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The Use of Minimal RNA Toeholds to Trigger the Activation of Multiple Functionalities

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Abstract

Current work reports the use of single-stranded RNA toeholds of different lengths to promote the reassociation of various RNA–DNA hybrids, which results in activation of multiple split functionalities inside human cells. The process of reassociation is analyzed and followed with a novel computational multistrand secondary structure prediction algorithm and various experiments. All of our previously designed RNA/DNA nanoparticles employed single-stranded DNA toeholds to initiate reassociation. The use of RNA toeholds is advantageous because of the simpler design rules, the shorter toeholds, and the smaller size of the resulting nanoparticles (by up to 120 nucleotides per particle) compared to the same hybrid nanoparticles with single-stranded DNA toeholds. Moreover, the cotranscriptional assemblies result in higher yields for hybrid nanoparticles with ssRNA toeholds.

Graphical Abstract

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The authors declare no competing financial interest

ASSOCIATED CONTENT

Supporting Information

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All sequences used in this project and experimental and computational data supplementary data (PDF)

Keywords

RNA nanotechnology; RNA nanoparticles; RNA interference; RNA–DNA hybrid reassociation

RNA interference (RNAi) is a cellular process that occurs post-transcriptionally to regulate gene expression.^{1–3} This pathway can be mobilized through the foreign introduction of small interfering RNAs (siRNA) allowing for the regulation of genes that contribute to the diseased state.^{4–6} In order to simultaneously target several genes inside the same diseased cell with multiple siRNAs, those siRNAs need to be codelivered in a controlled fashion. The field of therapeutic RNA nanotechnology aims to develop novel technologies facilitating this task through the introduction of programmable RNA-based nanoparticles amenable to functionalization with various therapeutics.^{7–26} These nanoparticles demonstrate a precise and reproducible formulation, modularity, and programmability. However, for further biomedical applications, additional control over their specific intracellular activation is desired in order to minimize any unwanted interactions in vivo. Recently, we introduced an approach that relies on the use of RNA–DNA hybrids for delivery of multiple split functionalities and their simultaneous intracellular activation.²⁷ The key idea behind the original methodology was to split functional RNA entities (e.g., RNAi activators) into two nonfunctional RNA–DNA hybrids. Each DNA strand was designed to be slightly (~12 nt) longer than its RNA counterpart. Thus, upon the formation of the RNA–DNA hybrids, these duplexes would have ssDNA toeholds that were complementary to each other. The presence of ssDNA toeholds on the extremities of the hybrids allowed for their mutual recognition, reassociation, and release of the functional RNA when they were in close proximity. This approach allowed an additional handle on the specificity of the site of action and conferred additional stability in vivo.²⁷ Indeed, serum degradation studies found less than 20% RNA–

DNA hybrid degradation after 24 h in human blood serum, far exceeding the stability of siRNA duplexes.²⁷ Previously, we showed that cognate RNA–DNA hybrids can, when delivered with a 24 h time delay between delivery of the two cognate hybrids, still lead to gene silencing.^{27,28} Because delayed timing makes it improbable that cognate RNA/DNA hybrids are colocalized in the same endosome, these results suggest that at least a substantial fraction of reassociation occurs in the cytoplasm. Furthermore, we showed that reassociation occurs within cells and not in medium. However, details of RNA–DNA internalization and reassociation events need to be investigated further.

