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Organometallic Activation of a Fluorogen for Templated Nucleic Acid Detection

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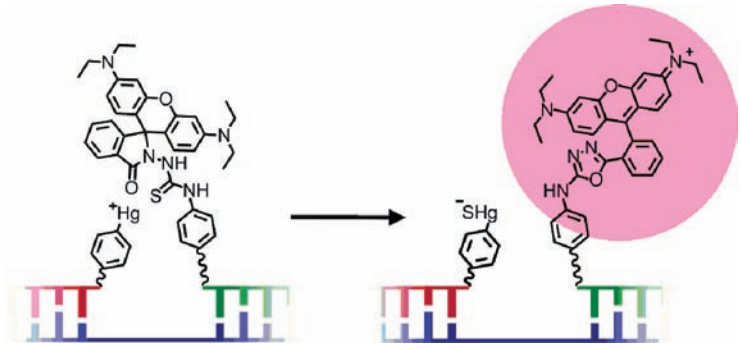
ABSTRACT



A nucleic acid detection scheme that employs DNA-mediated delivery of an organomercury activator to unmask a fluorophore is described. The approach relies on adjacent hybridization of two oligonucleotide conjugates containing organomercury and caged rhodamine functionalities. Postsynthetic conjugation of amino-modified DNAs enabled efficient preparation of these probes. Complementary DNA templates yielded fluorescence signals arising from metal-assisted rhodamine uncaging.

The biomedical importance of point mutations has generated a substantial demand for methods that detect nucleic acids with single nucleotide specificity. Detecting RNAs in vivo makes it possible to bypass amplification and purification steps and significantly reduce the costs and time associated with RNA detection. Possible applications of cellular RNA detection include pathogen identification and detection of cancer-associated mutations. Nucleic acid templated reactions that activate fluorescence are among the most sensitive and sequence-selective nucleic acid detection approaches[1–8] and can be applied to detecting RNAs in cells.[3,4] This strategy exploits the target strand as a template for a chemical reaction between two functionalized DNAs.[2] The probes hybridize to adjacent sites on the target sequence, increasing the effective concentration of the attached functionalities, and accelerating the reaction. Templated reactions have enabled the sensing of RNAs in both prokaryotes[3] and eukaryotic cells[4] using an S_N2 quencher displacement strategy.[5] However, substantial background emission from nonspecific interactions with cellular components currently limits in vivo detection to highly expressed RNAs.[4] Several other chemical

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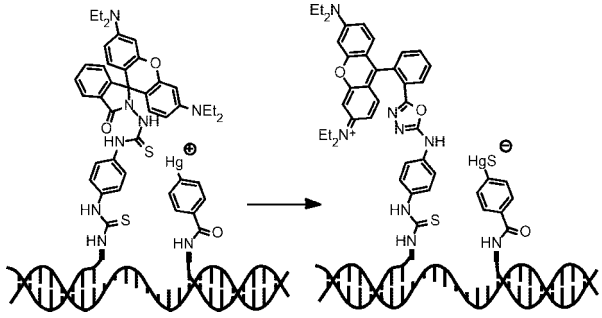
transformations such as native chemical ligation,[6] the Staudinger reaction,[7] and diamine-catalyzed aldol condensation[8] have been applied to DNA-templated activation of fluorescence. However, the design of fluorogenic probes that are active under physiological conditions but inert to nontarget cellular components continues to be a challenge.

Metal complexes may provide complementary functionalities for the templated activation of fluoresence. Krämer et al. have combined DNA templated chemistry with metal catalysis in vitro.[9] Also, the templated complementation of luminescent coordination complexes has been described.[10] However, the use of labile coordination complexes likely precludes the application of such probes in a cellular context.

Many organometallic reactants not only have unique chemical reactivities but also metal−ligand interactions that are inert to physiological conditions.[11] Organometallic molecules may provide stable, bioorthogonal reactant pairs that can be applied to templated fluorescence activation in living cells. This letter describes a DNA-templated reaction between an organomercury−DNA probe and a caged fluorophore conjugate. In the presence of a complementary target, the reaction generates a strong fluorescence turn-on signal.

