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Abstract

Peptide nucleic acids (PNAs) are among the most interesting and versatile artificial structural mimics of nucleic acids and exhibit peculiar and important properties (i.e. high chemical stability, and a high resistance to cellular enzymes and nucleases). Despite their unnatural structure, they are able to recognize and bind DNA and RNA in a very high, specific and selective manner. One of the most popular, easy and reliable method to measure the stability of PNA-DNA hybrid systems is the melting temperature but the thermodynamic data are obtained using a big quantity of materials failing to provide information on the kinetics of the interaction.

In the present work, the PNA decamer **6**, with the TCACTAGATG sequence of nucleobases, and the corresponding fluorescent PNA-FITU (fluorescein isothiourea) decamer **8** were synthesized with standard manual Boc-based chemistry. The interaction of the PNA-FITU with parallel and antiparallel DNA has been studied by stopped-flow fluorescence, which is proposed as an alternative technique to obtain the kinetic parameters of the binding.

The great advantage of using the stopped-flow technique is the possibility of studying the kinetics of the PNA-DNA duplex formation in a physiological environment. In particular, fluorescence stopped-flow technique has been exploited to compare the affinity of two PNA-DNA duplexes since it can discriminate between parallel and antiparallel DNA binding.

1. Introduction

Peptide nucleic acid (PNA) is one of the most studied and interesting DNA mimic with great potential for biomedical applications, both in diagnostics and therapy.[1] The chemical structure of PNA is quite different with respect to that of natural nucleic acids in that its backbone is composed of *N*-(2-

aminoethyl)glycine repeating units forming a pseudopeptide chain on which the four nucleobases (adenine or A, cytosine or C, guanine or G and or thymine T) are inserted as pendants (Figure 1). The neutral character of PNA molecules and the distance between two adjacent nucleobases (the same as that found in DNA and RNA) confer them stronger and more selective binding affinity for complementary nucleic acid (DNA and RNA) strands than natural nucleic acids.[2]

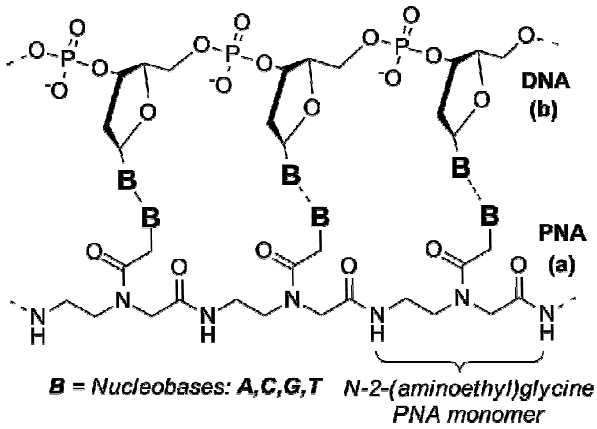


Figure 1. PNA oligomer (a) with its complementary DNA (b).

Though in DNA-DNA duplexes the two strands are always in an antiparallel orientation (with the 5'-end of one strand opposed to the 3'-end of the other), PNA:DNA adducts can be formed in two different orientations, termed parallel (in which the terminal amino group of PNA is opposed to the 5' end of DNA) and antiparallel (where, on the contrary, the amino group is opposed to the 3' end of DNA). However, the antiparallel binding is favored over the parallel one.[3] The binding of PNAs to DNA and RNA targets is stronger than that of DNA-DNA or RNA-RNA bindings.[4] This enhanced binding affinity is partially due to the uncharged property of the PNAs. Since PNAs are neutral in charge, the duplexes formed by PNA-DNA or PNA-RNA hybrids lack the electrostatic repulsion formed by DNA-DNA or RNA-DNA duplexes, resulting in a very stable duplex formation even at a relatively high temperature and a very high binding affinity.

An important aspect to be carefully evaluated in the characterization of PNA analogues is the determination of their affinity and specificity for DNA and RNA, that is if the structural modification has a beneficial or detrimental effect on the formation, specificity and stability of hybrids formed with natural nucleic acids.

One of the most popular, easy and reliable method to measure the stability of PNA-DNA hybrid systems is the melting temperature (T_m), that is the monitoring of the changes in UV absorption at about 260 nm as a function of temperature.[5] The higher affinity of PNA-DNA binding is expressed by the larger melting temperature values of PNA-DNA hybrids with respect to those of DNA-DNA ones. In addition to melting temperature measurements, isothermal titration calorimetry (ITC)[6] and atomic force microscopy (AFM) have been proposed as suitable methods.[7] But the thermodynamic data are obtained using a big quantity of materials failing to provide information on

the kinetics of the interaction. Surface Plasmon Resonance (SPR)[8] and SPR-enhanced fluorescence spectroscopy[9] have been used to investigate also the kinetics of DNA-DNA and PNA-DNA interactions but every technique shows some advantages along with some drawbacks and a detailed description of the phenomenon is thus limited.

