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# Chapter 16

## Site-specific spin-labeling of RNA for NMR and EPR structural studies

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## **Abstract**

Many RNA architectures were discovered to be involved in essential biological pathways acting as catalysts and/or regulators of gene expression, transcription, translation, splicing or viral infection. The key to understand their diverse biological functions is to investigate their structure and dynamic. Nuclear Magnetic Resonance (NMR) is a powerful method to gain insight into these properties. However, the study of high molecular weight RNAs by NMR remains challenging. Advances in biochemical and NMR methods over the recent years allow to overcome the limitation of NMR. In particular, the incorporation of paramagnetic probes, coupled to the measurement of the induced effects on nuclear spins has become an efficient tool providing long-range distances restraints and information on dynamic in solution. At the same time, the use of spin label enabled the application of Electron Paramagnetic Resonance (EPR) to study biological macromolecules. Combining NMR and EPR is emerging as a new approach to investigate the architecture of biological systems.

Here, we describe an efficient protocol to introduce a paramagnetic probe into a RNA at a specific position. This method enables various combinations of isotopic labeling for NMR and is also of interest for EPR studies.

**Key words :** RNA, NMR, EPR, paramagnetic

## 1. Introduction

RNAs play a crucial role in a wide range of biological processes in all organisms. The classical vision of RNA as genetic code carrier has completely evolved since the discovery of ribozymes [1–5], riboswitches [5, 6] and small regulatory RNAs such as small interfering RNAs (siRNAs), microRNAs or PiwiRNAs [7, 8]. RNAs achieve their biological functions through their remarkable ability to adopt various architectures and to undergo structural rearrangements upon binding to proteins, nucleic acids or small molecules [9–14]. The key to understand their diverse biological functions is to investigate their structure and dynamic. Nuclear Magnetic Resonance (NMR) is a powerful method to gain insight into these properties [15–18]. However, the study of high molecular weight RNAs by NMR remains challenging due to spectral crowding and broadened linewidths. Advances in biochemical and NMR methods over the recent years allow to overcome some limitations of NMR [16, 18–23]. In particular, the incorporation of paramagnetic probes, coupled to the measurement of paramagnetic relaxation enhancement (PRE) has become an efficient tool providing long-range distances restraints and information on dynamic in solution [24, 25]. The use of PREs allows the structural characterization of RNA and RNA-protein complexes [26–28]. The commonly used spins labels to study RNAs are commercially available nitroxides, such as 3-(2-Iodoacetamido)-proxyl (proxyl) and (1-Oxyl-2,2,5,5-tetramethyl- $\Delta^3$ -pyrroline-3-methyl) Methanethiosulfonate (MTSL).

Several techniques known as site-directed spin labeling (SDSL), have been developed to incorporate paramagnetic probes into RNA. The first main approach of SDSL relies on non-covalent spin-labeling strategies. Helmling *et al.* described the use of a short chemically synthesized spin-labeled RNA subsequently hybridized to a large *in vitro* transcribed RNA [29]. Non-covalent spin-labeling using abasic sites in DNA and RNA duplexes was also reported for introducing nitroxyde group [30]. More recently, a first example of SDSL of

unmodified RNA based on an aptamer approach has been described [31]. The second main method to incorporate spin labels into RNA uses covalent approaches. Several techniques were developed to attach a probe at the RNA 5'-end. The 2,2,6,6-tetramethyl piperidine 1-oxyl (TEMPO) radical phosphoramidite building block was grafted to the 5'-terminus of RNA, allowing PRE measurement by NMR [27]. Enzymatic methods to attach a spin label to a thio-modified nucleotide were also reported. A guanosine monophosphorothioate was introduced at the 5'-end of a RNA using *in vitro* transcription by T7 RNA polymerase, enabling the linkage with the paramagnetic probe [32]. Grant and Qin used the T4 polynucleotide kinase to attach a phosphorothioate group at the 5'-terminus of a RNA, allowing its reaction with iodomethyl derivative of nitroxide [33]. A variety of methods to link covalently a spin label at a chosen position into RNAs are also available. Spin-labeled phosphoramidites were synthesized and incorporated into the RNA during the solid-phase synthesis [34, 35]. The post-synthetic spin-labeling of convertible nucleosides introduced into RNAs was also described [36]. One approach using convertible nucleosides combined to DNA-catalyzed RNA ligation was reported to site-directed spin label a long riboswitch [37, 38]. In the case of large RNA, an enzymatic method combining SDSL and segmental labeling has been developed [39]. Another protocol using enzymatic ligation of 4-thiouridine modified small RNAs led to obtain large doubly spin-labeled RNA providing EPR long distance measurement [40]. More recently, a novel approach combining *in vitro* transcription and click chemistry enabled the measurement of distances and corresponding distance distributions by PELDOR between two nitroxide groups in RNA duplex [41]. Combining NMR and EPR is emerging as a new approach to investigate the architecture of biological systems [42, 43].

In this chapter, we provide a detailed protocol for site-directed spin-labeling RNA using a fully enzymatic method with yields compatible with NMR and EPR studies. By contrast with solid-phase chemical synthesis methods, the enzymatic approach can be applied to large RNA

molecules while maintaining the advantage of selective and segmental isotopic labeling with nitrogen-15 and carbon-13. In addition, this technique is of particular interest for EPR studies where two specific sites have to be labeled in order to monitor the dipolar interaction between two electronic spins. This method therefore enables various combinations of isotopic labeling and spin labeling for both EPR and NMR structural studies.

## 2. Materials

Prepare all solutions using ultrapure water (water purified on resin filters followed by deionization to achieve a resistivity of 18.2 MΩ.cm at 25°C). This will be indicated as “water” in the text. Work with specific precautions recommended for RNA manipulation [44].

### 2.1. Synthesis of RNAs by *in vitro* transcription

1. DNA templates resuspended in water at a concentration of 100 μM stored at –20°C (See **Note 1**).
2. Nucleotides triphosphates (rNTPs) supplied in aqueous solution titrated to a pH at 7.3–7.5 with NaOH (ThermoScientific for unlabeled rNTPs or Eurisotop for <sup>13</sup>C/<sup>15</sup>N labeled NTPs) stored at –20°C at a concentration of 100 mM (See **Note 2**).
3. 6-thioguanosine-5'-O-monophosphate (6-T-GMP, Biolog, Germany) dissolved in water at a concentration of 100 μM stored at –20°C.
4. T7 RNA polymerase (“in-house”) stored in aliquots at –20°C, in 20 mM sodium phosphate pH 7.7, 1 mM DTT, 1 mM EDTA, 100 mM NaCl and 50% glycerol.
5. Stock solutions stored at –20°C in single use aliquots: 50% (w/v) polyethylene glycol (PEG-8000), 1 M MgCl<sub>2</sub> and 0.28 M MgCl<sub>2</sub>.
6. Triton X-100 stored at room temperature.
7. 10X transcription buffer stored in single use aliquots at -20°C: 400 mM Tris-HCl pH 8.1, 10 mM spermidine, 0.1% (v/v) Triton X-100, 50 mM DTT [45, 46].
8. EDTA 0.5 M, pH 8.0, stored at 4°C.
9. Phenol:chloroform saturated solution at pH 4.7 stored at 4°C.
10. Ammonium acetate 3 M, pH 5.5, stored at 4°C.
11. Absolute ethanol stored at -20°C.

