EXCITING INSTRUMENTS

Rapid characterisation of pH-sensitive DNA nanoswitches on the EI-FLEX system

Application Note

Rapid characterisation of pH-sensitive DNA nanoswitches on the EI-FLEX system

In this application note, we showcase the use of single-molecule Förster Resonance Energy Transfer (smFRET) on the EI-FLEX in the development of a surface-immobilised, pH-dependent DNA nanoswitch. Dynamic DNA machines, like the DNA nanoswitch discussed here, can switch between conformational states in response to environmental conditions. For many applications, it is advantageous to immobilise these DNA machines on a surface for use in solid-state devices.

D'Rozario et al. used solution-based smFRET in tandem with circular dichroism spectroscopy to quantify the conditions required for conformation switching of a DNA nanoswitch prior to surface immobilisation. Once the proper conditions were determined and conformational switching was confirmed, the DNA nanoswitches were deposited on a surface in a monolayer to verify that surface immobilisation did not disrupt the conformational dynamics measured with the EI-FLEX[1].

Overview of this application note:

- Single-molecule FRET can rapidly resolve open, closed and mixed configurations of a DNA triplex nanoswitch at different pH values using picomolar concentrations
- smFRET can be used to calculate the solution-phase dissociation (pKa) pH
- Surface immobilisation of the nanoswitch does not affect pH-dependent behaviour

Glossary of terms used in this application note

FRET efficiency (E): A measure of how effectively energy is transferred from a donor dye to a nearby acceptor dye. It is determined from the ratio of acceptor emission to total emission detected when only the donor laser is active. High FRET efficiency indicates that the labelled sites are closer to each other, low FRET efficiency indicates they are further apart.

Alternating Laser EXcitation (ALEX): A measurement scheme in which a donor and an acceptor laser are switched on and off in rapid sequence, hitting each molecule multiple times with either laser. This allows emission arising from donor and acceptor excitation to be distinguished. This enables the calculation of stoichiometry, which is used to identify and separate doubly labelled molecules from donor-only and acceptor-only species.

Circular Dichroism Spectroscopy: An ensemble technique that can determine the helicity of DNA by measuring the absorbance of left and right circularly polarised light. This enables the identification of DNA in different forms, such as duplexes and triplexes.

The EI-FLEX System

The EI-FLEX brings a biophysics professor into any lab with one simple, confocal benchtop solution that rapidly reveals physiologically-relevant behaviour without immobilising targets or requiring large sample volumes, all at single-molecule precision. With easy-to-use acquisition and analysis protocols and fully automated, high-throughput options available, high-quality data and publication-ready figures can be generated with ease.

How did the EI-FLEX benefit this work?

- Provided rapid characterisation of a pH-dependent nanoswitch across a range of pH values, demonstrating conformational changes upon protonation
- ALEX and accurate FRET correction protocols enabled the calculation of correction factors to account for photophysical artefacts
- Provided highly complementary smFRET data to be used with other biophysical techniques to confirm observations and compare between in-solution and immobilised nanoswitches

The EI-FLEX single-molecule spectrometer

Resolving open, closed and mixed configurations of a DNA triplex nanoswitch at different pH values

To test the pH-responsiveness of a DNA triplex nanoswitch, the authors labelled their construct with a Cy3 fluorophore (donor) on the polypurine strand (nPu), and a Cy5 fluorophore (acceptor) on the polypyrimidine strand (nPy) (Figure 1). When in the closed formation, Hoogsteen interactions form alongside Watson-Crick base-pairing and the two fluorophores are brought into close proximity, promoting donor fluorescence energy transfer to acceptor fluorophores.