The stopped-flow absorbance spectroscopy and fluorescence intensity method[10,11] offers a powerful tool for a detailed kinetic analysis of interactions since it can monitor reactions continuously in real time and in a real physiological medium.[12,13] It has been used in the past to obtain a detailed understanding of locked nucleic acid (LNA)[14] and PNA[15] as a trapping strand in helicase-catalyzed unwinding of oligonucleotide substrates. But fluorescence stopped-flow spectroscopy has never been used for a detailed PNA interaction study with parallel and antiparallel DNA with the aim of measuring the different affinities of the formed duplexes. Moreover, Gangamani et al. have previously demonstrated that intrinsic fluorescent PNA analogues can be used to monitor PNA self-melting and PNA-DNA duplex transitions without affecting or perturbing their structures.[16] The objective of this work is to study PNA-DNA interaction from a kinetic point of view with a non-consuming and fast spectroscopic method. With this technique, with respect to steady-state spectroscopy, in a single experiment, the data that can be collected are many: a complete kinetic profile can be obtained as well as a thermodynamic description of the interaction.

In the present work, the PNA decamer **6**, with the TCACTAGATG sequence of nucleobases, and the corresponding fluorescent PNA-FITU (fluorescein isothiourea) decamer **8** were synthesized with standard manual Boc-based chemistry. The interaction of both decamers **6** and **8** with the complementary parallel and antiparallel DNA was followed by steady-state and stopped-flow fluorescence intensity. The kinetics of the PNA-DNA duplex formation has been studied. In particular, fluorescence stopped-flow technique has been exploited to compare the affinity of two PNA-DNA duplexes: using parallel and antiparallel DNA, such data would be very important for the evaluation and improvement of antisense reagents and of diagnostic probes based on PNA.

2. Experimental Section

2.1. Materials

The thymine monomer *aeg*-(T)PNA-COOH was synthesized according to the literature.[17] The other three monomers containing the nucleobases C, G and A were purchased from ASM Research Chemicals and were used as such without further purification. 11-Mercaptoundecanoic acid (MUA), 11-bromoundecanethiol, *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU), 1-[1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), fluorescein isothiocyanate isomer I (FITC), *N(α)*-Boc-*N(ε)*-Fmoc-*L*-lysine, trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA) and *m*-cresol were purchased from Aldrich. *N*-methyl-2-pyrrolidone

(NMP), *N*,*N*-diisopropylethylamine (DIPEA) and thioanisole were purchased from Fluorochem. Polystyrene bead carrying 4-methylbenzhydrylamine hydrochloride salt groups (MBHA resin, 0.63 mmol/g) was purchased from VWR International.

Parallel DNA (5'-3' sequence: CATCTAGTGA) and antiparallel DNA (5'-3' sequence: AGTGATCTAC) were purchased from Eurofins Genomics, Germany.

2.2. Experimental techniques

UV-Vis measurements for the evaluation of the PNA-FITU concentration and the FITC labelled and unlabelled PNA-DNA interaction studies were recorded using a Varian cary 300 Bio UV-Visible Spectrophotometer.

