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ARTICLE TYPE

Design of ultrasensitive DNA-based fluorescent pH sensitive nanodevices

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Here we tune the pH sensitivity of a DNA-based conformational switch, called the I-switch, to yield a set of fluorescent pH sensitive nanodevices with a collective, expanded pH sensing regime from 5.3 to 7.5. The expanded pH regime of this new family of I-switches originates from a dramatic improvement in the overall percentage signal change in response to pH of these nanodevices.

The availability of non Watson-Crick basepairing in DNA has led to the discovery of several functional DNA architectures which have been deployed *in cellulo* to yield insight on intracellular chemical environments.[1] In 1993, Gueron and co-workers found that the DNA sequence d(C_5T) can form a special tetraplex structure called the i-motif under acidic conditions where two parallel duplexes paired via C•CH+ pairs intercalated with each other in a head to tail orientation.[2, 3] It has applications as a pH reporter[4-9] in the context of synthetic DNA-based conformational switches,[6, 7] where an i-motif induced conformational change is transduced into a photonic output[8, 9] using FRET. One such nanodevice called the I-switch has been used to study pH of endocytic organelles in cells and *in vivo*. However, different intracellular organelles maintain a different resting pH that varies from pH 5.0 (lysosomes) to pH 8.0 (mitochondria) and thus, there is a need to engineer I-switches which can respond to the whole physiological range.

There is evidence that the pH responsive regime of i-motif based conformational switches may be tuned by increasing the number of cytosine in a stretch.[10, 11, 12] However, the cooperativity of folding also correlates directly with i-motif stability.[13, 14] As a result, along with the increase in the midpoint of the pH-induced structural transition (pH_{half}) there is an unavoidable increase in its cooperativity that narrows the overall pH sensitive regime.[14] A narrower pH regime is useful in certain contexts as it provides better pH resolution. It is however, highly desirable to alter the pH_{half} without overly affecting the cooperativity.

Cytosine hemiprotonation drives i-motif formation. The pK_a of Cytosine N3 is 4.45 and thus DNA_4 i-motifs are maximally stabilized at ~pH 5.0.[15] This typically results in I-switches with pH reporting capacity at 5.5<pH<7.0. We reasoned that introduction of chemically modified cytosines[16, 17] with a lower or higher pK_a such as 5'-Bromocytosine (pK_a 2.5) or 5'-Methylcytosine (pK_a 4.7) [18] could accordingly tune I-switch response by altering the

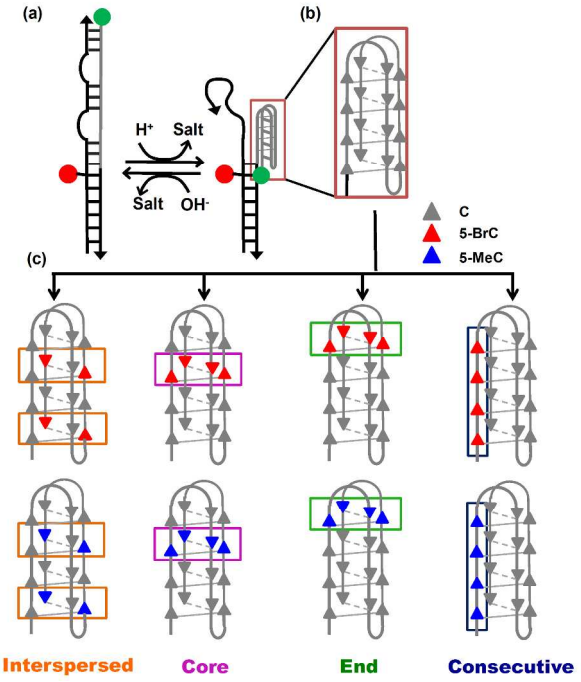

Fig 1. Schematic representation of the working principle of the I-switch and its various modifications. (**a**) Working principle of the I-switch; C-rich domain, donor and acceptor fluorophores are shown in grey, green and red respectively. (**b**) Schematic of i-motif formed in I-switches. Cytosine (grey triangles) modified cytosines (coloured triangles) positions are indicated. Triangle apices point towards the 3' strand terminus. (**c**) I-switch variants incorporating modified cytosines used in this study.

