*Supporting Information*

# Ultrasensitive Detection of β-Lactamase-Associated Drug-Resistant Bacteria Using a Novel Mass-Tagged Probe-Mediated Cascaded Signal Amplification Strategy

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**Table S1.** Accuracy and precision of QCs.

| Nominal concentration of QCs | 50.0 fM | 150 fM | 10.0 pM | 300 pM |
|---|---|---|---|---|
| **Mean** | 54.9 | 158 | 10.8 | 282 |
| **%Bias** | 9.7 | 5.6 | 8.4 | -6.0 |
| **Intra-day precision (%CV)** | 2.5 | 2.6 | 2.1 | 2.0 |
| **Inter-day precision (%CV)** | 15.2 | 10.9 | 7.3 | 5.5 |
| **n** | 18 | 18 | 18 | 18 |
| **Number of runs** | 3 | 3 | 3 | 3 |

# Supplementary Figures

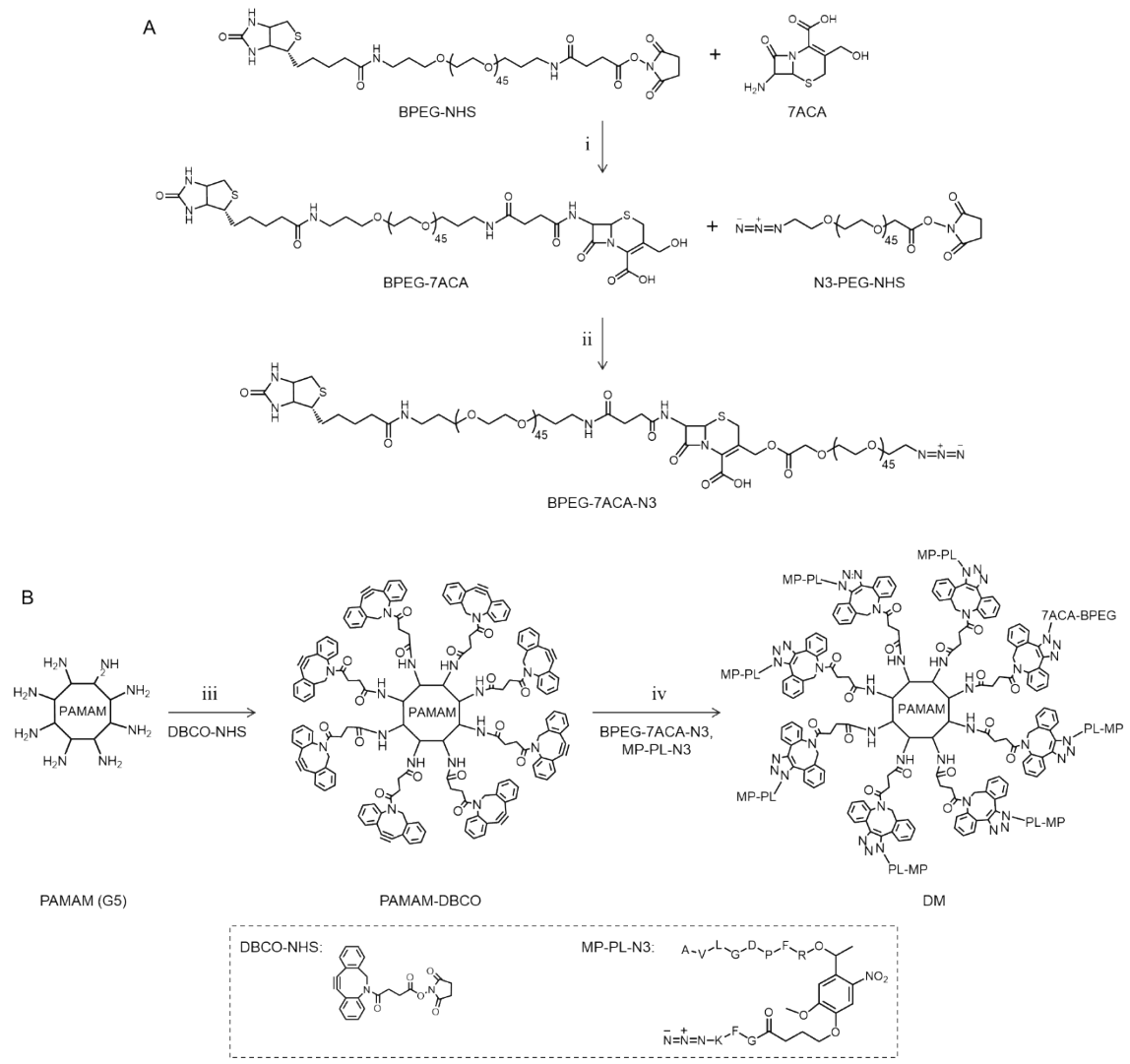


**Figure S1.** The synthesis routes for (A) BPEG-7ACA-N3 and (B) DM. The reaction conditions included (i) deionized water, TEA, pH 8.0, 30 °C, 24 h; (ii) DMSO, TEA, 30 °C, 24 h; (iii) DMSO, 30 °C, 18 h; and (iv) deionized water, 30 °C, 12 h. The detailed synthesis procedures were provided in the experimental section of the article.

