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

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The selectivity of nucleic acid hybridisation can be exploited to template chemical reactions, enabling materials discovery by chemical evolution. However, to date the range of reactions that can be used has been limited to those that are compatible with aqueous media, since the addition of organic co-solvents can have a large impact on the stability of nucleic acid duplexes. Peptide nucleic acids (PNAs) are promising in this regard because previous studies have suggested they may be stable as duplexes in high organic content solvent mixtures. Here, we use micro-differential scanning calorimetry (micro-DSC) to confirm for the first time that double-stranded PNA (dsPNA) is stable in *N,N*-dimethylformamide (DMF)/water mixtures up to 95 vol% DMF. Using fluorescence, we corroborate these results and show that the isothermal annealing of PNA in high DMF content solution is also rapid. These findings suggest that PNA could enable the use of a range of water-sensitive chemistries in nucleic acid templating applications.

Nucleic acids have the remarkable ability to recognise complementary strands. This selective hybridisation has been exploited to template the synthesis of oligomers<sup>1,2</sup> and for the production of combinatorial libraries<sup>3,4</sup> in an analogous manner to the genetically controlled biosynthesis of proteins.<sup>5</sup> Interestingly, this approach to organic synthesis has the potential to perform directed chemical evolution: whole libraries of DNA-tagged molecules can be screened in one pot, allowing the selection and identification of those that perform a particular function, such as catalysis or enzyme inhibition.<sup>6–10</sup>

One major limitation of this nucleic acid-templated synthesis (NATS) is the incompatibility of many chemical reactions with aqueous solution. Aqueous solution is required because nucleic acid duplexes are greatly destabilised by the addition of organic cosolvents.<sup>11</sup> Overcoming this limitation

would allow the use of a wider variety of chemical transformations, increasing the scope of NATS technology.

Peptide nucleic acids (PNAs) are DNA analogues in which the phosphate and sugar backbone is replaced by *N*-(2-aminoethyl)glycine linkages.<sup>12</sup> PNAs have been used as molecular tags to encode the identity of molecules in combinatorial chemical libraries,<sup>13</sup> and as templates in several examples of templated synthesis.<sup>14–16</sup> PNAs are capable of selective recognition through hybridisation and, in contrast to native DNA and RNA, are soluble in a range of organic solvents such as 1,4-dioxane, dimethylformamide (DMF), *N*-methylpyrrolidone (NMP) and dimethyl sulfoxide (DMSO).<sup>15</sup> Previous studies have also shown that double-stranded PNA (dsPNA) is stable in up to 70 vol% DMF/water mixtures.<sup>17</sup> They are therefore promising candidates to allow extension of NATS into high organic content mixtures, or even pure organic solvents.

The duplex melting temperature ( $T_m$ ) is the most commonly used proxy of the thermal stability of dsPNA, and is defined as the temperature at which half the possible duplexes have formed. There are three main techniques available to measure the  $T_m$  of nucleic acid duplexes (see Table 1): ultraviolet (UV) absorbance, fluorescence and calorimetry. The formation of dsPNA in hydrated organic solvent was studied by Sen and Nielsen through UV absorbance, and confirmed duplex stability up to 50<sup>18</sup> and 70 vol%<sup>17</sup> DMF. However, study of higher organic content mixtures was not possible because of DMF's interference with the UV absorbance measurements. This limitation also holds true for a wide range of organic solvents with high UV cut-offs. This problem can be avoided by

**Table 1** Main features of the three available techniques to determine the thermal stability of nucleic acid duplexes

	UV absorbance	Fluorescence	Calorimetry
Sensitivity	Moderate	<b>High</b>	Low
Chemical modification	<b>None required</b>	Required	<b>None required</b>
Solvent range	Narrow	<b>Wide</b>	<b>Wide</b>

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the use of fluorescence to measure  $T_m$ , by tagging one nucleic acid strand with a fluorophore and the other with either a fluorescence quencher or Förster resonance energy transfer (FRET) pair and following the change in fluorescence as hybridisation brings the two into close proximity.<sup>19</sup> However, this approach has proven effects on the  $T_m$ .<sup>20</sup>

Micro-differential scanning calorimetry (micro-DSC), meanwhile, has proved a useful tool to determine the thermal stability of macromolecular systems,<sup>21</sup> allowing quantification of small heat flow changes in dilute solutions. In contrast to fluorescence, it does not require the chemical modification of the macromolecules and unlike UV absorbance is compatible with a wide range of solvents – the only requirement is that the process being studied involves heat exchange.

Here, micro-DSC has been used to study the thermal stability of dsPNA across the whole range of aqueous-organic solvent compositions without any post-synthetic modification of the strands. DMF was chosen as the organic solvent due to the reported stability of dsPNA in DMF/water mixtures,<sup>17,18</sup> its high boiling point, miscibility with water and the fact that it would be a good solvent for PNA-templated synthesis in future applications. The findings were further corroborated by fluorescence measurements, based on fluorescence quenching, which also confirmed the rapid isothermal annealing of dsPNA in high organic content solvent mixtures.

