PNA based artificial nucleases displaying catalysis with turnover in the cleavage of a leukemia related RNA model†

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Several peptide nucleic acid based artificial nucleases (PNAzymes) are designed to create a bulge in the target RNA, which is a short model of the leukemia related bcr/abl mRNA. The target RNA is cleaved by the PNAzymes with a half-life of down to 11 h (using a 1 : 1 ratio of PNA-conjugate to target) and only upon base-pairing with the substrate. The PNA based systems are also shown to act in a catalytic fashion with turnover of substrate and are thus the first reported peptide nucleic acid based artificial RNA-cleaving enzymes.

Introduction

If oligonucleotide based artificial nucleases (OBANs) can become efficient enough they have many potential uses as tools in molecular biology, as well as in therapeutics. Obtaining high catalytic efficiency and specificity is, however, quite some challenge. Metal complexes linked to oligonucleotides can be reasonable catalysts for the cleavage of ribonucleic acids[1] and several studies demonstrate the potential for targeting biologically relevant RNAs with artificial ribonucleases.[2–5] Since it is known that RNA bulges are more predisposed to cleavage than fully duplexed RNA[6–9] we have approached the development of OBANs by creating systems that force the formation of a bulge in the target RNA.[10] This also provides a pocket for potential interaction with the cleaving agent and/or recognition elements. The initial 2′-*O*-methyloligoribonucleotide–zinc chelate systems based on this concept did give cleavage of a target RNA with catalysis and turnover.[10] Variation of the target RNA, with respect to the bulge formed, had a substantial influence on cleavage activity, as did the linker position.[10–11] Since the activity depends upon the target and OBAN sequence it would be advantageous to start developing OBANs towards a potential therapeutic target sequence before the catalyst is optimized for efficiency.

A potential therapeutic target is the junction point of the bcr/abl mRNA transcript from the Philadelphia chromosome (Ph), t(9;22).[12] This RNA has for a long time been associated with human cancer and is the cytogenetic hallmark of chronic myeloid leukemia (CML).[13] There have been several attempts to suppress expression of this oncogene.[14–22] An alternative approach is to direct oligonucleotide based artificial nucleases (OBANs) against the same mRNA target. An OBAN has built in catalytic activity and can be built to resist degradation, is more readily taken up into cells and has the potential of still being selective and able to give catalytic cleavage of the mRNA. OBAN systems developed so far are typically not efficient enough. Ribozymes and DNAzymes giving turnover of mRNA with half-lives of minutes to tens of minutes can be considered for therapeutic use and are comparable in the suppression of gene expression to an antisense approach dependent upon RNase H catalysis.[23] Thus, for the use of OBANs in a therapeutic setting the efficiency would need to get into that range. Until the efficiency is getting close to usability, a small manageable model system is preferable, since analysis is more accurate and the systems can be more readily varied to investigate modifications that influence the rate and selectivity of the cleavage.

Peptide nucleic acids (PNA) generally give more stable complexes with RNA than DNA or 2′-*O*-Me RNA do (see also the ESI†), are stable in biological fluids and are also readily conjugated by the use of peptide type chemistry.[24,25] However, PNA has been sparingly used as a carrier for RNA cleaving agents, and only when using an excess of the cleaver or giving stoichiometric cleavage without turnover of the target RNA, *i.e.*, without enzyme catalysis.[2,26–30] With our constructs with centrally placed bulges, however, we expected that we could create PNA based artificial nucleases that give catalytic cleavage of RNA with turnover of substrate. OBAN–target complexes can be constructed with a part of the junction of the bcr/abl mRNA (the b3a2 transcript variant) by choosing the appropriate sequence. Thus, several PNA based systems directed towards this model target (Fig. 1) were synthesized and the cleavage rates were determined.

