A bifunctional monomer for on-resin synthesis of polyfunctional PNA and tailored induced-fit switching probes

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Supporting Information

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**General**

The reagents were purchased from Sigma-Aldrich, Fluka, Merck, Carlo Erba, TCI Europe, ASM and used without further purification. DMF was dried over 4 Å molecular sieves and purged with nitrogen to avoid the presence of dimethylamine. THF was dried by distillation over sodium and stored over 3 Å molecular sieves. TLCs were run on Merck 5554 silica 60 aluminium sheets. Column chromatography was performed as flash chromatography on Merck 9385 silica 60 (0.040-0.063 mm).

NMR spectra were registered on a Brucker Avance 400. δ values are expressed in ppm relatively either to CDCl₃ (7.29 ppm for proton and 76.9 ppm for carbon) or DMSO-d⁶ (2.50 ppm for proton and 39.5 ppm for carbon). The following abbreviations are used to explain the multiplicities: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. Fluorescence spectroscopy was performed on a LS-55 Perkin Elmer Fluorescence Spectrometer connected to a Lauda ecoline RE104 temperature controller. Circular dichroism spectra were recorded with a Jasco J715 spectropolarimeter and a PTC 348 temperature controller unit. UPLC-ESI-MS was carried out by using a Waters Acquity Ultra Performance LC with Waters Acquity SQ Detector and with ESI interface, and equipped with a Waters Acquity UPLC BEH 300 (50x2.1 mm, 1.7 µm, C18) (UPLC1, 0.90 minutes in H₂O 0.2% FA, then linear gradient to 50% MeCN 0.2% FA in 5.70 minutes at a flow rate of 0.25 mL/min; UPLC2, 0.90 minutes in H₂O 0.2% FA, then linear gradient to 100% MeCN 0.2% FA in 5.70 minutes at a flow rate of 0.25 mL/min). PNA oligomers were purified with RP-HPLC using a Phenomenex Jupiler C18 (5 µm, 300 Å, 250x10 mm) (HPLC1, linear gradient from H₂O 0.1% TFA to 50% MeCN 0.1 % TFA in 30 minutes at a flow rate of 4.0 ml/min). HPLC-DAD-HR-MS were performed on a Dionex Ultimate 3000 with Thermo LTQ ORBITRAP XL detector equipped with a AERIS peptide (3.6 µm, XB-C18, 150x2.1 mm) (HPLC2, 5 minutes isocratic at 95% H₂O 0.2% FA 5% MeCN 0.2% FA, then linear gradient to 50% MeCN 0.2% FA in 30 minutenes).

2-(5-azidomethyluracil-1-yl)acetic acid was synthesized according to a procedure previously reported.¹
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Monomer synthesis

![Scheme S1: synthesis of the PNA monomer used in this study.](image)

N-methoxy-N-methyl-Nα-Boc-Nω-Fmoc-S-Lysinamide (s1) was synthesized using a variant of a previously reported procedure \(^2\) in a round bottom flask. Boc-L-Lys(Fmoc)-OH (3.78 g, 8.07 mmol) was solubilized in DMF (38 ml) and cooled to 0°C with an ice bath. HBTU (3.37 g, 8.88 mmol) and DIPEA (4.2 ml, 24.11 mmol) were subsequently added to the solution, which was stirred for 15 minutes at 0°C and further 15 minutes at RT. Then, N,O-dimethylhydroxylamine hydrochloride (1.58 g, 16.20 mmol) was added. After 3 hours the solvent was evaporated under reduced pressure and the resulting oil was taken up with EtOAc (200 ml), transferred in a separatory funnel and washed with saturated KHSO\(_4\) (2 x 400 ml), saturated NaHCO\(_3\) (2 x 400 ml) and brine (2 x 200 ml). The organic layer was dried over Na\(_2\)SO\(_4\) and the solvent removed under reduced pressure to yield s1 (4.25 g, 94%) as a white foamy solid. Characterisations are compatible with those previously reported \(^2\) and are listed for comparison. Corresponding original NMR spectra are reported below. **TLC** (EtOAc) Rf = 0.51; \(^1\)H NMR (CDCl\(_3\), 400 MHz) δ 1.40-1.48 (11H, m), 1.50-1.62 (3H, m), 1.65-1.75 (1H, m), 3.21 (5H, br s), 3.77 (3H, s), 4.22 (1H, t, J = 6.7 Hz), 4.39 (2H, d, J = 6.8 Hz), 4.69 (1H, s), 5.04 (1H, s), 5.30 (1H, d, J = 8 Hz), 7.32 (2H, t, J = 6.8 Hz), 7.40 (2H, t, J = 7.4 Hz), 7.60 (2H, d, J = 7.2 Hz), 7.77 (2H, d, J = 7.4 Hz); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) δ 22.5, 28.4, 29.3, 32.1, 32.6, 40.8, 47.3, 50.1, 61.6, 66.5, 79.6, 119.9, 125.1, 127.0, 127.7, 141.3, 144.0, 155.7, 156.5, 173.1; MS (ESI, MeOH) m/z calcd for [C\(_{28}\)H\(_{37}\)N\(_3\)O\(_6\)] 511.28824, found: 534 [M+Na\(^+\)]; HRMS (ESI, MeOH) found 534.2569 for [M+Na\(^+\)].