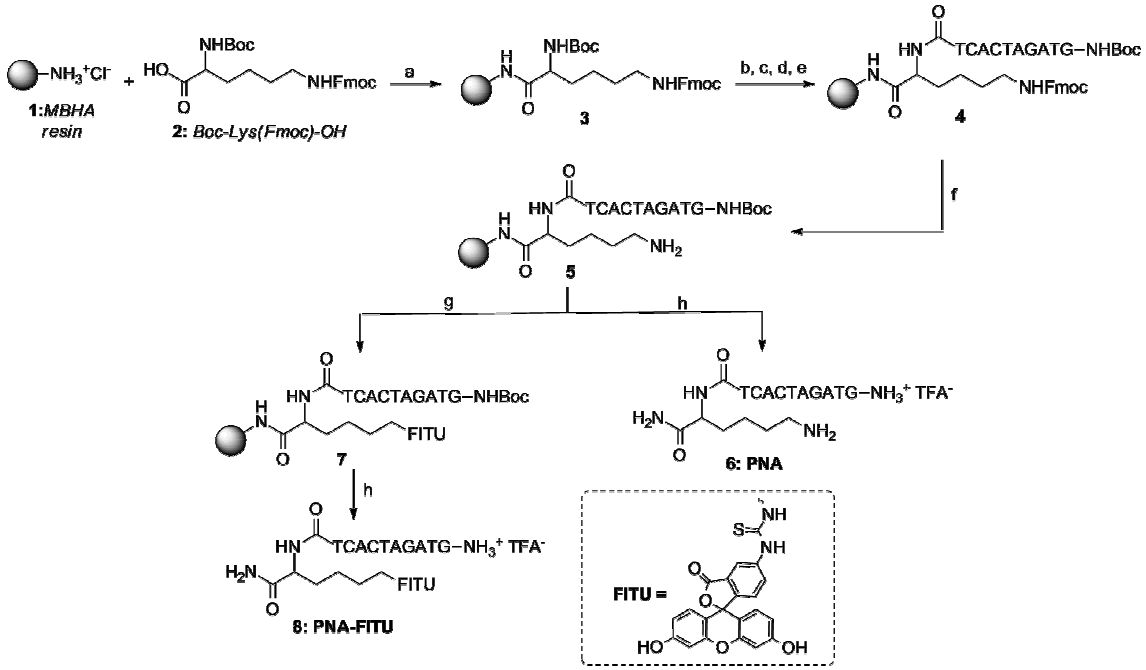
Steady state fluorescence measurements were recorded using a Fluorolog 2 from Jobyn Ivon. As a preliminary analysis for the stopped-flow binding experiments, steady-state fluorescence spectra were recorded in the range of 495–650 nm upon excitation at 480 nm, at 25 °C, in order to investigate the binding of PNA-FITU to DNA.

Fluorescence kinetics measurements were recorded using an Applied Photophysics SX20 stopped-flow spectrometer fitted with a 515 nm cut-off filter between the cell and fluorescence detector and equipped with a thermostat bath. The excitation wavelength was 480 nm and slits widths of the excitation monochromator were 1.0 mm. Data acquisition, visualisation and analysis were provided by Pro-Data software from Applied Photophysics Ltd.

2.3. Synthesis

The PNA decamer **6,** with the TCACTAGATG sequence of nucleobases, and the corresponding fluorescent PNA-FITU decamer **8** were synthesized with standard manual Boc-based chemistry using MBHA resin loaded with the *N(α)*-Boc-*N(ε)*-Fmoc-*L*-lysine in order to obtain functionalized resin **3** with loading 0.2 mmol/g. (Scheme 1).

2.3.1. Synthesis of PNA decamer 6



Scheme 1. Synthesis of PNA-FITU **8** and PNA **6**. a) HATU, DIPEA, NMP; b) TFA/*m*-cresol, 95:5; c) CH_2Cl_2/ DIPEA, 95:5; d) PNA monomer, HATU, DIPEA, NMP e) Ac_2O/Pyridine/NMP 1:25:25; f) Piperidine 20% in DMF; g) FITC, DIPEA, DMF; h) TFA/TFMSA/thioanisole/*m*-cresol 6:2:1:1 (v/v).

The MBHA resin **3** (100 mg, loading 0.2 mmol/g) downloaded with lysine monomer, was first swollen with CH_2Cl_2 for 30 min, and the *tert*-butyloxycarbonyl (Boc) group of the loaded lysine was removed by treatment with TFA/*m*-cresol (95:5). The resin was then rinsed with CH_2Cl_2 and a solution of DIPEA in CH_2Cl_2 (5% w/w). In a vial, a solution of DIPEA (35 µL, 0.2 mmol, 10 eq) and the thymine monomer (40.7 mg, 0.106 mmol, and 5.3 eq) in NMP was added to a solution of HATU (38.02 mg, 0.1 mmol, 5 eq) in NMP, and the resulting mixture was shaken for 2 min. The activated mixture was then added to the resin and shaken at room temperature for 2 h. After each coupling step, the resin was washed with NMP, and then treated with a solution of Ac_2O/pyridine/NMP (1:25:25, v/v) twice for 2 min. The cycle was repeated using the required monomers like cytosine (53.4 mg), adenine (56 mg) and guanine (57.6 mg) with the same protocol to afford the corresponding supported PNA 10-mer on resin **4**. Then, the Fmoc group was removed by treatment with a solution of piperidine in DMF (20% w/w) to give resin **5**, for which the presence of the free amino group was confirmed by the Kaiser test for amines. This resin was then washed with TFA, and subsequently stirred for 1 h with a mixture of TFA/TFMSA/thioanisole/*m*-cresol (6:2:1:1 v/v). The reaction mixture was filtered, and the resin washed with TFA. The filtrate was concentrated, and Et_2O was added to precipitate PNA as a white solid. Centrifugation of the slurry gave the product, which was washed with Et_2O and dried to afford the crude decamer **6** (28 mg). Purification of the crude PNA by RP-HPLC (tR = 6.2 min) afforded the PNA 10-mer **6** (7.4 mg) as a white solid. ESI/MS: found m/z: 2855.9 $[M]^+$, calculated for $C_{114}H_{149}N_{60}O_{31}^+$: 2855.83.

