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Noncovalent and site-directed spin labeling of duplex RNA†

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Cite this: *Chem. Commun.*, 2016, 52, 14442

Received 18th October 2016,
Accepted 23rd November 2016

DOI: 10.1039/c6cc08387k

www.rsc.org/chemcomm

An isoindoline-nitroxide derivative of guanine (Ǵ, "G-spin") was shown to bind specifically and effectively to abasic sites in duplex RNAs. Distance measurements on a Ǵ-labeled duplex RNA with PELDOR (DEER) showed a strong orientation dependence. Thus, Ǵ is a readily synthesized, orientation-selective spin label for "mix and measure" PELDOR experiments.

Electron paramagnetic resonance (EPR) spectroscopy is a magnetic resonance technique that has been used widely to investigate the structure and dynamics of biomolecules.[1–7] Although widely applicable for such studies, EPR spectroscopy relies on the detection of unpaired electrons. With the exception of biomolecules that contain paramagnetic centers, such as metal ions, appendage of spin labels is usually required. Aminoxyl radicals, also called nitroxides, are commonly used for this purpose. Practical methods for incorporation of radicals should enable their attachment at specific sites, referred to as site-directed spin labeling (SDSL), and have almost exclusively relied on linking radicals to the biomolecule through covalent bonds.[8–10] Covalent labeling can be performed either during the synthesis of the biopolymer or post-synthetically. Such labeling often requires extensive synthetic effort, can result in side-reactions and incomplete labeling and usually requires rather tedious purification of the spin-labeled biopolymer.[11] Moreover, for RNA, there are only a few general spin-labeling methods available for labeling internal sites.[12–18]

A spin-labeling method that requires less effort, synthetic expertise and time is noncovalent labeling, in which the biopolymer could simply be mixed with the spin label prior to EPR measurements. There are examples of noncovalent labeling of biopolymers, but many of those spin labels have limited binding affinity or specificity to the target molecule, such as nucleic acid intercalators.[19–21] For proteins, efficient and specific binding has been demonstrated to native binding sites by attaching spin labels to cofactors[10,22–26] or using encoded tags for high spin ions.[27] However, these approaches are limited to a relatively few number of proteins and only certain site(s). Multiple spin labels have also been delivered to nucleic acids through binding of the G–G mismatch-binding ligand naphthyridine carbamate dimer.[28,29]

Abasic sites in duplex nucleic acids have been used as ligand binding-sites for noncovalent labeling. Examples include fluorescent compounds[30–34] and adenine–acridine conjugates, some of which contain spin-labels.[35] We have previously used abasic sites in duplex nucleic acids for site-directed labeling of nucleic acids.[36–38] In particular, the spin label **ç** (Fig. 1), a derivative of cytosine, showed complete binding to abasic sites opposite to guanine in duplex DNAs at low temperatures.[36] However, later studies revealed that only a few flanking sequences showed complete binding[39] and incorporation of two binding sites into the same duplex resulted in incomplete binding.[40] In addition, only *ca.* 30% of **ç** was found to bind to abasic sites in RNA at low temperatures.[38] Among several pyrimidine-derived nitroxides

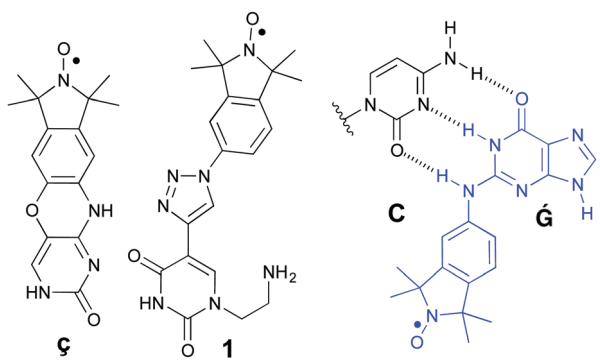

Fig. 1 Structure of the rigid spin label **ç** (left) and a triazole linked-nitroxide spin label **1** (middle). Proposed base-pairing of spin label **Ǵ** (blue) with **C** at an abasic site in duplex RNA.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6cc08387k

that were subsequently prepared and screened for binding to both DNA and RNA, only the triazole-linked nitroxide **1** (Fig. 1), which contains an amino group for increased affinity, showed nearly complete binding to abasic sites in RNA.[38] However, **1** was not a useful spin label because of its extensive non-specific binding to RNA.

