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RESEARCH ARTICLE

Non-Covalent Spin-Labeling of RNA With Short Hairpins Containing the Rigid Spin Label Çm

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ABSTRACT

Understanding the structure and dynamics of nucleic acids is essential for elucidating their complex biological functions. Electron Paramagnetic Resonance (EPR) spectroscopy is a valuable technique to extract such information. However, its application to nucleic acids requires the incorporation of spin labels, usually nitroxides. Rigid spin labels are more informative EPR probes than flexible ones, since they offer high-precision distance measurements and can provide information about the relative orientation of spin labels. However, the synthesis and incorporation of such rigid labels is nontrivial. Here we describe a strategy in which the rigid spin label **Çm** is incorporated into a small hairpin, which in turn can be used to label several different oligonucleotides noncovalently through helical stacking. We have studied the noncovalent assembly of spin-labeled RNA hairpins with RNA duplexes by both continuous wave (CW) and pulsed dipolar EPR spectroscopy, specifically pulsed electron-electron double resonance (PELDOR, also called DEER). Our data shows that short complementary overhangs facilitate efficient helical stacking, opening the possibility of using noncovalent labeling with **Çm**-modified hairpins in conjunction with pulsed dipolar EPR spectroscopy for structural studies of larger RNAs and RNA-protein complexes.

1 | Introduction

Electron Paramagnetic Resonance (EPR) spectroscopy has emerged as a valuable technique for studying paramagnetic molecules and molecular assemblies, for example, to gain insights into the structure and dynamics of complex biomolecules, such as proteins and nucleic acids [1–7]. Continuous wave (CW) EPR spectroscopy gives information about dynamics through line shape analysis [8] and can also be used to determine distances between paramagnetic centers in the range of 5 to 25 Å [9–11]. Pulsed dipolar EPR methods, such as pulsed electron-electron double resonance (PELDOR), also known as double electron–electron resonance (DEER), have been used to measure distances from 15–160 Å [12–16]. In addition to distance measurements, PELDOR can provide information about orientations of paramagnetic centers [17–19].

For the application of EPR spectroscopy to study nucleic acids, spin labels need to be incorporated into the biopolymer [20, 21]. Stable aminoxyl radicals, usually called nitroxides, are the most commonly used spin labels [20–22]. Short linkers between the nucleic acid and the spin label are desirable to limit the conformational ensemble of the label and thus give a narrow distance distribution [23–24]. Even better are rigid spin labels, such as the cytidine analogues **Ç** [25] and **Çm** [26] that can form base pairs with guanine in DNA and RNA helices, respectively (Figure 1A). In addition to providing precise inter-spin distances, PELDOR with such rigid labels also yields relative orientations

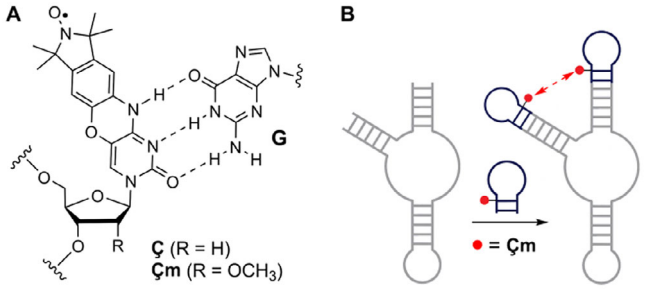

FIGURE 1 | **A**. The **Ç** and **Çm** spin labels are shown base-paired with guanine. **B**. Schematic representation of labeling a large RNA noncovalently through helical stacking with a **Çm**-labeled hairpin.

between the spin labels [18–19]. Thus, rigid spin labels give more detailed information about both the structure and dynamics of nucleic acids. However, both **Ç** and **Çm** have the drawback of requiring a labor-intensive multi-step synthesis and subsequent incorporation into nucleic acids using the phosphoramidite approach, which is limited to the synthesis of relatively short oligonucleotides [21, 24, 27].

Spin labels can also be incorporated into nucleic acids through noncovalent spin-labeling. One example is the binding of pyrimidine- and purine-derived nitroxides to abasic sites in DNA and RNA duplexes [28, 29]. However, such spin labels require the presence of abasic sites that need to be incorporated by chemical synthesis of oligonucleotides. Moreover, in some cases, they have poor affinity to the binding sites [28]. A ligand-binding RNA molecule (aptamer) has also been spin-labeled noncovalently through binding to its nitroxide-modified malachite green (MG) ligand [30]. This approach allows for spin-labeling of long RNAs containing the MG binding motif, prepared by transcription. However, the ligand-binding domain may interfere with the structure of the RNA under study. Base-pairing has also been used for noncovalent spin-labeling, where short, chemically synthesized oligomers containing a nitroxide label were hybridized to a segment of a large RNA [31]. Nonetheless, such an approach would require chemical synthesis of a spin-labeled oligomer of a certain sequence for each target RNA.

