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Imaging of mRNA in Live Cells Using Nucleic Acid-Templated Reduction of Azidorhodamine Probes

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Abstract: Nucleic acid-templated reactions leading to a fluorescent product represent an attractive strategy for the detection and imaging of cellular nucleic acids. Herein we report the use of a Staudinger reaction to promote the reduction of profluorescent azidorhodamine. The use of two cell-permeable GPNA probes, one labeled with the profluorescent azidorhodamine and the other with trialkylphosphine, enabled the detection of the mRNA encoding O-6-methylguanine-DNA methyltransferase in intact cells.

Introduction

While imaging technologies have had a tremendous impact on our understanding of protein function and dynamics,[1] analogous technologies for cellular nucleic acids are much less developed. Specific RNA sequences have generally been detected with molecular beacons[2,3] or with fluorescence in situ hybridization (FISH).[4] While several landmark studies have highlighted the importance of RNA visualization technologies,[5,6] a major limitation of the current state of the art is the poor membrane permeability of the oligonucleotide probes, thus requiring cell fixation, electroporation, or microinjection and high background signals, due in part to protein binding of the oligonucleotide probes.[7] Although alternative technologies have been reported, such as aptamers binding fluorophores[8] and reconstituted GFP fragments binding specific nucleic acids,[9] a general and practical method enabling real-time visualization of mRNA expression in a nondestructive or invasive fashion is warranted and would have a tremendous impact on our ability to dissect molecular processes at the cellular and organismic levels and ultimately in medical diagnosis. Toward this goal, nucleic acid-templated reactions yielding a fluorescent signal are emerging as an attractive alternative to molecular beacons or FISH. They have the potential to amplify the signal, by virtue of the fact that one template can catalyze multiple reactions, and to decrease the background signal, since unspecific binding does not give rise to fluorescence as long as the fluorophore activation is fully orthogonal to cellular chemistry.[10] Several reports have described elegant demonstrations of nucleic acid detection based on nucleophilic ligation accompanied by displacement of a fluorophore quencher,[11−15] native chemical ligation,[16−19] and Staudinger reaction.[20−23] In principle, it is preferable that the nucleic acid template catalyzes a transfer reaction rather than a ligation reaction, as the ligation is more likely to give a product with higher affinity for the template and thus lead to product inhibition.[24] Templated nucleic acid detection has been performed on bacteria to detect rRNA,[14,20] and there is one example of a templated reaction in live cells to detect mRNA.[12] However the lack of cellular permeability of the oligonucleotides required membrane perforation with streptolysin-O (SLO), a pore-forming bacterial toxin. Herein we report the use of cell-permeable guanidine-based peptide nucleic acid (GPNA)[25,26] for detection of mRNA in intact cells, without

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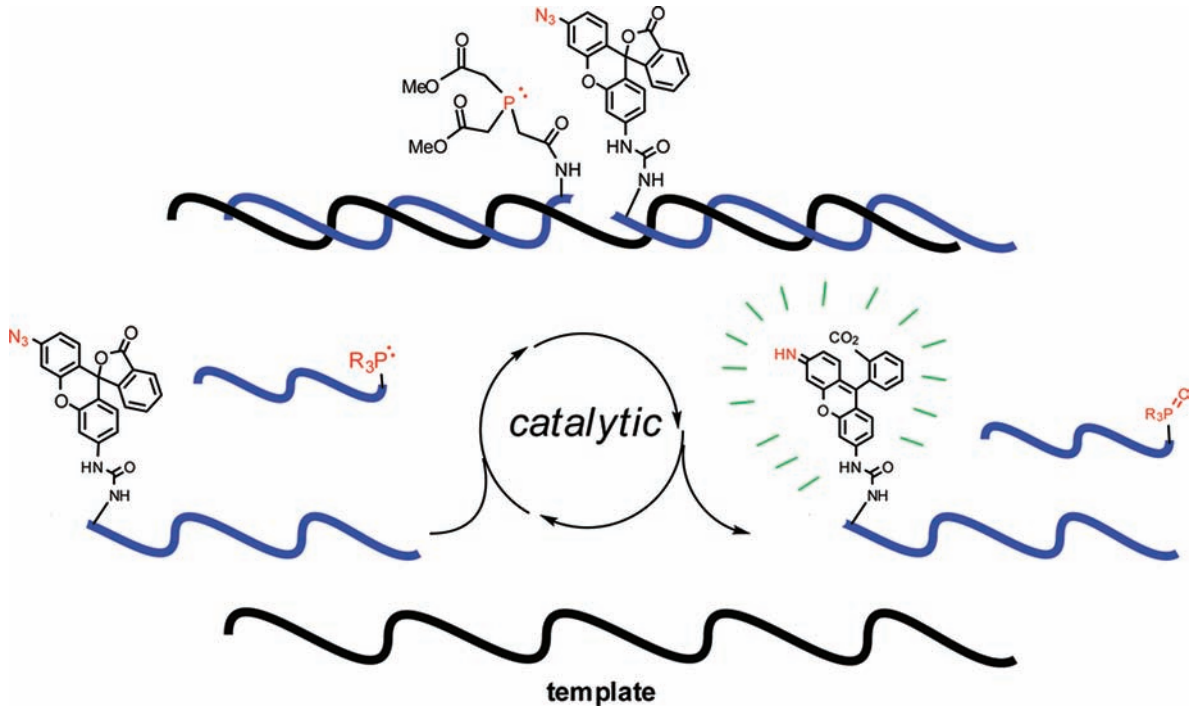


