Unusual RNA and DNA binding properties of a novel pyrrolidine–amide oligonucleotide mimic (POM)†

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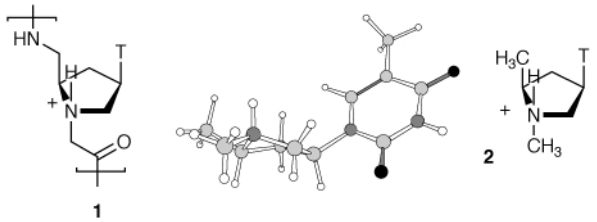
A pentameric thymidyl pyrrolidine–amide oligonucleotide mimic (POM) was synthesised and shown to bind with very high affinity to complementary single stranded RNA and DNA, whilst exhibiting kinetic binding selectivity for RNA over DNA.

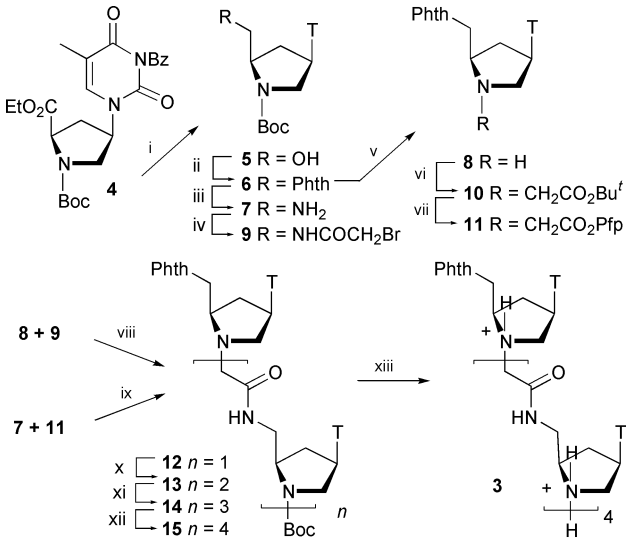
The sugar–phosphodiester backbone of nucleic acids has been replaced by many alternative neutral and anionic backbones.[1] There have also been reports of zwitterionic and cationic oligonucleotides with pendant aminoalkyl side chains attached to the sugar ring, pyrimidine base, or to various phosphorus internucleoside linkages.[2] Despite this, only a few *de novo* modified oligonucleotides have been reported with positively charged backbones.[3] Here we introduce a novel pyrrolidine–amide oligonucleotide mimic (POM) **1** (Scheme 1), which is derived by replacing the furanose sugar of native nucleic acids with a pyrrolidine ring which will be protonated and positively charged at physiological pH.[3d] An X-ray crystal structure[4] of a protonated pyrrolidine ring, which is stereochemically identical and both electronically and sterically similar to the pyrrolidine ring in **1**, closely resembles the northern (N) conformation of uridine in the crystalline state.[5] Semi-empirical quantum mechanical calculations (MOPAC 6.0) also revealed that the lowest energy conformation of a model pyrrolidine **2** closely resembles the preferred N-conformation of the ribose ring in native RNA. In addition, the rigid amide linkage is a viable replacement for the phosphodiester group in DNA resulting in modified oligonucleotides that form stable duplexes with RNA and DNA.[6]

To begin investigating the nucleic acid binding properties of POM a pentamer, T_5-POM **3**, was synthesised, in solution, from *trans*-4-hydroxy-L-proline *via* the ester **4**[7] (Scheme 2). Lithium borohydride reduction of the ester and benzoyl groups of **4** gave the alcohol **5**, which was subjected to a Mitsunobu reaction to afford the phthalimide derivative **6**. Removal of the phthalimide and Boc groups gave the amines **7** and **8** respectively, which were used as the building blocks for the construction of oligomers by an *N*-alkylation or *N*-acylation strategy. In the former approach primary amine **7** was treated with bromoacetic anhydride to give the bromoacetamide **9** which was coupled



Scheme 1

† Electronic supplementary information (ESI) available: UV thermal denaturation curves, Job plots and SPR sensograms for TS-POM **3** binding to DNA and RNA. See http://www.rsc.org/suppdata/cc/b0/b006903p/