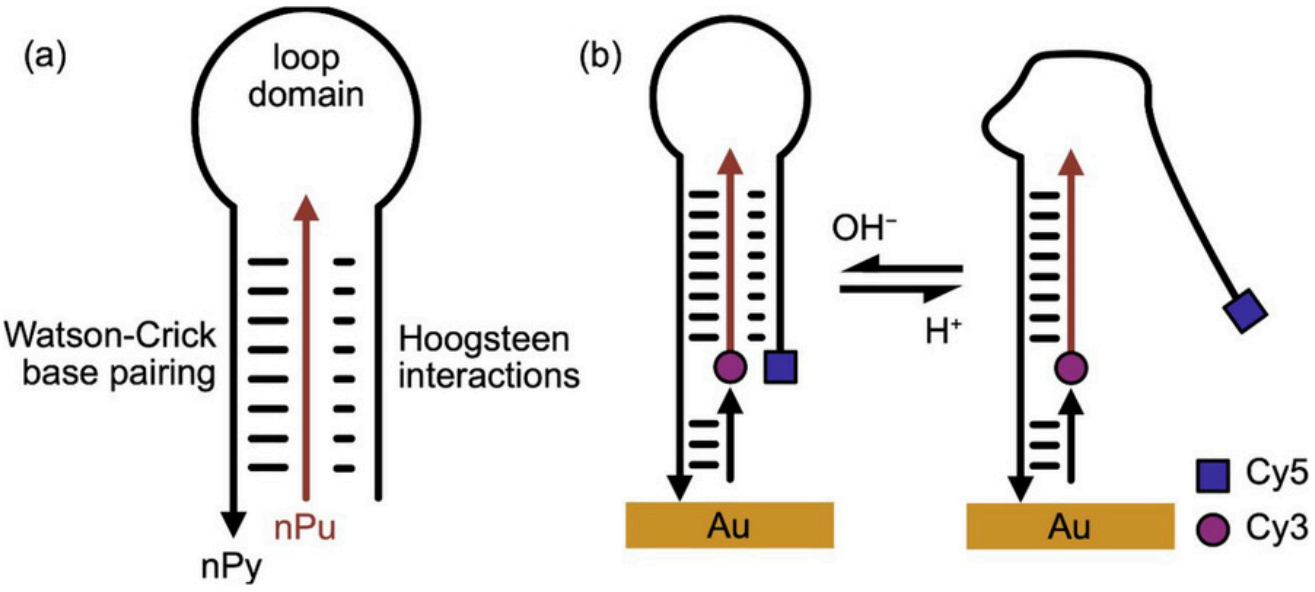

Figure 1 – Structure of the DNA nanoswitch
a) The overall structure of the DNA triplex containing both a polypyrimidine (nPy) and polypurine (nPu) strand
b) Closed (left) and open (right) configurations of the nanoswitch, labelled with the Cy3 donor and Cy5 acceptor fluorophores

smFRET experiments were performed on the EI-FLEX, using alternating laser excitation (ALEX) to calculate the stoichiometry of labelled molecules and ensure only doubly-labelled molecules were used for FRET efficiency calculations. FRET efficiencies for picomolar concentrations of the DNA nanoswitch were then quantified using a series of buffers ranging from pH 6 to pH 8.2 at a temperature of 20 °C.

Resolving open, closed and mixed configurations of a DNA triplex nanoswitch at different pH values

Changes in FRET efficiency were used to characterise the effect of protonation on the opening and closing of the DNA nanoswitch. Here, D'Rozario et al. found that the nanoswitch configuration was predominantly in a closed, high-FRET state (fluorophores close together) at pH 7.6, transitioning to an open, low-FRET state (fluorophores far apart) at pH 8.1 (Figure 2). Strikingly, smFRET assays permitted the identification of a heterogeneous state at pH 7.8, whereby approximately half the molecules were in open and closed states, respectively.

Another advantage of using smFRET to characterise nanoswitches in this manner is that any deviation from the expected binary outcomes of switch opening and closing can be easily identified. For example, if a mid-FRET state was identified, this could indicate that the nanoswitch experiences an off-path non-productive conformation, or that there is a previously unidentified intermediate that must be transitioned through.

To confirm that the transition from the open to closed conformation is indeed the formation of DNA triplexes from duplexes, the nanoswitch was also analysed by circular dichroism spectroscopy. The spectra at pH 7.2 and 8.8 showed patterns consistent with triple-stranded DNA and B-form duplex DNA, respectively, which is in agreement with the smFRET data that shows the nanoswitch was in triplex (closed) and duplex (open) conformations around those pH values.