Nα-Boc-Nω-Fmoc-S-Lysinal (s2) was synthesized using a variant of a previously reported procedure \(^2\) in a round bottom flask s1 (2.52 g, 4.93 mmol) was solubilized in THF (100 ml) and cooled down to 0°C with an ice bath. Under vigorous stirring, a 1M solution of LiAlH\(_4\) in THF (6 mL, 5.91 mmol) was added dropwise over 10 minutes and the reaction was periodically checked to control the disappearing of the starting material. After 40 minutes the reaction was quenched by adding saturated KHSO\(_4\) (65 ml), the organic layer was then removed under reduced pressure and the aqueous phase was extracted with EtOAc (200 ml). The organic phase was washed with saturated KHSO\(_4\) (2 x 200 ml), saturated NaHCO\(_3\) (2 x 200 ml) and brine (200 ml). The organic...
phase was dried over Na₂SO₄ and the solvent removed under reduced pressure to yield s2 (1.68 g, 75%) as a white foamy solid. Characterisations are compatible with those previously reported and are listed for comparison. Corresponding original NMR spectra are reported below. **TLC** (EtOAc) Rf = 0.60; **¹H NMR** (CDCl₃, 400 MHz) δ 1.35-1.50 (1H, m), 1.50-1.65 (3H, m), 1.82-1.97 (1H, m), 3.22 (2H, d, J = 6.1 Hz), 4.15-4.30 (2H, m), 4.43 (2H, d, J = 6.4 Hz), 4.93 (1H, br s), 5.23 (1H, d, J = 6.6 Hz), 7.34 (2H, t, J = 7.4 Hz), 7.42 (2H, t, J = 7.4 Hz), 7.61 (2H, d, J = 7.4 Hz), 7.78 (2H, d, J = 7.5 Hz), 9.59 (1H, s); **¹³C NMR** (CDCl₃, 100 MHz) δ 22.2, 28.3, 32.9, 40.4, 47.3, 59.6, 66.6, 80.2, 119.9, 125.0, 127.1, 127.7, 141.3, 143.0, 156.0, 158.2, 162.0, 174.0; **HRMS** (ESI, MeOH) m/z calcd for [C₂₉H₃₂N₂O₅]: 548.23313, found: 548 [M+Na⁺].

