

# Rapid Identification of Bacteria by Membrane-Responsive Aggregation of a Pyrene Derivative

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Supporting Information

**ABSTRACT:** An imidazolium-derived pyrene aggregation was developed to rapidly identify and quantify different bacteria species. When the nonemissive aggregates bound to the anionic bacteria surface, the sensor disassembled to turn on significant fluorescence. At the same time, ratiometric signals between pyrene monomer and excimer emission were controlled by different interactions with various bacteria surfaces. The resulted different fluorescent emission profiles then were obtained as fingerprints for various bacterial species. By converting emission profiles directly into output signals of two channels, fluorescence increase and ratiometric change, a two-dimensional analysis map was generated for bacteria identification. We demonstrated that our sensor rapidly identified 10 species of bacteria and 14 clinical isolated multidrug-resistant bacteria, and we determined their staining properties (Gram-positive or Gram-negative).



KEYWORDS: bacteria identification, multidrug resistant, pyrene derivative, aggregation, rapid identification

ntibiotic resistance is spreading faster than the A introduction of new antibiotics, causing bacterial infection, especially nosocomial infection, which represents a significant public health crisis.<sup>1</sup> With the overuse of antibiotics, multidrug-resistant (MDR) bacteria strains have developed a resistance to most common agents, such as methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococci (VRE) and certain Gram-negative bacilli (GNB).<sup>2</sup> The MDR profile of a bacterium is usually the combinational results of different mechanisms, including producing drug-inactivating enzymes, efflux pumps, and target-site or outer membrane modifications.<sup>3-5</sup> Because of the dynamic, random, and complex characteristics of antibiotic resistance, a one-off method monitoring the differences of a universal component of bacteria instead of single biomarker is more suitable for bacteria identification and, particularly, the screening and diagnosis of MDR species. Moreover, this method should be rapid and efficient to help doctors administer targeted treatments with reliable therapeutic effects.

Traditional "lock-key" sensors that are based on polymerase chain reaction (PCR) and immunological techniques recognize specific biomarkers with selective receptors (primers or antibody).<sup>6,7</sup> These sensors can confirm a clinical diagnosis accurately, but are powerless for bacteria with unknown

identities or antibiotic susceptibilities. MS-based omics approaches collect comprehensive data of proteins, DNA, or metabolomics for fingerprint analysis of bacterial dynamic changes.<sup>8</sup> However, the pretreatment is complex and timeconsuming. Surface-enhanced Raman spectroscopy (SERS) emerges as another fingerprint analysis method for microbial identification.<sup>9</sup> However, this method requires a SERS-active substrate and is limited by the poor substrate homogeneity and reproducibility.

Recently, fluorescent sensor arrays (FSA) have been developed to discriminate bacteria and even unknown species with rapid, easy-to-use, and highly sensitive characteristics.<sup>10–18</sup> As various bacteria have different surface physical chemical properties, differential pattern signals are generated from nonspecific interactions between diverse cross-reactive sensors and different bacteria surfaces. A recently reported FSA had the ability to identify 14 bacteria with variable genetic similarities.<sup>11</sup> However, the identification of MDR bacteria is still challenging.<sup>12</sup> In addition, more than three unspecific sensors should be reasonably designed and optimized. It will

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#### Scheme 1. Proposed Mechanism and Signal Output of PI-BactD for Rapid Identification of Bacteria

be ideal to develop a new method based on a single molecule with orthogonal sensing properties to identify various bacteria rapidly and efficiently. Although Wang et al. reported a conjugated polymer to discriminate three types of microbial pathogens,<sup>19,20</sup> to the best of our knowledge, there is no such a single sensor for identifying multiple bacterial species, especially for MDR ones.

In this paper, we reported a simple sensor that can rapidly identify 10 species of bacteria and 14 clinical isolated MDR bacteria, while determining their staining properties (Grampositive or Gram-negative) from visual interpretation. The structure of designed sensor PI-BactD is shown in Scheme 1. Imidazolium was selected as the binding site for its wide uses to recognize anionic bacteria surface through electrostatic interaction.<sup>21,22</sup> Pyrene, as a classic fluorophore that can emit both excimer and monomer pyrene fluorescence, was selected to give different ratiometric signals.<sup>23,24</sup> Herein, PI-BactD aggregated to form nanoparticles and the fluorescence was quenched by the aggregation effects. After the addition of bacteria, synergistic effects of electrostatic interaction and hydrophobic force caused competing binding between bacteria surfaces and sensor. This binding resulted in the disassembly of the sensor to give fluorescence turn-on signal. Meanwhile, PI-BactD bound bacteria surfaces and displayed both pyreneexcimer and pyrene-monomer fluorescence, which gave a ratiometric signal. Subsequently, based on output signals of these two channels, a two-dimensional analysis map could be established for visual interpretation.

