**Supporting Information**

# G-quadruplex unfolding in higher-order DNA structures

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## Materials and Methods

### DNA sample preparation

The $(T_2AG_3)_8T_2$ (Q2), $(T_2AG_3)_4T_2$ (Q1) and $(A_2TC_3)_4$ (SC1) oligonucleotides and the 2-aminopurine analogues of Q2 (Ap9 and Ap33) for fluorescence experiments were purchased from the Primm Company (Milan, Italy); stock DNA solutions were prepared by dissolving the lyophilized compounds in a buffer solution containing 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0. The stock solutions were then heated at 95 °C for 5 min and then slowly cooled to room temperature. The concentration of oligonucleotides was determined by UV adsorption measurements at 90 °C using the extinction coefficient values $\varepsilon_{(260\ nm)}$ of 505600 $M^{-1}$ $cm^{-1}$ (Q2), 261200 $M^{-1}$ $cm^{-1}$ (Q1), 236500 $M^{-1}$ $cm^{-1}$ (SC1) and 492600 $M^{-1}$ $cm^{-1}$ (Ap9 and Ap33).

### Polyacrilamide gel electrophoresis (PAGE)

Native gel electrophoresis experiments were performed on 20% polyacrilamide gel (29:1 acrylamide:bisacrylamide ratio) containing 100 mM KCl. Gels were run at 4 °C and 90 V for 5 h in 1× TBE (Tris-Borate-EDTA) running buffer. All the samples were prepared by dilution of the stock DNA solutions with the buffer solution (10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0). The Q2 and SC1 oligonucleotide concentrations were 20 μM and 40 μM, respectively, when loaded alone (lane 1 and 6 in Figure 4) or in their mixture at 1:2 molar ratio (lane 2, 4 and 5 in Figure 4). In their mixture at 1:1 molar ratio, the Q2 and SC1 concentrations were both 20 μM. For all samples, a solution of glycerol/TBE 1× 2:1 was added to facilitate sample loading in the wells. Bands were visualized by UV shadowing.

**Circular dichrosim (CD) spectroscopy**

CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI), and calibrated with an aqueous solution of 0.06% d-10-(1)-camphorsulfonic acid at 290 nm. The molar ellipticity [θ] (deg $cm^2$ $dmol^{-1}$) was calculated from the equation $[\theta] = [\theta]_{obs}/10 \times l \times C$, where $[\theta]_{obs}$ is the ellipticity (mdeg), $C$ is the oligonucleotide molar concentration, and $l$ is the optical path length of the cell (cm). The cell with 0.1 and 1.0 cm path length and oligonucleotide concentration in the range 2–20 μM was used to record CD spectra between 220 and 320 nm. A scan speed of 50 nm/min, 0.5 nm data pitch, and 2 nm bandwidth were used to acquire the data. The scan of the buffer was subtracted from the scan of each sample. Before to collect the CD spectra of the mixtures of the studied oligonucleotides, the samples were incubated at 20 °C for 4 hours to allow the corresponding hybridization reactions to occur. All the experiments were performed in 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

**CD kinetic experiments**

The unfolding kinetics of Q1 (single quadruplex forming sequence) was explored using CD spectroscopy by following the decrease of the signal at 298 nm on increasing time after adding SC1 to a solution containing Q1. The SC1 and Q1 concentration was 10 μM. The quadruplex concentration was assumed directly proportional to the CD signal at 298 nm as the DNA duplex contribution is negligible at this wavelength. Experiments were performed in 25-37 °C temperature range. The kinetic profiles were fitted to a single-exponential function to obtain the first-order kinetic constant. The activation energy was obtained by the linear fit of the plots of the logarithm of the rate constants as a function of $1/T$.

