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Controlling Hybridization Chain Reactions with pH

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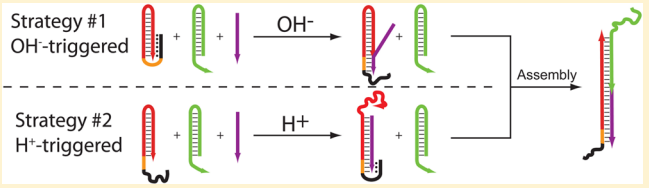
Supporting Information

ABSTRACT: By taking inspiration from nature, where self-organization of biomolecular species into complex systems is finely controlled through different stimuli, we propose here a rational approach by which the assembly and disassembly of DNA-based concatemers can be controlled through pH changes. To do so we used the hybridization chain reaction (HCR), a process that, upon the addition of an initiator strand, allows to create DNA-based concatemers in a controlled fashion. We re-engineered the functional units of HCR through the addition of pH-dependent clamp-like triplex-forming domains that can either inhibit or activate the polymerization reaction at different pHs. This allows to finely regulate the HCR-induced assembly and disassembly of DNA concatemers at either basic or acidic pHs in a reversible way. The strategies we present here appear particularly promising as novel tools to achieve better spatiotemporal control of self-assembly processes of DNA-based nanostructures.



KEYWORDS: *DNA nanotechnology, self-assembly, hybridization chain reaction, pH, DNA nanostructures*

The self-assembly of biomolecular species into supramolecular complex systems is a widespread phenomenon that has attracted the interest of chemists and bioengineers for a long time.[1−3] Nature employs peptides, proteins, nucleotides, and lipids to build higher-order assemblies and well-defined structures with functions that range from maintaining cellular regulation to containing and transporting cellular cargoes.[4−7] Nature achieves spatiotemporal control of supramolecular self-assembly by using a variety of mechanisms. That is, even in the presence of all the building blocks of the supramolecular object, further control and fine-tuning of its assembly/disassembly can be achieved using different external stimuli such as pH, temperature, or ionic gradient.[8] For example, the assembly of one of the most intriguing naturally occurring polymers, spider silk, is regulated by pH through pH-dependent domains in the silk precursors. This allows to achieve a remarkable finely tuned process of spider silk formation using pH gradients.[9,10]

Inspired by such regulated naturally occurring systems, recent efforts have been devoted to rationally recreate in vitro similar stimuli-induced self-assembly mechanisms in order to achieve control over the synthesis of new nanomaterials[11−15] or nanostructures.[16−18] Because of the unmatched programmability and simplicity of Watson−Crick interactions (compared to the more complex interactions present in proteins and peptides),[19] synthetic oligonucleotides have been extensively used to build sophisticated two- and three-dimensional structures[20−28] as well as functional nanomachines.[29−32] To achieve a spatiotemporal control of the self-assembly process of such nucleic acid based nanostructures, different approaches have been proposed. Among these, the use of hybridization chain reaction (HCR),[33] a process through which two metastable DNA hairpins react with each other to form concatemer-like nanowires in the presence of a triggering single strand, appears particularly promising. Using HCR in fact enables to control the catalytic self-assembly and disassembly of superior structures through prescribed pathways by using as a trigger an external DNA initiator molecule or a non-nucleic acid target.[33−37] A limitation associated with the above strategy is the fact that in the presence of the triggering input (initiator) the self-assembly reactions cannot be further controlled. Finding new ways to modulate the efficiency of the HCR process with a range of different stimuli (including environmental changes) would thus ultimately lead to a better spatiotemporal control of the assembly and disassembly of DNA nanostructures.

Motivated by the above arguments, we propose here a rational approach to control HCR with pH changes. We did so by taking advantage of the well-characterized pH sensitivity of the parallel Hoogsteen interactions in triplex DNA.[38,39] More specifically, the sequence-specific formation of a CGC^+ parallel triplet requires the protonation of the N3 of cytosine in the third strand (average pK_a of protonated cytosines in triplex structure is ∼6.5).[40,41] For this reason, DNA strands containing cytosines can only form a stable triplex structure at acidic pHs.[39] By re-engineering the HCR functional units to contain such triplex-forming pH-dependent domains, we have designed two different strategies that allow to trigger the HCR-induced

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assembly and disassembly of DNA concatemers at either basic or acidic pHs.