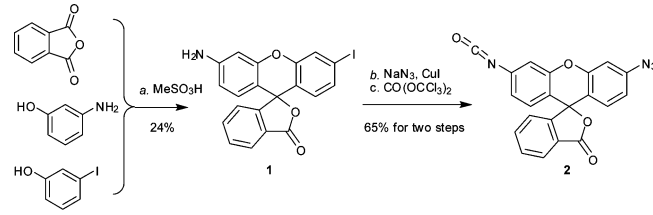
Figure 1. Schematic representation of the templated unmasking of azidorhodamine by Staudinger reduction.

recourse to membrane-permeabilization techniques, using a templated reduction of azidorhodamine (Figure 1).

Results and Discussion

We have previously reported that the fluorescence of coumarin can be quenched by replacement of the 7-amino group with an azide group and that this quenched fluorophore is suitable for template detection of DNA using two peptide nucleic acid (PNA) probes, one bearing a phosphine and the other the quenched azidocoumarin.[22] PNA[27,28] is an attractive choice for the probes, as its hybridization is more sensitive to mismatch and it is extremely robust toward enzymatic degradation. However, three limitations precluded the use of this first generation system for live-cell imaging: first and foremost, unsubstituted PNA are poorly membrane-permeable; second, their tendency to aggregate in physiological buffers gives erratic results; and third, the intrinsic fluorescence of cellular organelles in the coumarin excitation/emission wavelength region (λ_{ex} = 360 nm, λ_{em} = 455 nm) leads to high background fluorescence that severely compromises sensitivity. The present system, leveraged on cell-permeable GPNA with a green fluorophore (rhodamine), overcomes these previous limitations. To investigate the suitability of this system in living cells, we designed PNA sequences matching a segment of the mRNA corresponding to the UTR region of the O-6-methylguanine-DNA methyltransferase (MGMT) as a prototypical example, on the basis of the fact that it is known to be fairly highly expressed in HEK293 cells (http://plaza.ufl.edu/gshaw/293.xls)[29] and that the expression level of this protein has been found to correlate with the response to DNA-alkylating chemotherapeutics in several cancers.[30−32]

Scheme 1. Synthesis of Azidorhodamine **2** from Phthalic Anhydride, Aminophenol, and Iodophenol



It is known that the high quantum yield of rhodamine depends predominantly on one of the two amino groups and that the other can be substituted in the form of an amide, carbonate, or urea with only a marginal decrease in fluorescence.[33] Applying the same rationale as for the azidocoumarin, we reasoned that one of the rhodamine amino groups could serve as a handle to tether rhodamine to a PNA while the other could be substituted by an azide to quench its fluorescence. As shown in Scheme 1, quenched rhodamine isocyanate **2** can be prepared in three steps from inexpensive starting materials. While the condensation of aminophenol, iodophenol, and phthalic anhydride afforded the desired product in modest yield, this reaction lends itself to large-scale preparation, and gram quantities of the desired

