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β-lactamase triggered visual detection of bacteria using cephalosporin functionalized biomaterials†

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We report the conjugation of a chromogenic cephalosporin β-lactamase (βL) substrate to polymers and integration into biomaterials for facile, visual βL detection. Identification of these bacterial enzymes, which are a leading cause of antibiotic resistance, is critical in the treatment of infectious diseases. The βL substrate polymer conjugate undergoes a clear to deep yellow color change upon incubation with common pathogenic gram-positive and gram-negative bacteria species. We have demonstrated the feasibility of formulating hydrogels with the βL substrate covalently tethered to a poly(ethylene glycol) (PEG) polymer matrix, exhibiting a visible color change in the presence of βLs. This approach has the potential to be used in diagnostic biomaterials for point-of-care detection of βL-producing bacteria, helping combat the spread of drug resistant microbes.

Infectious diseases are among the leading causes of death worldwide. Treatment of infections is becoming increasingly difficult due to the spread of antibiotic resistant microbes. According to a 2019 report by the United States Centers for Disease Control and Prevention, resistant microbes cause over 2.8 million illnesses and 35,000 deaths annually in the US alone.[1] Production of β-lactamases (βLs) is one of the primary mechanisms of resistance to β-lactam antibiotics, especially for gram-negative bacteria.[2] βLs are enzymes that cleave the β-lactam ring and inactivate β-lactam antibiotics, which make up 65% of all prescribed intravenous antibiotics in the US,[3] including penicillins and cephalosporins.[4] The facile detection of βL-producing bacteria has the potential to improve prevention and treatment and reduce the spread of antibiotic resistant infections.

Conventional bacteria detection methods, including culture and DNA amplification, although highly sensitive are often not suitable for use without specialized instrumentation and trained personnel.[5] Recognizing the limitations of these methods, recent efforts in point-of-care infection diagnostics exhibiting visual detection of bacteria, have led to the development of paper strips that change color due to electrostatic interactions with bacteria[6] and wound dressings that change color in the presence of lipase-producing bacteria.[7] However, the stimuli causing color change in these materials are generally non-specific to bacteria, limiting their use. In this work, we focused instead on visually detecting βLs, which are only produced by bacteria and in particular, antibiotic resistant bacteria. Non-colorimetric βL hydrolysis indicators include luminescent ruthenium (i),[8] fluorescent,[9,10] chemiluminescent,[11] and fluorescence resonance energy transfer[12] probes, again requiring the use of external equipment. Alternatively, chromogenic βL substrates have been utilized for a visual indication in solution, on agar, or on discs, including commercially available CENTA,[13,14] PADAC,[15,16] and nitrocefin.[4]

Our goal was to synthesize a chromogenic βL substrate that can be readily conjugated to a range of polymers, providing building blocks for infection diagnostic biomaterials (**Scheme 1**). These biomaterials could be used in several different scenarios (e.g., contaminated surgical equipment and hospital surfaces, which are linked to increased risk of healthcare-associated infections,[17] or superficial bacterial infections), allowing for real-time *in situ* monitoring of βL-producing bacteria *via* a color change. Inspired by the structure of the chromogenic βL substrate, CENTA,[13,14] we synthesized a βL substrate by modifying a cephalosporin derivative, 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester (ACLE), with a chromophore, 4-nitrobenzenethiol (NBT), at the 3'-position, yielding compound **1** (**Scheme 1**; **SI Section 3** and **Figure S1**). In this substrate, the primary amine at the 7'-position of the cephem nucleus is readily modifiable and can be used to conjugate the substrate to compounds bearing any one of an abundance of amine reactive groups. Here, we modified the βL substrate with a maleimide moiety, allowing facile conjugation to a range of

