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Rational Control of Folding Cooperativity in DNA Quadruplexes

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Supporting Information

ABSTRACT: Availability of basic tools for engineering molecular systems with precisely defined properties is crucial toward progress in development of new responsive materials. Among such materials are systems capable of generating an ultrasensitive response (i.e., large relative changes in output in response to small changes in input). Herein, we focus on a rational design of DNA quadruplex based structures as ultrasensitive response elements. In particular, we demonstrate how addition of allosteric guiding elements can be engineered into H^+-responsive i-motif structure to yield maximized response sensitivity.

Recent progress in the development of DNA-based functional materials[1] fuels demand for responsive molecular systems with precisely defined operational characteristics. Response range and response sensitivity are the essential parameters representing the material's potential for a specific task. While approaches for rational control of response range in oligonucleotides have been rather well established,[2] the tools for deliberate regulation of response sensitivity are somewhat scarce. In particular, fundamental tools for rational design of ultrasensitive molecular systems (i.e., systems generating large relative changes in output in response to small changes in input)[3] appear to be in an especially high demand. The highly responsive systems are foundation of "digital" molecular devices such as sensors with "yes-or-no" output or ones geared up to quantify very small relative differences in biomarkers. The biocompatible highly responsive elements are critical for the design of precisely tailored drug delivery platforms. Also, they are prerequisites for the engineering of signal digitalization elements crucial for molecular computing and robotics.

While ultrasensitive response pattern is rather common in living systems, the purposeful design of artificial molecular devices with such a capability is contingent upon overcoming a fundamental limitation: a hyperbolic binding curve as a consequence of the classic Michaelis–Menten kinetics for receptor–ligand interactions.[4] Several molecular mechanisms underlying ultrasensitive response in protein world have been delineated and characterized so far,[3,5] with only two platforms rationally engineered into artificial oligonucleotides: molecular titration (sequestration)[2a,b,6] and cooperative binding.[2g,7] Sequestration is a viable approach for generating an ultrasensitive output; however, it relies on relatively complicated systems with multiple species at a meticulous level of control over probe affinities, ratios, and concentrations. Therefore, it is rather difficult to employ this approach in the design of robust elements capable to operate in complex conditions.

Positive cooperative binding as a strategy for achieving high response sensitivity has been employed in a rational design of multivalent oligonucleotide receptors responsive toward such targets as ions,[2g,7b,8] lectin,[9] small molecules,[7b] and single stranded oligonucleotides.[7a] However, the majority of the oligonucleotide-based cooperative systems described so far (especially those with more than two binding sites) operate with insufficient, suboptimal performance (Figure 1).[2g,8a,c,9,10] To

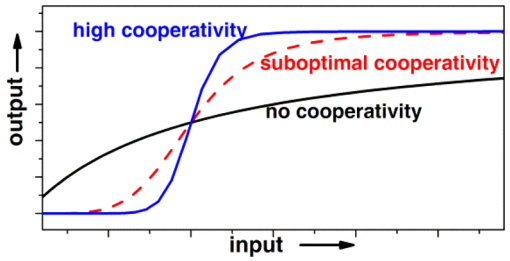

Figure 1. A transition with high positive cooperativity (blue) is characterized by a steeper increase in output in response to small changes in input as compared to a transition without cooperativity (black). Majority of engineered cooperative molecular systems, although showing some level of cooperativity, operate suboptimally (red).

the best of our knowledge, no systematic efforts either to delineate the cause of the suboptimality or to develop structural tools to rationally control it have been reported. Herein, using a DNA i-motif structure as a proof-of-concept model, we rationally designed and demonstrated an efficient strategy for maximizing transition cooperativity in quadruplex-based responsive molecular systems.