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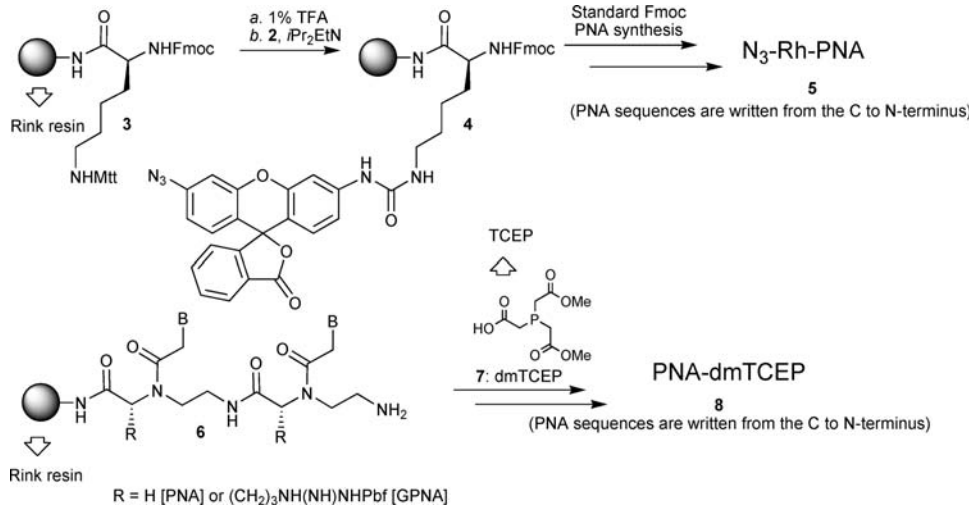
Scheme 2. Synthesis of Azidorhodamine−PNA Conjugate **5** and Phosphine−PNA Conjugate **8** from Rink Resin Using Standard Fmoc-Synthesis Protocols



rhodamine derivative **1** were readily isolated. The iodo group was then substituted by an azide in the presence of catalytic amount of copper,[34] and the aniline was converted to the isocyanate under the action of triphosgene to afford rhodamine derivative **2** in good yield.

We had previously tethered the fluorophore directly to the backbone of PNA, which required the synthesis of modified PNA monomers. To streamline the preparation of PNA−fluorophore conjugates, we opted to link the quenched fluorophore and PNA to a bifunctional linker. Thus, with a Rink resin derivatized with a lysine residue bearing orthogonal protecting groups (Mtt, Fmoc) as a starting material, the ϵ-nitrogen was deprotected (1% TFA) and coupled to isocyanate **2** (Scheme 2). Gratifyingly, the resulting azidorhodamine **4** was found to be perfectly stable toward TFA cleavage and standard Fmoc SPPS (hexamers to nonamers are routinely prepared) and could be used to access azidorhodamine−PNA conjugate **5**. The gain in fluorescence upon reduction of azidorhodamine for hexamers and octamers was then assessed. While the reduction of azidorhodamine−PNA (N_3-Rh−PNA) conjugates was found to be sluggish with triphenylphosphine, fast reaction kinetics were observed with alkylphosphines such as tris(2-carboxyethyl)phosphine (TCEP). During the course of our work, a similar fluorophore was reported by Abe and co-workers,[20] who showed that hydrolysis of the azaylide formed in the Staudinger reaction using triphenylphosphine was slow, thus resulting in a ligation reaction that prevented template turnover. As shown in Figure 2, treatment of N_3-Rh−PNA hexamer with excess TCEP led to a greater than 30-fold increase in fluorescence intensity, and the azaylide could not be isolated; this suggested that the hydrolysis is faster than with arylphosphine and should enable template turnover. Similar results were obtained for N_3-Rh−PNA octamers.

While TCEP is not membrane-permeable, it has been shown that the trimethyl ester of TCEP (tmTCEP)[35] is. On the basis of this observation, we reasoned that the dimethyl ester of TCEP (**7**) could be conjugated to the N-terminus of a PNA without compromising its cell permeability. Thus, TCEP was partially esterified[35] under acid catalysis (PS−SO_3H in MeOH) to obtain **7** (dmTCEP; Scheme 2), and the free carboxylate was activated (HBTU) and coupled to the N-terminus of a PNA on solid-phase **6**. Importantly, the PNA−dmTCEP conjugates **8** were found to be stable toward TFA cleavages, and the products could be stored for several months without significant oxidation (the phosphines are presumably obtained as TFA salts after cleavage). On the other hand, this conjugate was not compatible with further Fmoc deprotection, as rapid phosphine oxidation was observed under the basic conditions of Fmoc removal, and it thus had to be introduced at the last step prior to cleavage.