## ***2.2 Ligation of RNA segments***

1. DNA splint resuspended in water at a concentration of 100  $\mu$ M stored at  $-20^{\circ}\text{C}$ .
2. RNA fragments purified as described in 3.1.3 section.
3. T4 RNA Ligase 2 (New England Biolabs) supplied in 10 mM Tris-HCl pH 7.5, 50 mM KCl, 35 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 mM DTT, 0.1 mM EDTA and 50 % glycerol.
4. 10X reaction buffer: 500 mM Tris-HCl pH 7.5, 20 mM  $\text{MgCl}_2$ , 10 mM DTT and 4 mM ATP.

## ***2.3 Analytical Electrophoresis of RNA***

1. 10X TBE buffer: 0.9 M Tris-borate, 10 mM EDTA, pH 8.3, stored at room temperature.
2. 19:1 (w/w) Acrylamide/Bisacrylamide 40 % solution.
3. Analytical polyacrylamide gels electrophoresis under denaturing conditions (7 M urea) in 0.5X TBE (*see Note 3*).
4. Analytical polyacrylamide gels electrophoresis under native conditions in 0.5X TBE (*see Note 3*).
5. N,N,N,N'-tetramethyl-ethylenediamine (TEMED, Sigma).
6. Ammonium persulfate: prepare a 10 % solution (w/v) in water and freeze in single use aliquots at  $-20^{\circ}\text{C}$ .
7. 7 M urea solution.
8. 6X bromophenol blue/xylene cyanol loading dye: 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol blue and 30 % glycerol.
9. Staining Toluidine Blue solution: dissolved 0.6 g of Toluidine Blue in 240 mL ethanol, 6 mL acetic acid and complete with water to 600 mL.

## ***2.3 Purifications of RNA***



1. Gel-running buffer: 45 mM Tris-borate, 1mM EDTA, pH 8.3, stored at room temperature.
2. PAGE equipment.
3. Electroelution apparatus (Elutrap, Schleicher & Schuell).
4. Elution buffer: 45 mM Tris-borate, 1mM EDTA, pH 8.3, stored at room temperature.
5. Ammonium acetate 3 M, pH 5.5, stored at 4°C.
6. Absolute ethanol stored at -20°C.

## ***2.4 Spin labeling of RNA***

1. 3-(2-Iodoacetamido)-proxyl (Sigma).
2. RNA from step 2.3.
3. Reaction buffer : 100 mM sodium phosphate, pH 8.0.
4. Absolute ethanol stored at -20°C.

## ***2.5 NMR studies of RNA***

1. Dialysis tubes (Spectra Por 3, 18 mm, MW cut-off 3500).
2. Dialysis buffer: 50 mM sodium phosphate, pH 6.4.
3. Lyophilization apparatus.
4. 3 mm NMR microtube.
5. Deuterium oxide 100 % (Eurisotop).

## ***2.6 EPR studies of RNA***

1. Glass capillaries (Hirschmann ringcaps 20 up to 50 µL)
2. Hirschmann tube-sealing wax.
3. EPR tubes (Wilmad (Clear Fused Quartz) 2.97/1.98mm O.D/I.D)
4. 3-(2-Iodoacetamido)-proxyl (Sigma).

5. Dialysis buffer: 50 mM sodium phosphate, pH 6.4, supplemented by 10% v/v D<sub>2</sub>O

### 3. Methods

The method described here, is based on a segmental approach using ligation technique with T4 RNA ligase 2 [47–49]. Using a DNA splint, one transcribed RNA is ligated to a second RNA fragment in which the thio-modified nucleotide (6-T-GMP) is introduced at its 5'-terminus by *in vitro* transcription. These two RNA segments correspond each to a part of the studied full-length RNA. The procedure is described in Figure 1.

The first important step is the choice of an appropriate attachment site of the spin-label. The targeted nucleotide needs to be accessible and the probe should be placed in a region where the structure will not be modified upon SDSL. It is therefore recommended to have as precise as possible a NMR characterization of the RNA. In addition, the attachment site that corresponds to the segmentation position, has also to be chosen such as to optimize the transcription yield by the T7 RNA polymerase and therefore starting preferentially with a G nucleotide [45, 50].

#### 3.1. Synthesis of RNA segments by *in vitro* transcription using T7 RNA polymerase

##### 3.1.1. Optimization of *in vitro* transcription conditions

Transcription conditions are optimized with varying  $[\text{MgCl}_2]/[\text{NTPs}]$  ratio from 0.8 to 2.0 in 25  $\mu\text{L}$  reaction mixtures. The protocol below is detailed for  $[\text{MgCl}_2]/[\text{NTPs}]$  ratio equal to 1. All stock solutions, except the T7 RNA polymerase, are brought to room temperature prior to mixing to avoid precipitation.

1. Mix the appropriate volume of water and 5  $\mu\text{L}$  of 50 % PEG 8000 in 1.5 mL tubes (See **Note 4**).
2. Add 2.5  $\mu\text{L}$  of 10X transcription buffer and 1.4  $\mu\text{L}$  of  $\text{MgCl}_2$  (0.28 M).
3. Add appropriate volumes of each NTP and 6-T-GMP (only for RNA-2) (See **Note 4**).

4. Shake slowly the reaction mixture.
5. Add 2.5  $\mu\text{L}$  of each DNA strand (see **Note 5**).
6. Add 1  $\mu\text{L}$  of T7 RNA polymerase (final concentration: 0.04 mg/mL).
7. Incubate the reaction mixture at 37°C for 4 h 30.
8. Add 5  $\mu\text{L}$  of 0.5 M EDTA pH 8.0.
9. Vortex.
10. Centrifuge at room temperature, 2 min, at 9000 rpm.
11. Mix 5  $\mu\text{L}$  of the transcription reaction to 10  $\mu\text{L}$  of 7 M urea solution and 3  $\mu\text{L}$  of the 6X loading dye.
12. Pre-run the polyacrylamide denaturing gel for 15 min at 400 V in 0.5X TBE running buffer (See **Note 3**).
13. Rinse the wells thoroughly with 0.5X TBE solution prior to gel loading.
14. Load the sample.
15. Run the gel at 400 V in 0.5X TBE running buffer until the bromophenol blue reaches to the bottom of the gel.
16. Stain the gel with the Toluidine Blue solution for 15 min.
17. Remove the Toluidine Blue solution.
18. Destain the gel with water.
19. Evaluate the transcription yield (Figure 2).

### ***3.1.2. Preparative Transcriptions***

The protocol below is described at a 1.0  $[\text{MgCl}_2]/[\text{NTPs}]$  ratio in 5 mL reaction volume.

1. Mix the appropriate volume of water and 1 mL of 50% PEG 8000 in 15 mL Falcon tube (See **Note 4**).
2. Add 500  $\mu\text{L}$  of 10X transcription buffer and 80  $\mu\text{L}$  of 1M  $\text{MgCl}_2$ .