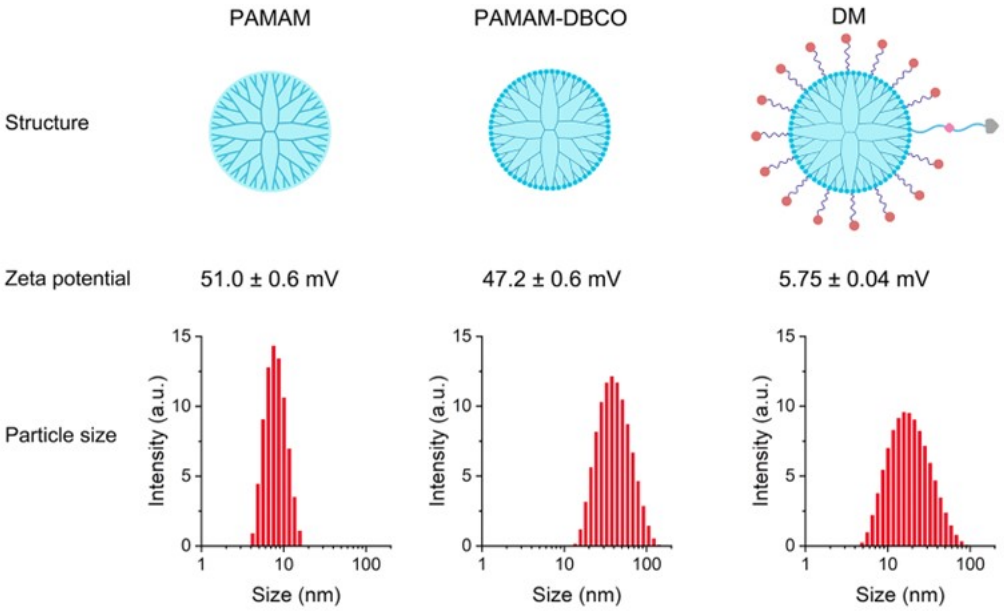


**Figure S2.** Characterization of PAMAM, PAMAM-DBCO and DM, including their structure, zeta potential and hydrodynamic particle size.

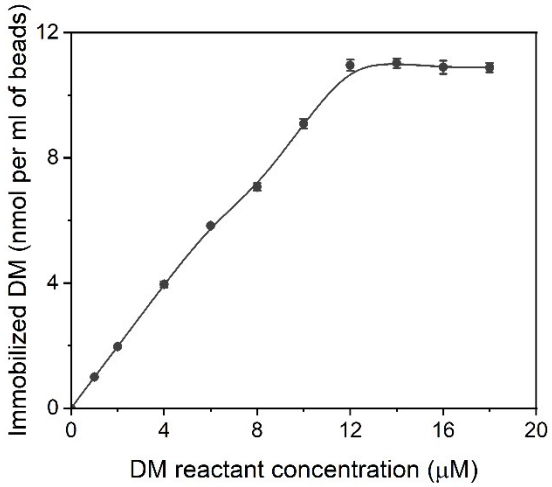


**Figure S3.** Optimization of the DM concentration. One milliliter of streptavidin agarose beads reacted with 1 mL of DM with concentrations of 0, 1.00, 2.00, 4.00, 6.00, 8.00, 10.0, 12.0, 14.0, 16.0 and 18.0 μM at 37 °C for 2 h. The error bars are indicative of the standard deviation of three measurement replicates of one mass tag sample.

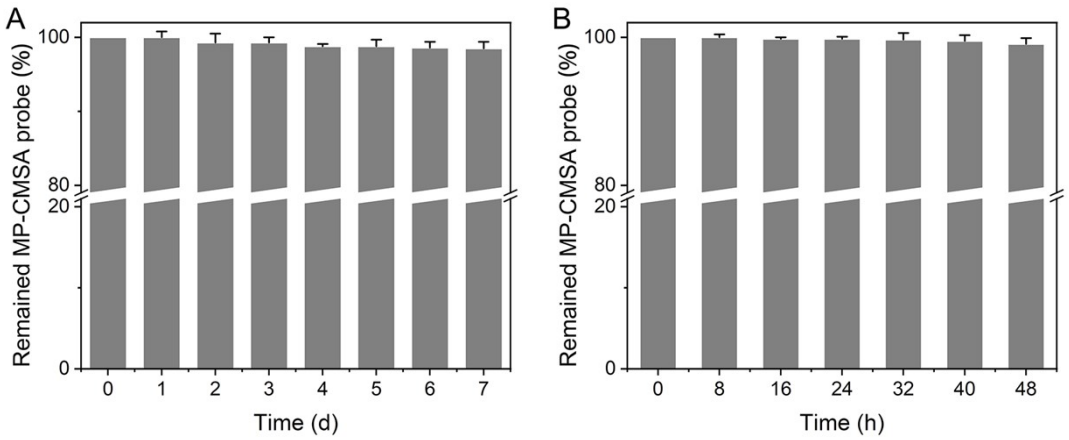


**Figure S4.** Stability of the MP-CMSA probe in (A) the storage condition (PBS, pH 7.4, 4 °C) and (B) the detection condition (PBS, pH 7.4, 37 °C), respectively. The error bars are indicative of the standard deviation of three measurement replicates of one mass tag sample.

