Site-directed spin labeling of 2′-amino groups in RNA with isoindoline nitroxides that are resistant to reduction†

Subham Saha,‡ Anil P. Jagtap‡ and Snorri Th. Sigurdsson*

Two aromatic isothiocyanates, derived from isoindoline nitroxides, were synthesized and selectively reacted with 2′-amino groups in RNA. The spin labels displayed limited mobility in RNA, making them promising candidates for distance measurements by pulsed EPR. After conjugation to RNA, a tetraethyl isoindoline derivative showed significant stability under reducing conditions.

Electron paramagnetic resonance (EPR) spectroscopy is a biophysical technique that is routinely applied for the study of the structure and dynamics of nucleic acids in order to gain insights into their mechanism of action. Structural information is usually derived from distance measurements, in particular using pulsed techniques, such as pulsed electron–electron double resonance (PELDOR), also known as double electron-electron resonance (DEER). Information about dynamics can be derived from line-shape analysis of continuous wave (CW) EPR spectra, from the width of distance distributions and by analysis of orientation-dependent PELDOR measurements.

Most EPR studies of nucleic acids require incorporation of paramagnetic reporter groups at specific sites, a technique referred to as site-directed spin labeling (SDSL). Aminoxyl radicals, usually called nitroxides, are common spin labels that can be attached to the desired site in the nucleic acid of interest with a covalent bond, although there are examples of noncovalent labeling. Two main approaches have been used for covalent spin-labeling of nucleic acids. The phosphoramidite method utilizes spin-labeled phosphoramidites as building blocks for automated chemical synthesis of the spin-labeled oligonucleotide. This strategy usually involves significant synthetic effort and the spin label is exposed to reagents used in nucleic acid synthesis that might reduce the efficiency of 2′-amino labeling. The other covalent SDSL approach involves a post-synthetic modification of the nucleic acid, wherein a spin-labeling reagent reacts with a specific reactive functional group within the nucleic acid. Post-synthetic spin-labeling usually requires less effort than the classical phosphoramidite approach and can often be performed with commercially available reagents.

Post-synthetic modification of 2′-amino groups in RNA is an efficient method for site-directed spin labeling of oligonucleotides. 2′-Amino-modified RNAs are commercially available or can alternately be prepared using commercially available phosphoramidites. This 2′-labeling method has been used to incorporate the paramagnetic 2′-ureido-TEMPO [(2,2,6,6-tetramethylpiperidin-1-yl)oxy] at specific sites by reaction of 2′-amino groups with 4-isocyanato-TEMPO. However, isocyanates are relatively reactive and, therefore, prone to hydrolysis and can react with other functional groups of the nucleic acid. Special care is required while handling this reagent and when carrying out the spin-labeling reaction. In addition, incomplete labeling has been observed for some long RNAs, presumably due to the formation of secondary structures under the spin-labeling conditions (–8 °C), which may slow down the spin-labeling reaction relative to the competing hydrolysis of the isocyanate. Therefore, it is of interest to find more suitable reagents to react with 2′-amino groups in oligonucleotides, which would make this spin-labeling strategy even more useful.

This report describes the spin-labeling of 2′-amino groups in RNA using isoindoline-derived aromatic isothiocyanates. Aromatic isothiocyanates are more stable than isocyanates and yet reactive enough to modify 2′-amino groups in RNA. We show here that the isothiocyanate spin labels react very efficiently with 2′-amino uridine in RNA, forming a stable thiourea linkage. Moreover, the spin-labeling reactions were carried out at 37 °C in the presence of a denaturing agent (DMF), which minimizes the formation of secondary structures that might reduce the efficiency of 2′-amino labeling.

