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Cu(II)-Based Paramagnetic Probe to Study RNA–Protein Interactions by NMR

Leah M. Seebald, Christopher M. DeMott, Srivathsan Ranganathan, Papa Nii Asare Okai, Anastasia Glazunova, Alan Chen^{iD}, Alexander Shekhtman, and Maksim Royzen^{*iD}

Department of Chemistry, University at Albany, SUNY, 1400 Washington Avenue, Albany, New York 12222, United States

Abstract

Paramagnetic NMR techniques allow for studying three-dimensional structures of RNA–protein complexes. In particular, paramagnetic relaxation enhancement (PRE) data can provide valuable information about long-range distances between different structural components. For PRE NMR experiments, oligonucleotides are typically spin-labeled using nitroxide reagents. The current work describes an alternative approach involving a Cu(II) cyclen-based probe that can be covalently attached to an RNA strand in the vicinity of the protein's binding site using “click” chemistry. The approach has been applied to study binding of HIV-1 nucleocapsid protein 7 (NCp7) to a model RNA pentanucleotide, 5′-ACGCU-3′. Coordination of the paramagnetic metal to glutamic acid residue of NCp7 reduced flexibility of the probe, thus simplifying interpretation of the PRE data. NMR experiments showed attenuation of signal intensities from protein residues localized in proximity to the paramagnetic probe as the result of RNA–protein interactions. The extent of the attenuation was related to the probe's proximity allowing us to construct the protein's contact surface map.

Graphical abstract

^{*}Corresponding Author. mroyzen@albany.edu.

ORCID

Alan Chen: 0000-0001-8246-2935

Maksim Royzen: 0000-0002-2930-2171

ASSOCIATED CONTENT

Supporting Information

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Simulation methods. X-ray crystallographic data collection and refinement parameters for **1**. HPLC trace of **RNA 1**. ¹H and ¹³C NMR of compounds **2–5**. Mass spectra of compounds **RNA 1**, **RNA 2**, and **NCp7** (PDF) X-ray crystallographic files in CIF format for the structure determination of **1** (CIF)

The authors declare no competing financial interest.

INTRODUCTION

There is a strong interest in the biochemical community to develop better fundamental understanding of how proteins interact with RNA.¹ RNA–protein interactions are ubiquitous in biology and critical at many regulatory steps of gene expression and stages of cell development. While X-ray crystallography continues to be an invaluable tool for structural biology, it is laden with the intrinsic difficulty of crystallizing ribo-nucleoprotein assemblies. Flexibility of the RNA component often presents a challenging barrier to crystallization. Consequently, two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy is an excellent alternative, and in many ways complementary, analytical method that allows to study the function, dynamic behavior, and regulation principles of RNA–protein complexes.²

Nuclear Overhauser enhancement (NOE) measurements have been the classical approach to macromolecular NMR structure determination, allowing characterization of contacts between protons separated by less than 6 Å.³ On the one hand, while this approach has been very successful for analysis of compact structures, such as globular proteins, it does not provide long-range structural information that can be extremely beneficial in the cases of RNA–protein complexes. On the other hand, paramagnetic relaxation enhancement (PRE) effect can detect interactions between an unpaired electron of a paramagnetic center and protons up to 35 Å away.⁴ PRE arises from magnetic dipolar interactions between an unpaired electron of a paramagnetic center and protein's nuclei, resulting in an increase in the relaxation rate of the nuclear magnetization.⁵ For an electron–nucleus pair separated by distance r , the magnitude of PRE is proportional to r^{-6} , a relationship analogous to that between the magnitude of the NOE and interproton distance. Because the magnetic moment of the unpaired electron is large, PRE effects are also large and can provide long-range distance information.

