Electronic supplementary information

Microcalorimetry and fluorescence show stable peptide nucleic acid (PNA) duplexes in high organic content solvent mixtures

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1. General methods

1.1. Design of the sequences

The 10-mer PNA sequences were designed similarly to previously reported relevant studies[1,2] and extended into 15-mer PNAs using NUPACK web application.[3] DNA sequences were purchased from Integrated DNA Technologies (IDT) with standard desalting and HPLC purification for the quencher labelled strand. A 5,6-carobxyfluorescein / Black Hole Quencher 1 donor-acceptor system was chosen for the fluorescence melting experiments of dsDNA. A tryptophan / dansyl donor-acceptor system was chosen for the fluorescence melting experiments of dsPNA thanks to its compatibility with organic solution.[4,5] We were unable to identify a synthetically accessible dye/quencher pair that allowed for fluorescence melting experiments of dsPNA in aqueous solution.

Table S1. DNA and PNA sequences used in this work.

Identifier	Sequence
[10]-PNA1	H - TCA CTA GAT G - NH_2
[10]-PNA2	H - CAT CTA GTG A- NH_2
[15]-PNA1	H - CGC CGT CAC TAG ATG - NH_2
[15]-PNA2	H - CAT CTA GTG ACG GCG - NH_2
[10]-DNA1	5’ - TCA CTA GAT G - 3’
[10]-DNA2	5’ - CAT CTA GTG A - 3’
[10]-DNA1-F	5’ - /56-FAM/ TCA CTA GAT G - 3’
[10]-DNA2-Q	5’ - CAT CTA GTG A /3BHQ_1/ - 3’
[10]-PNA1-F	H - Trp - TCA CTA GAT G - NH_2
[10]-PNA2-Q	Ac - CAT CTA GTG A - Lys(dansyl) - NH_2

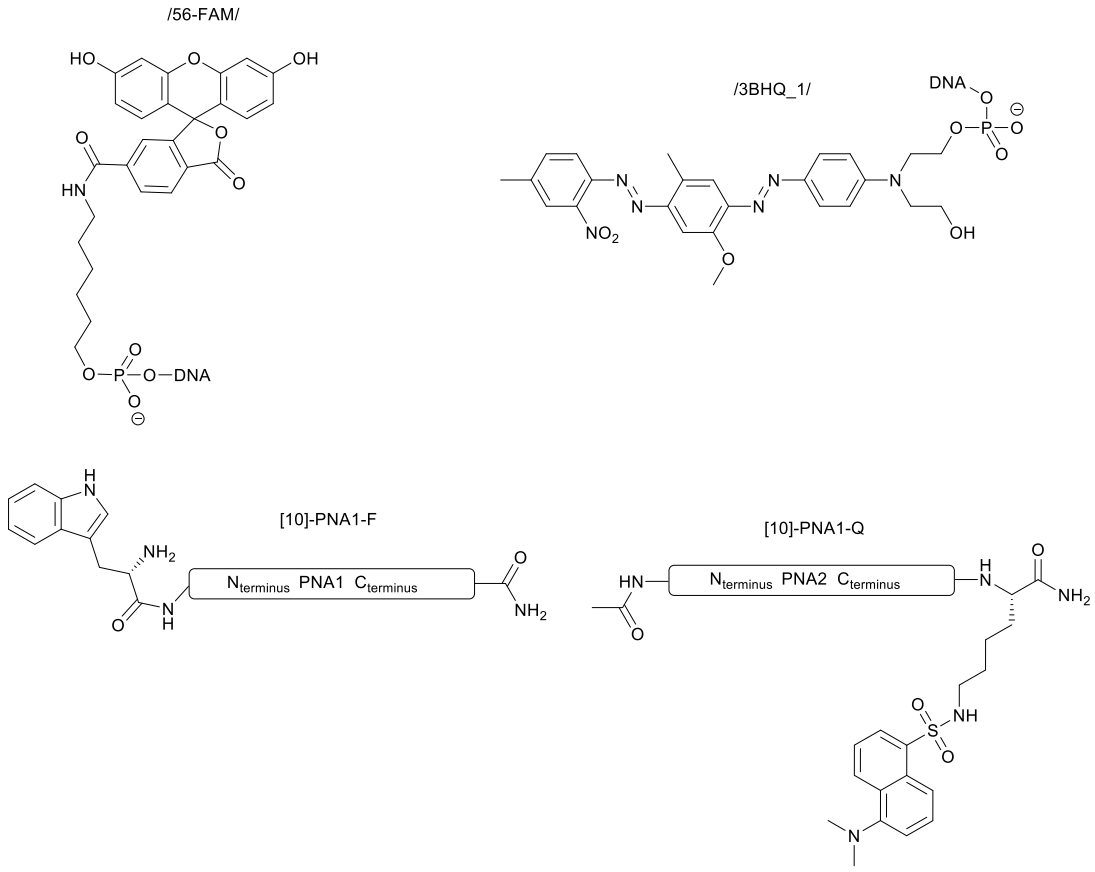

Figure S1. Chemical modifications used for fluorescence measurements.

1.2. Solid phase synthesis of PNAs

PNAs were prepared using a microwave-assisted Liberty Blue (CEM) automated synthesiser at a 10 µmol scale with a low-volume PTFE reaction vessel. Fmoc/Bhoc-protected monomers were purchased from Link technologies. A PAL-novaPEG (Novabiochem) 0.44 mmol·g^{-1} loading capacity or Rink amide ProTide(LL) (CEM) 0.19 mmol·g^{-1} loading capacity solid supports were interchangeably used. Peptide synthesis DMF was used as the main solvent (VWR) and the rest of the reagents were directly purchased from Sigma-Aldrich. The synthetic cycles are summarised below:

Table S2. Summary of the solid phase PNA synthetic cycles.