Initial templated reduction experiments showed that incorporation of GPNA monomers into the sequence (one every two or three residues) dramatically improved the water solubility of the PNA and enabled a reliable template reaction in physiologically relevant buffers (10 mM PBS, 154 mM NaCl, 25 mM $MgCl_2$, 0.05% Tween). As shown in Figure 3 (using GCCACACTCCTTGCCCTTTAA as a template), the reaction proceeded fastest when two to four nucleotides separated the sites of hybridization of the rhodamine- and phosphine-bearing PNAs. This observation is consistent with our previous results with azidocoumarin,[22] despite the slightly longer distance between the fluorophore and the PNA in the current system. We then turned our attention to the identified MGMT sequence: ACCACACTGGACAGCCCTTTG. A genomic analysis showed that two hexameric PNA probes would be sufficient to uniquely target this mRNA sequence, and our previous experience had

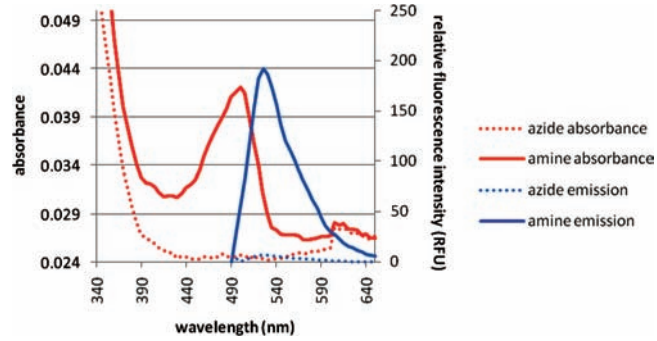


Figure 2. Absorption and emission spectra for N_3-Rh−C*GG*GA*AK (dotted line) and H_2N-Rh−C*GG*GA*AK (solid line) in PBS buffer (pH 7.4) at 23 °C. Each * denotes a residue where a GPNA monomer was used.

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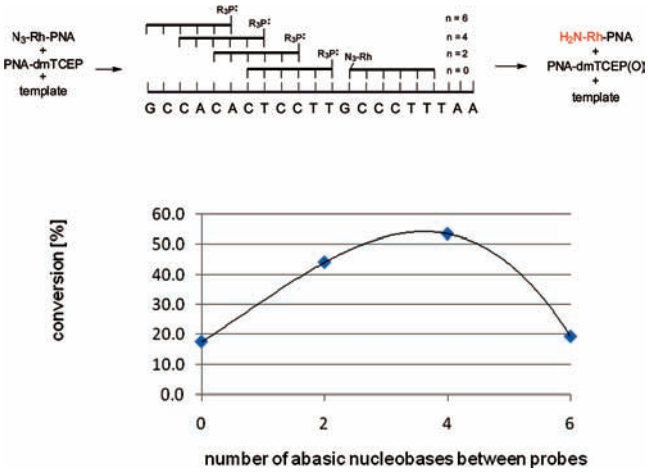

Figure 3. Distance dependence of the templated reduction: percentage conversion after 10 min using 1 equiv of DNA with 0, 2, 4, or 6 nucleotides between the reactive PNAs (template: GCCACACTCCTTGCCCTTTAA; N_3-Rh−C*GG*GA*AKwithKGA*GG*AA*−dmTCEP,KGT*GA*GG*−dmTCEP, KGT*GT*GA*−dmTCEP, or KCG*GT*GT*−dmTCEP).