Scheme 1. Chemistry of DNA-Templated Activation of Rhops Fluorescence by Phenylmercury−DNA Conjugates



The outlined reaction strategy involves two functionalized DNA probes. When hybridized to the nucleic acid target, one strand delivers a *p*-mercuriobenzoate (HgBA) group to a fluorogenic, mercury-sensitive reagent attached to the second strand (Scheme 1). The thiophilicity of mercury and the inertness of the Hg−C bond to aqueous conditions makes HgBA a promising organometallic reactant for bioanalytical applications. Rhodamine B phenylthiosemicarbazide[12] (Rhops) serves as the masked fluorophore. In the prescence of Hg^{2+}, the thiosemicarbazide functionality undergoes a cyclization reaction generating an oxadiazole. This transformation unlocks the rhodamine spirolactam ring and induces a substantial fluorescence increase. We hypothesized that Rhops would be sensitive to HgBA although with a reduced reactivity relative to inorganic Hg^{2+}. Rhops has excellent photophysical properties such as long wavelength emission and a high turn-on ratio; this fluorogenic reagent is biostable and is under investigation for cellular imaging of inorganic Hg^{2+}.[13] The sequences of the modified probes are complementary to adjacent positions at a single nucleotide polymorphism locus of the H-*ras* oncogene (Table 1).[14]

Table 1. Sequences of Probe[a] and Template[b] Strands Used for Fluorescence Activation Studies

strand	sequence
Rhops-DNA1	5′-Rhops-$(CH_2)_3$-OPO_3^--TGT GGG CAA GAG T-3′
Rhops-DNA2	5′-CCG TCG G-OPO_3^--$(CH_2)_3$-Rhops-3′
Hg-DNA1	5′-CCG TCG G-OPO_3^--$(CH_2)_3$-HgBA-3′
Hg-DNA2	5′-$(HgBACH_2)_2$CH-OPO_3^--TGT GGG CAA GAG T-3′
mut	5′-GC*A CTC TTG CCC ACA CCG ACG G*CG-3′
mut A	5′-GC*A CTC TTG CCC ACA* ACC *GAC GG*C G-3′
mut AA	5′-GC*A CTC TTG CCC ACA* AAC *CGA CGG* CG-3′
wt A	5′-GC*A CTC TTG CCC ACA* ACC *GCC GG*C G-3′

[a] Rhops = rhodamine B phenylthiosemicarbazide. HgBA = *p*-mercuriobenzamide. [b] Italic letters indicate probe hybridization sites. The underlined base in *wt A* specifies a single nucleotide mismatch.

The postsynthetic condensation of amino-modified DNAs with activated *p*-chloromercurio benzoate[15] allowed straightforward preparation of HgBA−DNA conjugates (Scheme 2). HgBA−DNA was stable to HPLC purification in CH_3CN/aqueous eluent systems as confirmed by MALDI-TOF mass spectrometry (Supporting Information).

Preparation of Rhops−DNA1 (Table 1) involved the postsynthetic conjugation of Rhops−isothiocyanate to amino-modified DNA (Scheme 2). The reaction of rhodamine B hydrazide with phenylene 1,4-diisothiocyanate afforded Rhops−isothiocyanate in 89% yield (Supporting Information). This caged dye−DNA conjugate was purified by HPLC and its identity confirmed by MALDI-TOF mass spectrometry.