pH_{half} of the structural transition while possibly maintaining the cooperativity. We observed that fully-brominated or fully-methylated cytosines in the C-rich domain of I4 failed to form i-motifs at room temperature in our hands.[16] Thus, we doped I4 with cytosine modifications at specific positions while keeping the total number of modified cytosines per I-switch constant (N=4) to see if this could alter the pH_{half} of the structural transition. The new family of I-switches incorporates a stretch of four, pH sensitive, C-rich segment $(C_4TAA)_3C_4$ that forms a mismatched duplex at neutral or basic pH with a partially complementary G-rich strand (*TTT*GTTA*T*G*T*GTTA*T*G*T*GTTA*T*), where *T* indicates mismatches. In this I-switch design, the C-rich segment bears

a donor fluorophore (green sphere, Alexa 488) and the mismatched duplex positions an acceptor fluorophore (red sphere, Alexa 647) far apart (Fig. 1a). At acidic pH, the mismatched duplex frays as the C-rich strand forms an i-motif, bringing the two fluorophores into a high FRET conformation that may be monitored by fluorescence spectroscopy. The perfect duplex domain (black) harbors a binding site for a recombinant antibody to enable targeting. Figures 1b&c shows only the i-motif domain of the I-switch where only the cytosine residues are represented as triangles, the apices of the triangles pointing towards the 3'end of the strand. The grey triangles represent unmodified cytosines while the red and blue triangles represent sites that incorporate either 5'-Bromocytosines (Br I-switches, red triangles) or 5'-Methylcytosines (Me I-switches, blue triangles) respectively in the modified I-switches. Figure 1c shows the four classes of modified I-switches we have investigated categorized on the basis of their relative positions in the i-motif domain of the I-switch. The first class incorporates either two all-bromo or two all-methyl modified C_m-H^+-C_m base pairs at positions 3, 9, 17, 23 that lie at the core of the resultant i-motif (**Core**) with the reasoning that these could possibly affect the nucleation event for i-motif formation. The second class incorporates either two all-bromo or two all-methyl modifications at the peripheral C_m-H^+-C_m base pairs at positions 1, 11, 15, 25 (**End**) with the reasoning that these could modulate i-motif fraying and thereby stability to possibly shift the pH responsive regime.[19] In the **Core** and **End** designs, the i-motifs have two modified C_m-H^+-C_m base-pairs on adjacent stacks. We therefore sought to modulate pH of responsivity differently by interspersing two modified C_m-H^+-C_m base pairs between unmodified C-H^+-C base pairs. We therefore introduced modifications at positions 15, 17, 23, 25 to yield the **Interspersed** variant shown in Fig 1c. All these variants have two all-bromo or all-methyl modified C_m-H^+-C_m base-pairs in different topologies. We then sought to modulate pH_{half} by instead incorporating four hemi-modified C_m-H^+-C base pairs, by modifying only one of the participating cytosines of a C-H^+-C base pair. We did this by introducing modifications on four consecutive cytosines at positions 1, 2, 3, 4 at the 5' end (**Consecutive**).