PRE measurements entail site-specific labeling of RNA or a protein with extrinsic paramagnetic probes, and additionally diamagnetic probes of similar structure are also site-specifically incorporated for determination of electron spin relaxation rate. Typically, oligonucleotide strands are spin-labeled using nitroxide reagents that are small to minimally perturb native structures, and these nitroxides can be easily reduced to act as diamagnetic controls.⁶ Spin labeling of nucleic acids has recently been reviewed.⁴ Some examples include Sonogashira coupling of 2,2,5,5-tetramethylpyrrolin-1-yloxy-3-acetylene (TPA) to 5-iodouridine⁷ and incorporation of 4-amino-TEMPO into phosphodiester backbone.⁸ Lippard and co-workers also utilized 4-amino-TEMPO spin label to investigate the solution structure of platinated double-stranded DNA using NMR.⁹ The technique termed site-directed spin labeling (SDSL) allowed Qin and co-workers to study RNA's dynamic behavior in solution.¹⁰

Paramagnetic metal ion-based probes can provide a valuable alternative to nitroxide labels. Suitable ligands can be designed for site-specific attachment of Mn(II), Cu(II), and Gd(III)-based paramagnetic labels with an isotropic g tensor.^{3a,11} This approach offers additional attractive features. For example, the RNA-bound probe can be designed to have multiple electrostatic interactions with the protein, thereby reducing the probe's flexibility that sometimes complicates the analysis of PRE data.¹² Unlike spin labels, paramagnetic metal-

based probes can be designed for in-cell NMR experiments, as the former will likely be reduced inside the cell.¹³

Transition-metal-based paramagnetic probes have previously been utilized to study protein structure and dynamics by NMR.¹⁴ A number of groups reported NMR structures of Cu(II)-containing metalloproteins.¹⁵ Rosnizeck et al. incorporated Cu(II) cyclen probe into human Ras protein to differentiate between its two conformational states.¹⁶ Meanwhile, Ni(II) has been explored by Led and co-workers to obtain the structural information on thioredoxin.¹⁷ Otting and co-workers genetically encoded Co(II)-binding amino acid, bipyridylalanine, into West Nile virus NS2B-NS3 protease.¹⁸ Lanthanide tags, such as DOTA and DTPA, have also been extensively explored for protein structure analysis.¹⁹ Work described herein is the first example where a paramagnetic transition-metal ion-based probe is applied toward understanding of binding interactions between an RNA and a protein.

RESULTS AND DISCUSSION

The Cu(II)-based paramagnetic NMR probe **1**, shown in Scheme 1, was designed with four objectives in mind: (a) thermodynamic and kinetic stability; (b) minimal steric perturbation to native RNA–protein complex; (c) facile attachment to RNA using “click” chemistry; (d) unsaturated coordination sphere that allows for additional point of contact with the RNA–protein complex. Cyclen is a macrocyclic ligand with a very high affinity for Cu(II) ($\log K = 23.4$).²⁰ Macrocyclic ligands are known to provide higher kinetic stability to the coordinating metals relative to the acyclic counterparts.²¹ The terminal alkyne group allows for efficient and site-specific attachment of the paramagnetic probe to RNA strands of interest using click chemistry. The reported Cu(II) complexes of the 12-membered N4 macrocycle, as well as modified cyclen ligands, have square-pyramidal geometry, with equatorial sites occupied by four secondary amino nitrogens of a tetradentate ligand and with the axial position by a counterion or a solvent.²² The metal is positioned above plane of the four nitrogen atoms. Consequently, the donor atoms impart less electron density to Cu(II) than larger N4 macrocycles, such as cyclam. This enhanced Lewis acidity of the Cu(II) makes it more reactive towards anionic Lewis bases, such as carboxylates.^{22b}

Compound **1** was synthesized in four steps, shown in Scheme 2, from commercially available cyclen ligand.²³ The synthesis commenced with protection of three secondary amines with Boc groups. The remaining secondary amine group was alkylated with propargyl amine. The Boc groups were cleaved with trifluoroacetic acid (TFA). Copper perchlorate was refluxed in the ethanolic solution containing compound **5** to form the Cu(II) cyclen complex **1**, which can be attached to an RNA strand of interest using click chemistry.