DNA i-motifs show intrinsic high sensitivity toward changes in proton concentration (pH).[8c] The sensitivity is based on positive cooperative binding of multiple protons by specifically designed sequences. Particularly, an i-motif is formed of cytidine-rich DNA fragments that fold into an H^+-mediated quadruplex (Figure 2a). It is well established for multivalent systems with positive binding cooperativity that the response sensitivity with respect to a target increases with increase in a number of binding sites.[11] However, as was recently demonstrated,[8c,10b] the observed apparent cooperativity in i-motifs is always suboptimal. A similar trend for suboptimal operation has been observed for other cooperative multivalent quadruplex[8a] and duplex systems.[2g,10a] A primary reason for the suboptimal cooperativity in the systems with high number of binding sites is strong propensity of those structures toward formation of stable misfolded/partially folded conformations.[8a] Indeed, existence of rather stable partially folded i-motifs

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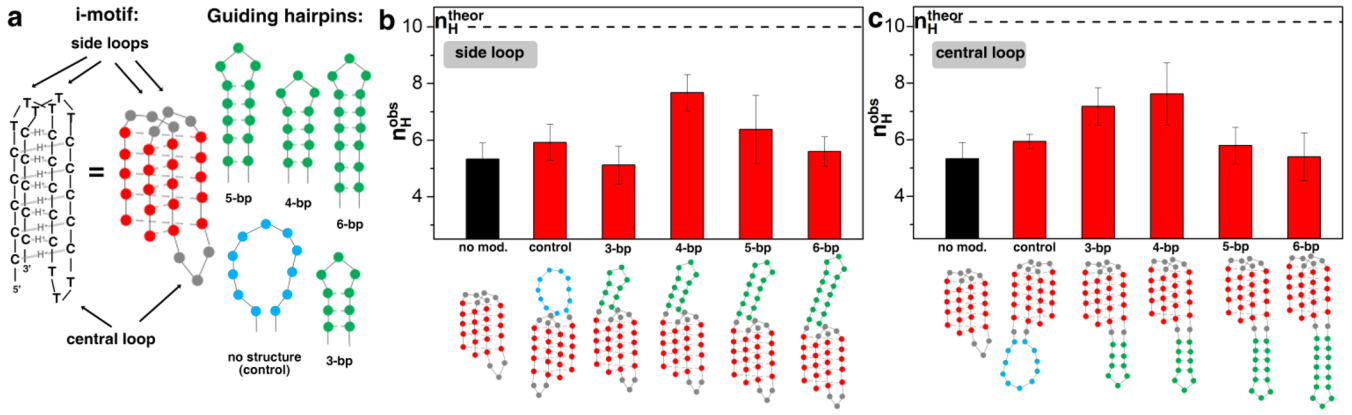

Figure 2. (a) A DNA i-motif is formed by single stranded oligonucleotides containing at least 4 tracts of cytosines separated by short loops. In the presence of protons the fragment can fold into an intramolecular tetraplex structure mediated by the hemiprotonated Cytidine−H^+−Cytidine base pairs. For the purpose of this paper the i-motif loops were modified with short guiding hairpins consisting of identical 3-bp (hairpin) loops and stems of various length. The apparent cooperativity of folding/unfolding transitions in i-motifs is affected by the modifications of side (b) and central (c) loops. The sharpness of transitions is characterized via Hill coefficient (y-axis). The dashed line indicates a value of maximum theoretically possible Hill coefficient (n_H^{theor}). All the sequences and experimental conditions are included in the Supporting Information.

has been recently demonstrated at a single-molecule level.[12] Therefore, we hypothesize that a promising strategy toward maximizing response sensitivity in such systems should be based on suppressing the formation of misfolded and partially folded quadruplex configurations.

Herein, we report how weaving rationally designed "guiding" elements into DNA quadruplexes can be utilized to suppress the formation of misfolded and/or stable partially folded conformations. The idea was inspired by various guiding elements such as hairpin stems, Watson−Crick double stranded fragments, and parallel-stranded DNA structures that have been recently utilized in controlling folding topology in a few artificial G-quadruplex based systems.[13] We hypothesized that the same principles might be instrumental in minimizing formation of misfolded/partially folded conformations in DNA i-motifs. In particular, we modified the quadruplexes with structural elements intended to prearrange the "correct" folding of the quadruplexes. Importantly, the elements must function in an "allosteric" manner in such a way that the induced control does not interfere with specificity of the primary interaction.