3. Add appropriate volumes of each NTP and 6-T-GMP (only for RNA-2) (See **Note 6**).
4. Shake slowly the reaction mixture.
5. Add 15  $\mu$ L of each DNA strand (stock: 100  $\mu$ M).
6. Add 20  $\mu$ L of T7 RNA polymerase (final concentration: 0.04 mg/mL).
7. Incubate the reaction mixture at 37°C for 4 h 30.
8. Add 0.5 mL of 0.5 M EDTA pH 8.0.
9. Mix.
10. Add 2.5 mL of phenol/chloroform pH 4.7.
11. Vortex.
12. Centrifuge 10 min at room temperature at 9000 rpm.
13. Transfer the aqueous phase in a 50 mL Falcon tube.
14. Add 2 mL of water to the phenol fraction.
15. Vortex.
16. Centrifuge 10 min at room temperature at 9000 rpm.
17. Combine the aqueous phase to the first one in the 50 mL Falcon tube.
18. Add 0.1 volume of Ammonium acetate.
19. Mix.
20. Add 2.5 volumes of cold absolute ethanol.
21. Vortex.
22. Place at -20°C overnight.

### ***3.1.3. Purification of RNA transcripts***

1. Centrifugation at 9000 rpm for 30 min at 4°C.
2. Remove the liquid gently.
3. Resuspend the pellet in 1 mL of a 7 M urea solution.

4. Prepare a polyacrylamide denaturing (7 M urea) gel at the appropriate percentage of acrylamide with a two-wells comb (See **Note 7**).
5. Pre-run the gel at a constant power of 40 W at about 50°C in 0.5X TBE running buffer for 30 min.
6. Rinse the wells thoroughly with 0.5X TBE solution prior to gel loading.
7. Mix 200 µL of 7 M urea solution and 40 µL of 6X loading dye.
8. Load the mixture in the smaller well-comb.
9. Load the pellet of RNA resuspended in 7 M urea solution (step 3) in the second well-comb.
10. Run the gel at a constant power of 40 W in 0.5X TBE running buffer until the bromophenol blue reaches to the bottom of the gel.
11. Remove the plates from the gel.
12. Place the gel on clean and clear cellophane over a silica chromatography plate.
13. Visualize RNA by UV shadowing at 256 nm.
14. Excise the band corresponding to the expected transcript RNA with a clean scalpel.
15. Elute the RNA from the gel slices using an Elutrap Electroelution System: recover the RNA in the sample chamber every hour, until the absorbance decreases.
16. Combine all fractions containing the desired RNA.
17. Add 0.1 volume of 3 M ammonium acetate pH 5.5.
18. Mix.
19. Add 2.5 volumes of cold absolute ethanol.
20. Place at -20°C overnight.
21. Centrifuge at 9000 rpm for 30 min at 4°C.
22. Wash the pellet with 70% ethanol.
23. Resuspend the pellet in water.

### 3.2. Ligation of RNA segments

The ligation by T4 RNA ligase 2 enables to link an acceptor RNA to a donor RNA modified at its 5'-end with 6-T-GMP using a DNA splint [49]. Prior ligation, the correct hybridization of the two RNA segments to the complementary DNA splint has to be verified to avoid the formation of side-products.

The procedure is described in Figure 1.

#### *3.2.1. Hybridization of RNAs to the DNA splint*

1. Mix equimolar amounts of the two RNAs fragments and the DNA splint at a final concentration of 10  $\mu$ M each, in the 1X T4 RNA ligase 2 buffer, in a 10  $\mu$ L reaction volume.
2. Heat the mixture at 95°C (2 min) and snap-cool at 4°C (5 min).
3. Centrifuge at 10 000 rpm for 5 min at room temperature.
4. Pre-run a native polyacrylamide gel for 15 min at 150 V in 0.5X TBE running buffer at 4°C (See **Note 3**).
5. Rinse the wells thoroughly with 0.5X TBE solution prior to gel loading.
6. Load the sample.
7. Run the gel at 400 V in 0.5X TBE running buffer until the bromophenol blue reaches to the bottom of the gel.
8. Stain the gel with the Toluidine Blue solution for 15 min.
9. Remove the Toluidine Blue solution.
10. Destain the gel with water.
11. Free oligonucleotides and RNAs/DNA hybrid are visualized (Figure 3).

### ***3.2.2. Ligation of RNA fragments***

1. Mix equimolar amounts of the two RNAs fragments and the DNA splint at a final concentration of 30  $\mu$ M each, in 1X T4 RNA ligase 2 buffer, in a 5 mL reaction volume (see **Note 8**).
2. Heat the mixture at 95°C (5 min) and snap-cool at 4°C (15 min).
3. Centrifuge at 10 000 rpm for 10 min at room temperature.
4. Add 2.25 kU of T4 RNA ligase 2.
5. Incubate at 37°C overnight.
6. Add 0.1 volume of 3 M ammonium acetate pH 5.5 and 2.5 volumes of cold absolute ethanol.
7. Mix.
8. Place at -20°C overnight.
9. Purified the ligation product as described in section 3.1.3.
10. Precipitate by addition of 0.1 volume of 3 M ammonium acetate pH 5.5 and 2.5 volumes of cold absolute ethanol.
11. Place at -20°C overnight.

### **3.3. Site-Specific Spin Labeling of RNA**

All the following steps have to be performed in the dark to prevent light-induced degradation.

1. Centrifuge at 9000 rpm for 30 min at 4°C.
2. Wash the pellet with cold 70% ethanol solution.
3. Dry the pellet.
4. Resuspend the pellet in 400  $\mu$ L of 100 mM sodium phosphate buffer pH 8.
5. Evaluate the concentration of the ligation product by UV-absorption using a Nanodrop Spectrometer.



6. Dissolve 3-(2-Iodoacetamido)-proxyl in absolute ethanol at a concentration of 0.15 M.
7. Add a 100-fold-excess of 3-(2-Iodoacetamido)-proxyl solution to the RNA.
8. Incubate at 40°C for 7 hours under stirring at 300 rpm.
9. Add again a 100-fold-excess of 3-(2-Iodoacetamido)-proxyl solution to the RNA.
10. Incubate at 40°C overnight under stirring at 300 rpm.
11. Precipitate by addition of 0.1 volume of 3 M ammonium acetate pH 5.5 and 2.5 volumes of ethanol.
12. Place at -20°C overnight.
13. Centrifuge at 9000 rpm at 4°C for 30 min.
14. Remove the supernatant.
15. Resuspend the pellet in water.
16. Dialyzed the spin-labeled RNA against the buffer used for NMR and EPR experiments.
17. Change the dialysis buffer four times.

### **3.4. NMR experiments**

All the following steps have to be performed in the dark to prevent light-induced degradation.

1. Concentrate the sample by lyophilization.
2. Resuspend in 90/10 H<sub>2</sub>O/D<sub>2</sub>O in 150 µL.
3. Transfer the sample in a 3 mm NMR tube.
4. Record one-dimensional NMR spectra (spectral width of 20 ppm) at several temperatures to check the sample quality (Figure 4; see **Note 9**).
5. Record all classical experiments for RNA structural studies by NMR and PRE measurements [16, 51, 52] (See **Note 9**).

