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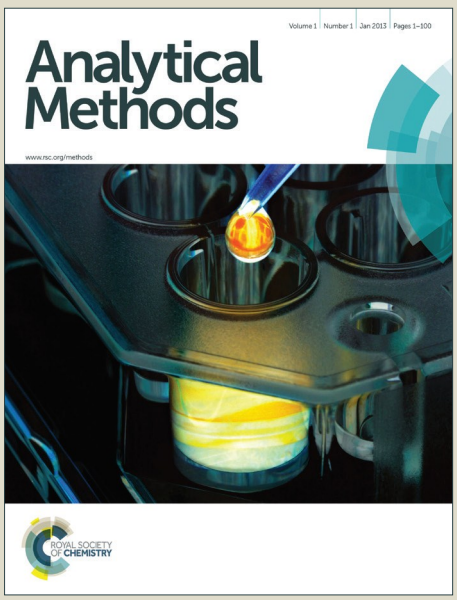

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PAPER

A Strategy for Minimizing Background Signal in Autoinductive Signal Amplification Reactions for Point-of-Need Assays

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Rapid point-of-need assays are used to detect abundant biomarkers. The development of *in situ* signal amplification reactions could extend these assays to screening and triaging of patients for trace levels of biomarkers, even in resource-limited settings. We, and others, have developed small molecule-based *in situ* signal amplification reactions that eventually may be useful in this context. Herein we describe a design strategy for minimizing background signal that may occur in the absence of the target analyte, thus moving this *in situ* signal amplification approach one step closer to practical applications. Specifically, we describe allylic ethers as privileged connectors for linking detection and propagating functionality in a small molecule signal amplification reagent. Allylic ethers minimize background reactions while still enabling controlled release of a propagating signal in order to continue the signal amplification reaction. This paper characterizes the ability of allylic ethers to provide an amplified response, and offers insight into additional design considerations that are needed before *in situ* small molecule-based signal amplification becomes a viable strategy for point-of-need diagnostics.

Introduction

Traditional approaches for signal amplification use enzyme or nanoparticle labels and multistep sequences (ELISA or bio barcode), conjugated polymers and electronic readers, or quantitative PCR, which is a multistep target amplification process.[1–9] Diagnostic assays that are designed for resource-limited environments (i.e., point-of-need diagnostics), however, typically do not employ these signal amplification strategies. For many point-of-need applications, the assays must maintain an appropriate balance of low cost, speed (assay times of minutes), and simplicity (ideally nearly anyone would be able to conduct the assay). Achieving this balance has resulted in rapid assays with modest sensitivity (e.g., dipsticks, lateral flow assays, and glucose meters) that are not capable of detecting trace levels of analytes, and therefore are ineffective for many rapid screening and triaging applications.[7, 10, 11]

Recent research has begun to explore new strategies for improving the sensitivity of point-of-need assays to expand their scope and versatility.[11–14] Important considerations in this context include maintaining (if possible) single step assays, ensuring low cost (to reduce barriers to use), and avoiding electronic equipment (which complicate assays and increase cost).[15] One strategy, which is the topic of this paper, involves *in situ* signal amplification reactions that are initiated only if the analyte is present, thus eliminating the need for multi-step assays (Fig. 1).

Small molecule reagents are key to this strategy, both for providing selectivity and signal amplification. Several variations of the general amplification strategy have been reported, including assays in which (i) a single small molecule reagent simultaneously mediates the self-propagating signal amplification reaction and provides the amplified readout for the assay (termed direct signal amplification; Fig. 1a);[8, 16–21] (ii) two or more small molecule reagents operate in concert to provide the amplified readout (indirect signal amplification; Fig. 1b);[22, 23] or (iii) an enzyme and one or more small molecule reagents are used in concert to enable amplification of the desired readout (enzyme-mediated indirect signal amplification; Fig. 1c).[13, 24–27] Collectively, these three variants have been used in assays that detect low levels of fluoride, hydrogen peroxide, palladium, thiols, glucose, β-D-galactosidase, choline, pencillin-G-amidase, and alcohol oxidase, but also should be amenable to a wide range of other analytes.[8, 12, 13, 16, 18–28] These variants largely employ thermally stable reagents, and thus should be functional in nearly any point-of-need setting.