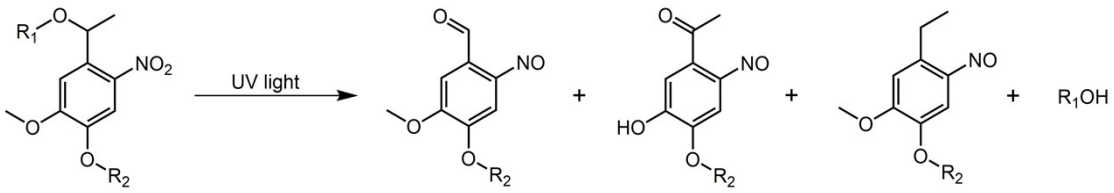


**Figure S5.** Photolysis mechanism of PL. As shown, the mass tag can ligate to both R1 and R2-positions of the benzene ring of PL. The results in this study indicated that several byproducts were produced after photolysis if the mass tag was conjugated at the $R_2$ position of PL, whereas the mass tag was the dominant product and could be completely released if it was conjugated to the $R_1$ position of PL. This observation was in agreement with the previous studies. [1, 2]

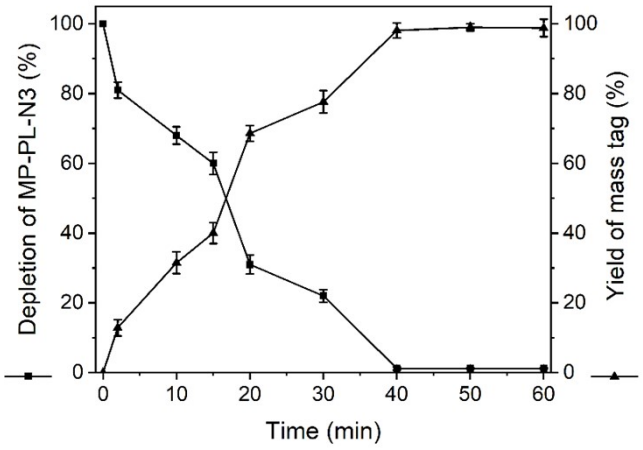


**Figure S6.** Optimization of the photolysis time of MP-PL-N3. The depletion of MP-PL-N3 (100 nM) and the corresponding yield of mass tag after UV exposure for various times. The error bars are indicative of the standard deviation of three measurement replicates of one mass tag sample.

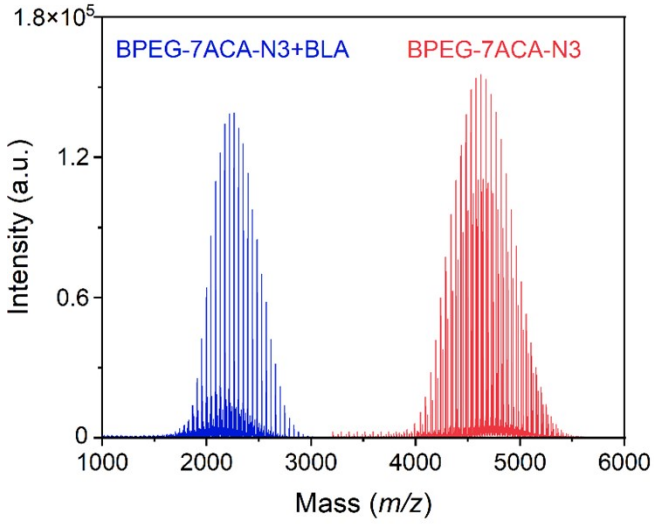


**Figure S7.** The MALDI-MS spectra of 50.0 nM BPEG-7ACA-N3 before and after the incubation with 200 nM BLA at 37 °C for 2 h.