### **3.5. EPR experiments**

EPR investigation is here performed to: (i) assess whether the spin-label (*i.e.* the proxyl) is covalently bound to the RNA molecule and if yes how the motion of the rotamer is restricted (Figure 5; See **Note 10**), (ii) detect the presence of potential free spin-label and define the proxyl-RNA coupling efficiency. All the following steps must be performed in the dark to prevent potential light-induced degradation.

### ***3.5.1. Preparation of RNA samples***

1. Load resuspended sample (from step 3.4) into 20  $\mu$ L glass capillaries sealed at both ends and place it into a EPR quartz tube (2.97/1.99 mm O.D./I.D.).
2. Place it into the EPR cavity so that the whole length of the cavity is filled by the sample.
3. Record conventional X-band field swept EPR spectra centered around  $g$  of *ca.* 2.0055. (Figure 6; see **Note 11**).
4. After potential baseline subtraction perform a double integration of the spectra in order to obtain the EPR intensity (a.u), which is directly proportional to the number of paramagnetic centers detected within the cavity.

### ***3.5.2. Spin labeling efficiency via nitroxide standards***

Proceed following an identical protocol as for RNA sample measurements.

1. Prepare several 3-(2-Iodoacetamido)-proxyl solutions into the buffer used for EPR and NMR investigations, with known concentrations around the expected RNA sample one.
2. Load each sample into 20  $\mu$ L glass capillaries sealed at both ends.
3. Transfer capillaries into EPR quartz tubes, placed into the EPR cavity.
4. Record conventional X-band field swept EPR spectra with the identical experimental parameters as for the RNA samples (Figure 6; see **Note 11**).
5. After baseline subtraction (if attended for the RNA sample) perform a double integration of the spectra in order to obtain EPR intensity (a.u), that can be compared to the RNA

sample intensities to assess their spin concentrations and therefore the coupling efficiency.

## Notes

### Note 1

DNA templates can be prepared according to three options: linearized plasmid, single stranded or double stranded synthetic DNA templates. Here, the protocol describes the use single stranded or double stranded synthetic DNA templates. DNA templates are purchased from SIGMA, IDT or Eurogentec. Milligan *et al.* first used synthetic DNA templates and T7 RNA polymerase for the synthesis of short oligonucleotides [45, 50]. The efficiency of the transcription depends on the specific sequence at the 5'-end of the RNA: one or more guanine residues are required to improve the transcription yield. Care should be therefore taken when designing DNA templates.

### Note 2

The full-length RNA can be uniformly labeled by using  $^{13}\text{C}/^{15}\text{N}$  rNTPs. Combinations of unlabeled and  $^{13}\text{C}/^{15}\text{N}$  labeled rNTPs can be also used to produce selectively labeled (A or U or C or G residues or any combinations). In addition, the ligation step in our protocol allows the segmental labeling of the RNA. This offers various possibilities of isotopic labeling schemes.

### Note 3

Polyacrylamide gels have to be prepared at an appropriate percentage according to the size of the RNA [53]. Analytical gels electrophoresis under denaturing conditions contains the appropriate percentage of acrylamide/bisacrylamide, 7 M urea in 0.5 X TBE. Analytical gels electrophoresis under native conditions contains the appropriate percentage of acrylamide/bisacrylamide in 0.5 X TBE.

**Note 4**

Nucleotides triphosphates (rNTPs) and 6-T-GMP stock solutions are stored at a concentration of 100 mM.

The first RNA segment (RNA-1) is transcribed in the presence each NTP at a concentration of 4 mM: add 1  $\mu$ L of each NTP. The appropriate volume of water is 6.1  $\mu$ L.

In the case of the 5'-thio-modified (6-T-GMP) RNA fragment (RNA-2), ATP, UTP and CTP are added at a concentration of 4 mM while the GTP and 6-T-GMP concentrations are respectively 3 mM and 9.1 mM: add 1  $\mu$ L of ATP, 1  $\mu$ L of UTP, 1  $\mu$ L of CTP, 0.8  $\mu$ L of GTP and 2.3  $\mu$ L of 6-T-GMP. The appropriate volume of water is 4  $\mu$ L.

**Note 5**

For transcription tests, the DNA strands stocks at 100  $\mu$ M are diluted to a concentration of 3  $\mu$ M in order to add 2.5  $\mu$ L of DNA. The final DNA template concentrations are 300 nM in 25  $\mu$ L reaction volume.

**Note 6**

Nucleotides triphosphates (rNTPs) and 6-T-GMP stock solutions are stored at a concentration of 100 mM.

The first RNA (RNA-1) segment is transcribed with each NTP at a concentration of 4 mM: add 200  $\mu$ L of each NTP. The appropriate volume of water is 2.57 mL.

In the case of the 5'-thio-modified (6-T-GMP) RNA fragment (RNA-2), ATP, UTP and CTP are added at a concentration of 4 mM while the GTP and 6-T-GMP concentrations are respectively 3 mM and 9.1 mM: add 200  $\mu$ L of ATP, 200  $\mu$ L of UTP, 200  $\mu$ L of CTP, 152  $\mu$ L of GTP and 456  $\mu$ L of 6-T-GMP. The appropriate volume of water is 2.16 mL.

### **Note 7**

For preparative gels, the percentage of acrylamide/bis acrylamide is adjusted according to the size of RNA. The size and the thickness of the gel are respectively 20 cm\*42 cm and 3 mm.

### **Note 8**

To optimize the ligation yield, a series of test experiments are performed in 10  $\mu$ L with varying reaction times and, RNA and enzyme concentrations.

### **Note 9**

Before PREs analysis, it is necessary to have as complete as possible the NMR chemical shift assignments of the free RNA. The spectra recorded for the spin-labeled RNA can next be compared to the free RNA to measure and analyze PRE effects.

One-dimensional NMR spectra recorded over a spectral width of 20 ppm allow the analysis of imino proton found between 9 and 15 ppm. They constitute a good probe of the overall secondary structure of the RNA molecule. Their observation points out their protection from exchange with the solvent and therefore their involvement in hydrogen bond. The resonances corresponding to those protons found between 12.0 and 15.0 ppm are characteristic of imino protons involved in Watson-Crick base pairs, whereas those found upfield (between 9.0 and 12.0 ppm) indicate the formation of non-canonical base-pairing. Assignment of these resonances provides thus precious informations about the folding of the RNA. The comparison of the spectra of the wild-type RNA and the spin-labeled RNA allows to evaluate: (i) the efficiency of the reaction, (ii) the quality of the sample and (iii) the impact of the paramagnetic probe on the global fold of the RNA. Then, all classical experiments for RNA structural studies by NMR and PRE measurements can be performed and compared to the RNA-wt [16, 51, 52]. As exemplified in Figure 4, the introduction of a spin label at position

G24 induces reduced intensities of G24, G25, G31, G32 and U33, while G3 and G5 remain unaffected.

