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New Trimodal Phenotypic Reporter of Extended-Spectrum β-Lactamase Activity

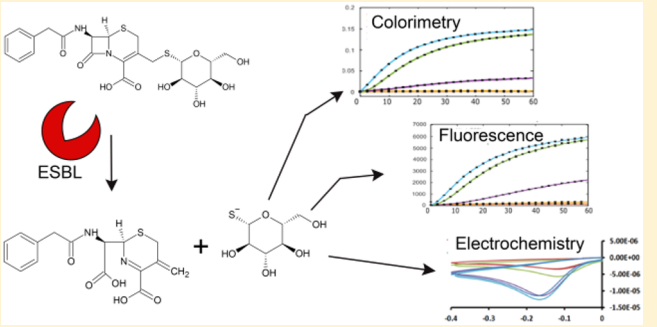
Hieu T. Nguyen,[†] Shweta Ganapati,[‡] David Watts,[‡] Imaly A. Nanayakkara,[†] Philip DeShong,[‡] and Ian M. White*[,†]

[†]Fischell Department of Bioengineering, University of Maryland, 8278 Paint Branch Drive, College Park, Maryland 20742, United States

[‡]Department of Chemistry and Biochemistry, University of Maryland, 8051 Regents Drive, College Park, Maryland 20742, United States

Supporting Information

ABSTRACT: Bacterial resistance to β-lactam antibiotics continues to grow as misadministration presents evolutionary pressure that drives bacteria to develop improved resistance enzymes. Known as extended-spectrum β-lactamases (ESBLs), these enzymes are capable of hydrolyzing advanced β-lactam antibiotics such as third-generation (and higher) cephalosporins. Phenotypic detection substrates can be used to rapidly identify a cultured patient sample prior to confirmation by more exhaustive but slower means, critically aiding in the antibiotic stewardship essential in maintaining the effectiveness of not only the cephalosporins but also indirectly the carbapenems, our last-resort β-lactams. To enhance the phenotypic detection arsenal, we have designed an ESBL detection substrate that releases a glucose molecule upon β-lactamase hydrolysis. Because many forms of detection for glucose exist, the substrate enables ESBL quantification via three modalities commonly found in the clinical laboratory: optical absorbance, for use with the most common microbiology platforms; fluorescence, for enhanced sensitivity; and electrochemistry, which offers the potential for integration into a hand-held platform similar to a personal glucometer. Moreover, we demonstrate that, as opposed to currently available phenotypic detection substrates, our new substrate is engineered to be resistant to older and narrower β-lactamases, thus enabling specific identification of newer and more dangerous ESBLs.



KEYWORDS: *ESBL, multidrug resistance, cephalosporin, biosensor*

Antimicrobial resistance (AMR) to β-lactam compounds poses a critical and mounting challenge in the treatment of community and hospital-acquired infections. The Center for Disease Control (CDC) listed extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* as one of the biggest threats in its 2013 Antimicrobial Resistance Threats Report.[1,2] Though enumerated under "serious threats", ESBL pathogens are direct evolutionary forerunners of carbapenem-resistant *Enterobacteriaceae*, declared as an "urgent threat" to public health. The etiology of resistant cases lies largely in Gram-negative *Enterobacteriaceae*, which produce and disseminate plasmid-encoded enzymes capable of hydrolyzing β-lactam antibiotics that range from older (*-cillins*) to more advanced (*cephalosporins, carbapenems*). Incorrect antibiotic treatment not only leads to poor patient outcomes but also places selective pressure on the pathogen, driving AMR advancement. Therefore, timely and accurate testing for antibiotic resistance is crucial for good patient outcomes and safeguarding public health. As recommended by the Clinical and Laboratory Standards Institute, the current standard in antimicrobial susceptibility testing relies on bacterial culture (disk diffusion susceptibility test, Etest, broth dilution).[3] Genotypic tests to identify ESBL resistance utilize polymerase chain reaction followed by sequencing and are thus not appropriate for rapid diagnostics in the clinic at this point. Furthermore, as these methods are designed to detect single nucleotide polymorphisms (SNPs), they have been limited to the TEM and SHV families of ESBLs, and only include a small handful of CTX-M variants.[4] Though TEM and SHV evolutionary expansion via SNPs represents a large portion of enzyme mutations that are conducive to resistance,[5] sequencing efforts are far from fully encompassing the widening scope of this rapidly evolving gene.

