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Assay Development

Using Smell To Triage Samples in Point-of-Care Assays**

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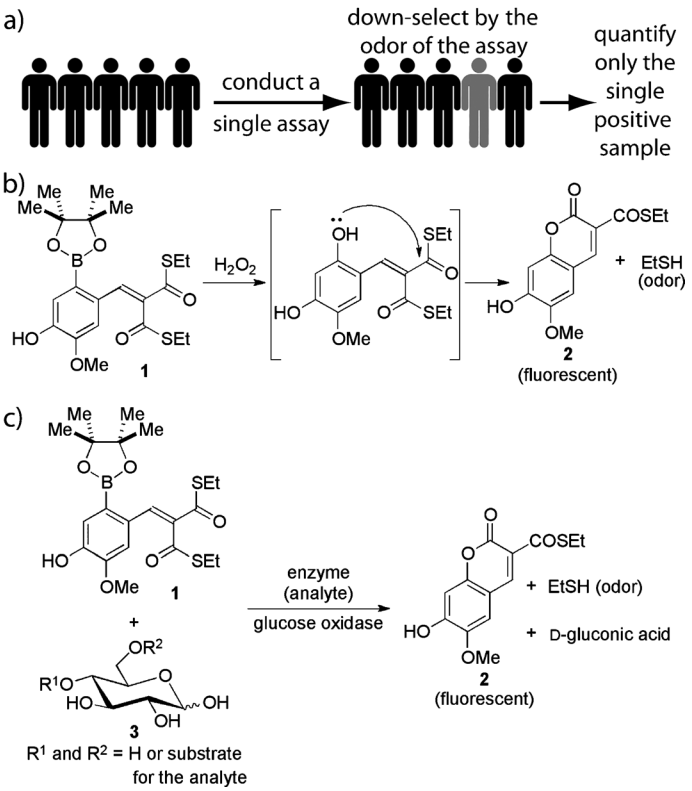
Quantification of an analyte in point-of-care assays is more time consuming, resource intensive, and expensive than conducting simple yes/no qualitative assays. Quantitative assays require highly engineered assays, electronic readers, and knowledgeable operators, whereas qualitative assays often involve simple colorimetric responses on a strip of paper.[1] In resource-limited environments, such as remote regions in the developing world, the challenges in conducting quantitative point-of-care assays become even more pronounced since many individuals must be tested quickly for the presence and (if appropriate) the quantity of a biomarker to determine the necessary treatment and dosage. To minimize the time commitment associated with quantitative point-of-care assays in these regions, we now describe a reagent and assay strategy that enables rapid triaging of samples followed by quantitative measurements of positive tests. These requirements are achieved by using the same single-step assay and does not require a quantitative measurement to be obtained on all samples (which is the standard approach). The triaging step is accomplished by smell (a process that requires little time), and the quantitative measurements are obtained by using fluorescence spectroscopy (Scheme 1). With this approach, the absence of the analyte in the triaging step means that the quantitative measurement can be omitted, thus saving time and effort.

This type of rapid down-selection process is made possible by small-molecule **1**, which enables both the qualitative and quantitative readouts in a single assay (Scheme 1a).[2] The qualitative readout (i.e., the odor of released ethanethiol)[3] matches the sensitivity of the quantitative readout (fluorescence); this feature is crucial for an effective down-selection process, particularly when detecting and quantifying analytes that are present in low concentrations.

Reagent **1** functions by reacting selectively with hydrogen peroxide[4] by oxidative cleavage of the arylboronate on **1** to generate an unstable intermediate phenol, which then undergoes a rapid 6-*exo*-trig cyclization to release ethanethiol (odor) and generate a 7-hydroxycoumarin derivative (**2**; fluorescent readout; Scheme 1b).



Scheme 1. A strategy for triaging samples in point-of-care assays by using reagent **1**. a) Depiction of the concept. b) Reaction of reagent **1** with hydrogen peroxide (a signal-transduction reagent) to form the dual readouts of smell (ethanethiol) and fluorescence (**2**). c) An assay strategy that involves the use of **1** and detection reagent (**3**) to detect enzyme analytes.

Hydrogen peroxide, however, is not the target analyte in this system. Instead, it is a signal-transduction reagent that enables a detection reagent (**3**) and the output reagent (**1**) (Scheme 1c) to operate in tandem in a single assay. With this approach a variety of analytes can be detected (and down-selected for) using essentially the same reagents and measurement procedures in all assays (only reagent **3** would need to be modified from one assay to the next). For example, hydrogen peroxide can be derived from specific activity-based detection events with enzyme biomarkers (Scheme 1c) when the presence of the enzyme causes release of glucose from the detection reagent (**3**). This glucose is then converted into hydrogen peroxide by glucose oxidase, which also is present as a reagent in the assay.