#### **Note 10**

In solution the dynamic of the spin-label, characterized by the tumbling of the molecule, is observable by EPR and characterized by the so-called rotational correlation time ( $\tau_r$ ) [54–56]. The lineshape of the nitroxide derivative EPR spectrum gives a direct indication whether the spin probe is free or attached to a much more weighted molecule. In the first case the molecule undergoes a rapid tumbling and its corresponding spectrum illustrates the isotropic limit that consists in three sharp lines of nearly equal intensities ( $\tau_r < 10^{-10}$  s, see Figure 6). If covalently attached to a much larger molecule the tumbling is slowed down accordingly as it is now restricted by the host molecule, potential steric hindrance and/or restriction of the potential rotamer configurations. In such case, the spin probe goes from fast- and slow-motion regime towards the rigid limit corresponding to the fully immobilized spin-label ( $\tau_r > 10^{-7}$  s, see Figure 6). Consequently, changes within the paramagnetic probe environment can be reflected by a change within the spectral nitroxide lineshape. To evaluate the rotational correlation time several methods are available ranging from the simulation of the experimental spectrum by using dedicated software/programs (*e.g.* the Easyspin toolbox under Matlab (Mathworks) environment [57, 58]) to various empirical methods [54, 55, 59]. It allows to define and deconvolute potential configuration of the spin-label rotamer *vs.* the host molecule (See Figure 7).

#### **Note 11**

All experiments are performed at room temperature. An example of the principal experimental parameters is: X-band EPR spectrometer (*e.g.* EMXplus Bruker Biospin

GmbH), equipped with a resonator (*e.g.* high sensitivity resonator (4119HS-W1, Bruker). The  $g$  factor is calibrated in the experimental conditions (*e.g.* using the Bruker strong pitch ( $g = 2.0028$ )). Center Field: *ca.* 3500 G, Sweep Width: *ca.* 15 mT, Microwave power: *ca.* 2 mW, Modulation Amplitude: *ca.* 0.5 G (usually taken at least 30 % inferior to the natural width of the free spin label to avoid overmodulation), Conversion Time: *ca.* 100 ms, Time Constant: *ca.* 50 ms (as a safe rule of thumb the time constant is usually set up inferior to 50% of the conversion time to avoid spectrum distortion). Several scan can be accumulated to achieve a reasonable Signal to Noise ratio.



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## Figure Legends

**Figure 1.** Description of the method for site-specific spin-labeling of RNA. (A) Choice of the attachment site. (B) Schematic representation of the approach to introduce a spin-label at a chosen position into a RNA.

**Figure 2.** Optimization of *in vitro* transcription yield. The best transcription yield is obtained at 1.2 [MgCl<sub>2</sub>]/[NTPs] ratio.

**Figure 3.** (A) Free RNAs, DNA splint and the RNAs/DNA hybrid are visualized on a native polyacrylamide gel. The hybrid is indicated by a star. (B) Free RNAs, DNA splint and the ligation reaction are visualized on a denaturing polyacrylamide gel.

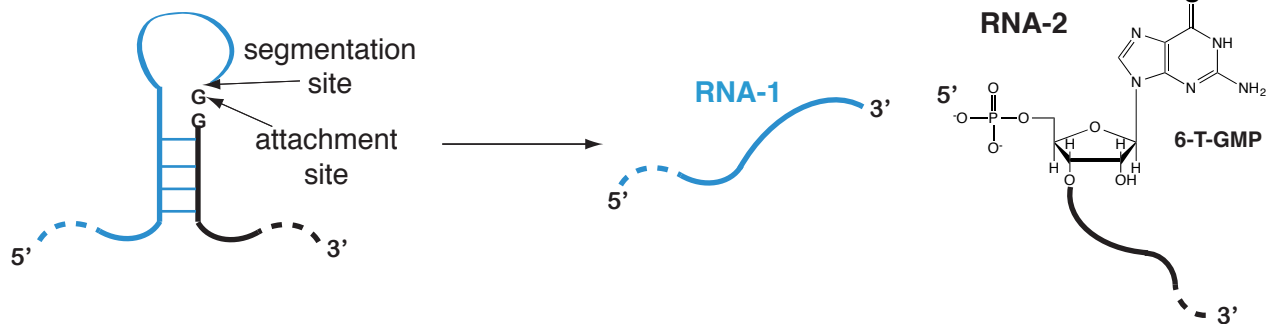
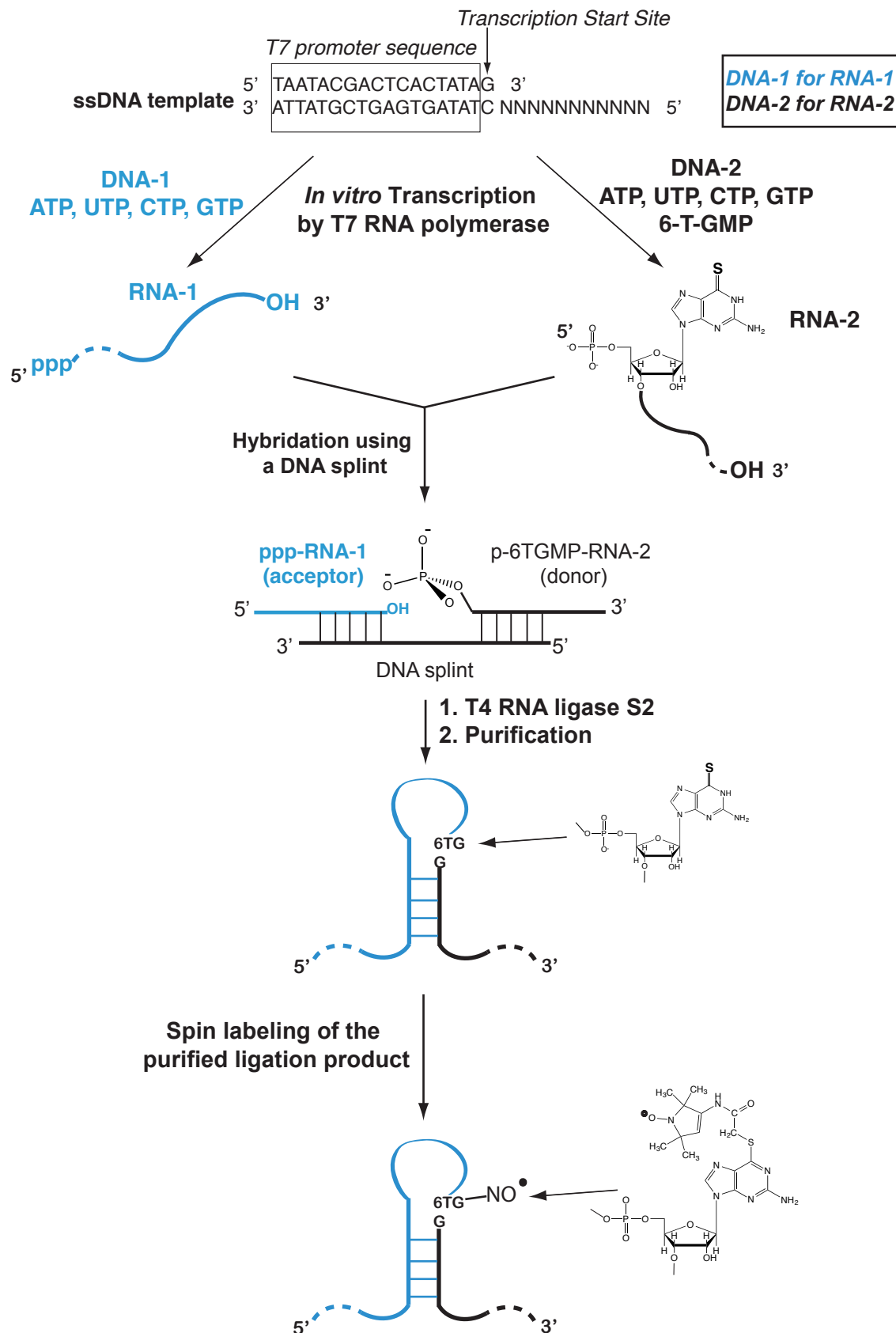
**Figure 4.** NMR of a site-specific spin-labeled RNA [39]. Imino-protons region of 1D spectra, recorded at 20°C, of the free RNA (black) and of the SL-RNA (blue; see **Note 9**).