Additional development is needed, however, before the anticipated benefits of these small molecule-based signal amplification strategies can be realized fully. For example, efforts to date have focused primarily on exploring (i) different mechanisms for signal amplification (such as autoinductive and autocatalytic reactions),[16, 20, 21, 24] (ii) different propagating signals for facilitating the self-propagating autoinductive or autocatalytic reactions,[17, 20, 21, 23] (iii) different types of readouts (i.e., colorimetric or fluorescent),[17, 20–22] and (iv) the degree of amplification that is possible within a fixed assay period.[16] These seminal studies provide the framework for small molecule-based signal amplification, but efforts moving forward must now focus on additional design elements that will help transform the initial concepts into practical assays. One of these design elements is the critical need to minimize background reaction(s) that may occur independent of a detection event. Background signal will become amplified along with the desired signal for the detection event, thus reducing the overall sensitivity for an assay. This paper reports a new approach for minimizing background reaction in signal amplification assays.

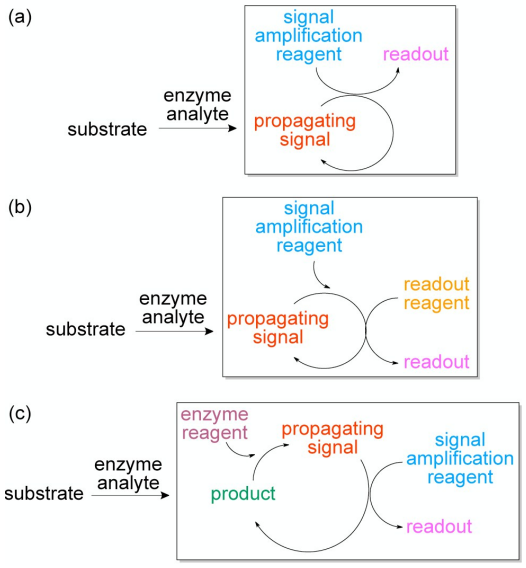

Fig. 1 Three variants of a general signal amplification strategy that employs thermally-stable small molecule reagents as the key mediators of amplification reactions occurring directly in an assay if the target analyte is present. The boxed sections represent the amplification reactions, while the sections outside the boxes depict detection events that can be linked, *in situ*, to an amplification reaction. (a) Direct signal amplification; (b) indirect signal amplification; and (c) enzyme-mediated indirect signal amplification.

Results and discussion

Design of a signal amplification reagent that minimizes the production of background signal

Our design for signal amplification (Fig. 2a) includes a plan for generalizing the signal amplification reaction for use in assays targeting specific enzymes, certain inorganic species, as well as reactive organic molecules. Thus, the top line in Fig. 2a depicts a small molecule activity-based substrate for a target enzyme analyte, in which the presence of the enzyme causes release of glucose. We have prepared this type of reagent already,[16] and have in hand reagents for targeting four major classes of enzymes.[19, 29] Thus, the focus of this work is on the bottom line in Fig. 2a, which represents the key signal amplification reaction that will be initiated when glucose is released during a detection event.

The design of this amplification reaction is a minimalistic version of Shabat's concept of dendritic chain reactions (Figure 1c), which employs an enzyme to facilitate the signal amplification reaction.[24] We used the enzyme glucose oxidase (an inexpensive, readily available, and relatively stable enzyme), and therefore needed a complimentary small molecule reagent that was capable of releasing glucose in response to a propagating signal, which, in this case, is hydrogen peroxide (Fig. 2a). The released glucose is oxidized by glucose oxidase to generate another equivalent of hydrogen peroxide as well as D-(+)-gluconic acid δ-lactone. The D-(+)-gluconic acid δ-lactone readily hydrolyzes in water to D-(+)-gluconic acid, increasing the acidity of the assay solution (as revealed by the change in color of a pH indicator dye; Figs. 2a,b). The new equivalent of hydrogen peroxide is free to react with another equivalent of the small molecule signal amplification reagent to continue the self-propagating reaction (Fig. 2a).