Rapid phenotypic tests including acidimetric and iodometric exist as convenient, quick indicators of β-lactamase presence by way of color change readouts. The acidimetric test relies on the generation of a carboxylic acid from the hydrolyzed β-lactam, which acidifies unbuffered systems, and the resultant pH

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change can be measured using aqueous phenol red in a tube or bromocresol purple in the form of strips.[6] The iodometric test is limited to the detection of penicillinase activity, as it relies on the production of penicilloic acid to reduce iodine, which changes color when in complex with starch.[7] Though rapid, these tests are susceptible to producing false positives as reduction of iodine and solution acidification may occur nonspecifically;[8] furthermore, the β-lactamase enzymes detected by these methods do not encompass the broad- or extended-spectrum genetic permutations.[8]

As more refined specificity is required to categorically identify a pathogen as ESBL-positive and differentiate from penicillinase-only producers, several β-lactam derived chromogenic compounds (Nitrocefin, pyridine 2 azo *p* dimethylaniline cephalosporin (PADAC), CENTA) have previously been described as "reporters" or "sensors" of the resistance phenotype to better serve as effective detection substrates in the study of ESBL activity.[9,10] Nitrocefin has been established as a clinical laboratory test and is rapidly hydrolyzed by a range of β-lactamases upon incubation with bacterial isolates from overnight culture. In the presence of β-lactamase, the amide bond of the β-lactam ring in Nitrocefin is hydrolyzed, resulting in a shift of the absorbance from 390 to 486 nm (neutral pH), which can be measured spectrophotometrically. In recommended protocols, access of the detection substrate to β-lactamase is further enhanced by CFU concentration via centrifugation and by cell wall disruption via sonication.[11] However, Nitrocefin is cleavable by most β-lactamases and therefore does not distinguish a given isolate explicitly as an ESBL producer (the same is true for CENTA as well).[9]

As fluorescence measurement is generally accepted to have higher sensitivities than chromogens, which rely on absorbance spectrophotometry, development of fluorogenic ESBL substrates has expanded the phenotypic detection repertoire with the promise of lower limits of detection.[12−17] They include substrates that have been designed to release well-characterized coumarins upon specific cleavage by advanced ESBLs including metallo-β-lactamases (MBLs), an improvement on substrates unable to distinguish β-lactamases with extended-spectrum profiles from narrow-spectrum β-lactamases.[18] Yet, as previously described methodologies require the specialization of a central microbiology lab facility, resistance testing away from equipped central laboratories, such as in disadvantaged regions, remains an underserved goal. Electrochemical readouts of a redox-active reporter have the potential to meet this paradigm, as demonstrated by the hand-held personal glucose meter (PGM). Amperometric measurements of hydrolyzed Nitrocefin have also been reported in the literature, with the aim toward rapid detection of ESBL activity using disposable screen-printed sensors with small sample volumes to enable robust, field-portable instrumentation.[19]

The description of multiple methods for resistance readout is promising as a multipronged approach to AMR detection, yet each of these testing methods requires specific substrates, with each their own optimal readout modality. A single ESBL substrate possessing the versatility to enable different aforementioned readout techniques would democratize AMR testing access, as standard laboratories tend to have equipment for optical absorbance measurement, and more specialized, central laboratories possess fluorescence enabled equipment for sensitive detection. Furthermore, though amperometric AMR detection is uncommon, establishing this methodology holds great promise toward the development of hand-held, portable detection modules for use outside of equipped central lab facilities.

Toward the accommodation of a wide scope of previously described detection methodologies, we have developed a new cephalosporin-derived detection substrate, Cep-glucose, that is capable of multimodal detection by colorimetric, fluorescence, and electrochemical means. Key to this multimodal detection is the use of thio-glucose release as the detection method. Once liberated from the substrate, the reactive thiol moiety of this reporter molecule allows for the conceptualization of any thiol-sensitive chemistries into downstream detection. Further, as thio-glucose is a substrate for glucose oxidase (GOx), established electrochemical detection methods allow for the easy reimagination of PGMs into ESBL quantifying detection devices. These important features are further enhanced by the promise of lowered production costs as compared to commercially available substrates, thanks to the ease of synthesis through straightforward thiol chemistry for alkylation and production of Cep-glucose, making it potentially commercializable as a low-cost substrate. Here we demonstrate the use of this new substrate for the phenotypic detection of ESBL activity with colorimetric, fluorescence, and electrochemical readout.

