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# Noncovalent spin-labeling of RNA: The aptamer approach

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**In the first example of site-directed spin-labeling of unmodified RNA, a pyrrolidine-nitroxide derivative of tetramethylrosamine (TMR) was shown to bind with high affinity to the malachite green (MG) aptamer, as determined by continuous-wave (CW) electron paramagnetic resonance (EPR), pulsed electron-electron double resonance (PELDOR) and fluorescence spectroscopies.**

The investigation of structure and dynamics of nucleic acids is a prerequisite for obtaining an in-depth understanding of their functions. In this regard, electron paramagnetic resonance (EPR) spectroscopy has become a valuable technique to gather structural information, usually by measuring distances between paramagnetic centers.[1] In particular, pulsed dipolar methods, such as pulsed electron–electron double resonance (PELDOR),[2, 3] also known as double electron-electron resonance (DEER),[4, 5] double-quantum coherence (DQC)[6, 7] and relaxation induced dipolar modulation enhancement (RIDME)[8-10] can measure long-range inter-spin distances between 20 to 100 Å.[2, 4, 11] Naturally occurring nucleic acids are intrinsically diamagnetic and, therefore, paramagnetic reporter groups (spin labels) are usually incorporated into the biopolymer at predetermined sites for EPR studies. The most commonly used spin labels are the bench-stable aminoxyl radicals, commonly called nitroxides.[12]

Spin labels have been incorporated at specific sites of interest using a number of techniques that are collectively known as site-directed spin labeling (SDSL),[13] usually attaching the spin labels to the desired sites via a covalent bond.[14, 15] For example, a nitroxide-derived nucleoside phosphoramidite can be used to incorporate a spin label at the position of choice in a nucleic acid by chemical synthesis.[16] However, synthesis of spin-labeled phosphoramidites usually involves substantial time and effort, as well as expertise in synthetic organic chemistry. Another drawback is the exposure of spin labels to the reagents used during the oligonucleotide synthesis, which may result in partial reduction of the nitroxide.[17, 18] Spin labels can also be covalently attached to nucleic acids post-synthetically, wherein a spin-labeling reagent reacts with a uniquely reactive functional group within the nucleic acid. Several examples of postsynthetic spin labeling are available for RNA.[19-27] However, potential drawbacks of this method include incomplete labeling and side reactions of the spin label with inherent functional groups of the nucleic acids that result in non-specific conjugation.[28]

Noncovalent spin-labeling circumvents the problems associated with covalent methods of spin labeling. For example, pyrimidine- and purine-derived nitroxides have been shown to bind to abasic sites in DNA and RNA duplexes.[29-32] However, this approach requires abasic sites that are incorporated during the chemical synthesis of the nucleic acid. Hence, all the techniques developed thus far for SDSL require a chemical modification of the nucleic acid. Here, we introduce a strategy to noncovalently spin-label an *unmodified* RNA using the malachite green (MG) aptamer (**Figure 1, left**).

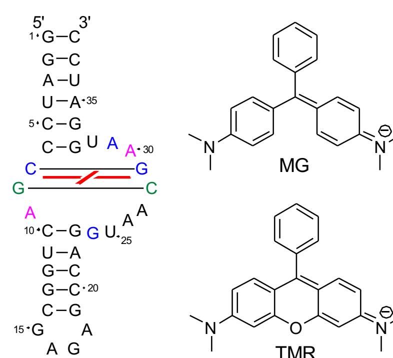


**Figure 1**. Secondary structure of the malachite green (MG) aptamer with the ligand-binding position shown in red (left). The key nucleotides in the binding pocket of the MGA consist of a base quadruple shown in blue (C7, G24, A31 and G29) and a Watson – Crick base pair shown in green (G8 and C28), both of which serve as a platform for stacking for the ligands MG and tetramethylrosamine (TMR) (right). In addition, nucleotides A9 and A30 (shown in pink) assist in almost complete closure of the binding pocket.

