Nucleic Acids Research, 2023, **51**, 11291–11297
https://doi.org/10.1093/nar/gkad802
Advance access publication date: 9 October 2023
RNA and RNA-protein complexes

Resolving altered base-pairing of RNA modifications with DNA nanoswitches

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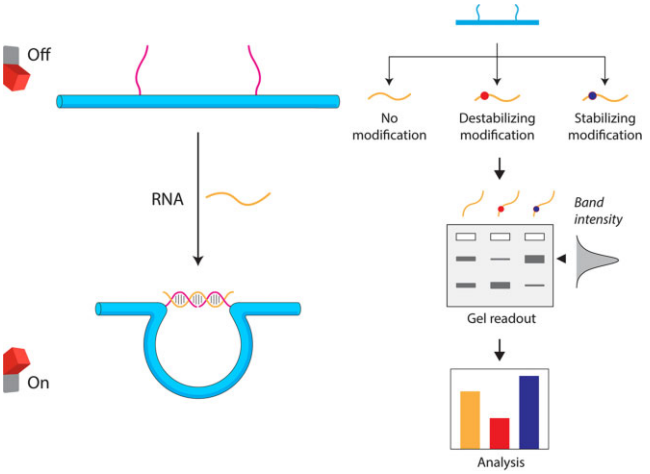
Abstract

There are >170 naturally occurring RNA chemical modifications, with both known and unknown biological functions. Analytical methods for detecting chemical modifications and for analyzing their effects are relatively limited and have had difficulty keeping pace with the demand for RNA chemical biology and biochemistry research. Some modifications can affect the ability of RNA to hybridize with its complementary sequence or change the selectivity of base pairing. Here, we investigate the use of affinity-based DNA nanoswitches to resolve energetic differences in hybridization. We found that a single m^3C modification can sufficiently destabilize hybridization to abolish a detection signal, while an s^4U modification can selectively hybridize with G over A. These results establish proof of concept for using DNA nanoswitches to detect certain RNA modifications and analyzing their effects in base pairing stability and specificity.

Graphical abstract



Introduction

RNA molecules are generally known to have four canonical bases, but chemical modifications of RNA provide a rich diversity in structure and function. There are >170 RNA modifications that are currently known (1), including methylations, thiolations and other chemical groups at different nucleoside positions (2). Modified RNAs can be present in many varieties of coding and non-coding RNAs, but our knowledge of their many locations and functions is still far from complete. Chemical modifications are generally moderated and regulated by enzymes including writers, readers and erasers, changing the biophysical properties of RNA in ways that can influence RNA generation, transportation, function and metabolization in a dynamic and reversible manner (3–5). Modified RNAs can influence normal biological processes as well as progression of diseases (6,7). For example, RNA modifications have been shown to participate in immune cell biology by altering activation of immune regulating dendritic cells (8) and play critical roles in cancer cells, such as proliferation, metastasis, metabolism, apoptosis and treatment resis-

Received: July 13, 2023. **Revised:** August 31, 2023. **Editorial Decision:** September 4, 2023. **Accepted:** September 19, 2023