Cycle	Description
1. Resin swelling	The resin was swollen with 3 mL of DMF for 5 min at 20 °C.
2. Fmoc deprotection	The resin was treated with 2 mL of 10 vol% piperidine in DMF for 10 min at 20 °C. Extensive DMF washes were applied after the Fmoc $UV_{301\ nm}$ quantification.
3. Coupling	0.5 mL of 0.1 M solution of Fmoc-PNA-OH monomer in DMF, 0.5 mL of 0.1 M Solution of PyBOP® in DMF and 0.5 mL of 0.1 M *N*,*N*-diisopropylethylamine 0.1 M 2,6-lutidine in DMF were added to the reaction vessel. The coupling step was performed for 1 h at 50 °C followed by two DMF washes.
4. Cleavage	The resin was transferred to a manual solid phase synthesis device, washed with 4 mL of MeOH, MeOH, DCM and DCM. Then, it was air dried and briefly dried under vacuum. The dry resin was transferred to a vial with 1 mL of 95 vol% TFA and 5 vol% of *m*-cresol and the solution was shaken at 20 °C for 1.5 h. The resin was removed by filtration and the supernatant was collected. The cleaved product was precipitated by addition of 2 volumes of diethyl ether and centrifuged at 4 °C for 30 min. The white pellet was rinsed further with 1 mL of diethyl ether and centrifuged at 4 °C for 15 min.

The products were quantified according to their UV absorbance at 260 nm using a Nanodrop spectrophotometer ND2000c (Thermoscientific), and the calculated molar extinction coefficient.[6]

Synthesis of [10]-PNA2-Q: 200 nmol of the unlabelled [10]-PNA2-Lys precursor were dissolved in 200 µL of DMF in a microcentrifuge tube. 11 mg (40 µmol, 200 eq) of 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride, Sigma-Aldrich) were dissolved in 200 µL of DMF and added over the PNA solution. 7 µL of DIPEA (40 µmol, 200 eq, Sigma-Aldrich) were then added and the resulting mixture was shaken in a thermal shaker overnight at 20 °C. The solvent was removed by freezing the solution with liquid nitrogen and placing the solid under high vacuum. The excess of reagent was removed by two consecutive precipitations of the product in 200 µL of DMF and 400 µL of diethyl ether. The product was pelleted by centrifugation at 4 °C and $21{\cdot}10^3$ rcf. Finally, the product was analysed by LCMS and HPLC fraction-collected (see Section 2). The conversion based in the chromatographic peak area was 66%.

1.3. HPLC analysis

High pressure liquid chromatography (HPLC) was performed on a modular Shimadzu instrument with the following modules: CBM-20A system controller, LC-20AD solvent delivery module, SIL-20AC HT autosampler, SPD-M20A photodiode array UV-Vis detector, RF-20A spectrofluorometric detector and a FRC-10 fraction collector. A Shim-pack GISS 5 µm C18 (4.6 × 125 mm) reversed phase column was used. The mobile phase consisted of a H_2O (18.2 MΩ·cm + 0.05 vol% formic acid)/MeCN (HPLC grade) system at 60 °C and a flow rate of 0.8 mL·min^{-1}. The elution of sequences with a tryptophan fluorophore was determined using the fluorescence detector (excitation wavelength 295 nm and emission wavelength 350 nm). The elution of sequences with Dansyl fluorophore was determined using the fluorescence detector (excitation 335 nm and emission wavelength 519 nm). A typical elution gradient is shown below:

Time	MeCN (vol%)
0	5
4	5
15	60
16	95
17	95
17.5	5
20	5

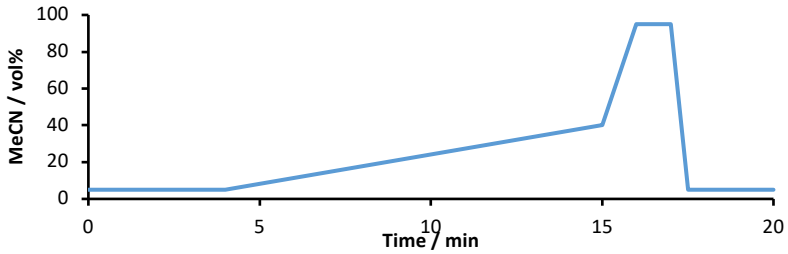

Figure S1. Summary of a typical HPLC gradient.

1.4. LC-MS analysis

Tandem liquid chromatography-mass spectrometry analysis was performed in a Xevo G2-XS QTof instrument (Waters) in negative ionisation mode. Reversed phase liquid chromatography was performed using an ACQUITY UPLC H-Class plus system with an ACQUITY UPLC BEH 130 Å 1.7 µm C18 (2.1×75mm) column. The mobile phase consisted on buffer A (75 mM triethylammonium acetate pH 7.0 buffer in H_2O) and buffer B (75 mM triethylammonium acetate pH 7.0 buffer in MeCN) at a flow rate of 0.2 mL ·min^{-1} at 60 °C. Leucine enkephalin was used as the reference for the LockSpray correction. The raw continuum data was deconvoluted using ProMass HR for MassLynx (Novatia) software to produce a complete zero charge masses spectrum.

1.5. T_m determination through UV absorbance

UV absorbance measurements were performed on dsDNA, PNA·DNA and dsPNA 2.5 µM solutions at 260 nm on an Evolution 360 UV-Vis (Thermoscientific) coupled to a PCCU1 Peltier control and cooling unit. The instrument was controlled by the ThermoInsight2 software. The sample was heated and cooled at a rate of 1 °C step , 40 s stabilisation time, with continuous stirring and the temperature was determined using a Compact transition joint thermocouple probe (Omega).

1.6. T_m determination through fluorescence

Fluorescence measurements were performed on labelled dsDNA, PNA·DNA and dsPNA 2.5 µM solutions at the optimal excitation and emission wavelengths for each fluorophore/quencher pairs, on a FS5 steady state spectrofluorometer (Edinburgh instruments) with a SC-25 temperature controlled holder coupled to an Integrated TC1 Temperature controller (Quantum Northwest). The instrument was controlled by the Fluoroclare software. The sample was heated and cooled in 0.5 °C steps with a 40 s stabilisation time before measurement and a 0.1 °C tolerance.

