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Bioorthogonal Tetrazine-Mediated Transfer Reactions Facilitate Reaction Turnover in Nucleic Acid-Templated Detection of MicroRNA

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Supporting Information

ABSTRACT: Tetrazine ligations have proven to be a powerful bioorthogonal technique for the detection of many labeled biomolecules, but the ligating nature of these reactions can limit reaction turnover in templated chemistry. We have developed a transfer reaction between 7-azabenzonorbornadiene derivatives and fluorogenic tetrazines that facilitates turnover amplification of the fluorogenic response in nucleic acid-templated reactions. Fluorogenic tetrazine-mediated transfer (TMT) reaction probes can be used to detect DNA and microRNA (miRNA) templates to 0.5 and 5 pM concentrations, respectively. The endogenous oncogenic miRNA target mir-21 could be detected in crude cell lysates and detected by imaging in live cells. Remarkably, the technique is also able to differentiate between miRNA templates bearing a single mismatch with high signal to background. We imagine that TMT reactions could find wide application for amplified fluorescent detection of clinically relevant nucleic acid templates.

Bioorthogonal tetrazine ligation reactions are well-known for their chemoselectivity, tunable kinetics, and fluorogenic nature.[1] By having fast kinetics at low concentrations in biological media, tetrazine ligations have been used for many applications requiring molecular tagging or labeling with a fluorophore. These reactions have proven to be powerful tools, as demonstrated in studies ranging from live-cell imaging to detection of proteins, in vivo imaging, and probing of glycosylation patterns.[2] Despite their many applications, tetrazine ligations have not been used for the in situ detection and imaging of low-abundance nucleic acids in biological samples.

Several methods for in situ detection and imaging of nucleic acids have been reported previously.[3] One powerful strategy that is frequently employed to achieve the requisite sensitivity and specificity is to use templated fluorogenic reactions with turnover-driven signal amplification.[4] In this strategy, fluorogenic antisense probes are designed so that, upon hybridization with a nucleic acid template, the reactive groups will be brought into proximity of one another. The increase in effective concentration drives the fluorogenic reaction, producing a detectable signal only in the presence of the template. Reacted probes can be displaced by unreacted probes to allow turnover of multiple reactions on a single template, resulting in the signal amplification required for detecting low-abundance targets. For this technique to work in practice, one needs highly fluorogenic reactive groups that do not degrade or cross-react with biological functionalities and sufficient turnover per template to achieve a low limit of detection.

The favorable properties of tetrazine reactions would be desirable in probes for templated nucleic acid detection; indeed, we have previously demonstrated that tetrazine ligation probes can be used to detect synthetic nucleic acid templates.[5] This approach could be improved using recently developed highly fluorogenic tetrazines that are quenched by through-bond energy transfer,[6] but the tetrazine ligation itself is a barrier to achieving a low limit of detection. The product of a tetrazine ligation has a higher affinity for the template than its precursors, inhibiting turnover of additional probes.[5] A nonligating tetrazine reaction would produce products with a similar affinity for the template as the reactive probes, facilitating reaction turnover and signal amplification by allowing reacted probes to be displaced (Figure 1). The use of highly fluorogenic tetrazine reactions that proceed by transfer of functionality (instead of ligation) has not previously been explored for biomolecule detection.

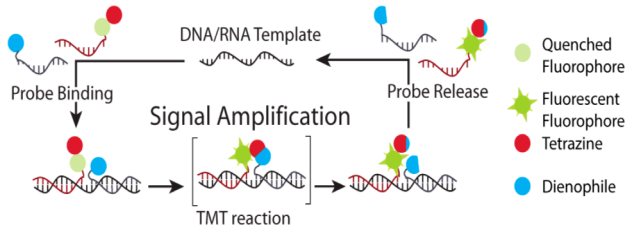

Figure 1. A possible method for nucleic acid-templated signal amplification using a tetrazine-mediated transfer (TMT) reaction.