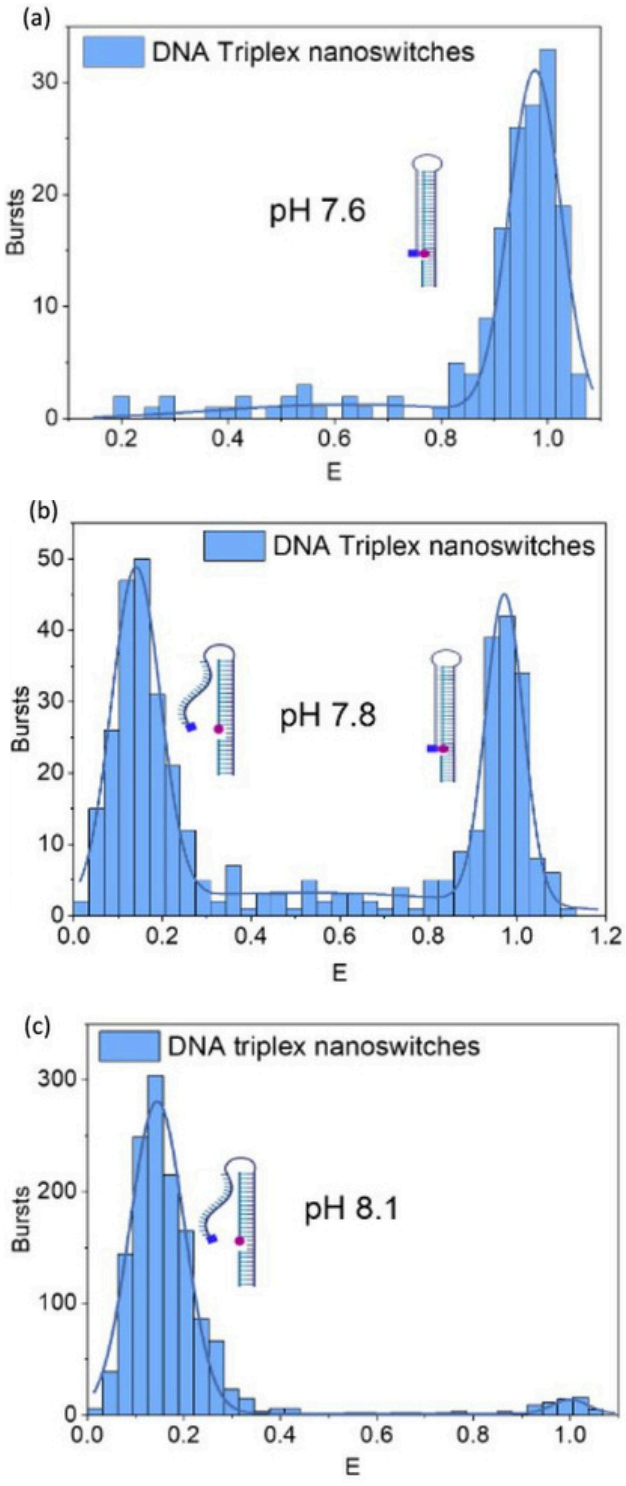

Figure 2 – smFRET and circular dichroism spectroscopy demonstrating the pH-dependent shift in nanoswitch conformation

a) High-FRET population at pH 7.6 (closed state)
b) double population at pH 7.8 (mixed conformations of open and closed states)
c) low-FRET population at pH 8.1 (open state)

Solution-phase and surface-immobilised pKa values are similar for the DNA triplex nanoswitch

These data were used to calculate the solution-phase dissociation (pKa) (the pH at which half of the nanoswitches are in the closed conformation), which was found to be pH 7.83 (Figure 3a). Following immobilisation onto a 2-dimensional DNA monolayer, the authors investigated the resultant behaviour of the nanoswitch using quartz crystal microbalance with dissipation monitoring (QCM-D). This technique captures the viscoelasticity of samples, whereby the rigidity of the immobilised nanoswitch can be detected; the closed nanoswitch is more rigid than the open nanoswitch.