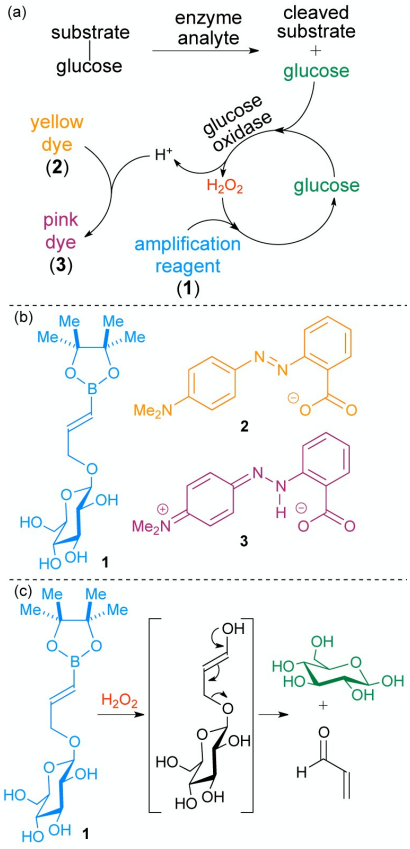

Fig. 2 (a) The amplification strategy employed in this work; (b) structures of specific reagents used in the assay; and (c) partial mechanism of response of the signal amplification reagent. The signal amplification reagent, **1**, is blue, the propagating signal, H_2O_2, is red, and the generated product, glucose, is green, which are colors that match the coding in Fig. 1.

In general, the ideal small molecule signal amplification reagent for this type of self-propagating reaction must (i) be synthetically accessible, (ii) respond selectively to the propagating signal mediating the self-propagating reaction (e.g., hydrogen peroxide), (iii) provide rapid and tunable rates of signal amplification, (iv) be soluble in water, (v) be thermally stable for long-term storage and use in environments that do not have refrigeration, and (vi) amplify signal without introducing background signal. Moreover, in order to minimize background reaction arising from the signal amplification reagent, glucose cannot serve as a substrate for glucose oxidase when attached to the small molecule reagent (i.e., compound **1** in Fig. 2a), and the bond connecting glucose to the signal amplification reagent must be hydrolytically stable.

We reasoned that attaching glucose to the signal amplification reagent via an ether linkage between the connector and the 2-position of glucose (Figs 2b, 3a) would satisfy both of these design goals since an ether bond should be more resistant to hydrolytic cleavage than other bonds used for attaching alcohols to pro-drugs and reaction-based indicators (such as carbonates or carbamates).[6, 9, 13, 30, 31] Moreover, glucose oxidase requires an alcohol at the anomeric position of glucose (i.e., the hemiacetal form) to act upon the substrate, which would not be available in an ether linkage. The challenge with this approach is the need for a pro-drug-like connector piece (Figure 3a) that links the anomeric position of glucose with a unit that reacts selectively with hydrogen peroxide, but is also capable of releasing glucose quickly.

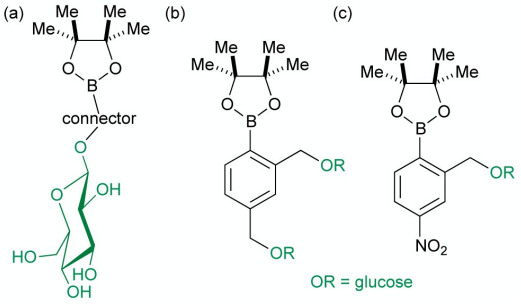

Fig. 3 (a) General structure of a small molecule signal amplification reagent that responds to hydrogen peroxide by releasing glucose. Two initial designs for connector groups are depicted in (b) and (c).

A common connector strategy is a benzylic group, such as those depicted in Figs. 3b,c. These connectors become phenols when the aryl boronate cleaves oxidatively upon reaction with hydrogen peroxide, and thus are designed to release benzylic groups via quinone methide chemistry. However, quinone methide-mediated (or even aza-quinone methide-mediated) release of alcohols that are attached as benzylic ethers was predicted to be exceedingly slow (e.g., days) for alcohols with pK_a values greater than ~11,[32] which we verified experimentally by preparing and testing the compounds in Figs. 3b,c. The hemiacetal in glucose has a pK_a value of 12.3 in water,[33] thus, as predicted, the reagents in Figs. 3b,c released glucose on a time scale of days when exposed to hydrogen peroxide, even in pH 10 media.

