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Reagents and assay strategies for quantifying active enzyme analytes using a personal glucose meter†

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This communication describes small molecule reagents and a rapid single-step assay for quantifying nanomolar levels of active enzyme analytes using a personal glucose meter.

A wide range of qualitative colorimetric activity-based assays have been developed over the years for detecting enzymes and other analytes *via* specific reactions between a substrate and the analyte.[1–3] Quantifying the colorimetric output, however, has proven challenging, particularly in point-of-care (POC) settings where few resources are available for conducting the assay. Specialized hand-held readers that enable quantification based on reflectance,[4] transmittance,[5] and absorbance[6] have been developed for this purpose, but they are produced at low volume, are expensive for many POC applications, and suffer from interference from background color and particulates that may be in a sample. Quantification using a camera-equipped cellular phone offers a lower cost alternative to specialized readers, but with added concerns associated with lighting conditions, focus, and differences between cameras that may affect the quantitative interpretation of an assay.[7–9] Consequently, the need remains for a simple and inexpensive approach for quantifying activity-based assays without relying on measurements of color, while still building on years of development in activity-based enzyme assays.[3] Herein, we describe such a strategy using a personal glucose meter.[10–18]

To enable the use of a personal glucose meter for activity-based assays, we developed reagents that release glucose when a target enzyme analyte is detected. Our single-step assay is illustrated in Fig. 1. When the target enzyme is present in a sample, it reacts with one of the reagents to release glucose, which is then quantified using a personal glucose meter. This assay requires only that the user deposit a test fluid into a pre-loaded tube containing a small molecule assay reagent, wait a fixed period of time, and then measure the quantity of glucose in the sample.[19] Thus, this strategy offers a combination of ease of operation and quantification that may be particularly useful in environments that lack refrigeration, electricity, and other resources, such as remote villages in the developing world.[20–23]

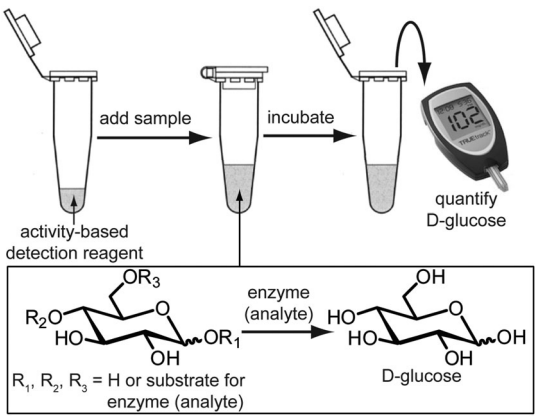

Fig. 1 A one-step assay strategy for quantifying enzyme analytes *via* their enzymatic activity in combination with a personal glucose meter.

The selectivity in the assay originates from the functionality on the small molecule reagent, which can be tuned easily for specific activity-based detection events.[3,24] Fig. 2 shows four general controlled release reagents that we prepared to illustrate four methods for releasing glucose in response to a target enzyme. These four reagents are not optimized for a specific target enzyme, but rather provide platforms on which four general classes of enzymes can be detected in this type of glucose meter-mediated assay. These classes include glycosidases (reagent **1a**), esterases (**1b**), phosphatases (**1c**), and proteases (**1d**). As demonstrated by the structures in Fig. 2, different classes of enzyme targets require different strategies for releasing glucose.

To target a specific enzyme within one of these classes, the substrate (blue portion in Fig. 2) that is covalently linked to glucose or the controlled release reagent (depending on the release strategy)

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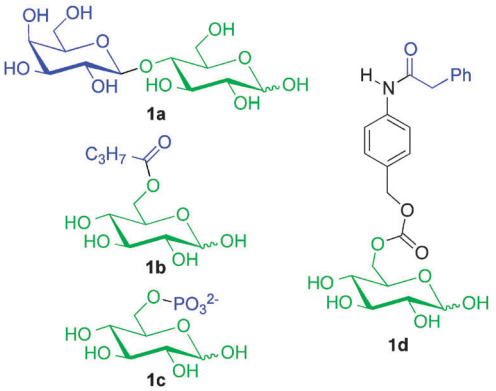