Here we describe the synthesis and evaluation of the spin label **Ġ** ("G-spin") for noncovalent binding to abasic sites in nucleic acid duplexes. This new spin label binds with high affinity and specificity to abasic sites in duplex RNA and shows extensive binding to abasic sites in duplex DNA. We also demonstrate the use of noncovalently labeled RNA for distance measurements by pulsed EPR spectroscopy. These experiments also show that the label has limited motion at the abasic site, as judged by a strong orientation dependence.

The new spin label **Ġ** is an isoindoline derivative of guanine. In contrast to previously reported spin labels for noncovalent and site-directed labeling of nucleic acids that require multi-step syntheses,[36–38] **Ġ** can be prepared in one step from readily available starting materials. The commercially available 2-bromohypoxanthine was simply heated with isoindoline nitroxide **3**[41] in DMF to give **Ġ** in moderate yield (Scheme 1).

The binding of **Ġ** to abasic sites in nucleic acids at different temperatures was investigated using continuous wave (CW)-EPR spectroscopy (Fig. 2 and Fig. S2, ESI†). The spectrum of **Ġ** in a phosphate buffer containing 30% ethylene glycol and 2% DMSO, showed a gradual broadening of the three narrow nitroxide lines as the temperature was lowered down to −30 °C, due to decreased tumbling of the spin label in solution (Fig. 2A, left column). When the EPR spectrum of **Ġ** was recorded for the same range of temperatures, in the presence of a DNA duplex containing an abasic site opposite to cytosine (C) (Fig. 2A, middle column), a slow-moving component started appearing in the spectrum at 10 °C. This component increased as the temperature was lowered. At −30 °C, the EPR spectrum showed full binding of the spin label to the abasic site. When this experiment was performed in the presence of an RNA duplex, containing an abasic site (Fig. 2A, right column), there was extensive binding of the spin label, even at 20 °C (>95%, $K_D = 6.15 \times 10^{-6}$ M[36]), at which temperature no binding to DNA was detected. At −20 °C, the spin label was fully bound to the abasic site in the RNA duplex. Thus, **Ġ** has higher affinity to abasic sites in RNA duplexes than DNA duplexes. Changing the identity of the bases immediately flanking the abasic site of the RNA duplex (5′-A_U or 5′-C_U instead of 5′-G_A) showed only a minor effect on the binding affinity to **Ġ**; the spin label was fully bound for all three sequences at −30 °C (Fig. S3, ESI†).

Since **Ġ** binds to the nucleic acid through noncovalent interactions, it was important to verify that it was binding to the abasic site rather than non-specifically, for example by intercalation or groove binding. An unmodified RNA duplex that was mixed with one equivalent of spin label **Ġ** showed barely detectable binding at −20 °C (<1%, Fig. S4, ESI†), which is the temperature required for full binding to the abasic site. Although slightly more non-specific binding was observed at −30 °C (<5%), the small amount of binding at −20 °C, where the spin label is fully bound to the abasic site, will ensure a high occupancy of the labeling sites for distance measurements. Specific binding to the abasic site was also probed by titration with hypoxanthine, which has been used to rescue the activity of a hammerhead ribozyme containing an abasic site.[42]

