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# A thermally-stable enzyme detection assay that amplifies signal autonomously in water without assistance from biological reagents†

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**This Communication describes a thermally-stable small molecule and a corresponding assay strategy that autonomously amplifies a colorimetric signal when a specific enzyme biomarker is detected.**

The World Health Organization (WHO) has identified selectivity, sensitivity, and thermal stability[1] as three of seven key features that are needed in ideal point-of-care tests for use in the developing world.[2] Designing appropriate assays that incorporate these three features, however, has proven to be a substantial challenge, especially for assays and reagents that must be transported to remote villages without refrigeration. Of particular difficulty in this context is the design of thermally-stable signal amplification reagents that enable trace-level detection.[3] In this Communication, we describe the first-generation design of a new thermally-stable small molecule reagent (**1**) for amplifying signal in water without assistance from enzymes or other thermally-unstable reagents.[4] We also describe an assay strategy that uses this signal amplification reagent in combination with a second reagent (a detection reagent; **2**) (Fig. 1) to detect and amplify signal for specific enzyme biomarkers. Both of these reagents operate cooperatively[4a,b,5] and autonomously when a specific enzyme analyte is detected, thus offering an alternative to traditional analyte detection and signal amplification reagents and assays that employ thermally-unstable components (*e.g.*, antibodies, enzymes, and/or nucleic acids).

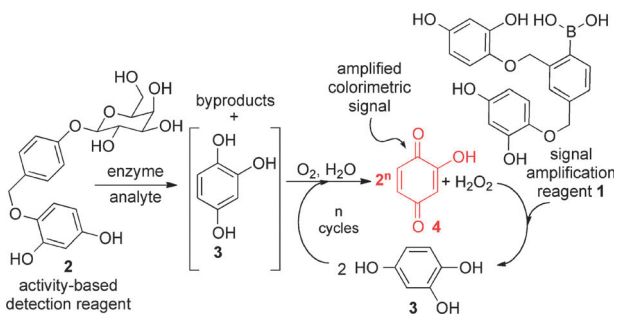


**Fig. 1** A one-pot, tandem assay strategy that uses two thermally-stable small molecules (**1** and **2**) to selectively detect an enzyme biomarker and autonomously amplify a colorimetric output signal (**4**).

Our signal amplification reaction generates a colorimetric readout in the form of 2-hydroxy-1,4-benzoquinone (**4**), which is an intense red-brown color (Fig. 1). During the assay, 2-hydroxy-1,4-benzoquinone (**4**) is obtained from the auto-oxidation of 1,2,4-trihydroxybenzene (**3**),[6] which itself is released from both the enzyme detection reagent (**2**) and the signal amplification reagent (**1**). This auto-oxidation reaction generates hydrogen peroxide, which serves as a "signal transduction reagent" in the tandem assay, thus enabling the detection event with **2** to initiate the signal amplification reaction with **1** *via* the highly selective oxidative cleavage reaction of hydrogen peroxide with aryl boronic acids.[7]

This reaction between hydrogen peroxide and the aryl boronic acid in **1** generates a phenol intermediate (not shown), which then undergoes two sequential quinone methide-mediated elimination reactions to release two equivalents of 1,2,4-trihydroxybenzene (**3**) (Fig. 1). Auto-oxidation of these two equivalents of **3** provides more hydrogen peroxide (thus perpetuating the signal amplification reaction *via* reaction with additional equivalents of **1**) and produces two equivalents of 2-hydroxy-1,4-benzoquinone (**4**) (the molecule that provides the colorimetric readout). Multiple self-perpetuating cycles of this reaction increase the quantity of **4** to levels where the color can be seen easily by eye. Quantification is readily achieved using a camera-equipped cellular phone in combination with image processing software in a process referred to as Telemedicine.[8]

In principle, only a single molecule of 1,2,4-trihydroxybenzene (**3**) must be released from **1** to perpetuate the signal amplification reaction if a quantitative yield of hydrogen peroxide is obtained from the auto-oxidation of **3**. Because we suspected that the yield of hydrogen peroxide would be less than 100%,[9] we designed **1** to release two equivalents of 1,2,4-trihydroxybenzene to increase the likelihood that at least one equivalent of hydrogen peroxide would be obtained from each molecule of **1** that reacts.