Another known noncovalent nucleic acid binding interaction is helical stacking of blunt-ended duplexes, observed by EPR spectroscopy [25, 32, 33]. In this paper, we describe end-to-end helical stacking of RNA for noncovalent spin-labeling with short hairpins containing the rigid spin label **Çm**. This approach minimizes the synthetic effort and cost of using rigid spin labels for RNA (Figure 1B). This involves the synthesis of a small **Çm**-labeled hairpin that can be used as a universal adaptor to spin label different RNAs noncovalently through helical stacking. Thus, this approach yields spin-labeled RNA without chemical modification of the RNA to be studied. We show that the affinity of stacking, and thereby the labeling efficiency, can be improved by using complementary overhangs.

2 | Results and Discussion

Although our strategy is for labeling long and structured RNAs, we used stacking of spin-labeled hairpins to duplexes for proof-of-principle experiments (Figure 2). The oligonucleotide constructs were designed such that the core sequences of the hairpins and duplexes were kept constant (Figure 2A). The hairpins were designed to minimize complementarity for duplex formation and contained the hairpin-stabilizing GNRA tetraloop [34]. We began by synthesizing a short blunt-ended hairpin ($\mathbf{I_{HP}}$) containing **Çm** [35] and its corresponding RNA duplex ($\mathbf{I_{DUPL}}$, Figure 2A). Incorporation of **Çm** into the hairpins was achieved through automated solid-phase synthesis as previously described [35]. The spin-labeled hairpins were enzymatically digested, and the digests were analyzed by high-performance liquid chromatography (HPLC) to demonstrate incorporation of intact **Çm** (Figure S1) [27, 35]. The spin-labeling efficiency was quantified by spin counting using CW-EPR spectroscopy (Figure S2).

Helical stacking in solution can be monitored by CW-EPR through line shape analysis [25]. Biomolecular assembly increases the rotational correlation time of spin-labeled RNAs and thereby the anisotropy and the associated line-broadening of the EPR spectra. Moreover, any association of two spin-labeled hairpins that brings two spin labels into close proximity (<15 Å) may alter the line shape through dipolar coupling between the nitroxide radicals if the rotational correlation time of the assembly is long, relative to the time scale of the EPR experiment [2, 25]. The EPR spectrum of the hairpin $\mathbf{I_{HP}}$ alone at -10°C shows some broadening and some irregular features (Figure 2B) that might arise from dipolar coupling due to self-stacking of the hairpins. Adding an unmodified duplex ($\mathbf{I_{DUPL}}$) resulted in the disappearance of the irregular features in the EPR spectrum, which might have been due to hairpin-duplex stacking and thereby reduced dipolar coupling. However, when the hairpin was titrated with an unmodified single-stranded RNA, a similar change in the line shape was observed (Figure S4C). This suggests that the disappearance of the irregular features may not solely be due to stacking between duplexes but also due to other nonspecific intermolecular interactions. The line shape of the EPR spectrum of $\mathbf{I_{HP}}$ alone at 20°C shows no broadening (Figure S3).

The stacking of two hairpins on the ends of the same duplex can be monitored by PELDOR. If $\mathbf{I_{HP}}$ stacks on each end of $\mathbf{I_{DUPL}}$, as in **I** (Figure 2A), the distance between the spin labels is expected to be ca. 3.0 nm, as predicted by molecular modeling (Figure S7, I, Table S2), ideal for a PELDOR measurement. However, the PELDOR data of **I** showed a broad distance distribution that was dominated by shorter distances (~ 2.2 nm) and some longer distances of much lower probability between 3 and 6 nm (Figure S6, B). The shorter distances are consistent with the modeled distance between two **Çm**s in a hairpin dimer (Figure S7, I, Table S2). Taken together, the data indicate that the blunt-ended $\mathbf{I_{HP}}$ prefers self-stacking rather than stacking with its duplex ($\mathbf{I_{DUPL}}$).