An X-ray crystallographic study was undertaken prior to utilizing the probe for PRE NMR experiments. Purple crystals suitable for X-ray analysis were obtained by slow vapor diffusion of ethyl ether into a 10 mM solution of **1** in methanol. The molecular structure, solved in monoclinic space group *Pnma*, was formulated as $[\text{Cu}(\mathbf{5})(\text{CH}_3\text{O})](\text{ClO}_4)$. An ORTEP representation of the X-ray structure is shown in Scheme 1. The pentacoordinate Cu^{2+} cation resides in a distorted square pyramidal geometry, which is similar to the previously reported Cu(II) cyclen complexes.²² As expected, the copper ion is bonded above

the plane of the four nitrogen atoms. The apical position is occupied by the solvent. The bond distances between Cu and the cyclen nitrogen atoms are 2.008, 2.009, 2.026, and 2.029 Å, respectively. The weakly coordinated solvent molecule could be exchanged with an appropriate amino acid residue of the RNA–protein complex.²⁴

As a proof-of-principle the paramagnetic probe was utilized to study binding of the RNA pentanucleotide sequence 5'-ACGCU*-3' (U* represents the labeled nucleotide) and HIV-1 nucleocapsid protein 7 (NCp7). The latter plays a number of key roles in the pathogenic lifecycle of the virus, including packaging of viral RNA into budding virions.²⁵ NCp7 has higher affinity for single-stranded DNA or RNA than for double-stranded DNA and has specificity for particular oligonucleotide sequences.²⁶ An unlabeled RNA pentanucleotide, 5'-ACGCU-3', was used as a diamagnetic control (**RNA 2**).

The azide group was introduced at the 3'-end of the RNA using previously reported compound **6**, immobilized on polystyrene resin (Scheme 3).²⁷ The RNA strand with the sequence 5'-ACGCU*-3' was synthesized by solid-phase synthesis. The paramagnetic probe was subsequently attached via click chemistry using conditions that minimize Cu(I)-catalyzed hydrolysis of the RNA's phosphodiester backbone.²⁸

There is a wealth of data describing oligonucleotide binding by NCp7, including an NMR structure of the protein's zinc finger domain bound to the single-stranded DNA pentanucleotide 5'-ACGCC-3'.²⁹ The key binding interaction in this structure is π -stacking between the indole ring of W37 (Figure 1A), inserted between the nucleobases of C2 and G3. Previously reported binding studies indicated that NCp7 binds equally tightly to the DNA and RNA pentanucleotide of the aforementioned sequence.²⁹ The reported structure further predicts that attachment of the paramagnetic probe at the 3' end of the pentanucleotide would place it in proximity of the protein, while minimally affecting its binding affinity. We replaced the cytidine residue at the 3' end of the pentanucleotide with uridine to simplify the synthesis.

Molecular dynamics (MD) simulations, based on the published NMR structure of the NCp7-d(ACGCC) complex,²⁹ were performed to predict which protein residues would be most affected by the paramagnetic probe placed at the 3'-uridine. Ten parallel MD simulations initiated with different starting structures of the protein–nucleic acid complex were performed, generating a total of 1 μ s simulation data. The simulation data were analyzed to assess the proximity of the paramagnetic probe to different amino acid residues of the protein. Figure 1B shows the predicted interaction surface map of NCp7 color-coded to reflect the proximity of different amino acid residues to the paramagnetic center. A distance cutoff of 0.6 nm between the metal ion and the protein backbone was used for this purpose. Lewis acidity of the copper ion correlates to its predicted proximity to the negatively charged regions (E42 & D48).

The proposed paradigm was tested by titrating ¹⁵N-labeled NCp7 with either **RNA 1** or an unlabeled RNA pentanucleotide **RNA 2**. For these experiments, ¹⁵N-labeled protein was overexpressed in BL21 pLysE cells grown in ¹⁵NH₄Cl-enriched media and purified by fast protein liquid chromatography (FPLC).³⁰ ¹⁵N heteronuclear single quantum coherence

(HSQC) NMR spectra were obtained by titrating substoichiometric amounts of RNA to 500 μM [U - ^{15}N] NCp7 (450 μL). The observed cross-peaks were assigned based on the values reported in literature.³¹

The observed HSQC spectra provided evidence that the paramagnetic probe minimally perturbed the native RNA–protein binding pocket. Previous studies have shown that the NCp7 residues, F16, W37, K38, and M46 are directly involved in nucleic acid binding.²⁹ The most important are π -stacking interactions between the indole ring of W37 stacked between the nucleobases of C2 and G3. In addition, W37 and M46 interact with the C-terminal zinc knuckle through hydrophobic contacts with the aromatic protons of C2, G3, and C4. Figure S3 draws comparison between HSQC spectra of ^{15}N -labeled NCp7 (green) overlaid with either NCp7-**RNA1** (blue) or NCp7-**RNA2** (red). The noncovalent interactions resulted in practically identical broadening of HSQC cross-peaks of the aforementioned residues in both spectra, indicating that the paramagnetic probe did not perturb the fidelity of RNA–protein binding.