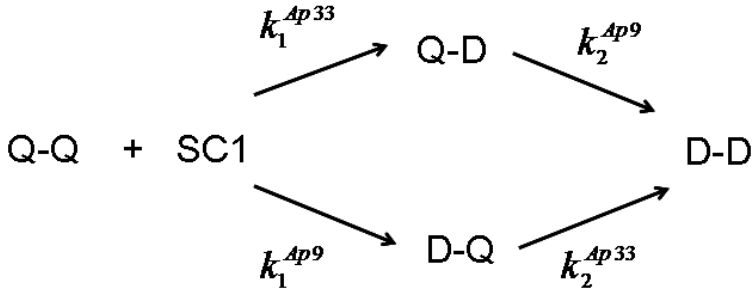
**Fluorescence spectroscopy**

Steady-state fluorescence measurements were performed on a Perkin Elmer LS50B fluorescence spectrometer. Both excitation and emission slits were set at 5 nm. A sealed quartz cuvette with a path length of 1 cm was used. The 2-aminopurine analogues Ap9 and Ap33, were excited at 305 nm, and emission spectra were recorded between 320 and 460 nm. The concentration of Ap9 or Ap33 was 0.5 μM both alone or in their 1:1 or 1:2 mixtures with SC1. Before to collect the fluorescence spectra of the mixtures of the 2-aminopurine analogues with SC1, the samples were incubated at 20 °C for 4 hour to allow the corresponding hybridization reactions to occur. All the experiments were performed in 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

**Fluorescence kinetic measurements**

The unfolding kinetics of each quadruplex unit in Q2 was explored by following the emission fluorescence signal at 370 nm of Ap9 or Ap33 on increasing time and in the selected temperature range, upon addition of the complementary SC1 strand. The concentration of Ap9 or Ap33 was 0.5 μM and the concentration of SC1 was varied in the range 5-30 μM. A 1.0 cm pathlength cuvette was employed. Experiments were performed in the 15-37 °C temperature range. All the experiments were carried out in a buffer solution containing 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

The fitting equations were derived starting from the following reaction model :




where Q-Q is the initial two-quadruplexes structure, D-D is the final complex formed by two duplexes whereas Q-D and D-Q are the quadruplex-duplex intermediates with the quadruplex at the 5' or 3'-end, respectively. According to this reaction model, and considering all steps as a first order reactions, the variation of the relative populations of the Q-Q, Q-D, D-Q and D-D species on changing time is given (at each temperature) by the following differential equations:

$$\frac{d\alpha_{Q-Q}}{dt} = -\left(k_1^{Ap9} + k_1^{Ap33}\right) \times \alpha_{Q-Q}$$

$$\frac{d\alpha_{Q-D}}{dt} = k_1^{Ap33} \times \alpha_{Q-Q} - k_2^{Ap9} \times \alpha_{Q-D}$$

$$\frac{d\alpha_{D-Q}}{dt} = k_1^{Ap9} \times \alpha_{Q-Q} - k_2^{Ap33} \times \alpha_{D-Q} \qquad \text{(eq. S1-S4)}$$

$$\frac{d\alpha_{D-D}}{dt} = k_2^{Ap9} \times \alpha_{Q-D} + k_2^{Ap33} \times \alpha_{D-Q}$$

where $\alpha_i$ represents the molar fraction of the i-specie. A Runge-Kutta-Fehlberg integration method[1] was used to solve equation S1-S4 for a given set of kinetic constants and the obtained solutions ($\alpha_i$) were used

to calculated the following model functions for the fluorescence decay of Ap9 and Ap33:

$$F^{Ap9} = F_{Q-Q}^{Ap9} \times \left(\alpha_{Q-Q} + \alpha_{Q-D}\right) + F_{D-D}^{Ap9} \times \left(\alpha_{D-Q} + \alpha_{D-D}\right) \qquad \text{for Ap9} \qquad \text{(eq. S5)}$$

$$F^{Ap33} = F_{Q-Q}^{Ap33} \times \left(\alpha_{Q-Q} + \alpha_{D-Q}\right) + F_{D-D}^{Ap33} \times \left(\alpha_{Q-D} + \alpha_{D-D}\right) \qquad \text{for Ap33} \qquad \text{(eq. S6)}$$

