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ARTICLE

## Influence of conjugation and other structural changes on the activity of Cu<sup>2+</sup> based PNAzymes

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We have previously shown that PNA-neocuproine conjugates can act as artificial RNA restriction enzymes. In the present study we have additionally conjugated the PNA with different entities, such as oligoethers, peptides etc and also constructed systems where the PNA is designed to clamp the target RNA forming a triplex. Some conjugations are detrimental for the activity while most are silent which means that conjugation can be done to alter physical properties without losing activity. Conjugation with a single oligoether close to the neocuproine does enhance the rate almost twofold compared to the system without the oligoether. The systems designed to clamp the RNA target by forming a triplex retain the activity if the added oligoT sequence is 5 PNA units or shorter and extends the arsenal of artificial RNA restriction enzymes. Changing the direction of a closing base pair, where the target RNA forms a bulge, from a GC to a CG pair enhances the rate of cleavage somewhat without compromising the selectivity, leading to the so far most efficient artificial nuclease reported.

### Introduction

Modified oligonucleotides and conjugates are useful tools for research but are also developed as drugs for treatment of patients with genetic diseases. Most oligonucleotide therapies, e.g., siRNA<sup>1,2</sup>, antisense<sup>3-5</sup> and splice-switching<sup>6,7</sup> approaches, target specific RNA sequences. Oligonucleotide therapy has potential for treatment of a broad range of diseases,<sup>8-11</sup> therefore the interest for oligonucleotide therapy is increasing. There are now a number of different possible approaches and miRNA's are also being explored as targets and clinical trials are even conducted with DNAzymes cleaving mRNA.<sup>12</sup> Much current research is focused on developing efficient cellular delivery of ON's, but other issues are stability, higher specificity towards the target and of course overall efficiency which includes efficient use of the oligonucleotide.

In oligonucleotide therapy the regulation of gene expression could be more efficient if turnover of the target RNA is obtained. This can occur if native enzymes (e.g., RNase H for antisense<sup>3-5</sup> and RISC complex for siRNA<sup>6,7</sup>) can recognise the relevant oligonucleotide complex. Most modifications of oligonucleotides are, however, not recognized by RNase H and gapmers are needed to take advantage of the cellular

enzyme.<sup>10-12</sup> If the antisense oligonucleotide could carry a group that causes cleavage of the target RNA upon hybridization this problem would be avoided and independence of native enzymes would be achieved. Substantial research has gone into development of artificial ribonucleases<sup>13-19</sup> that typically cleave RNA at the internucleoside phosphodiester linkages.

Use of an oligonucleotide based artificial nuclease (OBAN)<sup>20</sup>, if effective enough, could then lead to recognition and cleavage of RNA sequences responsible for genetic or viral diseases. Such artificial enzymes could allow for complete modifications that give stable oligonucleotides not degraded by host enzymes. Recognition of the target substrate would be reliably achieved through Watson-Crick base pairing and the concept should work in theory. In reality, the difficulty lies in developing a sufficiently efficient artificial nuclease. OBANs that carries non-metal ion catalysts<sup>19, 21-23</sup> have been developed but metal ion based oligonucleotide based artificial ribonucleases<sup>19, 20, 24-28</sup> are more common. The most efficient artificial ribonuclease developed so far is based on a peptide nucleic acid (PNA) carrying a catalytic Cu<sup>2+</sup>-neocuproine entity (Figure 1)<sup>29</sup>. When these PNAzymes bind the target an RNA bulge is formed (due to partial complementarity) and the RNA is cleaved specifically at one site with turnover of substrate, with the PNAzyme thus acting as an RNA restriction enzyme.<sup>29</sup>



Figure 1. Schematic presentation of a complex between RNA and a PNAzyme that act as an RNA restriction enzyme.<sup>29</sup>

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In this study we present further work on  $\text{Cu}^{2+}$  based PNAzymes where additional entities are added or the structure otherwise changed. For further development of PNAzymes it is important to see how these changes can influence the enzyme activity as we are ultimately searching for improved efficacy.