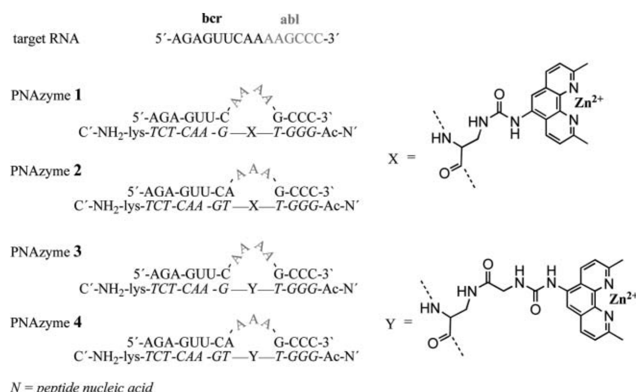

Fig. 1 Complexes of PNA based artificial nuclease enzymes (PNAzymes) **1–4** with the bcr/abl derived RNA model target.

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† Electronic supplementary information (ESI) available: Details of the bcr/abl RNA model purification, thermal melting analysis details, HPLC analyses of reaction turnover and plots from the experiments to determine rate constants. See DOI: 10.1039/b810106j

Results and discussion

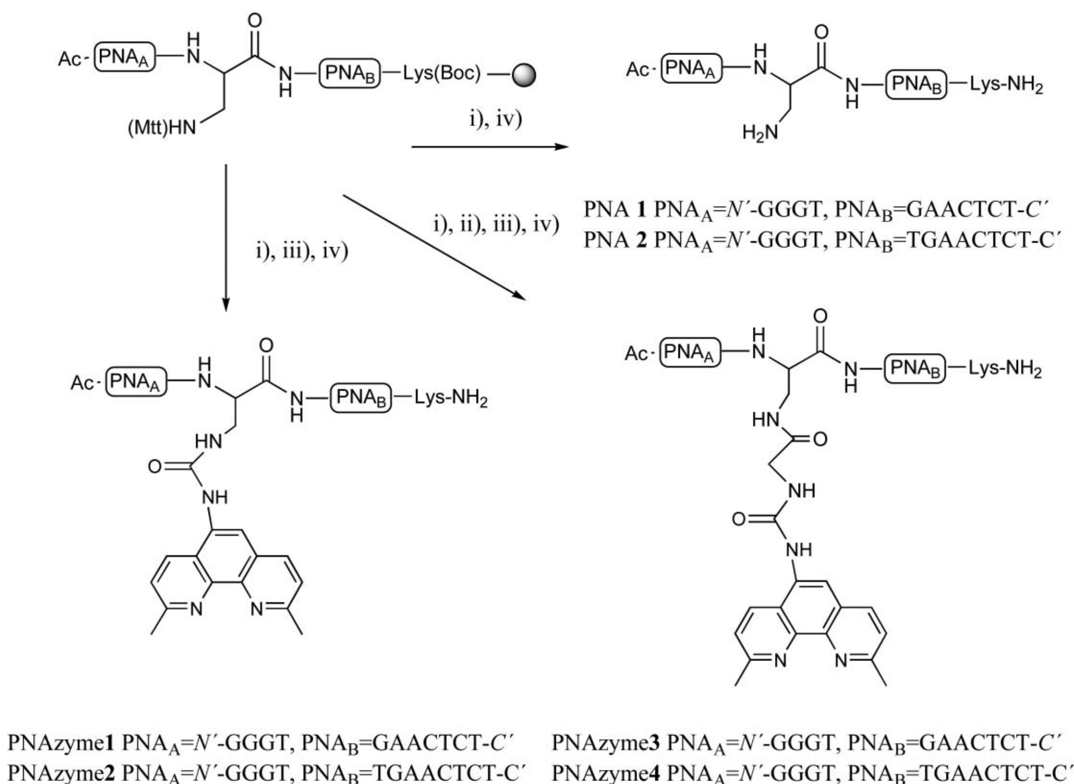
In the design of PNA based artificial nucleases we utilized PNA sequences with an internally placed diaminopropionic acid (Dap) unit in a position expected to be facing the bulge in the target upon complex formation (Fig. 1). The Dap unit also serves as an attachment point for the catalytic group *via* the side chain amino group. The PNA-Dap-PNA oligonucleotide analogues were prepared by Fmoc chemistry and then the Dap unit was deprotected to give the free amine. On solid support this was then reacted with phenyloxycarbonyl-5-amino-2,9-dimethylphenanthroline,[11,31,32] either directly or after extension of the Dap unit with a glycine moiety, to obtain the corresponding 2,9-dimethylphenanthroline (neocuproine) conjugates (Scheme 1). Deprotection and cleavage from the support then gave the PNA conjugates (PNAzymes) **1–4**. The thermodynamic stabilities of the different complexes between PNAzymes and target RNA (without Zn(II) present) were determined as the UV thermal melting points (T_m). Observed melting points for these PNA based OBANs were approximately 10 degrees higher than for the corresponding 2′-*O*-Me systems (see ESI†), T_m(PNAzyme **1**) = 55 °C, T_m(PNAzyme **2**) = 55 °C, T_m(PNAzyme **3**) = 52 °C and T_m(PNAzyme **4**) = 54 °C. Thus, the different complexes with the RNA target have very similar stabilities and the equilibrium should be almost completely shifted towards the complex at 37 °C when using a 1 : 1 ratio (4 μM conc. of each) of PNA conjugate and RNA.