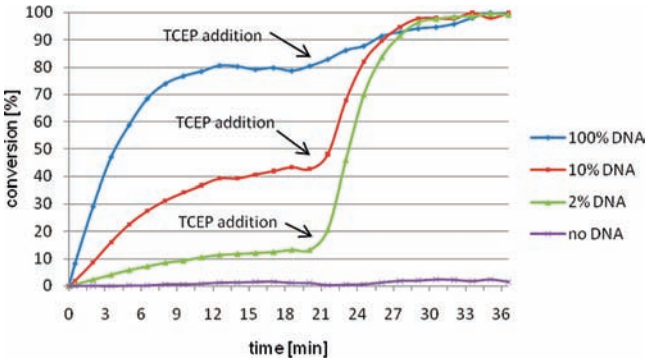

Figure 4. Templated reduction of azidorhodamine (N_3-Rh−C*GG*GA*AK, 200 nM) with phosphine (KGT*GT*GA*−dmTCEP, 800 nM) using stoichiometric and substoichiometric quantities (100, 10, and 2%) of MGMT template (AC**CACACT**GGACA**GCCCTT**TG) in buffer [10 mM PBS (pH 7.2), 154 mM NaCl, 25 mM $MgCl_2$, 0.05% Tween). Each * denotes a residue where a GPNA monomer was used.

shown that shorter nucleic acid probes afforded higher turnovers and better sequence specificity in the templated reactions.[22] Analysis of the sequence led to the selection of the PNAs GTGTGA−dmTCEP and N_3-Rh−CGGGAA, and a spacing of five nucleotides between the sites of hybridization was used on the basis of the fact that (*i*) such spacing still gave a fast template reaction, (*ii*) BLAST searches showed no other significant matching sequences for CACACT(N)$_x$GCCCTTA ($x = 1-6$), suggesting that PNA probes should uniquely target the MGMT mRNA, and (*iii*) these sequences do not contain hairpin or complementary motifs.

To investigate potential signal amplification by virtue of template turnover, the MGMT-templated reaction was run using various substoichiometric amounts of nucleic acid template. To quantify the percentage of conversion, all of the reactions were treated with a large excess of TCEP (1 mM, 5000 equiv) after 20 min, and the conversion was normalized to the maximum fluorescence observed. As shown in Figure 4, the rate of the templated reaction using 1 equiv of the template was extremely high, yielding 80% conversion in 10 min. Impressively, this reaction was slightly faster than the reduction with 1000 equiv of TCEP (100 μM), attesting to the high effective concentration achieved by the templated process (TCEP curve not shown). The reaction with 2% template still proceeded with useful kinetics, affording 14% conversion within 20 min, suggesting that the template statistically turned over 7 times within that time frame; the reaction using 10% template afforded over 40% conversion within the same time frame. It is interesting to note that the rate of the reaction tapered off in all cases, including the one with 1 equiv of template. We speculate that this reduction in reaction rate is due to the fact that the PNA hybridization is under equilibrium, and even with 1 equiv of template, the probability that an azidorhodamine−PNA (rather than an aminorhodamine−PNA) is hybridized on the same template strand as a phosphine−PNA (rather than a phosphine oxide−PNA) decreases as the reaction proceeds. Thus, the rate of the reaction inherently decreases as it progresses. It should also be noted that all of the reactions were carried out in a ventilated microtiter plate reader that was open to the air, so it can be assumed that the solutions were saturated with oxygen. It can thus be expected that the phosphine was oxidized by molecular oxygen over time, further decreasing the rate of the reaction. As the ultimate goal of this study was to perform these experiments in live cells, further experiments excluding oxygen from the system were not performed.

We then verified the sequence specificity of the templated reaction for the MGMT template (ACCACACTGGACAGC-CCTTTG). As shown in Figure 5, the PNA probes (200 nM N_3-Rh−PNA, 400 nM PNA−dmTCEP) reacted quickly in the presence of 10% MGMT sequence, whereas probes bearing a single mismatch or two mismatches reacted slowly (Figure 5, top). Conversely, when the same experiment was carried out with the MGMT template bearing two mutations (Figure 5, bottom), the PNA probe carrying the two complementary mutations reacted quickly whereas the perfect match to the MGMT sequence did not, thus showing that the lack of reactivity in the case of the PNA probes bearing the mutation was indeed due to the poorer efficacy of the templated process.

