An Efficient Lanthanide-Dependent DNAzyme Cleaving 2′-5′ Linked RNA

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Dedication ((optional))

Abstract: RNA can form two type of linkages. Aside from the dominating 3′-5′ linkage, 2′-5′ linked RNA is also important in biology, medicine, and prebiotic studies. Herein, in vitro selection was used to isolate a DNAzyme that specifically cleaves 2′-5′ RNA using Ce^{3+} as the metal cofactor, leaving the 3′-5′ counterpart intact. This Ce5 DNAzyme requires trivalent light lanthanide ions with a rate of 0.16 min^{-1} in the presence of 10 μM Ce^{3+}, and the activity decreases with heavier lanthanide ions. This is the fastest DNAzyme reported so far for this reaction, and it may enable applications in chemical biology. As a proof-of-concept, using this DNAzyme, the reactions between phosphorothioate-modified RNA and strongly thiophilic metals (Hg^{2+} and Tl^{3+}) are studied as a function of pH.

Keywords: DNAzymes • RNA • lanthanides • phosphorothioate • biosensors

While most natural nucleic acids have the 3′-5′ linkage, 2′-5′ linked RNAs are also important in biology and biotechnology. For example, bacterial cells have enzymes that catalyze 2′-5′ RNA ligation.[1] In prebiotic models, both types of linkages might exist, and functional RNAs are able to tolerate a certain fraction of the 2′-5′ linkage without losing binding or catalytic activity.[2] Many artificially evolved enzymes for RNA ligation produce the 2′-5′ linkage as well.[3,4] Synthetic 2′-5′ RNAs were tested as anti-sense agents.[5] The structural and biophysical aspects on this linkage were also studied.[6,7]

Given the importance of 2′-5′ linked RNA, it is useful to develop its analytical methods so that it can be quantified and distinguished from its 3′-5′ counterpart. RNase T2 digestion was used to confirm the presence of such a linkage, which requires radio-isotope labeling, expensive enzymes and long procedures.[8] For its quantification, a duplex with a cDNA is first formed and then incubated with high concentrations of Mg^{2+} at high pH (called alkaline hydrolysis). Under this condition, the 2′-5′ linkage is cleaved leaving the 3′-5′ linkage intact. This reaction however takes 4 days to complete since the cleavage kinetics is extremely slow.[8]

DNAzymes are excellent chemical biology tools,[9-11] and they have demonstrated applications in RNA cleavage,[12] ligation,[13] phosphorylation,[14] and labeling.[15,16] In particular, RNA-cleaving DNAzymes are useful in metal detection, chemical biology, and nanotechnology.[13,17-19] A few DNAzymes have been isolated with 2′-5′ RNA cleavage activity. For example, Ordoukhanian and Joyce selected a DNAzyme with a rate of 0.01 min^{-1} in the presence of 25 Mg^{2+}.[20] The Silverman group reported a number of RNA-ligating DNAzymes that also have 2′-5′ RNA cleavage activity.[3,4] However, with 40 mM Mg^{2+}, the rates were below 0.005 min^{-1}. The HDV ribozyme can also cleave 2′-5′ RNA but its rate is 100-fold slower compared to cleaving the 3′-5′ substrate.[21] It is interesting to note that these DNAzymes are much slower than those cleaving 3′-5′ RNA, which are often more than 0.1 min^{-1}. Probably because of their slow rates, DNAzymes cleaving 2′-5′ RNA have not been used as chemical biology tools. Herein, we aim to isolate faster DNAzymes for this reaction and demonstrate a preliminary example of application. If a similar rate can be reached, this study will also testify that 2′-5′ RNA is not intrinsically more difficult to cleave using DNAzymes.