To achieve turnover in templated tetrazine reactions, we utilized 7-azabenzonorbornadiene derivatives as novel strained dienophiles that can undergo tetrazine-mediated transfer (TMT) reactions. These dienophiles react with tetrazines through an irreversible inverse-electron-demand Diels–Alder reaction to release dinitrogen and form a dihydropyridazine coupling adduct.[7] In contrast to typical strained dienophiles, the dihydropyridazine spontaneously undergoes a retro-Diels–Alder reaction to aromatize and release the products (Figure 2A).[7,8] Along with loss of dinitrogen, the net result of this process is effectively a functional group transfer from the dienophile to the tetrazine. We hypothesized that the lack of a ligation product from a TMT reaction would make it ideal for enabling oligonucleotide template-driven turnover.

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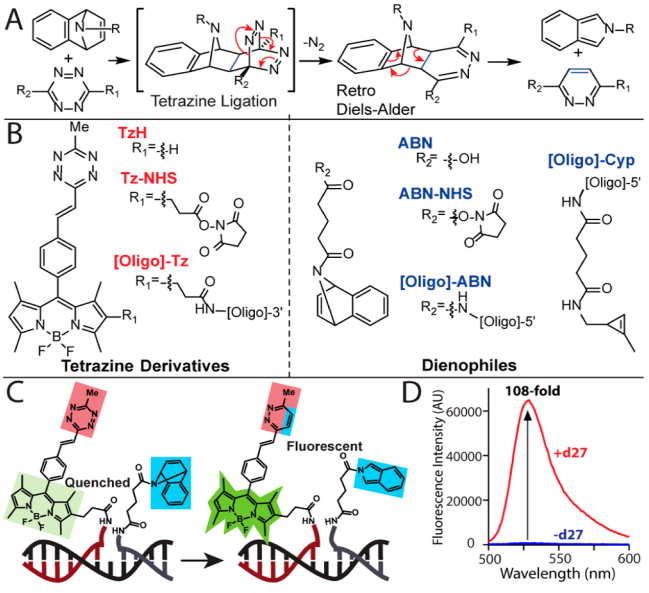

Figure 2. (A) Reaction mechanism for a TMT reaction showing tetrazine ligation followed by fragmentation by a retro-Diels–Alder process. (B) Structures of the tetrazines and dienophiles used. (C) Schematic of a templated transfer reaction with ABN and Tz probes. (D) Fluorescence spectrum (λ_{ex} = 480 nm, $\lambda_{max,em}$ = 530 nm) of 1 μM **d27′-Tz** and **d27′-ABN** with/without **d27** after 1.5 h.

We first synthesized the 7-azabenzonorbornadiene derivative **ABN** and assessed its reactivity with the previously described highly quenched tetrazine–BODIPY compound **TzH**[6a] in chloroform (see the Supporting Information (SI)). After the reaction reached completion, a highly fluorescent pyridazine product was isolated in 95% yield (λ_{ex} = 480 nm, $\lambda_{max,em}$ = 530 nm). This validated that the transfer reaction could elicit a fluorogenic response from quenched tetrazine probes. We designed **Tz-NHS** and **ABN-NHS** for oligonucleotide modification with NHS coupling chemistry. We reacted a series of 5′ or 3′ amine-modified oligonucleotides with **Tz-NHS** or **ABN-NHS** to produce tetrazine– or dienophile–oligonucleotide probes. Nucleic acid probes and products were characterized by ESI-TOF-MS. These antisense probes were designed so a tetrazine and dienophile would be brought into close proximity when the probes hybridized to a complementary template oligonucleotide strand.