where $F^{Ap9}$ is the observed fluorescence intensity for Ap9 whereas $F_{Q-Q}^{Ap9}$ and $F_{D-D}^{Ap9}$ are the fluorescence intensity before adding SC1 (when all the sample is in the Q-Q state) and at t= ∞ (when all the sample is in the D-D state), respectively. $F^{Ap33}$, $F_{Q-Q}^{Ap33}$, $F_{D-D}^{Ap33}$ represent the analogous parameters for Ap33. The parameters $F_{Q-Q}^{Ap9}$, $F_{D-D}^{Ap9}$, $F_{Q-Q}^{Ap33}$ and $F_{D-D}^{Ap33}$ were experimentally determined at each temperature and the kinetic constants were obtained by fitting the kinetic curves to equations S5 and S6. The kinetic constants reported in Table S2 and S3 are the best optimized parameters that simultaneously fit both Ap9 and Ap33 fluorescence decay. The corresponding activation energies were obtained by the linear fit of the plots of the logarithm of the rate constants as a function of $1/T$.

**Isothermal Titration Calorimetry (ITC) Measurements**

ITC measurements were used to measure the enthalpy changes for the hybridization reactions of Q2 or Q1 with SC1. The experiments were carried out at 37 °C using a high-sensitivity CSC-5300 Nano-ITC microcalorimeter from Calorimetry Science Corporation (Lindon, Utah) with a cell volume of 1 ml. Before each ITC experiment, the pH of each solution was checked, the reference cell was filled with deionised water, and the DNA solutions were degassed for 5 min to eliminate air bubbles. In each experiment the heat released over time was followed after a single injection of SC1 into the reaction cell. Care was taken to start the SC1 addition after baseline stability had been achieved.

In each experiments, appropriate volumes (in 10-125 µL range) of a solution containing SC1 in a 8-14 µM concentration range were injected into the cell solution (containing Q2 or Q1 alone, or a pre-equilibrated equimolar mixture of SC1 and Q2) to achieve the appropriate SC1 concentration in the reaction cell.

Particularly, to evaluate the enthalpy change ($\Delta H_1$) for the first reaction (Figure S6A), we measured the heat released after mixing SC1 with a large excess of Q2 (initial concentration in the reaction cell: Q2=1µM, SC1=0.1 µM), in this condition all the SC1 strand should form the 1:1 complexes (the Q-D structures). The enthalpy change ($\Delta H_2$) involved in the second hybridization event was measured in a

similar experiment (Figure S6B) by recording the heat released after adding SC1 (initial concentration of the free SC1 strand in the reaction cell=1 μM) to a solution containing a pre-equilibrated mixture of Q2 and SC1 at 1:1 molar ratio (1 μM each strand). In this experiments we assumed that the pre-equilibrated 1:1 mixture is formed by 100% of the Q-D structures. The total heat released by the two reactions ($\Delta H_{TOT}$) was measured by adding SC1 (initial concentration of the free SC1 strand in the reaction cell=2 μM) to a solution containing Q2 at 1 μM concentration (Figure S6C), in this conditions all Q2 forms the 1:2 complex (D-D structure). Finally, the enthalpy change for the hybridization of the Q1 single quadruplex with SC1 (Figure S7) was measured by recording the heat released after adding Q1 (0.15 μM) to a solution containing an excess of SC1 (1 μM). For each experiment, the heat produced by the SC1 dilution was evaluated by performing a control experiment adding the same amount of SC1 to the buffer alone. The interaction heat for each experiment was calculated by integration of the ITC profile after correction for the heat of the SC1 dilution. The enthalpy changes were reported per mole of quadruplex structure.

**Table S1.** Oligonucleotide sequences used in this study

| Name | Sequence |
|---|---|
| Q1 | 5’ TTAGGGTTAGGGTTAGGGTTAGGG TT 3’ |
| SC1 | 3’ AATCCCAATCCC AATCCCAATCCC 5’ |
| Q2 | 5’TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT AGGGTT 3’ |
| Ap9 | 5’TTAGGGTT**A$_P$**GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT AGGGTT 3’ |
| Ap33 | 5’TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT**A$_p$**GGGTTAGGGTT AGGGTT 3’ |

This table provides the nucleotide sequences used in this study. Singly substituted oligonucleotides containing 2-aminopurine (**Ap**) are shown with the position of the substitution in bold.