To obtain DNAzymes cleaving 2′-5′ linked RNA, *in vitro* selection was carried out using a library containing 50 random nucleotides bearing a single 2′-5′ RNA linkage (Figure 1A, see Table S1 for DNA sequences). The RNA linkage is the intended cleavage site since it is around one-million-fold less stable compared to the rest DNA linkages.[22] For RNA cleavage, multivalent metal ions are usually required to achieve a high catalytic efficiency,[23-25] such as Mg^{2+},[26] Pb^{2+},[27] UO_2^{2+},[28] and other metals.[29] While these metals produced fast DNAzymes for cleaving 3′-5′ RNA, all existing enzymes for 2′-5′ RNA are very slow. Recently, we isolated a few efficient DNAzymes using trivalent lanthanide ions.[30-32] Lanthanides have low toxicity and they were also used to assist RNA ligation,[33] DNA cleavage,[34] and RNA labeling.[16] Lanthanides were not previously used for cleaving 2′-5′ RNA. Therefore, in this work, Ce^{3+} was employed.

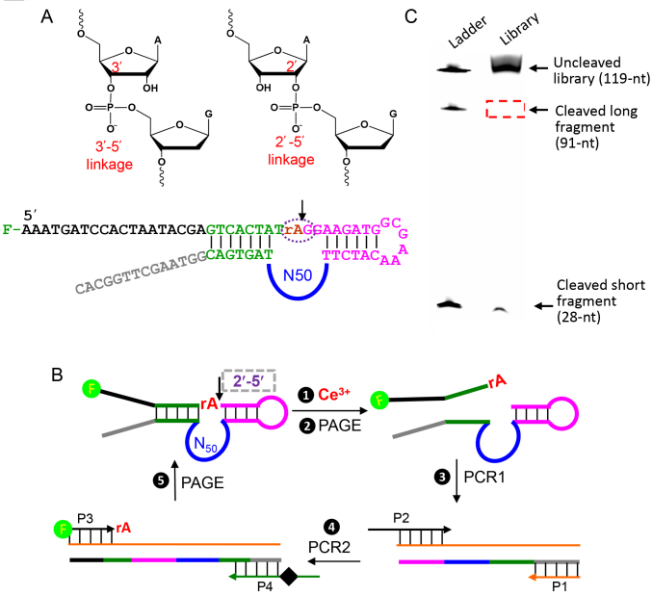

Figure 1. (A) The library design for selecting DNAzymes cleaving 2′-5′ linked RNA. The structures of both types of linkages at the rAG cleavage junction are shown. The 'F' attached to the 5′-end denotes for the carboxyfluorescein (FAM). (B) The scheme of *in vitro* selection with five main steps: 1: cleavage; 2: PAGE-based separation; 3: polymerase chain reaction 1 (PCR1) to produce the full-length library;

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4: PCR2 to introduce the FAM-label and the rA base; and 5: PAGE-based purification to isolate the positive strand of the PCR product. (C) A representative gel image showing step 2. The full-length library was 119-nt. After cleaving, 28-nt was removed, and the 91-nt position was excised (red box) as indicated by the DNA ladder.