First, we confirmed formation of i-motifs at acidic pH by the the C-rich domains of all the I-switch variants used in this study. This was done by monitoring the difference in Circular Dichroism (CD) spectra between pH 5.0 and pH 8.5 from 220 nm - 320 nm (Fig. S2). The difference spectra of pH 5.0 and pH 8.5 showed a positive band centered around 292 nm, and a negative band centered around 260 nm.[20] This type of spectrum is the CD signature of i-motif structure that is held together by C-H^+-C base pairs[21, 22, 23] This was further confirmed by CD spectroscopy at as a function of pH of all the new I-switches (Br I-switches and Me I-switches) (Fig. 2a&b). The change in molar ellipticity at 292 nm, where the C-H^+-C basepairs are known to absorb maximally was plotted as a function of pH. For uniformity, molar ellipticity of all I-switches was normalised from 0 to 1. As the pH increases, the structural transition from i-motif structure to duplex DNA occurs, hence, positive band at 292 nm decreases sigmoidally due to the decrease in C-H^+-C base pairs reflecting the pH induced denaturation of the i-motif. The different Br modified switches namely **Interspersed**, **Core**, **End** and **Consecutive** showed a pH_{half} of structural transition at pH 6.6 ± 0.2, 7.3 ± 0.2, 6.2 ± 0.2 and 6.8 ± 0.1 respectively. The different Me modified switches namely **Interspersed**, **Core**, **End** and **Consecutive** showed pH_{half} at pH 6.8 ± 0.2, 7.0 ± 0.3, 6.9 ± 0.2 and 7.0 ± 0.2 respectively. Bearing in mind that I4 showed a pH_{half} at pH 6.6 ± 0.2 and the pK_a of 5 methyl Cytosine is only 0.2 pH units higher than cytosine, this indicates that the Me-I-switches have been tuned according to the expectation, i.e., 0.3 – 0.5 pH units higher. The **End** variant of the Br-I switch was tuned only to 0.4 pH units lower. However, this is the only Br-I-switches, that was tuned in the right direction, and this point is discussed later. Nevertheless these modest changes in pH_{half} of the modified switches informed us that i-motif induced structural transitions were indeed confirmed in these assemblies before one proceeded to FRET reporters of the transition.[24]

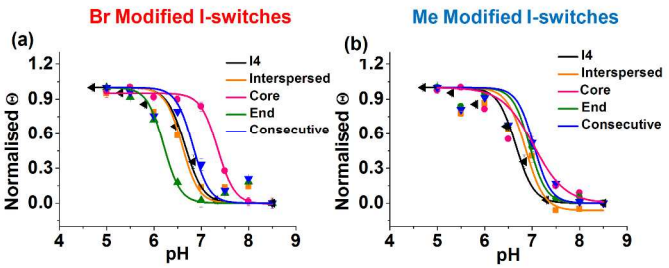

Fig. 2. *In vitro* characterisation of all I-switch variants. (**a&b**) Normalised ellipticity (Θ) at 292 nm of 1 µM native (I4) and (**a**) 5'-Bromocytosine modified (Br I-switches) I-switches (**b**) 5'-Methylcytosine modified (Me I-switches) in 1X clamping buffer is shown as a function of pH. All experiments were performed in duplicate at RT and shown as mean ± standard error of the mean.

To investigate the capability and performance of these modified I-switches as pH reporters we proceeded to investigate the pH-induced transition by fluorescence resonance energy transfer (FRET) using I-switch variants bearing donor and acceptor fluorophores as shown in Figure 1. At basic pH the labels are held far apart by a mismatched duplex showing low FRET and high D/A values, while at acidic pH the i-motif domains shorten the distances between the two fluorophores showing high FRET and consequenly low D/A values. The dually labeled I-switch variants (1X clamping buffer of desired pH, 120 mM KCl, 5 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) were excited at 495 nm and emission spectra were collected from 505 nm to 725 nm. Emission intensity at 520 nm from Alexa 488 (D) was divided by emission intensity at 669 nm from Alexa 647 (A) to obtain D/A ratios at various pH which was then normalised to pH 4.0 and plotted as a function of pH (Fig. 3a&b). This gives the characteristic pH responsive regime

and pH sensitivity of the given I-switch variant. The change in D/A ratios was a result of both decrease in Alexa 488 intensity and increase in the Alexa 647 intensity due to FRET(Fig. S3) yielding a characteristic sigmoidal curve.