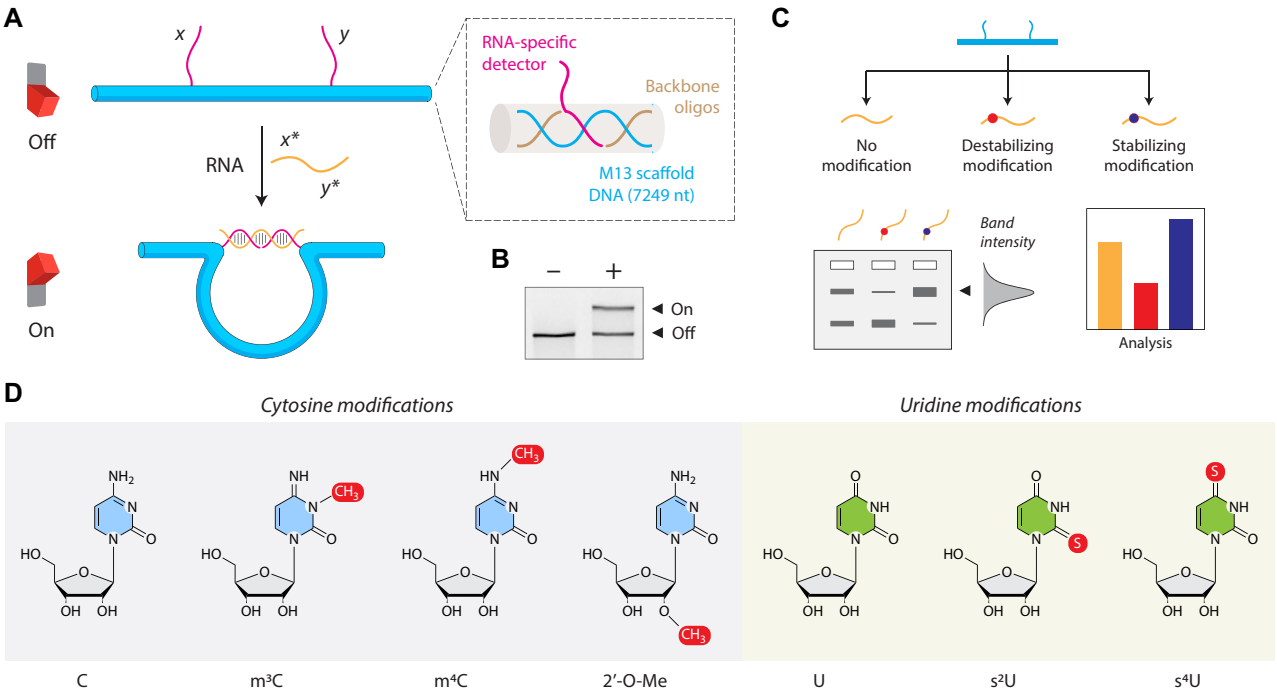


Figure 1. DNA nanoswitch design and operation to monitor base pairing changes in RNA modifications. (**A**) The DNA nanoswitch undergoes a predefined conformational change on binding a target RNA sequence, flipping the switch from a linear 'off' state to a looped 'on' state. (**B**) The two states of the nanoswitch can be identified using a gel electrophoresis readout. (**C**) The presence of RNA modification affects the looping efficiency of the nanoswitch, which can be resolved by measuring the looped band intensity in the gel readout. (**D**) Chemical structures of RNA modifications used in this study.

tance (9). Because of their wide influence, RNA modifications have gained interest for therapeutic potential (10), which has already been partly demonstrated in the use of modified nucleotides to improve RNA-based vaccines such as those used for COVID-19 (11,12).

Technology remains a major hurdle in understanding RNA modifications and in unlocking their potential (13). While several detection and analysis techniques have been reported (14), there is currently no method to report the full sequence of RNA with its diversity of modifications. Mass spectrometry techniques have been shown to identify specific modifications, but typically only at the nucleoside level when sequence information has been lost (15). Conversely, sequencing based methods retain sequence information but are limited in the modifications they can report (16,17). The push for new technologies in the RNA modification space has become increasingly vocal (13,18,19).

Here, we investigate the use of our DNA nanoswitch technology for measuring the presence and effect of RNA modifications. These nanoswitches work by arrangement of single-stranded 'detector' regions placed along a linear dsDNA, such that binding of two detectors to a single RNA or DNA sequence induces a conformational change to a looped structure (Figure 1a and b). Recent efforts from our laboratory demonstrated programmable DNA nanoswitches for the detection of nucleic acid sequences including microRNA and viral RNA (20–23). By altering the length and composition of the detector sequences, we previously showed that we could resolve a single-nucleotide variation (20). Here, we expand that work to investigate how nanoswitches can be designed to resolve small energetic changes brought by RNA modifications (Figure 1c).

