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Journal

Biochemistry, 61(16)

ISSN

0006-2960

Authors

Falco, Natalie
Garfio, Chely M
Spitalny, Leslie
et al.

Publication Date

2022-08-16

DOI

10.1021/acs.biochem.2c00270

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Author manuscript

Biochemistry. Author manuscript; available in PMC 2023 March 13.

Published in final edited form as:

Biochemistry. 2022 August 16; 61(16): 1665–1668. doi:10.1021/acs.biochem.2c00270.

A Fluorescent Reverse-Transcription Assay to Detect Chemical Adducts on RNA

Natalie Falco,

Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697, United States

Chely M. Garfio,

Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697, United States

Leslie Spitalny,

Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697, United States

Robert C. Spitale

Department of Pharmaceutical Sciences and Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States

Abstract

Herein, we detail a novel reverse-transcription (RT) assay to directly detect chemical adducts on RNA. We optimize a fluorescence quenching assay to detect RT polymerization and employ our approach to detect N^1-alkylation of inosine, an important post-transcriptional modification, using a phenylacrylamide as a model compound. We anticipate our approach can be expanded to identify novel reagents that form adducts with RNA and further explored to understand the relationship between RT processivity and natural post-transcriptional modifications in RNA.

RNA molecules perform key functions at the heart of many biological pathways and are significant drivers in the onset of many diseases.[1,2] RNA molecules are also prone to modifications, many of which have been characterized to control RNA structure, function, and RNA–protein interactions.[3,4] In addition, researchers have a growing interest in identifying RNA–small molecule interactions, with a specific focus on discovering small molecules that can bind to RNA and introduce ligand-dependent covalent adducts.[5,6] Subsequently, there is growing demand for the development of RNA-centric assays to directly detect chemical interactions or modifications on RNA.

Corresponding Author rspitale@uci.edu.

The authors declare the following competing financial interest(s): The authors have submitted a provisional application on the material disclosed herein.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00270.

Supporting figures and methods (PDF)

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Several biochemical assays have been developed to identify RNA–ligand interactions of post-transcriptional chemical modifications. Nuclear magnetic resonance and other spectroscopic techniques can report binding with high resolution but have severely limited scalability.[7,8] Chemical microarrays have proven their utility in screening a desired RNA for binding, but the chemical space is limited due to the available functional groups that are compatible with arrays.[9–11] Additionally, the use of fluorescent nucleotides and fluorescent ligand displacement assays has a limited scope because they report on only a predetermined site in a primary RNA sequence.[12–15] While these approaches are useful for identifying ligand–RNA interactions, they do not directly report covalent RNA adducts. The ongoing demand to exploit covalent modifications for functional control of biomolecules implored us to develop a system for the direct detection of RNA adducts.

Our first goal in developing an approach to detect chemical adducts on RNA was to utilize the unique characteristics of RT assays. A recent RT-based approach was published, but it relied on costly RNA sequencing to determine the site of adduct formation.[16] Instead, we developed a fluorescence protocol that could easily be performed in a lab setting using a more conventional readout.

We envisioned an assay that could use fluorescence to detect full length RT cDNA and RT truncation at the site of a covalent adduct (Figure 1). To achieve this, we implemented a molecular beacon design in which fluorescence is retained upon RT truncation or quenched upon full RNA–cDNA hybrid extension. Working toward this goal, we determined which fluorophore would be ideal for our turn-off system and then established how cDNA hybridization quenching was proximally tied to the conjugation site of the fluorophore on the RNA.

DNA molecular beacons are designed to rely on DNA–DNA intramolecular hybridization to control fluorescence and quenching. One part of the single-stranded DNA is appended with a fluorophore, and the other end with a fluorescent quencher.[17] Aiming toward the eventual utility of a RT reaction, we decided not to rely on attached quencher molecules, but the act of hybridization to quench fluorescence. We began by testing the physiochemical properties of a variety of fluorophores to understand their quenching capabilities.

We selected bodipy fl (BDP-FL), tetramethylrhodamine (TMR), and coumarin 343X (C343 X) for their known ability to be quenched using single-stranded beacons (Figure 2a).[18–20] We also utilized a ROX fluorophore as it is known to have increased fluorescence when in the proximity of double-stranded DNA upon hybridization.[21] We designed the 5′-end of the appended oligonucleotides to be C-rich as it has been observed that hybridizing DNA beacons in which fluorophores are brought into the proximity of G-rich ends results in efficient fluorescent quenching (Figure 2b).[18,22] This quenching is thought to be caused by a photoinduced electron transfer mechanism.[23] We attached the fluorophores to the 5′-end of a single-stranded 5′-amino DNA oligonucleotide using NHS ester chemistry. Reaction mixtures contained excess NHS ester fluorophore, 30% $NaHCO_3$, and 20% DMSO, and reactions were performed at room temperature overnight (Figures S1–S7).