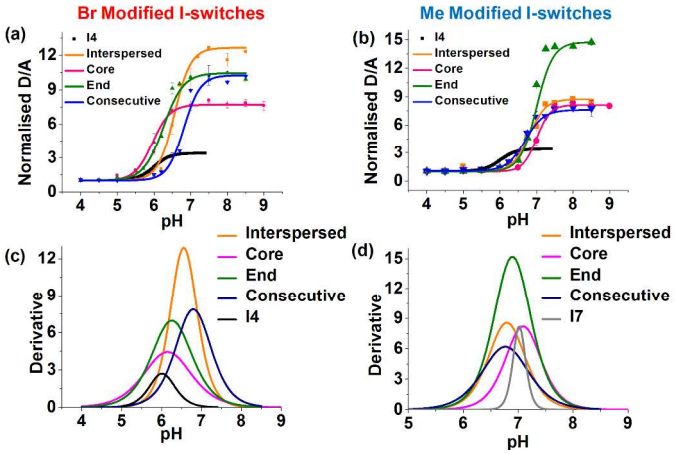

Fig. 3. (**a&b**) Donor (D) to FRET acceptor (A) ratio measurements of dually labelled I-switch assemblies as a function of pH. Normalised ratio of fluorescence intensities at 520 nm and 669 nm (λ_{ex} 495 nm) of 50 nM (**a**) Br I-switches and (**b**) Me I-switches in 1X clamping buffer is shown as a function of pH. All experiments were performed in triplicate at RT and shown as mean ± standard error of the mean. (**c&d**) First derivatives of normalised D/A v/s pH traces for (**c**) Br I-switches and (**d**) Me I-switches. Peak maxima denotes pH_{half}.

The Me I-switch variants **Interspersed**, **Core**, **End** and **Consecutive** all showed FRET pH_{half} values that were in fairly good correspondence with their CD pH_{half} values (Table 1). This was also the case with all the Br I-switches except the **Core** variant. This variant showed pH_{half} of 6.1 ± 0.1, in large discrepancy with the CD transition, but showed an overall shift of pH sensitivity in the expected direction. CD and FRET measure different parameters associated with the transition and it is not unusual to find discrepancies in pH_{half} between the two methods. The success in this strategy of tuning using nucleobase pK_a is evident from the the derivatives of the D/A vs pH traces (Figure 3c&d). When the number of cytosines increases from I4 to I7, although the pH_{half} of I7 changes to 7.03, the cooperativity increases to 6.3 and its pH responsive regime spans 0.5 pH units from Fig. 3d and Table 1. The **Core** Me-I-switch whose pH_{half} has been tuned to 7.1, with a cooperativity of only 3.3 (Table 1) spans a pH sensitive regime of 1.0 pH units.

The most notable feature of this new family of I-switches, which was unpredicted, was the phenomenal fold change in D/A ratio between the closed and open states. The overall percentage of signal change (%SC) of each of these variants dramatically increased ranging from 770% (Core Br-I-switch) to 1400% (End Me-I-switch). %SC of any reporter is one of the most important factors that determines its dynamic range. I4 and **End** Br-I –switch have very similar pH_{half} and cooperativity. However, due to the ~600% greater overall %SC in the Br-I-switches, it shows a wider regime of pH reporting capacity from pH 5.3-7.5. Dynamic pH range in which these nanodevices may be deployed is given in Figure 4 and obtained as discussed in the Supporting Information (Figure S6). While we are currently unaware of the structural basis of such high %SC, it is reasonable to assume that the introduction of substitutents on the cytosines likely distorts the i-motif structure in such a way that the fluorescent dyes are positioned optimally for FRET. Small changes in interfluorophore distances and the orientation factor can result in dramatic increases in FRET efficiency[25]. The Me-I-switches were predictably tuned to basic pH regimes by 0.3-0.5 pH units and coupled with the %SC this expands the sensitivity into mildly basic pH regimes. Interestingly, 5-Methylcytosines are also present naturally in CpG islands, telomeres[26] and several heterochromatin regions of human genome. This finding may open up considerations of possible structural transitions at such methylated sites harboring mismatches in the human genome.

Table 1. pH response characteristics of all the I-switch variants in this study.