We further demonstrated computationally²⁹ and experimentally³⁰ that multiple split RNA functionalities could be embedded in the same RNA–DNA hybrids, resulting in their simultaneous coactivation. This was achieved through the introduction, in a controlled fashion, of multiple functional entities, such as RNAi inducers (up to seven at once), FRET pairs for tracking of reassociation in real time, and RNA aptamers for fluorescence response on reassociation or specific cell targeting, just to name a few.^{27,30,31} However, the number of functionalities activated at once was limited by the total length of the DNAs and the longer DNA duplexes that resulted from reassociation became immunogenic.³⁰ To overcome these limitations, individual hybrids were introduced as the extremities of the compact RNA nanoparticles, nanorings,¹⁰ and nanocubes,⁹ which were previously designed and characterized as programmable RNA nanoscaffolds.^{7,32–34} For all of the approaches described above,³⁵ the ssDNA toeholds had to be specifically designed to avoid any internal secondary structures and any undesired interactions with the scaffold strands or split functions while triggering the reassociation of the hybrids at physiological temperature (37 °C). These limitations would potentially require computer-aided sequence optimization for each new generation of hybrid nanoparticles. The current methodology schematically depicted in Figure 1a relies solely on the use of RNA–DNA hybrids with ssRNA toeholds and demonstrates a significant simplification of all design principles in the existing technique. The overall size of the new nanoparticles amenable to intracellular activation becomes significantly smaller (up to 120 nts less per nanoparticle) compared to the older designs,¹⁰ and the current method allows better yields in the cotranscriptional assemblies of the hybrid nanoparticles. Also, a novel computational algorithm aiming at the prediction of multistranded hybrid secondary structures and their further reassociation was developed to confirm the experimental results.

Rational Design of RNA–DNA Hybrids.

As the proof of concept, we split the function of a Dicer Substrate RNA (DS RNA), designed to downregulate the production of green fluorescent protein (GFP)³⁶ that is stably expressed in model human breast cancer cells (MDA-MB231/GFP). The use of DS RNAs (as opposed to siRNAs) is required to ensure that once inside the cells, the individual hybrids will not be active in the RNAi pathway.³⁰ GFP DS RNAs were split between two RNA–DNA hybrids with the DNA strands being 8, 6, 4, and 2 nts shorter than their corresponding complementary RNAs, thus, providing the ssRNA toeholds for further reassociation. Later in the text and in the figures, we will refer to hybrids containing the sense strand of DS RNA as H_{sen} and to hybrids containing the antisense strand as H_{ant}. A scheme explaining the reassociation of new hybrids studied in this work is shown in

Figure 1a. All RNA and DNA sequences used in this project are listed in the Supporting Information.

Reassociation of RNA–DNA hybrids.

Four sets of cognate RNA–DNA hybrids with different ssRNA toehold lengths (2-, 4-, 6-, and 8-nt) were prepared and tested in parallel (Figure 1). The reassociation of the hybrids was first assessed by native-PAGE experiments (Figure 1c and Supporting S1). The results show that the extent of reassociation is dependent on the length of the ssRNA toeholds. In particular, only partial reassociations were observed for the hybrids with toeholds of 4-nt and less. As will be discussed later, *in silico* predictions based on a novel multistrand secondary structure prediction approach confirmed these results.

To trace the reassociation of hybrids in solution in real time, Förster resonance energy transfer (FRET) was measured. The kinetics of reassociation were studied using fluorescently labeled (with Alexa 488 and Alexa 546) RNAs entering the composition of the different hybrids. When two fluorescently labeled hybrids are mixed and incubated at 37 °C, their reassociation brings Alexa 488 within the Förster radius ($R_0 = 6.31$ nm) of Alexa 546 (Figure 1c). Consequently, when excited at 460 nm, the emission of Alexa 488 significantly drops while the fluorescent signal of Alexa 546 increases (Figure 1c and Supporting S2). The results indicate strong dependence of the extent of reassociation on the toehold lengths and confirm the previous observation.

Reassociation of RNA–DNA Hybrids in Human Cells.

