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Rational Design of Highly Responsive pH Sensors Based on DNA i-Motif

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Supporting Information

ABSTRACT: Availability of strategies for molecular biosensing over a finely adjustable dynamic range is essential for understanding and controlling vital biological processes. Herein we report design principles of highly responsive pH sensors based on a DNA i-motif where both response sensitivity and transition midpoint can be tuned with high precision over the physiologically relevant pH interval. The tuning is accomplished via rational manipulations of an i-motif structure as well as incorporation of allosteric control elements. This strategy delivers molecular sensing systems with a transition midpoint tunable with 0.1 pH units precision and with a total response range as narrow as 0.2 pH units which can be adjusted to a variety of outputs (e.g., fluorescent readout). The potential of the presented approach is not limited by pH sensing but may extend toward manipulation of other quadruplex based structures or the development of ultraresponsive elements for artificial molecular machines and signaling systems.

The significance of biocompatible molecular sensors with a rationally adjustable response range *and* sensitivity is universally recognized.[1] Real-time pH monitoring in biological media is an area that would especially benefit from the availability of such sensing systems, particularly those showing high response on small pH variations. Indeed, changes as small as 0.2−0.3 pH units in tightly regulated living organisms are attributed to a variety of severe cellular malignancies including a propensity toward neoplastic transformations and inhibition of apoptosis.[2] For example, increase in intracellular pH by 0.2−0.7 units via hyperactivation of a Na^+/H^+ exchanger (NHE1) induces transformation events such as cell proliferation.[3] Apoptosis, an important physiological feature of normal tissues and a target of multiple therapeutic developments, is also accompanied by slight alterations (usually acidification) of cytoplasmic pH (by 0.3−0.4 pH units).[2b,4]

Rational design of a highly responsive sensing system requires careful control of two parameters: *transition range* (i.e., positioning a transition midpoint within a required narrow response range) and *transition width* (Figure 1). To position a transition midpoint, conventional pH sensing strategies[5] typically utilize chemical modifications of the reporters' structural core to change the acidity/basicity of the reactive site.[6] Although this approach is viable, the precision of such manipulations is rather limited. The narrowing of response sensitivity is even more challenging. Simple molecular pH sensors are based on the equilibrium between protonated and nonprotonated species. Intrinsically, the response range of such sensors (i.e., readily detectable change between the ratio of protonated to nonprotonated species of 1:10 to 10:1, called ΔpH_{10-90} here) as derived from the Henderson−Hasselbalch equation is $pK_a \pm 1$ pH unit (or 2 pH units total). Among the few previous attempts to overcome this restriction and sharpen the response were the design of an NIPAM/DMAPAM copolymer based sensor with ΔpH_{10-90} of 0.7[7] and a micellar system with a ΔpH_{10-90} of ∼0.25 pH units.[8] The latter approach does deliver sharp response sensitivity; however, limited precision of a midpoint tuning (based on chemical modification of the pH-sensitive tertiary amine environment) and overall complexity may limit its practical utility. Here, we describe a general strategy toward sensors with a precisely defined transition midpoint (i.e., tuning precision of 0.1 pH units) and response sensitivity overcoming the Henderson−Hasselbalch limitation (i.e., ΔpH_{10-90} as narrow as 0.2 pH units) based on the DNA i-motif structure.

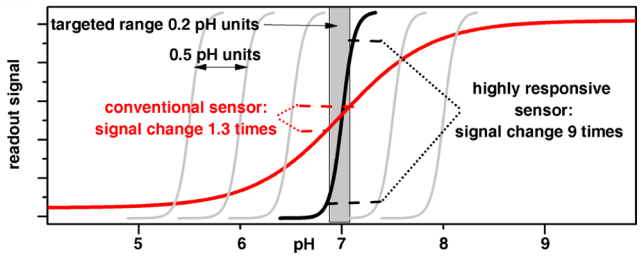

Figure 1. Design of a sensing system responding over a narrow target range (gray rectangle) requires precise tuning of both the *transition midpoint* (to position response curve inside the target range) and the *transition width* (to maximize the readout signal within the target range). The black curve represents a highly responsive sensing system with a transition midpoint tuned to fit a targeted range (e.g., pH 7 ± 0.1 pH units), and the red curve represents a generic conventional sensing system. Gray curves represent highly responsive systems positioned outside of the targeted range.

