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Fluorescent Monitoring of RNA Assembly and Processing Using the Split-Spinach Aptamer

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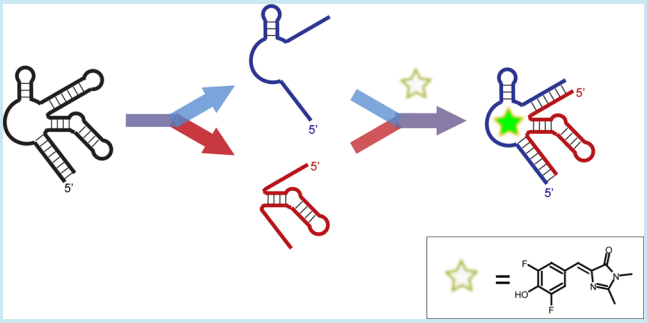
Supporting Information

ABSTRACT: As insights into RNA's many diverse cellular roles continue to be gained, interest and applications in RNA self-assembly and dynamics remain at the forefront of structural biology. The bifurcation of functional molecules into nonfunctional fragments provides a useful strategy for controlling and monitoring cellular RNA processes and functionalities. Herein we present the bifurcation of the preexisting Spinach aptamer and demonstrate its utility as a novel split aptamer system for monitoring RNA self-assembly as well as the processing of pre-short interfering substrates. We show for the first time that the Spinach aptamer can be divided into two nonfunctional halves that, once assembled, restore the original fluorescent signal characteristic of the unabridged aptamer. In this regard, the split-Spinach aptamer is represented as a potential tool for monitoring the self-assembly of artificial and/or natural RNAs.



KEYWORDS: *RNA self-assembly, RNA nanotechnology, RNA design, RNA monitoring*

RNA is an exceedingly important molecule in an array of cellular processes (i.e., catalysis, gene regulation, and metabolite recognition) beyond its traditionally recognized roles involving protein expression. This increased awareness of RNA's utility calls for new tools that can be used to study and monitor RNA self-assembly, structure, and cellular dynamics. Split-protein and nucleic acid systems, which rely on the reassociation of independent nonfunctional fragments to conditionally restore the desired whole and operative moiety,[1,2] represent usefuls tool for identifying and ascertaining important molecular interactions[3] and have the potential to provide new devices for synthetic biology and/or biomedical applications.[4] The benefits of these split systems hinge on at least two important factors: the involved fragments' collective ability to readily and stably reassociate into the functional complex and their ability to elicit the desired functional response or signal. In the first regard, nucleic acids provide an accommodating platform for choreographing self-assembling, predefined molecular interactions because of their ability to form predictable and precise hydrogen bonds between complementary nucleobases.[5−7] With respect to this second point, molecules that provide fluorescent outputs are thought to offer highly sensitive signals with desirable signal-to-noise ratios.

RNA's versatility has been made all the more useful in the developing fields of RNA synthetic biology and nanotechnology with the advent of directed evolution techniques that may be used to select for novel synthetic RNAs possessing virtually any preconceived functionality.[8] Using this approach, the previously reported Spinach aptamer was evolved and selected for its ability to produce a fluorescent signal when it is complexed with the small molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a mimic of the chromophore found in the green fluorescent protein.[9] The scope and utility of the Spinach aptamer have been expanded further to include the ability to report the presence of various metabolites and, more recently, as a means of quantitatively monitoring protein production.[10,11]

In our view, the bifurcation of the full-length RNA aptamer into two segments provides an additional set of future applications that include the ability to monitor and/or visualize dynamic self-assembly. Herein, we describe the design of the split-Spinach system for monitoring the formation of synthetic RNAs for use in the RNA interference (RNAi) pathway as well as their subsequent processing by the human recombinant dicer enzyme.

RESULTS AND DISCUSSION

Previous reports about the Spinach aptamer[10] demonstrated that the third stem (from the 5′ end of the full-length