The efficiency of PNAzymes **1–4** in promoting the cleavage of the RNA model was evaluated by analyzing aliquots from the reaction mixture by anion-exchange HPLC. This gave a higher resolution of the cleavage products than RP-HPLC and the PNA conjugate does not disturb the analysis since it is not retarded on the column (as it is with RP-HPLC). The RNA target is cleaved in all investigated systems at a rate comparable to that of 2′-*O*-methyloligoribonucleotide based systems carrying the same catalytic group and other Zn^{2+} based systems developed so far[1,10–11] (Table 1). The scission only takes place within the formed bulge and the maximum cleavage rate was observed for a 4-nt bulge forming system (PNAzyme **1**, $t_{1/2}$ = 11 h) with a direct linkage, closely followed by that observed with the 3-nt and 4-nt bulges

Table 1 Rate constants and half-lives for cleavage of the bcr/abl RNA model by PNAzymes at a 1 : 1 ratio (4 μM of each)[a]

	$k_{obs}/10^{-6}$ s^{-1}	$t_{1/2}$/h
PNAzyme **1**	17.6 ± 0.6	11
PNAzyme **2**	9.2 ± 0.2	21
PNAzyme **3**	16.0 ± 0.4	12
PNAzyme **4**	13.1 ± 0.7	15

[a] The experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES buffer, 0.1 M NaCl at pH 7.4, t = 37 °C. In the same buffer single stranded bcr/abl RNA model was cleaved at a rate of 2×10^{-6} s^{-1} and rates of cleavage of the bcr/abl RNA model in the presence of non-conjugated PNAs (PNA **1** and **2**) (1 : 1) were less than 1×10^{-6} s^{-1}. The stability constant for the complex with Zn(II) is only about 10^4, which means that an excess of zinc ions is necessary to keep the chelate nearly saturated.[10,33]



Scheme 1 Solid supported post-conjugation of PNA sequences. (*i*) 1% TFA–DCM for 1 min, repeated 5 times, (*ii*) Fmoc-Gly-OH, HBTU, HOBt, DIEA, followed by removal of Fmoc-protection by 20% piperidine–NMP, (*iii*) phenyloxycarbonyl-5-amino-2,9-dimethyl-1,10-phenanthroline, NMM, overnight in NMP, (*iv*) TFA–TIS–H_2O (95 : 2.5 : 2.5).

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(PNAzyme **3**, $t_{1/2} = 12$ h and PNAzyme **4**, $t_{1/2} = 15$ h) with the longer glycine linker.