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We first tested different fluorophores for their ability to efficiently quench when hybridized to G-rich antisense oligonucleotides. As shown in Figure 2c (and Figure S8), incubation of fluorophore-conjugated DNA with a G-rich antisense oligonucleotide resulted in varied quenching with BDP-FL, TMR, C343 X, and ROX. As predicted, hybridization resulted in an increase in ROX fluorescence. These results are consistent with other published reports demonstrating that BDP-FL fluorophores can be efficiently quenched by the interaction between themselves and a guanine.[23]

Having demonstrated that BDP-FL was quenched most efficiently, we tested if the linker distance between the C-rich 5′-end and the fluorophore could be shortened to enhance quenching by encouraging base stacking between the BDP-FL fluorophore and the hybridized guanosine nucleotides. Upon replacing the 12-carbon spacer with a 6-carbon spacer, we observed nearly 90% quenching when hybridized (Figure 2d and Figure S9). This result is also demonstrated by looking at the fluorescent spectra showing a dramatic increase in overall quenching, and that quenching was observed for the entire fluorescent spectra when we utilized the shorter linker connecting the fluorophore with the oligonucleotide (Figure 2e). These results demonstrate that BDP-FL fluorescence is efficiently quenched when hybridized with a G-rich oligonucleotide and that a shorter (6-carbon) linker increases the level of quenching.

Having optimized the conditions for hybridization that enable efficient nucleotide-based quenching, we turned our attention toward the RT-based assay. We first aimed to understand the conditions used for normal RT extension as well as those that would produce a truncated RT product. As shown in Figure 3a, we used an internal C18 spacer (labeled iSp18), which is known to inhibit other processive enzymes, but had yet to be demonstrated with RT.[24,25] We incubated single-stranded RNA with increasing amounts of RT primer to determine the equivalents necessary to efficiently extend. As shown in Figure 3b, we observed efficient quenching under conditions ranging from 1:1 to 8:1 molar equivalents (RT primer:RNA). We also determined that our approach could efficiently read through a structured RNA (PreQ1 riboswitch[11,26]) with similar fluorescent quenching (Figure S10).

To determine the conditions for comparing quenching and a lack of quenching, due to full length RT against truncation we used a carbon spacer, iSp18 [18-atom hexa-ethylene glycol spacer (Figure 3b)]. We also observed that RNA with an iSp18 spacer had undetectable quenching, suggesting that the iSp18 spacer is a good control for halting RT processivity. Overall, these experiments demonstrated that our approach is amenable to fluorescent quenching using an RT assay and that our assay can detect full length RT processivity and truncations due to altered RT extension.

We also desired to understand if our assay would be sensitive enough to detect adducts formed between an RNA and an electrophilic compound. To demonstrate this, we utilized the known reactivity between inosine and aryl acrylamide reagents.[27,28] Inosine formation is a naturally occurring post-transcriptional modification that can control RNA function through recoding open reading frames or altering RNA processivity.[29,30] Inosine's unique chemical structure makes it amenable to reactivity with electrophilic small molecules with high selectivity. Determining where inosine modifications occur is crucial to understanding

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how it relates to disease. We speculate that the unique chemical reactivity of inosine would make it amenable to small molecule-induced adduct formation for potential therapeutic benefits. As such, we aimed to use the specific chemical reactivity of inosine as a positive control for assay development.

We synthesized the known aryl acrylamide compound *N*-(4-ethynylphenyl)acrylamide (EPhAA) (Figure S11), which is an easy to synthesize aryl acrylamide that has been demonstrated to form adducts selectively with inosine (Figure 3c).[28] We rationalized that this EPhAA adduct would result in blocking of the W–C face of inosine, resulting in truncated RT products (Figure 3d). Consistent with this concept, incubation of RNA with an increasing molar excess of EPhAA resulted in the formation of truncated RT products as identified by denaturing gel electrophoresis (Figure 3e). We also observed a reduction in the level of fluorescent quenching in our RT assay using the same number of molar equivalents (Figure 3f). Overall, these results strongly convey that we are able to detect RT truncations using our RT assay and have demonstrated that this approach could be used to identify RNA adducts with a RT-based platform.

Herein, we have demonstrated the utility of RT-based reactions for fluorogenic detection of adducts between RNA and a small molecule. Fluorescence-based assays have been demonstrated to be widely used in drug discovery and basic biology, but there are limited examples of their applications for high-throughput discovery of RNA–small molecule interactions. We are optimistic that RT-based assays could pave the way for high-throughput measurements to detect these adducts in RNA. In future work, we will further our understanding of the design principles of such RT-based assays in the hope of spearheading this approach for RNA adduct detection in the drug discovery sphere.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank members of the Spitale lab for their careful reading and critique of the manuscript.