In our first strategy (OH^--triggered HCR) we have selected hairpins with size and sequences similar to those used by Pierce and Dirks in their seminal work about HCR.[33] We first confirmed with gel-electrophoresis and time-course fluorescence measurements that the conventional HCR system proceeds with very similar efficiency over a wide pH window (Figure S1). In fact, no significant differences in the polymerization efficiency can be noticed over the entire pH window we have tested (pH 4.5 and 9.0) thus demonstrating that the original HCR provides no pH-control over the polymerization reaction once the initiator is added to the metastable hairpins solution (Figure S2). To achieve OH^--triggered HCR process we have redesigned a hairpin that can be opened only under basic conditions ($tH1_{OH^-}$). To do so, we re-engineered the sequence of one of the hairpins used by Pierce and Dirks by adding a short nine-base tail (t) at the 5′-end. This tail has a homopyrimidinic sequence that can form an intramolecular triplex structure with the terminal portion of the stem duplex hairpin (Figure 1A). This triplex structure acts as a molecular trap sequestering the toehold domain and preventing initiator binding and the start of polymerization (Figures 1 and S3). Of note, this additional triplex-forming tail (t) does not affect the stability of the hairpin (Figure S4) and, as required for a HCR system, the mixture containing both hairpins ($tH1_{OH^-}$ and H2) remains stable (at acidic and basic pHs) in the absence of strand initiator over at least 48 h (Figure S5). We first demonstrated the pH-triggered opening of this switch using a fluorophore/quencher pair (Figure 1A, right). As expected, triplex formation occurs only under acidic pHs (condition at which Hoogsteen interactions are favored)[39] with a pK_a (average pK_a of protonated cytosines in the triplex structure) of 6.3. We then demonstrated that the formation of the triplex structure inhibits the strand-displacement reaction between the hairpin ($tH1_{OH^-}$) and the initiator (i.e., the first step of the HCR process). The initiator-induced strand-displacement reaction proceeds efficiently only at basic pHs, while at acidic pHs the initiator is not able to open and thus activate the hairpin ($tH1_{OH^-}$) (Figure 1B). A control experiment where the hairpin lacks the triplex forming portion confirms that the pH dependency observed is to be ascribed to the contribution of triplex formation (Figure S6).

Using this pH-dependent hairpin ($tH1_{OH^-}$) we then show that we can program an HCR process that is only triggered at basic pHs (Figure 2A). We first demonstrate this with time-course experiments performed at different pHs and under a fixed concentration of initiator (1 μM). As expected, HCR is completely inhibited at acidic pHs: no signal increase can be observed upon the addition of the initiator at pHs below 5.0, thus confirming that the initiator is not able to nucleate with H1 and start the HCR process (Figure 2B). Because the toehold domain is released at higher pHs, we achieve a gradual activation of the HCR process by gradually increasing the solution's pH (Figure 2B) until we reach an efficiency comparable to that observed in the classic HCR at pHs above 7.5 (see open dots in Figures 2B and S2). Of note, the final fluorescence signal obtained in these experiments is about 4-fold larger than that obtained in the absence of H2 (Figure 1B). This suggests that formation of the I·tH1·H2 complex leads to a complete opening of the tH1 hairpin thus resulting in a less efficient quenching of the fluorophore by the quencher due to a larger distance between them. To support the evidence