The differences in rates between these systems are not large. There is quite some flexibility in both the linkers and bulges, and it is not unlikely that proximity to the cleavage site is mostly governed by the metal–phosphate interaction, as suggested for the 2′-*O*-methyloligoribonucleotide based OBANs.[11] This also means that the intramolecularity, and hence overall rates, will be relatively modest. With a short linker and a 3-nt bulge a slightly more restricted system is obtained but this is less active. Thus, the system with PNAzyme **2** gives a significantly lower cleavage rate and it is likely that the geometric restrictions are such that it is energetically more demanding to position the catalytic group in a productive fashion in this system. The phosphate to metal ion interaction would, on its own, be neither strong enough to hold the metal ion in the vicinity of only one phosphate nor be able to force the bulge into a single conformation. This is also supported by the observation of more than a single cleavage site even in the more selective systems (Fig. 2). It is also clear that the cleavage at all sites is governed by the presence of the zinc–neocuproine moiety since the cleavage in the presence of non-conjugated PNA-Dap-PNA is very slow (Fig. 2b). There is a correlation between the site selectivity and the overall rate: the higher the cleavage rate is, the more site-selective the cleavage obtained. This could be an indication of a somewhat higher proximity at the more prominent cleavage sites. As can be seen in Fig. 2, cleavage takes place exclusively in the bulged out region.

As the next step we investigated if our bulge design concept would also enable catalytic behaviour in these PNA based systems, that form more stable complexes than the corresponding 2′-*O*-methylRNA based OBANs. To explore this we employed the PNAzyme **1** to carry out turnover experiments with an excess of RNA substrate (1 : 2, 1 : 4 and 1 : 10). This PNA based system is indeed capable of catalytically cleaving the target bcr/abl model RNA with turnover of the substrate present in excess and is thus truly an enzyme (Fig. 3).

The PNA based OBANs also display a dependence of cleavage rate on the PNAzyme concentration. Despite the higher reactivity of single stranded RNA, the background reaction (with zinc aquo ions alone) is also low, even when compared to the reaction with a 10-fold excess of RNA substrate relative to the PNAzyme **1** present (for HPLC analysis see ESI†). These are the first reported RNA-cleavers based on a PNA backbone that are catalytically active, *i.e.*, that do give turnover of substrate. These could be called PNAzymes in a similar fashion to DNAzymes and ribozymes that use Mg^{2+} as cofactors, as we have a modified PNA with a zinc ion cofactor.

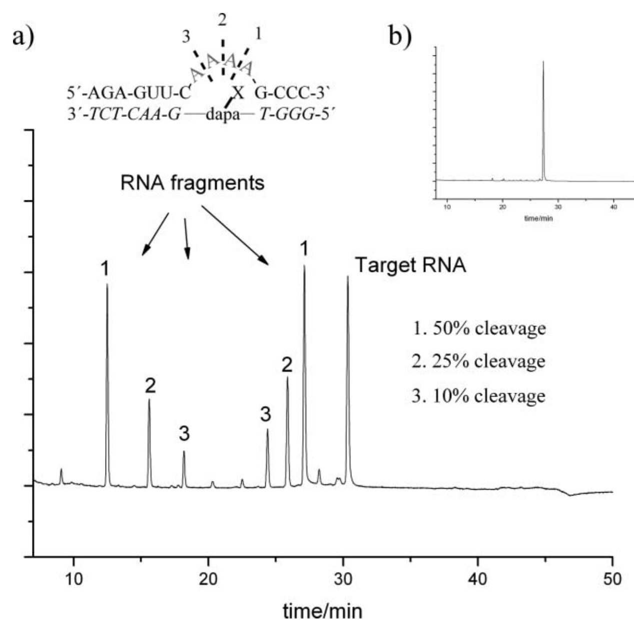

Fig. 2 (a) HPLC analysis of the PNAzyme **1** promoted cleavage of the bcr/abl RNA model, after 22 h at 37 °C. The trace shows the cleavage sites as determined by MS of fragments (ESI-TOF MS: site 1: 1529 + 3251; 2: 1857 + 2923; 3: 2593 + n.d.). (b) HPLC analysis of the background reaction with PNA **1** and the bcr/abl RNA model. Both reactions were carried out using the same conditions (100 μM Zn^{2+}) and were analysed at the same time points (22 h).