The paramagnetic probes also caused global attenuation of other cross-peak intensities. The extent of attenuation was found to be dependent on the predicted proximity of the protein's backbone to the paramagnetic center. Figure 2A shows an overlay of the HSQC spectrum corresponding to NCp7 bound to **RNA 1** and NCp7 bound to the unlabeled RNA, **RNA 2**. The peak volumes from the two spectra were normalized against the residue that was least affected by the paramagnetic probe, R29. The extent of attenuation, illustrated in Figure 2B, was quantitated by computing the ratios of each corresponding cross-peak area between the two spectra. The paramagnetic probe did not produce pseudocontact shifts (PCS). The largest observed shift was 0.051 ppm (K20), and the average shift was 0.016 ppm, both within the range of experimental error.

The observed attenuation is consistent with the MD predictions. The strongest attenuation of intensity was observed for the E42 residue, suggesting coordination of Cu(II) to the glutamic acid residue. Notable attenuation was also observed for the protein residues in spatial proximity to E42: K33, K34, C36, and K41. An arbitrary cutoff line was drawn to differentiate the residues that undergo significant attenuation. The graph bars were color coded to indicate the relative extent of attenuation indicative of the spatial proximity of the paramagnetic probe. On the basis of these results we were able to construct the protein backbone map, shown in Figure 2C, as well as the protein's interactive surface map (Figure S4), which are strikingly similar to that obtained by using molecular simulations (Figure 1B). Residues shown in red are the most attenuated and thus the closest to the paramagnetic probe, while the ones shown in blue are the least attenuated. There are three additional amino acid residues that experienced strong attenuation, G19, H23, and Q53. On the basis of the MD simulation, these residues are not in spatial proximity to the paramagnetic center. We believe that the observed attenuation of their cross-peak intensities could be due to conformational changes that NCp7 experiences as the result of coordination of Cu(II) to the glutamic acid residue.

CONCLUSIONS

This work describes a new approach for studying RNA–protein interactions using 2D correlation NMR techniques. We have shown that labeling a model RNA pentanucleotide with a transition-metal-based paramagnetic NMR probe allowed us to create a visual map describing the RNA's binding to HIV-1 nucleocapsid protein NCp7. The paramagnetic metal was found to coordinate to a glutamic acid residue, thus reducing flexibility of the probe and producing distance-dependent attenuation of HSQC cross-peaks corresponding to amide backbone of NCp7. The signal attenuation observed by NMR was consistent with the predictions obtained by MD simulation.

The structural data obtained from this proof-of-concept study is in agreement with the previously described NOE experiments that elucidated binding of the unlabeled pentanucleotide 5'-ACGCC-3' and NCp7.²⁹ This serves as a confirmation that the paramagnetic probe was attached just outside of the binding site, having minimal impact on the RNA–protein binding. The described system lacked a diamagnetic control that interacted with NCp7 similar to the paramagnetic probe. In the future, the system will be redesigned to have a metal-based diamagnetic control that would facilitate quantitation of PRE data. In addition, the transition-metal ion-based paramagnetic probe will be optimized to study RNA–protein interactions inside of live cells using in-cell NMR techniques.³²

EXPERIMENTAL DETAILS

All chemicals were received from commercial sources and used without further purification. Chromatographic purifications were conducted using SiliaSphere spherical silica gel 5 μm , 60 Å silica gel (Silicycle). Thin-layer chromatography (TLC) was performed on SiliaPlate silica gel TLC plates (250 μm thickness) purchased from Silicycle. Preparative TLC was performed on SiliaPlate silica gel TLC plates (1000 μm thickness). High-pressure liquid chromatography (HPLC) purification was performed using Phenomenex Luna 5u C18(2) semipreparative column (250 $\times$ 10 mm). ^1H and ^{13}C NMR spectroscopy was performed on a Bruker NMR at 400 (^1H) and 100 (^{13}C) MHz.