In order to establish how comparable the previously reported values for the  $T_m$  of dsPNA in DMF determined by UV absorbance<sup>17</sup> would be with those measured by micro-DSC, a comparative study was performed. The  $T_m$ s of 10-mer dsDNA, PNA-DNA and dsPNA duplexes with the same base sequence, that had previously shown good stability in DMF/water mixtures up to 70 vol% DMF, were determined in aqueous solution using UV absorbance, fluorescence and micro-DSC, and good agreement was found between the three techniques (Fig. 1). Measurement of the  $T_m$  of the dsPNA duplex by fluorescence was not possible as we were unable to find a synthetically accessible dye/quencher pair that gave sufficient sensitivity in aqueous solution. Nevertheless, UV absorbance and micro-DSC identified the following stability trend in aqueous solution: dsPNA  $\approx$  PNA-DNA > dsDNA. There was a maximum discrepancy between the  $T_m$ s measured by different techniques of approximately 7 °C in dsDNA, 8 °C in PNA-DNA and 5 °C in dsPNA. We hypothesise that these differences arose from the different accuracies in the control of the heating flow for each instrumental set up. The higher concentrations required for micro-DSC measurements (due to the lower sensitivity of this technique) may also have played a role. For fluorescence measurements, the conjugation of a fluorophore and quencher may have had an additional impact on the  $T_m$ .<sup>20</sup> Despite the differences in  $T_m$  determined through different techniques, micro-DSC and UV absorbance showed the same stability trend across the dsPNA, PNA-DNA, and dsDNA series. It was therefore concluded that micro-DSC would provide results that could be meaningfully compared with previously reported data obtained using UV absorbance.

We next moved on to study the stabilities of dsPNA duplexes in very high organic content solvent mixtures. Fig. 2



**Fig. 1** Comparison of three techniques for the measurement of the  $T_m$  of nucleic acid duplexes in aqueous solution: UV absorbance (UV, orange solid line), fluorescence (Fl., violet dashed line) and calorimetry (Cal., blue dashed-dotted line). The black dashed line indicates the 50% maximum intensity from which the  $T_m$ s were calculated. The duplexes studied were: (a) dsDNA, (b) PNA-DNA, (c) dsPNA. The calculated melting temperatures for each duplex are displayed in the bar charts in the right hand column. \*Fluorescence was not measurable. The normalised intensities (norm. int.) corresponded to: UV absorbance at 260 nm, fluorescence emission measured in counts and the numeric integral of the excess heat capacity. (dsDNA was excited at 495 nm and the fluorescence emission was measured at 512 nm. PNA-DNA was excited at 290 nm and the fluorescence emission was measured at 340 nm. Sigmoidal baselines were fitted and subtracted to produce the molar excess heat calorimetry signals.<sup>26</sup> Sequences: [10]-DNA/PNA1 5'/N – TCA CTA GAT G – C/3' [10]-DNA/PNA2 5'/N – CAT CTA GTG A – C/3'. For full sequences and modifications, see section 1 ESI.† For solvents, concentrations and heating rates see Table S3 ESI.†)

shows that for a short dsPNA duplex (10-mer) similar to the one studied previously by Sen and Nielsen<sup>17</sup> micro-DSC confirmed that stability was retained up to 90 vol% DMF. Interestingly, in previous reports an approximately linear relationship between  $T_m$  and DMF vol% was identified and it was hypothesised that the  $T_m$  would be slightly above 50 °C in





ensure thorough mixing, before being replaced in the instrument and measurements continued.

In 90 vol% DMF, annealing was found to be rapid (Fig. 3a), with full quenching observed by the time the cuvette had been replaced in the instrument following addition of the complementary strand (a delay of around 12 s). It was also found that the freshly dissolved PNA strands required a heating/cooling cycle in the desired solvent prior to isothermal annealing in order to display these fast kinetics, otherwise annealing took approximately 1.5 h to complete (see Fig. S14 ESI†). This was interpreted as the effect of kinetically trapped states present in the dry pellet that provided an additional barrier to dsPNA formation. The isothermally annealed PNA was subsequently thermally denatured and was observed to re-anneal upon cooling, demonstrating the full reversibility of the process (Fig. 3b). This supported the micro-DSC evidence of dsPNA formation in 90 vol% DMF and suggests that multistep PNA templated synthesis in organic solvents may be possible in the future.

In conclusion, it has been demonstrated that microcalorimetry allows reliable determination of the thermal stability of dsPNA in solvent mixtures where the widely employed UV absorbance method cannot be used as a result of the high UV cut-off of the solvent. This technique was subsequently used to study the stability of 10-mer and 15-mer dsPNA duplexes in 0 to 100 vol% DMF showing the formation of dsPNA in up to 95 vol% DMF. A remarkable stability in high organic solvent content was observed, which was directly dependent on the length of the PNA. Finally, it was shown that PNAs isothermally anneal rapidly in high DMF content solutions. Altogether, these findings highlight the potential of microcalorimetry to study duplex formation events in a wider range of solvent media and in the absence of a measurable spectrometric change. They also provide a good starting point for further applications of peptide nucleic acids as chemical templates in water-free media.

## Conflicts of interest

There are no conflicts to declare.

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