Scheme 2. *Reagents and conditions*: i, $LiBH_4$ (2 eq.), THF, 0 °C → rt, 15 h, 69%; ii, phthalimide, PPh_3, DEAD (all 1.3 eq.) in THF, −15 °C → rt, 15 h, 63%; iii, 25–30% aq. $MeNH_2$, 1 h, 40 °C, 89%; iv, bromoacetic anhydride (1 eq.), AcCN–CH_2Cl_2, −8 °C → rt, 5 min, 97%; v, CH_2Cl_2–CF_3CO_2H (2:1), 4 h, 86%; vi, *tert*-butyl bromoacetate (1.5 eq.), DIPEA (3 eq.), DMF, 0 °C → rt, 18 h, 92%; vii, CH_2Cl_2–CF_3CO_2H (4:1), 3 h, then pyridine (2 eq.), CF_3CO_2Pfp (1.2 eq.), DMF, 2 h, 78%; viii, **8** + **9** (1:1), DIPEA (3 eq.), DMF, rt, 18 h, 98%; ix, **7** + **11** (1:1) CH_2Cl_2, rt, 3 h, 100%; x, CH_2Cl_2–CF_3CO_2H (4:1), rt, 4 h, then DIPEA (5 eq.), DMF, **9**, rt, 97%; xi and xii, repeat conditions for **12** → **13**, 96 and 91%; xiii, MeOH–H_2O (1:1) saturated with $HCl_{(g)}$, rt, 2 h, 95%. DIPEA = diisopropylethylamine, Pfp = pentafluorophenyl, Phth = phthalimide, T = thymidyl. New compounds were characterised by ^1H and ^{13}C NMR, IR, UV, MS, mp, $[\alpha]_D$.

with the secondary amine **8** resulting in the dimer **12**. Boc deprotection of **12** and a second coupling with **9** results in the trimer **13.** These steps were repeated to give the tetramer **14** and pentamer **15**, which on treatment with HCl resulted in T_5-POM **3** as an highly water soluble HCl salt. Alternatively, treatment of the secondary amine **8** with *tert*-butyl bromoacetate followed by acidolysis and esterification with pentafluorophenyl trifluoroacetate gave the Pfp-ester **11** which was used to acylate primary amine **7** to give dimer **12**.

UV thermal denaturation experiments were then carried out with an equimolar mixture of T_5-POM **3** and poly(rA) (Table 1). At pH 7, 0.12 M K^+ a single hyperchromic shift was observed with a melting temperature (T_m) of 49 °C (*ca.* 10 °C per base). In comparison, native $d(T)_5$ showed no hyperchromic shift with poly(rA), above 8 °C under identical conditions, whilst $d(T)_{20}$ formed a duplex with poly(rA) with a T_m of 42 °C (2.1 °C per base). Peptide nucleic acid (PNA) lys-T_5-lysNH_2 exhibited only slightly higher affinity for poly(rA) (T_m = 56 °C). Furthermore, no hyperchromic shifts were observed for T_5-POM **3** with non-complementary poly(rC), (rG) and (rU), whilst Job plots of **3** with poly(rA) revealed a 1:1 binding stoichiometry consistent with the formation of a Watson–Crick base paired duplex.

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Table 1 Transition melting temperatures (T_m) of T_5-POM **3** with poly(rA) and poly(dA).

		T_m/°C	
$[K^+]$/M	pH	Poly(rA)[a]	Poly(dA)[b]
0.12	7.0	49 (56)[c]	57 (48)[c]
0.22	7.0	52	n.d.[d]
0.62	7.0	54	42, 66[e]
1.20	7.0	55	61
0.12	8.0	45	n.d.[d]
0.12	7.5	46	n.d.
0.12	6.5	54	n.d.
0.12	6.0	57	35, 64[e]

[a] T_5-POM **3** and poly(rA) (42 μM each in bases) in 10 mM K_2HPO_4(total volume 1 cm³) adjusted to the appropriate ionic strength and pH. UV absorbance (A_{260}) was recorded with heating at 5 °C min^{-1} from 25 to 93 °C, cooling at 0.2 °C min^{-1} to 15 °C and heating at 0.2 °C min^{-1} to 93 °C. The T_m was determined from the first derivative of the final slow heating curve. [b] **3** and poly(dA) (210 μM each in bases) in 10 mM K_2HPO_4 (total volume 0.2 cm³) were incubated for 48–96 h at 25 °C, diluted to 1 cm³ adjusting to the appropriate ionic strength and pH, cooled to 15 °C at 1 °C min^{-1}, heated at 0.2 °C min^{-1} to 93 °C from which the T_m was measured as above. [c] T_m values for lys-T_5-lysNH_2 PNA (PE Biosystems). [d] T_m not determined. [e] Two transitions observed.

Surprisingly however, increasing the salt concentration resulted in slightly higher T_m values. This is in contrast to other cationic modified oligonucleotides that show a marked decrease in duplex and triplex stability with RNA and DNA at higher salt concentration, which is attributed to a reduction in the electrostatic attraction between the oppositely charged backbones.[3] The T_m of **3** with poly(rA) was also highly dependent on pH with more stable duplexes formed at lower pH. This suggests that the extent of protonation of the nitrogen atom of the pyrrolidine ring, is important for binding to RNA. However, factors other than electrostatic attraction, perhaps conformational changes brought about by protonation, are more likely to be the cause of increased duplex stability.