**Nu-Boc-Ψ-(Noc-Fmoc-S-Lysin)glycine methyl ester (s3):** in a round bottom flask s2 (1.60 g, 3.53 mmol) and glycine methyl ester hydrochloride (1.33 g, 10.60 mmol) were solubilized in MeOH (40 ml) and cooled to 0°C with an ice bath. After 20 minutes NaBH₄CN (266 mg, 4.24 mmol) and acetic acid (0.24 mL, 4.24 mmol) were added and the reaction mixture was allowed to react for further 3 hours before evaporating the solvent under reduced pressure. The resulting solid was dissolved in EtOAc (250 ml), transferred in a separatory funnel and washed with saturated NaHCO₃ (2 x 150 ml) and brine (150 ml). The organic layer was dried over Na₂SO₄, the solvent removed under reduced pressure, and the residue was purified by flash chromatography (from AcOEt/hexane 7:3 to AcOEt) to afford s3 (1.05 g, 57%) as a yellowish foamy solid. **TLC** (EtOAc) Rf = 0.28; **¹H NMR** (CDCl₃, 400 MHz) δ 1.35-1.60 (14H, m), 1.89 (1H, br s), 2.56-2.73 (2H, m), 3.21 (2H, q, J = 5.8 Hz), 3.39 (1H, d, J = 17.5 Hz), 3.37 (1H, d, J = 17.3 Hz), 3.60-3.68 (1H, m), 3.72 (3H, s), 4.22 (1H, t, J = 6.5 Hz), 4.40 (2H, d, J = 6.2 Hz), 4.81 (1H, s), 5.04 (1H, s), 7.32 (2H, t, J = 7.4 Hz), 7.41 (2H, t, J = 7.3 Hz), 7.61 (2H, d, J = 7.2 Hz), 7.77 (2H, d, J = 7.4 Hz); **¹³C NMR** (CDCl₃, 100 MHz) δ 23.0, 28.4, 29.6, 32.8, 40.7, 47.3, 50.1, 50.7, 51.8, 53.0, 66.5, 79.2, 119.2, 125.1, 127.0, 127.7, 141.3, 144.0, 156.0, 156.5, 173.0; **MS** (ESI, MeOH) m/z calcd for [C₂₉H₃₀N₂O₅]: 528.2389, found 526 [M+H]+, 548 [M+Na]+. **HR-MS** (LTQ-Orbitrap, MeOH) m/z calcd for [C₂₉H₃₀N₂O₅]: 525.23389, found 526.29053 [C₂₉H₄₀N₃O₅]+.

**Boc-PNA (5L-Lys(Fmoc))-T(N3)-OMe (s4):** in a round bottom flask 2-(5-azidomethyluracil-1-yl)acetic acid (398 mg, 1.77 mmol), EDC-HCl (338 mg, 1.77 mmol), DhBtOH (288 mg, 1.77 mmol) and DIPEA (308 µL, 1.77 mmol) were solubilized in dry DMF (5 ml) and cooled down to 0°C with an ice bath. Then s3 was added to the mixture, which was stirred for 30 minutes, then warmed to RT and allowed to react overnight. The solvent was then evaporated under reduced pressure. The resulting solid was dissolved in EtOAc (250 ml), transferred in a separatory funnel and washed with saturated KHSO₄ (2 x 250 ml), saturated NaHCO₃ (2 x 250 ml) and brine (250 ml). The organic phase was dried over Na₂SO₄, the solvent removed under reduced pressure and the residue was purified by flash chromatography (gradient elution from EtOAc/hexane 9:1 to EtOAc), to yield s4 (874 mg, 81%) as a yellow foamy solid. **TLC** (EtOAc) Rf = 0.30; **¹H NMR** (CDCl₃, 400 MHz, major rotamer) δ 1.35-1.63 (15H, m), 3.15-3.25 (2H, m), 3.32-3.41 (1H, m), 3.45-3.53 (1H, m), 3.74 (3H, s), 3.98-4.09 (2H, m), 4.14 (1H, t, J = 7.0 Hz), 4.20-4.32 (2H, m), 4.40-4.89 (4H, m), 4.73 (1H, d, J = 8.3 Hz), 5.02 (1H, br s), 7.27 (1H, s), 7.33 (2H, t, J = 7.2 Hz), 7.42 (2H, t, J = 7.5 Hz), 7.61 (2H, d, J = 7.5 Hz), 7.78 (2H, d, J = 7.6 Hz), 9.12 (1H, s); **¹³C NMR** (CDCl₃, 100 MHz, major rotamer) δ 22.8, 28.3, 29.4, 31.9, 40.2, 40.7, 47.1, 47.3, 47.7, 49.0, 49.3, 52.4, 53.0, 66.6, 80.0, 109.4, 120.0, 125.0, 127.1, 127.7, 141.3, 143.6, 144.0, 150.7, 156.0, 156.7, 162.8, 167.2, 169.5; **MS** (ESI, MeOH) m/z calcd for [C₃₆H₄₄N₈O₉]: 732,32313, found 755 [M+Na]+, 771...
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[144.4, 145.9, 151.1] N