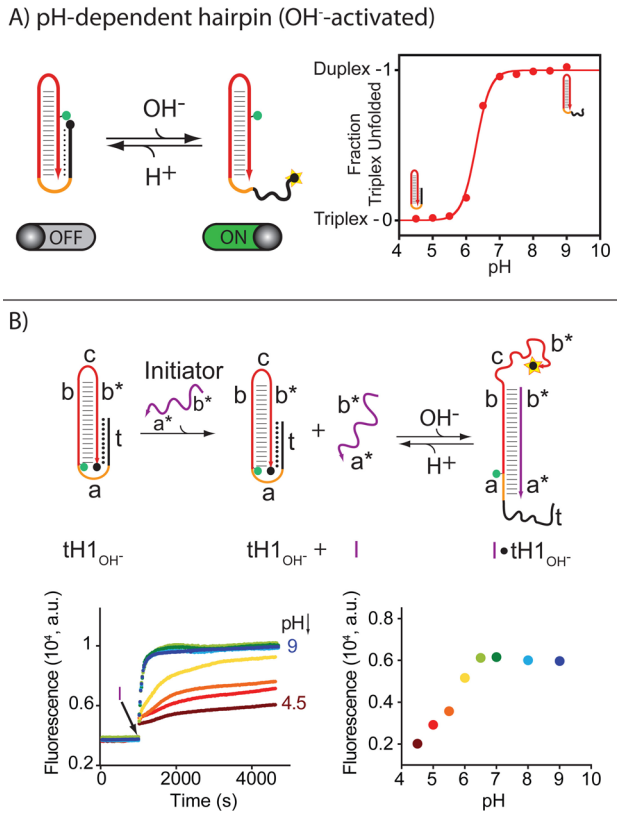

Figure 1. pH-dependent hairpin for OH^--triggered HCR strategy. (A) A nine-base tail (domain "t") is added to the 5′-end of the sequence of a hairpin. This tail can form a pH-dependent intramolecular triplex that acts as a molecular *trap* and sequesters the toehold portion to which the initiator should bind. To demonstrate the pH-dependence of this hairpin (named $tH1_{OH^-}$) we have labeled the hairpin with a pH-insensitive fluorophore (Alexa Fluor 488) and a quencher (Black-Hole Quencher 1, BHQ-1) (see cartoon). Triplex formation occurs under acidic pHs, a condition at which Hoogsteen interactions are favored, and starts to unfold at pH above 6.0 (right). Shown is the pH–titration curve of the hairpin (0.2 μM) achieved in the reaction buffer adjusted at different pHs. (B) Fluorescence kinetic experiments in the presence of a fixed concentration of initiator (1.0 μM) and $tH1_{OH^-}$ (0.2 μM) and at varying pHs demonstrate that optimal initiator-induced strand displacement reaction is only observed at basic pHs (pH > 6.5). Of note, in this case the hairpin is labeled with a fluorophore/quencher pair (see cartoon) to signal the opening of the duplex portion. Here and in the following figures, letters marked with * represent nucleotide sequences that are complementary to the sequences labeled with unmarked letters.

of concatemers formation we also performed gel-electrophoresis experiments at different concentrations of initiator. These experiments clearly show the formation of bands at higher molecular weights at basic pH (pH = 8.0), while no such bands are observed at acidic pH (pH = 5.0) (Figure 2C) thus further validating our pH-triggered HCR strategy.

To demonstrate the versatility of our approach, we also engineered a second strategy in which HCR assembly is activated at acidic pHs (H^+-triggered HCR). We started the design of this strategy using again two hairpins with size and sequences similar to those originally designed by Pierce and Dirks.[33] However, in this case we selected a hairpin (H1) with a toehold of only four bases, which, in normal conditions, is not long enough to allow initiator nucleation and an efficient HCR.[33,42] This results in a poor HCR efficiency over a wide

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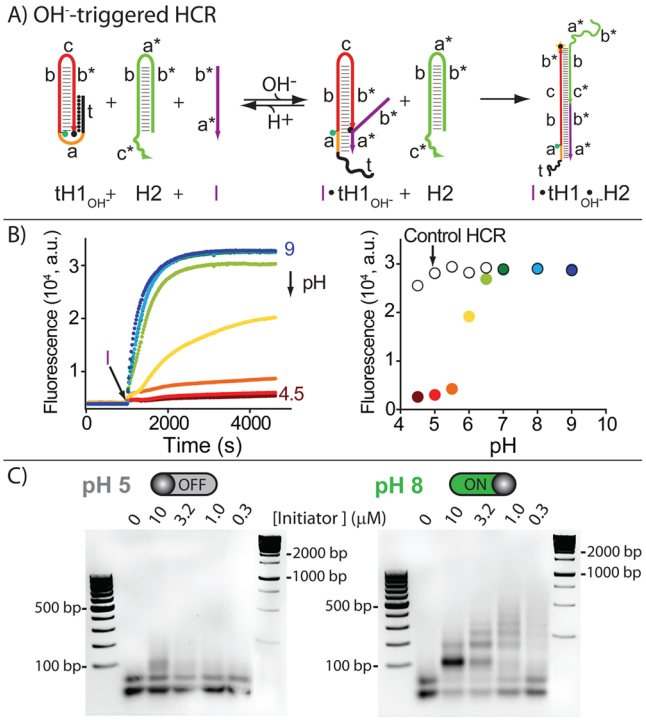