1.7. T_m determination through differential scanning calorimetry

DSC measurements were performed on a Nano Differential Scanning Calorimeter 602000 (TA instruments) at 3 atm pressure and with a heating and cooling rate of 1 °C·min^{-1} for diluted solutions (< 50 µM) or 0.5 °C·min^{-1} for concentrated solutions. A volume of 600 µL of solution was introduced into the sample and reference capillary cells. The instrument was controlled by the DSCrun software. The data was analysed with the NanoAnalyze software, corrected with a sigmoidal baseline and a two-state model was fitted to determine the melting temperature.[7]

2. Characterisation of PNA sequences

PNAs were synthesised according to the method in section 2.2, analysed by HPLC and by LC-MS. The HPLC fractions of labelled PNAs were fractionally collected prior to fluorescence measurements.

Table S2. Mass spectrometry results of PNA sequences.

Identifier	Sequence	Expected mass (Da)	Found (Da)
[10]-PNA1	H - TCA CTA GAT G - NH_2	2725.090	2725.130
[10]-PNA2	H - CAT CTA GTG A- NH_2	2725.090	2725.119
[15]-PNA1	H - CGC CGT CAC TAG ATG - NH_2	4060.611	4060.660
[15]-PNA2	H - CAT CTA GTG ACG GCG - NH_2	4100.618	4100.451
[10]-PNA1-F	H - Trp - TCA CTA GAT G - NH_2	2911.169	2911.189
[10]-PNA2-Q	Ac - CAT CTA GTG A - Lys(Dansyl) - NH_2	3128.247	3128.140

2.1. HPLC of PNA sequences

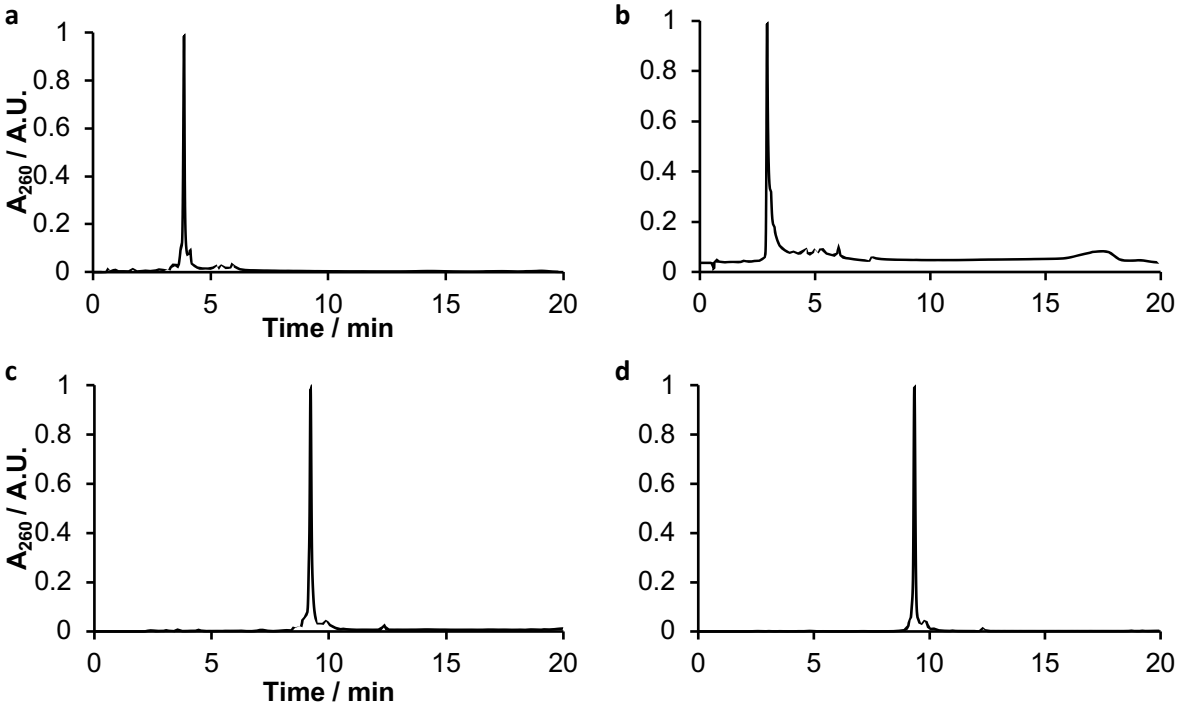

Figure S3. HPLC chromatograms of the unlabelled PNA strands. A) [10]-PNA1. B) [10]-PNA2. C) [15]-PNA1. D) [15]-PNA2.

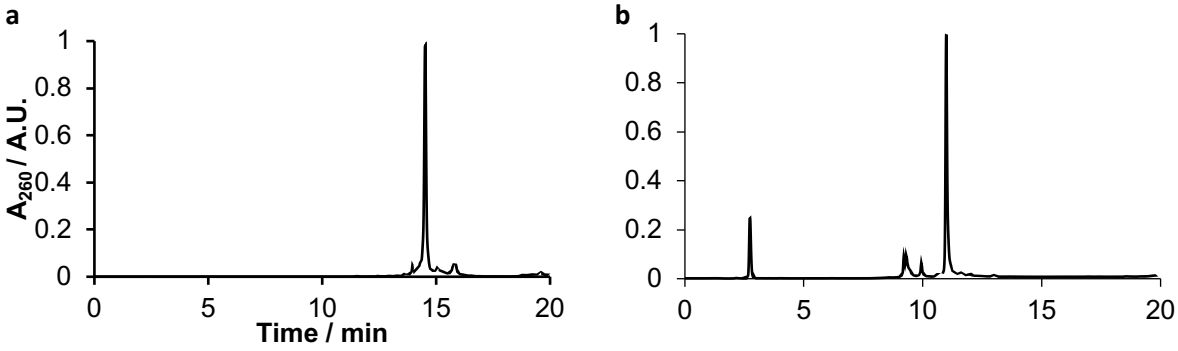

Figure S4. HPLC chromatograms of the labelled PNA strands. A) [10]-PNA1-F. The product eluting at 14.9 min was fraction-collected. B) [10]-PNA2-Q. The product eluting at 11.0 min was fraction-collected.