2.3.2. Synthesis of PNA-FITU 8

MBHA resin **5,** with the free amine group on lysine (100 mg), was directly reacted with fluorescein isothiocyanate [FITC]. In particular, a solution of DIPEA (34 µL, 0.2 mmol, 8 eq) and FITC (38.60 mg, 0.099 mmol, 4 eq) in DMF was added to the resin and shaken at room temperature for 12 h. Then the resin was washed twice with DMF, DCM, and finally with TFA, and then stirred for 1 h with a mixture of TFA/TFMSA/thioanisole/*m*-cresol 6:2:1:1. The reaction mixture was filtered, and the resin washed with TFA. The filtrate was concentrated, and Et_2O was added to precipitate PNA as a yellow solid. Centrifugation of the slurry gave a solid, which was washed with Et_2O and dried to afford the crude decamer **8** (26 mg). Purification of the crude PNA by RP-HPLC (tR = 12.9 min) afforded the PNA-FITU **8** (7 mg) as a yellow solid. MALDI-TOF MS: found m/z: 3246.2 [M]+, calculated for $C_{135}H_{160}N_{61}O_{36}S^+$: 3245.22.

2.4. Stopped Flow experiments

Due to the low solubility of both PNA and PNA-FITU, mother solutions (≈ 6 µM) were prepared by dissolving a certain amount of PNA and PNA-FITU powder in 0.1 M phosphate buffer pH 7. Then, the concentration was checked by UV-Vis spectroscopy both by means of the PNA (62348 $M^{-1}cm^{-1}$) and FITC (103500 $M^{-1}cm^{-1}$) molar extinction coefficients. Dilutions and working solutions were performed in 0.1 M phosphate buffer pH 7.

DNA mother solutions (100 µM) were prepared in MilliQ water while the dilutions were performed in 0.1 M phosphate buffer pH 7.

In stopped-flow experiments, PNA-FITU concentration (0.12 µM in 0.1 M phosphate buffer pH 7) was kept constant and several shots of different antiparallel DNA concentrations were performed over the range 0.8-15 µM in 0.1 M phosphate buffer. The concentrations used for parallel DNA were in the range 0.8-8 µM. The reported concentrations are all cell concentrations, i.e. the real concentration in the cuvette. Each experiment for each kind of DNA (whole concentration set) was repeated six times, each time using new mother solutions of PNA-FITU and DNA. For each dilution, at least five scans were acquired and averaged. Each experimental point is therefore an average of at least 30 shots. Raw data were analyzed and plotted to a single exponential function by using Pro-Data Viewer 4.0.17 and, from this data treatment, the observed rate constants (k_{obs}) were obtained.

2.5. Displacement experiments

The displacement experiment was performed according to Eccleston et al.[10] A solution containing a mixture of 1 µM FITC biolabelled PNA with 1 µM DNA was rapidly mixed with a large excess (50 µM) of unlabelled PNA. The displacement was repeated several times and the k_{off} obtained is an average value of these experiments.

2.6. Steady-state experiment

The steady-state experiment was performed by keeping PNA-FITU concentration constant (0.2 μM) and titrating an increasing concentration of antiparallel DNA in the concentration range 1.6-16 μM in order to reach saturation. The fluorescence intensity maxima of the spectra were obtained by exciting at 480 nm and collecting the spectra in the 480-650 nm range.

3. Results

PNA decamer **6** and fluorescein isothiocyanate (FITC) labelled PNA decamer **8** (PNA-FITU) were synthesised with a standard manual Boc-based chemistry (Scheme 1). *N(α)*-Boc-*N(ε)*-Fmoc-*L*-lysine loaded MBHA resin (loading 0.2 mmol/g) was used to build the TCACTAGATG PNA decamer sequence. The *(ε)* amino group of the lysine was used as anchoring point for the FITC. Therefore, in decamer **6** the *(ε)* amino group of the lysine residue is present as a free amino moiety, while in decamer **8** it is conjugated with the FITC, becoming PNA-FITU (Fluorescein isothiourea).

The interaction of both decamers **6** and **8** with the complementary parallel and antiparallel DNA was followed by steady-state and stopped-flow fluorescence intensity.