**Table S2.** Effect of SC1 concentration on the kinetic parameters for the opening of each quadruplex unit in Q2 obtained using fluorescence spectroscopy measurements at 30 °C.

| [SC1] (μM) | $k_1^{Ap9}$ ($min^{-1}$) | $k_2^{Ap9}$ ($min^{-1}$) |
|---|---|---|
| 5 | 0.14 ± 0.04 | 0.029 ± 0.004 |
| 15 | 0.19 ± 0.05 | 0.027 ± 0.005 |
| 30 | 0.16 ± 0.05 | 0.024 ± 0.005 |
| | $k_1^{Ap33}$ ($min^{-1}$) | $k_2^{Ap33}$ ($min^{-1}$) |
| | | **Ap33** |
| 5 | 0.22 ± 0.03 | 0.053 ± 0.005 |
| 15 | 0.25 ± 0.04 | 0.058 ± 0.005 |
| 30 | 0.28 ± 0.05 | 0.052 ± 0.006 |

This table provides the kinetic constants at 30 °C on varying the SC1 for the two hybridization reactions of Q2 with SC1 on changing SC1 concentration. The nomenclature of the kinetic constants is shown in Scheme 1 (in the main text of the manuscript).

**Table S3.** Effect of temperature on the kinetic parameters for the opening of each quadruplex unit in Q2 obtained using fluorescence spectroscopy measurements.

| T (°C) | $k_1^{Ap9}$ ($min^{-1}$) | $k_2^{Ap9}$ ($min^{-1}$) | $E_1^{Ap9}$ (kJ $mol^{-1}$) | $E_2^{Ap9}$ (kJ $mol^{-1}$) |
|---|---|---|---|---|
| | | **Ap9** | | |
| 15 | 0.023 ± 0.005 | 0.005 ± 0.001 | | |
| 20 | 0.038 ± 0.006 | 0.010 ± 0.002 | | |
| 25 | 0.064 ± 0.007 | 0.016 ± 0.003 | | |
| 30 | 0.14 ± 0.04 | 0.029 ± 0.004 | 88 ± 6 | 82 ± 5 |
| 35 | 0.24 ± 0.04 | 0.048 ± 0.005 | | |
| 37 | 0.29 ± 0.05 | 0.057 ± 0.006 | | |
| | $k_1^{Ap33}$ ($min^{-1}$) | $k_2^{Ap33}$ ($min^{-1}$) | $E_1^{Ap33}$ (kJ $mol^{-1}$) | $E_2^{Ap33}$ (kJ $mol^{-1}$) |
| | | **Ap33** | | |
| 15 | 0.017 ± 0.002 | 0.004 ± 0.001 | | |
| 20 | 0.034 ± 0.004 | 0.009 ± 0.002 | | |
| 25 | 0.087 ± 0.005 | 0.024 ± 0.004 | | |
| 30 | 0.22 ± 0.03 | 0.053 ± 0.005 | 125 ± 8 | 119 ± 7 |
| 35 | 0.49 ± 0.04 | 0.091 ± 0.006 | | |
| 37 | 0.61 ± 0.04 | 0.14 ± 0.02 | | |

This table provides the kinetic constants and the corresponding activation energies for the two hybridization reactions of Q2 with SC1. The nomenclature of the kinetic constants is shown in Scheme 1 (in the main text of the manuscript).