EXPERIMENTAL SECTION

Reagents and Solutions. Phosphate buffer (PB; 0.1 M; pH = 6.5) and all solutions were prepared with Milli-Q 18-MΩ water (Millipore purification system) and pH-adjusted with HCl. 4-Methoxybenzyl-3-chloromethyl-7-(2-phenylacetamido)-3-cephem-4-carboxylate was purchased from TCI America. 1-Thio-β-D-glucose was purchased from Cayman Chemical Company. Stock solutions of 50 mM Cep-glucose were prepared by dissolving the purified product in dimethyl sulfoxide (DMSO), and aliquots were stored at −20 °C. Working dilutions were prepared by dissolving the stock solution in PB. TEM-1 β-lactamase enzyme was purchased from ThermoFisher Scientific. Extended-spectrum β-lactamase (ESBL) was purchased from AG Scientific (β-lactamase-BS) and was tested to show hydrolytic activity against a broad range of β-lactam compounds including third, fourth, and fifth generation cephalosporins as well as carbapenems. Because of its reliable cleavage as determined by our enzyme kinetic characterization studies (Supporting Information, Figure S12), this ESBL "blend" was used as a standard in this study, as specified ESBLs are not commercially available. 5,5′-Dithiobis-[2-nitrobenzoic acid] (DTNB, Ellman's reagent) was purchased from Sigma-Aldrich, and a 100 mM solution was always freshly prepared in Milli-Q water and diluted into PB to a final working concentration of 250 μM. Amplex Red was purchased from Molecular Probes (Eugene, OR). Ceftazidime hydrate was purchased from Millipore Sigma and was freshly prepared in water from powder stock and filter sterilized prior to addition to culture broth.

New ESBL Detection Substrate. Briefly, Cep-glucose was prepared as follows: 4-methoxybenzyl-3-chloromethyl-7-(2-phenylacetamido)-3-cephem-4-caboxylate (PMB-Cep-Cl) was dissolved in acetone, and 1-thio-β-D-glucose was added to the reaction and allowed to stir at reflux for 4 h. After solvent removal *in vacuo*, the aqueous layer was extracted with dichloromethane to yield the *para*-methoxybenzyl-protected Cep-glucose. Deprotection was performed using standard trifluoroacetic acid deprotection procedures to yield the Cep-glucose detection substrate. Full NMR characterization of the

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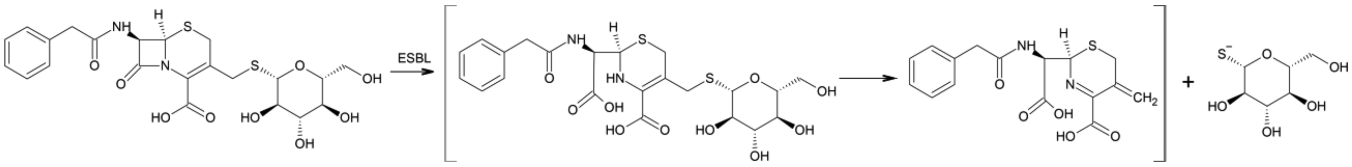
Scheme 1. ESBL Rapidly Cleaves β-Lactam Ring of Cep-Glucose Leading to Intermediate That Retains Thio-Glucose[a]



[a]Thio-glucose is subsequently released in a separate, rate-determining step.

Cep-glucose is presented in the Supporting Information, Figures S1−S10. For ESBL detection experiments, Cep-glucose was prepared from frozen stocks less than six months old. Moderate degradation in performance for longer-term storage and for storage at 4 °C is shown in the Supporting Information (Figure S11).

Apparatus. All electrochemical measurements were carried out on the Model 800B Series Electrochemical Detector (CH Instruments, Inc.) potentiostat interfaced to a PC system equipped with the CHI832B Electrochemical Analyzer version 9.02 software. Zensor TE100 (#ET077−40, EDAQ) screen-printed carbon paste electrodes possessing a Ag/AgCl reference electrode were used for replicates in cyclic voltammetric measurements (CV; scan rate of 100 mV/s), which were performed at 25 °C with a working volume of 100 μL. CV peak heights were determined using eL-Chem Viewer, a freeware package available online (www.lchem.cz/elchemviewer.htm).[20] All spectral measurements (optical density, fluorescence) were performed using the SpectraMax M5 (Molecular Devices) multiplate reader or a Tecan Spark Multimode Microplate Reader.

Colorimetric Assay with Ellman's Reagent. Ellman's reagent reacts with free thiols to form a colored product (absorbance peak at 412 nm) and thus was used to detect the release of thio-glucose upon cleavage of our Cep-glucose substrate. Measurements were carried out at 25 °C in standard 96-well microplates in a 100 μL volume, with each well containing 250 μM Ellman's reagent and 250 μM Cep-glucose substrate in PB (pH = 6.5).