Two spin-labeling reagents were prepared, isothiocyanates 1 and 2 (Scheme 1), in a single step using readily accessible starting materials. When isoindolines are utilized for spin-labeling, tetramethyl derivatives are normally used, but isoindoline 2 was included because tetraethyl derivatives have...
been shown to be more resistant towards reduction.\textsuperscript{17} 1,1,3,3-
Tetramethylisoindolin-5-amine-2-oxyl (3)\textsuperscript{17,18} and its corresponding
tetraethyl derivative (4)\textsuperscript{17b} were treated with thiophosgene to obtain
the isothiocyanate spin-labeling reagents 1 and 2 in 82% and 57% yields, respectively (Scheme 1). Unlike 4-isocyanato-TEMPO, aromatic
isothiocyanates 1 and 2 were found to be stable solids and did not
require special precautions when prepared or handled.

Spin-labeling reagents 1 and 2 were reacted with the 2'-amino modified RNA oligonucleotide 5'-GAC CUC G(2'-NH₂)₄ UCG UG (I)
at 37 °C, in borate buffer (pH 8.6) containing 50% DMF. Samples
were removed at specific intervals of time and analyzed by
denaturing polyacrylamide gel electrophoresis (DPAGE) analysis
(Fig. 1). A new product was formed in each reaction that
migrated slower than the parent oligonucleotide, thus indicating
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migrated slower than the parent oligonucleotide, thus indicating
successful covalent attachment of the spin labels to the RNA.

Tetramethyl-derivative 1 reacted faster than 2; the former fully
converted RNA I within 4 h and the latter in 8 h, to the corresponding spin-labeled derivatives. Selective reaction at the 2'-amino
group was verified by the lack of reaction between 1 and an
unmodified RNA, even after heating at 60 °C for 48 h (Fig. S3, ESI†).

The spin-labeled oligonucleotides II and III were purified by
DPAGE to give II and III in ca. 75–80% yields. It is noteworthy
that ethanol precipitation of RNA II gave material of the same
purity, as judged by EPR and DPAGE (Page S7, ESI†), making it
a very rapid spin-labeling method. MALDI-TOF analysis of the
oligonucleotides showed the mass expected for the spin-labeled
oligomers (Fig. S4, ESI†). Circular dichroism (CD) spectroscopy
of the corresponding spin-labeled RNA duplexes IV and V
showed negative and positive molar ellipticities at ca. 210 nm
and 262–264 nm, respectively (Fig. S5, ESI†), values that are
characteristic of A-form RNA duplexes.\textsuperscript{19} The thermodynamic
stabilities of the spin-labeled RNA duplexes were determined by
thermal denaturation (T_m) experiments (Table S3 and Fig. S6, ESI†).
Only minor destabilization of 1.2 °C and 2.0 °C were observed for the
tetramethyl- and the tetraethyl-derivative, respectively, relative to an
unmodified duplex. The corresponding TEMPO-labeled RNA duplex
VII, prepared by reaction of 4-isocyanato-TEMPO with oligo-
nucleotide I,\textsuperscript{1,3b} showed considerably less stable (ΔT_m = −5.3 °C).

The EPR spectra of II and III (Fig. 2) show broadening of the
EPR spectral lines relative to spin labels 1 and 2 (Fig S1 and S2,
ESI†), which is consistent with their covalent attachment to the
RNA. The EPR spectra of single stranded oligonucleotides II and III
were also compared with the corresponding TEMPO-derived oligo-
nucleotide VI, which had a noticeably narrower spectrum. The
narrow spectrum of VI presumably reflects in part the inherent
flexibility of TEMPO, in which the six-membered ring can sample
different conformations. The EPR spectra of the corresponding
RNA duplexes (Fig. 2, IV, V, VII) were considerably broader than for

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Scheme 1 Preparation of spin-labeling reagents 1 and 2 and their reaction with the 2'-amino modified RNA oligonucleotide I [5'-GAC CUC G(2'-NH₂)₄ UCG UG] to yield spin-labeled oligonucleotides II and III.