Figure 2. (A) In the OH^--triggered HCR strategy the formation of the DNA-based concatemers in the presence of the two hairpins and of the initiator only occurs at basic pHs. (B) Fluorescence time-course experiments (showing the opening of the optically labeled $tH1_{OH-}$ hairpin duplex upon initiator addition) performed in the presence of a fixed concentration of initiator (1 μM) added to a mixture of $tH1_{OH-}$ (0.2 μM) and H2 (0.24 μM) demonstrate that gradual activation of HCR process is achieved by gradually increasing the pH of the solution. Optimal signal (comparable to that obtained with a control non-pH-dependent hairpin, open dots and Figure S2) is observed at pHs above 7.0. (C) Gel-electrophoresis experiments in the presence of different concentrations of initiator added to a 1 μM mixture of $tH1_{OH-}$ and H2 confirm that bands at high-molecular weights are only observed at a basic pH. See Supporting Information for details on both gel-electrophoresis and fluorescence experiments.

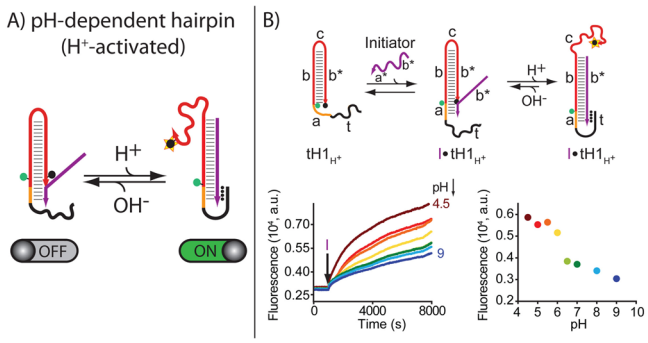

Figure 3. pH-dependent hairpin for H^+-triggered HCR strategy. (A) A nine-base tail that can form a triplex clamp-like structure with the initiator is added to the 5′-end of the sequence of a poorly behaving hairpin (toehold length of only 4 bases). The formation of a triplex clamp between this re-engineered hairpin (named $tH1_{H+}$) and the initiator provides an additional energetic contribution that supports efficient strand displacement process and thus efficient polymerization reaction. (B) Fluorescence kinetic experiments in the presence of a fixed concentration of initiator (0.32 μM) and $tH1_{H+}$ (0.2 μM) at different pHs demonstrate that optimal initiator-induced strand displacement reaction is only observed under acidic pHs. At pHs above 6.0, at which triplex formation is unfavored, we observe a signal that is comparable to that observed with the nontriplex original hairpin (Figure S12).

range of initiator concentrations and a wide range of pH (Figure S7). We confirmed the link between small toehold portion and weak HCR activity by showing that the same HCR system but with a hairpin H1 having a six-base toehold completely restores the HCR efficiency (Figure S8).

We achieved H^+-triggered HCR by adding to the 5′-end of this poorly behaving hairpin a nine-base tail (portion t), which is able to form a clamp-like triplex structure with the duplex formed by the initiator and the toehold portion (Figures 3A and S9). Of note, adding this triplex-forming tail does not affect the stability of the hairpin (Figure S10); also in this case in the absence of strand initiator, such modified hairpin, named $tH1_{H+}$, remains stable over 48 h in the presence of H2 (Figure S11). The Hoogsteen interactions in this re-engineered hairpin provides an additional energetic contribution that improves the efficiency of initiator's strand nucleation to the hairpin. We demonstrate this by studying the strand-displacement reaction between $tH1_{H+}$ and the initiator under different pH conditions (Figure 3B). In this case the reaction is favored by triplex formation, and thus, we observe efficient strand displacement only at pHs below 6.0. Using $tH1_{H+}$ we can therefore control HCR and trigger its activation at acidic pHs (Figure 4A). Fluorescence kinetic measurements, for example, show a gradual activation of HCR upon gradually decreasing the pH of the solution (Figure 4B). As the pH is increased to values where the Hoogsteen interactions can no longer promote initiator's nucleation (i.e., pH > 7.0) we observe signals that are comparable to those obtained under the same conditions but using a control hairpin lacking the triplex-forming domain (see open dots in Figures 4B and S7). In support of these results, we observe concatemers formation with gel-electrophoresis at pH 5.0, while no high-molecular weight polymers can be detected at pH 8.0 (Figure 4C).

