Published in final edited form as:

Chem Commun (Camb). 2020 September 25; 56(75): 11098–11101. doi:10.1039/d0cc04088f.

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

Dahlia Alkekhaia^{a,†}, Hannah Safford^{a,†}, Shashank Shukla^a, Russel Hopson^b, Anita Shukla^{a,§}

^aSchool of Engineering, Center for Biomedical Engineering, Institute for Molecular and Nanoscale Innovation, Brown University, Providence, RI 02912, USA

^bDepartment of Chemistry, Brown University, Providence, RI 02912, USA

Abstract

We report the conjugation of a chromogenic cephalosporin β -lactamase (β L) substrate to polymers and integration into biomaterials for facile, visual β L detection. Identification of these bacterial enzymes, which are a leading cause of antibiotic resistance, is critical in the treatment of infectious diseases. The β L 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 β L substrate covalently tethered to a poly(ethylene glycol) (PEG) polymer matrix, exhibiting a visible color change in the presence of β Ls. This approach has the potential to be used in diagnostic biomaterials for point-of-care detection of β L-producing bacteria, helping combat the spread of drug resistant microbes.

Graphical Abstract

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

[†]Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/d0cc04088f.

[§]Corresponding author; anita_shukla@brown.edu.

[‡]These authors contributed equally to this work

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 (β Ls) is one of the primary mechanisms of resistance to β -lactam antibiotics, especially for gram-negative bacteria.² β Ls 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 β L-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 bacteria⁶ 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 β Ls, which are only produced by bacteria and in particular, antibiotic resistant bacteria. Non-colorimetric β L hydrolysis indicators include luminescent ruthenium (i),⁸ fluorescent,^{9,10} chemiluminescent,¹¹ and fluorescence resonance energy transfer¹² probes, again requiring the use of external equipment. Alternatively, chromogenic β L 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 β L 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 β L-producing bacteria *via* a color change. Inspired by the structure of the chromogenic β L substrate, CENTA,^{13,14} we synthesized a β L 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 β L substrate with a maleimide moiety, allowing facile conjugation to a range of 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 β Ls, during chemical reactions and polymer functionalization.^{20,21} Covalent conjugation of the β L 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 β Ls. **2** was tested with β Ls produced by *Bacillus cereus* (β L-BC), *Pseudomonas aeruginosa* (β L-PA), and *Enterobacter cloacae* (β L-EC). β L-BC was selected due to its previous thorough characterization,^{2,22} while β L-PA and β L-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 β L concentration-dependent color change from clear to yellow when mixed with β Ls 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 β L, 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 β L concentration. The limit of detection was determined to be 4.6, 0.66, and 0.11 units (U)/mL ($\sim$ 0.05, 0.02, 12.86 μ M) for β L-BC, β L-PA, and β L-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 β L-BC were within a similar range of values previously reported with β Ls derived from *B. cereus*,^{9,13} as expected. The $k_{\text{cat}}/K_{\text{M}}$ values for **2** were slightly below those of CENTA and nitrocefin for β L-BC and β L-PA. With β L-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 β Ls tested are likely related to variation in substrate specificity for different β Ls.²¹ As a preliminary investigation of β L 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 μ g/mL) against *Staphylococcus aureus* 25923, which is a non- β L-producing strain,^{25,26} and *S. aureus* 29213, which produces low levels of β Ls,^{26,27} but showed no antibacterial effect at concentrations up to 128 μ g/mL against other bacteria 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 β Ls, 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 biocompatibility 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). ¹H-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 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 β L-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 β Ls. A decrease in the 345 nm absorbance signal of **4** after incubation with β L was also observed *via* SEC (Figure S16).

Upon conjugation to the hydrophilic PEG, aqueous solubility of the substrate was greatly improved facilitating detection of β L-producing bacteria in *in vitro* cultures at higher substrate concentrations. We incubated conjugate **4** at increasing concentrations (up to 2028 μ M) with β L-producing strains of *P. aeruginosa*,¹² *E. cloacae*,^{12,31} and *B. cereus*,⁸ as well as a non-pathogenic, non- β L-producing *E. coli* (DH5- α).^{8,31,32} As shown in Figure 2a, the conjugate changed color when incubated with β L-producing bacteria, even at the lowest concentrations of **4** tested (~127 – 254 μ M), 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 β Ls in bacterial suspensions using PEG modified **2**, we investigated the formation of a macroscale β L-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 β L 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 β L-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 β L responsiveness.