For each round of selection, 10 μM Ce^{3+} was added to the library to induce cleavage (Figure 1B). The cleaved sequences were shorter by 28-nt, so that they were readily harvested by polyacrylamide gel electrophoresis (PAGE, Figure 1C). The active sequences were then amplified by PCR to seed the next round of selection. The selection progress is shown in Figure S1, and almost no cleavage occurred in the first four rounds. An obvious cleavage was achieved at round 5, and the round 6 library (with ~6% cleavage yield) was analyzed by deep sequencing. We did not push for a higher yield since we hope to get a diverse sequence distribution. The 21,000 resulting sequences were aligned, and the twenty largest families were analyzed using Mfold (see Table S2 for their sequences and abundance).[35] Seven of them appear to form typical DNAzyme secondary structures with the cleavage site exposed to the potential enzyme loop, and these were converted to trans-cleaving constructs (Figure S2). An example of converting the Ce5 DNAzyme is shown in Figure S3. Further activity assays using the trans-cleaving DNAzymes indicated that three of them (Ce2, Ce4, Ce5) were capable of cleaving the 2′-5′ linked substrate quite efficiently, one was moderately active (Ce6), and the rest three were inactive (Figure S4A). Sequence alignment of these three most active DNAzymes indicates a few highly conserved nucleotides (in red, Figure S4B), while the nucleotides in purple can be mutated without affecting the activity. We chose Ce5 for further characterization (Figure 2A). Ce5 has a structure typical of most RNA-cleaving DNAzymes with a hairpin and a large bulge. Ce5 is highly specific for the 2′-5′ linked RNA with no noticeable cleavage using the normal 3′-5′ substrate (Figure 2B). For comparison, the well-studied 17E DNAzyme cleaves the 3′-5′ substrate,[26] but not the 2′-5′ substrate (except for a trace amount of cleavage with 0.1 mM Pb^{2+}, Figure 2D). In addition, 17E failed to cleave the 2′-5′ substrate with lanthanide ions in absence or presence 20 mM Mg^{2+} (Figure S5), suggesting that lanthanides alone cannot discriminate the 2′-5′ linkage from the 3′-5′. The DNAzyme scaffold is needed to guide the function of the metal ion.

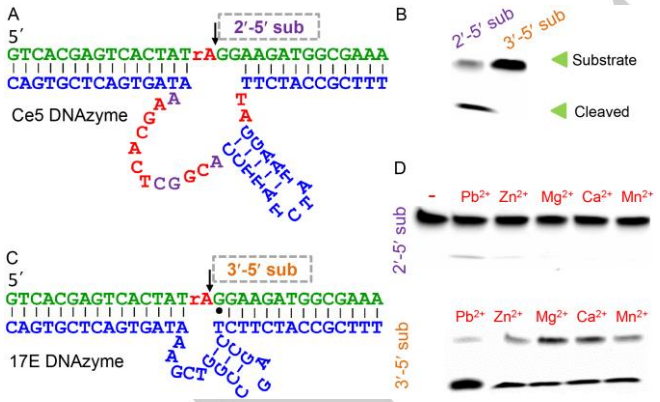

Figure 2. The secondary structure of (A) the Ce5 DNAzyme cleaving the 2′-5′ linkage, and (C) the 17E cleaving the 3′-5′ linkage. The red nucleotides in (A) are highly conserved. (B) A gel image showing Ce5 cleaves only the 2′-5′ linked RNA with 5 μM Ce^{3+}. (D) 17E cleavage assay of the two substrates with 100 μM Pb^{2+} or 10 mM other metal ions.

RNA cleavage usually employs the free –OH group in the ribose ring as the nucleophile. For cleaving 2′-5′ RNA, the 3′-OH is the nucleophile. The role of metal ions (in this case Ce^{3+}) is likely to be stabilizing the highly negatively charged transition state phosphorane and thus lower the activation energy.[36] Alternatively, the metal may act as a general base to help deprotonate the ribose –OH to make it a stronger nucleophile.[24]

Next, the biochemical properties of Ce5 were characterized. Ce5 was incubated with increasing concentrations of Ce^{3+}, and a linear increase in cleavage yield was observed with up to 2 μM Ce^{3+} (Figure 3A). With > 20 μM Ce^{3+}, an inhibition effect was observed, which may be explained by nonspecific interactions between DNA and Ce^{3+}.[36] Under the optimal Ce^{3+} concentration of 10 μM, a cleavage rate of 0.16 min^{-1} was obtained (Figure 3B, red trace). See Figure S6 for the kinetic traces at different Ce^{3+} concentrations. This rate is similar to most of our previously reported lanthanide-dependent DNAzymes cleaving the 3′-5′ RNA linkages.[30-32] Therefore, from this example, DNAzymes cleaving 2′-5′ RNA does not seem to be at a disadvantage. For comparison, the standard alkaline hydrolysis method with 100 mM Mg^{2+} at pH 9 and 37 °C yields a rate of 0.043 h^{-1} (Figure 3B, blue trace), which is 220-fold slower than that by the Ce5 DNAzyme. Ce5 is also over 10-fold faster than all previously reported DNAzymes for cleaving 2′-5′ RNA.[3,4,20] Therefore, Ce5 might be a useful alternative to characterize 2′-5′ linked RNA. We noticed that the maximal cleavage yield by Ce5 is ~70%, and the fraction that failed to cleave might due to DNAzyme misfolding (e.g. forming DNAzyme dimers or folded in structures different from that in Figure 2A).