The ability of the hybrids with ssRNA toeholds to enter and reassociate inside mammalian cells was assessed. Fluorescently labeled hybrids were cotransfected into human breast cancer cells and analyzed with confocal microscopy the next day (Figure 1d).^{9,10,27} The samples were excited at 488 nm, and the emission of Alexa546 was collected. To estimate the extent of intracellular FRET, Alexa546 sensitized emission was imaged as detailed in our previous work.²⁷ The FRET signal remaining upon bleed-through correction was calculated and is shown in blue (Figure 1d, images 1 + 4 and 5). The ssRNA-toehold driven intracellular reassociation of RNA–DNA hybrids was further confirmed by specific gene silencing experiments with human breast cancer cells stably expressing GFP. First, cells were cotransfected with only one hybrid at a time (H_ant), and 3 days later, the level of eGFP expression was analyzed with fluorescence microscopy and flow cytometry. All experiments were repeated in triplicate, and results demonstrated no GFP silencing caused by the individual hybrids (Supporting Figure S3). However, when the same cells were cotransfected with separately prepared complexes of Lipofectamine 2000 (L2K) and individual hybrids (H_sen/L2K and H_ant/L2K), significant GFP silencing was observed (Figure 1e). It was hypothesized that the longer ssRNA toeholds would be positively correlated with a higher level of reassociation and gene silencing. Interestingly, the extent of silencing was proportional to the ssRNA toehold lengths only at relatively low concentrations (1 nM) but not at the higher ones (30 nM). Even though the reassociation was not detected for hybrids with 2-nts toeholds at 1 μ M concentrations (Figure 1b,c), significant gene silencing was observed for the same hybrids at 30 nM concentrations. This discrepancy

can be explained by the use of the polycationic carrier (L2K) that electrostatically attracts high quantities of individual hybrids during the transfection incubation step (see Methods), and then releases them intracellularly in much smaller volumes (in endosomes), thus providing a significant increase in the “local” concentration of cognate hybrid. This phenomenon is being investigated further and will result in a separate report. But to test the feasibility of the existing hypothesis, several reassociation experiments at higher hybrid concentrations were carried out; these showed that at high enough concentrations (5 μ M or higher), the reassociation occurs even between the hybrids with 2 nts ssRNA toeholds (Supporting Figure S4).

To demonstrate the generality and the therapeutic potential of this approach, we tested two additional sets of hybrids designed against the full-length genomic HIV-1 RNA that also serves as the mRNA coding for the viral structural proteins and enzymes.^{37–40} Gag and LDR hybrids targeted the sequences coding the capsid domain and the amino terminus of the matrix domain of Gag, respectively (Figure 2a,b). Both hybrids were designed to have 8 nt ssRNA toeholds. Transfection of the individual hybrids showed no significant effect on relative virus release. However, the cotransfection of cognate hybrids reduced the production of virus by 65% to 75% (Figure 2c). The total amount of Gag was reduced on average by 65% with the reassociated hybrids, while the cellular expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were not affected.

Controlled Activation of RNAi by Nanorings Functionalized with RNA–DNA Hybrids.

To guarantee the simultaneous delivery and intracellular activation of multiple RNA–DNA hybrids, the previously characterized nanorings were employed.³⁴ Each nanoring was functionalized with six RNA–DNA hybrids of varying ssRNA toehold lengths (2, 4, 6, and 8 nts in length). The toeholds were oriented toward the nanoring scaffolds to improve the stability of ssRNAs (exonuclease degradation). All nanoparticles were produced cotranscriptionally,^{32,41–43} gel purified, recovered, and further characterized. The relative yields of the cotranscriptionally produced nanoparticles with ssRNA toeholds were higher than the yields of the previously characterized nanoparticles¹⁰ containing ssDNA toeholds (Supporting Figure S5). All assemblies were confirmed by native-PAGE, and their reassociation was visualized using fluorescently labeled cognate hybrids (Figure 3b). In agreement with previous results, the assemblies with 4 and 2 nts ssRNA toeholds demonstrated only limited reassociation, and the 6 and 8 nts toeholds were the most effective in forming the fully functionalized nanorings (with six DS RNAs). The formation of functional rings was further supported by cell transfection experiments (Figure 3c and Supporting S7).