Materials and methods

Reagents and oligonucleotides

DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting. For modified RNA oligomer synthesis, 2-Thio uridine CE phosphoramidite, 4-thio uridine phosphoramidite and 2′-*O*-methyl cytidine phosphoramidite were purchased from ChemGenes. N4-methyl-cytidine phosphoramidite and N4-benzyl-N3-methyl-cytidine phosphoramidite were synthesized in the laboratory based on the protocol we developed earlier (24,25). Oligonucleotide sequences used in this study are provided in Supplementary Table S1.

Synthesis of modified RNA oligonucleotides

All modified RNA oligonucleotides were synthesized in-house by standard solid phase synthesis using an automated Oligo-800 DNA synthesizer (24,25). After synthesis, oligonucleotides were cleaved and deprotected using AMA (a mixture of ammonium hydroxide and methylamine solution, 1:1) by heating the sample at 65°C for 45 min. The cleaved supernatant RNA samples were collected in separate Eppendorf tube and samples were fully dried in a speed vac evaporator. The dried RNA samples were dissolved in dimethylsulfoxide and triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$), and then heated at 65°C for 150 min to deprotect all tertbutyldimethylsillyl groups, before being subjected to ethanol precipitation. The crude RNA oligos were purified using 15% preparative denaturing polyacrylamide gel electrophoresis (PAGE). After butanol extraction, oligomers were desalted, concentrated and lyophilized before redissolving in RNase-free water.

The RNA oligonucleotide concentrations were determined by UV absorbance at 260 nm using a nanodrop (Thermo Fisher Scientific).

Mass spectrometry

Oligonucleotides synthesized in-house were characterized by LTQ Orbitrap Velos Mass spectrometry. Before injection, PAGE-purified 10 μM RNA samples were filtered through sigma syringe filter (0.45 μm). Then 1–5 μl volume of sample was injected for each analysis. Composition of buffers used for the LC were as follows: Buffer A: 100–400 mM Hexafluoro isopropanol+triethylamine (25 mM) + 0.4% MeOH (pH 7.8), Buffer B: 95% Acetonitrile and specifications of column were Waters BEH 300A C18 (300 μm × 10 cm). Data were processed using the Xcalibur software. Finally, raw text files were processed in the UniDec software to obtain deconvoluted spectra. Calculated and observed mass for all the modified RNA sequences are shown in Supplementary Table S2.

Design of DNA nanoswitches

The nanoswitch is formed using the backbone oligonucleotides that hybridize with the single stranded M13 scaffold to form a duplex (Supplementary Figure S1). In addition, a set of 'variable' strands are designed to bind to the M13 at the prospective positions of the detector strands. The variable strands are replaced by the detector strands for forming a nanoswitch of a particular loop size. The open gaps left by the replacement of the variable with detector strands are filled using the 'filler' strands (sequences are provided in Supplementary Table S3).

Construction of DNA nanoswitches:

Single-stranded circular M13 DNA (New England Biolabs) was linearized according to our previously reported protocol (26). Nanoswitches were constructed using the linearized M13 scaffold DNA, backbone oligonucleotides and other oligonucleotides according to our previously reported protocol (26,27). Briefly, the linearized single-stranded DNA was mixed with 10-fold excess of the backbone oligonucleotides, variable strands, detector strands and filler strands. The mixture was annealed from 90 to 4°C at 1°C/min in a T100 Thermal Cycler (Bio-Rad, USA). Assembled DNA nanoswitches were purified using liquid chromatography to remove excess oligonucleotides (28).

Analysis of modified RNA

To test RNA oligonucleotides containing modifications (target RNA), the LC purified nanoswitches (80 pM) were incubated with the 22 nt target RNA (2 nM) for 1 h at 20°C. Concentrations of final solutions were determined by the volume ratios during dilutions. Typical reactions were carried out in PCR tubes with 10-μl final volumes. To increase selectivity, we used various detector lengths (one detector was kept at a constant 11 nt and the other detector was 9, 10 or 11 nt).