RNA aptamers are RNA oligomers that bind to a variety of targets with high affinity and specificity,[33, 34] such as amino acids,[35] drugs,[36] proteins[37] and other small molecules.[38] The MG RNA aptamer is

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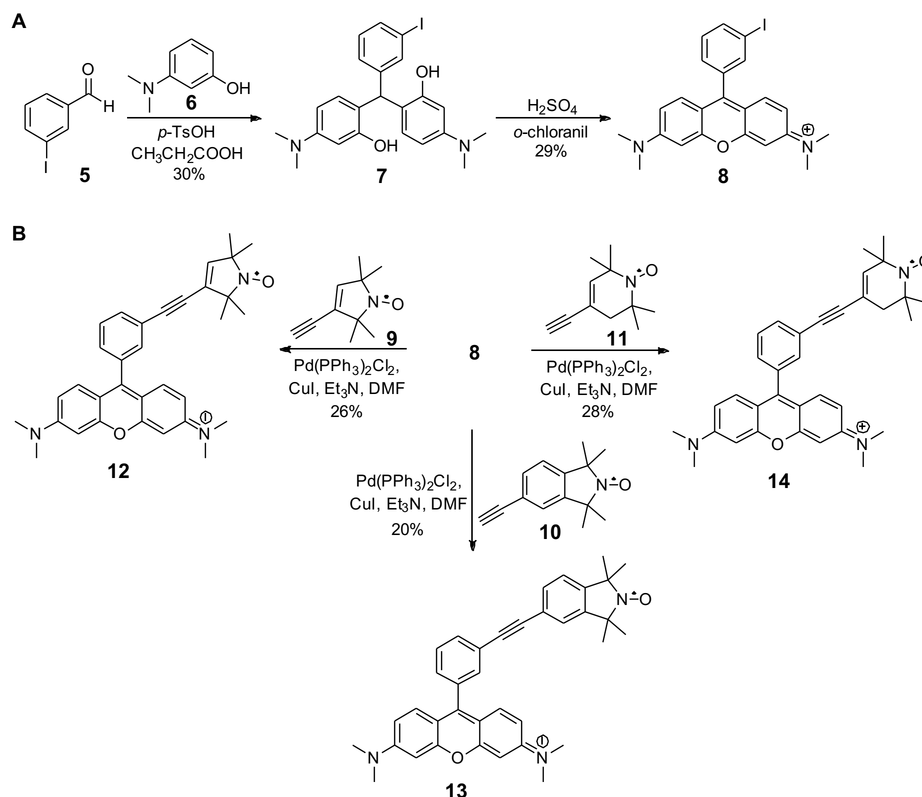
known to bind to the dyestuff malachite green (MG) (**Figure 1**) and its derivatives with dissociation constants ($K_D$) in the nanomolar range.[39]

The MG aptamer has a comparatively short sequence (38-nucleotides) and detailed structural information is available for the ligand-aptamer complex,[40, 41] which was used to guide the design of the spin label. The best known ligand for this aptamer is the cognate dye tetramethylrosamine (TMR) (**Figure 1**), which structurally differs from MG by a single oxygen atom that bridges two of the aromatic rings to form a partial planar structure.[40] Both X-ray[40] and NMR[41] structures of the TMR- and MG-bound aptamer complexes, respectively, revealed that the ligand binding-site in the aptamer was defined by an asymmetric internal loop flanked by a pair of helices (**Figure 1**). Various modifications in the ligands have been reported to be tolerated by the MG aptamer.[42] Based on the crystal structure of the MG aptamer with TMR, we introduced nitroxides at the meta-position of the non-nitrogen bearing aromatic ring of TMR with an acetylene tether.

A divergent synthetic strategy was followed to obtain three spin-labeled derivatives of TMR (**Scheme 1**). An iodide was introduced at the meta-position of the non-nitrogen bearing aromatic ring of TMR by condensing 3-iodobenzaldehyde (**5**)[43] with 3-(dimethylamino)phenol (**6**) to obtain triaryl intermediate **7**, which was further subjected to ring-closure to yield TMR derivative **8** (**Scheme 1A**). Compound **8** was a common substrate for a Sonogashira cross-coupling using three different acetylene-modified nitroxides as coupling partners: a five-membered pyrrolidine-based nitroxide **9**,[16] an isoindoline nitroxide **10**[44] and a six-membered piperidine-based nitroxide **11**,[45] to afford TMR-derived spin labels **12**, **13** and **14**, respectively, in moderate yields (**Scheme 1B**).