Computational Studies of RNA–DNA Hybrid Reassociation.

The estimated free energies of the non-reassociated hybrid state, the reassociated state, and a quadruplex intermediate state are shown in Figure 4a for different toehold lengths and for different concentrations. Interestingly, the computational thermodynamic predictions indicate that all toehold strand lengths should lead to reassociation, because the reassociated

state has a lower free energy compared to the RNA/DNA hybrids (Figure 4a). Estimating, however, the free energy of activation of the transition state (we used a model of a quadruplex conformation consisting of non-reassociated RNA/DNA hybrid helices but bound cognate toeholds), suggests that hybrid sequences with toehold lengths of less than 6 nt are prevented from reassociation because of an energetic barrier, possibly leading to kinetic frustration. Conversely, for higher concentrations and for toehold lengths greater than 4 nt (according to the computational model), there is no kinetic barrier with respect to the model transition state (Figure 4a–b). In other words, both the experimental and computational results suggest a saturation of the reassociation rate as a function of toehold length for toeholds longer than 4 nt. Similar tendencies have been found computationally for the case of RNA–RNA strand-invasion.⁴⁴

Furthermore, we computed structure predictions for the RNA nanoring scaffold sequences (Figure 4c). One can see, that the structure prediction corresponds to a ring structure connected by kissing loop interactions. A secondary structure prediction of the functionalized nanoring sequences with cognate DNAs (Figure 4d) shows the expected strand interactions with the exception of one kissing loop interaction.

Methods.

RNA/DNA Nanoparticles and Hybrid Assemblies and Native PAGE.

The designing principles and production of RNA strands entering the composition of the nanorings functionalized with six siRNAs is comprehensively described elsewhere.¹⁰ The full list of RNA and DNA sequences used is available in Supporting Information. Fluorescently labeled RNA molecules were purchased from Integrated DNA Technologies, Inc. RNA–DNA hybrids were assembled as detailed elsewhere,²⁷ and RNA/DNA nanorings were produced cotranscriptionally.⁴¹ Briefly, synthetic DNA molecules coding for the sequence of the designed RNA were purchased and amplified by PCR using primers containing the T7 RNA polymerase promoter. Corresponding DNA templates were mixed together with ssDNA required for hybrid formations and RNA–DNA nanoparticles were produced enzymatically by in vitro runoff transcription using wildtype T7 RNA polymerase. Transcription was performed in 80 mM HEPES-KOH, pH 7.5; 2 mM spermidine; 50 mM DTT; 25 mM MgCl₂; 1 mM NTPs; 0.2 μ M of DNA templates, 50 μ M of ssDNA for hybrids; and T7 RNA polymerase. The resulting cotranscriptionally assembled nanorings functionalized with six hybrids were purified by native-PAGE and further analyzed. Typically, all assembly experiments were analyzed at 10 °C on 7% (37.5:1) native polyacrylamide gels in the presence of 89 mM Tris-borate, pH 8.3, and 2 mM Mg(OAc)₂. ChemiDoc MP System was used to visualize fluorescently labeled (Alexa 488 and Alexa 546) and EtBr stained RNA, RNA–DNA, and DNA–RNA based assemblies.

FRET Studies.

Reassociation of cognate RNA–DNA hybrids in vitro was tracked with FRET measurements using a FluoroMax3 (Jobin-Yvon, Horiba). All fluorescently labeled RNAs are presented in Supporting Information (Fluorescently labeled molecules). For all the experiments, the excitation wavelength was set at 460 nm, and the excitation and emission slit widths were set

at 2 nm. Alexa 488 labeled constructs were first incubated for 2 min at 37 °C, and then Alexa 546 labeled constructs were added. Upon excitation at 460 nm, the emissions at 520 and 570 nm were recorded simultaneously every 30 s to follow the process of reassociation through FRET measurements. This experiment was also carried out with hybrids individually preincubated with L2K (Invitrogen) in the amounts relevant for the transfection conditions. In vitro fluorescent experiments show no reassociation of these complexes in solution (Supporting Figure S5); thus, the reassociation occurs only in cells.