To favor hairpin-duplex stacking over hairpin-hairpin stacking, overhangs were incorporated at the 3'-end of the spin-labeled hairpins ($\mathbf{II_{HP}}$-$\mathbf{VI_{HP}}$) and the corresponding duplexes ($\mathbf{II_{DUPL}}$-$\mathbf{VI_{DUPL}}$) (Figure 2A). The overhangs were one to five nucleotides long and complementary to overhangs on the corresponding duplexes. Significant broadening (indicated by asterisks in Figure 2B) became prominent at -10°C in the CW-EPR spectra of the **Çm**-labeled hairpins $\mathbf{II_{HP}}$-$\mathbf{VI_{HP}}$. These features are consistent with increased anisotropy due to intermolecular association. On

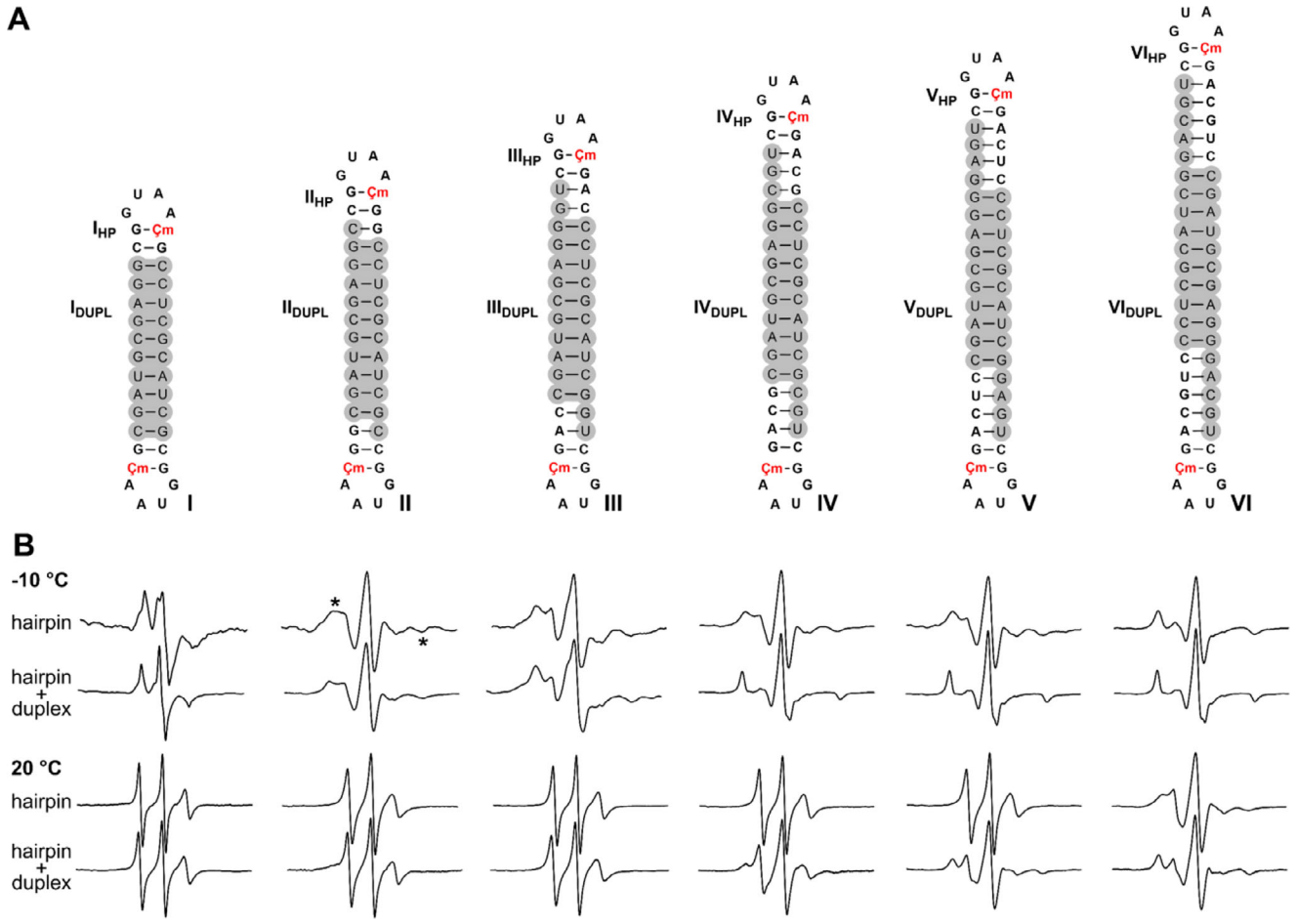

FIGURE 2 | **A**. The sequences of **Çm**-labeled hairpins ($\mathbf{I_{HP}}$-$\mathbf{VI_{HP}}$) shown stacked on their corresponding RNA duplexes ($\mathbf{I_{DUPL}}$-$\mathbf{VI_{DUPL}}$) (in gray), in the order of increasing number of nucleotides in the overhangs (0-5). The overhangs are on the 3'-end of both the hairpins and the duplexes. The hairpin-duplex assemblies are labeled **I**-**VI**. **B**. X-band EPR spectra of the hairpins ($\mathbf{I_{HP}}$-$\mathbf{VI_{HP}}$) in the absence and presence of their corresponding duplexes ($\mathbf{I_{DUPL}}$-$\mathbf{VI_{DUPL}}$) at -10°C and 20°C, recorded in a phosphate buffer (10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0; concentration of each **Çm**-labeled hairpin was 200 μM and its corresponding duplex 100 μM). Asterisks indicate broad features.

mixing the hairpins with their corresponding duplexes, the slow-moving features became more pronounced, in particular for three-, four-, and five-nucleotide overhangs (**IV**-**VI**), consistent with hairpin-duplex stacking. Moreover, the EPR spectra for the assemblies containing three-to five-nucleotide-long overhangs look nearly identical, suggesting a similar extent of stacking at -10°C. As expected, the EPR spectra for hairpins $\mathbf{II_{HP}}$-$\mathbf{V_{HP}}$ alone at 20°C showed no detectable broadening (Figure 2B), since overhangs are known to prevent RNA stacking [33]. However, the EPR spectrum of the hairpin with a five-nucleotide overhang ($\mathbf{VI_{HP}}$) at 20°C indicated intermolecular association.