Binding of the spin labels to the MG aptamer was studied by CW-EPR spectroscopy at 20 ˚C (**Figure 2**). The rotational correlation time of a nitroxide radical bound to a biomolecule, such as the MG aptamer, in solution is longer than for an unbound nitroxide. At a longer rotational correlation time, the anisotropic hyperfine coupling is only partly averaged out, resulting in a broadened CW-EPR spectrum. All the three nitroxides (**12**, **13** and **14**) showed binding to the aptamer, judged by broadening of the EPR spectra (**Figure 2**, middle). EPR spectra for two of the spin-labeled TMRs, **13** and **14**, showed the presence of a fast-moving component that implied either partial binding and/or persistence of some degree of mobility in the labels even after binding to the aptamer. In contrast, the EPR spectrum of the pyrrolidine-based TMR spin-label **12** in the presence of the MG aptamer predominantly displayed the slower, more anisotropic component, which indicated more extensive binding of **12** to the aptamer. The specificity of the binding of **12** to the MG aptamer was evaluated by replacing the aptamer with a non-binding mutant RNA (C7A) (**Figure 2**, right).[42] Although the spin labels showed broadened EPR spectra in the presence of the mutant RNA, indicating non-specific binding, the overall motion of the spin label was clearly faster than of the bound spin labels. This non-specific interaction is presumably due to electrostatic interaction of the cationic dyes with the negatively-charged RNA and/or hydrophobic interactions. Simulations of the EPR spectra of **12** itself, in the presence of the C7A mutant and in the presence of the MG aptamer are shown in **Figure S11**. The spectrum of **12** in the presence of the C7A mutant could only be adequately simulated by including ca. 10% of the free spin label **12**. The simulations yielded approximate rotational correlation times of 0.4 ns (**12**), 2 ns (**12**+C7A) and 10 ns (**12**+MG aptamer).




**Scheme 1**. Synthesis of *m*-iodo derivative of TMR **8** (**A**) and TMR-derived spin labels **12**, **13** and **14** (**B**).



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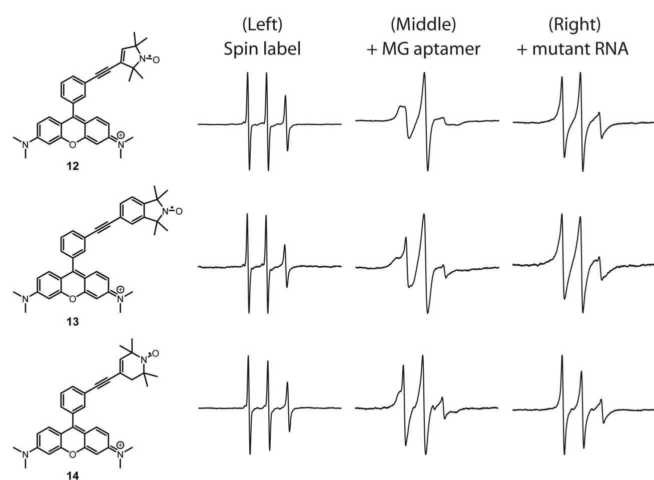


**Figure 2**. EPR spectra of the spin labels **12**, **13** and **14** (200 µM) without RNA (left), when bound to the MG aptamer (500 µM) (middle) and with non-binding mutant RNA (C7A, 500 µM) (right). All data were recorded at 20 °C in a buffered solution of 10 mM $Na_2HPO_4$, 100 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.0, containing 2% DMSO and 30% ethylene glycol.

The binding specificity of **12** to the MG aptamer was further verified by titrating the MG aptamer into a solution of spin label **12**; all of the label was bound, as observed by EPR, when equimolar ratios of **12** and the aptamer were used (**Figure S12**). In addition, a competition experiment was performed in which MG was titrated into a solution of a 1:1 complex of the MG aptamer and **12**. It could be inferred from the EPR spectra that the native ligand MG outcompeted **12** to occupy the binding pocket only when it was used in an excess (**Figure S13**). At 1:1 ratio, **12** was predominantly bound, thus indicating higher binding affinity than MG.

The fluorescence of MG-based dyes are known to increase several fold upon binding to the MG aptamer.[46, 47] Although nitroxides are known to quench fluorescence,[48-51] spin label **12** was found to be fluorescent (quantum yield = 0.58) despite being connected to a nitroxide, presumably because the acetylene tether separated the fluorescent triarylmethyl and the nitroxide moiety. Fluorescence of the unbound spin label **12** decreased by about threefold upon binding to the MG aptamer, which enabled determination of its $K_D$ to be 66 nM (**Figure S15**). For comparison, the $K_D$ of TMR (unmodified) when bound to the MG aptamer has been reported to be 40 nM.[40] Thus, the nitroxide modification does little to adversely affect the binding of **12** to the MG aptamer. The $K_D$s for both **12** and MG were also determined in the presence of 30% ethylene glycol, used for the EPR measurements, and gave similar values (see Supporting Information, **Figure S16**).