The RNA oligonucleotide synthesis was performed on a 1.0 μmol scale using MerMade 8 DNA synthesizer. All the natural nucleoside phosphoramidites (TBDMS as the 2'-OH protecting group) and the accessory reagents were purchased from ChemGenes. After synthesis, the unmodified RNA oligomer was cleaved from the beads and deprotected by the treatment with AMA solution (a 1:1 aqueous solution of methylamine and concentrated ammonium hydroxide) at 65 °C for 2 h. After the resulting solution was evaporated to dryness, the RNA was resuspended in dimethyl sulfoxide (DMSO; anhydrous, 100 μL). The 2'-TBDMS deprotection was performed using $\text{NEt}_3\cdot\text{HF}$ (1:1, 125 μL) solution at 65 °C for 2.5 h. The RNA was precipitated with addition of 25 μL of 3 M NaOAc from butanol (1 mL). The solution was cooled at -80 °C for 30 min, then centrifuged for 15 min at 12 500 rpm. The supernatant was removed, and the precipitate was dried and resuspended in H_2O . Prior to electrospray ionization mass spectrometry (ESI-MS) analysis RNA was desalted by ethanol precipitation with 5 M NH_4OAc .

The samples were analyzed on a Thermo Fisher Scientific (West Palm Beach, CA) LTQ Orbitrap Velos Mass spectrometer, using quartz capillary emitters. To facilitate spray optimization, 10% isopropyl alcohol was added to each sample prior to MS analysis. Cyclen derivatives were dissolved in MeOH and analyzed in the positive mode, and the RNA was analyzed in a solution containing 150 mM ammonium acetate in the negative mode.

All 2D NMR experiments were performed at 25 °C on a 500 MHz Bruker Avance III NMR spectrometer equipped with the TCI cryoprobe. To prepare the NMR sample, 0.15 M of [*L*-¹⁵N] NCp7 was dissolved in the NMR buffer of 10 mM potassium phosphate, pH 6.5, and 10% D₂O. Meanwhile, stock solutions of **RNA 1** and **RNA 2** (20 mM) were prepared. NCp7 was titrated with the **RNA 1** or **RNA 2** solutions to achieve molar ratios of 1:0.33, 1:0.67, 1:1, and 1:1.33 between the NCp7 and RNA. ¹H-¹⁵N HSQC with Watergate water suppression³³ was used to monitor protein chemical shift changes due to RNA binding. 1024 × 256 points in proton and nitrogen dimensions, respectively, were acquired with 128 transients. The spectra were processed by using Topspin 2.1 (Bruker Inc.) and analyzed by using Cara software. The peaks were assigned based on published results.³⁰ The peak volumes were normalized by dividing the peak volume of each residue by the peak volume of R29 for NCp7-RNA 1.

The peak intensity changes were quantified by using the ratio between the peak volumes:

$$\frac{\text{normalized peak volume of residue from RNA1}}{\text{normalized peak volume of residue from RNA2}} = \text{ratio of peak volume change}$$

Synthesis of 3

A solution of Boc anhydride (2.28 g, 10.45 mmol) in anhydrous CH₂Cl₂ (60 mL) was added dropwise over 1 h to the solution of cyclen (1.00 g, 5.81 mmol) and DIPEA (5.06 mL, 29.03 mmol), in CH₂Cl₂ (250 mL) under nitrogen atmosphere. After addition the mixture was cooled to -15 °C. A second solution of Boc anhydride (1.52 g, 6.97 mmol) in CH₂Cl₂ (60 mL) was added dropwise over 1 h. The solution was slowly warmed to room temperature (rt) and stirred for 18 h, washed with aqueous 0.5 M K₂CO₃ (2 × 150 mL), dried with Na₂SO₄, and concentrated under reduced pressure. The title product was obtained as a white foam by flash chromatography using a gradient of 5–10% MeOH in EtOAc. *R*_F = 0.82 in 1:4 MeOH/EtOAc with 0.1% Et₃N. Yield = 2.06 g (75.5%); ¹H NMR (CDCl₃, 400 MHz) δ 3.54 (br s, 4H), 3.36–3.06 (m, 8H), 2.74 (br s, 4H), 2.02 (br s, 1H), 1.37 (br s, 9H), 1.35 (br s, 18H); ¹³C NMR (CDCl₃, 100 MHz) δ 155.5, 155.2, 79.2, 79.0, 50.8, 49.5, 49.2, 49.0, 28.5, 28.3; HRMS (ESI) *m/z*: calcd. for C₂₃H₄₄N₄NaO₆ [M + Na]⁺ 495.3159; found 495.3139.