To study the kinetics of a DNA-templated TMT reaction, we synthesized probes **d27′-Tz** and **d27′-ABN** for the corresponding DNA template **d27** (see Figure 3 for sequences). When each probe (1 μM) was allowed to react in the presence of 1 μM **d27**, a reaction was immediately detectable by fluorimetry, whereas no significant reaction was detected in the absence of template. The fluorescence increased with an observed first-order rate constant of $(9.1 \pm 0.2) \times 10^{-4}$ s^{-1} and a reaction half-life of 12.7 min. The sample was found to have a fluorescence turn-on of 108-fold after reaction (Figure 2D). This is more than an order of magnitude improvement over previously described fluorogenic tetrazine oligonucleotide probes[5] and is comparable to some of the best visible-spectrum turn-on probes that have been utilized for oligonucleotide-templated chemistry.[4c,d,f,i]

We next turned our attention to studying template-driven reaction turnover. To allow probes to undergo dynamic strand exchange at physiological temperature (37 °C), we synthesized the shorter probes **d21′-Tz** and **d21′-ABN** for template **d21**.[9] We measured the melting temperatures (T_m) of these probes with **d21** to be 38.8 and 43.8 °C, respectively. We then incubated **d21′-Tz** and **d21′-ABN** with decreasing amounts of DNA template. The fluorescence increase above background was compared with the signal elicited from stoichiometric template to determine the extent of turnover. After 7 h we observed notable fluorescence signal amplification with substoichiometric template concentrations (10 nM to 500 fM), and the ratio of fluorescence relative to control increased as the template concentration decreased (Figure 3A). This is similar to previous observations and is likely due to the increased ratio of probe to template.[4c,e,h,10] Template could be discriminated from background down to a remarkable 500 fM. Mismatch discrimination assays demonstrated the sequence-specific nature of this effect (Figure S3 in the SI). We compared the transfer probe **d21′-ABN** with a ligation probe **d21′-Cyp**, a 3′-cyclopropene probe we previously used for DNA-templated reactions.[5] As expected, the reaction of **d21′-Cyp** with substoichiometric template resulted in a much smaller increase in fluorescence relative to the reaction with **d21′-ABN** (Figure S4). This is further evidence that TMT reactions facilitate strand exchange leading to signal amplification.[4c,9b] We verified the biological stability of this