Fluorometric Assay with Amplex Red Reagent. Each 100 μL volume of PB (pH 6.5) contained final concentrations of the following reagents: 10 μM Amplex Red, 0.5 U GOx, 100 μU HRP, and 250 μM Cep-glucose. Just prior to reading, experimental dilutions of ESBL or only PB were added in 10 μL volumes to 90 μL of the previously described solutions. Oxidation of Amplex Red to resorufin within experimental samples was measured at 25 °C over the course of 60 min at 30 s intervals in standard 96-well flat bottom microplates using the kinetic fluorescent read setting on the microplate reader, with excitation at 530 nm and emission measurement at 590 nm.

HRP Enzyme Inhibition Assay. Electrochemical measurements were performed using carbon screen printed electrodes from Zensor (EDAQ). Experiments were performed in a 0.1 M phosphate buffer (pH 6.5) containing 0.5 M KCl to increase the stability of buffer solution against the Ag/AgCl reference electrode. Then 1.25 mM hydroquinone was used as a mediator with 12 μM hydrogen peroxide. Each 100 μL reaction volume contained 1 U HRP, which was added after a 15 min incubation of various dilutions of ESBL with 500 μM of our Cep-glucose substrate and pipetted to mix prior to measurement. CVs were run under a potential range of −0.4 to 0.6 V, a 100 mV/s sweep rate, with a sampling rate of 0.01 V. All samples were run in triplicate using new identical sensors as each replicate. An inhibitor calibration curve with thio-glucose as the inhibitor was produced to relate the current to released thio-glucose upon substrate cleavage following incubation.

Detection of ESBL-Producing *E. coli*. SHV-4 ESBL-producing *E. coli* (strain J53−2 pUD21, *ATCC* product number *BAA-200*) were initially streaked onto tryptic soy agar (Difco) plates containing 10 μg/mL ceftazidime as a selection agent and allowed to incubate overnight at 37 °C to produce single colonies. From this, one colony was selected and allowed to grow overnight (16 h, shaking at 37 °C) in liquid tryptic soy broth (10 mL volume) containing 10 μg/mL ceftazidime. CFU dilutions were prepared directly in PBS from the turbid culture. In a 96-well microplate, various conditions were prepared in 100 μL total volume of 0.1 M PBS (pH = 7.4): bacteria only, bacteria with 250 μM Ellman's reagent, and 10-fold dilutions of bacteria (including a no bacteria control) with 250 μM Cep-glucose detection substrate and 250 μM Ellman's reagent. Samples were incubated in the microplate at 25 °C, and absorbance measurements at 412 nm were taken at 2 and 4 h using a microplate reader.

RESULTS AND DISCUSSION

Synthesis, Verification, and Characterization of Cep-Glucose Detection Substrate. The Cep-glucose molecule is presented in Scheme 1. As the state of research on detection of glucose and hydrogen peroxide is mature and reliable, we employed a thiolated form of glucose, 1-thio-β-D-glucose (thio-glucose), as the signaling molecule that is released upon hydrolysis of the substrate by ESBL. Importantly, as thiol chemistry would allow for a simple, one-step synthesis methodology followed by well-established acid-mediated deprotection protocols, we incorporated the thiolate in S_N2 displacement of the halogenated cephalosporin starting material, PMB-Cep-Cl. The starting material was selected based on prior synthesis methodology by van Berkel et al.,[21] who produced fluorogenic substrates for the detection of metallo-β-lactamases. Within this context, we expect that the starting material, through the addition of specific side chains, can be generalized to producing detection substrates cleavable by ESBLs hydrolyzing later-generation cephalosporins. As compared to Nitrocefin or CENTA, which are cleavable by enzymes that inactivate first-generation cephalosporins, our starting material has a benzyl group where CENTA and Nitrocefin have a thiophene, implying that our Cep-glucose molecule will be more selective. Following synthesis and purification, we verified the structure of our substrate through standard procedural analysis of spectral profiles to confirm the formation of a cephalosporin derivative covalently attached to thio-glucose (Supporting Information Figures S1−S10).