Preliminary UV-Vis and steady-state fluorescence experiments were performed in order to check the complex formation. The steady-state experiment was performed by keeping PNA-FITU concentration constant (0.2 μM) and titrating an increasing concentration of antiparallel DNA in the concentration range 1.6-16 μM in order to reach saturation (Figure 2a). The fluorescence intensity maxima of the spectra were plotted against DNA concentration and the data were fitted to a rectangular hyperbola (Figure 2b) to get the thermodynamic binding constants.[18,19]

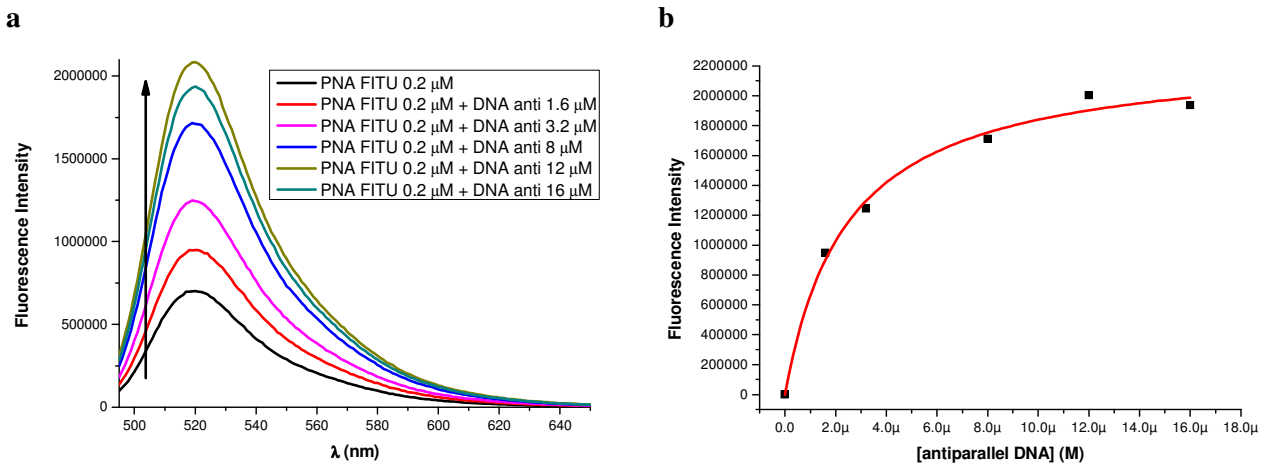


Figure 2. (a) Titration curves of the PNA-FITU *vs* antiparallel DNA interaction followed by steady-state fluorescence and (b) plot of the fluorescence intensity maxima (black squares) against DNA concentration and non linear fitting to hyperbole (red curve) to get the thermodynamic binding constants.

Once it was evaluated that the fluorescent biolabeling did not interfere in the binding, a kinetic binding experiment was made by stopped-flow fluorescence. The binding was investigated under *pseudo*-first order condition[20] ([DNA] >> [PNA-FITU]) by monitoring the fluorescence changes

after the formation of the complex. We need to work under this condition because only in this range of concentration the kinetic parameters can be calculated (i.e. equation 1 is valid). The two interactions (PNA-FITU versus both parallel and antiparallel DNA) were studied by keeping PNA-FITU concentration fixed at 0.12 μM and changing the DNA concentration over the range 0.8-8 μM. Figure 3 shows that on mixing 0.12 μM PNA-FITU with 4 μM DNA (cell concentration) there is a decrease of the signal which reaches a plateau after few ms. Raw data were analyzed and plotted to a single exponential function providing the so called observed rate constant (k_{obs}). Figure 4 shows the dependence of the k_{obs} of DNA (both parallel and antiparallel) binding to PNA. The different behavior when comparing parallel and antiparallel DNA is evident: in the case of antiparallel DNA binding to PNA-FITU, the k_{obs} evaluation is reproducible and the dependence with concentration is pronounced. Moreover, the repeatability is very good with a standard deviation for each concentration point being extremely small. A completely different scenario is recorded for parallel DNA where the fitting of the single curves to get k_{obs} is more difficult and the fitting to get any insight on the association and dissociation constants of the overall interaction results nearly impossible (Figure 4b). The Watson-Crick duplex formation (anti-parallel duplex formation) is the preferred formation for the PNA bindings than the parallel duplex structure,[4] and we can suppose a "loosely-bound state"[21] for PNA-FITU with parallel DNA. On the other hand, the preference for the antiparallel binding orientation seems to be a general feature of mixed sequence PNA-DNA duplexes. Kinetic binding studies, for example employing capillary gel electrophoresis, have shown that PNA-DNA duplex formation is very fast (<30 s) for antiparallel hybrids, whereas considerably slower kinetics were observed for parallel complexes.[22] In any case, we can observe that k_{obs} dependence from [DNA] is, in term of slope of the line, comparable for both parallel and antiparallel DNA. The fact that the antiparallel DNA interaction is stronger and more reproducible is also in agreement with the T_m measurements.[23]