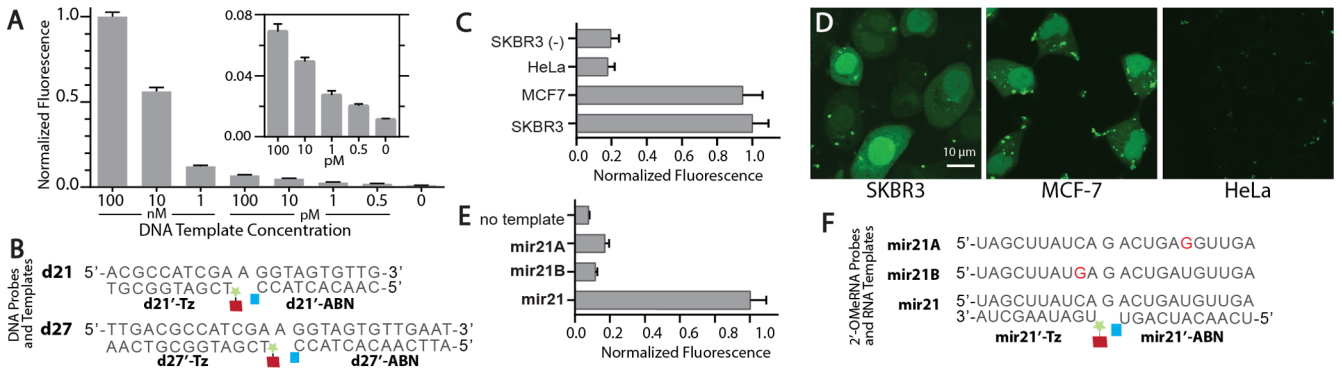

Figure 3. (A) Normalized fluorescence from 100 nM **d21′-Tz** and 200 nM **d21′-ABN** at various **d21** concentrations. The inset shows detail for low **d21** concentrations. (B) Sequences for **d21** and **d27** with corresponding probes. The colored shapes correspond to the probes in Figure 2C. (C) Normalized fluorescence from cell lysates for SKBR3, MCF-7, and HeLa cells. SKBR3(–) had 10× probe added that lacked reactive groups as a competitive inhibitor for endogenous mir-21. (D) Live-cell miRNA detection in human cancer cell lines. (E) Distinguishing single-mismatch templates **mir21A** and **mir21B** from perfect match **mir21** using **mir21′-ABN** and **mir21′-Tz**. (F) Sequences for mir-21 probes and templates. In all graphs, error bars show standard deviations of three replicates.

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system by incubating the probes (100 nM **d21′-Tz,** 200 nM **d21′-ABN**) with large excesses (50×) of reductants, oxidants, and thiol nucleophiles. After a 7 h incubation, the system provided nearly full fluorogenic response when 1% template (1 nM final concentration) was added compared with an untreated control (Figure S5).

We next turned our interest to applying this technique to the detection of endogenous microRNA (miRNA).[11] MiRNAs are a class of single-stranded noncoding regulatory RNAs that can regulate many biological processes through the degradation of mRNA targets. Altered miRNA levels have been linked to large number of human diseases and disorders.[12] Thus, there is tremendous interest in methods to reliably detect, quantitate, and profile miRNA levels.[11,13] Methods with high sensitivity and specificity that can be used for in situ miRNA quantification have a high likelihood of translation into clinical settings.[13a,14] As a proof of concept, we explored the use of TMT reactions to detect microRNA-21 (mir-21), a 22 nucleotide (nt) miRNA that was one of the first oncogenic miRNAs (oncomirs) to be identified.[15] Mir-21 expression is associated with a variety of human cancers,[16] notably human breast cancer cells.[13b,17] Furthermore, cancers that develop resistance to chemotherapy show an increase in mir-21 expression.[17a] Thus, a robust method to profile mir-21 in cell samples could allow mir-21 expression to be a valuable diagnostic marker. Because of the importance of mir-21 in human oncology, it has been commonly targeted by alternative detection techniques.[18]

To develop probes to detect endogenous miRNA, we first synthesized Tz- and ABN-modified oligonucleotides using 2′-*O*-methyl-RNA (2′-OMeRNA), which is structurally similar to RNA but displays better discrimination between matched and mismatched RNA targets and has faster kinetics of hybridization to complementary targets.[19] 2′-OMeRNA is also highly resistant to degradation by nucleases, which can rapidly degrade **mir21′-Tz** and **mir21′-ABN** that contain sequences complementary to mir-21. We studied the turnover of this reaction using a synthetic mir-21 template, **mir21**, in the same manner as with a DNA template, but $MgCl_2$ was omitted from the buffers as it was found to accelerate RNA degradation. We observed excellent signal amplification after 7 h with **mir21′-Tz** and **mir21′-ABN** using multiple concentrations of **mir21** (Figure S6), with a detection limit of ∼5 pM. Shorter incubation periods also resulted in notable signal amplification, but with a lower absolute limit of detection (Figure S7).

A significant challenge in detecting miRNA in biological samples is discriminating between similar miRNA sequences.[13a] It has been shown that miRNAs within a family can differ by a single base.[11,12,20] Thus, probes designed to detect a specific miRNA need to be able to distinguish between templates bearing a single mismatch. We tested the ability of **mir21′-Tz** and **mir21′-ABN** to discriminate between **mir21** and two alternative templates, **mir21A** and **mir21B**, bearing single mismatches separated from the site of reaction by 5 and 1 nt, respectively (Figure 3E). Remarkably, at 1% equivalent template concentration, our probes showed high discrimination against both **mir21A** and **mir21B**, with background-corrected perfect match/mismatch (PM/MM) ratios of 9.9 and 24.4, respectively. This mismatch selectivity is also reflected in the decreased T_m of probes with mismatched templates relative to the matched template. The PM/MM ratio was higher with **mir21B** than with **mir21A**, in line with previous work demonstrating that templated chemical reactions can be more sensitive to mismatches near the site of reaction.[21] The sensitivity to a single mismatch is particularly impressive and this technique provides useful signal-to-background at long time points without the need for stimuli or external enzymes; comparable performance by other highly selective RNA-templated methods can only be achieved at short time points because background signal increases over time.[10,21,22]