Following synthesis of the new Cep-glucose molecule, we examined the adduct's activity as a biochemical substrate for ESBL. The kinetic characterization of Cep-glucose hydrolysis

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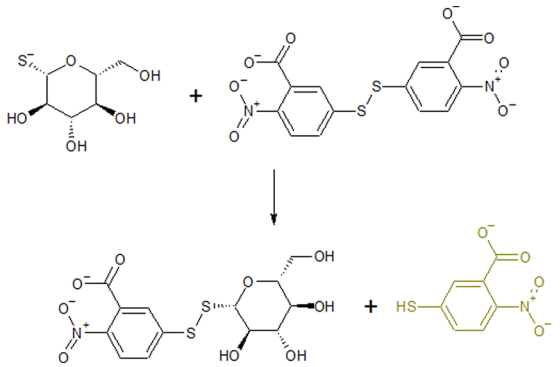
(presented in the Supporting Information, Figure S12) was performed by continuously recording the absorbance variation at 260 nm, as described in previous literature.[22] Using the ESBL blend, we determined that the ESBL and Cep-glucose substrate have a Michaelis constant K_M of 572.65 μM and a catalytic constant k_{cat} of 43.9 s^{-1}, with catalytic efficiency k_{cat}/K_M of approximately 0.077 s^{-1} μM^{-1} (V_{max} = 0.1655 s^{-1}, MW estimated as 35 kDa). This measurement determines only the kinetics of the β-lactam ring opening, not the expulsion of the thio-glucose. While the results presented later in this work demonstrate expulsion of the thio-glucose for signal generation, liquid chromotography-mass spectrometry (LC−MS) results verified the presence of the intermediate, shown in Scheme 1 (Supporting Information, Figures S13−S20). The LC−MS results demonstrate that the stability of this intermediate is the dominant factor in the release kinetics of the Cep-glucose sensing molecule.

Colorimetric Detection of ESBL Enzymes. We designed our detection substrate to enable colorimetric readout via absorbance spectrophotometry to align with standard procedures and equipment found in clinical laboratories. The CENTA substrate directly releases the colored molecule 5-thio-2-nitrobenzoic acid (TNB) upon hydrolytic cleavage; similarly, we employed 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB, also known as "Ellman's reagent"), a symmetric molecule composed of two TNB halves linked by a disulfide bond, in our colorimetric readout scheme (Scheme 2). When

Scheme 2. Ellman's Reagent Participating in Thiol Exchange Reaction, Resulting in Mixed Disulfide and Colored Compound (TNB^{2-})



the β-lactam ring is hydrolyzed, electronic rearrangement allows for the release of the attached thio-glucose, which may then participate in reactions enabling downstream readout modalities. As Cep-glucose releases thio-glucose upon cleavage by ESBL, which readily reacts with DTNB via its thiolate moiety to break the disulfide bond, it produces a mixed disulfide and colored TNB. The corresponding absorbance peak shift due to the resultant TNB can be measured at 412 nm.[9,23]

We first used the colorimetric method to prove the specificity of our Cep-glucose substrate for ESBL enzymes over older, less aggressive β-lactamases. The CENTA substrate, which is cleavable by enzymes that inactivate first-generation cephalosporins, has a thiophene where our starting material has a benzyl group, which requires a larger binding pocket to cleave. Figure 1 compares the hydrolysis of Cep-glucose with 10 mU/mL ESBL enzymes and 100 mU/mL TEM 1 β-lactamase. The colorimetric signals clearly demonstrate that

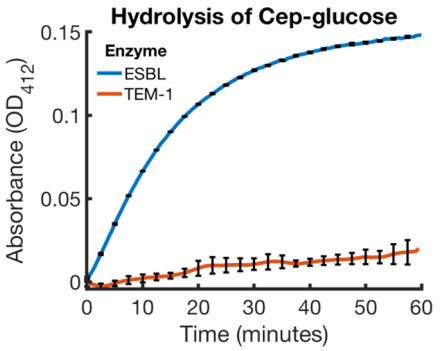


Figure 1. Specificity of the Cep-glucose substrate for ESBL enzymes. As compared to 100 mU/mL TEM-1, 10 mU/mL ESBL much more readily hydrolyzes the Cep-glucose substrate (250 μM). Colorimetric signal visualized using 250 μM DTNB.

ESBL much more readily hydrolyzes the Cep-glucose. In comparison, Figure S21 (Supporting Information) shows that 10 mU/mL ESBL and 100 mU/mL TEM-1 hydrolyze the CENTA substrate somewhat comparably.