In this case, the pH value at which half of the nanoswitches were in the closed conformation was calculated using the shift in dissipation (how much energy is being lost due to molecule dragging caused by high hydration). This was determined to be at pH 8, which is in close agreement with the pKa value derived from the smFRET data (Figure 3b).

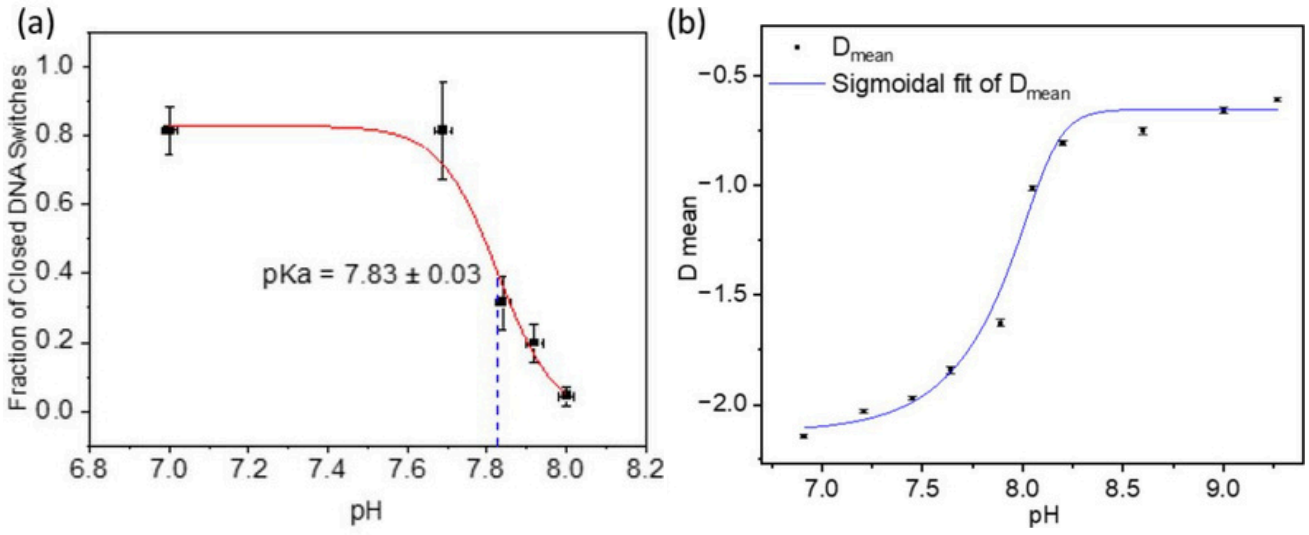

Figure 3 – Calculation of solution-phase dissociation (pKa) value and on-surface triplex dissociation
a) smFRET data was used to calculate the pKa value by plotting the fraction of closed nanoswitches against a range of pH values
b) A sigmoidal curve demonstrating the dissociation of the DNA triplex from the surface as pH value increases

Summary

In summary, smFRET can rapidly resolve the open, closed and mixed conformations of pH-dependent DNA nanoswitches, illustrating that the constructs are behaving in a binary manner as designed. In combination with circular dichroism spectroscopy, the change in nanoswitch conformation was confirmed to be the production of a DNA triplex from a duplex. The use of single-molecule, in-solution FRET prior to immobilisation enabled swift characterisation of the pH-dependency of the nanoswitch, which was then seen to be preserved once the construct was anchored on a surface.

For a deeper dive on the techniques used in this application note, we recommend exploring our Resource Library. Discover a range of applications for smFRET and the EI-FLEX system on our website.

References

1. D'Rozario, F. et al. Electronic Actuation of Surface-Immobilized, pH-Responsive DNA Nanoswitches. ACS Appl. Mater. Interfaces 18, 18039–18048 (2026).

All data used in this application note was generated by the authors cited in this publication.

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