Transfection of Human Breast Cancer Cells MDA-MB-231.

MDA-MB-231 cells either with or without eGFP were grown in a 5% CO₂ incubator in DMEM (Gibco BRL) supplemented with 10% FBS and penicillin–streptomycin. All transfections in this project were performed using L2K. 50× solutions of hybrids were individually preincubated at room temperature with L2K for 30 min. RNA–DNA nanorings were cotransfected with their cognate RNA–DNA hybrids (at 6-fold higher concentrations). To avoid reassociation in media, cognate RNA–DNA hybrids were preincubated with L2K separately. Prior to each transfection, the cell media was swapped with OPTI-MEM mixed with prepared 50× of hybrids/L2K complexes to the final concentration of 1X. The cells were incubated for 4 h followed by the media change (DMEM, 10% FBS, 1% pen-strep).

9,10,27

Confocal Microscopy.

The intracellular reassociation of RNA–DNA hybrids was assessed through FRET.^{9,10,27} All measurements were performed using a LSM 710 confocal microscope (Carl Zeiss) with a 63×, 1.4 NA magnification lens. All images were taken with a pinhole adjusted to 1 airy unit. Fluorescently labeled hybrid nanoparticles and cognate hybrids were individually preincubated with L2K and cotransfected into cells. On the next day, the samples were fixed by incubation in 4% paraformaldehyde for 20 min at room temperature. Images of the cells were then taken to assess the appearance of FRET within the sample. For Alexa 488 imaging, the 488 nm line of an argon laser was used as excitation, and the emission was collected between 493 and 557 nm. For Alexa 546 imaging, a DPSS 561 laser was used for excitation, and emission was collected between 566 and 680 nm. In order to evaluate the sensitized emission through FRET, images were taken exciting the sample with the 488 nm line and collecting emission between 566 and 680 nm. Because of spectral overlap, the FRET signal is contaminated by donor emission into the acceptor channel and by the excitation of acceptor molecules by the donor excitation wavelength. This bleed-through was assessed via measurements performed with samples transfected with individual dyes and mathematically removed from the images of FRET (Supporting Figure S9).

Flow Cytometry Experiments.

MDA-MB-231 cells expressing GFP were grown in 12-well plates (1.0×10^5 cells per well) and lifted with a cell dissociation buffer (Gibco BRL). The level of GFP was measured by fluorescence-activated cell sorting (FACS) analysis on a FACScalibur flow cytometer (BD Bioscience).^{45–48} At least 20 000 events were collected and analyzed using the CellQuest software. For statistical analysis, the geometric mean fluorescence intensity (gMFI) and standard error of the mean (SEM) were calculated and plotted.^{9,10}

HIV-1 Experiments.