As a consequence, we considered other strategies for releasing alcohols. Floreancig's vinyl boronates, which respond to hydrogen peroxide to release amines via allylic carbamates, releases carbamates 6× faster than more traditional aryl boronate–quinone methide chemistry.[31] Thus, we wondered whether an allylic moiety could be an effective connector group for triggered release of allylic ethers, and in particular whether such a design could affect the triggered release of glucose when connected as an allylic ether at the anomeric position of glucose (Fig. 2c). These considerations led to the design of reagent **1** (Figs. 2b,c).

Synthesis and reactivity of reagent 1

Reagent **1** is readily accessible via the short route depicted in Figure 4, which was modified slightly from the procedure reported by Davis.[34, 35] With reagent **1** in hand, we tested whether exposure of the reagent to hydrogen peroxide would release glucose. Thus, treatment of **1** (5 mM in distilled water) with hydrogen peroxide (25 mM, 5 equiv) yielded measureable readings for glucose that increased over time when the sample was probed using a commercial glucometer, which is designed to measure the level of glucose in blood (Fig. 5a). In the absence of hydrogen peroxide, no measurable reading was obtained over the same assay period. These results are significant for two reasons: they demonstrate that glucose oxidase, which is used in the glucometer assay, does not react with reagent **1**, at least not within the measurement limits of the meter; and they illustrate the ability of the reagent to release glucose.

Release of glucose also can be inferred from ^{1}H NMR experiments in which we monitored formation of acrolein, which is the expected product of the oxidative cleavage reaction of the vinyl boronate in **1** and subsequent elimination of glucose (Fig. 2c). As expected, time-dependent ^{1}H NMR spectra (Fig. 5b) clearly reveal peaks for acrolein that grow in intensity when a solution of reagent **1** (17 mM in deuterated water) was exposed to hydrogen peroxide (170 mM, 10 equiv).

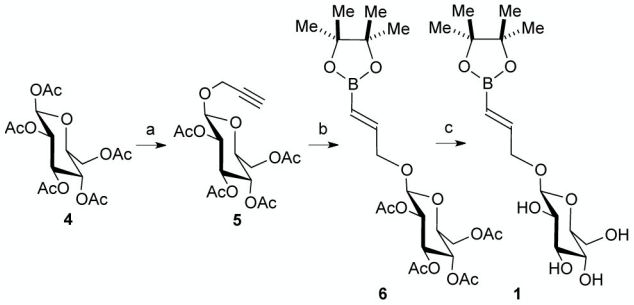

Fig. 4 Synthesis of amplification reagent **1**. Conditions: a, propargyl alcohol, $BF_3{\cdot}Et_2O$ (89%); b, pinacolborane, 100 °C (37%); c, (i) NaOMe, MeOH, CH_2Cl_2; (ii) Dowex H^+ (80%).

Testing the colorimetric readout for the assay

Since reagent **1** is capable of releasing glucose in response to hydrogen peroxide, we proceeded to develop the proof-of-concept signal amplification assay depicted in Fig. 2a, with the primary goal of evaluating whether the vinyl boronate in **1** is effective at minimizing background reaction. This proof-of-concept assay relies on the amplified acid (D-(+)-gluconic acid) to increase the acidity of the assay medium, where the change in acidity is revealed by conversion of yellow **2** to pink **3** (Fig. 2b).

The selection of pH indicator is important in this context for maximizing the sensitivity of the assay. In our proof-of-concept demonstration, we chose methyl red (**2**), which provides a yellow solution at pH values greater than 6.2, but switches to pink below pH 4.2. The increased concentration of D-(+)-gluconic acid δ-lactone during the amplification reaction with **1** will hydrolyze readily to D-(+)-gluconic acid, thus decreasing the pH of the assay medium and causing a change in color of the solution from yellow to pink. We verified this predicted change in color by exposing **2** (0.05 mM in distilled water) to increasing concentrations of D-(+)-gluconic acid δ-lactone and by tracking the red-shifting of the wavelength of maximum absorption in UV/vis spectra (Fig. 6).