Synthesis of 4

Potassium carbonate (2.40 g, 17.40 mmol) and propargyl bromide (~80% toluene, 193 μL, 2.54 mmol) were added to a solution of **3** (2.06 g, 4.35 mmol) in anhydrous acetonitrile (55 mL). The solution was stirred at reflux under nitrogen for 18 h. The insoluble salts were filtered, and the solution was concentrated under reduced pressure. The title product was obtained as a white foam by flash chromatography using a gradient of 5%–80% EtOAc in hexanes. *R*_F = 0.86 in 7:3 EtOAc/Hexanes with 0.1% Et₃N. Yield = 1.64 g (73.5%); ¹H

NMR (CDCl₃, 400 MHz) δ 3.45–3.01 (m, 14H), 2.61 (br s, 4H), 2.08 (s, 1H), 1.30 (br s, 9H), 1.28 (br s, 18H); ¹³C NMR (CDCl₃, 100 MHz) δ 155.7, 155.4, 154.9, 79.3, 79.1, 78.9, 77.3, 73.5, 54.0, 93.0, 49.6, 47.6, 47.4, 46.7, 46.2, 38.7, 28.5, 28.3, 28.2; HRMS (ESI) m/z : calcd. for C₂₆H₄₆N₄NaO₆ [M + Na]⁺ 533.3315; found 533.3303.

Synthesis of 5

Compound **4** (1.64 g, 3.08 mmol) was dissolved in a 1:1 mixture of TFA and CH₂Cl₂ (15 mL) and stirred for 1.5 h at room temperature. The solution was concentrated under reduced pressure and coevaporated with MeOH (5 × 10 mL). The TFA salt was dissolved in a 1:9 mixture of MeOH/CH₂Cl₂ (50 mL) and washed with aqueous 1 M NaOH (150 mL), and brine (150 mL). The organic layer was dried with Na₂SO₄ and concentrated under reduced pressure. The title product was obtained as a light yellow solid. Yield = 0.36 g (53%); ¹H NMR (CD₃OH, 400 MHz) δ 3.46 (d, J = 2.7 Hz, 2H), 2.77–2.75 (m, 4H), 2.68–2.62 (m, 9H), 2.59–2.56 (m, 4H); ¹³C NMR (CD₃OH, 100 MHz) δ 79.5, 74.6, 51.3, 47.3, 46.6, 45.01, 44.1; HRMS (ESI) m/z : calcd. for C₁₁H₂₃N₄ [M + H]⁺ 211.1923; found 211.1892.

Synthesis of 1

(Caution! Perchlorate salts of metal complexes with organic ligands are potentially explosive. They should be handled in small quantity and with caution.) Copper(II) perchlorate hexahydrate (105.77 mg, 0.286 mmol) was added to a solution of **5** (50.0 mg, 0.238 mmol) in EtOH (5 mL) and refluxed for 1.5 h. The Cu(II) complex was precipitated with ether and filtered. The crude product was dissolved in MeOH (1 mL). X-ray quality crystals were obtained by slow diffusion of ether. HRMS (ESI) m/z : calcd. for C₁₁H₂₂CuN₄ [Cu(**5**)]²⁺ 136.5570; found 136.5559; IR (neat) 3496.8, 3278.0, 2954.1, 2442.6, 2159.9, 1977.5, 1636.6, 1481.2, 1465.2, 1435.6, 1340.8, 1301.8, 1291.3, 1276.8, 1241.5, 1052.0.