## Results and discussion

### PNAzymes conjugated to oligoethers or His-containing peptides

Most interesting for further development of artificial nucleases is that metal ion catalyzed cleavage of phosphate diesters can be up to  $10^{12}$  times faster in methanol than in aqueous solution.<sup>30</sup> It is of course not feasible to have this environment *in vivo* but it could be possible to create a local solvent effect around the cleavage site. If we can obtain only a small fraction of the enhancement found in methanol we could still obtain a substantial increase in cleavage rate. In our first attempts we are conjugating the PNAzymes with oligoethers in the hope of forming a partial cage, at least a fraction of the time, around the active site that would render the environment less aqueous. In the development of methods for oligoether conjugation to PNA we synthesized a PNA-neocuproine oligoether conjugate carrying five oligoethers.<sup>31</sup> However, the solubility of this derivative was problematic and therefore RNA cleavage studies could not be performed.

With other PNA-neocuproine oligoether conjugates (PNA 1 and PNA 3, Figure 2) we could, however, perform cleavage studies. When comparing RNA cleavage as accomplished with PNA 1 and PNA 2 there is a modest but significant difference (Figure 3). The PNAzyme with the oligoether conjugated close to the neocuproine (PNA 1) cleaves RNA with an approximate half-life that is almost twofold shorter than when the corresponding position is unoccupied (PNA 2) (Table 1). It is not a large effect and it is not possible at this stage to adduce that the difference is due to solvation. It is, however, encouraging that a single oligoether conjugation enhances the rate of cleavage of RNA as promoted by a  $\text{Cu}^{2+}$ -neocuproine based PNAzyme.

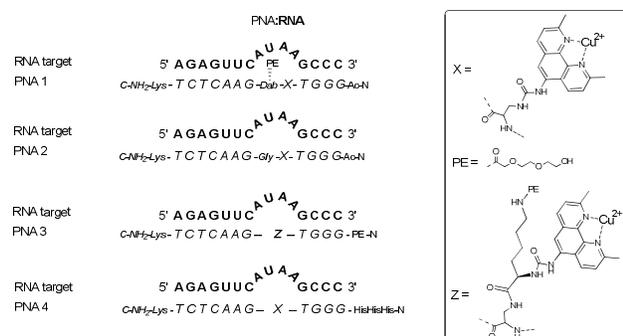


Figure 2. Complexes between RNA-target and PNA-neocuproine constructs, with further conjugation to oligoethers or a peptide.

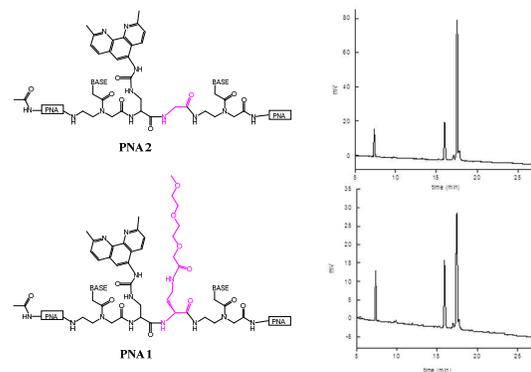


Figure 3. Ion exchange HPLC-chromatograms showing extent of cleavage of the RNA-target AGAGUUCAUAAGCCC after 80 min incubation with PNA 1 (lower chromatogram) and PNA 2 (upper chromatogram) in a 1:1 ratio, at 37°C, pH 7.

Table 1. Full length RNA remaining at different times after incubation of AGAGUUCAUAAGCCC with PNAs 1-4 (4  $\mu\text{M}$  of each).

PNA \ % full length RNA after	10 min	30 min	80 min
<b>PNA 1</b>	93,5	79,6	56,6
<b>PNA 2</b>	96,2	89,5	74,4
<b>PNA 3</b>	100	100	100
<b>PNA 4</b>	100	100	100

A somewhat different construct containing two oligoethers, one at the amino terminus of the PNA and one extending from a lysine branching (PNA 3, Figure 2), was on the other hand inactive as an RNA-cleaver (Table 1). Extension of the linker to neocuproine with an additional amino acid (Gly) did not change the activity much in previous work<sup>28</sup> but the result here demonstrates that the PNAzymes are sensitive to relatively small structural changes as has also been shown when varying the RNA-sequence in the bulge region.<sup>29</sup> It is then, without a decent 3-D structure, difficult to predict how changes will alter the activity.