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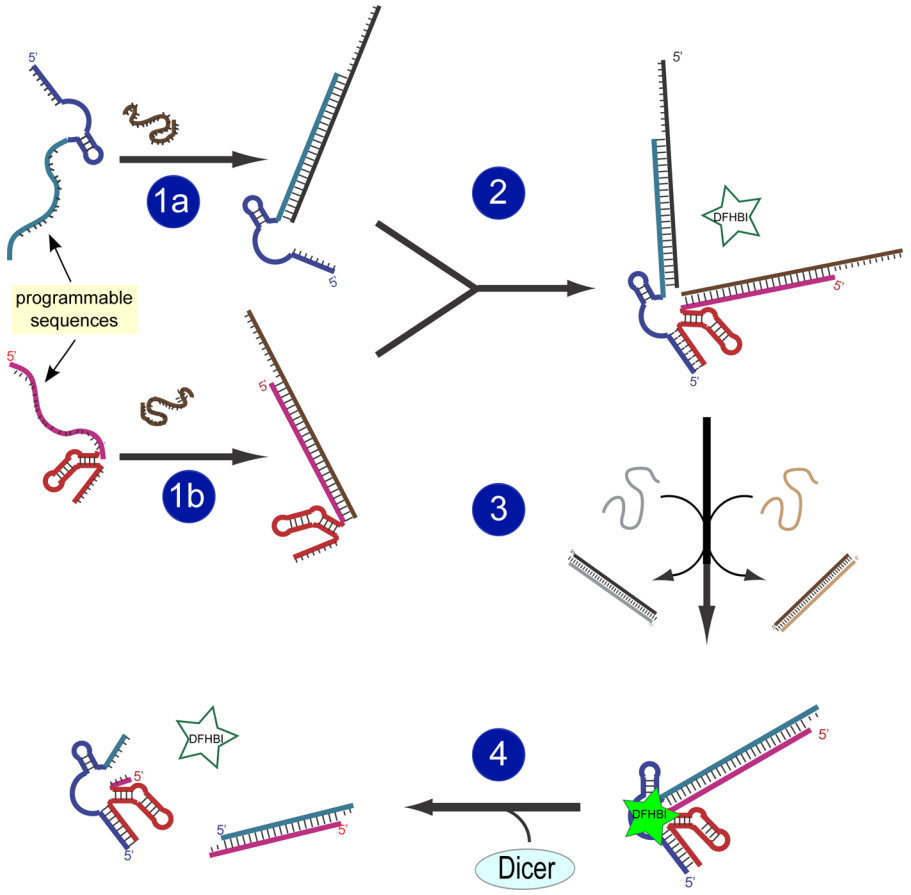

Figure 1. Schematic showing the methods used to control the assembly and processing of the split-Spinach system. In steps 1a and 1b, single strands of the aptamer were annealed to a complementary DNA blocker sequence on the elongated stem. In step 2, the products of steps 1a and 1b are combined to form the full Spinach aptamer with DNA blocker strands in place. In step 3, DNA toehold strands complementary to their respective DNA blocker strands "unzip" the DNA from the aptamer by maximizing Watson–Crick base pairs, allowing the elongated stem to bind with itself and form the DFHBI binding site and DFHBI to bind and fluoresce. In step 4, dicer cuts the dsRNA 21 nucleotides from the 3′ two-nucleotide overhang, destabilizing the binding site and ending fluorescence.

molecule) tolerates a range of different sequences, including the appendage of various aptamer moieties, without too much loss of function (70–80% signal strength of the original Spinach aptamer). Thus, we hypothesized this stem to be a suitable point for bifurcation of the full-length Spinach aptamer. As a way to selectively control the formation of the bipartite complex and demonstrate its functionality, we placed the split-Spinach aptamer in the context of a self-assembling RNA–DNA hybrid system, similar to that of Afonin et al.[12,13] The bifurcated stem of the aptamer complex was extended using different short interfering RNA (siRNA) sequences (see Figure SI1 of the Supporting Information). Three siRNA sequences were tested on the basis of previously reported work[4,14] (see the Supporting Information). Using the RNA–DNA hybrid system, complementary DNA "blocker" strands were annealed to individual RNA strands to prevent the formation of a functional split-Spinach aptamer (Figure 1). The "blocking" DNA strands could be selectively displaced by the addition of fully complementary "unblocking" DNA strands that could associate with the blocker strands through the 12-nucleotide toehold sequences, leaving the split-Spinach blocked stem of the aptamer free to assemble and form the required DFHBI binding pocket.

Analysis of split-Spinach aptamer assembly was conducted using polyacrylamide gel electrophoresis (PAGE) and fluorescence spectroscopy. Conformational studies via PAGE show that the two aptamer segments, when annealed, produce a single band that migrates through the gel at a rate comparable to that of the full-length Spinach aptamer in native salt concentrations (Figure 2). In terms of functionality, the DFHBI fluorescence was observed only in the presence of both aptamer strands (i.e., DFHBI did not fluoresce in the presence of either split-Spinach aptamer alone). Furthermore, the split-Spinach aptamer showed fluorescence comparable to that of the full-length Spinach aptamer in the presence of DFHBI, indicating that bifurcation of the aptamer does not substantially reduce the binding affinity for DFHBI and that the bifurcated aptamer conforms to a tertiary structure analogous to that of the full-length Spinach aptamer.