Testing the signal amplification reaction using reagent 1

After demonstrating that **1** and **2** function as designed, we next combined them (2 mM **1**, 0.05 mM **2** in distilled water) with glucose oxidase (50 U mL^{-1}) and, in separate assays, exposed the solutions to various quantities of glucose (0.1–2 mM), which in practice would arise from a specific detection event (Fig. 2a). Measurements of absorbance (525 nm) over time revealed the ability of the system to provide an amplified colorimetric response, as expected (Fig. 7a). A key diagnostic indicator of a successful signal amplification reaction is the observation that substoichiometric quantities of glucose relative to **1** provide amplified colorimetric readouts of equal intensity to when 1 equiv of glucose was added to the assay solution, which is the result that we observed in this system.

Figure 7a also reveals the presence of background signal that was amplified, thus reducing the potential for the system to enable trace level detection of a target analyte. Since we designed reagent **1** to be stable to hydrolytic cleavage of glucose, we hypothesized that the background reaction might have originated from trace levels of glucose contamination in reagent **1**, although we were unable to detect such contamination by ^{1}H NMR or through use of the glucometer.

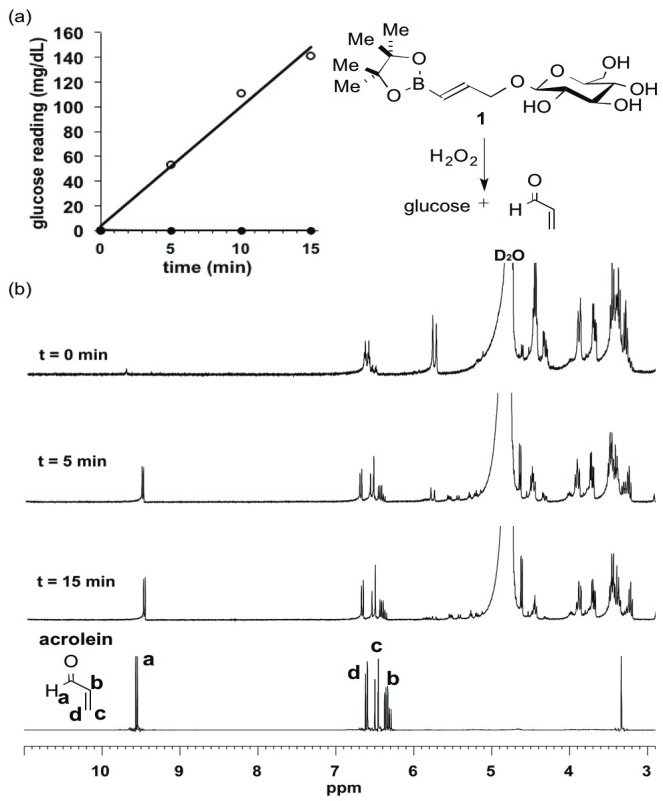

Fig. 5 Demonstration that **1** is capable of releasing glucose in response to hydrogen peroxide. (a) Time-dependent glucometer measurements of glucose concentration when **1** (5 mM) is treated with either 0 equiv or 5 equiv of hydrogen peroxide in water. The open circles represent 5 equiv of hydrogen peroxide and the closed circles depict the results for 0 equiv of hydrogen peroxide. (b) Conversion of reagent **1** (17 mM) to acrolein and glucose when **1** was exposed to 10 equiv of hydrogen peroxide in deuterated water (D_2O). The partial ^{1}H-NMR spectra show the time-dependent production of acrolein, as expected from the reaction scheme depicted in (a).

Thus, to test this hypothesis, we treated **1** with magnetic beads bearing glucose oxidase and catalase to remove glucose and hydrogen peroxide, respectively. The bead-bound enzymes were removed by magnetic filtration, and any unbound enzyme (if present) was removed by microcentrifugation. Purified reagent **1** was again used in signal amplification reactions (Fig. 7b). Now, in the absence of glucose, less than 10% background signal was observed after 15 h of incubation as opposed to ~75% background signal at 15 h for the original sample of **1**. The use of both enzymes proved necessary to achieve this minimal level of background signal. Thus, these results support our hypothesis that trace quantities of glucose were present in the synthetic sample. As might be expected, removing trace quantities of glucose from reagent **1** also slowed the rate of the amplification reaction. This result suggests that purposeful standard addition of fixed quantities of glucose into an assay medium will enable faster amplification reactions, if desired, but at the expense of increased background signal as well. Moreover, a standard addition strategy should enable assays from trace levels to abundant analytes.