In order to confirm our hypothesis that the rationally applied guiding elements can control folding cooperativity in quadruplexes with multiple binding sites, we designed a series of i-motifs modified with short hairpins. For these proof-of-concept studies we focused on an i-motif consisting of 5-cytidine stretches and 3-nt loops. This fragment possesses high intrinsic folding cooperativity, and, at the same time, demonstrates suboptimal performance of the transition.[8c] However, the conclusions/trends derived from these studies can be extended toward other i-motifs and, more generally, toward other quadruplex structures such as DNA and/or RNA G-quadruplexes. The parent i-motif sequence was modified with hairpins of various stem lengths (i.e., 3-, 4-, 5- and 6-bp with an identical 3-bp (hairpin) loop). The modifications were incorporated in one of the two positions: either central or side loop of the parent i-motif (Figure 2a). The guiding hairpins were designed to be stable at room temperature and unlikely to form unintended secondary structures (such as stable duplex stretches) with the i-motif core as modeled with *mfold*. Additionally, we obtained control oligonucleotides modified with single stranded fragments of the length identical to a 4-bp stem guiding hairpin but with no secondary structure. We monitored folding/unfolding transitions of the parent (unmodified), control, and modified i-motifs in response to changes in input (H^+ concentration) using UV absorbance at 295 nm where quadruplex denaturation is exclusively observed.[14] We also confirmed all structural transformations via circular dichroism (CD) spectroscopy (Table S1 in the SI). The transitions were characterized with respect to cooperativity using Hill equation (details in the SI) The derived Hill coefficient (n_H^{obs}) is an established quantitative measure of cooperativity.[3] The values were evaluated against theoretical Hill coefficients (n_H^{theor}) derived based on the maximum number of ligands the receptor can bind (i.e., n_H^{theor} for an i-motif consisting of 4 stretches of 5 cytosines is 10). All the experimental details are included in the Supporting Information.

In excellent agreement with our main hypothesis, our data shows that the i-motif's loop modification with a hairpin does result in increase in its apparent folding cooperativity, compared to the parent unmodified structure. Indeed, the parent i-motif demonstrates a suboptimal folding cooperativity (n_H^{obs} = 5.33 ± 0.57 vs n_H^{theor} = 10). Modification of either loop with an unstructured single stranded oligonucleotides (control) did not cause a statistically significant difference in folding cooperativity (n_H^{obs} = 5.94 ± 0.25 for central loop modification and n_H^{obs} = 5.92 ± 0.64 for side loop, Figure 2b,c). In contrast, modification of the i-motif loop with a 4-bp stem hairpin results in a sharp increase in the Hill coefficients characterizing the i-motif transition (n_H^{obs} = 7.62 ± 1.10 for the central loop modification and n_H^{obs} = 7.68 ± 0.64 for a side loop modification) (Figure 2b,c). These results provide strong support to our hypothesis that modification of i-motif loops with guiding hairpins is instrumental for control over its folding cooperativity.

Adding hairpin to a loop also affected pH transition midpoint (pH_T) of modified i-motifs compared to a parent (Table S1 in the SI): the sequences with stable hairpins within looping regions shifted pH_T toward higher values of pH (lower H^+ concentration). At the same time, controls with unstructured loops shifted the pH_T toward higher H^+ concentrations (lower pH). The results are in agreement with recently reported trends that (i) stable hairpins inside i-motif loops stabilize the structure[15] (shifting pH_T toward higher values), and (ii) longer unstructured loops may cause decrease in the overall structure stability therefore causing shifting pH_T toward lower values.[16]