HeLa cells were cultured in a 24-well plate, in a 5% CO₂ incubator, in antibiotic-free DMEM (Gibco BRL) supplemented with 5% FBS (Atlanta biologicals). Nucleic acids and L2K were mixed at concentrations higher than recommended by the manufacturer; for each well, 0.66 μ g HIV-1 infectious clone pNL4-3,⁴⁹ 0.05 μ g *Gaussia* luciferase reporter plasmid pGLuc (NEB) and either 2.5 or 10 pmol of RNA/DNA hybrids, were combined in 15 μ L of Optimem. 2.4 μ L of L2K was mixed with 15 μ L of Optimem and incubated for 5 min before combining with the nucleic acids. To avoid reassociation in media, cognate RNA–DNA hybrids were preincubated with L2K separately. The transfection mix was incubated for 30 min at room temperature then added dropwise to cells. After incubation for 8 h, the media were removed and replaced with DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Gibco BRL). After 48 h, supernatant was collected and supplemented with 0.5% Triton X-100 to inactivate infectious HIV-1 particles. Samples of supernatant were assayed for *Gaussia* luciferase activity, as a reporter for transfection efficiency and toxicity, using Biolum (NEB) following the manufacturer's instructions; and for reverse transcriptase (RT), a component of the HIV-1 virion, as described previously.⁵⁰ Virus release was calculated as the ratio of RT to luciferase signal. Cells were also harvested in 2 $\times$ Laemmli buffer (20% glycerol, 4% SDS, 0.01% bromophenol blue, 700 mM β -mercaptoethanol, 100 mM Tris pH 6.8) and probed by Western blotting with HIV immunoglobulin (NIH AIDS Reagent Program Catalog no. 3957) and monoclonal mouse anti-GAPDH (clone 6C5, Santa Cruz), and species-specific horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific). Bands were revealed using chemiluminescence, and the signal was recorded using a Chemidoc XRS+ system (Biorad). Band volumes were determined using Image Lab software (Biorad); to determine Gag expression, volumes for p55, p41 and CA were summed, and then divided by the corresponding GAPDH volume.

In Silico Predictions of RNA–DNA Hybrid Reassociation.

In the novel computational approach called HyperFold (described in more detail in Bindewald et al. Multistrand Structure Prediction of Nucleic Acid Assemblies and Design of RNA Switches. Accepted to *Nano Lett.*), an estimated free energy of a large variety of secondary structure states of the four involved sense and antisense RNA and DNA strands is computed. The free energy contributions of base pairing are based on base pair stacking energies for RNA/RNA, DNA/DNA, and RNA/DNA interactions. Entropy changes with respect to the unfolded state are estimated using a geometric distance-based model. In this approach, for each pair of nucleotides, maximally attainable residue–residue distances are computed for each examined secondary structure state. Each formed base pair can lead to a reduction of these maximally extended conformations and thus to a reduction in entropy. This distance-based approach is a general model for estimating entropy contributions that is not limited by the number of involved nucleotide strands and is applicable to pseudoknotted structures.²⁹ From this list of states, concentrations of the RNA/DNA hybrids, the reassociated RNA and DNA duplexes as well as a variety of intermediate states can be estimated. The free energy of activation from the state of RNA/DNA hybrid duplexes to the quadruplex intermediate state is estimated by estimating the free energy change of the formation of the first duplex between the cognate toehold regions (without unfolding of any

bases pairs of the RNA/DNA hybrid start state). In other words, for an RNA/DNA hybrid structure with 6 nt toeholds, the free energy change of forming a 6 nt helix between the cognate toehold regions of the nonreassociated RNA/DNA hybrids strands is estimated as shown in Figure 4b for different toehold lengths and strand concentrations. Other strand configurations (such as complexes involving 3 strands) were ruled out as important transition states because they are predicted to have unfavorable free energies. For the case of the hexameric ring with 6 RNA strands and 6 cognate DNAs, the program was run with command-line options that reduce the search space by restricting the considered pseudoknots to “inter-strand pseudoknots”, in other words the considered secondary structures are such that they would be pseudoknot-free if one would remove the interstrand interactions. Details of the used program parameters are listed in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

RNA toeholds initiate the reassociation of RNA/DNA hybrids that leads to activation of RNAi and FRET. (a) Schematic representation of reassociation for RNA/DNA hybrids with 10 nts RNA toeholds. (b) Native-PAGE demonstrates the reassociation (1 h at 37 °C) of fluorescently labeled hybrids with 2, 4, 6, and 8 nts RNA toeholds (at 1 μM final). (c) In vitro analysis of hybrids with 2, 4, 6, and 8 nts RNA toeholds reassociation (but no Dicing) using FRET. Emission of Alexa-488 was measured every 30 s. Hybrids containing Alexa-546 were added 2 min after the incubation. (d) FRET experiments: cells were cotransfected with cognate hybrids, with 8 nts RNA toeholds (100 nM final), labeled with Alexa546 and Alexa488, and images were taken on the next day. 3D chart representation of zoomed fragment indicated by a white box of bleed-through corrected FRET image. (e) GFP knockdown assays for GFP expressing human breast cancer cells. Three days after the cotransfection of cells with hybrids, GFP expression was statistically analyzed with flow cytometry experiments. Hybrid concentrations (in nM) used in the silencing experiments are indicated. Error bars denote ± SEM.