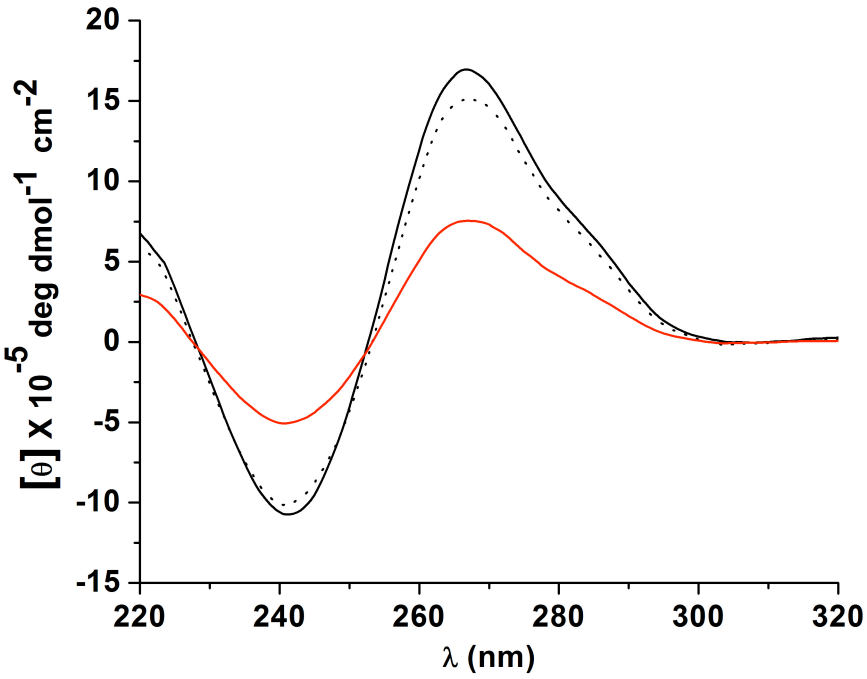



**Figure S1.** CD spectra of the Q2 (10μM) and SC1 (20μM) mixture at 1:2 molar ratio (black line) and of the duplex Q1/SC1 (red line). The CD spectrum of the duplex Q1/SC1 (10 μM each strand) multiplied by two is also shown for comparison (dotted line). All the spectra were collected after equilibration of the samples at 20 °C. All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

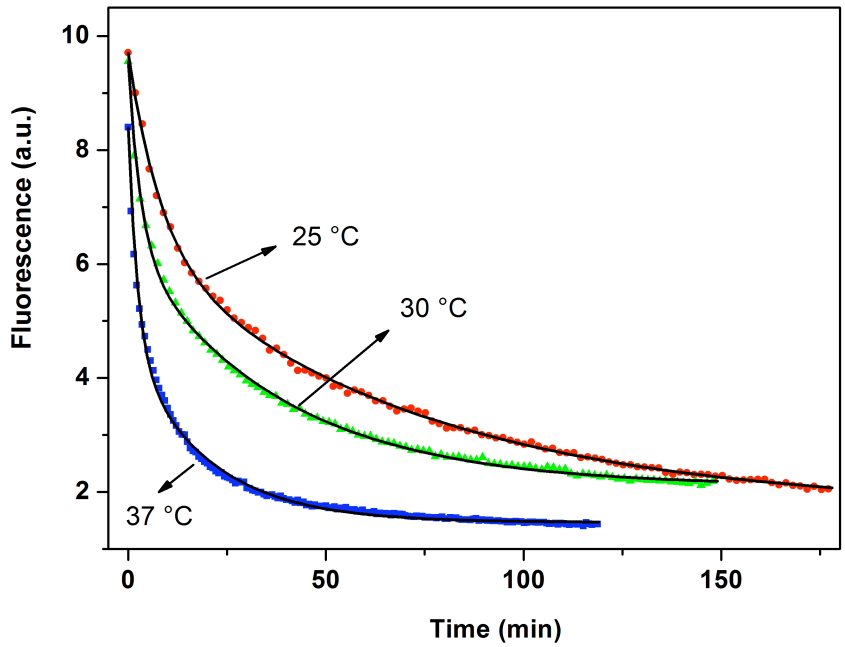



**Figure S2.** Kinetic curves for the hybridization of Ap9 with a large excess of the SC1 strand at 25, 30 °C and 37 °C. The concentrations of Ap9 and SC1 were 0.5 μM and 5 μM, respectively. Fitting curves are also shown (black solid lines). All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0

