOAc washings was collected separately, and the presence of excess unreacted spin label was monitored. TLC (silica gel, 20% EtOAc:pet. ether, R_f (**2** or **3**) = 0.8) could only be used to detect the presence of spin label in the first two rounds of extraction. In addition, EPR spectroscopy could be used to monitor the whole extraction process; the last EtOAc washing should not show any EPR activity.

(3) In spite of the washings in step 2, we have observed traces of unattached spin contaminants in the spin-labeled RNA (especially using **2**), which were removed by EtOH precipitation: (NaOAc (5 μL, 3 *M*, pH 4.6) and EtOH (300 μL), −80 °C, 4 h) to yield 30–34 nmol of spin-labeled RNA. Note: Further purification of the spin-labeled RNA from the precipitation by DPAGE yielded a product which was of similar purity as the precipitated RNA as judged by EPR and DPAGE.

As mentioned in the spin-labeling protocol of **1**, DPAGE is a useful method to ascertain the extent of RNA spin labeling with **1**, **2**, and **3**. It is also useful for determining the time course of a spin-labeling reaction, just as TLC is useful for monitoring the extent of chemical reactions. Figure 7 shows a DPAGE analysis of samples taken from the spin-labeling reaction mixtures (1 μL) after specific intervals of time. The spin-labeled oligonucleotide showed reduced mobility as compared to the starting 2′-amino RNA, owing to its increased mass. For example, the sample containing **2**, which

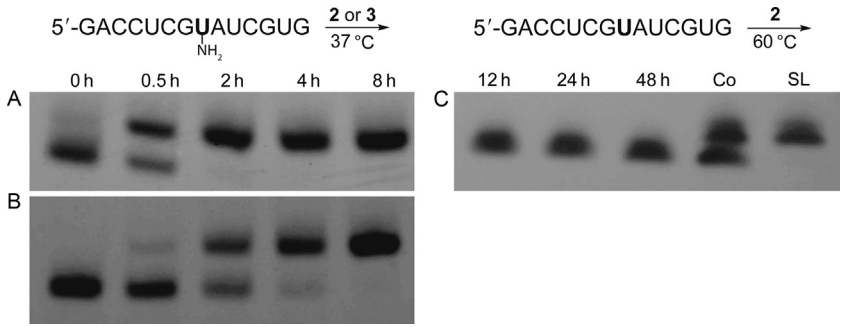


Figure 7 (A) Time course of spin-labeling reaction of RNA sequence **I** with **2**. (B) Time course of spin-labeling reaction of RNA sequence **I** with **3**. (C) Control reaction on unmodified sequence for checking out specificity of the labeling reaction with isothiocyanate spin label **2**. Lane SL contains spin-labeled RNA **III**, and Co is an equimolar mixture of SL and reaction mixture after 48 h.

was removed from the reaction mixture after 0.5 h, clearly showed two bands (Fig. 7A), indicating that the reaction was still not complete. However, the band corresponding to the starting oligonucleotide had disappeared after 2 h, showing that RNA **I** had been converted to its spin-labeled derivative **III**. In contrast, when tetraethyl-derivative **3** was used as the labeling reagent, 90% of the same RNA **I** was converted to **IV** in 4 h (Fig. 7B), showing that **2** was more reactive than **3**. All of the RNA for both reagents had fully reacted after 8 h.

One of the potential drawbacks of postsynthetic labeling is nonspecific reaction of reagents at unwanted sites in RNA. For example, reacting aliphatic isocyanates with unmodified RNA at 37 °C yields modified RNA (Sigurdsson & Eckstein, 1996). To determine specificity of the 2′-amino spin labeling with aromatic isothiocyanates, isothiocyanate **2** was reacted with an unmodified RNA oligonucleotide of the same sequence as 2′-amino-labeled oligomer **I**. Although the spin-labeling reactions of **I** were performed at 37 °C, the unmodified RNA was heated with **2** at 60 °C and reacted for 48 h to assess the degree of potential nonspecific labeling. Figure 7C shows no detectable conversion of the unlabeled RNA to slower moving products, demonstrating the selectivity of **2** for 2′-amino groups in RNA.