We hypothesized that the DNA i-motif would be a suitable scaffold for tunable pH sensor design because it possesses three essential characteristics: (i) *potential for tunability of the transition range* since an i-motif's stability depends on molecular structure and pH[9] and therefore can be tuned; (ii) *high intrinsic cooperativity of an i-motif folding* (as opposed to other systems such as a recently reported tunable DNA-based pH sensing

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platform utilizing Hoogsteen interactions[10]); and (iii) *potential for the cooperativity adjustment* as a tool to tune the response sensitivity. It is known that the folding cooperativity of DNA-based structures can be controlled via structural manipulations.[11] We propose that it is the cooperativity, a unifying feature in the two examples of highly responsive systems mentioned above, that should be instrumental in the control of response sensitivity.

An i-motif is formed of cytidine-rich DNA fragments that fold into a H^+-mediated quadruplex (see discussion and Scheme S1 in the Supporting Information (SI) for more details).[12] i-Motifs have been previously employed in the design of sensing systems,[13] artificial DNA-based nanomachines,[14] logic gates,[15] and for controllable assembly of multimeric supramolecular structures.[16] As DNA-based systems, i-motifs are compatible with a variety of signal transduction platforms including fluorescence. For example, i-motif based fluorescent sensors were proposed for pH monitoring inside living cells[17] and living organisms.[18] In such environments, the sensors remained stable for up to 8 h (half-lifetime) and yielded subcellular resolution and application-relevant kinetics.

To estimate the potential of transition range and response sensitivity tunability, we evaluated pH-triggered folding/unfolding transitions of single stranded oligonucleotides containing an i-motif forming sequences: continuous stretches of 4−5 cytidines ("C-tracts") with intermediate stretches of 3−4 A and T bases ("i-loops", Figure 2). Since we targeted pH

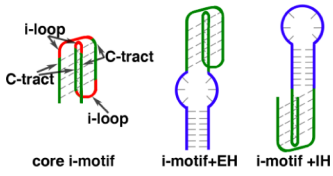

Figure 2. Oligonucleotide structures evaluated: *a core i-motif* ("C-tract" = continuous stretch of cytidines, "i-loop" = a stretch of A or T bases between the C-tracts); *an i-motif with an external hairpin* (i-motif + EH); and *an i-motif with an internal hairpin* (i-motif+IH). Exact sequences of all oligonucleotides evaluated in this work are shown in Table S1.

monitoring over a physiological range, we purposely aimed at oligonucleotides undergoing transitions within the 6.4 to 7.2 pH interval. The resulting molecular systems can serve as foundations for sensing modules *per se*, can be extrapolated to cover different pH range(s), and/or be combined with other elements to expand their practical usefulness.

To evaluate how precisely the folding/unfolding midpoint (pH^T) can be tuned, we followed pH-triggered transitions of i-motifs via UV absorption. "Core i-motifs" consisting of only C-stretches and i-loops as well as ones fitted with additional allosteric control elements ("i-motif+EH") were evaluated. To our surprise, even simple structural manipulations of a core i-motif composition yielded oligonucleotides with pH^T transitions different from each other by as little as 0.15 pH units (Figure 3A). To enable further fine-tuning, we took advantage of an allosteric control mechanism recently investigated by us.[19] The mechanism allows manipulating i-motif stability in a way that, for any given core i-motif structure, the transition midpoint can be additionally finely adjusted. For example, in the case of the i-motif $(C_5T_3)_3C_5$, appending of the EH with different stem and loop lengths yielded a series of oligonucleotides with pH^T varying in about 0.1 pH unit increments (Figure 3B).

Having established the rules for adjustment of the transition midpoint, we next attempted to tune the response sensitivity

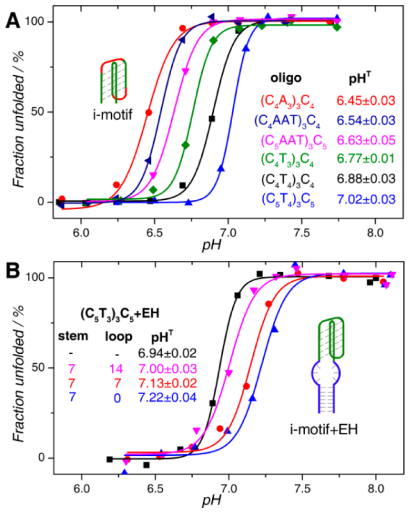

Figure 3. Transition midpoint (pH^T) of i-motifs can be tuned via manipulation of the number of cytidines in C-tract (A), the sequence of an i-loop (A), and/or via appending an external allosteric hairpin (B, structure "i-motif + EH" in Figure 2). The experimental points were plotted and fitted with sigmoidal fits (solid lines). The data points, fitting lines, sequence indicators, and transition midpoints are color coordinated. The stem and loop lengths in EH are shown in graph B. Experimental data are compiled in Table S2.