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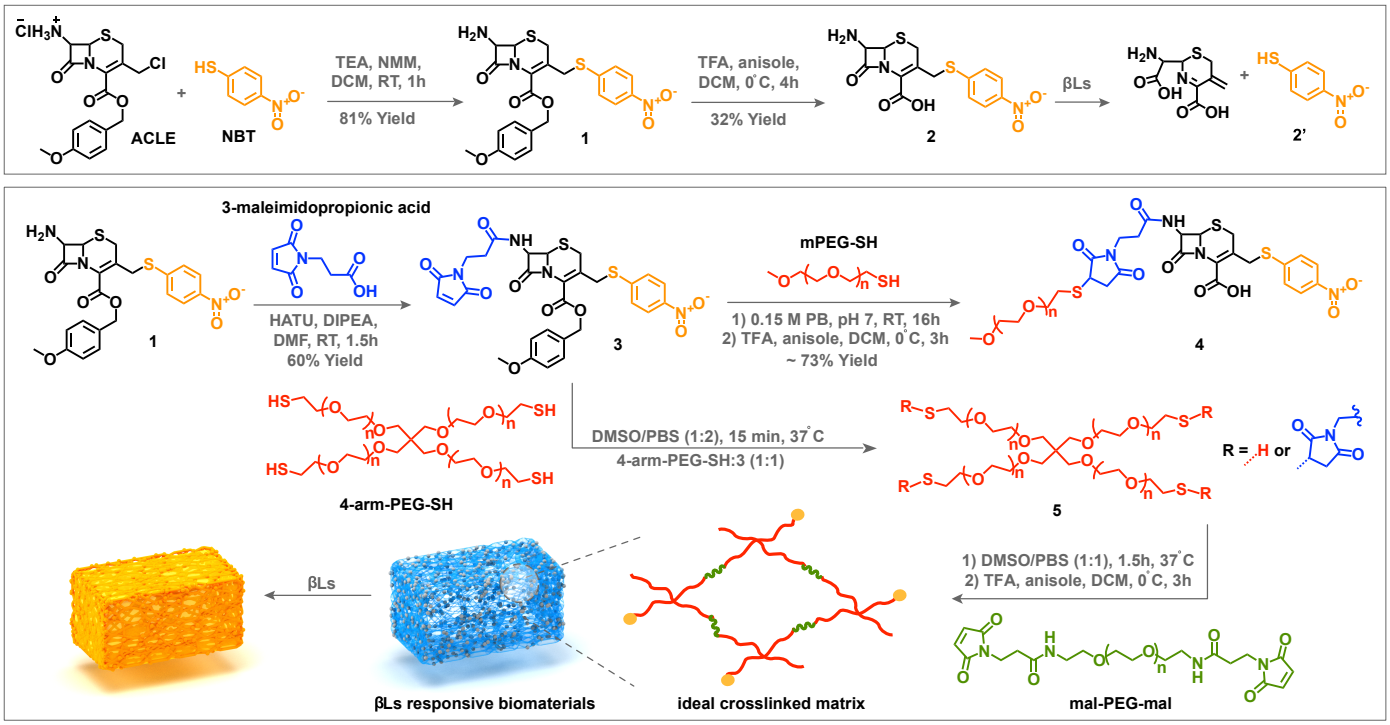

Scheme 1 Synthetic scheme for βL substrate and substrate-polymer conjugates and illustration of βL detecting hydrogel.

ACLE: 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid p-methoxybenzyl ester; NBT: 4-nitrobenzenethiol; TEA: triethylamine; NMM: 4-methylmorpholine; DCM: dichloromethane; TFA: trifluoroacetic acid; βLs: β-lactamases; HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA: N,N-diisopropylethylamine; DMSO: dimethyl sulfoxide; DMF: dimethylformamide; PB: sodium phosphate buffer; PBS: phosphate buffered saline; mPEG-SH: methoxy-poly(ethylene glycol)-thiol; 4-arm-PEG-SH: thiol modified 4-arm-PEG; mal-PEG-mal: bis-maleimide-PEG.

molecules and macromolecules *via* modular click-chemistry under mild conditions.[18,19] The *p*-methoxybenzyl protecting group prevents modification of the carboxyl on the cephem core, which is a key enzyme-substrate recognition site for many βLs, during chemical reactions and polymer functionalization.[20,21] Covalent conjugation of the βL substrate to biomaterial forming polymers enables the substrate to remain tethered to the biomaterial backbone preventing its premature release (*via* diffusion, for example).

Prior to modification with polymers, **1** was deprotected to yield **2** (**Scheme 1**; **SI Section 4** and **Figure S2**) in order to characterize the response of the substrate to βLs. **2** was tested with βLs produced by *Bacillus cereus* (βL-BC), *Pseudomonas aeruginosa* (βL-PA), and *Enterobacter cloacae* (βL-EC). βL-BC was selected due to its previous thorough characterization,[2,22] while βL-PA and βL-EC were examined due to the clinical relevance of the pathogenic, gram-negative bacteria that produce them.[2,23,24] Substrate **2** was found to exhibit a βL concentration-dependent color change from clear to yellow when mixed with βLs from any of the three bacterial species (**Figure 1a**).