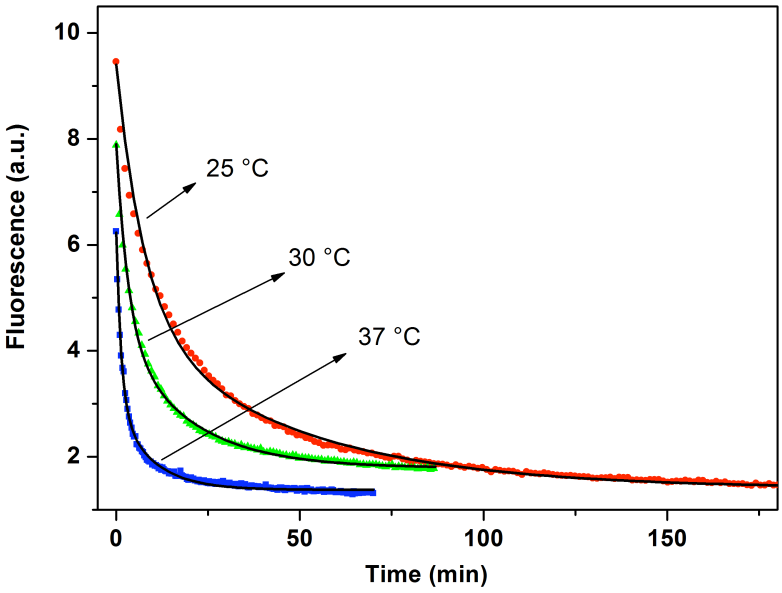



**Figure S3.** Kinetic curves for the hybridization of Ap33 with a large excess of the SC1 at 25, 30 °C and 37 °C. The concentrations of Ap33 and SC1 were 0.5 μM and 5 μM, respectively. Fitting curves are also shown (solid lines). All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0

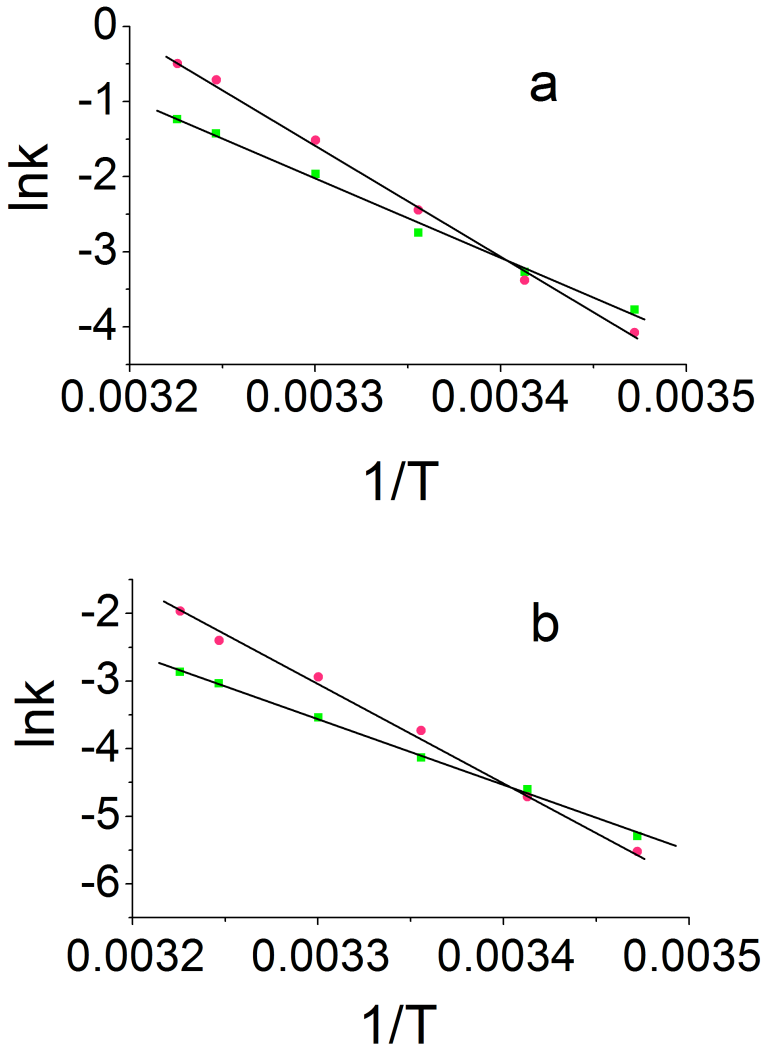



**Figure S4** (a) Arrhenius plots for the first hybridization reaction for Ap9 (■) and Ap33(●). (b) Arrhenius plots for the second hybridization reaction for Ap9 (■) and Ap33(●).