We then examined the detection performance of Cep-glucose as a colorimetric ESBL reporter. We incubated 250 μM Cep-glucose and 250 μM DTNB with varying unit concentrations (10 mU/mL to 1 μU/mL) of ESBL in 100 μL of 0.1 M phosphate buffer (pH 6.5 at 25 °C) and monitored the absorbance peak over the course of 60 min (Figure 2). Relying on optical density readout, we were able to easily distinguish a concentration of 100 μU/mL from lower amounts. The limit of detection was calculated to be approximately 19 μU/mL ESBL (approximated to be 70 pM assuming an average enzyme molecular weight of 35 kDa and a

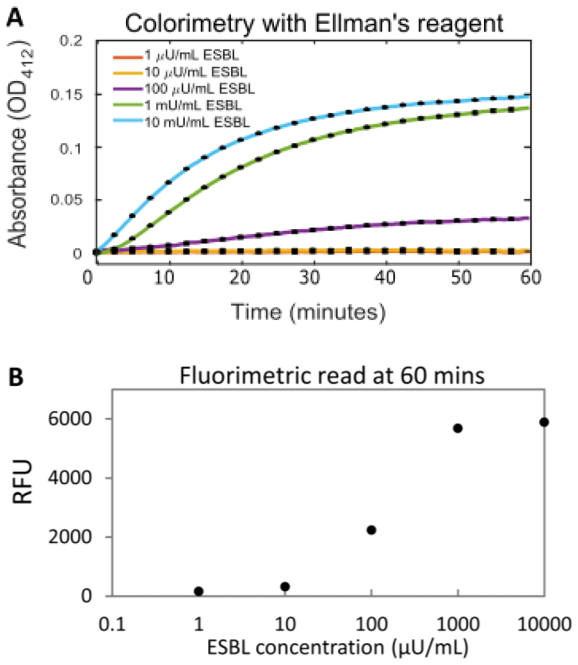


Figure 2. (A) Real-time absorbance change showing thio-glucose release in samples of varying unit concentrations of ESBL containing 250 μM DTNB, 250 μM Cep-glucose substrate in 0.1 M PB (pH = 6.5). (B) Signal change versus ESBL concentration after 60 min of reaction time (all data are background-subtracted; error bars included but not visualizable).

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specific activity of 5.87 U/mg as provided by the vendor), corresponding to three standard deviations above the background signal, or OD_{412} of 0.07.

Additionally, we verified the Cep-glucose colorimetric sensor system with cultured bacteria. Cep-glucose was added to cultured *E. coli* that produces the SHV-4 ESBL enzyme; SHV-4 is known to convey resistance to third generation cephalosporins.[24] As shown in Figure 3, within 2 h of direct

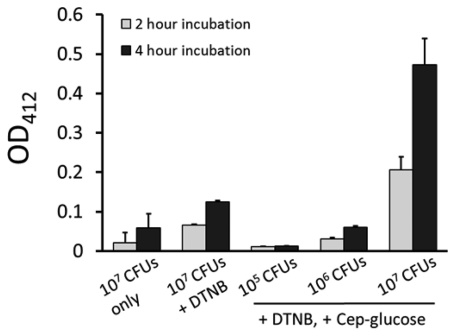
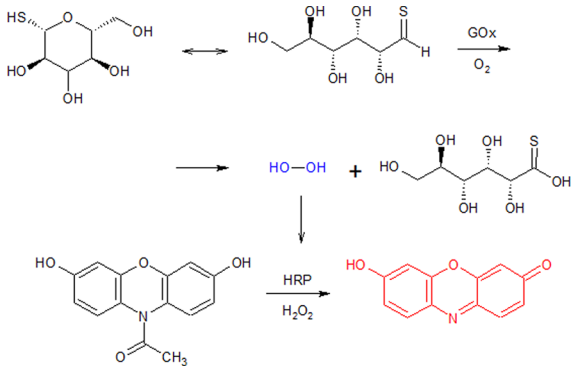


Figure 3. Colorimetric signal change for Cep-glucose with cultured *E. coli* that produces SHV-4 ESBL. As compared to 0 CFU (used as background for subtraction), 10^7 CFU with Cep-glucose and Ellman's reagent generated an easily measurable signal. 10^7 CFU samples with and without Ellman's reagent (no Cep-glucose) were also included as negative controls.

incubation of 10^7 CFU *E. coli* with the Cep-glucose detection substrate and DTNB, a significant increase in absorbance readout (larger than 10 mU/mL ESBL blend, Figure 2) was observed; the signal continued to increase rapidly over the next 2 h as well (data is background subtracted using 0 CFU/mL with Cep-glucose and DTNB). As compared to the blank sample (i.e., no bacteria), minimal signal was observed for the DTNB control, which was included to test for any background chromogenicity (i.e., thiolates arising as metabolic byproducts). Together, these results demonstrate that the Cep-glucose molecule can be utilized in a simple colorimetric assay, that it enables detection of ESBL enzymes, and that it can distinguish between these promiscuous ESBL enzymes and older β-lactamase enzymes.