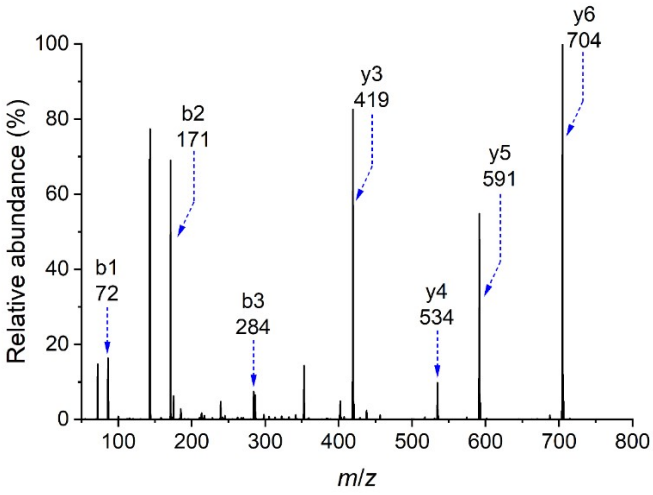


**Figure S8.** The product ion spectrum of the mass tag AVLGDPFR.

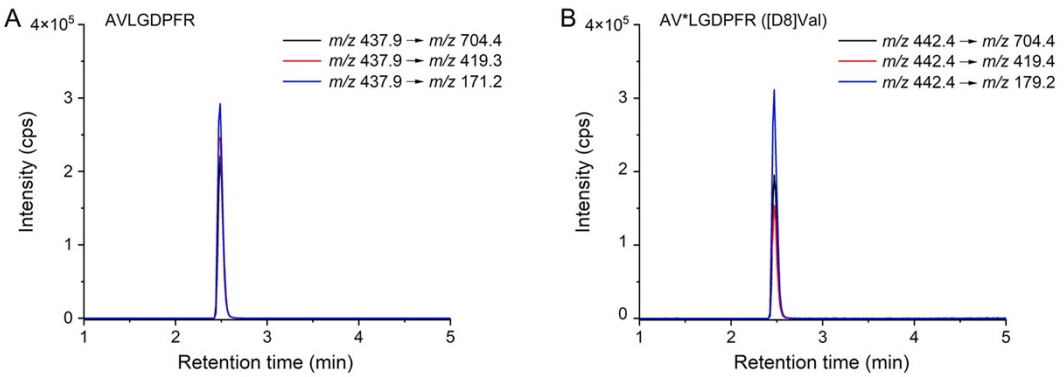


**Figure S9.** The LC-MS/MS chromatograms of (A) AVLGDPFR and (B) its corresponding isotope-labeled internal standard AV*LGDPFR ([D8]Val). MRM transitions are *m/z* 437.9 → *m/z* 704.4, *m/z* 437.9 → *m/z* 419.3 and m/z 437.9 → *m/z* 171.2 for AVLGDPFR, and *m/z* 442.4 → *m/z* 704.4, *m/z* 442.4 → *m/z* 419.4 and *m/z* 442.4 → *m/z* 179.2 for AV*LGDPFR ([D8]Val).

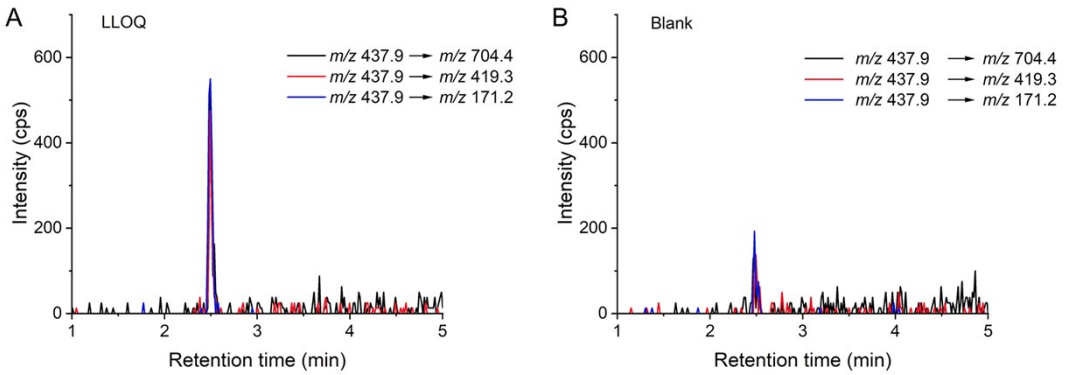


**Figure S10.** The LC-MS/MS chromatograms of (A) the mass tag released from the MP-CMSA probe at LLOQ (50.0 fM) of BLA and (B) the matrix blank (5% BSA).

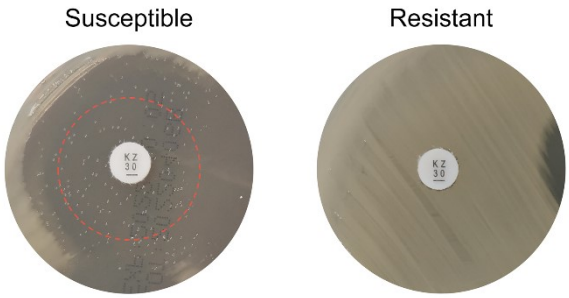


**Figure S11.** The representative AST results. The samples were obtained from two patients diagnosed with *Klebsiella pneumoniae* infections. Left: Bacteria were susceptible to a β-lactam antibiotic Cefazolin (KZ). Right: Bacteria were resistant to KZ. The susceptible breakpoints of zone diameter (red dotted box) of KZ for *Klebsiella pneumoniae* recommended by the Clinical and Laboratory Standards Institute Standards (CLSI) are listed as follows: the zone diameter ⩾ 23 mm was defined as susceptible, and the zone diameter < 19 mm was defined as resistant.[3]