In the NMR determined structure of a five nucleotide RNA bulge,<sup>32</sup> which was the starting point for our initial bulge design,<sup>20</sup> as well as in models where this structure was altered and cut,<sup>33</sup> the 5' end (or amino terminus for PNA) is relatively close to the bulge. Although there will be an entropic penalty due to the flexibility of the bulge as well as of N-terminal linked entities, it is reasonable to assume that additional functionality added at the N-terminal could reach the bulge and influence the catalysis. In order to combine the copper ion chelate with a group having the potential to act as a Brønsted acid/base catalyst we conjugated the PNA with a peptide carrying three histidines (PNA 4, Figure 2). This PNA-neocuproine-peptide conjugate was, however, inactive as an RNA cleaver (Table 1). It is plausible that one or more histidines complexes to the neocuproine bound copper ion thereby making the metal ion inaccessible to the

phosphodiester functions. A similar effect was found when two neocuproines were conjugated to the same PNA.<sup>34</sup>

### PNAs designed to form a triplex forming PNA-clamp

A possibility to add entities in proximity of the RNA bulge in a more controlled fashion could be if the PNAzyme forms a clamp around one of the stems of the RNA to reach back to the centrally positioned bulge. The target sequence would then have to be chosen so that a triplex, where one strand is RNA and two are PNA, can be formed, i.e., a bis-PNA targeting RNA. Little has been done with respect to two PNA strands forming a triplex with RNA so we first made sure that non-conjugated PNA with an additional sequence designed to form a triplex gives stable complexes with an RNA target. BisPNA's 1-4 (Figure 4) were synthesized and thermal melting points for the complexes with the respective RNA's were determined. Complexes with 5 or 6 TAT motifs (BisPNA 1-3) formed stable complexes with thermal melting of 60-61 °C while the construct with only four TAT motifs (BisPNA 4) was considerably less stable with a  $T_m$  of 46 °C (Figure 4).

A number of different BisPNA constructs conjugated to neocuproine (Figure 5) were synthesized and evaluated with respect to their ability to cleave target RNA sequences. The reactions with BisPNA 5 and 6 were difficult to analyse as the RNA seemed to complex or precipitate also during HPLC-analysis (see Supporting information) but reaction was clearly slower. With BisPNA 6 we did manage to perform turnover experiments with excess of RNA showing that this BisPNA acts as an enzyme. The melting temperatures of the RNA complexes with BisPNA 1, 2 and 3 do not suggest that the aggregation with BisPNA 5 and 6 is due to stronger RNA complexes. However, with neocuproine conjugated this could be the case and perhaps the release of the cleaved substrate then becomes rate-limiting. The PNAzymes with 5 and 4 TAT motifs (BisPNA 7 and 8) did perform well also at 1:1 ratio to the RNA, giving half-lives for the RNA between 30 and 60 minutes (Table 2) which is comparable to the fastest non-clamping systems with other sequences in the 5'-stem of the RNA<sup>29</sup>.

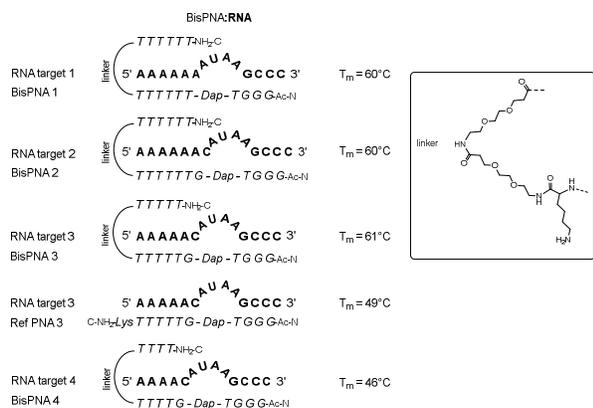


Figure 4. Non-conjugated Bis-PNAs for determination of complex stability.

When a non-clamping PNAzyme (Ref PNA 7, Figure 5) targeting the same RNA as BisPNA 7 was investigated the rate of cleavage was considerably lower showing that the TAT-clamp is essential for this particular target sequence (Table 2).