Ultraviolet–visible (UV-vis) spectroscopy was used to monitor the kinetics of substrate **2** cleavage. As the β-lactam ring is hydrolyzed by the βL, its characteristic absorbance at 260 nm decreases, and the chromophore is expelled (**Scheme 1**; **2'**), causing a clear red shift in the maximum absorbance (λ_{max}) from 345 to 410 nm (**Figure S3**). As expected, the absorbance at λ_{max} is enzyme concentration-dependent (**Figures 1b** and **S4**). **Figure 1c** shows the absorbance at 410 nm versus βL concentration. The limit of detection was determined to be 4.6, 0.66, and 0.11 units (U)/mL (~0.05, 0.02, 12.86 μM) for βL-BC, βL-PA, and βL-EC, respectively, for the concentration of **2** tested (**Figure S5**); these values are similar to the lowest enzyme concentration leading to a color change discernible by eye (**Figure 1a**). Utilizing the Michaelis-Menten model, we compared the enzyme kinetics of substrate **2** to CENTA and nitrocefin (**Figure S6**), summarized in **Table 1**. The kinetic parameters obtained for CENTA and nitrocefin with βL-BC were within a similar range of values previously reported with βLs derived from *B. cereus*,[9,13] as expected. The k_{cat}/K_M values for **2** were slightly below those of CENTA and nitrocefin for βL-BC and βL-PA. With βL-EC, an extremely high enzyme concentration was required for substrate hydrolysis yielding a negligible k_{cat}, indicating low turnover number. The differences observed in these kinetic parameters between the βLs tested are likely related to variation in substrate specificity for different βLs.[21] As a preliminary investigation of βL specificity, substrate **2**, nitrocefin, and CENTA were also incubated with bacteria-produced collagenases; no color change was observed (**Figure 1d**).

To examine potential antibacterial effects of the substrate, which could limit its utility in bacteria detection, the minimum inhibitory concentration (MIC) of **2** was measured against different bacterial species (**Table S1**). Compound **2** exhibited antibacterial effects (MIC = 8 μg/mL) against *Staphylococcus aureus* 25923, which is a non-βL-producing strain,[25,26] and *S. aureus* 29213, which produces low levels of βLs,[26,27] but showed no antibacterial effect at concentrations up to 128 μg/mL against other bacte-

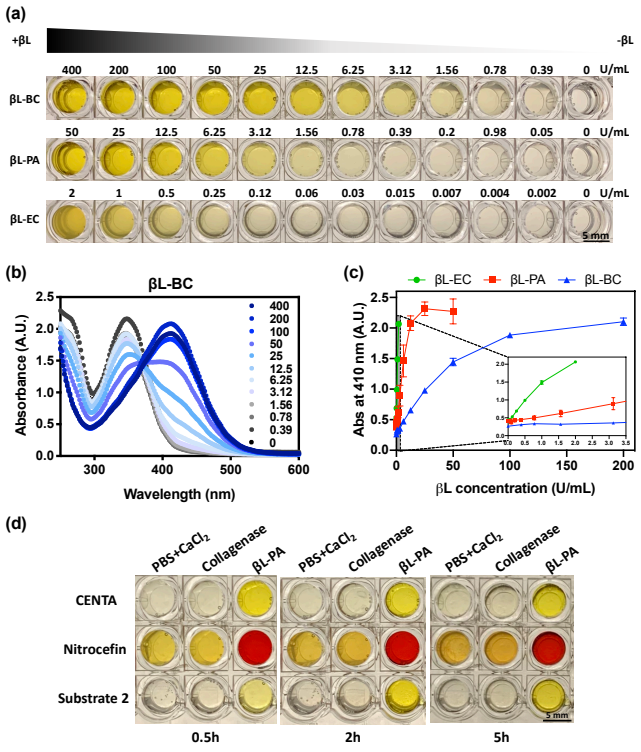

Fig. 1 (a) Substrate 2 (544 μM) incubated with βL-BC, βL-PA, or βL-EC (U/mL) for 45 minutes at 37°C. (b) Absorbance spectra of 2 incubated with βL-BC (U/mL). (c) Absorbance at 410 nm versus βL concentration for solutions in (a). (d) Images of CENTA, nitrocefin and 2 (all at 544 μM) incubated in 1× PBS with 1 mM $CaCl_2$ alone or with 1 mg/mL collagenases or with 30 U/mL of βL-PA in PBS for 0.5, 2, or 5 hours at 37°C. Note, 1 U is defined as hydrolyzing 1.0 μmole of benzylpenicillin per min at pH 7.0 at 25°C.

ria investigated (methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, *E. cloacae*, and *B. cereus*). These results are comparable to CENTA, nitrocefin, and PADAC, which have been shown to lack antibacterial activity against most gram-negative species but do exhibit activity against *S. aureus* and some *Streptococci*.[14,15]