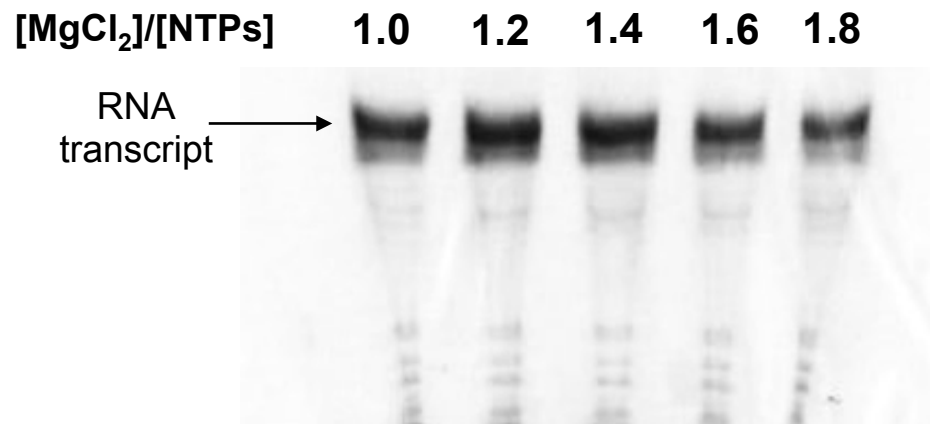
**Figure 5.** EPR spectra of free proxyl taken at various concentrations ranging from 10 (black curve) to 200 µM (light gray curve) and compared to proxyl-labeled RNA (light blue curve). All spectra were acquired in phosphate buffer (50 mM, pH = 6.4) supplemented by 10 % v/v D<sub>2</sub>O with identical experimental parameters. In this case, the concentration of the spin-labelled RNA was found to be  $150 \pm 15$  µM.

**Figure 6.** Evolution of the EPR lineshape for a nitroxide radical as a function of isotropic rotational correlation time ( $\tau_r$ ) ranging from 10 ps to 100 ns. This schematic representation highlights the four dynamical regime accessible by continuous wave X-band EPR ranging

from the isotropic limit (*e.g.* free spin-label) and fast motion regime to the slow motion regime and rigid limit (corresponding to fully immobilized nitroxide). Simulations were achieved using Easyspin toolbox [54].

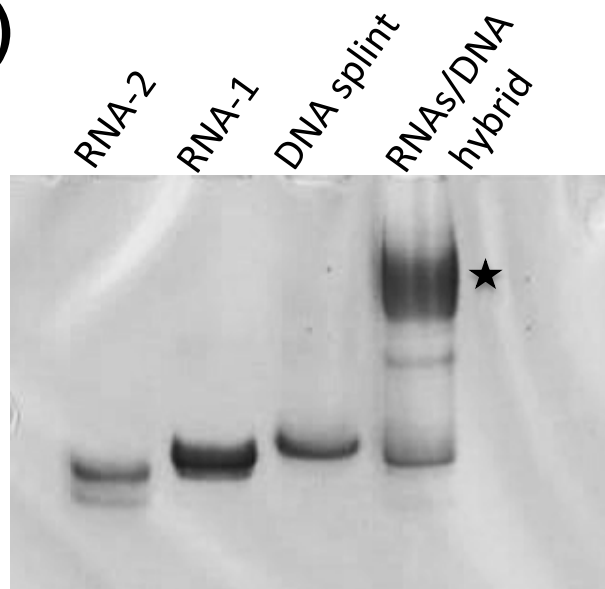
**Figure 7.** Experimental EPR spectrum of proxyl-labeled RNA (light blue curve) and corresponding simulation (red curve). Simulation investigation allows to deconvolute the overall EPR fingerprint as the superposition of two spin systems at distinct dynamical regimes. It corresponds to two rotamer configurations of the proxyl, characterized by two rotational correlation times ( $\tau_r$ ) of *ca.*  $4 \pm 0.5$  ns (slow motion regime, upper blue curve) and  $0.8 \pm 2$  ns (fast motion regime, lower blue curve) with a ratio 4:1 respectively. Noteworthy, no free spin-label was detected, testimony of the spin-labeling efficiency.