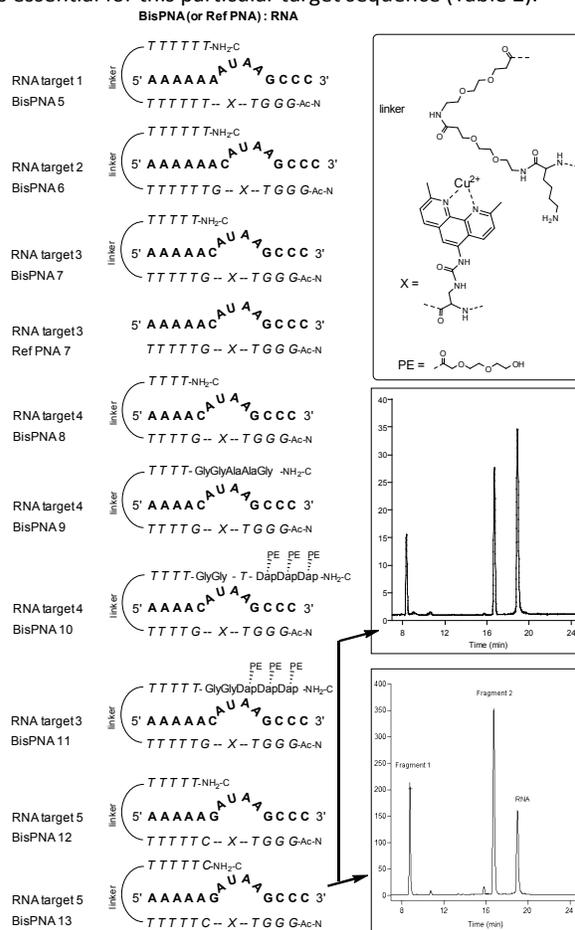


Figure 5. BisPNA neocuproine conjugates designed to clamp the RNA target by triplex formation at the 5'-stem part of the RNA. IE-HPLC chromatograms after incubation of RNA target 5 with BisPNA 13; Middle insert: 30 min. 4  $\mu$ M each of PNAzyme and RNA; Lower insert: 4  $\mu$ M of BisPNA13 and 20  $\mu$ M RNA target 5 after 48 h (MS analysis of Fragment 1: Calc = 1527.27, Found = 1526.55; Fragment 2: Calc = 2954.44, Found = 2953.54).

Table 2. Full length RNA remaining at different times after incubation of RNA with BisPNA/PNAs 5-13 (4  $\mu$ M each, 10  $\mu$ M Cu<sup>2+</sup>, pH 7.0, 37°C).

PNAzyme	15 min	30 min	60 min (or *45 min)
BisPNA 5	Complex properties made analysis difficult		
BisPNA 6	Complex properties made analysis difficult (Turnover experiments with PNA:RNA 1:5 in SI)		
BisPNA 7	81.7	66.9	44.4
Ref PNA 7	95.1	91.7	86.4
BisPNA 8	77.7	61.7	38.2
BisPNA 9	79.7	60.3	36.4
BisPNA 10	78.3	65.4	39.6
BisPNA 11	79.4	63.9	39.3
BisPNA 12	72.4	51.4	27.1
BisPNA 13	59.9	43.0	*33.6

The lower rate with Ref PNA 7 as compared to with BisPNA 7 could possibly be due to weaker binding to the target with only AT-pairs in one stem, leading to that the RNA is subsaturated with PNAzyme. However, this seems unlikely since the thermal melting of Ref PNA 3 with RNA target 3 (Figure 4) is well above the temperature of incubation. This would indicate that the clamping actually perturbs the structure in a fashion that enhances cleavage.

Clearly the clamping of a part of the RNA target by the PNAzymes is acceptable without compromising the activity and turnover. BisPNA 7 was also shown to turnover the RNA when this was present in excess (see Supporting information). The next question was if we, by conjugating entities at end of the triplex forming part of the PNA, can influence the cleavage, *e.g.*, by replacing water molecules with other functionalities to obtain a different solvation. Thus, BisPNA 9 extended with a GlyGlyAlaAlaGly peptide and BisPNA 10-11 (Figure 5) extended with oligoether containing peptides were synthesized. Although the physical properties of these constructs are expected to be somewhat different the action of these PNAzymes in cleavage of RNA was essentially unchanged as compared to the unconjugated BisPNA 7 and 8 (Table 2). Even though the initial direction of the peptides is guided by the triplex formation it may still be that the peptide is flexible enough to mostly reside in the surrounding water rather than interacting with the bulge and replace water molecules in the vicinity of the cleavage site.