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We also observe that the length of an i-motif stem affects its apparent transition cooperativity. Thus, the Hill coefficient in the case of side loop modification with a 3-bp stem hairpin is 5.12 ± 0.67, with a 4-bp stem −7.68 ± 0.64, 5-bp stem −6.38 ± 1.20, and with a 6-bp stem −5.60 ± 0.52 (Figure 2b). A similar trend is observed for the central loop modification, with the highest apparent cooperativity obtained with a 4-bp stem hairpins (Hill coefficient of 7.62 ± 1.10) and correspondingly lower values found for 3-bp, 5-bp and 6-bp stem hairpins (Hill coefficients of 7.18 ± 0.66, 5.80 ± 0.64, and 5.40 ± 0.85, respectively; Figure 2c). As demonstrated below, the primary reason for the higher cooperativity increase when transitioning from 3-bp to 4-bp stem hairpin likely results from the increased stability of the 4-bp hairpin, a prerequisite for an appropriate assembly prearrangement. The subsequent decrease in cooperativity while transitioning from a 4-bp to 5-bp and further to 6-bp stem hairpin might originate from variety of reasons. Steric hindrances associated with i-motif folding is one possibility. The folded i-motif is a very compact structure;[17] and therefore, attached bulky fragments may interfere with the proper i-motif folding. It is also likely that longer hairpin stems would result in higher propensity to forming more stable intermediate structures (e.g., self-dimers) or stabilize misfolded conformation in other ways. Further detailed structural studies are necessary to find out exact causes of these phenomena.

In order to demonstrate that "guiding" hairpin stability indeed affects the folding cooperativity, we designed and evaluated a series of i-motifs modified with hairpins of identical stem/loop length (4-bp stem +3-nt loop) but with different thermodynamic stability. In order to modulate the thermodynamic stability, we varied GC content in the hairpin stem. The experiments demonstrated an evident trend: folding cooperativity of an i-motif increased with increase in the stem stability for both modification positions (Figure 3 and Figure S1 in the SI), i.e., the higher the stability of the guiding hairpin (more negative ΔG°), the higher the value of Hill coefficient for transition cooperativity was observed. Interestingly, for both modification positions it appears that for low stability stem the cooperativity is actually lower than that of a parent and control. While the differences between the values are rather insignificant, they may originate, as we hypothesize, either from the different flexibility of loops stabilizing misfolded structures or from the higher propensity of structures containing Watson−Crick duplexes (hairpin stems here) to form self-dimers. Another observed trend to be mentioned is that lower Hill coefficients appeared for an i-motif modified with a more stable hairpin ($\Delta G^\circ = -2.22$ kcal mol^{-1}, n_H^{obs} = 3.37 ± 0.80) as compared to one modified with a less stable hairpin ($\Delta G^\circ = -0.04$ kcal mol^{-1}, n_H^{obs} = 4.43 ± 0.40, Figure 2a). However, these values, when statistically compared to each other, are not significantly different.

Hairpin position is another factor that potentially can influence cooperativity of the proton binding transition. However, we do not observe any statistically significant differences between modifications with identical elements in different positions (side or central loop) (Figure 2).

From the presented results, it is clear that "guiding" hairpins can be rationally applied to control and maximize folding cooperativity in DNA quadruplexes; however, the mechanism of this phenomenon requires further discussion. A recently proposed mechanism for i-motif folding[17a] suggests that the overall process involves two consecutive steps: an initial "fast" folding (rate constants ~0.5−1 s^{-1}) followed by a slower bond rearrangement (rate constants ~0.01 s^{-1}) (Figure S2 in the SI). Our hypothesis is that guiding hairpins fold on a time scale that is several orders of magnitude faster than the time scale of the initial binding step (for example, the rate constant for Watson−Crick hairpins is $k \sim 10^3$−10^4 s^{-1} (ref 18)). Therefore, they serve to "nucleate" subsequent "correct" quadruplex folding (i.e., the closed hairpin prearranges distal cytidines for a precise folding conformation during the initial binding step). This fast prefolding of guiding hairpins helps to minimize the number of misfolded species therefore resulting in a system involving mostly properly folded conformation. Similar seeding role of a hairpin has been conclusively demonstrated for folding G-quadruplex-hairpin hybrid.[19]