Ultimately, the results in Fig. 7b demonstrate that allylic ethers are effective connector units for mediating the controlled release of glucose with minimal background reaction. Thus, allylic ethers should be considered a useful motif when designing small molecule-based signal amplification reagents.

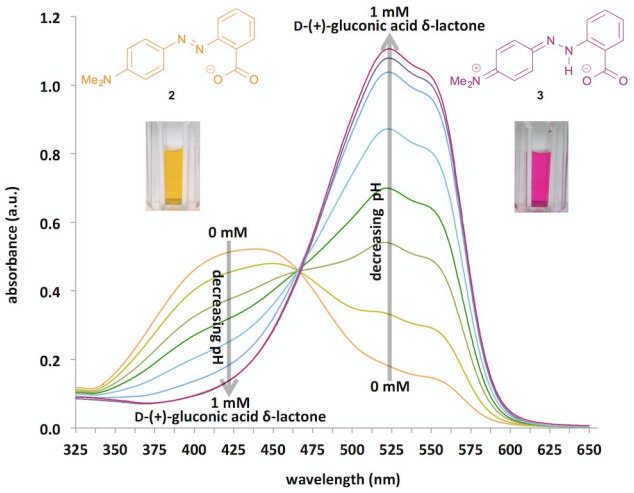

Fig. 6 Stacked UV/vis spectra (25 °C) of methyl red solutions (0.05 mM **2** in water) that were exposed to different concentrations of D-(+)-gluconic acid δ-lactone (0–1 mM).

Experimental

General materials and methods

Reagent **1** was prepared according to a known procedure.[34, 35] Photographs to illustrate the change in color during the assays were acquired using a Nikon digital camera (D40, D3100). Proton nuclear magnetic resonance (^{1}H-NMR) spectra and carbon nuclear magnetic resonance spectra (^{13}C-NMR) were recorded using a Bruker DRX-400 (400 MHz), Bruker CDPX-300 (300 MHz), Bruker DPX-300 (300 MHz) or AV-360 (360 MHz) at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to chloroform ($CDCl_3$, 7.26 ppm), or deuterated water (D_2O, 4.27 ppm). UV/vis spectroscopic data was obtained using a Beckman Coulter DU 800 spectrometer.

Measurements of released glucose

In a 2 mL vial was added 10 μL of a solution of reagent **1** (20 mM in distilled water), 10 μL of hydrogen peroxide (0.1 M), and 20 μL of distilled water. Final concentrations of reagents were: 5 mM of reagent **1** and 25 mM hydrogen peroxide (5 equiv). The release of glucose was measured using a glucometer (CVS TRUEtrack™).

^{1}H NMR experiment for monitoring the release of glucose

A solution of hydrogen peroxide (0.17 M, 10 equiv) was added to a solution of reagent **1** in deuterated water (17 mM). The combined solution was vortexed for 60 s and ^{1}H-NMR spectra were acquired at various intervals.

Titration of 2 with D-(+)-gluconic acid δ-lactone

Stock solutions of methyl red sodium salt (**2**, 0.015 mg mL^{-1}, 0.05 mM) and D-(+)-gluconic acid δ-lactone (0–1 mM) in deionized water were equilibrated for 30 min. To a 1.5 mL plastic cuvette was added an aliquot of the methyl red solution (315 μL) and an aliquot (185 μL) of the D-(+)-gluconic acid δ-lactone solution. The new solution was vortexed for 30 s and equilibrated at room temperature for 15 min before acquiring UV/vis spectra at 25 °C.