Complementary blocker and unblocker DNA strands were introduced to demonstrate selective immobilization and assembly of the split-Spinach aptamer (Figure 2). PAGE and fluorescence spectroscopy show that the addition of complementary blocking DNA strands prior to the split-Spinach aptamer's assembly induces the bifurcated aptamer to form a nonfunctional DNA–RNA hybrid moiety that prevents the formation of the third stem that is critical for aptamer formation. Thus, the split-Spinach aptamer does not fully assemble or fluoresce in the presence of the blocker strands but assembles and exhibits fluorescence with the removal of the

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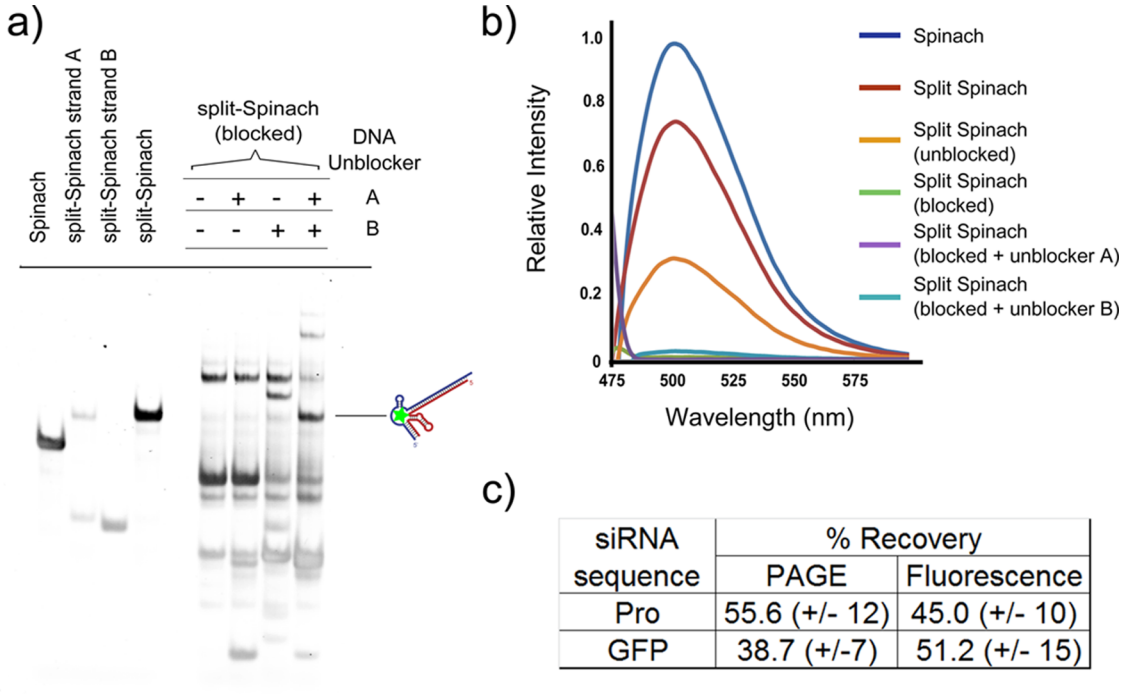

siRNA sequence	% Recovery	
	PAGE	Fluorescence
Pro	55.6 (+/- 12)	45.0 (+/- 10)
GFP	38.7 (+/-7)	51.2 (+/- 15)

Figure 2. Monitoring the assembly and fluorescence of the controlled split-Spinach system. (a) A representative native PAGE gel (40 mM HEPES buffer and 1 mM Mg^{2+}) confirms that the split-Spinach aptamer forms from the blocked complex only when both unblocker strands are added. Data represent the Pro-siRNA sequences. See the Supporting Information for sequence details. (b) Corresponding fluorescence data confirm that the unblocked split-Spinach system fluoresces. The split-Spinach system fails to fluoresce when blocker strands are added, but fluorescence occurs when both unblockers are added. (c) The percent recoveries (following the addition of the DNA unblocking strands) of the two programmable sequences tested were analyzed in triplicate by native PAGE and fluorescence microscopy.

blocker strands via strand displacement when complementary unblocker DNA strands are introduced (Figure 2). Of the three siRNA sequences tested (validated sequences targeting GFP; two targeting HIV, protease (Pro) and Ldr3), only two proved to be suitable for study. The Ldr3 sequence contained a series of four GA repeats that were prone to mispair. In the case of the other two sequences, the percent recovery of the functional aptamer upon unblocking at 37 °C was between 38 and 50%, which was confirmed by analysis of the assembly on native PAGE gels and fluorescence spectroscopy. We postulate that the split-Spinach system can be improved by tailoring the length and specific sequence of the toehold with the length of the desired siRNA sequence. We also saw that we could increase the overall recovery to >60% by assembling at 45 °C (see Figure SI2 of the Supporting Information).