We then decided to change the direction of the bulge closing GC base pair to a CG pair, which would enable a CGC triplex (although the triplex part would only have one hydrogen bond at physiological pH). Hence we synthesized BisPNA 12 without and BisPNA 13 (Figure 5) with an additional cytidine that could possibly interact with the G in the RNA. Just swapping the GC pair to a CG did actually improve the efficacy of the PNAzyme (BisPNA 12) to give a construct that cleaved the RNA target at a higher rate than any of the previous BisPNA's. The construct with the additional cytidine (BisPNA13), for possible further interaction with the RNA, gave an even higher rate of RNA cleavage. The rate of cleavage at a 1:1 ratio of the PNAzyme BisPNA 13 to RNA is in fact the most efficient observed so far in any artificial ribonuclease construct reported with a half-life between 15 and 30 minutes (Table 2). The PNAzyme, BisPNA 13, also displayed turnover of RNA target 5 (Figure 5 lower insert). The cleavage site was identified by MS to be between the two Adenosine adjacent to the GU wobble base pair (which is equivalent to the site identified with the non-triplexing artificial RNA restriction enzymes)<sup>29</sup>. As in that study the 5'-end fragment formed carries a 2',3'-cyclic phosphate at the 3'-end formed from intramolecular cyclisation.

## Experimental

### Material and Methods

PNA sequences were assembled automatically on a solid support (Rink Amide resin) using the manufacturer's protocol

for the Alstra Initiator Biotage peptide synthesizer with 9-fluorenylmethoxycarbonyl (Fmoc)-chemistry using *N,N'*-diisopropylcarbodiimide (DIC) as coupling agent and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as additive. PNA building blocks were from Link Technologies Ltd (Strathclyde, UK). Fmoc- $\alpha$ -N-Dap( $\beta$ N-Mtt)OH, Fmoc- $\alpha$ -N-Lys( $\epsilon$ N-Boc)OH, HN-Lys( $\epsilon$ N-Boc)OH and Fmoc-His(Trt)-OH were from Iris Biotech GmbH (Marktredwitz, Germany). High-resolution mass spectrometry (HRMS) was performed on a Micromass LCT electrospray time-of-flight (ES-TOF) mass spectrometer in acetonitrile–water 1:1 (v/v), 0.1% formic acid solutions. The molecular weights of the oligoribonucleotide and peptide nucleic acid conjugates were reconstructed from the *m/z* values using the mass deconvolution program of the instrument (Mass Lynx software package). The RNA substrate was purchased purified from ThermoScientific. Thermal melting analysis was determined from an absorbance vs. temperature profile measured at 260 nm on a Varian Cary 300 UV–vis dual beam spectrophotometer (Varian). Concentrations of both RNA and PNA were determined by UV absorption at 260 nm and calculated from extinction coefficients obtained by the nearest neighbor approximation.<sup>35</sup> All chemicals used in the RNA cleavage assays were of molecular biology grade. PNA 1 and PNA 2 and PE unit (2-(2-(2-(benzoyloxy)ethoxy)ethoxy)acetic acid) were synthesized as previously reported.<sup>31</sup>

**Post-conjugation of polyethers 2-(2-(2-(benzoyloxy)ethoxy)ethoxy)acetic acid (PE) and 5-phenoxycarbonylamino-2,9-dimethyl-1,10-phenanthroline to PNA (PNA 3).** The terminal Fmoc was cleaved off by subjecting the respective PNA (2 mg, 1.1  $\mu$ mol) to 20% piperidine in NMP for 25 min, followed by washing several times with DCM and NMP. Then compound PE (11.4 mg, 37.5 eq), HBTU (15.3 mg, 35.6 eq) and HOBt (5.4 mg, 35.6 eq) were dissolved in 97.5  $\mu$ L of NMP and NMM (8.25  $\mu$ L, 65 eq) and agitated for 5 min to preactivate the mixture, then added to the solid support and shaken for 30 min. The support was filtered and washed with NMP and DCM. Once the terminal polyether arm was conjugated, the *N* <sup>$\beta$</sup> -methyltrityl protection was cleaved off by subjecting the solid support to 1% trifluoroacetic acid in DCM 5 times for 1 min, followed by washing with DCM and NMP. One HN-Lys( $\epsilon$ N-Mtt)OH was coupled to the PNA using the manufacturer's protocol for the Alstra Initiator Biotage peptide synthesizer and a second unit of PE was coupled on the  $\epsilon$ N of the Lys. After removal of the  $\epsilon$ N-methyltrityl protection of the Lys, the 5-phenoxycarbonylamino-2,9-dimethyl-1,10-phenanthroline (3.1 mg, 9  $\mu$ mol), NMM (5  $\mu$ L, 50  $\mu$ mol) and NMP (150  $\mu$ L) were added to the support and left to react for 3h at 60°C in the microwave cavity of the Alstra Initiator Biotage peptide synthesizer. The PNA conjugate was deprotected using a 20% NH<sub>3</sub>/MeOH solution for 5 h at 50 °C and cleaved from support by TFA/TIS/water (95/2.5/2.5) (200  $\mu$ L) for 2 h, freeze dried and HPLC-purified. PNA 3 was purified with an Ascentis Express Supelco Peptide ES-C18 (2, 7  $\mu$ m 150  $\times$  4.6mm) column at 60 °C using a flow rate of 1mL/min and a