We investigated the fluorescence properties of Rhops−DNA1 and its changes upon reaction with Hg−DNA1. Incubation of 1 μM Rhops−DNA1 with 5 μM Hg−DNA1 in the prescence 1 μM of the complementary template *mut AA* (15 h at 37 °C in 70 mM tris-borate and 10 mM $MgCl_2$, pH 7) generated a strong fluorescence signal with an emission maximum at $\lambda_{em} = 600$ nm when excited at $\lambda_{ex} = 530$ nm

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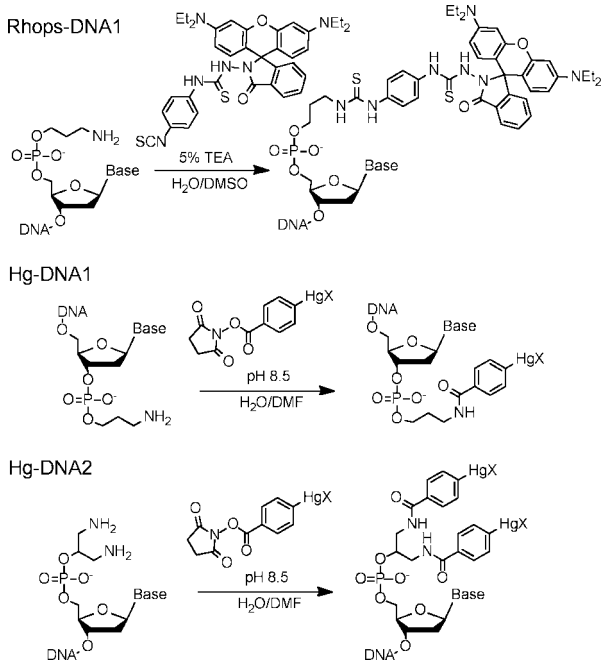
(15) Warning: *p*-chloromercuriobenzoic acid is acutely toxic!

Scheme 2. Post-Synthetic Preparation of DNA−Reactant Conjugates[a]



[a]DNA sequences are summarized in Table 1.

(Figure 1). The emission intensity of Rhops−DNA1 increased ca. 150-fold upon uncaging, which is comparable to the best current DNA-templated fluorescence activation designs.

Next, we assessed the template dependence of the reaction between Rhops−DNA1 and Hg−DNA1. The fluorescence signal of the DNA probes incubated at 37 °C in the presence of various DNA targets was monitored as a function of time (Figure 2a). Each sample consisted of 0.2 μM Rhops−DNA1 and template oligonucleotide, respectively, and 0.6 μM Hg−DNA1. The fluorescence emission after 110 min incubation served for quantitative comparison of the reaction rates (Figure 2b).

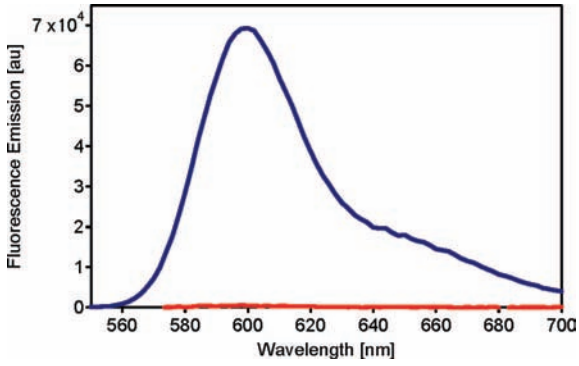


Figure 1. Fluorescence spectra (λ_{ex} = 530 nm) of 1 μM Rhops−DNA before (red trace) and after reaction with 5 μM Hg-DNA1 in the presence of 1 μM of the template *mut AA* (blue trace).