To further prove that spin label **12** was bound specifically to the binding site of the aptamer, PELDOR was used to measure a distance from the nitroxide radical of the bound spin-labeled ligand **12** to a nitroxide that was covalently tethered to the aptamer. The covalent labeling was achieved by a post-synthetic labeling of a 2′-amino uridine (U36) of the aptamer with a tetraethylisoindoline-based nitroxide spin-label (**Figure 3A**).[26] Tikhonov regularization of the PELDOR time trace using *DeerAnalysis*[52] yielded an inter-spin distance of 3.3 nm (**Figure 3B**). *In silico*, two inter-spin distances of 3.3 nm and 3.6 nm were obtained, as the covalently-attached isoindoline nitroxide can potentially sample two different rotamers. Therefore, the experimentally obtained distance was found to be in good agreement with that obtained from the molecular models, which further confirmed that spin label **12** bound specifically to the binding pocket of the aptamer.

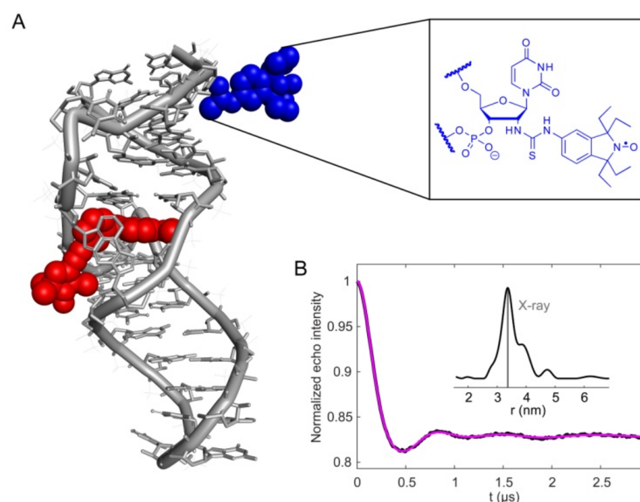


**Figure 3**. **A**. A molecular model of the MG aptamer used for the PELDOR studies, showing the positions of spin labels **12** at the binding site (red) and the tetraethylisoindoline nitroxide (blue). **B**. Background-corrected PELDOR time trace (black) and fit obtained by Tikhonov regularization (magenta). The distance distribution is shown in the inset. The grey line shows a distance obtained from molecular models based on the X-ray structure.[40] For experimental details and primary PELDOR data, the reader is referred to the ESI (**Figure S14**).

In conclusion, a new spin-labeling strategy using noncovalent interactions between the MG aptamer and a spin-labeled derivative of TMR has been described. This is the first example of site-specific spin labeling of a completely unmodified RNA. Spin label **12** had high affinity to the RNA aptamer even at ambient temperature. Distance measurement by PELDOR between the noncovalent spin-label **12** and a spin label that was covalently attached to the MG aptamer was performed to assert specificity of the ligand-aptamer binding. This easy, “mix and measure” spin-labeling approach will open new doors for site-directed spin labeling of long RNAs,[53-56] that are exclusively prepared by enzymatic approaches. The MG domain is unlikely to be found in biologically relevant RNAs. However, the structure of the MG aptamer is similar to a helix and, therefore, it may be possible to replace helices or stem-loops in RNAs with the MG domain for EPR studies. Spin labeling with the aptamer approach may also be combined with other spin labeling methods. For example, a covalently-labeled strand could be annealed to a different region of an RNA containing one MG aptamer motif for noncovalent labeling. Singly-labeled domains of RNAs or RNA-protein complexes may also find use in paramagnetic relaxation enhancement (PRE) experiments, conducted by NMR spectroscopy. Applications with the aptamer spin-labeling strategy will be reported in due course.

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characterization of the compounds prepared, K. R. Oskarsson for his assistance with collecting fluorescence data and Dr. T. Halbritter for his assistance in generating the molecular models. The authors thank members of the Sigurdsson research group for critical reading of the manuscript and for helpful discussions.

## Conflicts of interest

There are no conflicts to declare.

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