After characterizing substrate **2** and its response to βLs, we investigated its responsiveness following conjugation to poly(ethylene glycol) (PEG). PEG was chosen as our model polymer due to its extensive use in biomaterials such as hydrogels, nanoparticles, and coatings given its relative biocompatiblity and tunability.[28,29] We first functionalized **1** with a maleimide utilizing 3-maleimidopropionic acid, yielding **3** (**Scheme 1**; **SI Section 9** and **Figures S7 - S13**). **3** was then conjugated to a linear, short chain methoxy-PEG-thiol (mPEG-SH) (1.7 kDa) *via* Michael-type addition forming conjugate **4** (**Scheme 1**; **SI Section 10**). ^{1}H-NMR showed the disappearance of the maleimide protons at 7.00 ppm and the appearance of a new proton signal at 4.02 ppm in **4**, indicating formation of the thiol-maleimide adduct (**Figure S14**). Additional protons on the adduct which would likely appear in the region of 2.4 - 3.2 ppm,[30] were obscured by the PEG repeat unit protons. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry also confirmed PEG conjugation, demonstrating an increase

Enzyme	Substrate	[E][a] (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
βL from *B. cereus*	Substrate **2**	0.32	866.80 ± 141.40	4.99 ± 0.57	0.0058 ± 0.0011
	CENTA[b]	0.32	345.30 ± 36.08	16.50 ± 0.88	0.048 ± 0.0056
	Nitrocefin[c]	0.32	450.70 ± 69.55	49.67 ± 4.35	0.11 ± 0.012
βL from *P. aeruginosa*	Substrate **2**	1.19	58.01 ± 9.54	0.66 ± 0.02	0.011 ± 0.002
	CENTA	0.47	12.63 ± 6.84	0.42 ± 0.02	0.033 ± 0.018
	Nitrocefin	0.47	178.80 ± 32.71	5.14 ± 0.37	0.029 ± 0.0056
βL from *E. cloacae*	Substrate **2**	116.9	864.6 ± 123.4	-[d]	-[d]
	CENTA	1.17	68.88 ± 11.83	1.48 ± 0.062	0.021 ± 0.0038
	Nitrocefin	0.58	315.20 ± 26.75	9.04 ± 0.38	0.029 ± 0.0027

Table 1 Kinetic parameters of substrate 2, CENTA, and nitrocefin hydrolysis by βLs
[a] Enzyme concentration
[b] Previously reported: (1) [E] = 0.014 μM, k_M = 330 μM, k_{cat} = 50 s^{-1}, k_{cat}/k_M = 0.15 $\mu M^{-1}s^{-1}$;[13] (2) [E] = 0.001 μM, k_M = 135.9 ± 16.4 μM, k_{cat} = 8.9 s^{-1}, k_{cat}/k_M = 0.06 $\mu M^{-1}s^{-1}$.[9]
[c] Previously reported: (1) [E] = 0.014 μM, k_M = 70 μM, k_{cat} = 45 s^{-1}, k_{cat}/k_M = 0.64 $\mu M^{-1}s^{-1}$;[13] (2) [E] = 0.001 μM, k_M = 8.1 ± 1.0 μM, k_{cat} = 14.9 s^{-1}, k_{cat}/k_M = 1.8 $\mu M^{-1}s^{-1}$.[9]
[d] -; negligible

in the molecular weight of **4** compared to unmodified mPEG-SH (**Figure S15**). **4** and unmodified mPEG-SH were also compared using size exclusion chromatography (SEC), where mPEG-SH and **4** had comparable elution times (measured *via* a refractive index detector; data not shown) and only **4** had an absorbance signal at 345 nm (**Figure S16**), indicating successful conjugation. In the presence of βL-BC, **4** changed color from clear to a bright yellow, accompanied by a decrease in absorbance at 260 nm and the expected red shift in λ_{max} (**Figure S17**), confirming that **4** remains responsive to βLs. A decrease in the 345 nm absorbance signal of **4** after incubation with βL was also observed *via* SEC (**Figure S16**).

Upon conjugation to the hydrophilic PEG, aqueous solubility of the substrate was greatly improved facilitating detection of βL-producing bacteria in *in vitro* cultures at higher substrate concentrations. We incubated conjugate **4** at increasing concentrations (up to 2028 μM) with βL-producing strains of *P. aeruginosa*,[12] *E. cloacae*,[12,31] and *B. cereus*,[8] as well as a non-pathogenic, non-βL-producing *E. coli* (DH5-α).[8,31,32] As shown in **Figure 2a**, the conjugate changed color when incubated with βL-producing bacteria, even at the lowest concentrations of **4** tested (~127 - 254 μM), while no color change was observed for *E. coli* DH5-α. For *B. cereus*, at the highest concentration of **4** (>5.8 times the highest concentration of **2** investigated for antibacterial effects), no visible color change was achieved prior to growth inhibition (suggested by solution clearing).