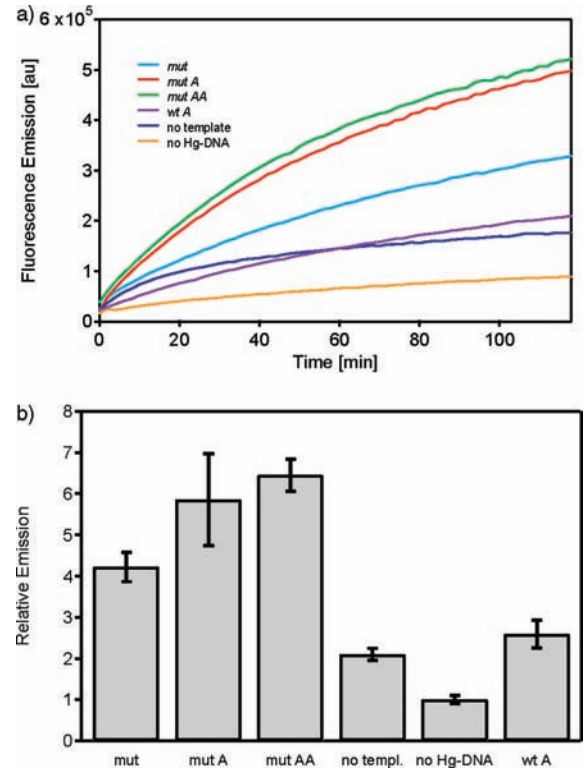


Figure 2. Template dependence of Rhops−DNA1 fluorescence activation by Hg−DNA1. (a) Representative time courses of fluorescence emission at λ_{em} = 600 nm (λ_{ex} = 575 nm). (b) Relative Rhops−DNA1 fluorescence signal in the presence of various templates after 110 min reaction with Hg−DNA1. Error bars are standard deviations of triplicate experiments.

To optimize probe alignment, we determined the rate of reaction between Rhops−DNA1 and Hg−DNA1 in the presence of complementary target strands with probe hybridization sites separated by 0 to 2 nucleotides (*mut*, *mut A*, and *mut AA*). All three templates accelerated the reaction significantly. The fluorescence emission of Rhops−DNA1 in the presence of *mut A* and *mut AA* after 110 min was 5.9 and 6.4 times higher, respectively, than the emission in the absence of Hg−DNA1. In the presence of *mut*, Hg−DNA1 was less efficient in unmasking Rhops−DNA1 (4.2-fold relative to no Hg−DNA) than in the presence of *mut A*. Short gaps between the sites of hybridization thus enable the optimal alignment of the probes.

Altering a single nucleobase at the center of *mut A*'s Hg−DNA1 binding site (*wt A*) significantly decreased the reactivity of Hg−DNA1 toward Rhops−DNA1. The emission of Rhops−DNA1 after 110 min in the presence of *wt A* (Table 1) was 2.6 times the emission measured without Hg−DNA1. This result translated into a ∼2-fold reduction of the reactivity between Hg−DNA and Rhops−DNA1 compared to *mut A*. A control sample containing Hg−DNA1 but no template exhibited a similar emission after 110 min as a sample containing the mismatched strand *wt A*.

We hypothesized that DNA probes with two HgBA functionalities might exhibit enhanced reactivity to Rhops−