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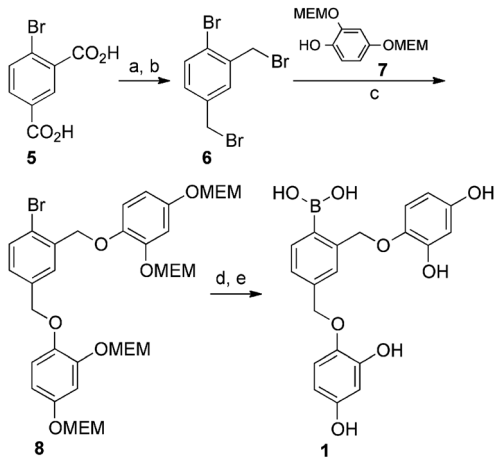


(a) $BH_3$·THF, THF, 66 °C, 96%; (b) HBr, $H_2SO_4$, $H_2O$, 122 °C, 82%; (c) $K_2CO_3$, acetone, 57 °C, 94%; (d) (i) *n*BuLi, THF -78 °C; (ii) $B(OMe)_3$, -78 °C to rt; (iii) 0.1 M HCl, 49% (e) PTSA·$H_2O$, EtOH, 35 °C, 39%

**Scheme 1** Synthesis of signal amplification reagent **1**.

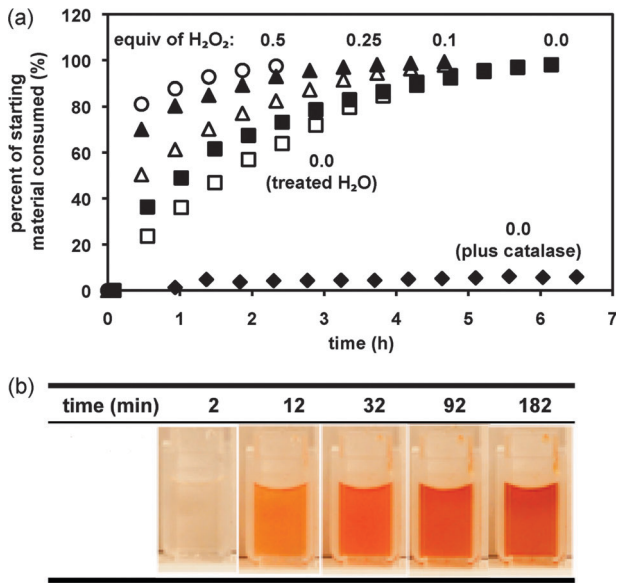


**Fig. 2** Self-perpetuating response of **1** when exposed to hydrogen peroxide. (a) Consumption of **1** over time for experiments using 2.5 mM **1** and different quantities of hydrogen peroxide. The percentage of **1** consumed during the reaction was measured at 254 nm using an LC-MS. ''Treated water'' refers to water that was treated with catalase to remove trace quantities of hydrogen peroxide. The ''plus catalase'' label refers to an experiment in which catalase was included in the assay. (b) Photographs over time after addition of 0.5 equivalents of hydrogen peroxide to a 2.5 mM solution of **1** in 0.1 M phosphate buffer containing 1.5% DMF (pH 7.3) at 23 °C.

We prepared amplification reagent **1** using the route shown in Scheme 1, which includes a late-stage introduction of the aryl boronic acid to avoid self-perpetuating decomposition reactions that could otherwise hinder the preparation of this autoinductive reagent. When stored dry at 40 °C, **1** is stable for at least 21 days.[10]

When exposed to hydrogen peroxide, all of **1** (2.5 mM in 0.1 M phosphate buffer containing 2% MeCN, pH 7.3, 34 °C) was consumed, even when only 0.1 equivalents of hydrogen peroxide is present (Fig. 2a). This result is consistent with the expectation that **1** is capable of self-perpetuating the signal amplification reaction by regenerating hydrogen peroxide *via* auto-oxidation of 1,2,4-trihydroxybenzene. In the absence of hydrogen peroxide, a background reaction is observed eventually, which we believe arises from slow hydrolysis of 1,2,4-trihydroxybenzene from **1**, followed by auto-oxidation to generate hydrogen peroxide.