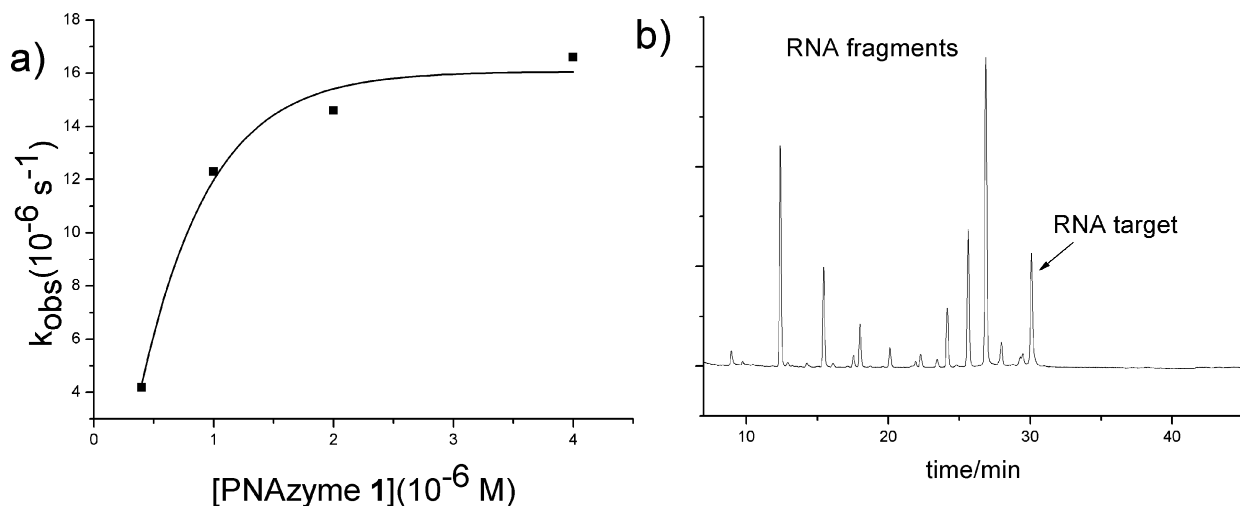

Fig. 3 (a) PNAzyme concentration dependence of the rate constant for cleavage of the bcr/abl RNA model with PNAzyme **1**. (b) HPLC analysis of bcr/abl RNA model cleavage by PNAzyme **1** when using a 1 : 4 ratio of PNAzyme to substrate (44 h reaction time).

Is then the cleavage action of these PNAzymes really dependent on forming a complex with Watson–Crick base pairing? We have shown that the PNAzymes can act as enzymes, giving turnover of substrate, that the reaction is dependent upon enzyme concentration and that the background reaction is substantially slower. Considering that PNA is somewhat prone to aggregation one could still argue that there is a non-specific interaction between the PNA-Dap-PNA chelate and the RNA, which gives a complex that is cleaved. Another issue is that an artificial nuclease towards a specific target should not cleave other sequences, whether containing four adjacent adenosines or not. To investigate this matter we incubated PNAzyme **1** with two other RNA sequences, one with a four adenosine stretch and both with limited possibilities for base-pairing (one fully complementary to the short stem on one side of the bulge). In the presence of PNAzyme **1** neither of these sequences were cleaved beyond background (Fig. 4) clearly showing that the action of the PNA based artificial nucleases developed is dependent on forming the complex governed by Watson–Crick base-pairs.