Gel electrophoresis

Samples were mixed with a solution containing 2:1 mixture of a Ficoll-based loading dye and 1× GelRed stain (Biotium) and run on 0.8% agarose gels. Gels were cast from molecular biology grade agarose (Fisher BioReagents) dissolved in 0.5× Tris-borate EDTA (TBE) (Ultra-pure grade, Amresco). Gels were typically run at 75 V (constant voltage) at room temperature for 45 min. Gels were imaged with a Bio-Rad Gel Doc XR+gel imager and analyzed using the gel analysis tool in the Image Lab software package available with Bio-Rad Gel Doc XR+.

Results

To demonstrate detection of RNA strands containing modifications, we first chose methylated nucleotides, which are the most common natural modification and have been shown to play key biological roles in cellular RNAs including regulation of RNA-protein recognition, stabilization of tRNA structure, reinforcement of the codon-anticodon interaction and wobble base pairing, as well as messenger RNA (mRNA) reading frame maintenance (29). RNA methylations are also involved in the translation process in both cap-dependent and cap-independent mechanisms (30). As proof of concept, we first chose three modifications of cytosine with methylations at different positions: N^3-methylcytidine (m^3C), N^4-methylcytidine (m^4C) and 2′-O-methyl (2′-O-Me) (Figure 1d). Our previous biophysical characterization of such modified RNAs showed that even a single m^3C modification in a 12-bp duplex reduces the thermal melting temperature by ∼20°C (31). We designed a set of RNA oligonucleotides derived from the let-7b microRNA sequence that was used in our prior work to show that we can distinguish microRNAs containing even a single nucleotide mismatch (20). For this work, we modified this sequence to contain native cytidines or modified cytidines. We previously reported the synthesis of m^3C and m^4C phosphoramidites (24,25) as well as solid phase synthesis of RNA oligonucleotides containing these modifications (31,32). Here, we used this optimized oligonucleotide synthesis procedure to synthesize a 22 nt native RNA as well as RNA containing one or two m^3C, m^4C and 2′-O-Me modifications (see Methods). We purified the in-house synthesized RNA oligonucleotides using denaturing PAGE and analyzed them using mass spectrometry before using them with the DNA nanoswitches (Supplementary Figures S2–S11).

We designed a DNA nanoswitch with two detectors that are complementary to each half (11 nt each) of the native RNA oligonucleotide. We constructed the DNA nanoswitches by mixing the 7249 nt M13 scaffold strand, backbone oligonucleotides and the pair of detectors specific to the RNA, followed by a thermal annealing step (20). We then purified the nanoswitches using a UPLC-based method we reported earlier (28). We incubated the DNA nanoswitches with ∼2 nM target RNA for 1 h at 20°C in a solution containing tris-HCl and 10 mM magnesium chloride.

To observe the effect of methylation at different positions in the nucleotide, we tested our nanoswitch against the native RNA and the doubly modified RNA with two m^3C, m^4C and 2′-O-Me modifications (Figure 2a and b). We found that only the m^3C modified strand prevented base pairing while the other two modifications had binding similar to the native strand (Figure 2b). This result was expected since the N^3-methyl group can disrupt the canonical base pairing with G (Figure 2c).