In summary, we have demonstrated a modular approach to covalently attach a chromogenic β L substrate to polymers which exhibit a color change specifically in the presence of β Ls. To the best of our knowledge, this is the first report of polymer- β L substrate conjugates for direct and facile colorimetric detection of β L-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Office of Naval Research [grant number N00014-17-1-2120]. The authors thank Dr. Tun-Li Shen at the Mass Spectrometry Facility at Brown University for his technical support, Dr. Vicki Colvin at Brown University for the use of her PerkinElmer Lambda 950 spectrophotometer, and Dr. Chao Yu at Brown University for support with illustrations. Research was made possible by the use of the MALDI-TOF MS available through the Rhode Island Institutional Development Award (IDeA) Network of Biomedical Research Excellence from the National Institute of General Medical Sciences of the National Institutes of Health [grant number P20GM103430].

References

1. Centers for Disease Control and Prevention, Antibiotic resistance threats in the United States, 2019.
2. Bush K, Antimicrobial Agents and Chemotherapy, 2018, 62, year.
3. Bush K and Bradford PA, Cold Spring Harbor Perspectives in Medicine, 2016, 6, a025247. [PubMed: 27329032]
4. Livermore DM and Brown DFJ, Journal of Antimicrobial Chemotherapy, 2001, 48, 59–64.
5. Váradi L, Luo JL, Hibbs DE, Perry JD, Anderson RJ, Orena S and Groundwater PW, Chemical Society Reviews, 2017, 46, 4818–4832. [PubMed: 28644499]
6. Creran B, Li X, Duncan B, Kim CS, Moyano DF and Rotello VM, ACS Applied Materials and Interfaces, 2014, 6, 19525–19530. [PubMed: 25318086]
7. Singh H, Li W, Kazemian MR, Yang R, Yang C, Logsetty S and Liu S, ACS Applied Bio Materials, 2019, 2, 2028–2036.
8. Shao Q and Xing B, Chemical Communications, 2012, 48, 1739–1741. [PubMed: 22057185]
9. Cain R, Schofield CJ, Brem J, Fishwick CWG, van Berkel SS, Owens RJ, Spencer J, Rydzik AM, Salimraj R and Verma A, Journal of Medicinal Chemistry, 2013, 56, 6945–6953. [PubMed: 23898798]
10. Xie H, Hassounah H, Rao J, Cirillo JD, Cheng Y, Graviss EA, Kong Y and Sule P, Angewandte Chemie International Edition, 2014, 53, 9360–9364. [PubMed: 24989449]
11. Maity S, Wang X, Das S, He M, Riley LW and Murthy N, Chemical Communications, 2020, 56, 3516–3519. [PubMed: 32101196]
12. Chan HL, Lyu L, Aw J, Zhang W, Li J, Yang H-HH, Hayashi H, Chiba S and Xing B, ACS Chemical Biology, 2018, 13, 1890–1896. [PubMed: 29595947]
13. Bebrone C, Moali C, Mahy F, Rival S, Docquier JD, Rossolini GM, Fastrez J, Pratt RF, Frere J-M and Galleni M, Antimicrobial Agents and Chemotherapy, 2001, 45, 1868–1871. [PubMed: 11353639]
14. Jones RN, Wilson HW, Novick WJ, Barry AL and Thornsberry C, Journal of Clinical Microbiology, 1982, 15, 954–958. [PubMed: 7047560]
15. Jones RN, Wilson HW and Novick WJ, Journal of Clinical Microbiology, 1982, 15, 677–683. [PubMed: 6978350]
16. Kobayashi S, Arai S, Hayashi S and Sakaguchi T, Antimicrobial Agents and Chemotherapy, 1988, 32, 1040–1045. [PubMed: 3263833]