Interestingly, the hairpins with three-, four-, or five-nucleotide overhangs ($\mathbf{IV_{HP}}$-$\mathbf{VI_{HP}}$) showed significant spectral broadening upon addition of their corresponding duplexes ($\mathbf{IV_{DUPL}}$-$\mathbf{VI_{DUPL}}$) at 20°C, indicating that hairpin-duplex stacking at this temperature requires a minimum of 3-base overhangs for base-pairing. Indeed, this broad feature increases with the length of the overhang.

The hairpin-duplex stacking of assemblies **II**-**VI** was further studied by PELDOR. The depth of the oscillation pattern (modulation depth) in the PELDOR time traces (Figure 3A) reflects the extent of dipolar coupling between interacting spin pairs [36]. Therefore, a larger modulation depth would indicate more double-stacking of the **Çm**-labeled hairpins on the duplex ends. However, modulation depth also depends on the excitation bandwidth, as strong dipolar coupling at very short spin-spin distances can lead to incomplete excitation of all spin pairs, resulting in a lower observed modulation depth [14, 37]. However, the modulation depth does reflect the same general trend as observed in the CW-EPR data: it increases as the length of the overhang increases. The time traces for oligonucleotides with three- and more overhangs (**IV**, **V,** and **VI**) display the highest modulation depth (Figure 3A), suggesting efficient hairpin-duplex stacking. For one- or two-nucleotide overhangs (**II**, **III**), the modulation depth is noticeably smaller. This is presumably due to a very strong dipolar coupling resulting from hairpin self-association, which contributes only partly to the modulation depth. To partly compensate for the limited excitation due to dipolar coupling, we recorded PELDOR time traces using short (strong) pulses to broaden the excitation bandwidth (Figure S5A). The stronger pulses yielded a higher modulation depth for **III** than **II**, presumably reflecting a high degree of self-association for **II**.