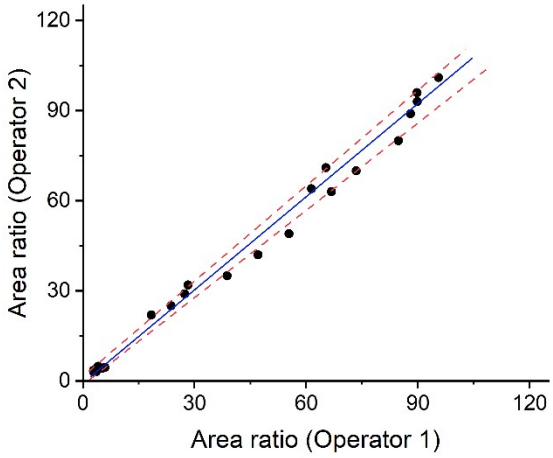


**Figure S12.** Passing-Bablok regression analysis of the results from two operators. The solid blue line corresponds to the regression curve. Dashed red lines represent the 95% confidence interval for the regression curve. The estimate confidence intervals of the slope and the intercept were (0.959 – 1.06) and (-0.433 – 0.626), respectively.

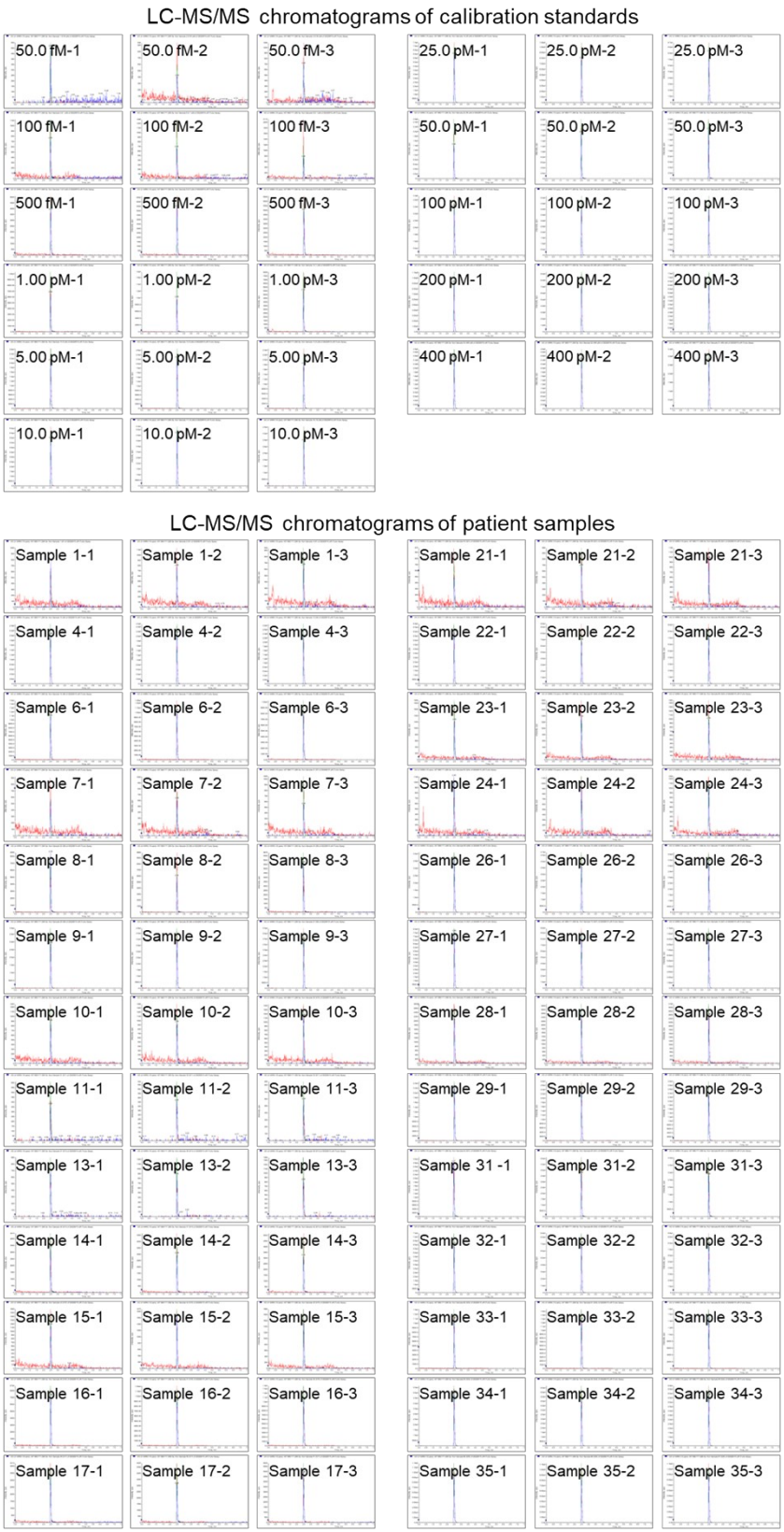


**Figure S13.** The LC-MS/MS chromatograms of calibration standards and the patient samples.

# Supplementary Material

## S1. Materials and reagents

*α*-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) and *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) were bought from Sigma-Aldrich (Shanghai, China). Biotin-poly(ethylene glycol)-succinimidyl-carboxymethyl-ester (BPEG-NHS, $M_w$: 2.00 ± 0.20 kDa) and azido-poly(ethylene glycol)-succinimidyl-carboxymethyl-ester (N3-PEG-NHS, $M_w$: 2.00 ± 0.20 kDa) were purchased from Jenkem Technology Co., LTD (Beijing, China). PAMAM dendrimer (generations 5, $M_w$: 28.8 kDa) was obtained from Sigma-Aldrich (Shanghai, China). Hydromethyl-7-aminocephalosporanic acid (7ACA) was purchased from Energy Chemical (Shanghai, China). Dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (DBCO-NHS) was purchased from Shanghai Haohong Biomedical Technology Co., Ltd. (Shanghai, China). The peptide AVLGDPFR ($M_w$: 875 Da), stable isotope-labeled internal peptide (AV*LGDPFR ([D8]Val), $M_w$: 884 Da) and azido-modified photo-sensitive peptide, MP-PL-N3 (AVLGDPFR-PL-GFK-N3, $M_w$: 1.57 kDa), were synthesized by ChinaPeptides Biomedical Technology Co., Ltd. (Shanghai, China). Streptavidin agarose beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fluorescein isothiocyanate (FITC), potassium clavulanate, β-lactamase (BLA, TEM-1), acetonitrile (ACN), dimethyl sulfoxide (DMSO), deuterated DMSO (DMSO-*d6*), deuterated chloroform ($CDCl_3$), triethylamine (TEA), formic acid (FA) and methanol were purchased from Aladdin Reagent (Shanghai, China). Bovine serum albumin (BSA) was purchased from

Biofroxx (Einhausen, Germany).

## S2. Instruments

$^1$H nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a JEOL 400YH 400 NMR spectrometer (JEOL, Japan) using DMSO-*d6* or $CDCl_3$ as the solvent. The fluorescence images were obtained on a Lecia DIM3000B fluorescence microscope (Lecia, Germany). The hydrodynamic particle size and zeta potential of PAMAM, PAMAM-DBCO and DM were determined by dynamic light scattering on a Malvern Zs90 Zetasizer (Malvern, UK). Bacteria were lysed by an XL-2000 Series probe sonicator (MISONIX, USA).