(response width). Our design strategy was based on deliberate manipulation of the i-motif folding cooperativity. The cooperativity could be quantitatively estimated in terms of Hill coefficient (n), and the response sensitivity, in terms of ΔpH_{10-90}.[20] When using these parameters, more narrow transitions are characterized both by larger n values and by smaller ΔpH_{10-90} values.

Initially we investigated the effect of the core i-motif structure on folding cooperativity and, as a consequence, on response sensitivity. We found some consistent qualitative *trends*. First, among oligonucleotides with 4-cytidine C-tracts and 3-nt i-loops, those with AAT loops yielded the narrowest transitions (n = 6.6; ΔpH_{10-90} = 0.29 pH units) whereas those with TTA loops showed the widest transitions (n = 5.3; ΔpH_{10-90} = 0.39 pH units) (Figure 4A). Second, the i-motifs with longer C-tracts yielded narrower transitions (n = 7.6; ΔpH_{10-90} = 0.25 pH units for $(C_5T_4)_3C_5$) relative to the structures with shorter C-tracts (n = 6.4; ΔpH_{10-90} = 0.31 pH units for $(C_4T_4)_3C_4$) (Figures 4B, S1). From a theoretical standpoint, this was an expected outcome since the folding transition of an oligonucleotide containing 5-cytidine C-tracts requires 2 more protons than the one with 4-cytidine C-tracts. However, one would anticipate a decrease in cooperativity for longer C-tracts based on the higher propensity of those structures toward formation of stable misfolded conformations (as previously described for RNA G-quadruplex folding).[11b] Therefore, in the case of DNA i-motifs with 4- and 5-cytidine C-tracts, the negative effects from potential misfolding do not seem to override the positive cooperativity contribution from binding additional protons. More narrow transitions were observed for 4-nt i-loops (n = 6.4; ΔpH_{10-90} = 0.31 pH units for $(C_4T_4)_3C_4$) than for 3-nt loops (n = 6.2; ΔpH_{10-90} = 0.33 pH units for $(C_4T_3)_3C_4$) (Figure 4B). Overall, we demonstrated that, similar to tuning the dynamic range, simple structural manipulations of a core i-motif can be instrumental in tuning its response sensitivity.

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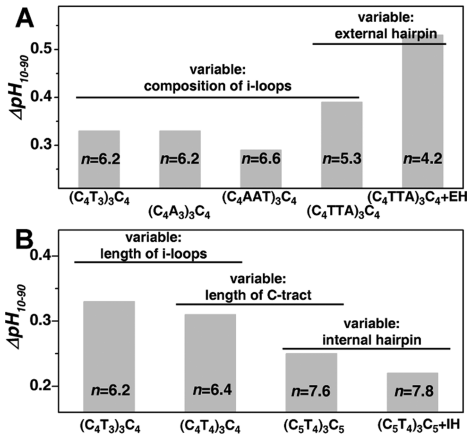

Figure 4. Effect of the structure of i-motif on its folding/unfolding cooperativity (in terms of Hill coefficient, *n*) and transition width (in terms of ΔpH_{10-90}, Y axis). The effects of i-loop composition (A) and length (B), of C-tract length (B), and of the appending allosteric internal (B) and external (A) hairpins on folding/unfolding cooperativity were derived from pH-triggered denaturations. Experimental data are compiled in Table S2.