3. UV absorbance, fluorescence and calorimetry comparison

Here, the main experimental details corresponding to the comparative thermal stability study are outlined in Table S3. The temperature at the maximum signal point corresponds to the melting temperature in Figure S5.

3.1. Experimental details

Table S3. Additional information of the comparative study between UV absorbance, fluorescence and calorimetry thermal denaturalisation. The error in the melting temperature corresponds to the standard deviation.

		dsDNA	DNA·PNA	dsPNA
UV Absorbance	*Concentration*	2.5 μM	2.5 μM	2.5 μM
	Solvent	PBS (100 mM, pH 7.2)	18.2 MΩ·cm water	18.2 MΩ·cm water
	Sequences	[10]-DNA1 [10]-DNA2	[10]-PNA1 [10]-DNA2	[10]-PNA1 [10]-PNA2
	Heating rate	0.5 °C / step – 60 s	0.5 °C / step – 60 s	0.5 °C / step – 60 s
	Melting temperature	39.5 ± 1.4 °C	51.9 ± 3 °C	56.1 ± 1.5 °C
Fluorescence	*Concentration*	2.5 μM	2.5 μM	Not determined
	Solvent	PBS (100 mM, pH 7.2)	18.2 MΩ·cm water	
	Sequences	[10]-DNA1-F [10]-DNA2-Q	[10]-PNA1-F [10]-DNA2-Q	
	Heating rate	0.5 °C / step – 60 s	0.5 °C / step – 60 s	
	Melting temperature	47.2 ± 2.6 °C	59.7 ± 2.0 °C	
Calorimetry	*Concentration*	20 μM	40 μM	40 μM
	Solvent	PBS (100 mM, pH 7.2)	18.2 MΩ·cm water	18.2 MΩ·cm water
	Sequences	[10]-DNA1 [10]-DNA2	[10]-PNA1 [10]-DNA2	[10]-PNA1 [10]-PNA2
	Heating rate	1 °C / min	1 °C / min	1 °C / min
	Melting temperature	44.6 ± 1.8 °C	60.5 ± 0.7 °C	60.9 ± 1.0 °C

3.2. Differential plots

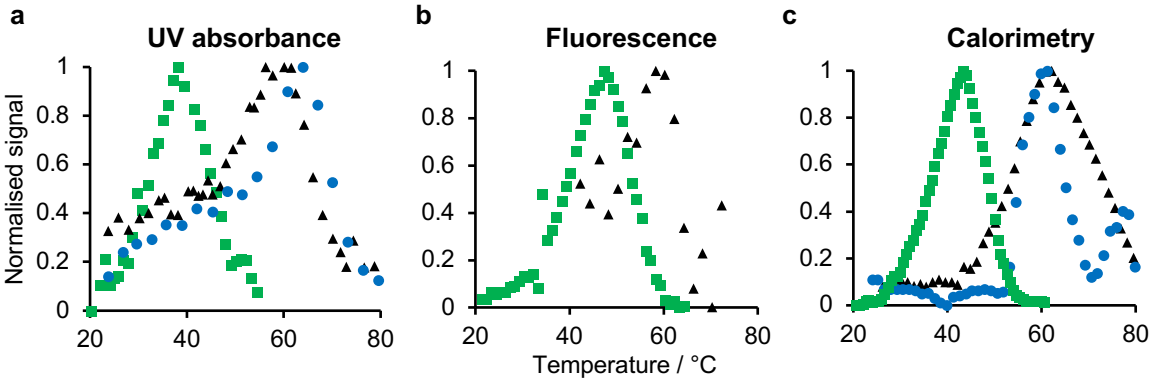

Figure S5. Plot of the derivative of each melting curve of dsDNA (■), PNA·DNA (▲) and dsPNA (●). A) The signal corresponds to the first derivative of the Absorbance at 260 nm with respect to the temperature B) The signal corresponds to the derivative of the Fluorescence intensity with respect to the temperature. C) The signal corresponds to the normalised excess heat capacity.

4. Micro-DSC study of PNA·DNA and dsPNA thermal stability in DMF:H_2O solutions

In order to prevent instability of the micro-DSC baseline, the DMF:H_2O solutions were degassed by sonication for 10 minutes. The 10-mer dsPNA and PNA·DNA duplexes were prepared at 500 µM while the 15-mer dsPNA solutions were prepared at 200 µM. Micro-DSC was performed across a temperature range of 0 to 90 °C, at a heat flux of 0.5 °C·min^{-1}. Each sample was subjected to two heating-cooling cycles. The T_m was determined as the average temperature at the maximum molar excess heat capacity of the melting transition, for the first cooling and the next heating and cooling ramps. The molar excess heat capacity was numerically integrated and normalised showing the progress of the melting process at each DMF content (see Figure S9 and S11).

4.1. Representation of the evolution of the T_m as function of the DMF vol%

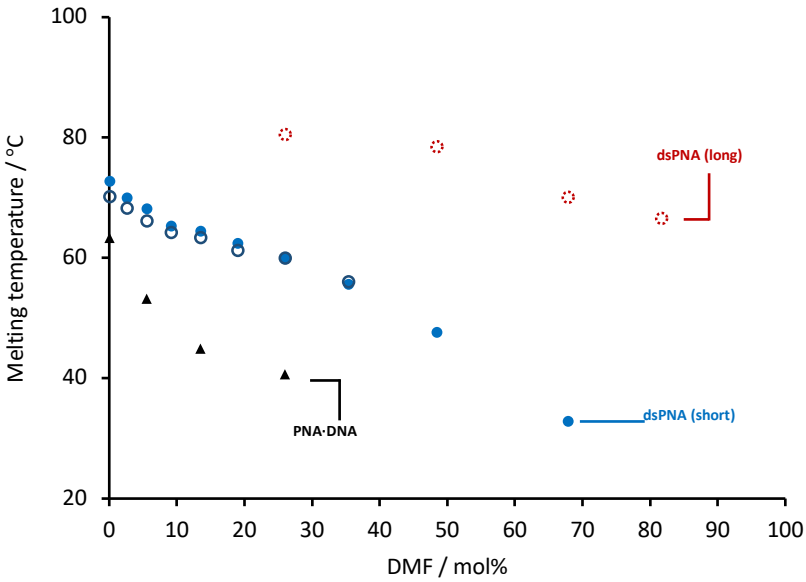

Figure S6. Evolution of the melting temperature (T_m) of dsPNA as function of the molar percentage of DMF. 15-mer dsPNA micro-DSC measurements (open dashed red circle), 10-mer dsPNA micro-DSC measurements (solid blue dot), 10-mer dsPNA UV absorbance reported data (open blue circle) and 10-mer PNA·DNA hybrid duplex micro-DSC measurements (black triangle).