In addition to its ability to monitor RNA self-assembly processes, the split-Spinach system can also be used to monitor RNA processing. The aptamer was subjected to selected degradation by the recombinant human dicer enzyme, targeting the 3′ overhang on the third stem, generating a siRNA (Figure 3). Independent analysis of the predicted product of the split-Spinach aptamer after dicing demonstrated no ability to fluoresce (data not shown). In the same way that blocking and unblocking DNA strands selectively induce fluorescence, dicing of the split-Spinach complex can selectively disrupt the split-Spinach aptamer's functionality, which was corroborated by the absence of fluorescence. Moreover, we show a direct correlation among the dicer concentration, siRNA formation, and the reduction of the fluorescence signal, further suggesting that the aptamer was selectively degraded by dicer processing of the bifurcated stem.

The split-Spinach aptamer complex, in the context of the DNA–RNA hybrid system, provides a successful demonstration of the bifurcated Spinach aptamer *in vitro*. We show that the modification of a previously reported RNA aptamer and fluorophore offers a promising tool for monitoring RNA assembly and RNA processing. Given that the split system reduces the magnitude of the signal by only ∼20% compared to that of the full-length sequence *in vitro*, it is anticipated that the split-Spinach aptamer could be used for *in vivo* applications similar to those previously reported for the original aggregate Spinach aptamer.[9−11] Alternatively, we hypothesize that the recently reported Spinach2 aptamer, which offers >3 times the fluorescence intensity of the first-generation Spinach aptamer, could be modified in the same fashion to create a programmable bifurcated aptamer.[15] The improved sensitivity of the Spinach2 aptamer is attributed to the elimination of mismatches in the first and third stems, which thereby improved the overall thermostability and folding efficiency of the Spinach aptamer.[15] Using the improved stem sequences associated with the Spinach2 aptamer to facilitate programmable assembly in the split-Spinach system (as the core of the aptamer sequence remains unchanged) would likely provide a better testing ground for future *in vivo* applications. Regardless of the precise stem sequences used, we posit that the split-Spinach concept offers a new tool for investigating a variety of RNA assembly and RNA–RNA interactions.[16,17] For example, the split construct has potential as a fluorescent signaling device in RNA-based sensors and/or programmable circuitry as well as the assembly of RNA nanostructures similar to how the binary malachite green aptamer has been previously reported[12,18,19] but without the reported toxicity.[20]