Both the strategies we have developed here allow an external control over the HCR process by using pH changes. To further characterize these strategies and demonstrate the reversibility of our approach we performed different fluorescence and gel experiments in the presence of the initiator under initial inhibiting or activating conditions. We first demonstrate the pH-dependent HCR-induced assembly by adding a fixed concentration of initiator under initial inhibiting conditions for both strategies (Figure 5, left). Under these conditions the initiator is not able to nucleate with tH1 and thus we do not observe any signal increase due to the opening of the metastable hairpins nor formation of high molecular DNA complexes. Upon addition of either H^+ (H^+-triggered HCR, pH jump from 8.0 to 5.0) or OH^- (OH^--triggered HCR, pH jump from 5.0 to 8.0), we were able to trigger the nucleation of the initiator to tH1 and to start the polymerization-like reaction (Figure 5, left). Gel-electrophoresis experiments, once more, support the proposed mechanism as we observe formation of high-molecular weight complexes only under pH activating conditions. We also studied the reversibility of our pH-controlled HCR strategies by rapidly changing the pH of the solution from activating to inhibiting pH conditions during the assembly process (Figure 5, right). In addition to stopping the assembly of the polymer, these pH-jumps also trigger the disassembly of the polymers (the signal decrease is associated with the closing of the hairpins) (Figure 5, right). Gel-electrophoresis experiments, once more, support the proposed

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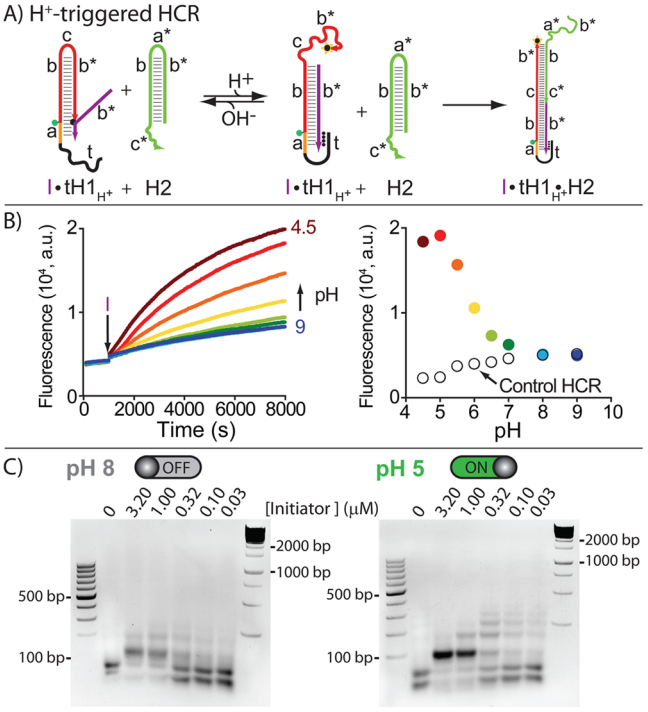

Figure 4. (A) In the H^+-triggered HCR the formation of the DNA-based concatemers in the presence of the two hairpins and the initiator only occurs at acidic pHs. (B) We performed fluorescence time-course experiments at different pHs in the presence of a fixed concentration of initiator (0.32 μM), $tH1_{H+}$ (0.2 μM), and H2 (0.24 μM). We observe optimal hairpin opening (fluorophore-quencher separation) at pHs below 5.5. In contrast, at high pHs the efficiency of HCR is poor and comparable to that obtained with a control hairpin H1 that lacks the triplex forming domain (open dots and Figure S7). (C) Gel-electrophoresis experiments show the formation of high-molecular weight complexes at acidic pH (pH = 5.0) and less at basic pH (pH = 8.0). See Supporting Information for details on both gel-electrophoresis and fluorescence experiments.