H NMR (DMSO-d$_6$, 400 MHz, major rotamer) $\delta$ 1.10-1.55 (15H, m), 2.90-3.02 (2H, m), 3.2-3.6*, 3.90 (1H, d, $J=17.0$ Hz), 4.01 (1H, d, $J=17.0$ Hz), 4.07 (2H, s), 4.21 (1H, t, $J=6.5$ Hz), 4.29 (2H, d, $J=6.6$ Hz), 4.52-4.83 (2H, m), 6.80 (1H, d, $J=8.8$ Hz), 7.2-7.30 (1H, m), 7.34 (2H, t, $J=7.4$ Hz), 7.42 (2H, t, $J=7.4$ Hz), 7.59 (1H, s), 7.69 (2H, d, $J=7.3$ Hz), 7.89 (2H, d, $J=7.6$ Hz), 11.58 (1H, br s); $^{13}$C NMR (DMSO-d$_6$, 100 MHz, major rotamer) $\delta$ 23.3, 28.6, 29.7, 31.6, 39.2-40.8**, 47.1, 47.2, 48.5, 49.3, 50.5, 51.9, 66.6, 78.4, 107.7, 120.6, 125.6, 127.9, 128.1, 141.2, 144.4, 145.9, 151.1, 156.1, 163.9, 168.0, 170.9; MS (ESI, MeOH) m/z calcd for [C$_{35}$H$_{42}$N$_{9}$O$_{9}$]: 718.30747, found 741 [M+Na]$^+$, 757 [M+K]$^+$. HR-MS (LTQ-Orbitrap, MeOH) m/z calcd for [C$_{35}$H$_{42}$N$_{9}$O$_{9}$Na]$^+$

* Integral not defined due to signal overlap with water peak.
** Signal overlap with DMSO.

PNA synthesis

PNA 1-13: the synthesis of the PNA probes was performed with standard manual Boc-based solid-phase synthesis with HBTU/DIPEA as coupling mixture, using the preformed monomer 1 in addition to commercially available Boc-PNA -OH monomers. MBHA resin was first loaded with Fmoc-Gly-OH as first monomer (0.2 mmol/g). Modifications on the lysine side chain were carried out removing Fmoc protective group with 20% piperidine solution in DFM (8 minutes, 2 times) and subsequently coupling the desired molecule using standard HBTU/DIPEA strategy. Modification of the nucleobase side was obtained by preliminary reduction of the azide group using an established solid phase Staudinger reaction with 0.33 M trimethylphosphine in THF/water 1:1 mixture (10 minutes, 2 times), followed by condensation with desired carboxylic acid using 10 equivalents and DIC/DhBrOEt as activating mixture (15 minutes pre-activation and overnight coupling). Cleavage was performed using a TFA/TFMSA/thioanisole/m-cresol (6:2:1:1) cocktail. The purity and identity of the PNAs were evaluated by cleavage of a part of the resin and analysis by UPLC-ESI/MS. PNA 8 and PNA 13 were the products of final steps which were completely deprotected, cleaved entirely from the resin, and purified. PNA 8 (No Fmoc): 19%; $t_1$: 3.18 min (UPLC1); ESI-MS: $m/z$ calcd 3604.57 [M]: 1225.7 [MH$_3$$^+$], 919.5 [MH$_4$$^+$], 735.8 [MH$_5$$^+$], 613.3 [MH$_6$$^+$], 525.9 [MH$_7$$^+$]; PNA 13: 13%; $t_1$: 4.70 min (UPLC2); ESI-MS: $m/z$ calcd 3877.75 [M]: 1287.6 [MH$_3$$^+$], 965.9 [MH$_4$$^+$], 772.9 [MH$_5$$^+$], 644.3 [MH$_6$$^+$], 552.3 [MH$_7$$^+$].