In point-of-care settings, the quantitative fluorescence assay would be accomplished by using a handheld fluorescence spectrometer (a variety of relatively inexpensive

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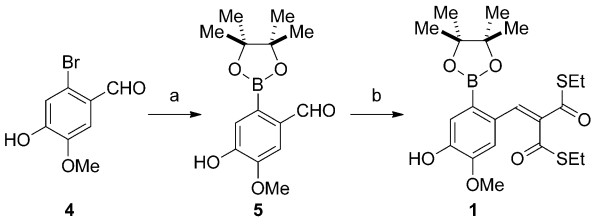
commercial versions are available), or by using an even less expensive paper-based microfluidic device that contains internal fluidic batteries to power on-chip fluorescence assays.[5] In this study, we demonstrate the performance of the reagent by using a laboratory fluorescence spectrometer. Because of the key role of **1** in the down-selection assay, the focus herein is on the design, synthesis, and performance of **1**. We also demonstrate model quantitative assays for the enzymes β-D-galactosidase[6] and alkaline phosphatase[7] to illustrate that multiple classes of enzymes can be detected and measured with this reagent. Future reports will describe fully developed down-selection assays conducted in settings outside of the laboratory.

We prepared reagent **1** in two steps from commercially available reagents (Scheme 2). The sequence of synthetic



Scheme 2. Synthesis of sensor reagent **1**. Reaction conditions: a) Bis-(pinacolato)diboron, KOAc, $[PdCl_2dppf]\cdot CH_2Cl_2$, 1,4-dioxane, 80 °C (84%). b) $CH_2(COSEt)_2$, DABCO, molecular sieves (3 Å), THF (35%).

steps involved a Miyaura borylation reaction[8] of **4** followed by a base-catalyzed Knovenagel condensation[9] to transform **5** into **1**. Although this Knovenagel condensation proceeded in only modest yield (35%), the remainder of the material consisted primarily of recovered starting material.

When treated with 5 equivalents of hydrogen peroxide, **1** (0.5 mM in 1:1 MeOH/10 mM phosphate buffer, pH 7.4, 20 °C) was consumed within minutes and **2** was formed immediately (Figure 1). In fact, the cyclization reaction to

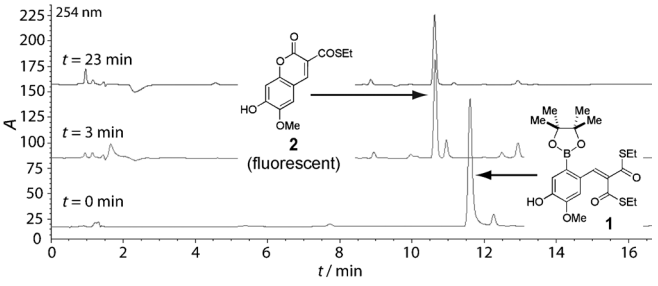


Figure 1. Overlayed LC–MS spectra for three sequential injections of aliquots taken from the reaction of **1** (0.5 mM) with 5 equivalents of H_2O_2 in 1:1 MeOH/10 mM phosphate buffer, pH 7.4 at 20 °C.

form **2** (Scheme 1b) is sufficiently rapid that none of the oxidative cleavage product (i.e., the phenol) was detectable by LC–MS. The presence of a characteristic thiol odor during these reactions confirmed the release of ethanethiol.

The progress of the reaction of hydrogen peroxide (1.1 equiv) with **1** (20 μM, 10 mM phosphate buffer containing 1% MeOH, pH 7.4) was monitored by using fluorescence spectroscopy; the emission intensity between 500 nm and 570 nm increased over a period of 12 minutes as **1** was converted into **2** (Figure 2). The rapid increase in fluorescence emission in this spectral region further confirms the formation of **2**.