17. Chemaly RF, Simmons S, Dale C, Ghantaji SS, Rodriguez M, Gubb J, Stachowiak J and Stibich M, *Therapeutic Advances in Infectious Disease*, 2014, 2, 79–90. [PubMed: 25469234]
18. Lutolf MP and Hubbell JA, *Biomacromolecules*, 2003, 4, 713–722. [PubMed: 12741789]
19. Gandini A, *Progress in Polymer Science*, 2013, 38, 1–29.
20. Morandi F, Caselli E, Morandi S, Focia PJ, Blázquez J, Shoichet BK and Prati F, *Journal of the American Chemical Society*, 2003, 125, 685–695. [PubMed: 12526668]
21. Bush K and Bradford PA, *Nature Reviews Microbiology*, 2019, 17, 295–306. [PubMed: 30837684]
22. Imsande J, Gillin FD, Tanis RJ and Atherly AG, *Journal of Biological Chemistry*, 1970, 245, 2205–2212.
23. Kazmierczak KM, Rabine S, Hackel M, McLaughlin RE, Biedenbach DJ, Bouchillon SK, Sahm DF and Bradford PA, *Antimicrobial Agents and Chemotherapy*, 2016, 60, 1067–1078. [PubMed: 26643349]
24. Davin-Regli A and Pagès J-M, *Frontiers in Microbiology*, 2015, 6, 392. [PubMed: 26042091]
25. Khan S, Sallum UW, Zheng X, Nau GJ and Hasan T, *BMC Microbiology*, 2014, 14, 84. [PubMed: 24708478]
26. Lee S, Kwon KT, Kim H-I, Chang HH, Lee J-M, Choe PG, Park WB, Kim NJ, Oh M-D, Song DY and Kim S-W, *Microbial Drug Resistance*, 2014, 20, 568–574. [PubMed: 25000230]
27. Kernodle DS, McGraw PA, Stratton CW and Kaiser AB, *Antimicrobial Agents and Chemotherapy*, 1990, 34, 420–425. [PubMed: 2334154]
28. Zhu J, *Biomaterials*, 2010, 31, 4639–4656. [PubMed: 20303169]
29. Hamley IW, *Biomacromolecules*, 2014, 15, 1543–1559. [PubMed: 24720400]
30. Northrop BH, Frayne SH and Choudhary U, *Polymer Chemistry*, 2015, 6, 3415–3430.
31. Aw J, Widjaja F, Ding Y, Mu J, Liang Y and Xing B, *Chemical Communications*, 2017, 53, 3330–3333. [PubMed: 28067350]
32. Boschi L, Mercuri PS, Riccio ML, Amicosante G, Galleni M, Frère JM and Rossolini GM, *Antimicrobial Agents and Chemotherapy*, 2000, 44, 1538–1543. [PubMed: 10817705]
33. Kim J, Kong YP, Niedzielski SM, Singh RK, Putnam AJ and Shikanov A, *Soft Matter*, 2016, 12, 2076–2085. [PubMed: 26750719]

Fig. 1.

(a) Substrate 2 (544 μ M) incubated with β L-BC, β L-PA, or β L-EC (U/mL) for 45 minutes at 37 $^{\circ}$ C. (b) Absorbance spectra of 2 incubated with β L-BC (U/mL). (c) Absorbance at 410 nm versus β L concentration for solutions in (a). (d) Images of CENTA, nitrocefin and 2 (all at 544 μ M) incubated in 1 $\times$ PBS with 1 mM CaCl_2 alone or with 1 mg/mL collagenases or with 30 U/mL of β L-PA in PBS for 0.5, 2, or 5 hours at 37 $^{\circ}$ C. Note, 1 U is defined as hydrolyzing 1.0 μ mole of benzylpenicillin per min at pH 7.0 at 25 $^{\circ}$ C.

Fig. 2.

(a) Conjugate 4 incubated with β L-producing (*P. aeruginosa*, *E. cloacae*, *B. cereus*) or non- β L-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 $\times$ PBS with and without 400 U/mL of β L-BC for <10 minutes.

Scheme 1. Synthetic scheme for β L substrate and substrate-polymer conjugates and illustration of β L 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; β lactamases: 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.

Table 1Kinetic parameters of substrate 2, CENTA, and nitrocefin hydrolysis by β Ls

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

^aEnzyme concentration^bPreviously reported: (1) [E] = 0.014 μ M, k_M = 330 μ M, k_{cat} = 50 s⁻¹, k_{cat}/k_M = 0.15 μ M⁻¹s⁻¹; ¹³ (2) [E] = 0.001 μ M, k_M = 135.9 $\pm$ 16.4 μ M, k_{cat} = 8.9 s⁻¹, k_{cat}/k_M = 0.06 μ M⁻¹s⁻¹.⁹^cPreviously reported: (1) [E] = 0.014 μ M, k_M = 70 μ M, k_{cat} = 45 s⁻¹, k_{cat}/k_M = 0.64 μ M⁻¹s⁻¹; ¹³ (2) [E] = 0.001 μ M, k_M = 8.1 $\pm$ 1.0 μ M, k_{cat} = 14.9 s⁻¹, k_{cat}/k_M = 1.8 μ M⁻¹s⁻¹.⁹^d-; negligible