Next, we investigated the reaction with RNA extracted from cells. As the PNA probe carrying the single mutation was a perfect match for the mRNA of vinculin, which has a high expression level in HEK293 cells,[29] the PNA probe carrying two mutations was used as a negative control [a genomic analysis by BLAST did not reveal significant matches for CATTCT(N)$_x$GCCCTTA ($x = 1-6$)]. Gratifyingly, we found that the target mRNA could be detected in the crude extract, whereas the reaction with the two mismatches did not afford notable conversion (Figure 6).

We then turned our attention to the kinetics of cell permeability for the PNA hexamers bearing three guanidinium groups (GPNA) in a HEK293 cell line. It should be noted that while such GPNA have been reported to be cell-permeable, the reported work used longer PNA sequences with long incubation (>12 h). For the purpose of mRNA imaging and in view of the fast kinetics of the reaction, shorter incubation times would be desirable. We thus incubated the cells at 37 °C with 1 μM N_3-Rh−PNA for 5, 15, 30, or 60 min and then washed the cells and treated them with tmTCEP (10 μM) for 60 min. The cells were then cooled to 4 °C, and the fluorescence intensity and cellular distribution were assessed by fluorescence microscopy. As a control for unspecific membrane association, the same experiments were carried out with the incubation of PNA at 4 °C, which would provide higher fluorescence if the PNAs simply aggregate around cells by nonspecific interactions. Very low fluorescence was observed in the samples incubated at 4 °C (data not shown), while a gradual increase in fluorescence was