4.2. Relationship between vol% and mol%

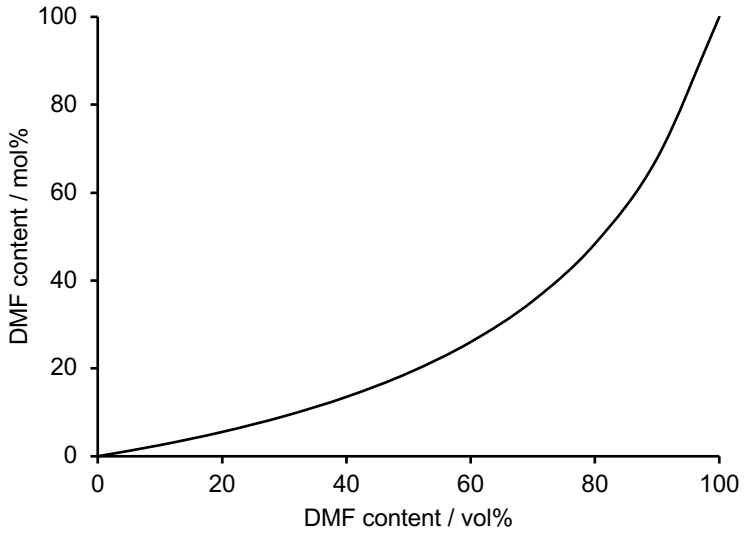

Figure S7. Plot of the molar percentage of DMF in DMF:H_2O mixtures, as function of the volumetric percentage of DMF.

4.3. 10-mer dsPNA in DMF:H_2O solutions

Table S4. 10-mer dsPNA melting temperature results obtained from micro-DSC. The standard deviation was calculated from three consecutive runs.

DMF (vol%)	T_m (°C)	SD (°C)
0	72.8	0.2
10	70.0	0.1
20	68.2	0.1
30	65.3	0.2
40	64.5	0.1
50	62.5	0.4
60	59.9	0.4
70	55.7	0.3
80	47.7	0.5
90	32.9	0.5

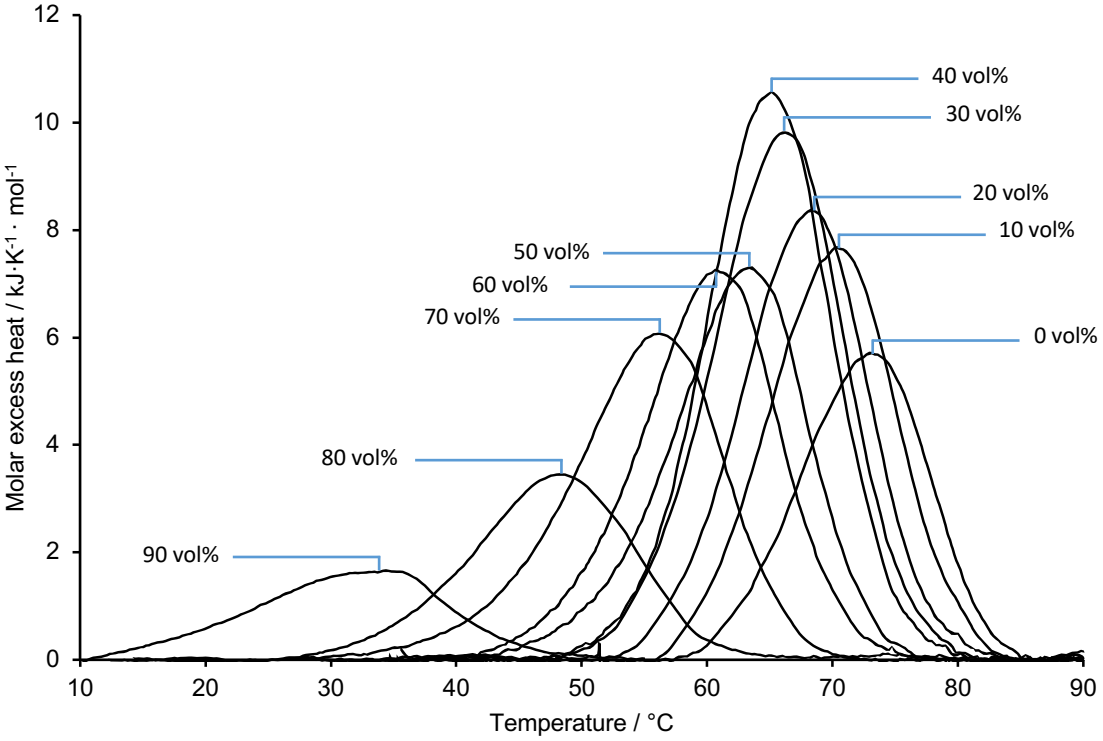

Figure S8. Sigmoidal baseline subtracted micro-DSC data of [10]-PNA1·[10]-PNA2 thermal melting in DMF:H_2O solutions.