Fig. 2 Structures of general activity-based controlled release reagents designed for assays that detect four classes of enzymes by releasing glucose. The classes are: glycosidases (**1a**), esterases (**1b**), phosphatases (**1c**), and proteases (**1d**). The green color-coding denotes glucose that will be released upon reaction of the reagent with the target enzyme, and blue represents the substrate for the enzyme. Selective assays should be possible by modifying the substrate (the blue portion of the general reagent) for the target enzyme.[3]

could be changed to match the substrate preference for the desired enzyme analyte.[3] For example, in the case of **1d** (which is used for detecting proteases; penicillin-G-amidase is the model protease in this study), the substrate for penicillin-G-amidase is connected to glucose through a self-immolative linker, which is necessary to ensure that cleavage of the C-terminus of the amide in **1d** can be translated into release of glucose without competing with non-specific background release in the time frame of the assay. Once penicillin-G-amidase cleaves the C-terminus of the peptide bond, the resulting aniline proceeds through azaquinone methide to release carbon dioxide and glucose. In the absence of the enzyme analyte, glucose oxidase (in the commercial strips that accompany personal glucose meters) is not able to process the glucose bound to the small molecule detection reagent, thus avoiding a possible source of background signal.

Detection reagent **1d** was accessed through a convergent route that involved preparing the peptide substrate and the glucose components separately, and then bringing them together through a carbonate linkage (Scheme S1, ESI†). This convergent approach offers the opportunity to modify the peptide substrate as needed to target a desired protease.

Treatment of **1d** with penicillin-G-amidase (0.5 U mL^{-1}) in 0.1 M phosphate buffer (pH 7.5, 0.5% (v/v) Tween 20, 20 °C) led to nearly instantaneous consumption of **1d** (see the stacked LCMS traces in Fig. S1, ESI†) and formation of glucose, which was measured using a personal glucose meter. In the absence of penicillin-G-amidase, **1d** decomposed only 2% after exposure to the assay solution for 1 h, and 7.5% after 5 h of exposure[25] (Fig. S1, ESI†), the latter duration being 5× longer than is needed for conducting the first-generation assay (Fig. S2, ESI†). Since reagent **1d** would be stored and supplied dry before conducting an assay, we tested its stability in this context as well. Heating the dry reagent at 40 °C open to the air caused no detectable decomposition after 24 h, and only 6% decomposition after heating for 7 days (Fig. S3, ESI†), thus illustrating its stability for use in certain types of point-of-care environments.

Exposure of **1d** to various quantities of penicillin-G-amidase provided a reproducible dose-dependent response (Fig. S2, ESI†) that provides a useful calibration curve for quantifying penicillin-G-amidase in a sample. Similar calibration curves were obtained for β-D-galactosidase (Fig. S4, ESI†), esterase (Fig. S5, ESI†), and alkaline phosphatase (Fig. S6, ESI†) using their respective small molecule assay reagents (Fig. 2). The sensitivity provided by these initial assays is comparable to colorimetric assays,[26] but with the benefit that the color of the sample does not interfere with the readout provided by the personal glucose meter (as it would for a colorimetric assay). A case in point (which is highlighted in Fig. S6, ESI†) is alkaline phosphatase.[26] At normal levels, alkaline phosphatase is present in blood between 30–120 U L^{-1}, whereas the concentration is greater than 120 U L^{-1} in patients with impaired liver function.[1] The sensitivity of the assay shown in Fig. S6 (ESI†) is sufficient for differentiating individuals with normal and elevated levels of alkaline phosphatase. Likewise, reagent **1c** (which is used for detecting alkaline phosphatase) is similar in structure to the standard colorimetric reagent (*p*-nitrophenol phosphate[27]) that is used for clinical analyses, and thus can be expected to display a similar level of selectivity for alkaline phosphatase.

Given these promising results, we reasoned that shorter assay times would further improve the convenience of the assays for point-of-care applications. Lu,[10–13] Xiang,[15–17] and Yang[18] employed a signal amplification step in their antibody, aptamer, and nucleic acid-based assays to increase signal and ultimately reduce assay times when using a personal glucose meter, but here we simply mix 62 μg of glucose (for a 200 μL assay) with the desired detection reagent prior to adding the sample that contains the desired analyte. In other words, we use pre-loaded assay tubes that contain enough glucose to provide a low-level reading on the glucose meter. The presence of the target enzyme then simply elevates this reading within the detection window of the glucose meter. The consequence of this standard addition approach is 2–12× shorter assays times (Fig. S7–S9, ESI†) compared with our first-generation assays (Fig. S2, S4–S6, ESI†), since time is not wasted waiting for the enzymatic detection event to accumulate levels of glucose (*i.e.*, millimolar levels of glucose) to enable a reading using the glucose meter.