Two control experiments support this hypothesis: First, we pre-treated the water used in the experiment with catalase (480 U $mL^{-1}$) for 15 min to remove traces of hydrogen peroxide in the water that might initiate the background reaction. After removing the catalase by filter centrifugation, we dissolved **1** in this purified water and monitored the consumption of **1** over time (Fig. 2a). The rate of consumption of **1** in the purified water was nearly identical to the rate with untreated water, indicating that trace levels of hydrogen peroxide that may be present in the water are not a significant contributor to the background signal. Second, when catalase (64 μg, 480 U $mL^{-1}$) was included with **1** (2.5 mM in 0.1 M phosphate buffer containing 2% MeCN, pH 7.3, 34 °C), only a gradual loss of **1** (10% after 15 h) was observed, which is consistent with the notion that trace background hydrolysis of **1** to release 1,2,4-trihydroxybenzene is the major cause of the background reaction in the catalase-free reaction.[11]

A graph similar to Fig. 2a was obtained when the intensity of the amplified color (rather than the consumption of **1**) was monitored over time when **1** was exposed to varying equivalents of hydrogen peroxide (Fig. S1a, ESI†). In this experiment, the color of the signal amplification reaction was obtained by photographing the samples and then quantifying the intensity of the images using Adobe®Photoshop®. Fig. 2b shows an example of the intense red-brown color that is generated when **1** (2.5 mM in 0.1 M phosphate buffer containing 1.5% DMF, pH 7.3, 23 °C) is exposed to 0.5 equivalents hydrogen peroxide.

The colorimetric response shown in Fig. S1a (ESI†) suggested that a quantitative assay might be possible. Indeed, a fixed assay time of 30 min provides a predictable dose response curve (Fig. S2, ESI†) for detecting hydrogen peroxide. Fig. S2b (ESI†) highlights the linear region of the 30 min assay, which has a limit of detection of 54 μM hydrogen peroxide.

This data illustrates that hydrogen peroxide should serve as an effective signal transduction reagent in the type of enzyme-detection assay depicted in Fig. 1. To demonstrate this concept, we designed a one-pot assay that uses reagent **1** as the signal amplification reagent and reagent **2** to detect β-D-galactosidase[12] (Fig. 3). The colorimetric signal for this tandem reaction (using **1** and **2**) is larger and increases more quickly (once the assay has proceeded for 6 h) than the signal obtained when only **2** is exposed to the enzyme. Background signal for **2** is minimal in the absence of the analyte.

Likewise, when **1** is exposed to the enzyme, the signal is negligible for 10 h, which, when compared to the more substantial background signal observed in the absence of surfactant (Fig. S1a, ESI†), suggests that the surfactant in the assay slows the rate of the signal amplification reaction relative to assays in the absence of surfactant. Kinetics experiments (Fig. S1b, ESI†) in the presence of surfactant (but absence of β-D-galactosidase) support this hypothesis.[13]