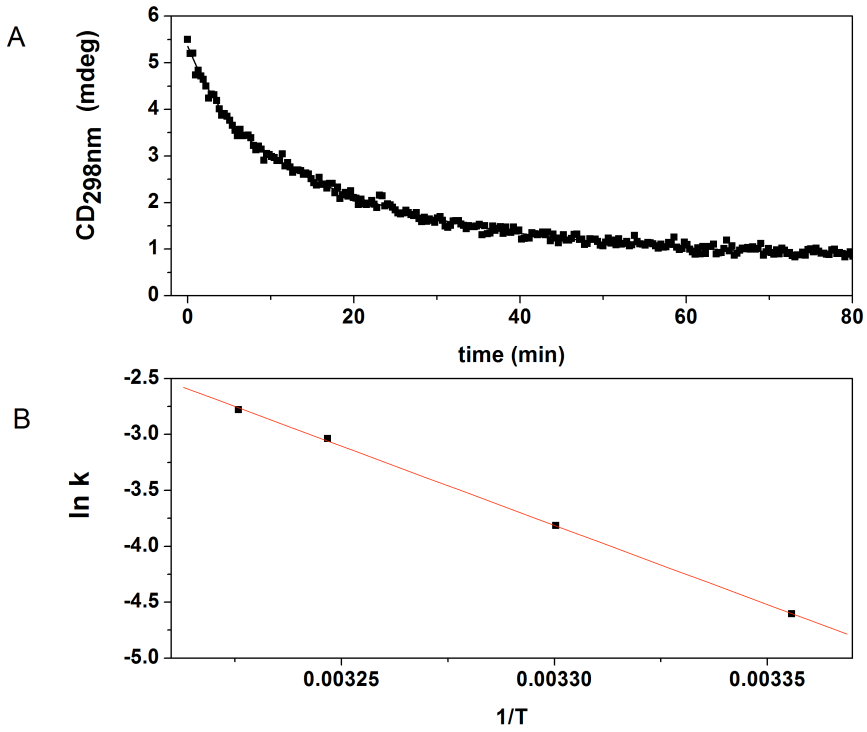



**Figure S5.** (A) Variation of the CD signal (298 nm) at 37 °C as function of time after mixing Q1 and SC1 in equimolar ratio (10 μM each strand). (B) Arrhenious plot for the hybridization reaction. All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0