Fig. S7–S9 (ESI†) show three calibration curves generated using this second generation approach. The calibration curves are for penicillin-G-amidase (using **1d**) (Fig. S7, ESI†), β-D-galactosidase (using **1a**) (Fig. S8, ESI†), and alkaline phosphatase (using **1c**) (Fig. S9, ESI†). For a 30 min assay, the limit-of-detection for penicillin-G-amidase is 0.01 U mL^{-1}, and for a 15 min assay, the limit-of-detection for β-D-galactosidase is 4 U mL^{-1} (38 nM). This latter level of sensitivity compares favorably with previous personal glucose meter-based assays that employ signal amplification reactions as part of the assay.[10,11] In our enzymatic assays, however, a secondary signal amplification step is unnecessary, and the assay times remain short (minutes). The dynamic range for these standard addition assays also is noteworthy. In the case of a 5 min alkaline phosphatase assay (Fig. S9, ESI†), the dynamic range is 1.5 U L^{-1} to 160 U L^{-1} (1.3 nM to 140 nM), with a limit of detection of 1.5 U L^{-1} (1.3 nM).

This range is within the clinically-relevant window for the enzyme analyte.[1]

While this approach of providing a base-line quantity of glucose to the assay improves the sensitivity and dynamic range of the assay, it also could increase the prevalence of undesired background signal arising from either decomposition of an assay reagent (*e.g.*, **1d**) or from glucose that may be present in a sample. As mentioned previously, **1d** decomposes ~6% when heated at 40 °C open to the air for 7 days. In the context of an assay for 0.06 U mL^{-1} penicillin-G-amidase, however, reagent **1d** that was heated at 40 °C for 7 days provided nearly equal assay results (using the personal glucose meter and the calibration curve in Fig. S7, ESI†) as a comparison assay using freshly-prepared reagent **1d**: *e.g.*, 0.05 ± 0.00 U mL^{-1} penicillin-G-amidase for the sample heated to 40 °C for 7 days *vs.* 0.07 ± 0.01 U mL^{-1} for freshly-prepared **1d**. Assuming that the 6% of decomposed **1d** releases glucose, we would expect a meter reading that is ~5% higher than the reading when **1d** is not decomposed. The accuracy of the personal glucose meter, however, is within 20% of standard laboratory assays, therefore this small level of decomposition of **1d** is not detectable within the sensitivity limits of the meter.

We next tested whether the assays could be conducted in a complicated fluid such as serum. Fig. S10 (ESI†) reveals that a 5 min assay for alkaline phosphatase (using **1c**) enables a dynamic range of 2 U L^{-1} to 160 U L^{-1} (2 nM to 140 nM), with a limit of detection of 2 U L^{-1} (2 nM), which is a range and level of sensitivity that is nearly identical to the calibration curve generated in buffer (Fig. S9, ESI†). We also purposely added glucose to the serum to simulate samples that contain different initial quantities of glucose. In each of these tests, the final assay solution contained 20 U L^{-1} alkaline phosphatase and either 0 mM, 6.6 mM, or 3.3 mM of added glucose (in addition to the glucose that was present already in serum). By conducting two assays on each sample (one with and one without reagent **1c**), we were able to account for glucose in a sample and still enable quantitative assays using the calibration curve in Fig. S10 (see Table S10, ESI†).

In conclusion, this communication describes a convenient assay for measuring trace levels of enzyme analytes. The assays are reproducible and easily implemented, rapid, operate in complex fluids such as serum, and use small molecule reagents, thus making them amenable for use in point-of-care settings that lack refrigeration. The assays also enable measurements of active enzymes rather than simply the total level of a target enzyme antigen, regardless of whether it is active or not. This type of differentiation should prove useful in the context of liver function tests, quantifying enzymes associated with the presence of an infectious disease, and for a variety of other applications where a rapid, quantitative point-of-care test will be valuable. With the advent of cell-phone-based personal glucose meters,[28] this new assay strategy may offer a powerful alternative to colorimetric activity-based assays that are quantified using cell phone cameras and telemedicine. Future efforts will build on these proof-of-concept studies to develop thorough and specific analytical assays for target analytes by modifying the general controlled release reagents so that they are selective for a desired enzyme analyte.

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