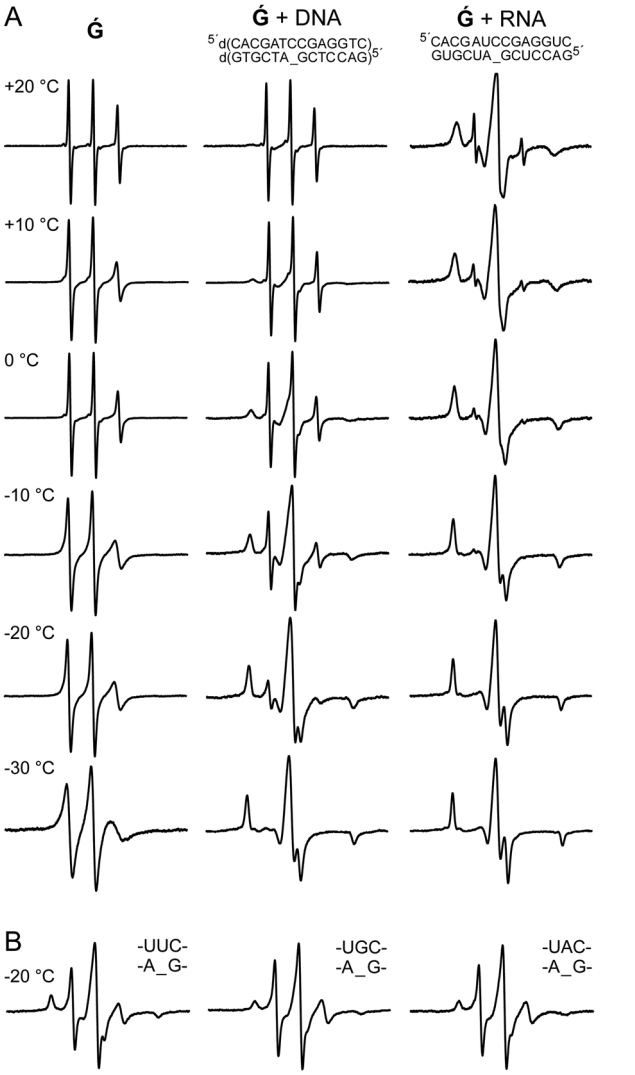
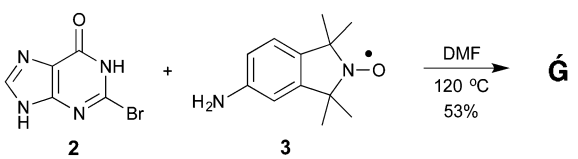


Fig. 2 (A) X-band CW-EPR spectra of the spin label **Ġ** alone (200 μM, left column), in the presence of abasic DNA (400 μM, middle column), and in the presence of abasic RNA (400 μM, right column). The temperature of each measurement is listed on the left. (B) CW-EPR spectra of **Ġ** in presence of abasic RNAs containing non-complementary bases (U, G and A) opposite to the abasic site, denoted by "_". All EPR spectra were recorded in a phosphate buffer (10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0) containing 30% ethylene glycol and 2% DMSO, using the same number of scans. The spectra were phase-corrected and aligned with respect to the height of the central peak.



Scheme 1 Synthesis of the guanine-derived nitroxide spin label **Ġ**.

Although hypoxanthine has lower affinity for the abasic site than Ġ, the spin label was clearly displaced from the duplex as the concentration of hypoxanthine was increased (Fig. S5, ESI†).

To investigate the nature of the binding interactions between the spin label and the abasic site, binding of Ġ to four RNA duplexes containing different orphan bases (A, U, G and C) on the strand opposite to the abasic site were investigated. Less binding was observed for the orphan bases A, G and U (Fig. 2B) than for C, which showed full binding under these conditions (Fig. 2A). Slightly more binding was observed for U than for the two purines. The same trend was observed for DNA (Fig. S1, ESI†). Taken together, these experiments indicate that Ġ binds to the abasic site of both DNA and RNA duplexes by forming hydrogen bonds with the orphan base C.

Pulsed electron–electron double resonance (PELDOR, also called double electron–electron resonance or DEER) can be used to measure medium to long-range distances in RNA between two spin labels.[1,4,5,7,43,44] To determine if noncovalent labeling could be used to measure interspin distances in RNA, a self-complementary 22-mer duplex containing two abasic sites was mixed with two equivalents of Ġ. CW-EPR spectroscopy showed *ca.* 80% binding of the spin label at 20 °C and full binding at −30 °C (Fig. S6, ESI†). A four-pulse PELDOR experiment[45] was subsequently performed, in which a series of time-traces was collected as a function of the frequency offset (40–90 MHz) between the pump and probe pulses (Fig. 3B and Fig. S7, ESI†). There was a striking variation in both the frequency and damping of the oscillations, consistent with strong orientation dependence. In other words, this experiment shows that there is very limited mobility of the spin label when bound to the abasic site in RNA. Summing up the time traces and performing Tikhonov regularization gave a distance of 31.3 ± 3.5 Å similar to the distance of *ca.* 29 Å, based on simple modeling (Fig. 3C; see also ESI,† including Fig. S8).