Conclusions

In the presence of zinc ions, PNA-Dap-PNA neocuproine conjugates (PNAzymes) cleave a target RNA sequence that is a model of the leukemia related bcr/abl mRNA. For real use in disease therapeutics the overall rate of RNA cleavage is likely to be insufficient for the efficient suppression of gene expression. However, it is a starting point for the development of PNAzymes that when efficient enough could become potential therapeutic agents.

The reaction is dependent on the concentration of the PNAzyme, and is catalytic with turnover of the substrate when this is present in excess. We have thus developed the first peptide nucleic acid based artificial RNA-cleavers that really act as enzymes (PNAzymes). Considering both this work and our previous studies on 2′-*O*-methylOBANs, it seems that the OBAN sequence can be altered according to the specific target with cleavage rates essentially retained as long as the base-pairing in the stems next to the bulge is governing the formation of the complex. However, if the base-pairing is not retained the rates fall down to background,

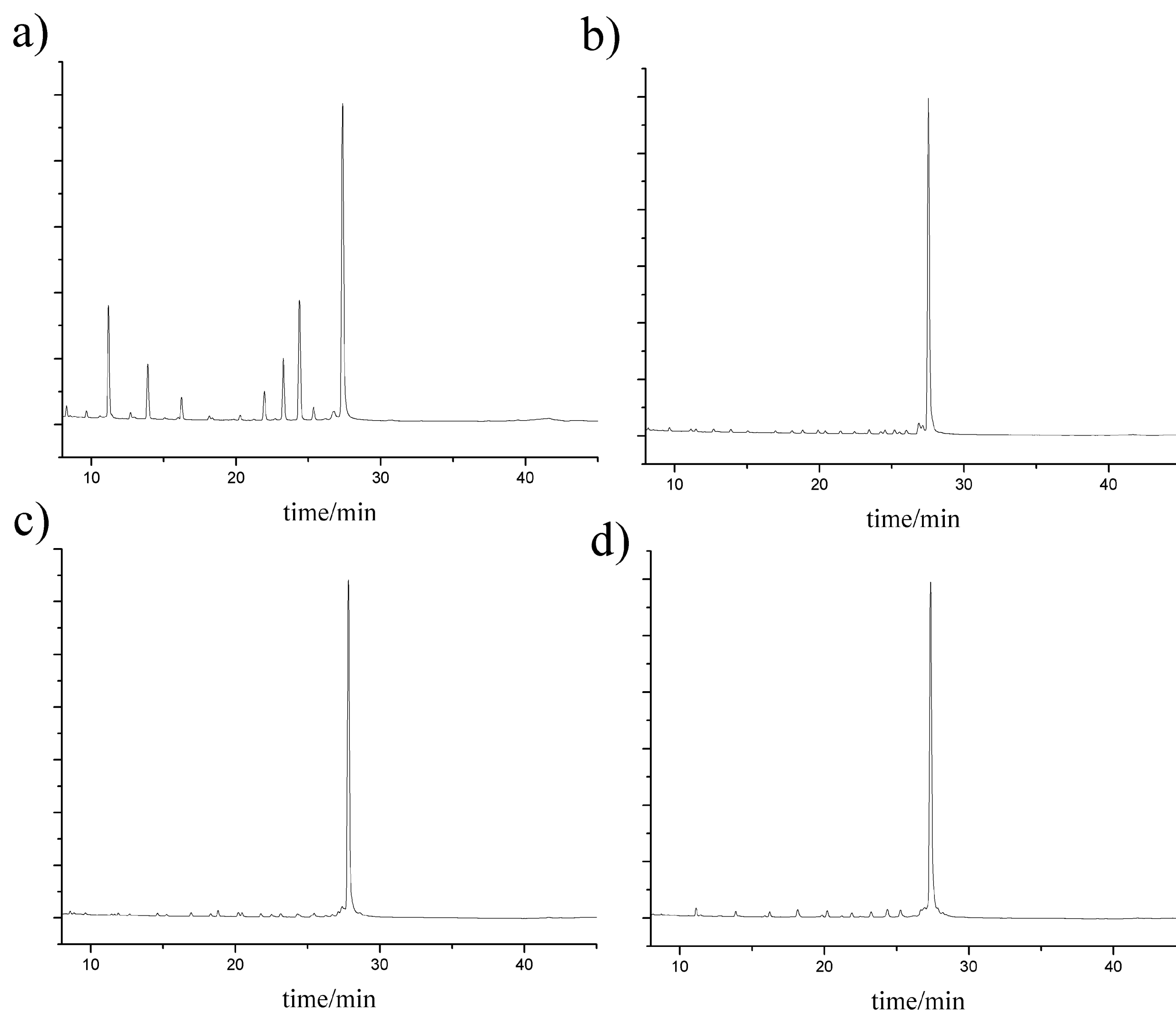

Fig. 4 HPLC analysis of the incubation of different RNA sequences with PNAzyme **1** and 100 μM $Zn^{2+}$for 11 h at 37 °C. (a) PNAzyme **1** and the bcr/abl RNA model 5′-AGAGUUCAAAAGCCC-3′, (b) PNAzyme **1** and RNA 5′-AUAAGGAAGAAGCCC-3′ (short model of the bcr/abl mRNA b2a2 transcript variant), (c) PNAzyme **1** and RNA 5′-UCUCGGUAAAAGCGC-3′, (d) only the bcr/abl RNA model 5′-AGAGUUCAAAAGCCC-3′.

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which means that it is possible to create OBANs/PNAzymes that are selective with respect to the target sequence. It seems that PNAzymes can provide all the basic properties needed to create artificial nucleases for use as tools and, with more progress, as potential regulators of gene expression. In addition they are rock stable in biological fluids and it certainly seems worthwhile developing these systems further (including the use of other metal ions) in order to obtain higher rates of cleavage as well as improvements in selectivity. Such studies, which also include the sequence dependence of the cleavage rate, are in progress.