Figure 2.

Inhibition of HIV-1 gene expression by RNA/DNA hybrids with 8 nts RNA toeholds. HeLa cells were transfected with an HIV-1 infectious clone, pGluc, and nucleic acids as indicated. The control dsRNA targets mRNA of the cellular glutathione S-transferase P1 gene.¹³ Samples were harvested after 48 h. (a) Cell lysates were probed by Western blotting for HIV-1 Gag protein and the constitutively expressed cellular protein GAPDH. (b) Band intensities were measured, and Gag expression was calculated as the sum of p55Gag, p41, and CA divided by GAPDH for each sample. These values were expressed relative to HIV-1 in the absence of nucleic acid, and the results of four independent experiments were plotted $\pm$ SEM. (c) Samples of culture media were assayed for the RT activity as an indicator of HIV-1 particle release and for the presence of Gaussia luciferase. The RT value was normalized to the Gaussia luciferase value and expressed relative to HIV-1 in the absence of nucleic acids and the results of four independent experiments were plotted. Hybrid concentrations (in nM) used in these gene silencing experiments are indicated. Error bars denote $\pm$ SEM.

Figure 3.

RNA toeholds initiate the reassociation of RNA/DNA based nanoparticles (nanorings or NR) and cognate RNA/DNA hybrids. (a) Schematic representation of reassociation of NR and RNA/DNA hybrids with RNA toeholds. (b) Co-transcriptionally assembled (at 37 °C for 4 h) and gel purified NR with six hybrids visualized with total staining native-PAGE (left) and reassociation (1 h at 37 °C) of nonlabeled NR with six hybrids (with 8, 6, 4, and 2 nts RNA toeholds) traced through the incorporation of fluorescently labeled (with Alexa488 RNA) strands of cognate to NR hybrids. DNA strands are not labeled. The trace amounts of fluorescence at the duplex site of the 8 nt NR lane can be attributed to the excess of hybrid duplexes added to NR. (c–d) Intracellular reassociation of hybrid nanorings (10 nM) and cognate hybrids triggers the GFP silencing. Three days after the cotransfection of cells with nanorings decorated with hybrids and cognate hybrids, GFP silencing was confirmed by fluorescent microscopy and statistically analyzed with flow cytometry experiments. Error bars denote ± SEM.

Figure 4.

In silico prediction of RNA/DNA hybrids reassociation initiated by ssRNA toeholds. (a) Predicted free energies for individual RNA/DNA hybrids with different lengths of ssRNA toeholds (2, 4, 6, and 8 nts), for their transition states and for their reassociated states (dsRNAs and dsDNAs). Shown are results for concentrations of 0.03, 0.25, 1, 5, and 10 $\mu\text{mol/L}$. (b) Predicted free energies of toehold activation for each transition state model. For high energies of activation the nonreassociated state becomes kinetically trapped. This is the case for 2 and 4 nt toeholds, unless high concentrations are employed. We are using a model of a transition state as consisting of the nonreassociated RNA/DNA hybrid helices combined with binding of the RNA toeholds. The shown labels indicate toehold lengths. (c–d) The circular plot depicts intrastrand base pairs as blue arcs and interstrand base pairs as red arcs. Nanorings are strands A–F, and cognate DNAs are strands G–L. (c) Predicted ring scaffold for RNA sequences without toehold arms. (d) Predicted ring with hybrid arms and bound cognate DNAs (before reassociation).