Synthesis of PNA 14

PNA 14 was synthesized on a 5 µmol scale using the preformed monomer 1 and the above-reported procedures with a slightly modified scheme as follows (Scheme S2). Once the whole sequence was synthesized, the first amine group was unmasked on the nucleobase (see Scheme S2, step a) by reduction of the azide group under by the solid phase Staudinger reaction, using 0.33 M trimethylphosphine in THF/water 1:1 mixture (10 minutes, 2 times). The Fmoc protective group on the lysine side chain was then removed to liberate a second free amine function (Scheme S2, step
b), using 20% piperidine in DMF (8 minutes, 2 times). The doubly pyrene-functionalized PNA 14 was obtained by subsequent coupling of 1-pyreneacetic acid using standard HBTU/DIPEA strategy (Scheme 2S, step c) (29.9 mg 1-pyreneacetic acid, 100 μmol; 43.6 mg HBTU, 100 μmol, 40 μl DIPEA, 200 μmol). PNA cleavage and purification was performed as described above and in the general section, and characterized by HPLC-DAD-HR-MS, which gave positive ions consistent with the final product 4736.04156 [M]: m/z found 1580.30523 [MH]+, 1185.48085 [MH]+, 949.58621 [MH]+, 790.65637 [MH]+, 677.84913 [MH]+. Quantification by UV-Vis spectroscopy (ε260 M⁻¹ cm⁻¹ 8600, C 6600, A 13700, G 11700, pyrene 6338) of the PNA dissolved in 1 mL of water gave a concentration of 695 μM, corresponding to 695 nmol (13.9 % yield). Yields around 20% or less, after purification, are common in PNA chemistry.

![Scheme S2](image)

Scheme S2. Solid-phase synthesis of the doubly pyrene-modified PNA probe. a) PMe₃ in H₂O/THF; b) piperidine 20% in DMF; c) 1-pyreneacetic acid, HBTU/DIPEA in DMF.

PNA15 was synthesized on a 5 μmol scale using standard Fmoc-based solid-phase synthesis with HBTU/DIPEA as coupling mixture, using commercially available monomers.

**PNA 15:** 4.2%; tᵣ: 2.91 (UPLC1); ESI-MS: m/z calcld 4168.0 [M]: 1390.3 [MH]+, 1043.1 [MH]+, 834.6 [MH]+, 695.9 [MH]+, 596.5 [MH]+, 522.1 [MH]+.

**Circular dichroism and melting experiments**

Circular dichroism spectra were obtained using annealed solution at 1 μM strand concentration in pH 7.0 PBS buffer (100 mM NaCl, 10 mM Na₂HPO₄) at 2% acetonitrile concentration, scanning the 200÷500 nm range at 50 nm/min. Spectra are obtained as sum of 5 different scans, background corrected and recorded at 25 °C and 50 °C.

Thermal denaturation profiles were measured by monitoring both the ORD and HT signal at 260nm from 18 °C to 90 °C with a heating rate of 1 °C/min. Melting temperatures were calculated from the first derivative of the 10⁶ order polynomial that best fitted the experimental data (using Matlab script).
Where mentioned, $T_m$ values were obtained with a UV spectrophotometer using the same buffer described above, and using the same experimental parameters.