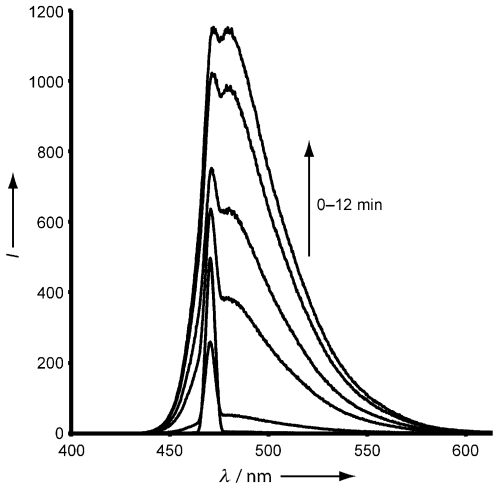
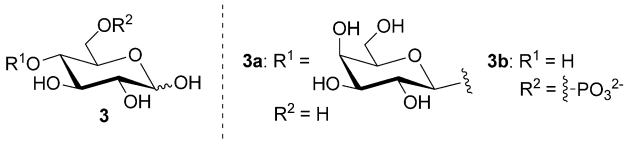


Figure 2. Time-dependent fluorescence emission (*I*) when **1** ($[\mathbf{1}]_{final} = 20$ μM in 10 mM phosphate buffer containing 1% MeOH (v/v), pH 7.4) was treated with hydrogen peroxide ($[H_2O_2]_{final} = 22$ μM) to form **2**. The excitation wavelength (λ_{ex}) was 470 nM and the experiment was conducted at 20 °C. Wavelength scans were acquired every 2 minutes. The initial scan and the data were acquired at 2 minutes overlap.

When **1** is exposed to different concentrations of hydrogen peroxide in a fixed-time assay, the intensity of the fluorescence emission signal obtained is proportional to the concentration of hydrogen peroxide (Figure 3). This correlation between intensity of fluorescence signal and concentration of hydrogen peroxide suggests that hydrogen peroxide should operate effectively as a signal-transduction reagent in a two-reagent assay for detecting enzyme biomarkers, as depicted in Scheme 1c.

To test this idea, we set up two separate two-component assays for selectively detecting either β-D-galactosidase or alkaline phosphatase (Scheme 3).The goal was to demonstrate that: 1) hydrogen peroxide does in fact serve as an effective signal-transduction reagent, 2) by using reagent **1** enzyme analytes can be detected qualitatively at levels that are relevant to the quantitative assay, and 3) the assay strategy is amenable to a variety of classes of enzyme analytes.



Scheme 3. Specific detection reagents **3a** and **3b** that are used in combination with **1**, as shown in Scheme 1c, to detect and quantify β-D-galactosidase and alkaline phosphatase, respectively.

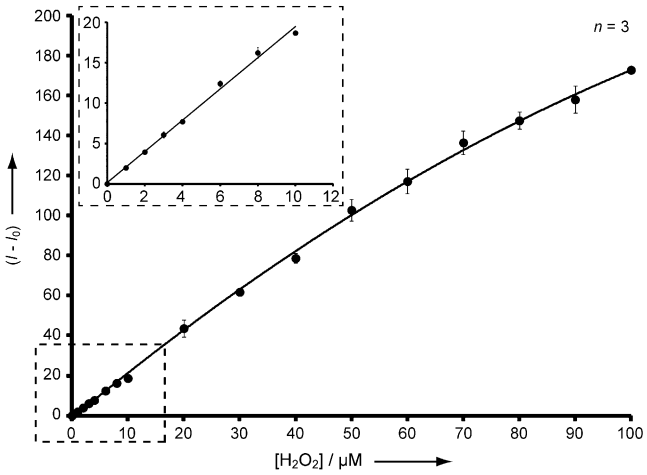


Figure 3. Calibration curve for the detection of hydrogen peroxide by using **1** in a 10 minute assay. The final concentration of **1** in the assay was 20 μM in 10 mM phosphate buffer, pH 7.4 containing 1% (v/v) MeOH. The fluorescence emission intensity (I) was measured at 510 nm with $\lambda_{ex} = 470$ nM. I_0 denotes the fluorescence intensity obtained for $[H_2O_2] = 0$ μM. The inset graph reveals the linear region defined by the dotted rectangle. All data points represent the average of three independent measurements. The limit-of-detection (LOD) (defined as 3 × standard deviation of 1 μM H_2O_2/slope of the line in the inset) is 0.3 μM.