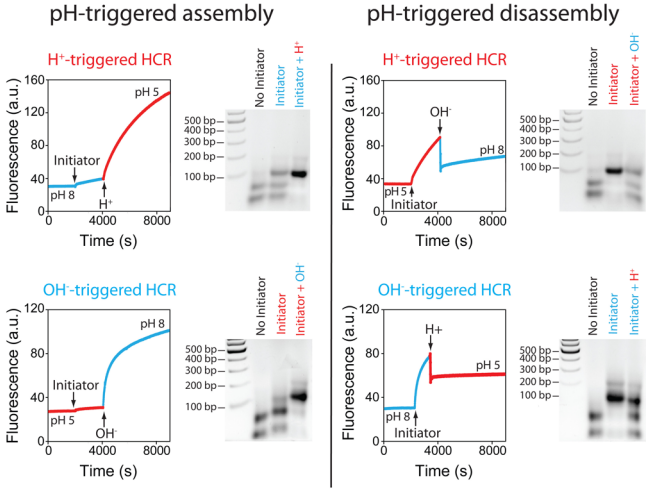

Figure 5. Our pH-triggered HCR strategies enable to control the assembly/disassembly through simple pH changes. We demonstrate this with both our strategies (top, H^+-triggered HCR; bottom, OH^--triggered HCR). (Left) We first demonstrated the pH-triggered assembly by adding the initiator under initial inhibiting conditions. No increase in fluorescence signal and no formation of higher molecular weight products (gel experiments) was observed under these conditions for both strategies. Upon pH change to activating conditions (pH = 5.0 for H^+-triggered HCR; pH = 8.0 for OH^--triggered HCR) we observe both an increase in fluorescence and concatemers formation. (Right) We also demonstrated the reversibility of the pH-triggered HCR assembly by rapidly changing the pH during the course of the polymerization reaction. For example, we raised the pH to 8.0 during a H^+-triggered HCR (top, right) and decreased the pH to 5.0 during a OH^--triggered HCR (bottom, right) and observe a significant reduction (~70% and ~58%, respectively) in fluorescent signal correlated with the disappearance of the high-molecular weight products in gel experiments. See Supporting Information for details on both gel-electrophoresis and fluorescence experiments.

mechanism as we observe formation of two bands corresponding to the two hairpin monomers, only under deactivating pH conditions. As a further proof we have also performed the same pH jump experiments after 24 h of HCR reaction. Gel electrophoresis experiments once again demonstrate the occurred assembly and disassembly of DNA based concatemers (Figure S13). Finally, we also demonstrate with time-course fluorescence experiments the possibility to reversibly assemble/disassemble the DNA based concatemers through pH cycles (Figure S14).

Here we have rationally designed triplex-forming metastable hairpins that can be used to control self-assembly and disassembly of DNA-based polymers through pH changes. We did so by re-engineering the functional units of the hybridization chain reaction,[33] through the addition of clamp-like triplex-forming domains that can either inhibit or activate the polymerization reaction in a pH-dependent manner.

Previous examples have recently demonstrated the possibility of using pH changes to control the assembly of DNA-based nanostructures[43−51] using the nonduplex pH-dependent $C{\cdot}C^+$ interactions of the *i-motif*. These examples, however, are not without drawbacks. These include sequence-dependency limitations (only C-rich sequences with a certain consecutive content of cytosines can form *i-motif*), structural constraints (formation of the complete tertiary structure is required in order to allow a pH-control), and a limited and quite narrow pH dynamic range (this usually spans not more than 2 orders of magnitude).[52,53]

In response to the above limitations the use of triplex-forming domains as an additional tool to control reactions in DNA-based nanotechnology can provide several advantages. For example, although homopurine sequences are needed to form triplex structures, the sequence-dependent limitations of our approach are far less strict than those related to the use of *i-motifs*. Our strategy thus appears more easily adaptable to pre-existing DNA-based self-assembly processes. We also note that the high-specificity of triplex-forming sequences[38] could allow to employ in the same solution different pH-dependent sequences that can be used to build more complex structures based on several branched reactions.[37] Finally, we have recently demonstrated that by simply changing the CGC^+ content we can modulate the pH-dependence of a triplex DNA switch over more than 5 orders of magnitude[41,54] making the use of triplex structure highly tunable over a wide range of pH.

The possibility to assemble/disassemble DNA structures through a simple change of the solution's pH may open the door to many interesting applications in DNA nanotechnology. For example, this approach could be used to hierarchically trigger, through a simple pH change, the formation of DNA nanostructures and DNA origami[55,56] or to actuate in a pH-controlled fashion DNA-based nanomachines[57] that could be ultimately used as imaging or drug-delivery tools in diseases

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characterized by pH-dysregulation (an inverted pH gradient between the inside and the outside of cells).[58]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.5b02123.

Sequences, materials and methods, and additional experiments. (PDF)

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Notes

The authors declare no competing financial interest.

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