## S3. Preparation of stock solutions, calibration standards, and quality controls (QCs)

Stock solutions (1 mM) of the substrate mass tag and the mass tag were first prepared in deionized water and stored at -20°C in amber glass tubes. The isotope-labeled synthetic peptide mass tag was weighed and used as internal standards. Its stock solution (100 μM) was also prepared in deionized water. Internal standard solutions (10.0 nM) were prepared by serially diluting the stock solutions using deionized water containing 0.1% FA. The calibration standard of peptide mass tag was prepared by serially diluting the stock solutions using 5% BSA as surrogate matrix. The concentrations of calibration standards were 50.0 fM, 100 fM, 500 fM, 1.00 pM, 5.00 pM, 10.0 pM, 25.0 pM, 50.0 pM, 100 pM, 200 pM and 400 pM.

Correspondingly, the QC standards (i.e., lower limit of quantification (LLOQ), low QC, mid QC and high QC) were set at 50.0 fM, 150 fM, 10.0 pM and 300 pM and frozen prior to use. Each prepared QC solution was divided into three aliquots, and frozen and stored at -20°C. The accuracy and precision of QCs were evaluated by measuring the response of QCs in three validation runs over several days. Each validation run consisted of a calibration curve (in duplicate), six replicates of QC samples and several blank samples.[4]

**S4. LC-MS/MS condition**

LC-MS/MS analyses were carried out on a QTRAP 5500 triple quadrupole mass spectrometer equipped with ExionLC AD (AB SCIEX, USA). An Agilent SB C18 column (2.7 μm, 30 mm × 2.1 mm, Agilent, USA) was used for liquid chromatography separation. Solvent A (0.1% FA in deionized water) and solvent B (0.1% FA in ACN) were employed as mobile phases with a flow rate of 0.3 mL/min at 40 °C. The linear gradient was as following: B 10% (0 min) → 35% (3 min) → 90% (4 min) → 90% (8 min) → 10% (10 min). The mass spectrometer equipped with electrospray ionization (ESI) source was operated in positive ion mode and multiple reaction monitoring (MRM) mode. The MS parameters were applied as follows: curtain gas, 35 psi; nebulizer gas (GS1), 55 psi; turbo gas (GS2), 55 psi; ion spray voltage, 5500 V; source temperature, 550 °C; and dwell time, 150 ms. Data acquisition and analyses were conducted using AB SCIEX Analyst® Software (version 1.6.3). The peak area ratio of the analyte over the internal standard was used

for quantification.