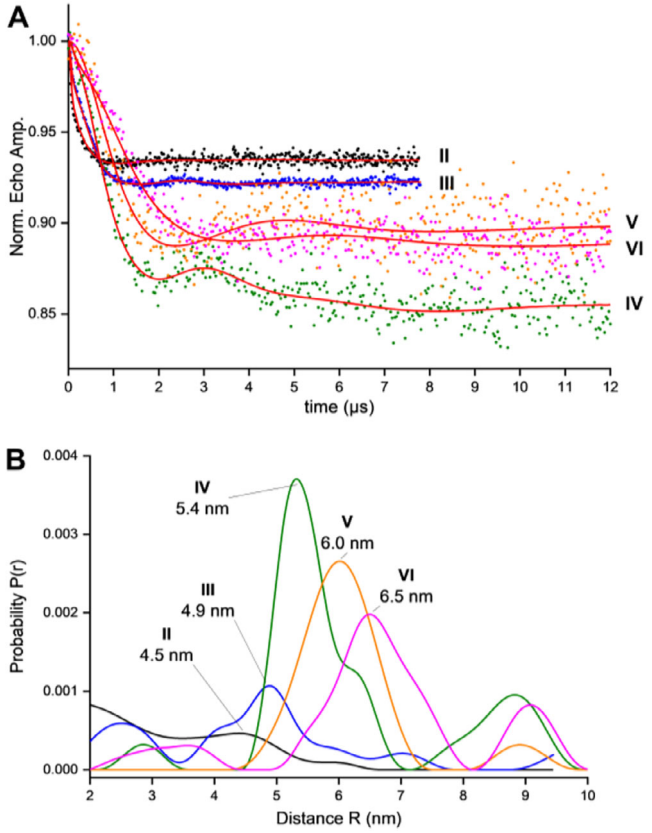

FIGURE 3 | **A**. PELDOR time-traces for the **Çm**-labeled hairpins with one to five nucleotide overhangs, mixed with their complementary RNA duplexes (**II**-**VI**). (10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM Na_2EDTA, 20% ethylene glycol, pH 7.0) **B**. Distance distribution derived from a Tikhonov regularization of the background-corrected PELDOR time traces using DeerAnalysis [38].

Although the modulation depth for assemblies **V** and **VI** is lower than for **IV**, it is not due to less stacking. The short transversal relaxation time only allowed a short time trace to be collected, relative to the longer oscillation of the PELDOR time trace [36]. This can lead to artifacts, such as a too-short distance distribution and wrong background correction, which in turn affects the modulation depth. As a result, the modulation is not as deep and the estimated distance is less accurate. However, hairpin-hairpin association can be excluded with certainty.

Tikhonov regularization of the time traces yielded the corresponding distance distributions (Figure 3B), further supporting the trends observed in the modulation depths. Assembly **II** and **III** show prominent short-distance populations (<3.5 nm), suggesting hairpin-hairpin association rather than stacking to the duplex ends. However, longer distances of 4.5 and 4.9 nm also appear, which agree with the expected modeled distances with the hairpins stacked on the duplex ends (3.9 and 4.6 nm, respectively) (Figure S5B). For the assembly with three- to five-nucleotide overhangs (**IV**-**VI**), the short-distance population is negligible, and a dominant distance distribution appears at ~5.4 nm, 6.0 nm, and 6.5 nm, respectively, close to that of the modeled distances of 5.0 nm, 5.5 nm, and 6.2 nm, respectively (Figure S5B). Overall, the PELDOR data aligns well with the CW-EPR results, further asserting that hairpins with three- or more overhangs exhibit the most efficient stacking.

3 | Conclusion

In conclusion, we have described a noncovalent spin-labeling strategy with the rigid spin label Çm that utilizes end-to-end helical stacking of RNAs. While blunt-ended hairpins did not result in appreciable stacking on RNA duplexes, CW-EPR and PELDOR experiments showed that inclusion of three or more complementary overhang nucleotides on the hairpins and duplexes resulted in efficient assembly. A great advantage of this spin-labeling strategy is that it circumvents the need to chemically incorporate a rigid spin label into each target RNA. Although the spin-labeled hairpin was prepared by chemical synthesis, each preparation can be used for multiple labeling; the spin-labeled RNA was prepared on a µmol scale, while only a few nmols are required for each PELDOR experiment. Thus, this labeling strategy reduces the synthetic effort and cost associated with rigid spin labeling.

Although the proof-of-principle experiments described here utilized hairpin-duplex stacking, this approach should be applicable for long RNAs produced by *in vitro* transcription, provided that accessible RNAs contain helix-loops that could be replaced with Çm-labeled hairpins. Potential applications include the study of helical arrangements within complex RNAs and interactions of RNA with other biomolecules, such as proteins. This labeling strategy can also be extended to other spectroscopic probes, such as fluorophores. Applications of this noncovalent spin-labeling approach, in conjunction with PELDOR, with structured RNAs will be reported in due course.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

The authors have cited additional references within the Supporting Information [38, 39]. **Supporting File:** chem70626-sup-0001-SuppMat.docx