Remarkably no melting was observed between T_5-POM **3** and equimolar poly(dA) under identical conditions. Only after a five-fold increase in concentration of both **3** and poly(dA) followed by an extended period of incubation (48–96 h) was it possible to observe melting, suggesting T_5-POM binds much more slowly to poly(dA) than poly(rA). On the other hand the affinity of **3** for poly(dA) was considerably higher than for poly(rA) (T_m = 57 °C, pH 7, 0.12 M K^+), whilst lys-T_5-lysNH_2 PNA exhibited a lower affinity for poly(dA). Noticeably upon increasing the salt concentration (0.62 M K^+) or lowering the pH to 6, two melting temperatures were observed consistent with triple helix to duplex and duplex to single strand transitions. Job plots of **3** with poly(dA) indicated a 2:1 (T:A) binding stoichiometry consistent of triplex formation.

To investigate the difference in the association kinetics of T_5-POM **3** with DNA and RNA, the change in A_{260} with time was recorded immediately following mixing of equimolar amounts of the polyadenylates with **3** (Fig. 1). With poly(rA) at pH 7, 0.12 M K^+ and a base concentration of 42 μM for each oligomer, a 29% hypochromic shift was observed with a $t_{1/2}$ for association of *ca*. 7 min. Under identical conditions no hypochromic shift was observed with poly(dA) even after 15 h. However, increasing the concentration of both T_5-POM **3** and poly(dA) fivefold resulted in a moderate 6% hypochromic shift with a $t_{1/2}$ of at least 30 min. This clearly shows that T_5-POM **3** binds much more slowly to poly(dA) than (rA). It was also apparent from these experiments that T_5-POM binds faster to poly(rA) at lower pH and salt concentration, suggesting that electrostatic attraction increases the rate of association.

The high affinity, sequence specific binding and relative rates of association of T_5-POM **3** with DNA and RNA were confirmed using surface plasmon resonance (SPR). In these experiments 5′-biotinylated $d(A)_{20}$, $r(A)_{20}$ and a mixed sequence DNA 30–mer were immobilised *via* streptavidin into a dextran matrix upon a gold surface. A solution of T_5-POM **3** was then injected across each surface and the SPR response was measured against time (see ESI†). This revealed that **3** does bind strongly to both $d(A)_{20}$ and $r(A)_{20}$ but associates faster with $r(A)_{20}$ than $d(A)_{20}$. Significantly, the response sensogram of the mixed sequence DNA was identical to the control non–derivatised surface.

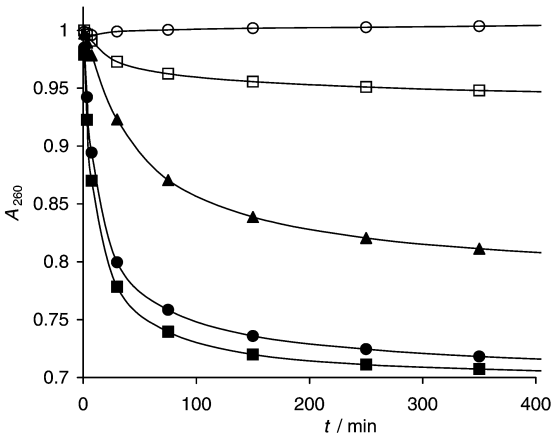

Fig. 1 Normalised UV absorbance (A_{260}) of T_5-POM **3** with poly(rA) and (dA) *vs.* time at 25 °C. **3** and poly(dA) (42 μM each in bases), 0.12 M K^+, pH 7 (○); **3** and poly(dA) (210 μM), 0.12 M K^+, pH 7 (□); **3** and poly(rA) (42 μM), 0.22 M K^+, pH 7 (▲) ; **3** and poly(rA) (42 μM), 0.12 M K^+, pH 7 (●); **3** and poly(rA) (42 μM), 0.12 M K^+, pH 6 (■).

In conclusion we have introduced a novel class of modified nucleic acids with a pyrrolidine–amide backbone and shown that the pentamer T_5-POM **3** binds sequence specifically to both ssDNA and ssRNA with an affinity that is much higher than native nucleic acids. Furthermore, T_5-POM binds much faster to ssRNA than ssDNA. Other oligonucleotides such as 2′,5′-linked RNA and DNA exhibit a thermodynamic binding selectivity for native ssRNA over ssDNA,[8] but as far as we are aware T_5-POM is the first modified oligonucleotide that can kinetically discriminate between the two. This kinetic preference may be due to folding of the polyadenylates induced by base pairing with T_5-POM, given that RNA would be expected to fold more readily than DNA. In addition the formation of tertiary interactions could also explain the high stability of T_5-POM complexes with complementary nucleic acids. The synthesis of longer mixed sequence POMs, using solid phase methods, is underway in order to explore the generality of these findings.

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