Switch[a]	pH_{half}[b]	pH_{half}[c]	FC[d]	Co-operativity[e]
Inter-spersed	6.60 ± 0.04	6.6 ± 0.2	12.5	2.4
Core	6.1 ± 0.1	7.3 ± 0.2	7.7	2.2
End	6.30 ± 0.03	6.2 ± 0.2	10.5	2.0
Conse-cutive	6.8 ± 0.1	6.8 ± 0.1	10.5	3.1
Inter-spersed	6.8 ± 0.3	6.8 ± 0.2	8.6	2.1
Core	7.1 ± 0.2	7.0 ± 0.3	7.8	3.3
End	6.9 ± 0.1	6.9 ± 0.2	14.7	3.2
Conse-cutive	6.7 ± 0.1	7.0 ± 0.2	8.0	3.3
I4	6.1 ± 0.2	6.6 ± 0.2	4.1	2.2
I7	7.03 ± 0.04	6.8 ± 0.2	3.5	6.3

[a]Red and blue fonts indicate Br and Me I-switch variants respectively. [b] pH_{half} is given by mid point of the normalised D/A v/s pH traces. [c] pHhalf obtained from normalised ellipticity v/s pH traces. [d] Fold Change (FC) values obtained from the ratio of the D/A value of the I- switch variant at pH 4.0 and pH 8.5. [d]Numbers indicate Hill slope obtained from normalised D/A v/s pH trace.

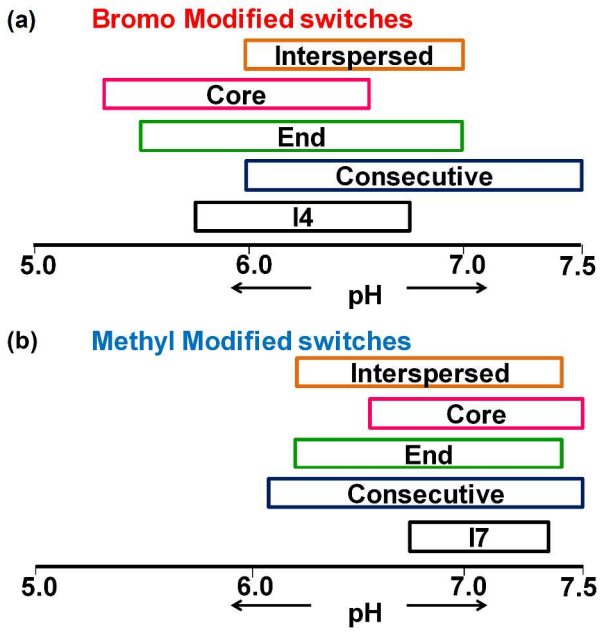

Fig. 4. pH tuning of the various I-switches. Dynamic range (10%-90% of total signal change) based on fluorescent studies of - (**a**) Br modified switches compared with native (I4) switch and (**b**) Me modified switches compared with native (I7) switch. The colour of the boxes indicate the position of the modification in the I-switches as described in Fig. 1(c).

The Br-I-switches on the other hand, showed evidence of I-motif stabilization both by CD and FRET, except for the End variant. Halogens such as Br and I are known for their large size to invoke several inter-atomic contacts and thereby promote crystallization[27]. In the i-motif, the nucleobases of two duplexes are intercalated leading to a very tightly packed structure. It is possible that despite the low pK_a of the bromocytosines, the bromo moieties could stabilize the i-motif resulting from hemiprotonated cytosines by simultaneously invoking a large number of weak contacts.

In summary, we describe the characterization of a family of I-switches whose pH responsivities have been expanded into more acidic ranges using 5-Bromocytosine and mildly basic pH using 5'-Methylcytosines. This was achieved largely due to a dramatic percentage signal change in this family of I-switches while maintaining low cooperativity in the pH-induced transition. The advantange of using low-cooperativity, high %SC I-switches, is that one can deploy them in biological scenarios such as screens where there could be large deviations of pH from the expected value. High cooperativity reporters as described by Nesterova et al, are useful when mapping very subtle pH changes in a given assay with a previously well-estimated pH. Given the fact that i-motif loop sequence is a critical determinant in the kinetics of i-motif formation[28, 29] we believe that the next challenge for DNA-based pH switches will be the design of high-sensitivity I-switches that are super-fast folders to yield pH sensitive nanodevices with fast response times.

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Notes and references

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†Electronic Supplementary Information (ESI) available: [Materials and methods, Supplementary figure 1 to 6]. See DOI: 10.1039/b000000x/.
‡ Footnotes should appear here.

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