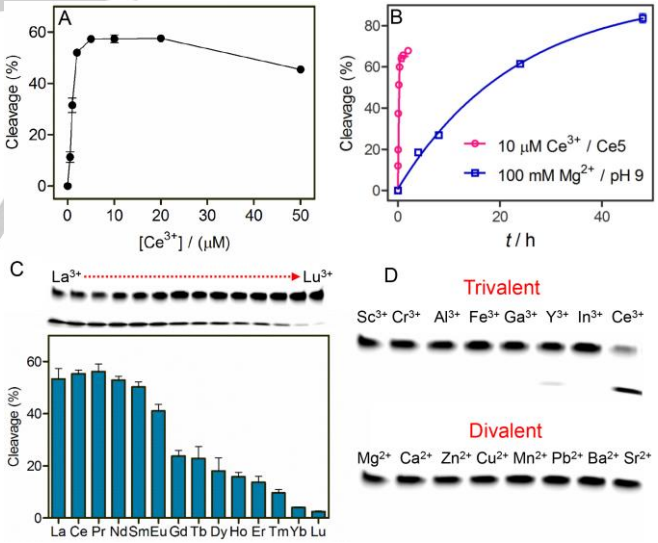

Figure 3. (A) Fraction of substrate cleavage by the Ce5 DNAzyme with various concentrations of Ce^{3+} (pH 6.0, 50 mM K^+, 1 h). (B) Cleavage kinetics by Ce5, and the same substrate cleaved by the alkaline hydrolysis method. (C) Gel image showing cleavage with 5 μM different lanthanides (upper panel) and its quantification (lower panel). (D) Metal selectivity test of Ce5 with 50 μM divalent or 5 μM trivalent metal ions. All assays in triplicate and error bars represent the standard deviation.

Ce5 was next studied for its metal specificity. While Ce^{3+} was used for the selection, all the trivalent lanthanide ions were active after 1 h incubation (Figure 3C). The first six light lanthanides have similarly high activity, while a quick descending trend is observed for the heavy ones starting from Gd^{3+}. We next studied its activity in presence of

other metal ions (Figure 3D). Among these, none showed any activity (except for a trace amount cleavage by Y^{3+}, which has a similar size to Ho^{3+}), indicating that Ce5 is quite specific toward lanthanides. It is easy to understand the preference for Ce^{3+} (and other light lanthanides) since Ce^{3+} was used during the selection. The DNAzyme sequence was evolved to use Ce^{3+}, while other divalent and trivalent metal ions cannot play the same role due to differences in size, charge, pK_a of metal bound water, and coordination geometry.

So far, we demonstrated that Ce5 is capable of selectively cleaving 2′-5′ linked RNA, and 17E is specific for the 3′-5′ linkage. With these two DNAzymes as chemical biology tools, it might be possible to analyze the two types of RNA linkages. RNA plays an essential role in biology. The "RNA world" hypothesis has been supported by many important discoveries including ribozymes and riboswitches.[37] Since the nucleophilicity of the 2′ and 3′ hydroxyl groups on the ribose is similar, chemically ligated RNA often generates a mixture of 2′-5′ and 3′-5′ linkages. Therefore, such DNAzyme-based chemical biology tools might be useful. As a proof-of-concept study to use this new DNAzyme, we next studied the reaction between a phosphorothioate (PS)-modified RNA and strongly thiophilic metal ions.