Next we focused on optimizing our detection of m^3C. In earlier work detecting single nucleotide mismatches in microRNAs, we decreased the detector length on the mismatch side until the energetic change of a single mismatch was sufficient to completely abolish our signal (20). Here we took a

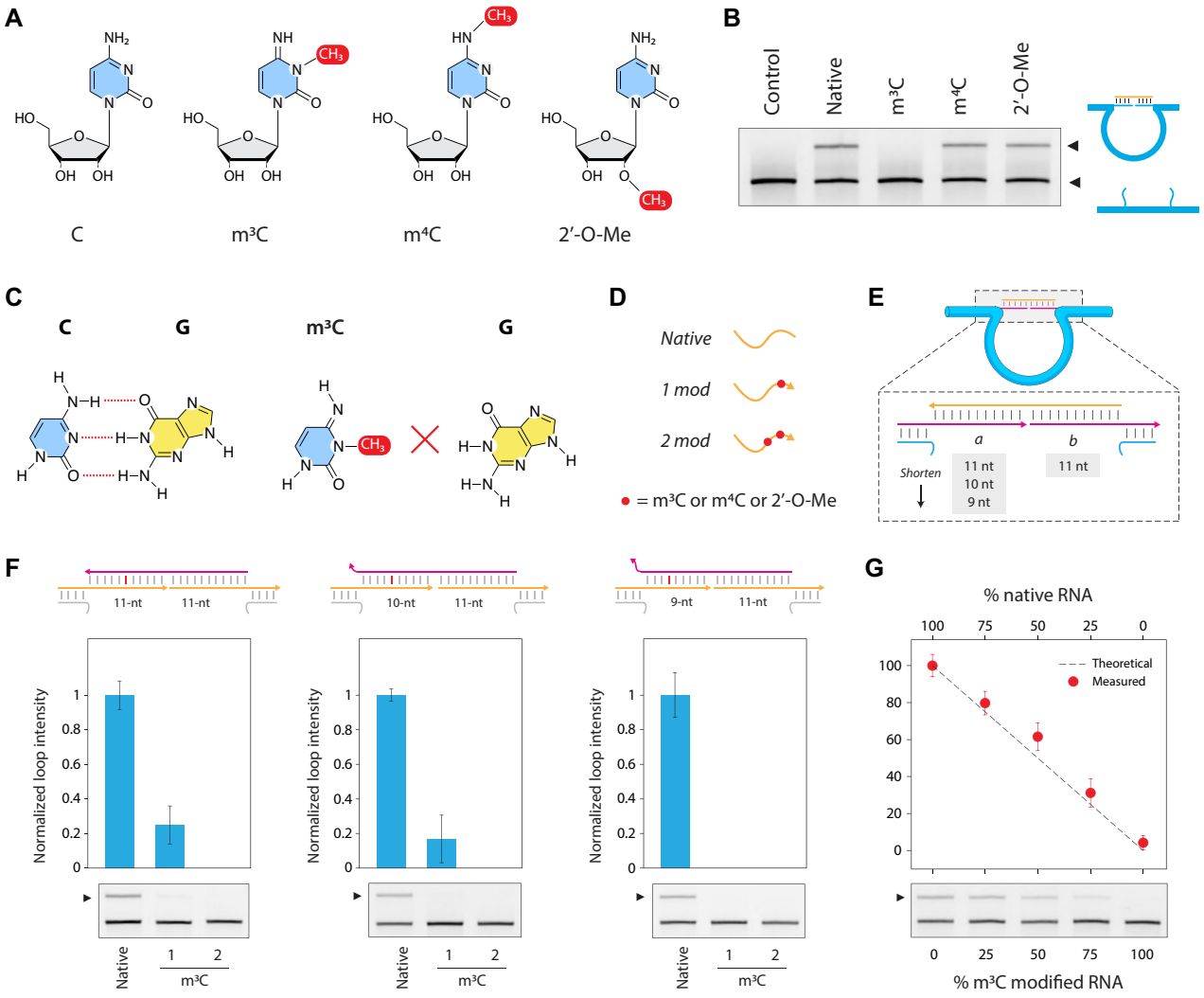


Figure 2. Detection of m^3C modifications by altered base pairing. (**A**) Chemical structures of cytidine modifications tested here, (**B**) analysis of nanoswitch looping with modified target RNA, (**C**) base pairing between C and G and effect of m^3C on C:G pairing, (**D**) RNA strands with different number of chemical modifications tested in this study, (**E**) cartoon of the nanoswitch with different detector lengths, (**F**) change in detection signal with one or two m^3C modifications for different nanoswitch designs and (**G**) signal reduction dependent on the fraction of the strands containing m^3C modifications.