Experimental section

Materials and methods

Peptide nucleic acid monomers were from Applied Biosystems. TentaGel S Rink Amide resin was purchased from Fluka. HBTU, HOBt and diaminopropionic acid and glycine derivatives were purchased from Novabiochem. Solvents and reagents for solid-phase synthesis were synthesis grade from Applied Biosystems. Other solvents were purchased from Merck Eurolab. High-resolution mass spectrometry (HRMS) was performed on a Micromass LCT electrospray time-of-flight (ES-TOF) mass spectrometer in CH_3CN–H_2O 1 : 1 (v/v) solutions. The molecular weights of the oligonucleotide- 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 from Dharmacon and was first purified by semi-preparative IE-HPLC (ion exchange high performance liquid chromatography) and then purified with RP-HPLC (see ESI†). 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 neighbour approximation.[34] All chemicals used in the kinetics experiments were of molecular biology grade.

Synthesis of PNAzymes 1–4

PNA sequences (PNA-Dap-PNA precursors to PNAzymes, here named PNA **1–2**) were assembled automatically on a solid support (TentaGel S Rink Amide resin) using the manufacturer's protocol for the Applied Biosystems 433A peptide synthesizer with 9-fluorenylmethyloxycarbonyl (Fmoc)-chemistry and HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as the condensation agent. Non-conjugated PNAs (for controls) were purified with a Phenomenex Jupiter Proteo (4 μm 250 × 4.6 mm) column at 60 °C using a flow rate of 1ml min^{-1} and a linear gradient of 22.5% B for 30 min. (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50% MeCN. HPLC retention times: PNA **1**, rt 24 min; PNA **2**, rt 25 min. PNAs were lyophilised three times before use and stored as frozen solutions. ES-TOF: mass calculated for PNA **1** $C_{130}H_{169}N_{69}O_{37}$ [M], 3290; found, 3291; PNA **2** $C_{141}H_{183}N_{73}O_{41}$ [M], 3557; found, 3557.