Fluorometric Detection. With the intent of achieving lower limits of detection, we investigated the incorporation of our Cep-glucose substrate in a biochemical scheme to allow for measurable fluorometric readout. To accomplish this, we utilized thio-glucose's ability to serve as a substrate for glucose oxidase (GOx), producing 1-thio-β-D-gluconic acid and H_2O_2[25] (Scheme 3). For our fluorogenic molecule, we chose *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) as it has been well-characterized as a stable and sensitive reporter of H_2O_2, producing highly fluorescent resorufin (excitation maximum at 563 nm, emission maximum at 587 nm) in an irreversible enzyme-catalyzed oxidative reaction mediated by HRP.[26] Various concentrations of ESBL (1 μU/mL to 10 mU/mL) were incubated with 250 μM Cep-glucose substrate and 10 μM Amplex Red in the presence of 0.5 U GOx and 100 μU HRP in a 100 μL volume of 0.1 M PB (pH 6.5) at 25 °C, and the generation of an emission signal was continuously recorded at 590 nm with excitation at 530 nm over the course of 1 h (Figure 4). With the fluorometric assay, we estimate the limit of detection to be 0.98 μU/mL ESBL, which generates a signal that is larger than three standard deviations above the

Scheme 3. Reaction of Thio-Glucose with GOx, Producing Hydrogen Peroxide, Which Oxidizes Amplex Red to Resorufin in the Presence of HRP



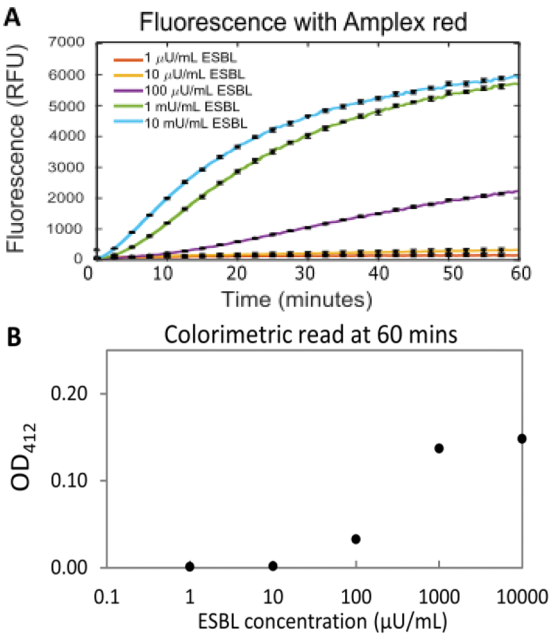


Figure 4. (A) Real-time fluorescence read showing production of hydrogen peroxide in samples of varying unit concentrations of ESBL, incubated with 0.5 U GOx, 1 mU/mL HRP, 10 μM Amplex Red in 0.1 M PB (pH = 6.5). (B) Fluorescence signal change versus ESBL concentration after 60 min of reaction time (all data are background-subtracted; error bars included but not visualizable).

background signal. Though the fluorometric read-out provides higher sensitivity than the colorimetric read-out, one aspect of the assay design prevents the difference from being much greater. HRP, which catalyzes the conversion of Amplex Red to fluorescent resorufin, is inhibited by free thiols.[27]

Electrochemical Detection by Sulfide Inhibition. Previous reporting of ESBL-producing *E. coli* strains through the amperometric detection of Nitrocefin cleavage[19] and methods of nonglucose target detection through engineered glucose generation schemes led us to explore Cep-glucose's potential in expanding ESBL detection beyond the central lab by utilizing electrochemical methods. Though thio-glucose generated from Cep-glucose cleavage can in theory serve as a source of electrochemically active H_2O_2, we found that employing the sulfide detection scheme based on the sulfide inhibition of HRP previously described by Savizi et al. provided an electrochemical method with better performance

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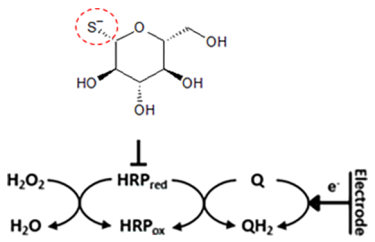
(Scheme 4).[27] This scheme utilizes the inhibitory effect of thiols on HRP to decrease its participation in a redox cycling