Before mass spectrometric analysis, the supernatants were desalted by a MicroSpin C18 column (The Nest Group, Inc., MA, USA) according to the manufacturer's protocol. Briefly, the column was pretreated by applying 100 μL of deionized water and 100 μL of ACN before sample loading. Afterward, the mixture was transferred to the column and the column was washed with 100 μL of 5% ACN containing 0.1% TFA and eluted with 100 μL of 80% ACN containing 0.1% FA. Finally, the eluent was subjected to LC-MS/MS detection.

**S5. MALDI-TOF MS analysis**

The $M_w$ of molecules was acquired on a MALDI-TOF MS (UltrafeXtreme, Bruker Daltonics, USA) equipped with a SmartBeam laser by using 2,5-dihydroxybenzoic acid (DHB) as a matrix for PAMAM-DBCO, *trans*-2-[3-(4-*tert*-butyl-phenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) for DM and *α*-cyano-4-hydroxycinnamic acid (CHCA) for the other molecules. CHCA was dissolved in ACN/deionized water (30/70, *v/v*) containing 0.1% TFA at a concentration of 20.0 mg/mL. DHB was dissolved in ACN/deionized water (50/50, *v/v*) containing 0.1% TFA at a concentration of 10.0 mg/mL. DCTB was dissolved in tetrahydrofuran at a concentration of 40.0 mg/mL. 1 μL of each analyte at a concentration of 10.0 mg/mL in methanol was mixed with 1 μL of the matrix. The sample was then spotted directly onto an MTP 384 ground steel MALDI target plate. Once the matrix was dry, mass spectra were obtained by MALDI-TOF MS. The conditions for the spectra acquired

from *m/z* 50 to *m/z* 5000 in the positive ion mode and reflectron mode were as follows. SmartBeam laser: 1000 Hz, laser power: 70~75%, ion source 1: 20 kV, ion source 2: 17.6 kV and lens: 7 kV. The conditions for the spectra acquired from *m/z* 5000 to *m/z* 500000 in the positive ion mode and linear mode were as follows. SmartBeam laser: 2000 Hz, laser power: 95%, ion source 1: 20 kV, ion source 2: 18.3 kV and lens: 8.25 kV. FlexAnalysis software 3.4 (Bruker Daltonik GmbH, Bremen, Germany) was used for data analysis.

**S6. Synthesis of FITC-labeled DM-immobilizing beads**

FITC-labeled DM was prepared by the conjugation of FITC and PAMAM in DM molecule *via* a click reaction between the primary amine of PAMAM and the isothiocyanate (NCS) of FITC according to the previous report.[5, 6] In brief, 500 μL of 15.0 mM DM in deionized water and 500 μL of 25.7 mM FITC in DMSO were mixed and stirred at 600 rpm at room temperature in the dark overnight. Subsequently, the product was purified by dialysis bag with a MWCO of 10.0 kDa in deionized water for 72 h in the dark. Finally, FITC-labeled DM was obtained by lyophilization.

Subsequently, the FITC-labeled DM-immobilizing beads were prepared by immobilizing FITC-labeled DM on streptavidin agarose beads. In brief, streptavidin agarose beads were washed with PBS three times. Then, 1 mL of streptavidin agarose beads was mixed with 1 mL of 200 nM FITC-labeled DM and incubated under 37 °C for 2 h. Subsequently, the beads were washed with PBS three times and stored at 4 °C in PBS.

## S7. Determination the number of mass tags in each DM

The amount of conjugated mass tags can be estimated from the released mass tags after DM was exposed to UV light for 40 min. The number of mass tags per DM was estimated as the amount of conjugated mass tags divided by the amount of DM, which was equivalent to that of the reactant PAMAM-DBCO.

$$\text{Number of mass tags per DM} = \frac{\text{Number of conjugated mass tags}}{\text{Number of DMs}} \quad \text{(S1)}$$

## S8. Determination of the number of DMs in each bead

The amount of immobilized DM can be estimated from the released mass tags in the supernatant. The number of immobilized DM molecules per bead was calculated as the amount of immobilized DM molecules divided by the number of beads in reaction.

$$\begin{aligned}&\text{Number of DMs per bead}\\ &= \frac{\text{Number of immobilized DMs}}{\text{Number of beads}}\\ &= \frac{\text{Number of conjuated mass tags}/\text{Number of mass tags per DM}}{\text{Number of beads}}\end{aligned} \quad \text{(S2)}$$