**Table S1**: melting temperatures of PNA 14 and PNA 15 in presence of different DNA and RNA sequences.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence</th>
<th>PNA</th>
<th>$T_m$ (°C) HT</th>
<th>$T_m$ (°C) CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>PNA 14</td>
<td>63.4$^a$</td>
<td>&lt;18</td>
</tr>
<tr>
<td>DNA-FM</td>
<td>5’-AGCTACATTGTCTGC-3’</td>
<td>PNA 14</td>
<td>69.8</td>
<td>69.3</td>
</tr>
<tr>
<td>DNA-MM</td>
<td>5’-AGCTAGTTGTCTGC-3’</td>
<td>PNA 14</td>
<td>67.2</td>
<td>63.4</td>
</tr>
<tr>
<td>DNA-RDM</td>
<td>5’-GGTGAATGAGTAAC-3’</td>
<td>PNA 14</td>
<td>62.9$^a$</td>
<td>&lt;18</td>
</tr>
<tr>
<td>RNA-FM</td>
<td>5’-AGCUACAUUGUCUGC-3’</td>
<td>PNA 14</td>
<td>69.6</td>
<td>70.6</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>PNA 15</td>
<td>63.4$^b$</td>
<td>n.d.</td>
</tr>
<tr>
<td>DNA-FM</td>
<td>5’-AGCTACATTGTCTGC-3’</td>
<td>PNA 15</td>
<td>71.9</td>
<td>72.7</td>
</tr>
<tr>
<td>DNA-MM</td>
<td>5’-AGCTACGTTGTCTGC-3’</td>
<td>PNA 15</td>
<td>64.8</td>
<td>63.9</td>
</tr>
<tr>
<td>RNA-FM</td>
<td>5’-AGCUACAUUGUCUGC-3’</td>
<td>PNA 15</td>
<td>74.4$^b$</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$ Transition only visible in the HT curve of the CD spectropolarimeter, not corresponding to a duplex melting (as inferred from CD spectra and melting)

$^b$ Measure obtained using UV spectrometer

**Fluorescence measurements**

Fluorescence emission spectra were recorded at $\lambda_{exc} = 343$ nm (slit$_{exc} = 5$ nm, slit$_{em} = 10$ nm) for working volumes of 200 µL, using quartz cuvettes (0.3 x 1 cm path lengths). Aqueous solutions of PNA 14 were obtained by diluting the PNA and DNA stock solutions in PBS (87.6 mM NaCl, 8.76 mM phosphate, pH = 7) to final concentrations either 1 or 5 µM (containing 0.7% acetonitrile). An equimolar ratio 1:1 PNA 14 : DNA (FM, MM, RDM) or RNA (FM) was always employed. Fluorescence intensity is normalized as described in the figure caption, either at maximum intensity or to fluorescence intensity at 381 nm (monomer emission)
NMR spectra

Fig. S1: $^1$H-NMR and $^{13}$C-NMR of compound s1.
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Fig. S2: $^1$H-NMR and $^{13}$C-NMR of compound s2.
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Fig. S3: $^1$H-NMR and $^{13}$C-NMR of compound s3.
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Fig. S4: $^1$H-NMR and $^{13}$C-NMR of compound s4.
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**Fig. S5:** $^1$H-NMR and $^{13}$C-NMR of compound 1.
Characterization of PNA intermediates.

Note for intermediate characterization: according to literature, the use of azide function is not suitable in Boc-based solid phase synthesis when thiol-containing scavengers are used. For this reason, all the intermediates analyzed in this studies that contain an azide function do not show the expected molecular weight. A list of signals generated from azide containing probes is as follows (reported as difference from the expected MW): -26, +64, +80, +114, +204 m/z.

These changes have been observed in all cases when azide was cleaved from the resin without previous reduction to amine, followed by derivatisation (PNA 1, PNA 3, PNA 4, PNA 5, PNA 7, PNA 10, PNA 11). Possible assignment for some of these peaks could be: -26 (azide reduction), +80 (azide reduction and addition of m-cresol), +114 (azide reduction and addition of oxidized thioanisole), m/z.
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Fig. S7: mathematical deconvolution of the multicharged signals of ESI-MS spectra for PNA 1-4 after cleavage from the solid support.
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Fig. S8: mathematical deconvolution of the multicharged signals of ESI-MS spectra obtained for PNA 5-8 in the intermediate steps of the synthesis of PNA 8. TOP: scheme of synthesis of PNA 8: i) azide reduction; ii) Fmoc deprotection; iii) 5(6)-carboxyfluorescein coupling; iv) Fmoc-AEEA coupling. BOTTOM: mathematical deconvolution of the multicharged signals of the intermediates (predicted MW indicated in the boxes)
Fig. S9: mathematical deconvolution of the multicharged signals of ESI-MS spectra obtained for PNA 9-13 in the intermediate steps of the synthesis of PNA 13. TOP: scheme of synthesis of PNA 13: i) Fmoc deprotection; ii) 5(6)-carboxyfluorescein coupling; iii) PNA elongation; iv) azide reduction; v) 1-pyreneacetic acid coupling; vi) Fmoc deprotection; B-Rhodamine coupling. BOTTOM: mathematical deconvolution of the multicharged signals of the intermediates (predicted MW indicated in the table)
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UPLC/HPLC-MS chromatograms of pure PNAs