Having demonstrated successful response to βLs in bacterial suspensions using PEG modified **2**, we investigated the formation of a macroscale βL-responsive chromogenic biomaterial using **3**. As shown in **Scheme 1**, **3** was reacted with thiol functionalized 4-arm-PEG to decorate on average one of the polymer arms with the βL substrate (**5**). Subsequently, a bis-maleimide-PEG cross-linker was used to cross-link the hydrogel network. Control non-β-lactam hydrogels were formed similarly omitting the addition of **3**. An increased swelling was observed in the β-lactam-PEG hydrogels compared to control PEG hydrogels (**Figure 2b**) likely resulting from increased network defects due to incomplete cross-linking at sites where **3** was conjugated.[33] Finally, when both hydrogels were incubated with βL-BC, only β-lactam containing hydrogels showed a rapid and distinct color change from clear to

yellow (**Figure 2b**), indicating that these materials successfully maintain βL responsiveness.

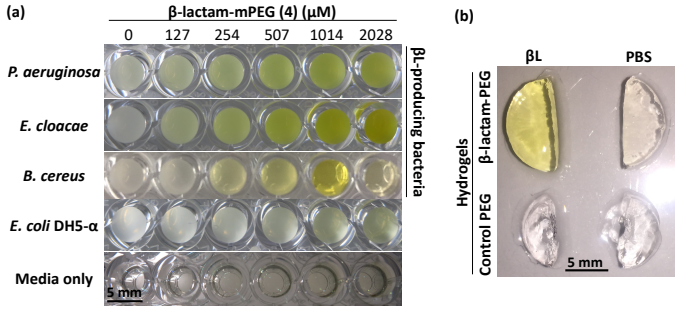

Fig. 2 (a) Conjugate 4 incubated with βL-producing (*P. aeruginosa*, *E. cloacae*, *B. cereus*) or non-βL-producing bacteria (*E. coli*), or in media for 18-24 hours at 37°C. (b) Representative images of 20% (w/v) β-lactam-PEG and non-responsive PEG hydrogels incubated in 1× PBS with and without 400 U/mL of βL-BC for <10 minutes.

In summary, we have demonstrated a modular approach to covalently attach a chromogenic βL substrate to polymers which exhibit a color change specifically in the presence of βLs. To the best of our knowledge, this is the first report of polymer-βL substrate conjugates for direct and facile colorimetric detection of βL-producing bacteria, involved in several difficult to treat infections. While we have demonstrated the feasibility of conjugating this substrate to PEG and PEG derivatives, this versatile substrate has the potential to be readily modified by a broad range of additional polymers. These conjugates could potentially be used for the detection of bacteria in a range of environments (e.g., medical devices, household surfaces) and may ultimately be incorporated into multifunctional biomaterials for the enhanced prevention, detection, and treatment of bacterial infections.

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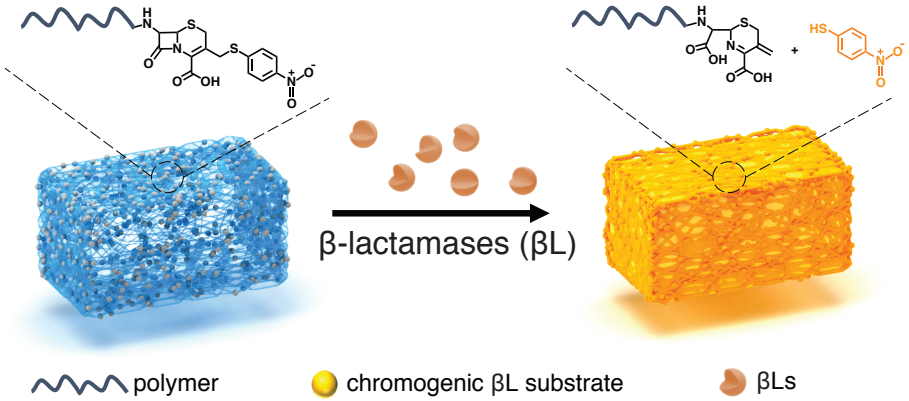

A chromogenic substrate of β-lactamases, key antibiotic resistance enzymes, is readily conjugated to polymers and incorporated into biomaterials providing visual detection of bacteria