similar approach and reduced the detector length from 11 to 9 nt to better resolve a single m^3C modification (Figure 2d and e). In the nanoswitch with 11 nt detectors, we observed looping of the nanoswitch for the native RNA and an ∼80% reduction in looping signal even for a single m^3C modification and no looping for the RNA with two modifications (Figure 2f and Supplementary Figure S12). The 10-nt version resolved the single modification better, with a ∼90% reduction in looping signal and the 9-nt detector completely abolished the signal. From these results, we can resolve differences between the native RNA and RNA with a single m^3C modification.

In a biological context, it can be common for modifications to occur on only some strands, resulting in a mixture of strands with and without the modification. To more closely resemble this situation, we incubated our nanoswitches (10 nt detector) with mixtures containing different ratios of the native and m^3C modified RNA (mixture containing 0, 25, 50, 75 and 100% modified RNA). We observed that the looping efficiency scaled almost perfectly with the amount of m^3C modification present in the sample, with the 100:0 mixture of native:modified sample giving the highest signal and 0:100 mixture giving no looped signal (Figure 2g and Supplementary Figure S13). These results demonstrate that our method can both detect the m^3C modification and also detect the amount of strands containing the modification in a heterogenous mixture. We similarly tested RNA containing one or two m^4C and 2′-O-Me with the various detector lengths and observed similar looping for these modifications, consistent with the fact that these modifications do not disrupt normal C:G base pairing (Supplementary Figure S12).

Next we turned to a thiol modification, another relatively common modification found in bacterial RNAs. Thiol modifications within the anticodon stem loop of tRNA enhance decoding of mRNA codons in the ribosome and stop frameshifting during translation (33). For this modification, we chose uridine due to some of the unique properties of thiolated uridine. Uridine is known to base pair with adenine but can also form a wobble base pair with guanine (Figure 3a). Thiolation at different positions in uridine has been shown to affect the selectivity of uridine for either adenine or guanine (34).