**Fig. S10**: UPLC1-MS of PNA 8. UPLC-MS trace (top), MS spectrum of the corresponding peak at 3.18 min (center) and mathematical deconvolution of the multicharged signals (bottom).
Fig. S11: UPLC-MS of PNA 13. UPLC-MS trace (top), MS spectrum of the corresponding peak at 4.70 min (center) and mathematical deconvolution of the multicharged signals (bottom).
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Fig. S12: HPLC-DAD-HR-MS chromatogram of purified PNA 14. HPLC-UV at 254 nm trace (top), MS spectrum of the corresponding peak at 23.8 min (center) and mathematical deconvolution of the multicharged signals (bottom).
Fig. S13: UPLC-MS of PNA 15. UPLC-MS trace (top), MS spectrum of the corresponding peak at 2.91 min (center) and mathematical deconvolution of the multicharged signals (bottom).
Fig. S14. Kinetic effects in the PNA 14:DNA complexation. 

a) Normalized (to maximum intensity) fluorescence emission spectra of a 5 µM aqueous solution of PNA 14, T = 25°C in PBS buffer with 0.7% acetonitrile. Spectra are recorded for PNA 14 initially alone, and at 120 min and 1 day after addition of full match DNA (DNA-FM); the time course showed in figure 2 of the main text is continued and after one day the excimer band results further reduced; 
b) fluorescence emission spectra at different times after addition of the mismatch sequence (DNA-MM), scaled to figure S14a; 
c) excimer/monomer fluorescence intensity ratio vs time, red profile = DNA-MM, blue profile = DNA-FM. The mismatched base in DNA-MM was in correspondence of the pyrene-modified unit of PNA 14. A slight fall of the excimer band was observed, which is however less pronounced than the one obtained for the full-match target; also in this case the excimer emission was lowered, but with slower kinetics. 
d) Normalized (to maximum intensity) fluorescence emission spectrum of a 1 µM aqueous solution of PNA 14, T = 25°C alone (0h), and at 120 min and 1 day after addition of full match DNA (DNA-FM) in PBS buffer with 0.7% acetonitrile; 
e) effect of ionic strength on the hybridization kinetics for PNA 14 (1 µM) with DNA-FM: red line: phosphate saline buffer (0.1 M NaCl) pH = 7.0, blue line: phosphate buffer with no NaCl.
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**Fig. S15:** a) Fluorescence emission spectrum of a 5 µM aqueous solution of PNA 14, T = 25° C with 3.75% acetonitrile; b) trend of fluorescence emission spectra at different times after addition of the full match sequence to the PNA 14, T = 25° C; c) trend of fluorescence emission spectra over time when adding a single mismatch sequence to the PNA switching probe PNA 14, T = 25° C; d) excimer/monomer fluorescence intensity ratio vs time, red profile = single mismatch DNA, black profile = full match target. Spectra are scaled to maximum intensity.
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**Fig. S16.** a) Fluorescence emission spectrum of a 1 µM aqueous solution of PNA 14, T = 40°C with 0.7% acetonitrile; b) evolution of relative fluorescence emission spectra over time when adding the full match sequence to PNA 14, T = 40°C; c) evolution of fluorescence emission spectra of PNA 14 over time in presence of a single mismatch sequence; T = 40°C; d) kinetics of the interaction between the PNA probe and the DNA sequences (black profile = full match, red profile= mismatch), expressed in terms of the excimer/monomer intensity ratio vs time. Spectra are scaled to maximum intensity.