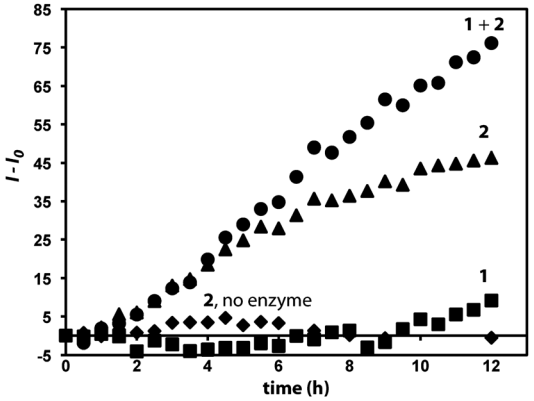


**Fig. 3** Normalized intensity of color for assays exposed to 194 nM β-D-galactosidase. *I* corresponds to the intensity of the colorimetric response and $I_0$ refers to the colorimetric signal at $t = 0$. The concentration of **1** and **2** were 2.5 mM in 0.1 M phosphate buffer (pH 7.3) containing 2.5% DMF and 0.5% Tween-20 (v/v); the assays were conducted at 23 °C. The surfactant in these assays slowed the rate of the signal amplification reaction as well as the background reaction with **1** in comparison to the conditions described in Fig. S1a (ESI†).

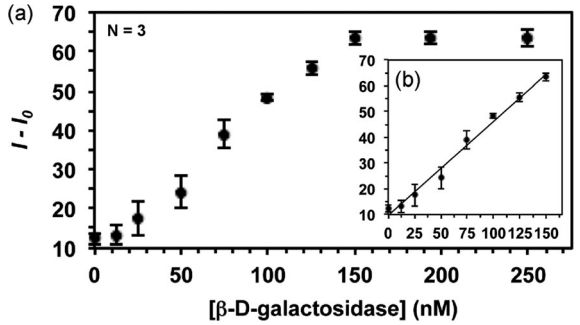


**Fig. 4** Calibration curve for a 10 h assay in which 2.5 mM **1** and **2** in 0.1 M phosphate buffer (pH 7.3) containing 2.5% DMF and 0.5% Tween-20 (v/v) were exposed to various concentrations of β-D-galactosidase at 23 °C. The intensity of color for each experiment was obtained using image analysis of photographs. The data points represent the averages of three measurements. (b) Expanded view of the linear region in (a).

The combined results from Fig. 3 and Fig. S1b (ESI†) indicate that (i) the assay provides selectivity for the detection event (*via* **2**), and (ii) the enzyme detection event is capable of initiating the signal amplification reaction with **1**. More importantly, the combination of **1** and **2** provides greater signal than the use of **2** alone.

Motivated by the results in Fig. 3, we next generated a quantitative assay for β-D-galactosidase by imaging the intensity of the colorimetric response (using a camera) after 10 h of exposure of **1** and **2** to solutions of the enzyme. The resulting calibration curve (Fig. 4) provides a dynamic range between 12 nM and 150 nM β-D-galactosidase and a limit of detection[14] of 12 nM (Fig. 4b), thus confirming the idea that the one-pot detection and signal amplification reaction shown in Fig. 1 is capable of providing quantitative measurements of an enzyme biomarker using only thermally-stable small molecules as reagents in the assay.

In conclusion, this communication describes a first-generation, thermally-stable signal amplification reagent (**1**) that amplifies signal for enzymatic detection events in water. Both the selectivity and sensitivity of assays using **1** (in combination with an activity-based detection reagent, such as **2**) are achieved without using thermally-unstable biological reagents, thus pointing to a new strategy for realizing three of the seven criteria put forth by the WHO for ideal diagnostic assays for use in the developing world. Future efforts will focus on increasing the rate of the signal amplification reaction and decreasing the background signal. Success in these efforts should enable the creation of highly developed assays for enzyme biomarkers associated with infectious disease, water quality, as well as other pressing diagnostic problems.

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1 The exact terminology used by the WHO is ''robust''.
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10 21 days was the duration of the experiment.
11 In this latter experiment, any hydrogen peroxide arising from background hydrolysis should be consumed by catalase, thus substantially slowing the consumption of **1**.
12 The enzyme β-D-galactosidase is a general marker of fecal contamination in drinking water. See: C. M. Davies and S. C. Apte, *Environ. Toxicol.*, 1999, **14**, 355.
13 We will study the mechanistic role of the surfactant on the rate of the signal amplification reaction in due course.
14 The limit of detection was calculated as $3 \times (sd/s)$ where *sd* is the standard deviation at 0.0 mM β-D-galactosidase and *s* is the slope of the calibration curve.

 