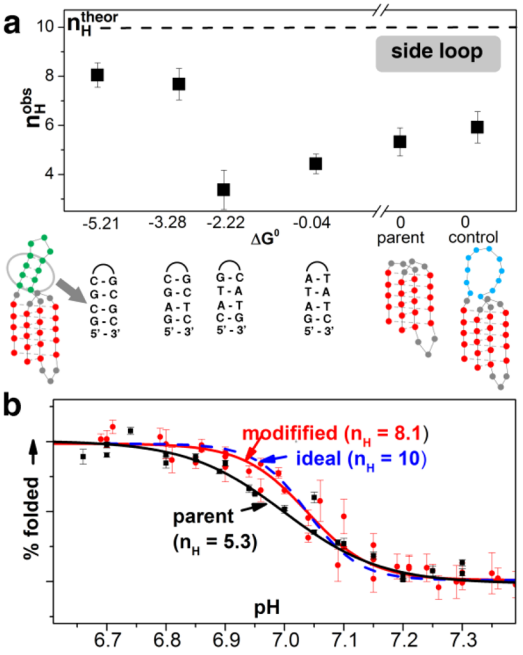

Figure 3. (a) The folding/unfolding cooperativity (in terms of n_H^{obs}) depends on hairpin's stability (ΔG°). Sequences of stems used to generate guiding hairpins of various stability are included along with data points. The 4-bp stem/3-bp loop guiding hairpins were inserted into the side loop of the i-motif (Figure 2a). (b) Folding/unfolding transition of an i-motif modified with 4-bp stem hairpin ($\Delta G^\circ = -5.21$ kcal mol^{-1}, red points and line) is much steeper than that of an unmodified (parent) i-motif (black) and is approaching an "ideal" transition behavior (assuming n_H^{theor} = 10, blue dashed line). All the oligonucleotide sequence information is included in Table S1 in the SI.

Overall, the extent of cooperativity enhancement induced by introducing a "guiding" hairpin depends on its dimension and stability: short thermodynamically stable hairpins yield maximum enhancement in folding cooperativity. Bulkier fragments, even when more stable, do not cause increase in folding cooperativity. For example, although ΔG° of a 6-bp stem hairpin is −5.56 kcal mol^{-1} (vs −3.28 kcal mol^{-1} for a 4-bp stem hairpin in Figure 2b), the Hill coefficient for the i-motif modified with the 6-bp stem hairpin in the side loop was 5.60 ± 0.52, which is substantially lower than the corresponding coefficient for the 4-bp hairpin modification (7.68 ± 0.64). Similar trend was also observed for central loop modification (Figure 2c).

We hypothesize that the developed structural tool will be instrumental for controlling folding cooperativity in other quadruplex-based systems. Indeed, we preliminarily demonstrated a similar effect for another i-motif system (Figure S3 in the Supporting Information). In addition, we expect that similar principles will work for G-quadruplexes. This assumption is

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supported by a recently reported effect of a hairpin in a central loop of G-quadruplex on increasing its folding cooperativity.[19]

In conclusion, we reported a rational approach for the control over folding cooperativity in quadruplex-based DNA systems. The control is accomplished via modification of the quadruplexes with allosteric "guiding" elements. The guiding elements are short fragments (i.e., hairpins) that fold on a faster time scale and "pre-arrange" correct folding of quadruplex, thus avoiding formation of partially folded/misfolded species. The approach yielded apparent folding cooperativity reaching ideal theoretical values for the used proof-of-concept model (i-motif with theoretical Hill coefficient of 10). The obvious significance of this finding can be in the area of DNA nanotechnology applications, such as design of highly responsive switching and sensing systems. In addition, the proposed mechanism may have biological significance, especially in view of recently established (i) role and therapeutic value of i-motifs in living systems,[15,20] and (ii) role of internal loop hairpins on formation of naturally existing i-motifs.[15,20j] Therefore, our study is an essential step toward better understanding of dynamic properties of various i-motif-containing systems and the factors affecting their properties.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06645.

Description of experimental procedures, Table S1, and Figures S1−S3. (PDF)

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Notes

The authors declare no competing financial interest.

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