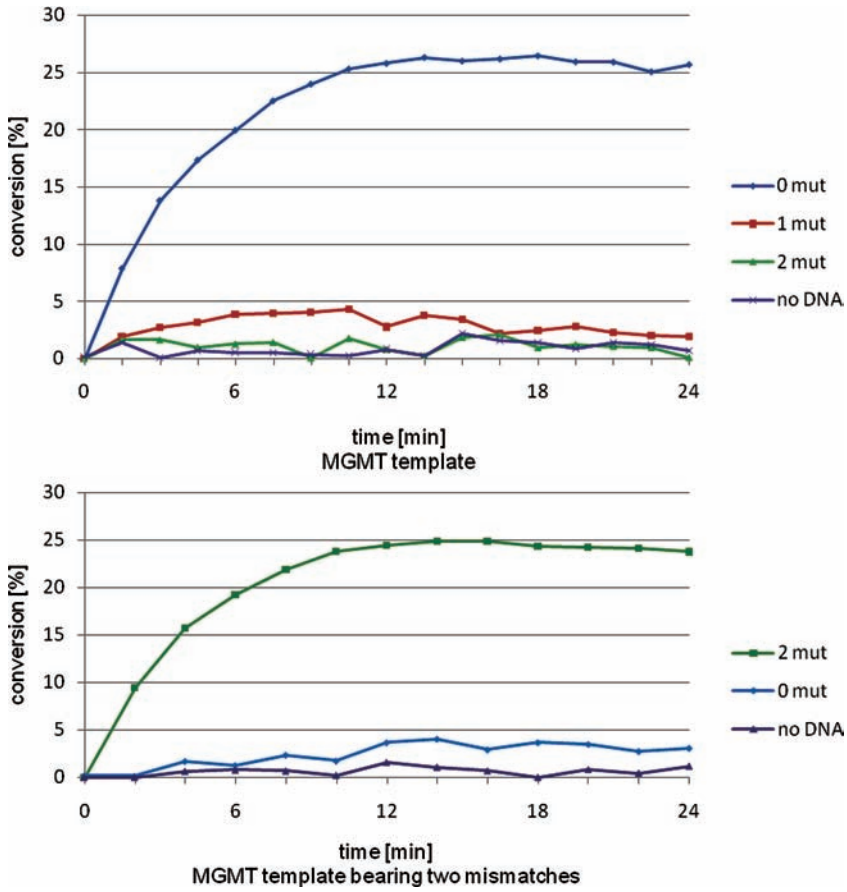

Figure 5. Sequence specificity of the templated reduction reaction using (top) the MGMT template and (bottom) a mutated template (mutations underlined). (top) Templated reduction using 10% template (AC**CACACT**GGACA**GCCCTT**TG) with azidorhodamine (N_3-Rh−C*GG*GA*AK, 200 nM) and phosphine [KGT*GT*GA*−dmTCEP, 400 nM (0 mut); KGT*G<u>A</u>*GA*−dmTCEP, 400 nM (1 mut); or KGT*<u>AA</u>*GA*−dmTCEP, 400 nM (2 mut)] in buffer [10 mM PBS (pH 7.2), 154 mM NaCl, 25 mM $MgCl_2$, 0.05% Tween). (bottom) Templated reduction using 10% template (AC**CA<u>TT</u>CT**GGACA**GCCCTT**TG) with azidorhodamine (N_3-Rh−C*GG*GA*AK, 200 nM) and phosphine [KGT*GT*GA*−dmTCEP, 400 nM (0 mut) or KGT*<u>AA</u>*GA*−dmTCEP, 400 nM (2 mut)] under the same conditions as above. Each * denotes a residue where a GPNA monomer was used.

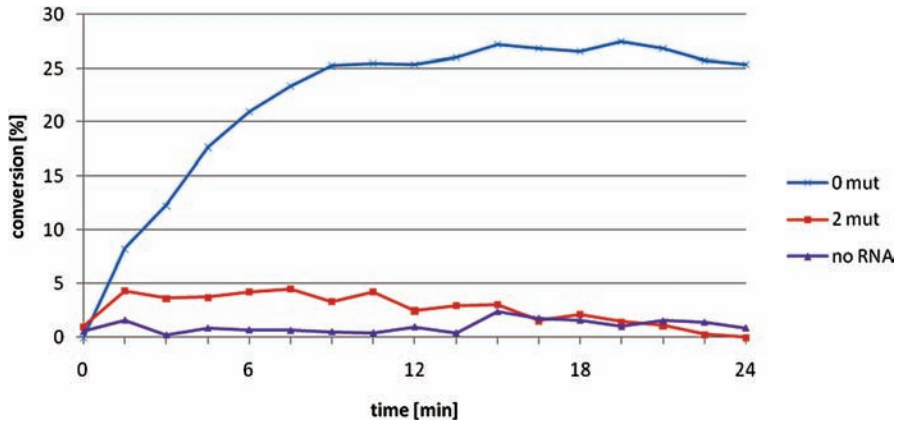

Figure 6. Templated detection of specific nucleic acid sequences within crude mRNA extracts. RNA extracts [5 μL in 480 μL of buffer containing 200 nM N_3-Rh−C*GG*GA*AK and 800 nM KGT*GT*GA*−dmTCEP (0 mut) or KGT*<u>AA</u>*GA*−dmTCEP (2 mut)].

observed at 37 °C, resulting in good cellular distribution. As shown in Figure 7, the increase of fluorescence reached a plateau at ∼30 min, suggesting a rapid uptake of PNA by the cells at 37 °C. Once this relatively fast uptake of PNA was established, HEK cells were incubated with N_3-Rh−PNA and PNA−dmTCEP matching the MGMT template, along with the mismatched sequence as a negative control. To minimize any background reaction, cells were first incubated with the N_3-Rh−PNA, then washed, and finally treated with the PNA−dmTCEP prior to imaging. Three N_3-Rh−PNA concentrations were evaluated (100, 250, and 500 nM) with 2 equiv of PNA−dmTCEP. As a positive control, the same experiments were carried out with tmTCEP (10 μM) to assess the sensitivity of detection at these lower N_3-Rh−PNA concentrations. The intensity of the fluorescence signal using 100 nM azidorhodamine incubation was deemed too weak, but satisfactory signal-to-noise ratios were obtained at 250 and 500 nM. However, the fluorescence intensity obtained in the experiments using 500 nM N_3-Rh−PNA did

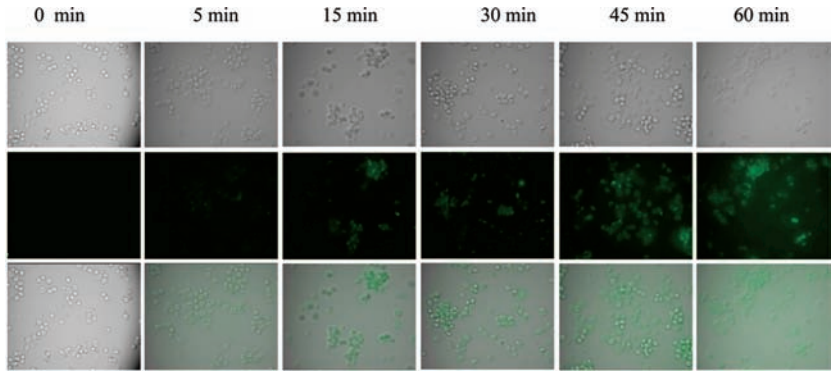

Figure 7. (top) White-light, (middle) fluorescence, and (bottom) composite images of cells incubated with profluorescent N_3-Rh−PNA (N_3-Rh−C*GG*GA*AK) for 0, 5, 15, 30, 45, and 60 min and then treated with tmTCEP.