The EPR data show that the spin label Ġ binds with high enough affinity to enable PELDOR measurements on RNA. While it is true that this spin-labeling method is applicable for duplexes and not for single-strands, most functional RNA molecules contain two or more duplexes as structural scaffolds. Therefore, labeling duplex regions will provide valuable information about the tertiary structure and dynamics of such RNAs as well as conformational changes associated with binding to biomacromolecules or small-molecule ligands.[46] The secondary structures of complex RNAs can be determined accurately,[47] thereby identifying suitable labeling sites without any prior knowledge about the RNA tertiary structure. It should also be noted that this method is suitable for spin-labeling long RNAs (>100 nt); RNAs containing abasic sites can be readily synthesized using commercially available phosphoramidites or purchased directly from companies that provide custom synthesis of oligonucleotides. These modified RNAs can subsequently be ligated to other chemically synthesized RNAs, or RNAs prepared by transcription, using standard methods for RNA ligation.[48,49]

In conclusion, we have demonstrated that the nitroxide Ġ, prepared by a one-step synthesis from readily available starting materials, is an efficient spin label for noncovalent and site-directed

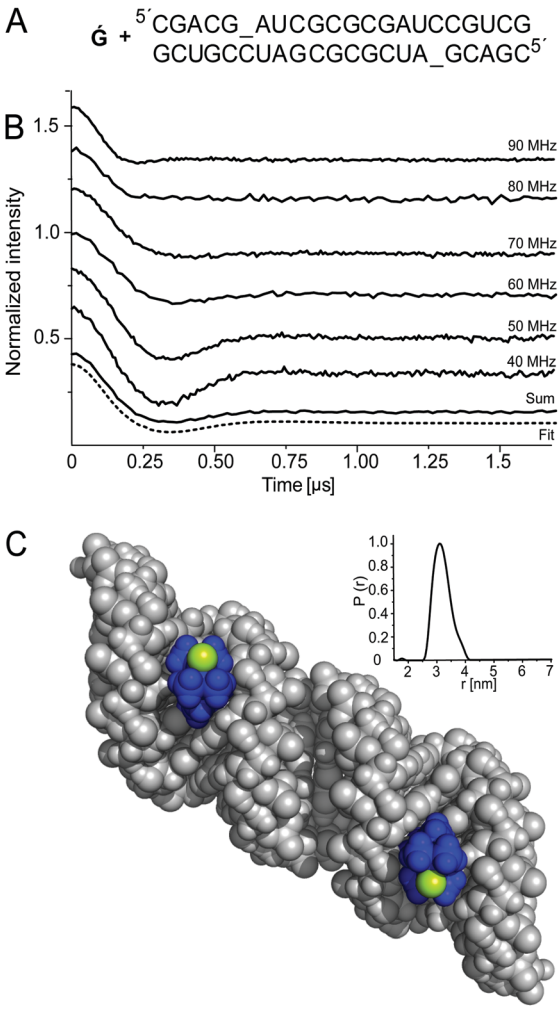


Fig. 3 (A) A 22-mer RNA duplex with two abasic sites, denoted as "_". (B) Multi-frequency X-band PELDOR experiments after background correction (original spectra are shown in Fig. S7, ESI†). The lowest solid trace shows the sum of all the offset measurements, while the dotted trace shows the fit obtained with Tikhonov regularization. Traces have been shifted vertically for better visibility of individual traces. (C) A model of two Ġs bound to the two abasic sites of the 22-mer, along with the distance distribution of the summed PELDOR time traces (inset), obtained with DeerAnalysis2013.[50]

spin labeling of nucleic acids, in particular for RNA. The new spin label binds with specificity and unprecedented affinity to abasic sites of duplex RNA, where it appears to form hydrogen bonds to the orphan base. The spin label Ġ should facilitate structural investigations of RNA by EPR spectroscopy due to the ease of spin labeling, as the label is simply added to a solution of the nucleic acid containing abasic sites. The PELDOR distance measurements also showed a strong orientation dependence, similar to that obtained with rigid spin labels.[40] This orientation dependence yields additional structural information, but the analysis will require more details of how Ġ binds to the abasic site. Those details are under investigation and will be reported in due course.

This work was supported by the Deutsche Forschungsgemeinschaft DFG (SFB 902 Molecular Principles of RNA-based

Regulation), the Icelandic Research Fund (141062051) and by a doctoral fellowship to N. R. K. from the University of Iceland Research Fund. We thank Dr S. Jonsdottir for assistance in collecting analytical data for structural characterization of the compounds.

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