We recently observed that an oligonucleotide containing a single PS-modified RNA undergoes three reactions when mixed with highly thiolphilic metal ions (e.g. Hg^{2+} and Tl^{3+}): cleavage, desulfurization, and isomerization (Figure 4A).[38,39] In all the products, the sulfur atom is removed and replaced by oxygen, as indicated by mass spectrometry (the sulfur containing species were not detected).[39] The uncleaved substrate became the phosphodiester (PO) linkage. Similar studies with a dinucleotide junction were previously performed with less thiophilic metals, and the mechanism of such reactions were also described.[40] Briefly, $Hg(OH)^+$ is recruited to the PS site, and the mercury bound –OH serves as a general base to activate the ribose –OH to make it $–O^-$ (a better nucleophile). This nucleophile attacks the phosphorus center and a penta-coordinated species is formed. Depending on the leaving group, different products are generated. Typically, with Hg^{2+} or Tl^{3+}, <16% of the substrate is cleaved,[38,39] and the rest undergo desulfurization (forming 3′-5′ linkage) and isomerization (forming 2′-5′ linkage). We are interested in understanding the distribution of the two types of RNA linkages produced under different conditions.

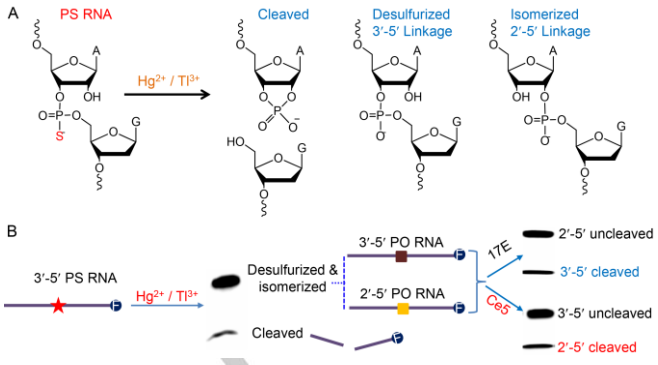

Figure 4. (A) A PS-RNA reacting with Hg^{2+} or Tl^{3+}, yielding three types of products. In all the cases, the sulfur atom was removed. (B) A scheme for characterizing the products using the 17E and Ce5 DNAzymes.

Our experiment design is shown in Figure 4B. Using the free PS RNA substrate as an example, after adding Hg^{2+}, the reaction products were separated by gel electrophoresis (see the gel). The top band contained the desulfurized/isomerized mixture (the original substrate length maintained). This band was harvested and then split into two samples. Each sample was respectively hybridized with 17E (using Mg^{2+}) or Ce5 (using Ce^{3+}). We observed cleavage in both, indicating the presence of both types of RNA linkages.

To quantify the fraction of each type of linkage, two calibration curves were built. The PO substrates bearing the 3′-5′ and 2′-5′ linkages were mixed at different molar ratios. The mixtures were then annealed with Ce5 or 17E to form the DNAzyme complexes. The cleavage was initiated by adding their respective metal cofactor (10 μM Ce^{3+} for Ce5; 10 mM Ca^{2+} for 17E). After 1 h, the reaction products were quantified by gel electrophoresis (Figure 5A, B). We chose 1 h reaction time to ensure cleavage reaching plateau, so that the yield is proportional to their initial ratio. Indeed, a linear relationship exists for both components.