Funding

Work in the Spitale lab is supported by the University of California, Irvine, and the Pew Biomedical Scholar program (R.C.S.).

ABBREVIATIONS

BDP-FL	bodipy fl
C343 X	coumarin 343X
EPhAA	*N*-(4ethynylphenyl)acrylamide
RT	reverse transcription
TMR	tetramethylrhodamine

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iSp18	internal C18 spacer

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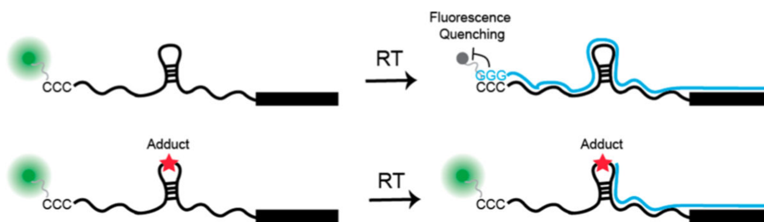

Figure 1.

Schematic of a fluorescence RT assay. In this study, a fluorescence RT assay in which the absence of an adduct on a fluorophore-conjugated oligonucleotide allows RT to occur has been developed. If full RT elongation occurs, fluorescence is attenuated. If an adduct is present on the oligonucleotide, RT is unable to proceed and the fluorescence signal remains. In this proof-of-concept paper, an internal C18 spacer and a clicked phenylacrylamide molecule are shown to stop RT elongation and prevent fluorescence attenuation.

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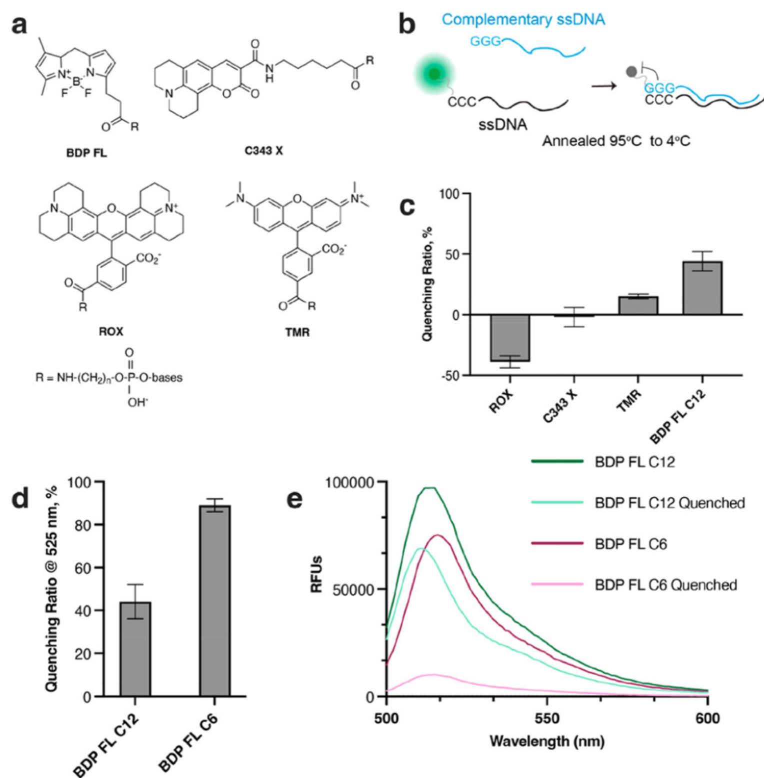

Figure 2.

Development of a fluorescence RT assay using DNA oligonucleotides. (a) Chemical structures of the fluorophores used in this study. (b) Schematic of the hybridization experiment in which antisense annealing quenches fluorescence. (c) Quantification of the percent quenching of annealed DNA oligonucleotides with their respective fluorophores. Error bars represent the standard deviation (SD) ($n = 3$). (d) Quantification of the percent quenching of the annealed BDP FL-DNA oligonucleotide with different linker lengths. Error bars represent the SD ($n = 3$). (e) Raw spectral data of the experiment quantified in panel d.