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Fig. 1 A time-course of the spin-labeling reactions between the 2'-amino oligonucleotide I and the aromatic isothiocyanates 1 (A) and 2 (B). Reaction conditions: 1 mM RNA, 50 mM 1, 50 mM borate buffer (pH 8.6), 50% DMF.

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Fig. 2 EPR spectra of the spin-labeled oligonucleotides at 10 °C (10 mM phosphate, 100 mM NaCl, 0.1 mM Na₂ EDTA, pH 7.0). U⃗{\textsuperscript{K}} indicates the position of the spin-labeled uridine and roman numerals under the spectra identify the oligonucleotides (see Table S1, ESI†).
the single strand and again, the EPR spectra of the isoindoline-derived duplexes (IV and V) were broader than that of the TEMPO-modified duplex (VII). It was somewhat surprising to see how broad the spectra for isoindoline nitroxide-labeled duplexes IV and V were, with both the high- and low-field peaks splitting at 10 °C (see Fig. S7, ESI† for other temperatures), given the fact that rotation is possible around bonds in the linker. Since the thiourea can be regarded as a stiff tether, flexibility is restricted to rotation between two single bonds, namely the one connecting the 2′-C and the 2′-N as well as the bond between the urea and the isoindoline. Molecular modeling (Fig. 3) showed that there is only one low-energy rotamer for the C–N bond, in which the large sulfur atom is lodged between two oxygen atoms on the spin-labeled nucleotide: the 3′-oxygen and the oxygen of the tetrahydrofuran ring, resulting in a snug fit for the sulfur atom. Otherwise, the label is projected away from the nucleic acid; the limited mobility indicates that there is restricted rotation around the bond connecting the isoindoline to the urea, as might be expected because of conjugation.

In-cell EPR spectroscopy has emerged as a promising technique to study nucleic acids in vivo.20 Pyrrolidine- and piperidine-based nitroxides have very limited stabilities in reductive environments21 and are thus considered to be ineffective spin labels for in-cell EPR studies. On the other hand, isoindolines have shown higher stability towards reduction, especially tetraethyl derivatives.17 The stabilities of the spin-labeled duplexes IV, V and VII were tested in the presence of ascorbic acid, which is a known cellular reducing agent and often used to evaluate the stability of nitroxides.17b,21a,22 Fig. 4 shows a normalized EPR signal as a function of time. There was a striking difference in the stability of the different spin labels: the TEMPO label was fully reduced within 10 min and the tetramethyl isoindoline within an hour, while ca. 90% of the tetraethyl isoindoline label still remained intact after 10 h (Fig. 4, inset). It is also noteworthy that the stabilities of the nitroxide radicals were slightly higher after being conjugated to the RNA oligonucleotides. For example, under identical conditions, 5% of simple tetramethyl isoindoline derivatives remained after 2 h,17b while 12% of RNA duplex IV (Fig. S9, ESI†) still had an intact spin label. Taken together, these ascorbate experiments indicate that the tetraethyl derivative is a promising spin label for in-cell EPR studies. However, a more detailed study of spin-label stability under cellular conditions, where other reducing agents (e.g. glutathione) are present, will be conducted and reported in due course.

In summary, we have described an efficient method for post-synthetic spin-labeling of 2′-amino groups with aromatic isothiocyanates using two new isoindoline-derived spin labels. This divergent synthetic approach can be used for a variety of isoindoline spin labels and has three major advantages over the previously described 2′-TEMPO derivative. First, the new spin labels have only a minor effect on the thermal stability of RNA duplexes. Second, the isoindoline labels have limited mobility independent of the nucleic acid duplex to which they are attached, which should make them useful for distance measurements. Third, the tetraethyl isoindoline conjugated to RNA exhibits high stability towards reduction, making it a promising candidate for in-cell EPR studies. This spin-labeling strategy should also be useful for spin-labeling long RNAs, either through direct derivatization of 2′-amino groups or by ligation of oligonucleotides containing the tetraethyl spin label, which is carried out in the presence of a reducing agent.

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