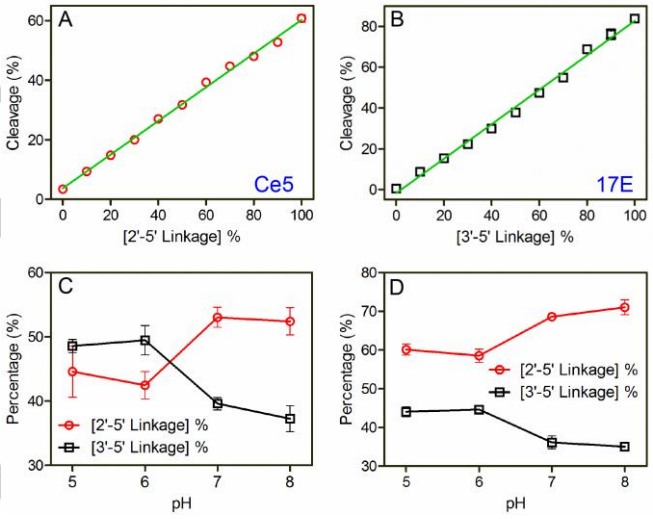

Figure 5. Calibration curves of the (A) 2′-5′ and (B) 3′-5′ linkage RNA by using Ce5 and 17E as the analytical tools, respectively. Quantification of the product of the free PS RNA substrate after treated with (C) Hg^{2+} and (D) Tl^{3+} at different pH's. All assays in triplicate and error bars represent the standard deviation.

Using these calibration curves, we studied the effect of pH in the presence of Hg^{2+} or Tl^{3+}. The free PS RNA substrate was treated with Hg^{2+} or Tl^{3+} at pH 5 to 8. The uncleaved products were then used for the DNAzyme-based analysis. At slightly acidic pH, after the Hg^{2+} treatment, the percentage of each type of the RNA linkage is quite similar, both approaching ~50% (Figure 5C). It is interesting to note that the 2′-5′ product increased slightly at higher pH, reaching 55% at pH 8. The Tl^{3+} cleaved products biased more towards the 2′-5′ linkage (Figure 5D). In particular, around 70% 2′-5′ linkage was produced at pH 8. Therefore, this simple assay can be used to obtain new information regarding nucleic acids chemistry.

Conclusions

In summary, we reported a DNAzyme that can selectively cleave the 2′-5′ RNA linkage with a high catalytic rate (faster than all previously reported DNAzyme for this reaction). Ce5 is active in the presence of light trivalent lanthanides and it can cleave the substrate in 1 h, by which the speed is significantly improved compared to the traditional alkaline hydrolysis method taking a few days. Compared to DNAzymes that cleave 3′-5′ RNA junctions using also Ce^{3+},[31,32] Ce5 has a similar rate, indicating that 2′-5′ RNA can be cleaved similar to 3′-5′ RNA. The application of Ce5 in studying the reaction between PS RNA and thiophilic metal ions as a function of pH is reported, and this has added new insights into an important system for analytical chemistry and nanotechnology. Further studies will focus on selecting of new DNAzymes that can cleave full-RNA substrates bearing such a linkage.