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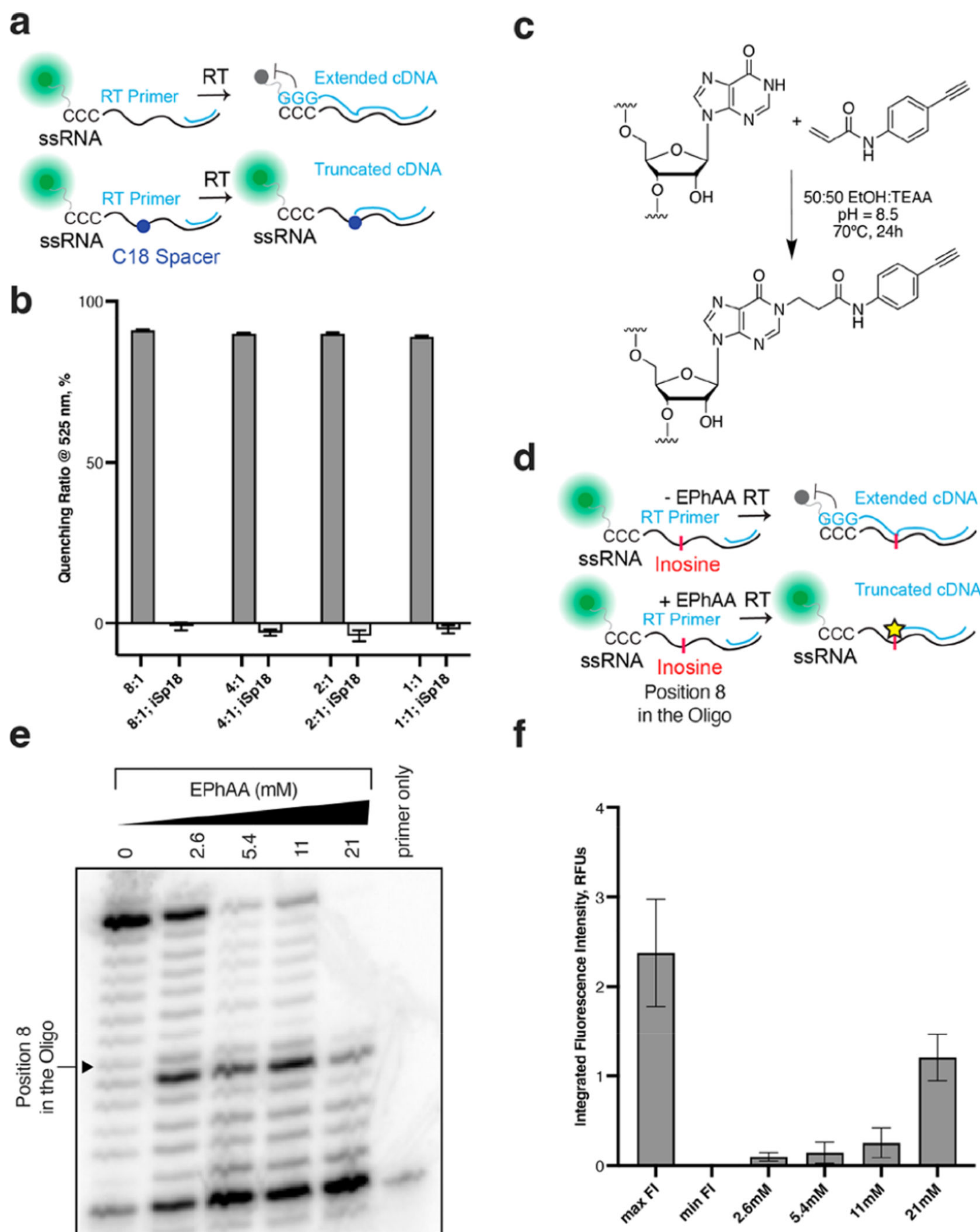

Figure 3.

Development a fluorescence RT assay using RNA oligonucleotides. (a) Schematic of the RT experiment in which the absence of an adduct on the RNA oligonucleotide allows RT to proceed, thereby attenuating the fluorescence intensity. Schematic of an RNA oligonucleotide that contains an internal C18 spacer inhibiting RT processivity and maintaining fluorescence. (b) Quantification of the percent quenching of a fluorophore-conjugated RNA oligonucleotide that has undergone RT. A RT primer titration was carried out from 1:1 to 8:1 (RT primer:RNA). RNA with an internal C18 spacer was used in

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the control lanes. Error bars represent the SD ($n = 3$). (c) Reaction scheme of EPhAA with inosine. (d) Schematic of the RT experiment in which an inosine-containing RNA oligonucleotide is reacted in the presence and absence of EPhAA. In the absence of the EPhAA reaction, RT elongation occurs, attenuating the fluorescence intensity, and in the presence of the EPhAA reaction, RT processivity is halted and the fluorescence intensity remains. (e) Radioactive gel of the data shown in panel d. Note that RT stops at the nucleotide preceding the inosine–EPhAA site. (f) Integrated fluorescence intensity of EPhAA-incubated inosine-modified RNA oligonucleotides after RT. EPhAA was incubated with an inosine-modified RNA oligonucleotide at various concentrations in a 50:50 EtOH/TEAA mixture at 70 °C for 24 h. Error bars represent the SD ($n = 3$).