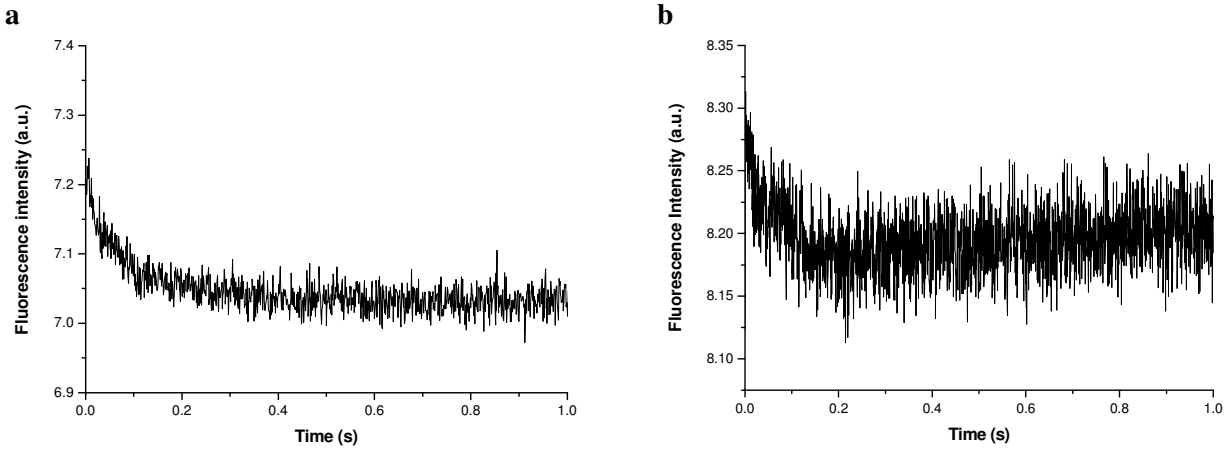


Figure 3. The stopped-flow fluorescence intensity record of the binding of 0.2 μM PNA-FITU to (a) 4 μM antiparallel DNA and (b) 4 μM parallel DNA. The reported concentrations are real cell concentrations.

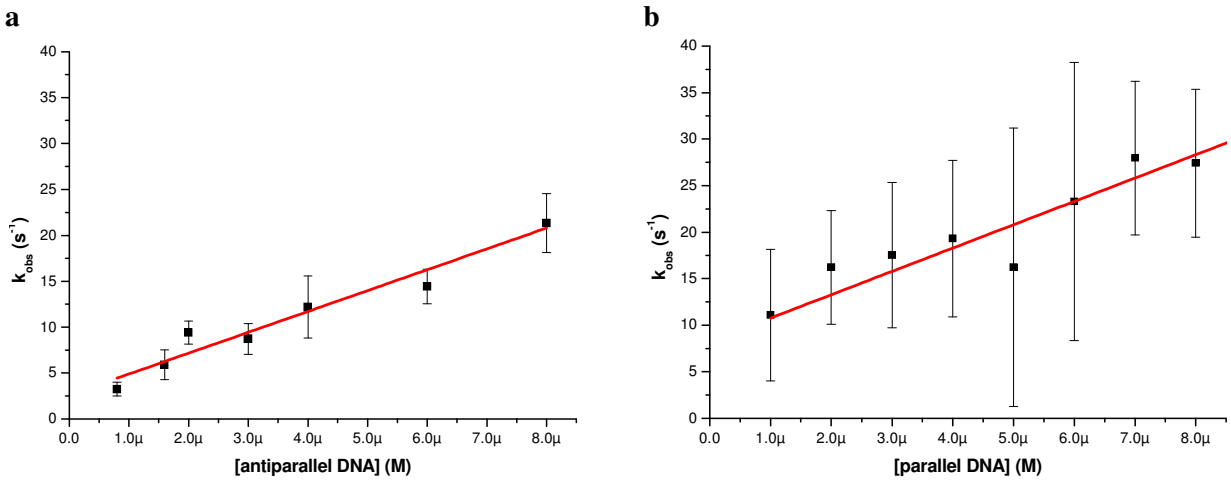


Figure 4. k_{obs} dependence for antiparallel DNA (a) and parallel DNA (b). Note that the same magnitude is used for a better comparison.