## S9. Evaluation of probe stability

The MP-CMSA probe in PBS was stored at 4 °C for different times (1, 2, 3, 4, 5, 6 or 7 days) or incubated at 37 °C for different times (4, 8, 16, 24, 32, 40 or 48 h). At each time point, 50 μL of probe solution was withdrawn, washed with PBS three times, and exposed to UV light at 365 nm for 40 min. Finally, the supernatant was

analyzed by LC-MS/MS. The remained MP-CMSA probe was calculated according to the following equation:

Remained MP-CMSA probe (%) =

$$= \frac{\text{Amount of released mass tag}}{\text{Amount of mass tag in MP-CMSA probe}} \times 100\% \quad \text{(S3)}$$

**S10. BLA hydrolysis of BPEG-7ACA-N3**

Fifty microliters of 50.0 nM BPEG-7ACA-N3 were mixed with 50 μL of 200 nM BLA. The mixture was stirred at 100 rpm at 37 °C for 2 h, transferred into an ultrafiltration centrifuge tube with a MWCO of 10.0 kDa and centrifuged at 8000 rpm at room temperature for 10 min. Subsequently, the filtrate was obtained and analyzed by MALDI-TOF MS.

**S11. Bacterial strains, culture and analysis**

Non-BLA-expressing bacteria strain *Escherichia coli* (*E. coli*, ATCC 25922), and BLA-expressing bacteria strains including TEM-1-expressing *Escherichia coli* (*E. coli*/TEM-1, ATCC 35218), SHV-18-expressing *Klebsiella pneumoniae* (*K. pneumonia*/SHV-18, ATCC 700603) and KPC-3-expressing *Klebsiella pneumoniae* (*K. pneumonia*/KPC-3, ATCC BAA-2814) were employed (Beijing Baio Bowei Biotechnology Co., LTD, Beijing, China). As previously reported, the BLA concentration in $10^5$ colony-forming units (CFU)/mL BLA-expressing bacteria was in the picomolar range.[7] Bacteria were grown in lysogeny broth (LB) and plated on LB

agar plates at 37 °C and 100 rpm during log-phase growth. The bacteria concentration in CFU/mL were measured by detecting the optical density at 600 nm ($OD_{600}$).

For bacterial analysis, bacteria were precipitated at 12000 rpm at 4 °C for 8 min, resuspended in 100 μL of PBS and sonicated for 20 s using an XL-2000 Series probe sonicator set at 5 W and 22 kHz. Subsequently, 100 μL of lysate with $10^6$ CFU/mL was mixed with 100 μL of 10.0 μM MP-CMSA probe and incubated in the dark at 37 °C for 2 h. Thereafter, the sample was centrifuged at 1000 rpm for 5 min, and the pellets were washed with 100 μL of PBS three times. The collected supernatants were combined and exposed to UV light for 40 min and then subjected to LC-MS/MS analysis.

**S12. BLA inhibition assay**

First, 50 μL of 20.0 pM BLA was pretreated with or without 50 μL of 100 pM potassium clavulanate at 37 °C for 20 min. Then, the sample was mixed with 100 μL of 10.0 μM MP-CMSA probe and incubated for 2 h at 37 °C. After treatment, the sample was centrifuged at 1000 rpm for 5 min, and the pellets were washed with 100 μL of PBS three times. The collected supernatants were combined and exposed to UV light for 40 min and then subjected to LC-MS/MS analysis.

**S13. Clinical blood collection**

Blood collection in the current work was approved by the Institutional Review Board of Nanjing Medical University. The methods were carried out by the approved

guidelines. Thirty-five discarded blood samples from patients with bacterial infection were collected consecutively between May 2020 and August 2021 from Zhongda Hospital of Southeast University, Nanjing, China (Age range: 27-85 years; mean age: 63.2 ± 1.37 years; age ≥ 65 ($n$ = 18), age < 65 ($n$ = 17)). All of the patients belong to the Han Chinese ethnic group from Jiangsu province in China without being biologically related. Informed consent was obtained from the subjects. BLA-associated DRB of the samples was originally defined by hospital pathologists using gold-standard AST (disc diffusion test, K-B method, **Figure S11**) following the CLSI.[3]

## S14. Disc diffusion test (K-B method)

The K-B method was issued by the CLSI for susceptibility test as the following[3]: (1) Select 5 well-isolated colonies of the same morphological type from the agar plate grown at 37 °C overnight. Transfer the bacteria growth to a tube containing 3 mL LB medium. Allow the broth culture to incubate at 37 ˚C until it achieves or exceeds the turbidity of 0.5 McFarland standard. Adjust the turbidity with sterile broth to 0.5 McFarland standard ($OD_{600}$ ~ 0.080-0.135). (2) Within 15 min after adjusting the turbidity of the inoculum suspension, transfer the growth to a Muller-Hinton agar plate tube. (3) Place the antibiotic disks evenly (no closer than 24 mm from center to center) on the surface of the agar plate. (4) Invert the plate and place them in an incubator at 37 °C within 15 min after disks are applied. After 16-18 h of incubation, examine each plate and measure the diameter of the zones of complete inhibition.

# Notes and References


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