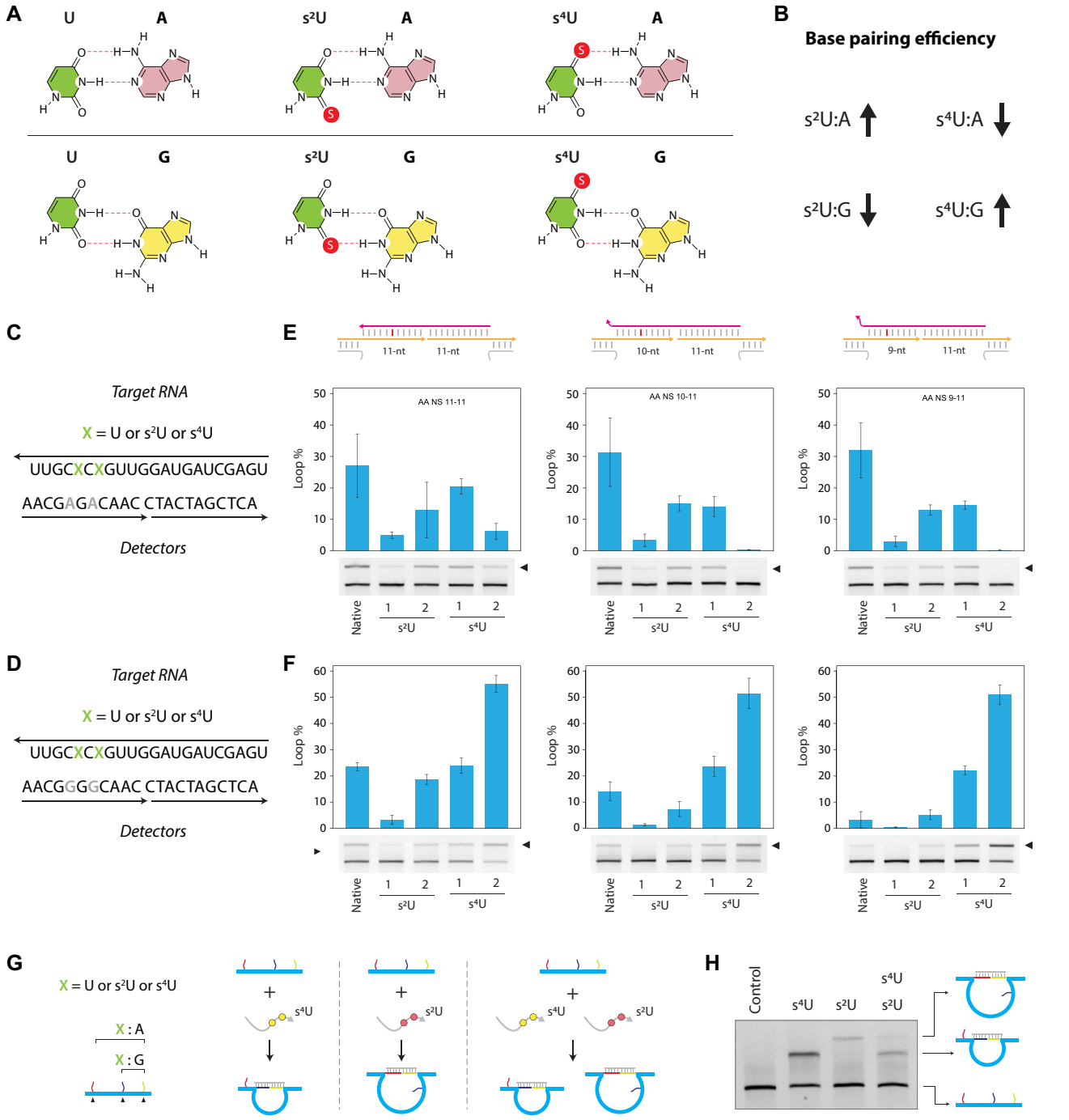


Figure 3. Analysis of s^2U and s^4U modifications by altered base pairing. (**A**) Chemical structure and base pairing of U, s^2U and s^4U with A and G, (**B**) base pairing efficiency of thio-modified RNA with A and G, (**C**) a cartoon of the detectors with A, (**D**) cartoon of the detectors with G, (**E**) change in detection signal with one or two modifications for different nanoswitch designs containing A:U pairing, (**F**) change in detection signal with one or two modifications for different nanoswitch designs containing G:U pairing, (**G**) Schematic of multiplexed analysis of s^2U and s^4U modifications and (**H**) gel showing base pairing preferences of s^2U and s^4U modified RNA in a multiplexed analysis.

In particular, an s^2U modification prefers A over G while an s^4U modification prefers G over A (Figure 3b) (35). To test the effect of thiolation, we synthesized four new RNA strands based on our native sequence with s^2U or s^4U modifications in either one or two locations. We also prepared a set of nanoswitches formulated for Watson-crick base pairing (with U-A), and with wobble pairing (with U-G) (Figure 3c and d). For the native nanoswitches, we found that s^4U modifications had reduced binding that was most evident with the double modifications where no detection was observed when using the 9- or 10-nt detectors (Figure 3e and Supplementary Figure S14). The s^2U modification exhibited reduced binding for one modification and similar binding for two modifications. It has been shown that in RNA:RNA duplexes, s^2U favors C3' endo confirmation that prefers A type helix and forms a more stable s^2U:A base pairing than U:A (35). In this study, the du-

plex made by a DNA:RNA hybrid (detectors and target RNA) might affect s^2U:A base pairing, resulting in reduced binding efficiency for the RNA containing one modification (36). For the wobble nanoswitches, we found that s^4U had increased binding while s^2U had reduced binding (Figure 3f and Supplementary Figure S14).