For the PNA-FITU/antiparallel DNA interaction, the values of the kinetic parameters (k_{on} and k_{off}) can be calculated from the slopes and intercepts of the linear plots of k_{obs} versus increasing concentration of DNA (see equation 1)[10].

$$k_{obs} = k_{on}[DNA] + k_{off} \qquad (1)$$

The slope of the straight line is the k_{on} (second-order rate constant or rate constant of association process; units, M^{-1} s^{-1}) and the intercept on the ordinate is the k_{off} (first-order rate constant or rate constant of dissociation process; units, s^{-1}). For the PNA-FITU interacting with antiparallel DNA, the second-order rate constant k_{on} is $2.27 \cdot 10^6 \pm 1.12 \cdot 10^5$ $M^{-1}s^{-1}$. As it can be seen, the intercept on the ordinate has a very small value (2.62 ± 0.56 s^{-1}). This means that the interaction is nearly completely shifted toward the formation of the complex. This interaction was also studied at higher concentrations of antiparallel DNA (up to 15 µM) to further monitor the interaction and data are shown in Figure 5 obtaining almost the same kinetic constants.

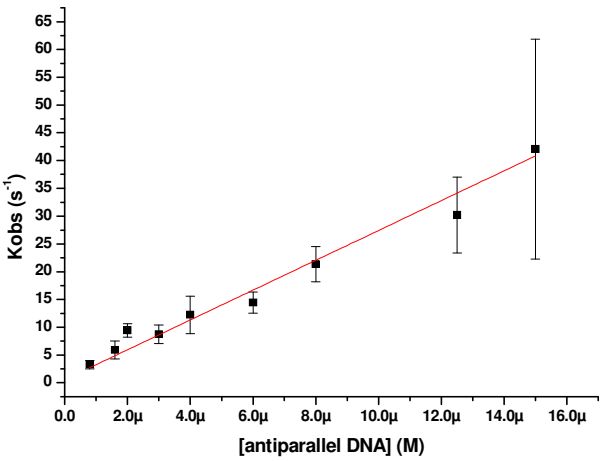
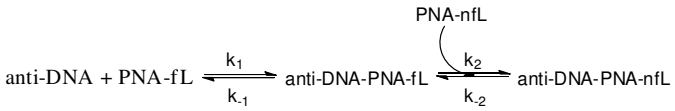


Figure 5. k_{obs} dependence for antiparallel DNA in the 0.8-15 µM range.

Since the dissociation rate constant value seems to be small, the k_{off} obtained from the intercept cannot be reliable. To gain more information and to measure k_{off} value accurately, a displacement experiment was executed as proposed previously for a protein-protein interaction.[12] Briefly, in the displacement experiment (Scheme 2), a solution containing fluorescent PNA-FITU (PNA-fL) and DNA is mixed together. The concentration of PNA-FITU is chosen to get a saturation of DNA, then a high concentration of unlabeled PNA (PNA-nfL) is added so that PNA-FITU dissociates from DNA and cannot re-associate. In this way the rate constant of the observed process is determined only by the k_{-1} in Scheme 1 which is what we measure directly by the displacement of PNA-FITU from its complex with DNA using an excess of unlabeled PNA. Experimentally, the increasing fluorescence intensity was monitored during the process and the corresponding record was fitted to give directly an average rate constant k_{off} value of 32.2 s^{-1} (Figure 6).



Scheme 2. Scheme of the displacement experiment.

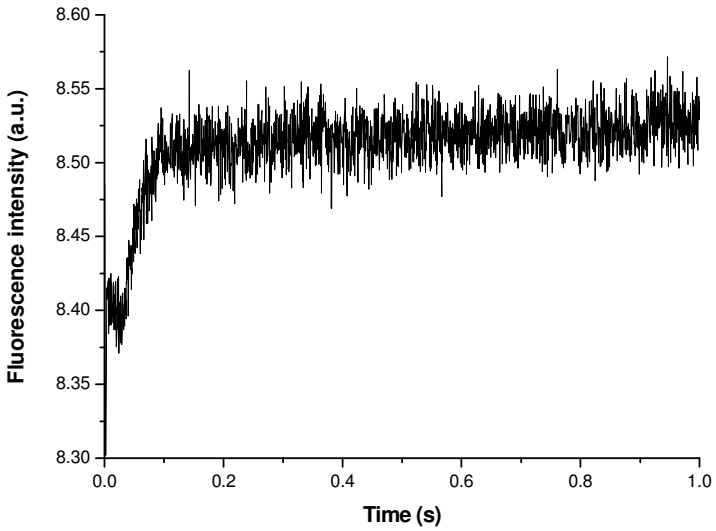


Figure 6. Displacement experiment: 50 μM of unlabelled PNA (PNA-nfL) is used to displace DNA (1 μM) to PNA-FITU (PNA-fL, 1 μM).