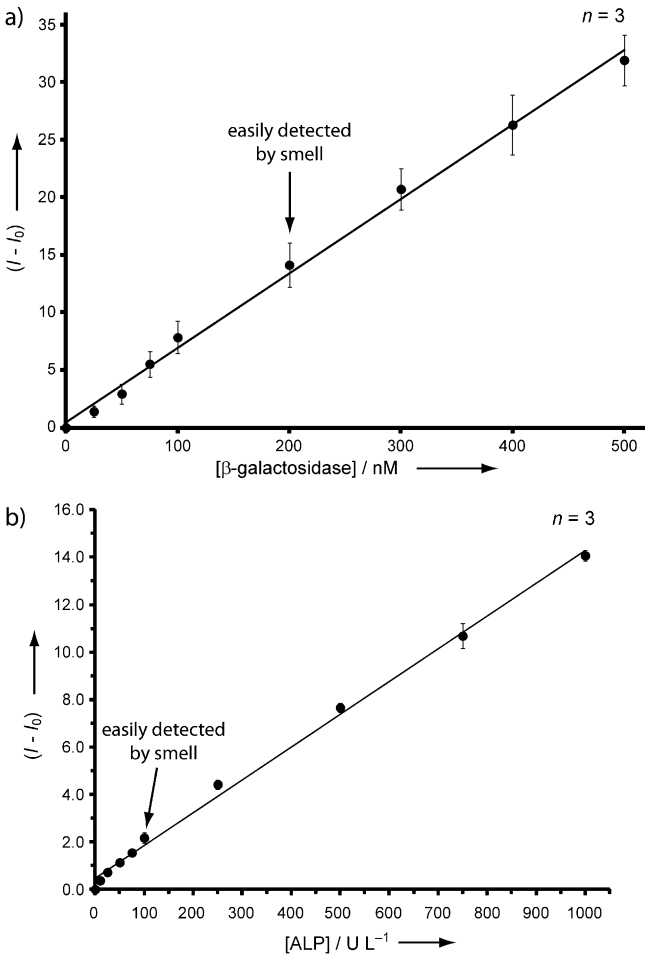


Figure 4. Calibration curves for quantitative detection of β-D-galactosidase (a) and alkaline phosphatase (b) by using reagents **1** and **3** (Scheme 3). a) The final concentration of **1** was 20 μM in 10 mM phosphate buffer, pH 7.4 containing 1% (v/v) MeOH. The concentration of lactose (**3a**) and glucose oxidase were 10 mM and 1000 U L^{-1} respectively. b) The final concentration of **1** in the assay was 20 μM in 50 mM Tris buffer, pH 7.4 containing 1% (v/v) MeOH. The concentration of D-glucose-6-phosphate (**3b**) and glucose oxidase were 10 mM and 1000 U L^{-1} respectively. In both (a) and (b), the fluorescence emission intensity (I) was measured at 510 nm using $\lambda_{ex} = 470$ nM, and the assay time was 1 h. I_0 denotes the fluorescence intensity obtained in the absence of the enzyme analyte. All data points represent the average of three independent measurements.

We prepared two calibration curves, one for β-D-galactosidase and one for alkaline phosphatase, (Figure 4) from results obtained from 1 h assays. The calibration curve for β-D-galactosidase (Figure 4a) reveals a limit-of-detection (LOD) of 21 nM (enzyme activity of 2.2 U mL^{-1}), with a linear dynamic range extending up to 500 nM β-D-galactosidase.

To demonstrate the effectiveness of the smell-based down-selection process, we conducted double-blind tests as follows: ten individuals each were given four different samples, two of which were control assays. The other two assays were chosen randomly from a selection that contained either 0 nM or 200 nM β-D-galactosidase. By using only smell as an indicator, 80% of samples were identified correctly either as being the same as the control or as containing β-D-galactosidase based on a distinct thiol odor. This level of sensitivity in the qualitative assay is perfectly matched with the sensitivity of the quantitative assay, which indicates that the former is an effective down-selection tool for the latter.

The calibration curve for alkaline phosphatase (Figure 4b) revealed a LOD of 10 U L^{-1} alkaline phosphatase, with a dynamic range extending to 1000 U L^{-1}. In this case, when assays containing either 0 U L^{-1} or 100 U L^{-1} alkaline phosphatase were tested, 70% of samples were identified correctly by smell either as being the same as the control or as containing a distinct thiol odor. This result further confirms the match in sensitivity of the qualitative and quantitative readouts for the assay.

In conclusion, we have developed a new, dual-readout-assay strategy that enables triaging of samples based on smell, followed by quantitative fluorescent measurements of the positive samples. Future studies will ascertain whether the odor will be as apparent in real samples under typical conditions encountered in resource-limited settings. Likewise, a larger sample pool will be used to determine the limit of sensitivity for the qualitative portion of the assay. The initial results outlined herein demonstrate that appropriate reagent design enables rapid down-selection before quantitative fluorescence assays are conducted. This strategy should facilitate the time-consuming process of performing quantitative assays in resource-limited environments.

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