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[1] C. L. Greer, B. Javor, J. Abelson, *Cell* **1983**, *33*, 899.
[2] A. E. Engelhart, M. W. Powner, J. W. Szostak, *Nat Chem* **2013**, *5*, 390.
[3] A. Flynn-Charlebois, Y. Wang, T. K. Prior, I. Rashid, K. A. Hoadley, R. L. Coppins, A. C. Wolf, S. K. Silverman, *J. Am. Chem. Soc.* **2003**, *125*, 2444.
[4] A. Flynn-Charlebois, T. K. Prior, K. A. Hoadley, S. K. Silverman, *J. Am. Chem. Soc.* **2003**, *125*, 5346.
[5] M. Wasner, D. Arion, G. Borkow, A. Noronha, A. H. Uddin, M. A. Parniak, M. J. Damha, *Biochemistry* **1998**, *37*, 7478.
[6] J. Sheng, L. Li, A. E. Engelhart, J. Gan, J. Wang, J. W. Szostak, *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 3050.
[7] L. Li, J. W. Szostak, *J. Am. Chem. Soc.* **2014**, *136*, 2858.
[8] R. Rohatgi, D. P. Bartel, J. W. Szostak, *J. Am. Chem. Soc.* **1996**, *118*, 3340.
[9] R. R. Breaker, G. F. Joyce, *Chem. Biol.* **2014**, *21*, 1059.
[10] S. K. Silverman, *Acc. Chem. Res.* **2009**, *42*, 1521.
[11] Y. Lu, *Inorg. Chem.* **2006**, *45*, 9930.
[12] K. Schlosser, Y. F. Li, *Chem. Biol.* **2009**, *16*, 311.
[13] R. L. Coppins, S. K. Silverman, *J. Am. Chem. Soc.* **2004**, *126*, 16426.
[14] Y. Li, R. R. Breaker, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 2746.
[15] D. A. Baum, S. K. Silverman, *Angew. Chem. Int. Ed.* **2007**, *46*, 3502.
[16] L. Büttner, F. Javadi-Zarnaghi, C. Höbartner, *J. Am. Chem. Soc.* **2014**, *136*, 8131.
[17] J. Liu, Z. Cao, Y. Lu, *Chem. Rev.* **2009**, *109*, 1948.
[18] Y. Lu, J. Liu, *Acc. Chem. Res.* **2007**, *40*, 315.
[19] X.-B. Zhang, R.-M. Kong, Y. Lu, *Annu. Rev. Anal. Chem.* **2011**, *4*, 105.
[20] P. Ordoukhanian, G. F. Joyce, *J. Am. Chem. Soc.* **2002**, *124*, 12499.
[21] I. H. Shih, M. D. Been, *RNA* **1999**, *5*, 1140.
[22] Y. Li, R. R. Breaker, *J. Am. Chem. Soc.* **1999**, *121*, 5364.
[23] R. K. O. Sigel, A. M. Pyle, *Chem. Rev.* **2007**, *107*, 97.
[24] W. L. Ward, K. Plakos, V. J. DeRose, *Chem. Rev.* **2014**, *114*, 4318.
[25] Y. Lu, *Chem. Eur. J.* **2002**, *8*, 4588.
[26] S. W. Santoro, G. F. Joyce, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262.
[27] R. R. Breaker, G. F. Joyce, *Chem. Biol.* **1994**, *1*, 223.
[28] J. Liu, A. K. Brown, X. Meng, D. M. Cropek, J. D. Istok, D. B. Watson, Y. Lu, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 2056.
[29] Z. Liu, S. H. J. Mei, J. D. Brennan, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 7539.
[30] P.-J. J. Huang, M. Vazin, Ż. Matuszek, J. Liu, *Nucleic Acids Res.* **2015**, *43*, 461.
[31] P.-J. J. Huang, M. Vazin, J. Liu, *Anal. Chem.* **2014**, *86*, 9993.
[32] P.-J. J. Huang, J. Lin, J. Cao, M. Vazin, J. Liu, *Anal. Chem.* **2014**, *86*, 1816.
[33] F. Javadi-Zarnaghi, C. Hobartner, *J. Am. Chem. Soc.* **2013**, *135*, 12839.
[34] V. Dokukin, S. K. Silverman, *Chem. Sci.* **2012**, *3*, 1707.
[35] M. Zuker, *Nucleic Acids Res.* **2003**, *31*, 3406.
[36] M. Vazin, P.-J. J. Huang, Ż. Matuszek, J. Liu, *Biochemistry* **2015**, *54*, 6132.
[37] R. F. Gesteland, T. R. Cech, J. F. Atkins, *The RNA World (2nd Edition)*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York **1999**.
[38] P.-J. J. Huang, M. Vazin, J. Liu, *Anal. Chem.* **2015**, *87*, 10443.
[39] P.-J. J. Huang, F. Wang, J. Liu, *Anal. Chem.* **2015**, *87*, 6890.
[40] M. Ora, M. Peltomaki, M. Oivanen, H. Lonnberg, *J.Org.Chem.* **1998**, *63*, 2939.