An additional strategy we attempted for manipulating the i-motif folding cooperativity involved appending allosteric control elements. Initially we evaluated the effects of adding a hairpin into the middle i-loop (oligonucleotide "i-motif+IH", Figure 2). The design was prompted by recent observations that folding of a structurally similar hairpin/G-quadruplex system[21] proceeds with a minimized number of intermediate states owing to an internal hairpin in the middle loop. Therefore, we hypothesized that a similar hairpin/i-motif system would fold/unfold with higher cooperativity. Indeed, incorporation of a 7-bp stem/5-nt loop hairpin (oligonucleotide $(C_5T_4)_3C_5$+IH) into the middle i-loop of a corresponding core i-motif resulted in higher values for the Hill coefficient *n* (Figure 4B) and, as a consequence, a more narrow ΔpH_{10-90} compared to the parent i-motif (i.e., n = 7.6, ΔpH_{10-90} = 0.25 pH units for $(C_5T_4)_3C_5$; n = 7.8, ΔpH_{10-90} = 0.22 pH units for $(C_5T_4)_3C_5$+IH). Whereas this qualitative trend seems to be valid here, establishing its generality would require more detailed quantitative confirmations and mechanistic evaluations.

In contrast to the effect of an *internal* hairpin, incorporation of an *external* hairpin (structure "i-motif + EH", Figure 2) showed an opposite effect: a decrease in the i-motif folding cooperativity. Indeed, addition of the external hairpin resulted in the decrease of Hill coefficient (from 5.3 to 4.2) and in the increase in ΔpH_{10-90} (from 0.39 to 0.53 pH units) in the case of the $(C_4TTA)_3C_4$ and $(C_4TTA)_3C_4$+EH pair (Figure 4A). We surmise that the reason for the cooperativity decrease relates to transient stabilization of partially folded and/or misfolded species via an external hairpin.

To demonstrate how the obtained information can be used in practical sensor design, we prepared three fluorescent sensors with high response on small pH variations within the target neutral pH range of 7.0−7.2. All three sensors were based on the single stranded oligonucleotides described above. To convert the conformational folding/unfolding into a fluorescent readout, the oligonucleotides were labeled with a fluorescence donor (Rhodamine Green, RG) and a fluorescence acceptor (Dabcyl) in such a way that in the sensor's folded conformation both dyes would be in close proximity to each other (thus causing quenching of the RG emission), and upon unfolding the RG emission would be restored (Figure S2 in the SI). Importantly, the emission characteristics of the donor/quencher pair itself are not affected by pH over the evaluated range.[22] The molecular structures of the three sensors were adjusted to deliver "high", "intermediate", and "low" response sensitivities. The "high" sensitivity sensor ($(C_5T_4)_3C_5$+IH^{FL}) consisted of a narrow ΔpH_{10-90} i-motif core with an *internal* hairpin element (to further *increase* the folding cooperativity). The "low" sensitivity sensor ($(C_4TTA)_3C_4$+EH^{FL}) consisted of the i-motif core with the widest (among evaluated) response range and incorporated an *external* hairpin element (to further *decrease* the folding cooperativity). The "intermediate" sensitivity sensor was an i-motif $(C_5T_3)_3C_5^{FL}$ without additional structural modifications. The sensors' response (i.e., changes in fluorescent emission) to pH was evaluated over the pH range of 6.4 to 7.8 (Figures 5 and

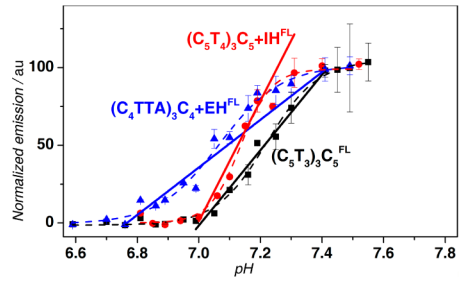

Figure 5. Fluorescent response on pH changes of 3 i-motif based sensors that are designed to operate over overlapping transition range with different response sensitivities. The widest response sensitivity is achieved for the $(C_4TTA)_3C_4$+EH^{FL} (blue), and the narrowest, for $(C_5T_4)_3C_5$+IH^{FL} (red). An "intermediate" response sensitivity (black) is for $(C_5T_3)_3C_5^{FL}$. The experimental points were fitted with sigmoidal (dash) and linear (solid) fits. Error bars represent standard deviations of signal from the measurements of 3 parallel preparations for each data point. Experimental data are summarized in Figures S3−S6.