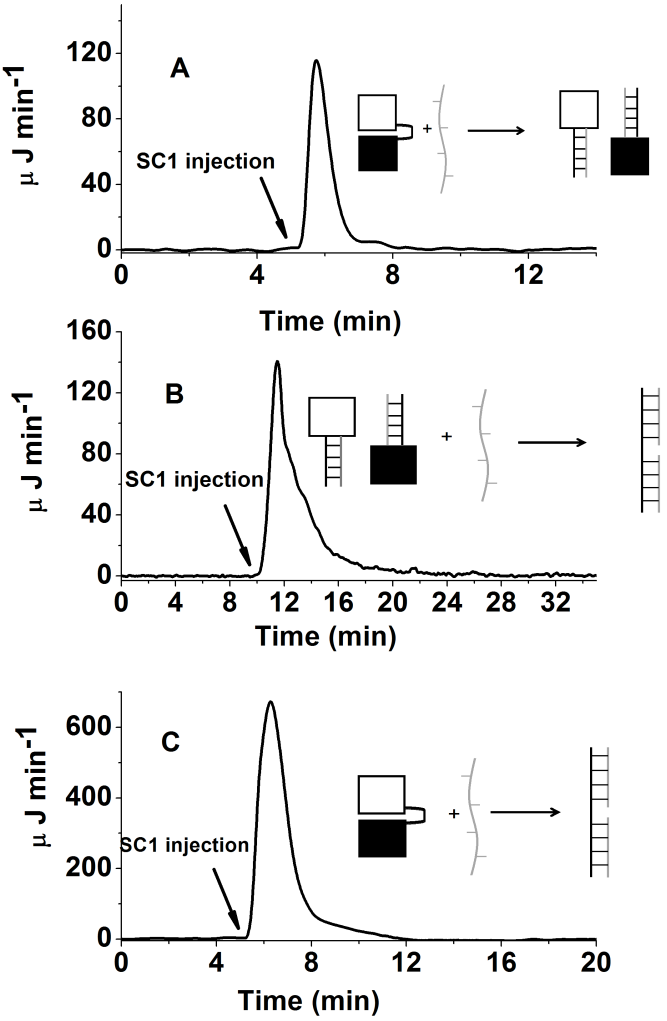



**Figure S6.** ITC profiles for the hybridization reactions of SC1 with Q2. Heat released as function of time after adding: (A) a small amount of SC1 to a Q2 solution (initial concentration in the reaction cell: Q2=1μM, SC1=0.1 μM), (B) SC1 (initial concentration of the free SC1 strand in the reaction cell=1 μM) to a solution containing a pre-equilibrated mixture of Q2 and SC1 at 1:1 molar ratio (1 μM each strand), (C) SC1 (initial concentration of the free SC1 strand in the reaction cell=2 μM) to a solution containing Q2 at 1 μM concentration. All the experiments were performed at 37 °C. Schematic illustrations of the hybridization reactions are also shown. All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0

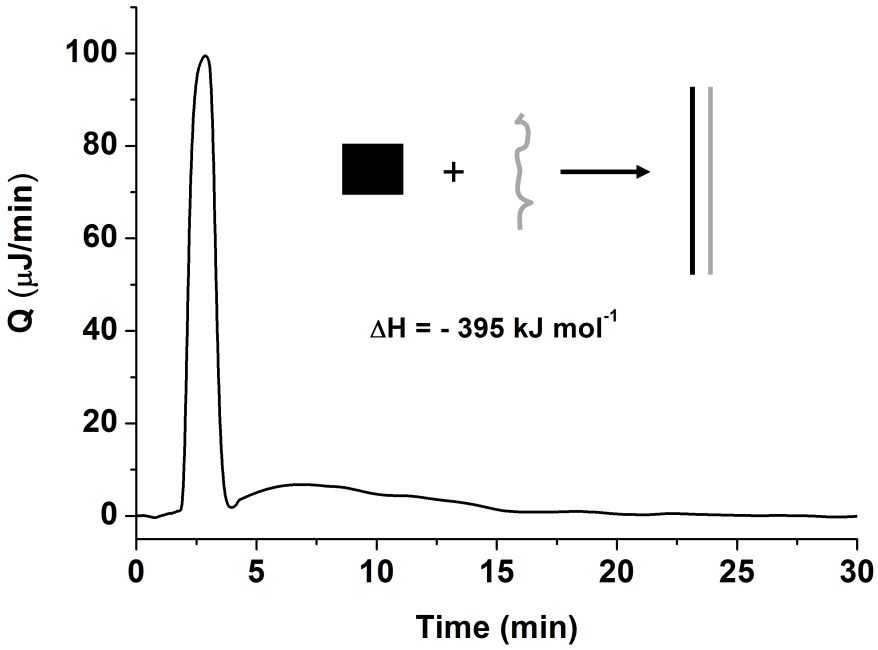



**Figure S7.** ITC profile obtained after mixing Q1 (0.15 μM) to an excess of SC1 (1 μM). The enthalpy value is reported per mole of Q1 added. The error on ΔH value is within 5 %. The experiment was performed at 37 °C. All the solutions contain 20 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0

## References

[1] P. Dormand P. and L.F. Shapire, *J Comp Appl Math*, 1980, **6**, 19.