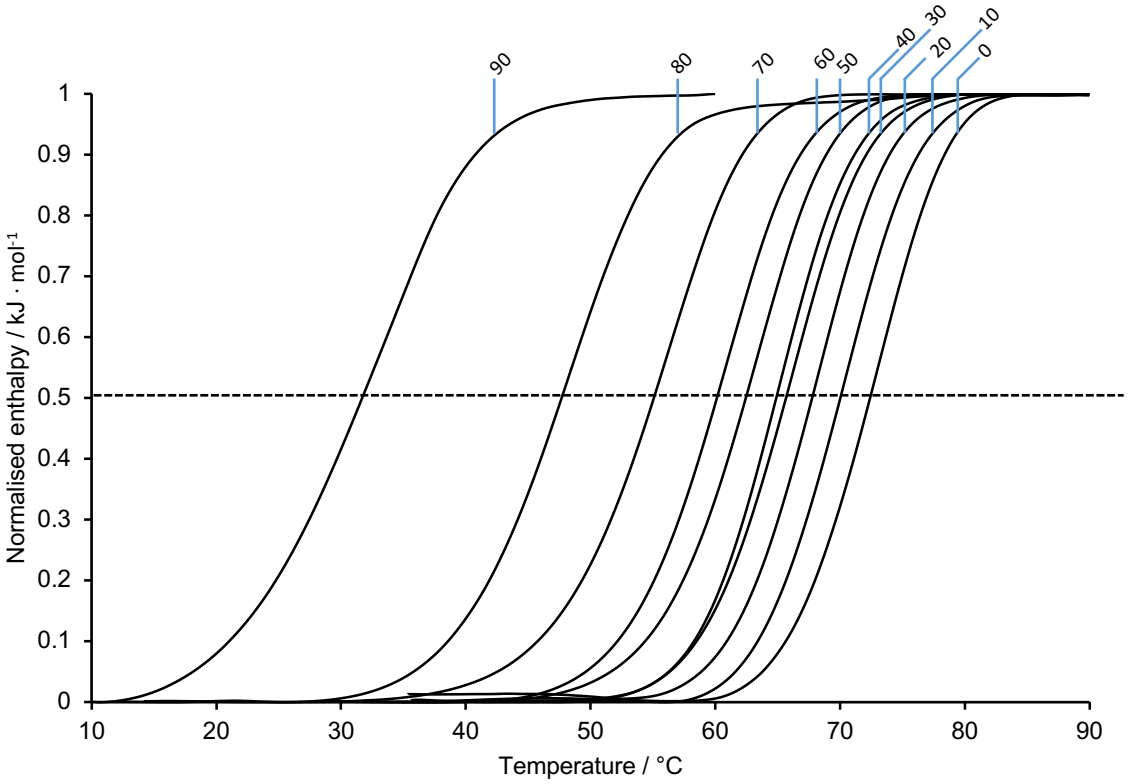

Figure S9. Normalised integrated molar heat capacity micro-DSC data of a 10-mer dsPNA in DMF:H_2O solutions. DMF (vol%) is indicated on top of each plot. The horizontal dashed line indicates the 50% maximum intensity, from which the T_m is calculated.

4.4. 15-mer dsPNA in DMF:H_2O solutions

DMF (vol%)	T_m (°C)	SD (°C)
95	66.7	0.6
90	70.1	1.0
80	78.5	1.4
60	80.6	0.6

Table S5. 15-mer dsPNA melting temperature results obtained from micro-DSC. The standard deviation was calculated from three consecutive runs.

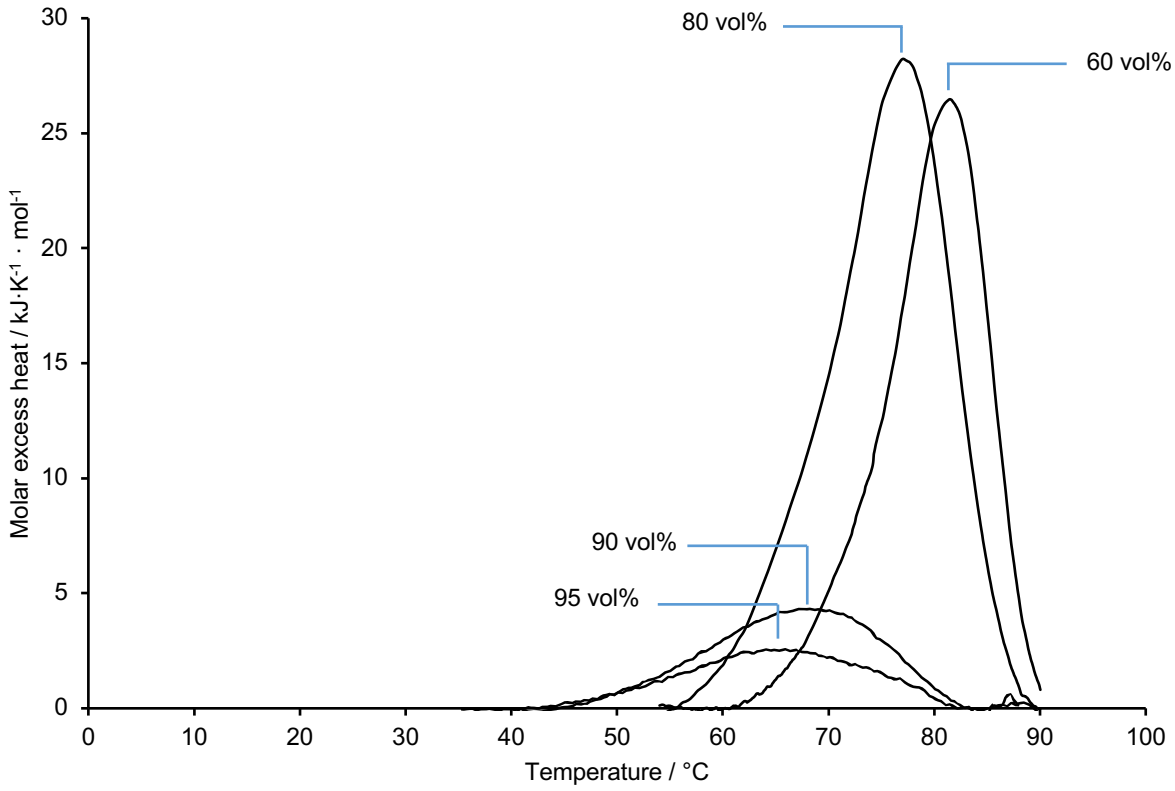

Figure S10. Sigmoidal baseline subtracted micro-DSC data of [15]-PNA1·[15]-PNA2 thermal melting in DMF:H_2O solutions