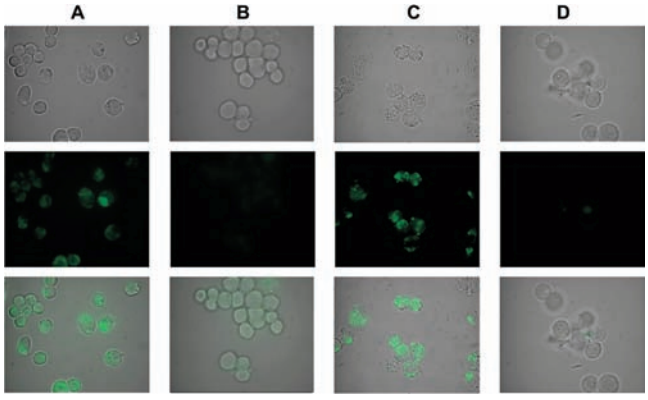

Figure 8. (top) White-light, (middle) fluorescence, and (bottom) composite images of cells incubated with profluorescent N_3-Rh−PNA (N_3-Rh−C*GG*GA*AK) and then treated with (A) tmTCEP, (B) nothing, (C) PNA−dmTCEP matching MGMT (KGT*GT*GA*−dmTCEP), and (D) PNA−dmTCEP mismatched to MGMT (KGT*AA*GA*−dmTCEP).

not significantly surpass that obtained using 250 nM, but more background fluorescence from the azidorhodamine was present. The lack of a higher signal at the higher concentration is not surprising, as the kinetics of the reaction is limited by template concentration, which was the same in the two experiments. The optimal conditions thus involved the use of 250 nM N_3-Rh−PNA. Incubation times of 15−90 min were evaluated, and it was found that the signal intensity obtained increased significantly between 15 and 30 min of incubation while only modest increases were observed for longer incubation times. As shown in Figure 8, when the optimal conditions (250 nM N_3-Rh−PNA, 90 min incubation) were used, a well-distributed fluorescence signal was obtained for the positive control (Figure 8A), while no signal was observed when the tmTCEP was omitted (Figure 8B). Incubation of the cells with the probes matching the MGMT mRNA afforded a fluorescence intensity comparable to the positive control (Figure 8C), while the cells incubated with the probes containing the two mismatches did not afford a significant fluorescence signal (Figure 8D). It is important to note that we previously established that the mismatched PNA probes reacted as fast as the matched PNA probes when presented with the right template. The results obtained in the whole cells are thus consistent with the results obtained with the purified DNA templates and the crude mRNA extracts.

Conclusion

We have shown that the reported azide-quenched rhodamine fluorophore is suitable for catalytic templated Staudinger reduction with alkylphosphines and that the use of GPNA enables the detection of nucleic acids in live cells. The azide-quenching strategy was thus extended from coumarin to a new fluorophore and is likely to work for other fluorophores that depend on a donating amino group, such as cresyl violet,[36] analogous far-red fluorophores,[37,38] or even chemiluminescence probes.[39] Furthermore, the availability of azide-based protecting groups such as Azoc[40] could be used as an alternative to the azide in order to unquench a fluorophore or to unmask bioactive small molecules. While other strategies have been reported to unmask fluorescence upon Staudinger reaction,[21,41] the use of azidorhodamine described herein is preferable, as it is insensitive to parasitic phosphine oxidation. The rapid kinetics and good sequence fidelity of nucleic acid-templated Staudinger reactions should make them useful for other applications, such as in computing systems for logical control of biological processes[42] or the design of synthetic networks.

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Supporting Information Available: Experimental procedures for the synthesis of Fmoc-protected GPNA, characterization of PNAs, and experimental procedures for templated reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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