Solid-Phase RNA Synthesis

Compound **6** was synthesized and immobilized on GE Healthcare Custom Primer Support Amino resin (GE Healthcare catalogue No. 17-5214-98) by following the previously reported procedure.²⁷ The functionalized resin was packed into empty Bioautomation MerMade columns (MM-1000–1; 4.00 mg of resin per column). The RNA oligonucleotide synthesis was performed as described above. After synthesis, the RNA was cleaved from the resin as previously described.²⁷ The synthetic RNA strands were purified from failure strands by reverse-phase (RP) HPLC using Phenomenex, Luna 5u C18(2) 100A column. Buffer A (100 mM TEAA and 5% acetonitrile); buffer B (100 mM triethylammonium acetate (TEAA) 50% acetonitrile). The purification was achieved using an 18 min gradient of 0–85% buffer B. After purification the solvents were lyophilized, and the sample was desalted, resuspended in water, and quantified using nanodrop. **7**–HRMS (ESI) m/z : calcd. for C₄₉H₆₁N₂₁O₃₃P₄ [M–2]^{2–} 797.6346; found 797.6353. **RNA 2**–HRMS (ESI) m/z : calcd. for C₄₇H₅₈N₁₈O₃₃P₄ [M–2]^{2–} 763.1182; found 763.1222.

Clicking the Probe with RNA

Click was done following the previously reported procedure.^{28a} Reaction progress was monitored by ESI-MS. Upon completion, the reaction mixture was lyophilized, resuspended

in H₂O, and purified by RP-HPLC using Phenomenex, Luna 5u C18(2) 100A column. Buffer A (100 mM TEAA and 5% acetonitrile); buffer B (100 mM TEAA 50% acetonitrile). The purification was achieved using an 18 min gradient of 0–85% buffer B. After purification the solvents were lyophilized, and the sample was desalted, resuspended in water, and quantified using nanodrop. Yields of the click reactions ranged between 89 and 91%. **RNA 1**–HRMS (ESI) *m/z*: calcd. for C₆₀H₈₁CuN₂₅O₃₃P₄ [Cu(**RNA 1**)-4]²⁻ 933.1837; found 933.1829. Prior to the 2D NMR experiments, the following ion exchange procedure was performed with all synthetic RNA strands: The RNA strands were dissolved in 300 μL of aqueous 100 μM KCl. After 20 min of equilibration, the solutions were loaded into SpinOUT columns (G biosciences GT-100, catalogue No. 786–866), and the supernatants containing RNA were collected after centrifugation at 1000 G. RNA concentrations were determined by nanodrop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Amino acid sequence of NCp7. The reported NMR structure, as well the computational studies described in this work, are based on the amino acid residues 13–53 that encompass the two zinc knuckle domains. (B) Superposition of 10 structures of NCp7-**RNA 1** complex predicted by MD simulation. The superimposed structures show affinity of the paramagnetic probe to negatively charged amino acid residues E42 and D48.

Figure 2.

(A) Overlay of ^{15}N HSQC spectrum for NCp7-**RNA 2** (blue) and NCp7-**RNA 1** (red). The residue experiencing the strongest attenuation is shown in a box. Asterisks correspond to side chain amides. (B) Ratio of peak volume change of the cross-peak intensities. (●) Residues that are lowered in intensity upon binding the unlabeled **RNA 2** (F16, W37, K38, M46). They were excluded from the analysis. (◆) Residue (E42) that was excessively broadened due to proximity to **RNA 1** and also removed from the analysis. The horizontal line differentiates the residues experiencing the strongest attenuation. (C) Protein backbone map based on the previously reported NMR structure. The amino acid residues are color-

coded based on the extent of attenuation of the HSQC cross-peak intensities. The yellow circles represent the two zinc atoms bound to zinc finger domains of the protein.

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Scheme 1. Paramagnetic NMR Probe 1 and the Ortep Representation^a of Its Crystal Structure

^aShowing 50% thermal ellipsoids and selected atom labels. Hydrogen atoms and disordered solvents were omitted for clarity.

Scheme 2. Synthesis of the “Clickable” NMR Probe 1^a

^a(a) Boc_2O , Et_3N , CH_2Cl_2 . (b) Propargyl bromide, K_2CO_3 , MeCN . (c) TFA , CH_2Cl_2 . (d) $\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$, EtOH , 75°C .

Scheme 3. Solid-Phase Synthesis^a

^aSynthesis of the RNA pentanucleotide and subsequent modification with the paramagnetic probe **1** resulted in **RNA 1**. Unlabeled RNA, **RNA 2**, was also synthesized as a diamagnetic control.