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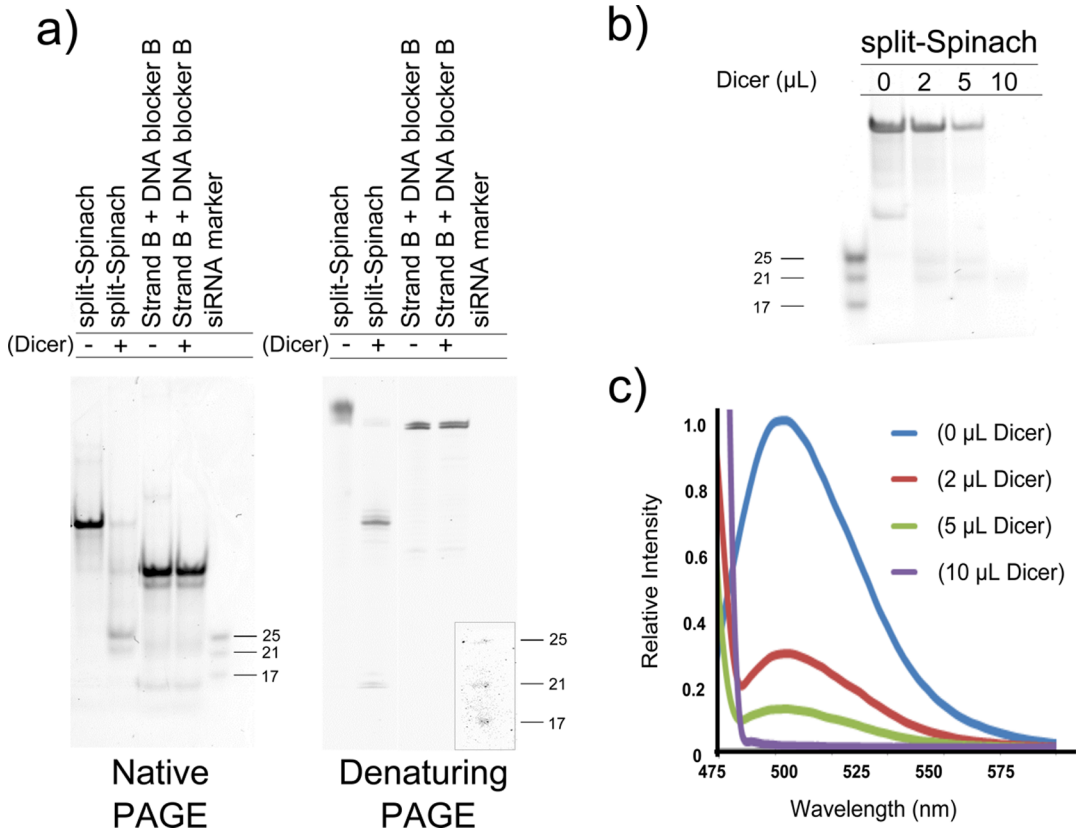

Figure 3. Monitoring dicer processing. (a) PAGE gels demonstrate that dicer selectively processes double-stranded RNA in the third stem of the split-Spinach aptamer. The denaturing gel indicates that the dicer cuts the programmable stem of the assembled the split-Spinach system into ~21-nucleotide segments. No dicing was observed when RNA is annealed to the blocker strand. Note that the contrast was increased for the ladder portion of the denaturing PAGE gel to increase visibility. (b, c) As the concentration of the dicer increases, the concentration of the split-Spinach system decreases and fluorescence decreases.

METHODS

Synthesis of the Aptamer and Fluorophore. DNA sequences and primers of interest were purchased from Integrated Data Technologies (IDT). RNA constructs were synthesized by *in vitro* transcription with T7 RNA polymerase from polymerase chain reaction-generated double-stranded DNA templates ordered from Integrated DNA Technologies (IDT), followed by 8 M urea−10% polyacrylamide gel electrophoresis (PAGE) purification. DFHBI was synthesized according to previously published protocols.[9] RNA and DNA sequences used here can be found in the Supporting Information.

Nucleic Acid Assembly Experiments. To control the assembly of the split-Spinach complex, individual RNA sequences were annealed to complementary blocker DNA strands by being slowly cooled from 95 °C. Blocked RNA strands were mixed in a 1:1 ratio and incubated together for 20 min at 37 °C. Mixtures containing unblocker DNA strands and their controls were left to incubate at 37 °C for 25 min before being loaded into a 7% polyacrylamide gel of 1× HEPES (40 mM HEPES) buffer and 1 mM $MgCl_2$. Gels were run at 8 W for 2−3 h at 4 °C. Aliquots of samples run on gel were also characterized by fluorescence spectroscopy. Identical assembly experiments were conducted using 10 to 12% polyacrylamide gels containing 8 M urea. Denaturing gels were run at 40 W for 1−1.5 h at room temperature. Gels were stained with Sybr Green II (Invitrogen) and imaged using ChemiDoc MP (Bio-Rad) or FluoroChemQ (Protein Simple).

Fluorimeter Studies. The fluorescence of the fully assembled and unblocked Spinach aptamer was confirmed with an LS 55 luminescence spectrometer (PerkinElmer). Samples from reaction mixtures in assembly and dicer experiments were incubated with 1 mM DFHBI at 37 °C for 20 min and then loaded into a 40 μL quartz cuvette (Starna Cells, Inc.). Samples were excited at 469 nm and emission spectra recorded at 509 nm.

Dicer Experiments. The recombinant human dicer enzyme kit was purchased from Genlantis. Samples were incubated overnight at 37 °C in the supplied dicer reaction buffer (Genlantis) or HEPES according to the manufacturer's suggested protocol. Dicing reactions were analyzed by PAGE on a 1 mM $MgCl_2$ native 7% PAGE or 8 M urea−10% PAGE gel and by fluorescence spectroscopy as described above.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

T.A.R. and G.E.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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