S3−S6). In excellent agreement with our design scheme, we found a response sensitivity (ΔpH_{10-90}) of 0.31 pH units for the "intermediate" sensor $(C_5T_3)_3C_5^{FL}$, 0.23 pH units for the "high" sensitivity sensor $(C_5T_4)_3C_5$+IH^{FL}, and 0.48 pH units for the "low" sensitivity sensor $(C_4TTA)_3C_4$+EH^{FL} (Figures 5 and S6). The values translated into a linear fluorescent response (R > 0.95) over a range of 0.42 pH units for the "intermediate" sensor $(C_5T_3)_3C_5^{FL}$, 0.31 pH units for the "high" sensitivity $(C_5T_4)_3C_5$+IH^{FL}, and 0.65 pH units for the "low" sensitivity $(C_4TTA)_3C_4$+EH^{FL} sensors. Thus, these sensors can report small pH variations within the working range of 0.31 ("high" sensitivity) to 0.65 ("low" sensitivity) pH units, much below the Henderson−Hasselbalch limitation of conventional molecular pH sensors. Practical applications, however, will require further improvements of the experimental system in order to reduce signal fluctuations (typical %RSD 1−20%, some data points up to 30%) observed in the current study.

As evident from the reported results, the i-motif based systems exhibit high folding cooperativity with respect to proton concentration (pH). Theoretically, if an i-motif folding transition is *absolutely* cooperative, the *ideal* Hill coefficient for an "x"-cytidine C-tract system would be "2x". The observed Hill coefficients were in the range 4.2−7.8. Probable reasons for deviations from the "ideal" Hill coefficients are effects from proton−proton repulsion preventing all the protonic sites from being occupied simultaneously and/or increased stability of misfolded species. The fact that the folding cooperativity can be

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controlled using simple structural manipulations significantly expands the applicability of the i-motif based systems.

Overall, i-motif based platforms have the potential to become versatile tools for assessing pH in living systems. A strong advantage of the platform is its tunability and reversibility that expand the potential temporal resolution of the approach. It is important for potential *in vivo* applications that the i-motif folding is compatible with DNase-resistant modifications (i.e., phosphorothioate[23] or LNA[24]). The compact single stranded nature of the presented system may likely circumvent concerns over bioavailability common for oligonucleotide-based systems. However, additional systematic investigations are necessary to address the system behavior in biological media. We have evaluated the pH-triggered transition of sensor $(C_5T_4)_3C_5$+IH^{FL} in the presence of blood serum. While the sensor's performance was similar in both conditions, in the presence of serum the transition midpoint pH^T shifted to somewhat higher pH values (by less than 0.1 pH unit), and its response width was slightly more narrow (Figure S7). The results agree with the previous reports that intramolecular i-motif structures are additionally stabilized in the presence of molecular crowding[25] but do not conform to another report of identical response curves for an i-motif based sensor evaluated *in vitro* and intracellularly.[17a] Other variables such as the presence of cationic polymers used to deliver DNA into cells may influence device switching properties. Obviously, additional systematic evaluations of the sensing system behavior in biological media are necessary to gain a clear understanding of its properties.

In conclusion, we demonstrated that the rational manipulation of an i-motif composition can be utilized in designing pH sensing elements with a specifically defined (i) transition midpoint (precision of 0.1 pH units) and (ii) response sensitivity (0.2 pH units and above). Furthermore, for the first time we demonstrated how core structure manipulations and incorporation of allosteric control elements can be utilized in tuning i-motif folding cooperativity. The unique advantage of the i-motif based systems reported herein over the conventional strategies employed for pH sensing is the capability to tune both the response range and/or response sensitivity with the high precision. The potential utility of the reported systems is not limited to pH sensing but may be extended to applications that would benefit from a narrow (more digital) response (i.e., molecular logic)[26] or toward the development of biocompatible ultrasensitive motifs for artificial molecular signaling networks. It appears likely that similar approaches may be employed for manipulating folding cooperativity of other quadruplex structures such as RNA and/or DNA G-quadruplexes.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, general information on i-motif structure and properties, additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(20) Cooperativity and response sensitivity are interconnected: Zhang, Q.; Bhattacharya, S.; Andersen, M. E. *Open Biol.* **2013**, *3*, 130031. However, we report and discuss quantitative estimates for both parameters, as one (cooperativity, evaluated via Hill coefficient) is illustrative of a folding mechanism while another (sensitivity, evaluated via ΔpH_{10-90}) is an analytical figure of merit for the quantitative estimation of a sensing system.

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