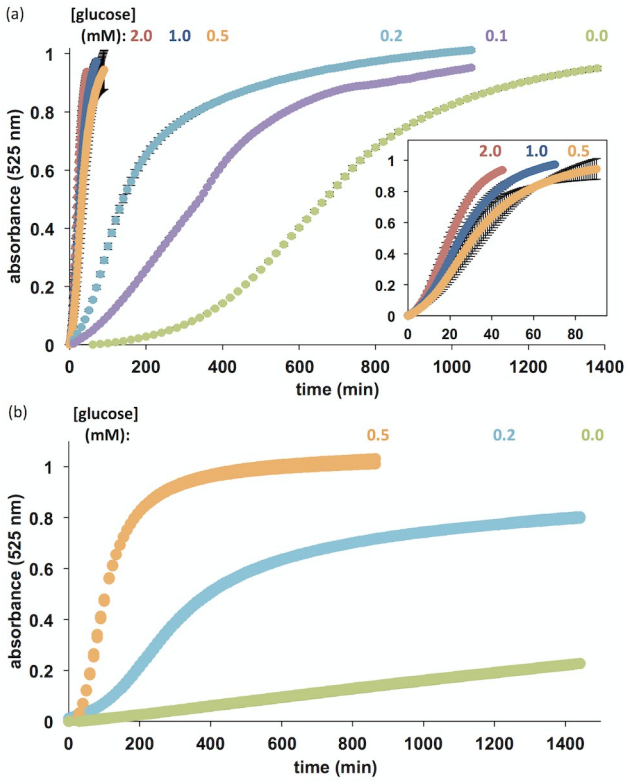

Fig. 7 (a) Measurements of the amplification kinetics for reagent **1** using UV/vis spectroscopy. The assay contained methyl red sodium salt (**2**, 0.015 mg mL^{-1}, 0.05 mM), amplification reagent **1** (2 mM), glucose oxidase (50 U mL^{-1}), and various concentrations of glucose (0.1–2 mM) in water. The progress of the signal amplification reaction was monitored at 525 nm at 25 °C. (b) Nearly identical measurements to (a), but the results in (b) were obtained after **1** was pre-treated with bead-bound glucose oxidase and catalase. The data points in (a) are the average of three measurements and the error bars (which often are smaller than the data points) represent the standard deviations of these averages. Duplicate experiments were performed in (b), therefore all of the data are plotted.

UV/vis measurements of the amplified response

We first prepared separate stock solutions of reagent **1** (20 mM), glucose oxidase (GOX) from *Asperigillus niger* (200 U/mL), and glucose (10 mM and 100 mM) in deionized water. To a 1.5 mL plastic cuvette was added a solution of methyl red sodium salt (**2**, 315 µL, 0.015 mg mL^{-1}, 0.05 mM), reagent **1** (50 µL, 2 mM), and GOX (125 µL, 50 U mL^{-1}). The solution was vortexed for 30 s and a solution of glucose (0.1–2 mM, 0.05–1 equivalents relative to reagent **1**) was added (the final volume of the solution was 500 µL). The new solution was vortexed for 30 s and the reaction progress was monitored using UV/vis spectroscopy at 525 nm at 25 °C.

The following procedure was used to remove glucose contamination from reagent **1**: In a 20 mL scintillation vial, 500 µL of reagent **1** in deionized water (20 mM) was treated with magnetic bead-bound GOX (75 µL, 0.25% w/v) and magnetic bead-bound catalase (100 µL, 0.25% w/v).[36] The mixture was stirred for 30 min, and the solution of reagent **1** was collected after a magnet was placed under the vial to remove the magnetic beads. The resulting solution was microcentrifuged using a 100 kDa filter for 10 min to remove traces of enzymes. The solution of **1** was used immediately for the UV/vis kinetics experiments.

Conclusions

The design of signal amplification reactions for use in point-of-need diagnostics is a topic of immense interest,[1–14, 16–30] but also of significant hurtles, particularly when operational simplicity for an assay is placed at a premium. Small molecule-based signal amplification reactions offer one potential route for achieving an amplified readout directly in an operationally straightforward assay. Before implementing these types of signal amplification reagents, however, the design criteria must be further clarified. Herein we demonstrate allylic ethers as a new motif for enabling triggered release of glucose with only minimal background reaction, which is an important step forward towards effective signal amplification assays. This simple design provides a colorimetric readout that we monitored using UV/vis spectroscopy, but in principle, the color could be quantified using camera-equipped cell phones and image processing software as well.[37–41] Looking forward, additional design elements must be discovered before practical signal amplification assays will be available. An important next step in this context involves developing strategies to accelerate the amplification reactions while maintaining minimal background reaction. Success in these efforts should make small molecule-based signal amplification a viable contender for achieving simple, yet sensitive *in situ* signal amplification in point-of-need assays.

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Notes and references

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Electronic Supplementary Information (ESI) available: synthetic procedures for **1** and copies of ^{1}H NMR spectra. See DOI: 10.1039/b000000x/

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