This k_{off} value together with that obtained for the k_{on} allowed us to calculate the thermodynamic dissociation constant K_d (i.e. k_{off}/k_{on}) value of 14.2 μM for PNA-FITU/antiparallel DNA interaction, being in the same order of magnitude compared to the K_d obtained by steady-state fluorescence in the same buffer (2.5 μM) (Figure 2).

A further study on the temperature dependence of the binding constants was undertaken. The same interaction experiments were repeated at 50 °C for both DNAs. The dependence of k_{obs} on parallel and antiparallel DNA concentration was again investigated in the range 0.8-8 μM and is reported in Figure

7. As is evident, in the case of parallel DNA, there is no temperature dependence of the interaction. On the contrary, the behavior of PNA/antiparallel DNA interaction is different when working at 25 or 50 °C. In this case, it is possible to express numerically the strength of the two interactions, as reported in Table 1. As expected, the binding constant increases by increasing the working temperature.

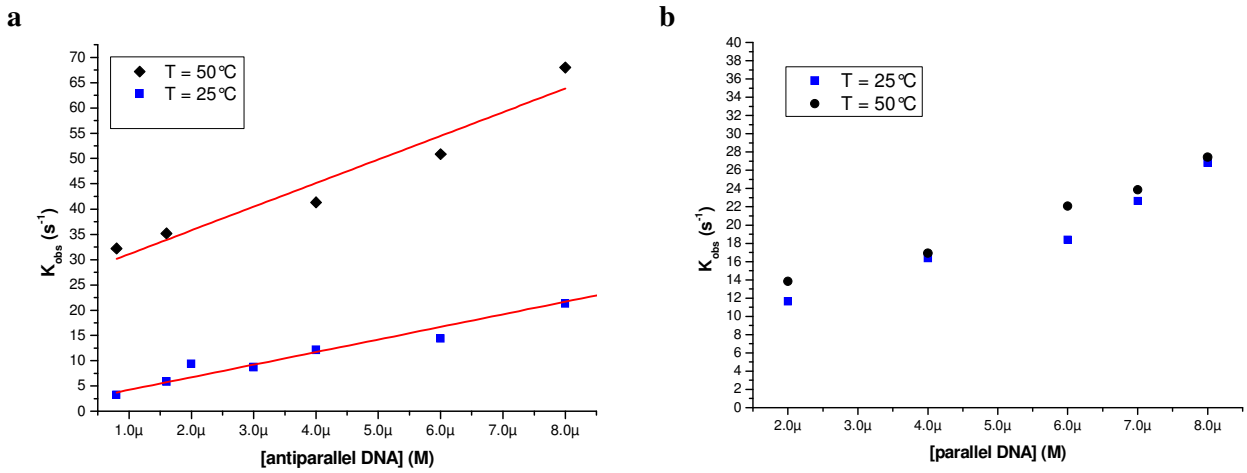


Figure 7. Temperature dependence of k_{obs} for (a) antiparallel DNA and (b) parallel DNA.

Table 1. Thermodynamic and kinetic binding constants for PNA-FITU *vs* antiparallel DNA interaction at different temperatures.

Temperature (°C)	K_a ($\cdot 10^4$) M^{-1}	K_d ($\cdot 10^{-6}$) M	K_{on} ($\cdot 10^6$) M^{-1} s^{-1}	K_{off} s^{-1}
25	7.05	14.18	2.27	32.2
50	17.69	5.65	4.67	26.4

Conclusions

The PNA decamer **6** and the corresponding fluorescent PNA-FITU decamer **8** were synthesized with standard manual Boc-based chemistry. The interaction of both decamers with the complementary parallel and antiparallel DNA was followed by steady-state and stopped-flow fluorescence intensity. In particular, fluorescence stopped-flow technique has been exploited to compare the affinity of two PNA-DNA duplexes: using parallel and antiparallel DNA, such data would be very important for the evaluation and improvement of antisense reagents and of diagnostic probes based on PNA.

Stopped-flow fluorescence showed the poor binding of PNA with its parallel DNA but was very useful for the determination of the binding constants for the PNA-antiparallel DNA interaction. The kinetic and thermodynamic constants obtained by stopped-flow fluorescence were in complete agreement with those obtained by steady-state measurements.

To conclude and to answer to the question present in the title, the stopped-flow has proved to be a reliable, non-consuming, in terms of time and sample, and fast method to discriminate the different

behavior between the parallel and antiparallel DNA. In the case of the interaction between PNA and antiparallel DNA, stopped-flow fluorescence has revealed its power, with respect to the melting temperature, in the possibility of working in a physiological environment with a real mimic of the interaction.

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