Scheme 4. Sulfide Inhibition of HRP by Released Thio-Glucose Reduces Redox Cycling of Hydroquinone Mediator and Electron Transfer to the Electrode[a]



[a]Resultant current decrease allows for amperometric quantification.

scheme that produces current via a hydroquinone (HQ) mediator. We expected that the release of thio-glucose from the cleavage of Cep-glucose by ESBL would provide a source of inhibitory free thiols and sought to demonstrate a dose-dependent current decrease with respect to ESBL. Varying amounts of ESBL were incubated for 15 min under identical conditions and HRP was added just prior to cyclic voltammetry measurement (time points up to 60 min were tested; however, no improvement in detection sensitivity was observed). The depth of the oxidation peak was measured (Figure 5), showing a decrease in oxidation current for

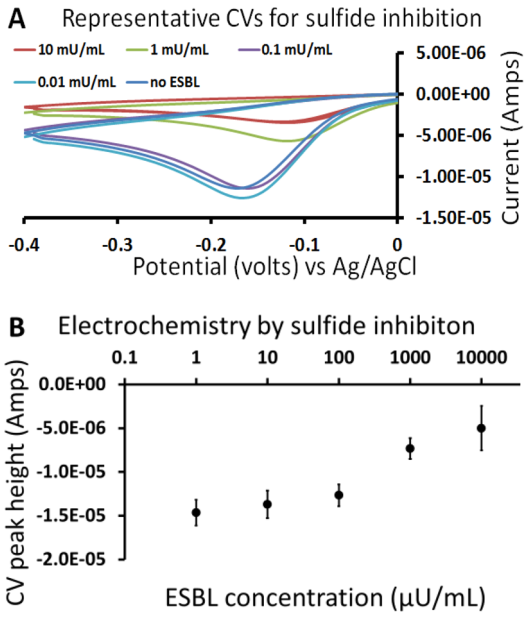


Figure 5. (A) Overlay of cyclic voltammetric traces corresponding to varying concentrations of ESBL recorded on a potentiostat. (B) Plot of current values measured from CV peak heights.

increasing concentrations of ESBL. A limit of detection of approximately 66 μU/mL was calculated (3σ above background). We produced a calibration curve measuring varying known amounts of thio-glucose. Back calculations using this calibration curve (Supporting Information, Figure S22) indicate an estimated release of 147 ± 3.3 μM thio-glucose after incubation of Cep-glucose with 10 mU/mL ESBL.

CONCLUSION

In this study, we have shown that our new detection substrate Cep-glucose can enable three different readout modalities (colorimetric, fluorometric, amperometric) for the detection of varying levels of ESBL enzymes spanning at least three orders of magnitude in concentration. Our colorimetric detection scheme, using Ellman's reagent, allows for use of common absorbance spectrophotometry techniques. Our fluorescence detection scheme using Amplex Red, despite greater assay complexity, provided lower limits of detection for greater sensitivity. Additionally, we engineered an amperometric means of detection to illustrate the possibility of antibiotic resistance testing outside of the central laboratory. In fact, because glucose is generated as a product, we expect that personal glucose meters with at most minor modifications may be able to detect ESBL-producing pathogens with our Cep-glucose substrate.

Additionally, as compared to the commercially available substrates CENTA and Nitrocefin, we have demonstrated that we can engineer the Cep-glucose substrate for specificity against ESBLs. We hypothesize that additional detection precision within the category of ESBLs can be engineered into the substrate through further modifications to the starting material. In particular, the variable group at the C-7 position of the cephem ring can be modified with side chains characteristic of different cephalosporin families that loosely generalize into cephalosporin generations. The emergence of a family of Cep-glucose molecules could potentially enable better precision in selecting the most appropriate cephalosporin or carbapenem that balances the health of the patient with the goals of antimicrobial stewardship.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.9b00138.

NMR and LC−MS characterization of synthesized compounds, enzyme kinetic characterization data of Cep-glucose compound, calibration data for electrochemical characterization (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: ianwhite@umd.edu.

ORCID

Shweta Ganapati: 0000-0003-1955-2772

Ian M. White: 0000-0003-4426-9311

Notes

The authors declare no competing financial interest.

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DEDICATION

P.D. dedicates this manuscript to his friend and colleague Professor Steve Weinreb on the occasion of his birthday.

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