linear gradient of 40% B for 30 min (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50 % MeCN. PNA 4, calculated mass, 3906; found ESI[M+], 3906.

**Post-conjugation of 5-phenoxy-carbonylamino-2,9-dimethyl-1,10-phenanthroline and H-His(Trt)-OH to PNA (PNA 4).** The N<sup>β</sup>-methyltrityl protection was cleaved off by subjecting the correspondent PNA on support (2 mg, 1.1 μmol) to 1% trifluoroacetic acid in DCM, 5 times for 1 min, followed by washing with DCM and NMP, then the 5-phenoxy-carbonylamino-2,9-dimethyl-1,10-phenanthroline unit (3.1 mg, 9 μmol) was coupled as previously reported. The terminal Fmoc was cleaved off by subjecting the support to 20% piperidine in NMP for 25 min, followed by washing several times with DCM and NMP. The polyHis peptide was synthesized on the terminal part of the PNA using the manufacturer's protocol for the Alstra Initiator Biotage peptide synthesizer. PNA 4 was cleaved from support by TFA/TIS/water mixture (95/2.5/2.5) (200 μL) for 2 h, freeze dried and purified as PNA 3. PNA 4, calculated mass, 3918; found ESI[M+], 3918.

**Post-conjugation of 5-phenoxy-carbonylamino-2,9-dimethyl-1,10-phenanthroline to PNA (Ref PNA 7, BisPNA 5, 6, 7, 8, 9, 12 and 13).** The N<sup>β</sup>-methyltrityl protection was cleaved off by subjecting the correspondent PNA on support (2 mg, 1.1 μmol) to 1% trifluoroacetic acid in DCM, 5 times and leaving to react for 1 min, followed by washing with DCM and NMP then the 5-phenoxy-carbonylamino-2,9-dimethyl-1,10-phenanthroline unit (3.1 mg, 9 μmol) was coupled as previously reported. The PNA conjugates were cleaved from support by TFA/TIS/water mixture (95/2.5/2.5) (200 μL) for 2 h, freeze dried. Ref PNA 7 was purified with an Ascentis Express Supelco Peptide ES-C18 (2, 7 μm 150 × 4.6mm) column at 60 °C using a flow rate of 1mL/min and a linear gradient of 40% B for 30 min (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50 % MeCN. Ref PNA 7, calculated mass, 3284; found ESI[M+], 3285.

The other PNAs were purified using a linear gradient from 5% till 40% B for 27 min. (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50 % MeCN. BisPNA 5, calculated mass, 5176; found ESI[M+], 5175. BisPNA 6, calculated mass, 5465; found ESI[M+], 5463. BisPNA 7, calculated mass, 4932; found ESI[M+], 4930. BisPNA 8, calculated mass, 4402; found ESI[M+], 4402. BisPNA 9, calculated mass, 4712; found ESI[M+], 4712. BisPNA 12, calculated mass, 5143; found ESI[M+], 5143. BisPNA 13, calculated mass, 4892; found ESI[M+], 4893.