To further demonstrate how these results could be used to detect and analyze different RNA modifications, we used a multiplexed nanoswitch design (37). The multiplexed design can be accomplished by placing detectors at different locations on the nanoswitch, such that different targets can produce different loop sizes. These different loop sizes can then be resolved as different bands in the agarose gel. Here, we constructed a multiplexed nanoswitch that can detect Watson–Crick pairing with a large loop (U–A pairing) and wobble pairing with a small loop (U–G pairing) (Figure 3g). When using the most selective detector length of 9 nt and the doubly modified strands, our multiplexed nanoswitch was able to detect s^2U only in the Watson–Crick loop and s^4U only in the wobble loop. The combination of both modified strands produced both bands (Figure 3h). Even without complex quantification, our results clearly show that U strongly prefers A over G, s^4U strongly prefers G over A and s^2U is intermediate in its preference.

Discussion

Here we demonstrate for the first time that our DNA nanoswitches can be used to detect biophysical changes due to RNA modifications. We envision that these results can be used in two different ways: first, as a potential mechanism to enable detection of certain RNA modifications within their native sequence, and second, as an analytical method to probe the energetic changes in duplex formation.

As an RNA modification detection scheme, we have shown that the technique can clearly distinguish certain modifications. However, it is unlikely to be scalable to detect many different types of modification, as some may have similar effects or no effect at all. It is also relatively difficult to disentangle whether an energetic disturbance is caused by all strands being modified or only some fraction. For these reasons, we suspect this approach could be more useful for answering specific questions regarding modifications that are known to affect duplex stability.

As an analytical method, the DNA nanoswitch can clearly and sensitively resolve small energetic differences. Alternative approaches to measure these effects include thermal melting using UV-Vis spectroscopy, isothermal titration calorimetry or PAGE. These approaches tend to use a lot of material and require instrumentation and often technical skill. Our method by contrast is inexpensive and can resolve changes in energy for duplexes with approximately fmol or pg scale material (nM concentrations at microliter volumes), nearly 1000 times less than some other methods. Calorimetry and spectroscopy approaches typically require at least micromolar concentrations and volumes of ∼1 ml, whereas nanopore direct sequencing requires a large amount of RNA (∼500 ng). Also, our detection method is relatively fast compared to other existing methods, with <2 h for detection and gel analysis for multiple measurements. Calorimetry and spectroscopy techniques can take several hours per measurement and cannot be as easily multiplexed.

Future work will certainly be needed to improve both detection and analysis using this method, as well as to expand the range of modifications that can be detected or analyzed. However, this first proof of concept shows how our DNA nanoswitches and perhaps DNA nanotechnology more generally may have a useful role to play in the detection and measurement of RNA modifications.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary data

Supplementary Data are available at NAR Online.

Acknowledgements

Author contributions: A.R.C., J.S. and K.H. conceived the project. J.S. and K.H. supervised the project. I.A.T. synthesized oligonucleotides, designed and conducted experiments, analyzed data and wrote the paper. A.R.C. designed experiments, visualized the results and wrote the paper. J.A.P. designed experiments and wrote the paper. S.M. synthesized phosphoramidites. P.H. synthesized and characterized oligonucleotides. C.B. conducted experiments. J.S. edited the paper. K.H. designed experiments and wrote the paper.

Funding

Research reported in this publication was supported by the National Institutes of Health (NIH) through the NIGMS under award R35GM124720 to K.H. and GM143749 to J.S. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. Funding for open access charge: National Institutes of Health.

Conflict of interest statement

K.H. and A.R.C. have intellectual property related to DNA nanoswitches. All other authors declare that they have no competing interests.

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Received: July 13, 2023. **Revised:** August 31, 2023. **Editorial Decision:** September 4, 2023. **Accepted:** September 19, 2023