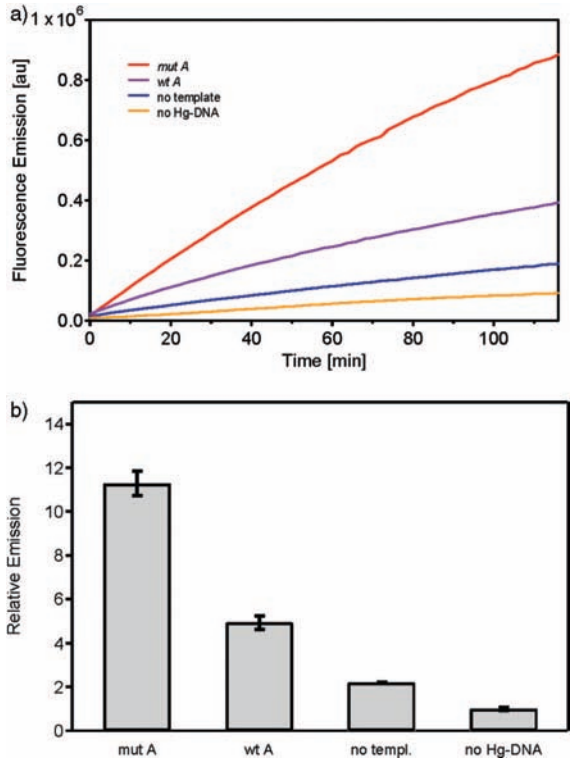


Figure 3. Template dependence of Rhops−DNA2 fluorescence activation by the doubly modified Hg-DNA2. (a) Representative time courses of fluorescence emission at $\lambda_{em} = 600$ nm ($\lambda_{ex} = 575$ nm). (b) Relative Rhops−DNA2 fluorescence signal in the presence of various templates after 110 min reaction with Hg−DNA2. Error bars are standard deviations of triplicate experiments.

DNA relative to Hg−DNA1. Rate acceleration could result either from an increased effective concentration of HgBA or from the concerted reaction of two HgBA groups with Rhops.

The preparation of the bis-HgBA probe Hg−DNA2 (Table 1) involved 5′-diamino modification followed by postsynthetic DNA conjugation of activated HgBA in analogy to Hg−DNA1 (Scheme 2). The synthesis of the 5′-diamino modifier required two steps. The reaction of 1,3-diamino-2-propanol with trifluoroacetic anhydride afforded 1,3-bis(trifluoroacetamido)-2-propanol in 83% yield. Treatment of the amino-protected alcohol with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite provided the 5′-diamino modifier (Supporting Information). The introduction of bis-HgBA at the 5′-terminus facilitated the preparation of Hg-DNA2. The associated DNA−fluorogen conjugate Rhops−DNA2 (Table 1) was prepared in analogy to Rhops-DNA1.

The reaction of Hg−DNA2 with Rhops−DNA2 was investigated as outlined for the reaction between Hg−DNA1 and Rhops−DNA1 (Figure 3). Hg−DNA2 accelerated the template-dependent Rhops−DNA2 activation approximately 2-fold relative to the monosubstituted Hg−DNA1. After 110 min, the fluorescence in the presence of the complementary template *mut A* was 11.3 times the fluorescence of background uncaging (no Hg−DNA2) compared to a 5.9-fold increase for the experiments with Hg−DNA1. In addition, the relative rate of reaction with Hg−DNA2 in the presence of the mismatched template *wt A* increased 4.9-fold relative to spontaneous Rhops−DNA unmasking (as compared to 2.6-fold for Hg−DNA1). On the other hand, the effect of a second organomercury group in absence of template was insignificant. This outcome demonstrates that two appended HgBA residues yield improved efficiency and selectivity for this templated reaction.

In summary, we have described a new DNA-templated reaction that uses an organometallic reactant. In the presence of a DNA target strand, an organomercury-functionalized DNA probe induces the fluorogenic unmasking of a Rhops−DNA conjugate. This system could be useful as a fluorogenic reporter of nucleic acid sequence in solution, and it gives readable signals with single nucleotide discrimination in minutes. The fluorescence turn-on ratio of the Rhops probe is superior to most current templated detection schemes. However, the mismatch sensitivity, while significant, is only moderate compared to previous methodologies. It is possible that a different mercury-sensitive caged fluorophore will afford a more predictable template dependence than Rhops. We are currently investigating alternative organometallic reactants for the development of template-dependent fluorescence activation schemes that may be applied to the sensing of nucleic acids with single mismatch discrimination in vitro and in vivo.

It merits mention that in addition to this novel templated reaction scheme, the approach we have developed for phenylmercury conjugation may find applications in derivatization of DNA. For example, organomercury compounds react with thiols under very mild conditions, forming inert but reversible Hg−S bonds.[16] Organomercury functionalities may further enable postsynthetic radiolabeling[17] or functionalization by metal-catalyzed cross-coupling reactions.[18]

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Supporting Information Available: Synthetic procedures and analytical characterizations; protocols for postsynthetic probe modification including MALDI-TOF characterization; supporting kinetics experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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