**Synthesis of BisPNA 10 and 11.** The synthesis of the PNA sequence was interrupted just before the conjugation of the last Fmoc-αN-Dap(BN-Mtt)OH in order to manually remove (as previously described) the N<sup>β</sup>-methyltrityl protections on the first three Dap monomers on support. Three PE units were then conjugated and the PNA sequence completed using the Alstra Initiator synthesizer. Once the last N<sup>β</sup>-methyltrityl protections had been removed, the 5-phenoxy-carbonylamino-2,9-dimethyl-1,10-phenanthroline unit (3.1 mg, 9 μmol) was

coupled as previously reported. The PNA conjugate was deprotected using a 20% NH<sub>3</sub>/MeOH solution for 5 h at 50 °C and cleaved from support by TFA/TIS/water (95/2.5/2.5) (200 μL) for 2 h, freeze dried and purified. BisPNAs 10 and 11 were purified using a linear gradient from 5% till 40% B for 27 min. (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50 % MeCN. BisPNA 10, calculated mass, 5476; found ESI[M+], 5476. BisPNA 6, calculated mass, 5476; found ESI[M+], main peak 5490 [M+2Na].

**RNA cleavage assay using Anion Exchange (IE) HPLC.** The reactions were performed in 0.3 ml glass tubes with screw cap immersed in a water bath at 37 °C. Incubation was carried out in a buffer with the following recipe: 10 μM CuCl<sub>2</sub> (from SigmaAldrich atomic spectroscopy standard concentrate), 10 mM Hepes, 100 mM NaCl at pH 7.0. Reactions were in general performed in equimolar concentrations (4 μM) of substrate RNA and PNAzyme, except the turnover experiments that were performed with the stated excess of RNA. The samples were prepared at room temperature adding the buffer, RNA and the amount of water necessary to achieve the right concentration, incubating the mixture for 10 min and then adding the aliquot from the PNAzyme stock solution. The final volume of the sample was 120 μl and 3 aliquots were collected at specific times (40 μl each one) and quenched with 70 μl of 300 μM EDTA 30% MeCN aqueous solution. The samples were frozen and stored at -18 °C until being analyzed by IE-HPLC. The samples were analyzed by anion exchange HPLC (IE-HPLC) equipped with a Dionex NucleoPacPA-100 (4 × 250 mm) column. The samples were analysed at 60 °C using a flow rate of 1.5 mL/min and a linear gradient of 0-45% over 30 min of buffer B where (A) is 20 mM NaOAc in 30% aqueous acetonitrile and (B) is 20 mM NaOAc, 0.4 M LiClO<sub>4</sub> in 30% aqueous acetonitrile. UV detection was carried out at 260 nm. For MS analysis of fragments formed upon cleavage of RNA target 5 with BisPNA 13 fractions at (9 and 17 min, see Figure 5) from IE-HPLC were collected. These samples were desalted by running RP-HPLC (on a Supelco Discovery BioWide Pore C18-5, 4.6x250mm) with a linear gradient of (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate in 50% aqueous acetonitrile (pH 6.5). A gradient of 0-40% buffer B over 40 min was used with a flow rate of 1 mL/min, at room temperature and UV detection at 260 nm. The desalted oligonucleotide fractions were then directly subjected to MS analysis by a Micromass LCT ESI-TOF instrument.

## Conclusions

We have further investigated PNA-neocuproine conjugates that can act as artificial RNA restriction enzymes. New constructs carrying oligoethers or peptides as well as systems where the PNA clamps the target RNA were made. The specific cleavage at a single site was retained for all active constructs with all studied RNA substrates, thus extending the arsenal of RNA restriction enzymes. Constructs decorated with entities that possibly could give enhanced activity due to a solvation

effect mostly gave a similar rate as for constructs where such entities were not included. However, conjugation with a single oligoether close to the neocuproine did enhance the rate almost twofold compared to the system without the oligoether which may or may not be due to influence on solvation. BisPNA constructs are efficient PNAzymes if an added clamping oligoT sequence is 5 PNA units or shorter. Changing the direction of a bulge closing base pair and adding the possibility of a CGC triplex enhances the rate of RNA cleavage without compromising the selectivity. It is not a huge jump in efficacy but nevertheless it is an improvement and this PNAzyme is thereby the so far most efficient artificial ribonuclease reported. It is a difficult and slow process but we are continuing to develop and extend the arsenal of RNA restriction enzymes with the new clamping construct giving additional possibilities in targeting.

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