Before post-conjugation on solid support the 4-methyltrityl (Mtt) protection was cleaved off (five times treatment with 1% TFA in dichloromethane for 1 min), and for synthesis of the PNAzymes **3** and **4** an extension with a glycine moiety on the Dap side-chain, using peptide coupling (according to Applied Biosystems protocol), was then carried out. Solid support bound PNA-Dap-PNA, with or without the glycine extension, was then reacted with phenyloxycarbonyl-5-amino-2,9-dimethyl-1,10-phenanthroline in the presence of *N*-methyl morpholine (NMM).[32] The PNAzymes were purified on a Phenomenex Jupiter Proteo (4 μm 250 × 4.6 mm) column at 60 °C using a flow rate of 1ml min^{-1} and a linear gradient of 10% B for 20 min, then 10–30% B for 15 min. (A) 0.1% TFA–aq., (B) 0.1% TFA–aq., 50% MeCN. HPLC retention times: PNAzyme **1**, rt 34 min; PNAzyme **2**, rt 35 min; PNAzyme **3**, rt 34 min; PNAzyme **4**, rt 34 min. PNAzymes were lyophilised three times before use and stored as frozen solutions. MS-analysis (ES-TOF): mass calculated for PNAzyme **1** $C_{145}H_{180}N_{72}O_{38}$ [M], 3540; found, 3540; PNAzyme **2** $C_{156}H_{194}N_{76}O_{42}$ [M], 3806; found, 3807; PNAzyme **3** $C_{147}H_{183}N_{73}O_{39}$ [M], 3597; found, 3597; PNAzyme **4** $C_{158}H_{197}N_{77}O_{43}$ [M], 3863; found, 3863.

Assay for RNA cleavage reactions

The reactions were carried out in sealed tubes immersed in a thermostatted water bath (t = 37 °C). Reactions with equimolar concentrations of substrate RNA and PNAzyme were performed with 4 μM of each. These kinetics experiments were performed in 100 μM Zn^{2+}, 10 mM HEPES (Sigma) buffer, 0.1 M NaCl at pH 7.4. Turnover experiments were done with 0.4, 1 or 2 μM PNAzyme while keeping the concentration of the substrate RNA at 4 μM, and were carried out in 90 μM Zn^{2+}, 40 μM EDTA, 10 mM HEPES (Sigma) buffer, 0.1 M NaCl at pH 7.4. For these experiments stock solutions (conc. 1.25 times the final concentration used) of Zn^{2+}–HEPES–NaCl–EDTA were mixed and the pH was adjusted to 7.4 with $NaOH_{(aq)}$. Appropriate amounts of substrate RNA, PNAzyme and water were added to achieve the final concentrations (*i.e.*, with 100 μM Zn^{2+} for the 1 : 1 experiments and effectively 50 μM accessible zinc ions for the turnover experiments) after which the reaction vials were incubated at 37 °C in a water bath. Immediately after the addition of all components and at appropriate time intervals, 40 μL aliquots were withdrawn from the reactions and were quenched by adding 80 μL of a solution of 100 μM EDTA in 30% MeCN–aq. and filtered through a Millipore Millex-GV (4 mm) syringe driven filter before IE-HPLC analysis.

The samples were then analysed by anion exchange HPLC (IE-HPLC). The buffers used were buffer (A) 20 mM NaOAc in 30% aqueous acetonitrile and buffer (B) 20 mM NaOAc, 0.4M $LiClO_4$ in 30% aqueous acetonitrile. A linear gradient of 0–35% buffer B over 35 min was used with a flow rate of 1 ml min^{-1} at 50 °C. A Dionex NucleoPac PA-100 (4 × 250 mm) column was used and UV detection was carried out at 260 nm.

First-order rate constants for the cleavage of RNA substrates were obtained by quantification of the remaining RNA and the sum of all the formed fragments detected in the HPLC analysis. The natural logarithm of the inverse of the remaining fraction of substrate RNA was plotted against time and the first-order rate constants (k) were obtained by least-squares linear fitting.

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