Having established the viability of using **mir21′-Tz** and **mir21′-ABN** to detect mir-21, we attempted to use these probes to detect endogenous mir-21 in live cells. We used our probes to detect mir-21 in SKBR3 and MCF-7 breast cancer cells, which have shown high mir-21 expression in previous studies.[13b,17] We also used our probes in HeLa cells, a cervical cancer cell line, which has been shown to have lower mir-21 expression than MCF-7.[10] Prior to microscopy, the cell lines were transfected with 200 nM **mir21′-Tz** and **mir21′-ABN** using 0.02 mg/mL Lipofectamine 2000 (Invitrogen, CA, USA). After incubation for 2 h, the cell nuclei were counterstained with Hoescht 33342 and imaged using bright-field and fluorescence confocal microscopy (Figures 3D and S9). Both MCF-7 and SKBR3 cells displayed strong fluorescent staining, indicating the presence of mir-21, whereas much less staining was observed in the HeLa cells. Some of the observed background could be attributable to probes coming into proximity in formed lipoplexes. Without **mir21′-ABN**, minimal background fluorescence was observed (Figure S10). The nonligating nature of the TMT reaction prevents definitive subcellular imaging, unlike techniques such as RNA FISH. For example, the nuclear fluorescence observed is not likely from high mir-21 levels; previous work has shown the tendency for small oligonucleotide probes to be shunted to the nucleus.[23]

To verify that the observed differences were not caused by differential probe uptake between cell lines, we tested **mir21′-Tz** and **mir21′-ABN** in cell-derived lysates. After a 1.5 h incubation at 37 °C, lysates from SKBR3 and MCF-7 exhibited a ∼5-fold increase in mir-21 signal compared with lysate from HeLa cells (Figure 3C). To verify the specificity of the fluorogenic signal to mir-21, **mir21′-Tz** and **mir21′-ABN** were incubated along with a 10-fold excess of unmodified oligonucleotide probes with identical sequences as competitive inhibitors. Competition reduced the signal by ∼5-fold, demonstrating that that the fluorescent signal is due to the mir-21-templated TMT reaction (Figure 3E). The ability to detect mir-21 in cell-derived lysates is a distinct advantage of this technique compared with alternatives such as Northern blot and qPCR, which require careful extraction of RNA before detection.

We have developed a fluorogenic tetrazine-mediated transfer reaction using 7-azabenzonorbornadiene derivatives and have utilized this reaction to detect oligonucleotides with high sensitivity and sequence specificity. Critical to achieving signal amplification is template-driven turnover of antisense probes, which is enabled by spontaneous diazine release after the initial tetrazine ligation takes place. The use of a highly quenched alkenyl-fluorogenic tetrazine enables a >100-fold increase in fluorescence in response to the TMT reaction. By using RNA to template this transfer reaction with antisense probes, we were able to detect mir-21 down to low-picomolar levels with the specificity to distinguish single-base mismatches in miRNA templates. The probes were capable of detecting endogenous mir-21 both in live cells and cell lysates. We believe that oligonucleotide-templated fluorogenic TMT reactions will be useful for many applications that require detection of specific nucleic acids in either live cells or biological samples. These

probes are likely to be applicable for profiling of endogenous miRNA levels in living cells, circulating exosomes, and tissues.[13a]

■ ASSOCIATED CONTENT

Supporting Information

Synthetic methods and characterization data for all compounds, oligonucleotide sequences, and other experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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