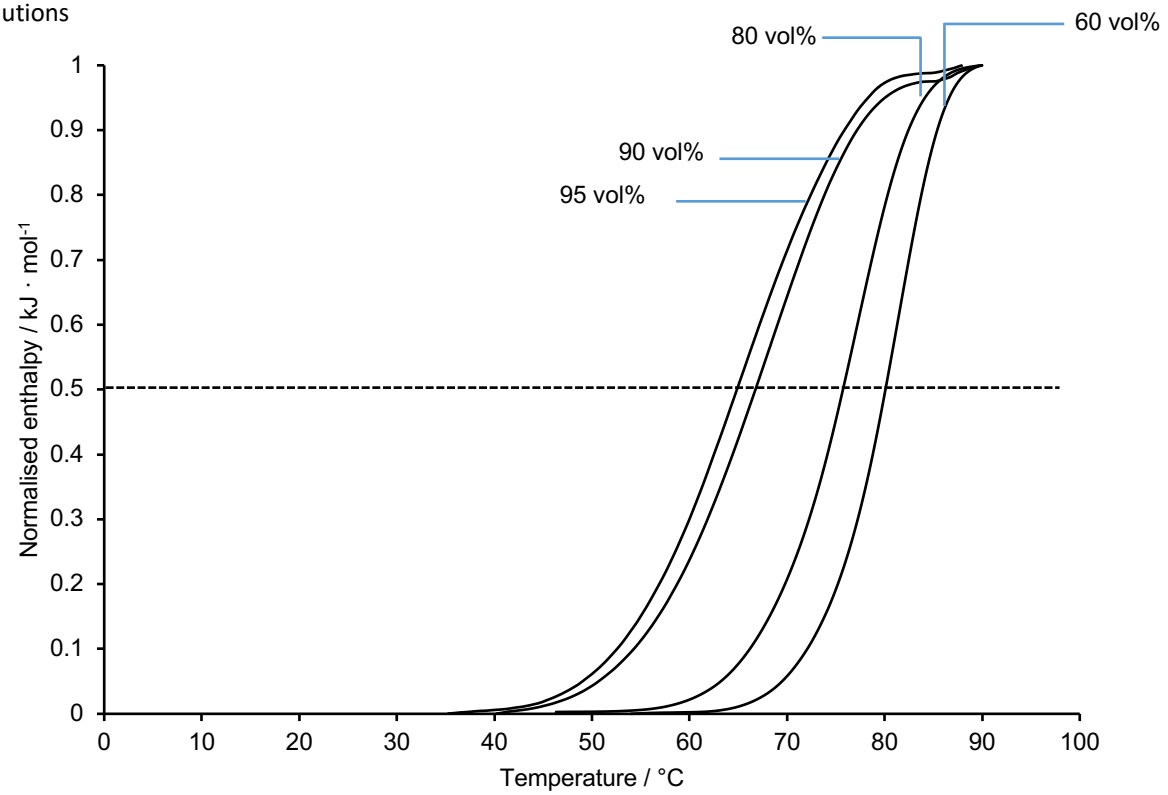

Figure S11. Normalised integrated molar heat capacity micro-DSC data of a 15-mer dsPNA in DMF:H_2O solutions. DMF (vol%) is indicated on top of each plot. The horizontal dashed line indicates the 50% maximum intensity, from which the T_m is calculated.

4.5. 10-mer PNA·DNA in DMF:H_2O solutions

Table S5. 10-mer PNA·DNA melting temperature results obtained from micro-DSC. The standard deviation was calculated from three consecutive runs.

DMF (vol%)	T_m (°C)	SD (°C)
0	63.3	0.2
20	53.2	0.4
40	45.0	1.0
60	40.7	1.1

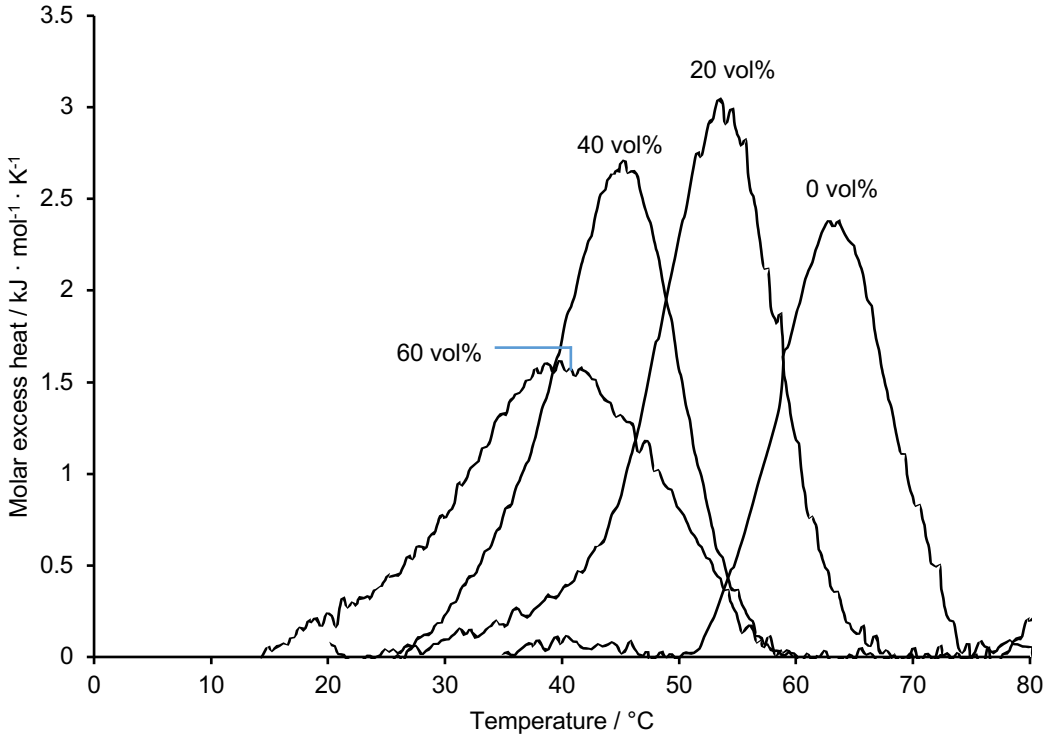

Figure S12. Sigmoidal baseline subtracted micro-DSC data of a 10-mer [10]-PNA1·[10]-DNA2 thermal melting in DMF:H_2O solutions