**(A)****(B)****Figure 1**

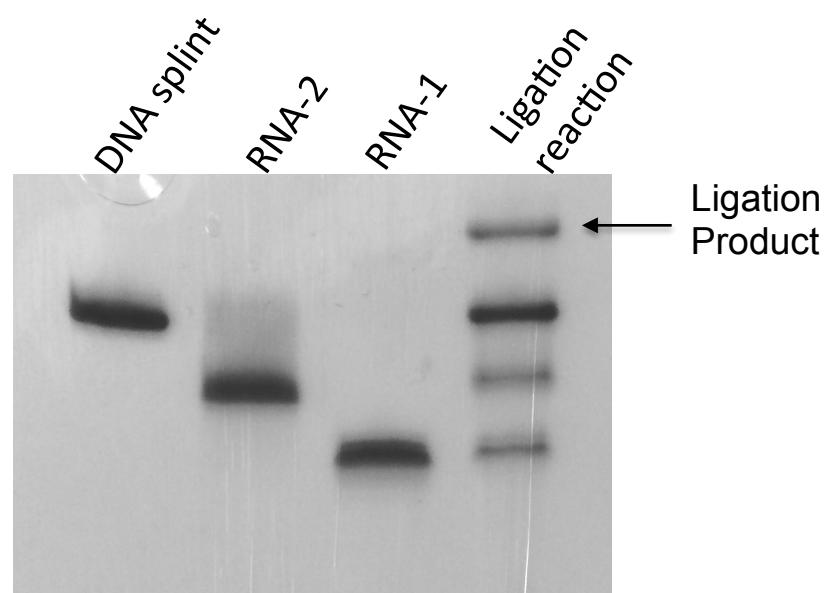


**Figure 2**

**(A)**

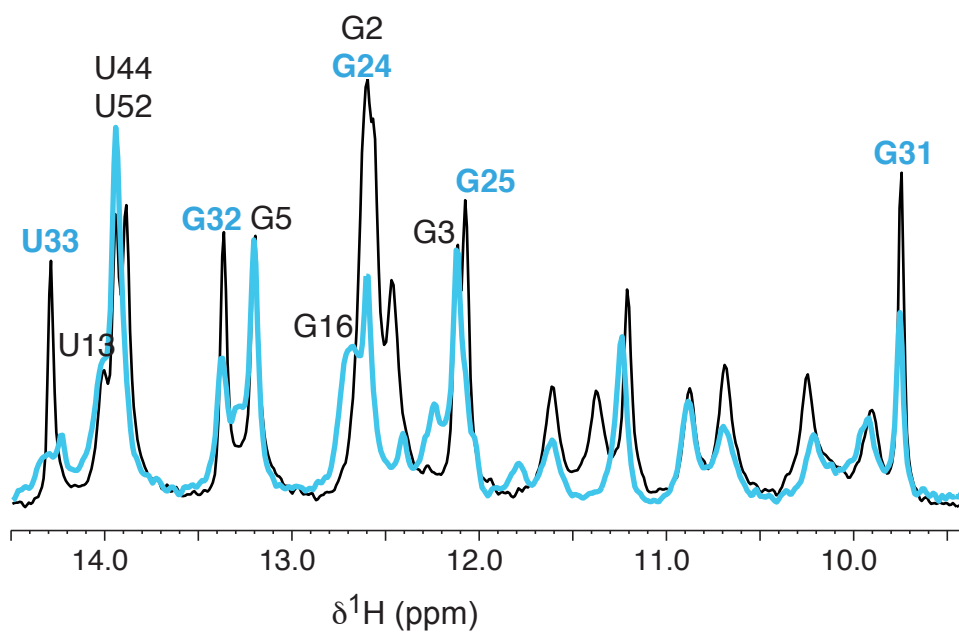
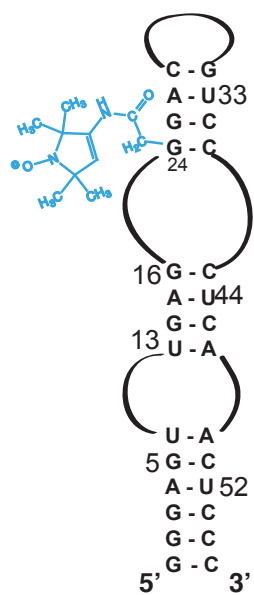