2.4 Analysis of Spin-Labeled Oligonucleotides

After the reaction of a 2′-amino-modified oligonucleotide with a spin-labeling reagent and isolation of the product, incorporation of the spin label into the RNA should be verified. Several techniques are routinely used for this purpose. Analysis by DPAGE and HPLC can be used to verify that the oligonucleotide has been modified, but other methods must be used to verify incorporation of an intact spin label (Edwards & Sigurdsson, 2014). Even mass spectrometry (MALDI-TOF) cannot distinguish between a nitroxide and its hydroxylamine derivative, which may result from an unlikely reduction of the spin label. Digestion of the oligonucleotide, followed by HPLC analysis and coinjection with an authentic sample of the spin label lesion, is a useful technique for that purpose (Edwards & Sigurdsson, 2014). However, the most direct method for detecting radicals is EPR spectroscopy.

Oligonucleotides labeled with a nitroxide radical show a characteristic three-peak pattern by EPR. EPR can also be used to detect and quantify free spin label contaminants. A free spin label tumbles rapidly in solution, giving a narrow EPR spectrum, but after attachment to RNA, the EPR lines

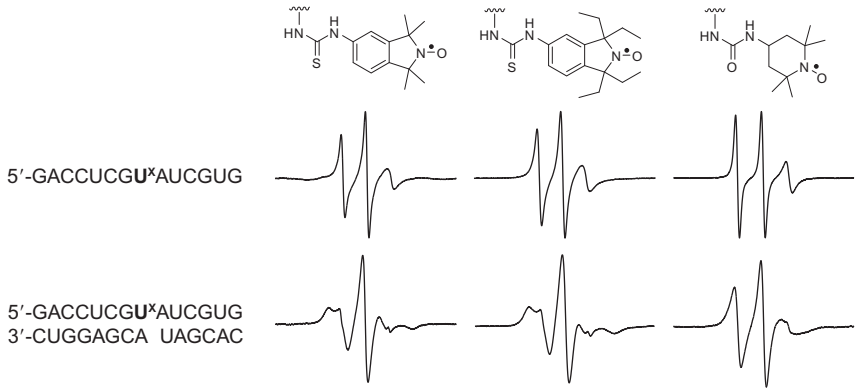


Figure 8 EPR spectra of the spin-labeled oligonucleotides at 10 °C (10 m*M* phosphate, 100 m*M* NaCl, 0.1 m*M* Na_2EDTA, pH 7.0). $\mathbf{U^X}$ indicates the position of the spin-labeled uridine.

become broader due to slower tumbling in solution. In the spin-labeling reaction with **2**, we detected the presence of an importunate unattached spin contaminant by EPR, which was still present after DPAGE purification. This impurity was removed by performing repeated ethyl acetate washes after the spin-labeling reaction, followed by ethanol precipitation. EPR can also be used to perform a spin-count experiment that quantifies the amount of nitroxide, which can be compared to the amount of oligonucleotides determined by UV spectroscopy.

In addition to verifying spin label incorporation, EPR spectroscopy gives valuable information about the mobility of the spin label, independent of the nucleic acid. Figure 8 shows the EPR spectra of oligonucleotides labeled with **1**, **2**, and **3**. It is noteworthy that the spectra of the isoindoline-derived spin labels are broader, compared to the TEMPO derivative, especially for the RNA duplexes. This shows that the isoindoline spin labels are less mobile and should, therefore, be more useful for studies of the structure and dynamics of nucleic acids.

3. SUMMARY AND CONCLUSIONS

Gaining understanding of RNA function through studies of structure and dynamics is an active area of research. SDSL, in combination with EPR spectroscopy, is fast turning out to be a valuable method for such studies. There are two main approaches for spin labeling, the phosphoramidite method and postsynthetic spin labeling. Among these, the latter strategy

requires minimal effort and is less time consuming. In this chapter, we have described postsynthetic spin labeling of 2′-amino groups in RNA using two classes of spin labels, aliphatic isocyanates and aromatic isothiocyanates. The aromatic isothiocyanates are particularly useful and do not suffer from any of the potential drawbacks associated with the postsynthetic labeling strategy, for example, incomplete and/or nonspecific labeling. Spin labeling with isothiocyanates is easy to perform and gives quantitative yields in a short period of time, with no detectable nonspecific labeling. These isoindoline-based spin labels are promising candidates for use in distance measurement with pulsed EPR as they showed reduced mobility by EPR, compared to a TEMPO-based spin label. Moreover, the isoindoline-derived spin labels are stable under reducing conditions (Saha et al., 2015), which makes them promising candidates for in-cell EPR spectroscopy.

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