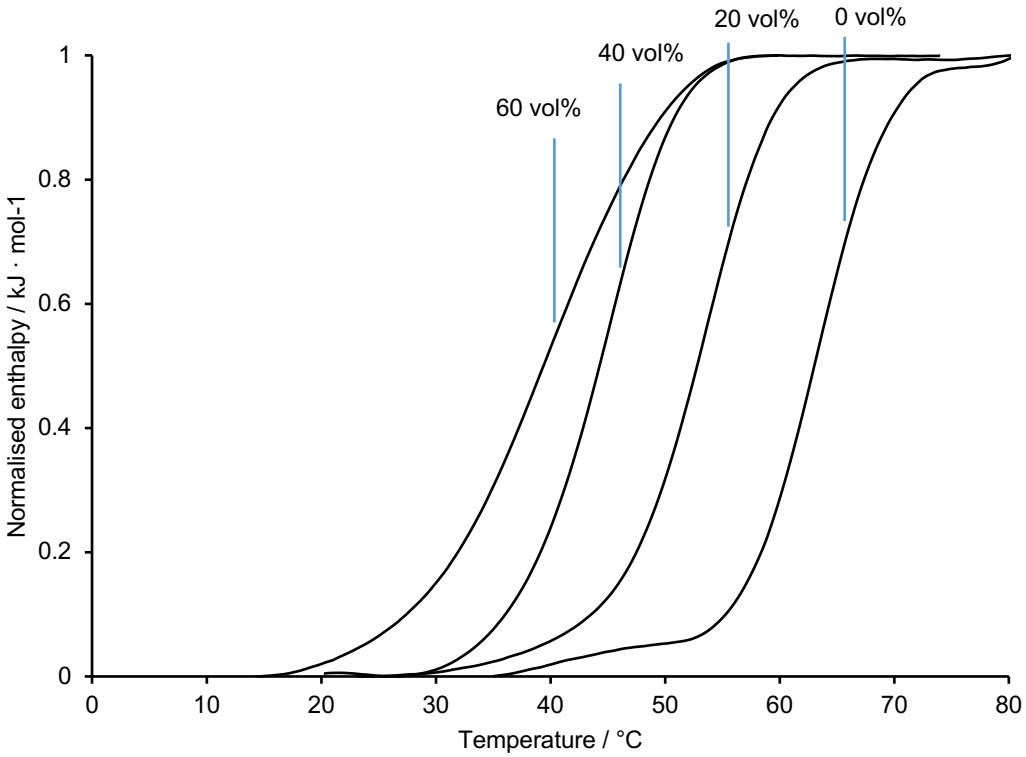

Figure S13. Normalised integrated molar heat capacity micro-DSC data of a 10-mer [10]-PNA1 [10]-DNA2 PNA·DNA hybrid duplex in DMF:H_2O solutions. DMF (vol%) is indicated on top of each plot. The horizontal dashed line indicates the 50% maximum intensity, from which the T_m is calculated.

5. Isothermal annealing of a 10-mer dsPNA in H_2O and in 90 vol% DMF

After dissolving [10]-PNA1-F solid pellet in 90 vol% DMF, it was found that a heating and cooling cycle, between 80 and 10 °C, was required before the addition of [10]-PNA2-Q for faster annealing kinetics (for comparison, see main manuscript Figure 3). This was performed in a thermal cycler with a heated lid to prevent evaporation. Then, the solution was placed in a fluorescence quartz cuvette and monitored until a stable baseline was obtained, corresponding to the unquenched fluorescence emission. Finally, the cuvette was removed from the spectrofluorometer, the complementary quencher strand was added and the mixture was briefly shaken before returning the cuvette to the cell holder to monitor the annealing process. During the analysis, the temperature of the solution was adjusted to 10 °C with a pre-equilibrated circulating water bath. The final concentration of dsPNA was 10 μM.

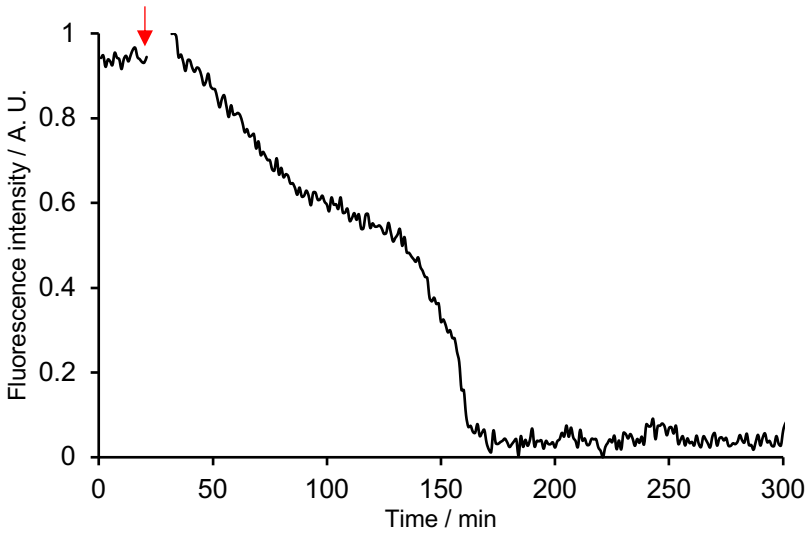

Figure S14. Isothermal annealing of [10]-PNA1-F and [10]-PNA2-Q in 90 vol% DMF at 10 μM concentration in the absence of a pre-anneal heating cycle. The addition of the quencher is marked with a red arrow.

6. References

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