**Fig. S17.** Normalized fluorescence emission spectra of a 1 µM aqueous solution of PNA 14, T = 50°C in PBS buffer at pH = 7.0 (0.6% acetonitrile) and its evolution over time when adding the full match sequence (DNA-FM), Mismatched DNA-MM, random DNA-RDM, and full match RNA-FM to PNA 14; C: a) after 120 min; b) after annealing at 90°C and cooling to 50°C; Annealing ensures that kinetic effects are less relevant in the final measurement. The use of a random sequence (DNA-RDM) at this temperature does not induce a significant change in the excimer/monomer ratio, thus confirming the sequence-dependence of the excimer/monomer switch at equilibrium. Spectra are normalized to monomer emission at 381 nm.
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Circular dichroism studies

Figure S18. Circular dichroism spectra at 25°C of single stranded PNA 14 (solid thick line), fully complementary ssDNA-FM (dashed line), PNA 14 : DNA-FM (dotted black line) and unmodified PNA15 : DNA-FM (blue solid line), in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. The conformation assumed by PNA 14 : DNA duplex is similar to that of unmodified PNA; thus the modified monomer conformation is expected to be rearranged to adapt to this structure.

Figure S19. Circular dichroism spectra at 25°C of singly mismatched ssDNA-MM (blue dashed line), PNA 14 : DNA-MM (black solid line) and PNA 14 : DNA-FM (red dotted line) in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. The conformation assumed by PNA14 : DNA duplex in the presence of a single mismatch is similar to that fully complementary DNA; this explains why both DNA-FM and DNA-MM both give rise to a fluorescence response of PNA 14 (though with different kinetics).
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Figure S20. Circular dichroism spectra at 25°C of a) ssDNA-5MM (with 5 mismatches, 5’-ACG TAC GTT GTC TCG-3’ dashed line) and PNA:DNA-5MM mixture b) ssDNA-RDM (dashed line) and PNA:DNA-RDM mixture in PBS buffer, pH = 7.0; c = 1 µM of each strand. The conformation assumed by PNA14 : DNA duplex is similar to that of unmodified PNA; binding of PNA 14 to DNA is as expected not present or very weak with non-complementary sequences.

Figure S21. Circular dichroism spectra at 25°C fully complementary ssRNA-FM (dashed line) and PNA 14 : RNA-FM (blue line), in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. This indicates that PNA 14 forms a duplex also with the complementary RNA.
Figure S22. Circular dichroism spectra at 50°C of single stranded PNA 14 (solid thick line), fully complementary ssDNA-FM (dashed line), PNA 14 : DNA-FM (dotted black line) and unmodified PNA15 : DNA-FM (blue solid line), in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. At this temperature, the PNA:DNA duplex is still formed.

Figure S23. Circular dichroism spectra at 50°C of singly-mismatched ssDNA-MM (blue dashed line), PNA 14 : DNA-MM (black solid line) and PNA 14 : DNA-FM (red dotted line) in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. The results suggest that also this complex is still formed at this temperature.
Figure S24. Circular dichroism spectra at 50° fully complementary ssRNA-FM (dashed line) and PNA 14 : RNA-FM (blue line), in PBS buffer, pH = 7.0 (2% acetonitrile); c = 1 µM of each strand. PNA 14 duplex also with the complementary RNA is still formed at 50°C.
Figure S25. Circular dichroism melting experiments profiles. a) PNA 14 : DNA-FM; b) PNA 15 : DNA-FM; c) PNA 14 : DNA-MM; d) PNA 15 : DNA-MM; e) PNA 14 : DNA-RDM; f) PNA 14 : RNA-FM. Denaturation profiles were recorder at 1 µM strand concentration in PBS buffer, pH 7 (2% acetonitrile).
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**Static 3D model of PNA 14 ss and PNA:DNA duplex**

Figure S26. Model representation of the two states. a) Model of a (capped) PNA monomer with conformation from MD trajectory, modified as in PNA 14, and subsequently geometry optimized using UFF force field; b) and c) PNA:DNA 10-mer corresponding to the central sequence of PNA 14, designed on the basis of known PNA:DNA sequences. Both geometries (extended and folded) of the side arms were built in order to have staggered geometry for each C-C bond; the side arms are geometry optimized, and structures are not energy minimized. Argus-Lab software was used for geometry optimization.