**(B)**

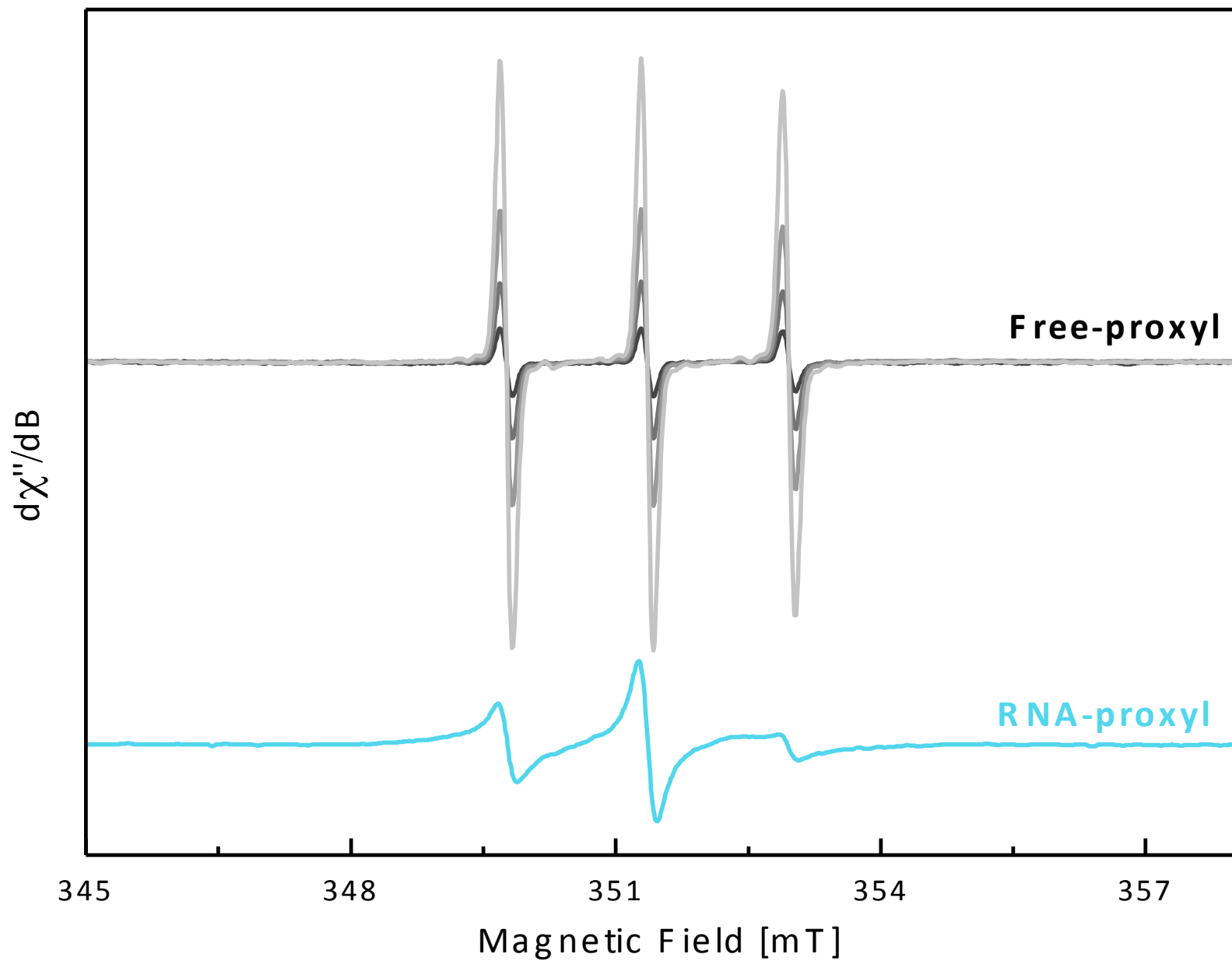


**Figure 3**

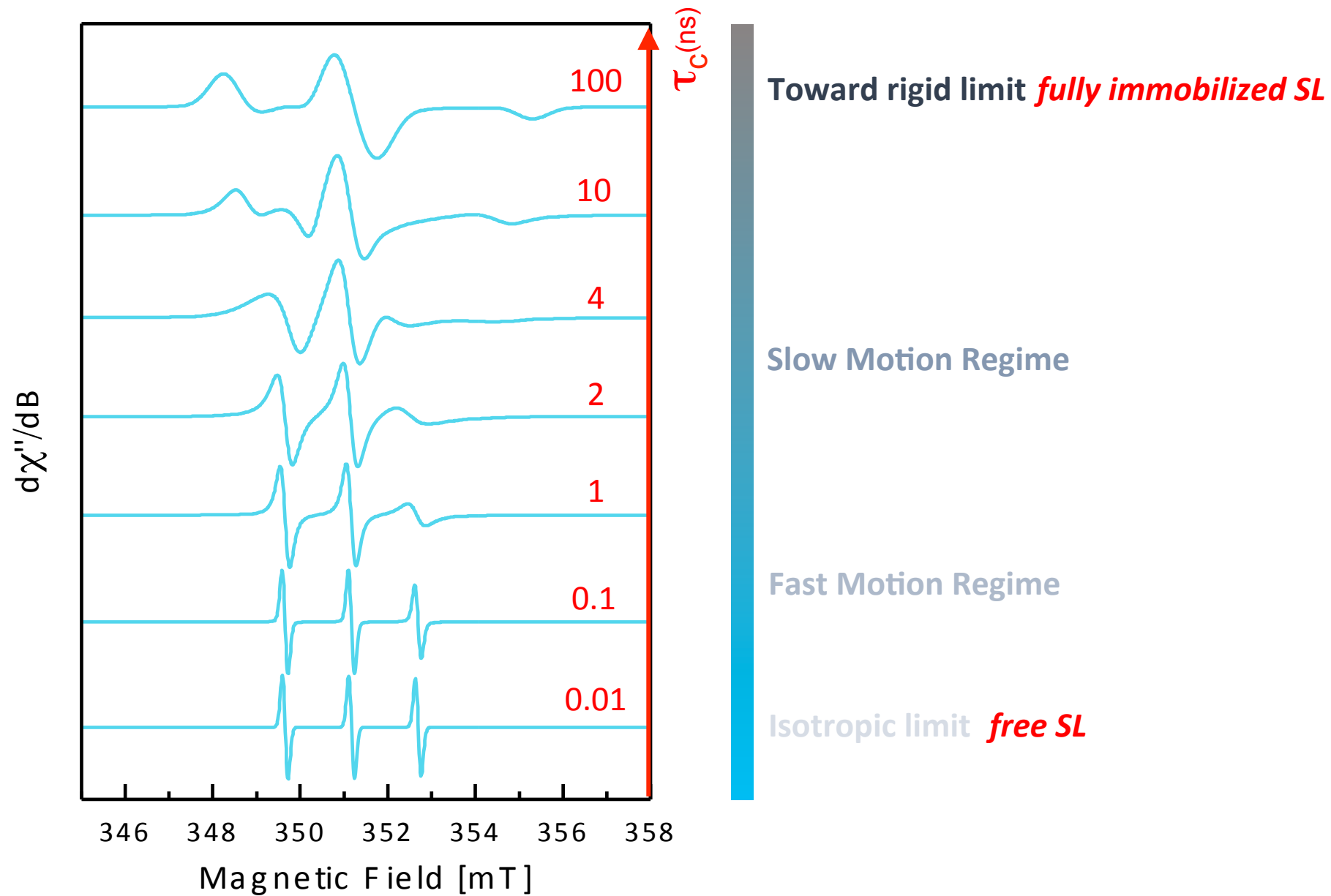




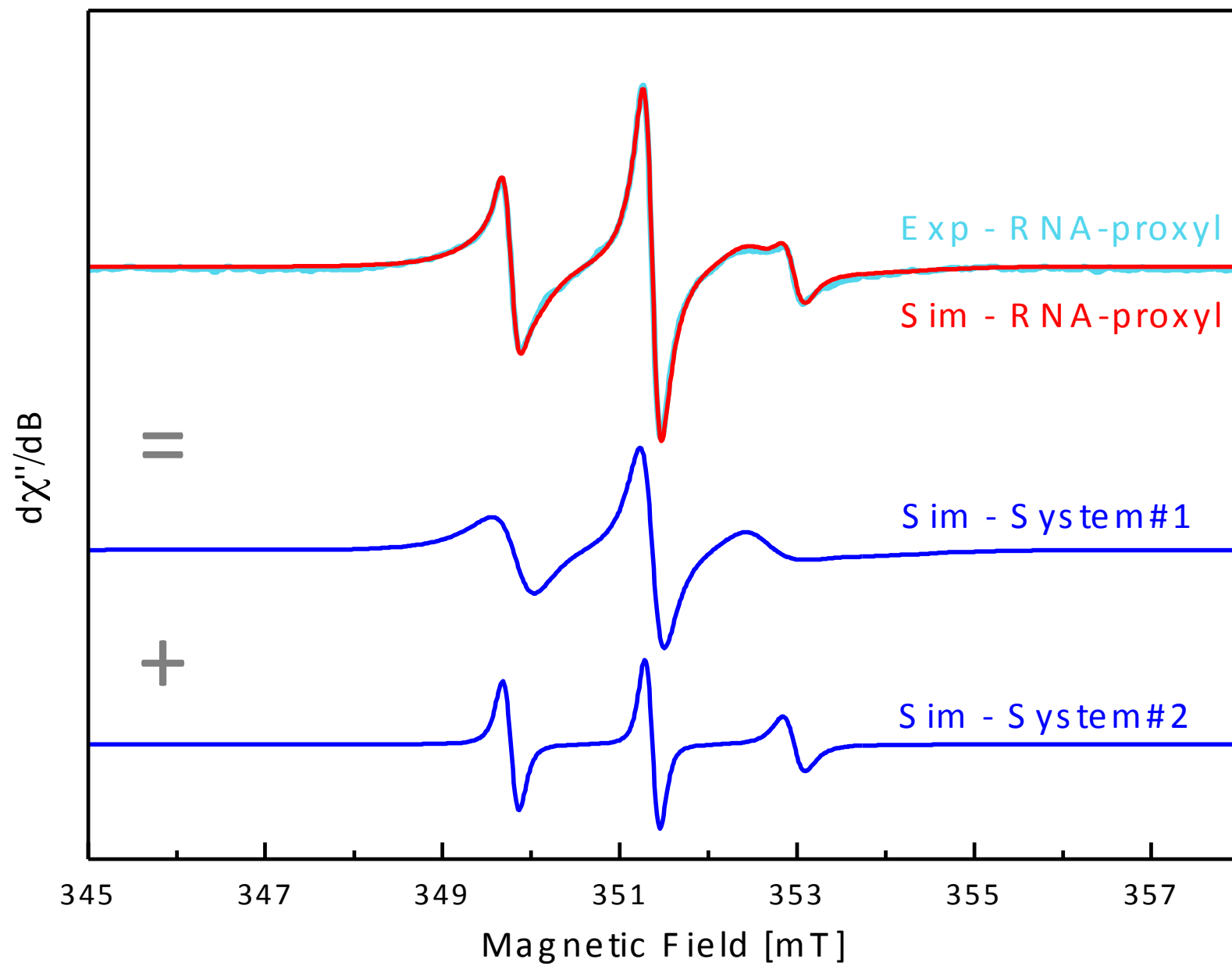
**Figure 4**



**Figure 5**



**Figure 6**



**Figure 7**