We first investigated the aggregation property of PI-BactD. In DMSO solution, PI-BactD emitted pyrene monomer emission centered at 375 nm, indicating that PI-BactD was uniformly dispersed. With the addition of water, the fluorescence was gradually quenched. With 99.5% water content, the emission was even hardly detectable (Figure 1a). The dynamic light scattering (DLS) and transmission electron microscopy (TEM) experiments confirmed the formation of nanoparticles (Figure 1b). We attributed the fluorescence quenching to the aggregation effects as a result of pyrene-pyrene stacking. The slight blue shift and obvious decrease in the absorption spectra also indicated the formation of aggregation (Figure S1 in the Supporting Information). It is also interesting to note that the size of the formed nanoparticles increased as the sensor concentration increased (Figure S2 in the Supporting Information).

To verify the feasibility of the sensor, we next detected the fluorescence responses of our sensor to surfactant micelles. These micelles were recognized as model<sup>25,26</sup> of bacterial cell surface (see Figure 1c, as well as Figure S3 in the Supporting



**Figure 1.** Assembly disassembly of **PI-BactD**. (a) Fluorescence spectra of **PI-BactD** (10  $\mu$ M) in DMSO/H<sub>2</sub>O mixtures ( $\lambda_{ex}$  = 345 nm); (b) DLS and TEM analysis of 10  $\mu$ M **PI-BactD**; (c) Fluorescent responses of the sensor to the presence of SDS (8 mM), BS-12 (1.8 mM), DTAB micelles (16 mM), BSA, GST, Her2, Trypsin proteins, and other ions (ATP, ADP, AMP, PPi, SO<sub>4</sub><sup>2-</sup>, PO<sub>4</sub><sup>3-</sup>, NO<sub>3</sub><sup>-</sup>, HPO<sub>4</sub><sup>2-</sup>, CO<sub>3</sub><sup>2-</sup>, CH<sub>3</sub>COO<sup>-</sup>, Br<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup>) in HEPES (20 mM, pH 7.4) (inset shows binding patterns of **PI-BactD** to zwitterionic BS-12 (left) and anionic SDS micelle (right)); (d) DLS and TEM analysis of **PI-BactD** in the presence of 400  $\mu$ M SDS.

Information). Since the cell walls of bacteria are negatively charged with the isoelectric point of  $\sim 2-4$ ,<sup>27</sup> anionic surfactants and zwitterionic surfactants are considered to mimic two extreme conditions on the surface of bacterial membranes: only negative charges or net neutral charges, respectively. As shown in Figure 1c, the addition of the sensor to the solution of SDS micelle (anionic) induced significant excimer emission. While in the solution of BS-12 micelle (zwitterionic), both monomer and excimer emissions were observed. Cationic DTAB micelle did not lead to any fluorescence changes. These results suggested that the anions of surfactants interacted with imidazolium and disassembled the sensor to recover the fluorescence. The DLS analysis provided direct evidence of the disassembly process. The sizes of the aggregates decreased from 494.3 nm (Figure 1b) to 190.7 and 357.3 nm in SDS (Figure 1d) and BS-12 (Figure S4) solutions (concentrations lower than CMC), respectively. The slight red shift and obvious increase in the ultraviolet-visible (UV-vis) absorption of **PI-BactD** after binding with micelles

also corroborated the disassembly process (Figure S5 in the Supporting Information). The presence of only excimer emission of PI-BactD bound with SDS indicated that the stronger electrostatic interaction preferred pyrene  $\pi - \pi$ stacking. The cationic repulsive force from zwitterionic BS-12 would separate pyrenes and, hence, induce monomer emission. Thus, both monomer and excimer emissions were coexisting in the case of BS-12. The micelle-dependent binding modes of **PI-BactD** (insets of Figure 1c) inspired us to apply it in the following bacteria identification. If we use anionic and zwitterionic surfactant micelles as a simple bacterial surface model, we can expect that the fluorescence profiles upon bacteria identification should be in the shadow part of Figure 1c. In addition, highly concentrated phosphate-containing anions, such as ATP, etc., cannot disassemble the sensor, which allows the sensor to detect bacteria without the interference with environmental factors. However, the sensor showed weak fluorescence enhancements with BSA and GST, as shown in Figure 1c. Since some of the proteins carry negative charges in a neutral environment, the sensor may interact with these proteins by electrostatic interaction. During the experimental process of bacterial sensing, we have washed the bacteria twice to avoid interference of these proteins.

The sensor was then applied to identify bacteria (see Figure 2, as well as Table S1 in the Supporting Information). Based on the electrostatic interaction and hydrophobic force between anionic bacteria surface and imidazoliums, bacteria dis-



Figure 2. Identification of bacteria. (a) Fluorescence spectra of the sensor (10  $\mu$ M) treated with 10 species of bacteria (OD<sub>600</sub> = 0.5; the solid line represents Gram-negative bacteria, while the dotted line represents Gram-positive bacteria); (b) 2D map obtained from output signals of two channels: fluorescence increase ( $\Delta S/S_0$ ) and fluorescence ratiometric changes ( $I_{482}/I_{375}$ ). Each species have five replicates.

assembled the sensor to emit strong fluorescence (E. coli as an example; see Figure S6 in the Supporting Information). The fluorescence turn-on response makes our sensor a good fluorogenic dye for bacteria staining. More importantly, the sensor exhibited different emission profiles with these 10 bacterial species (Figure 2a), which demonstrated high sensitivity of the probe. As expected, these fluorescence responses appeared in the shadow part of Figure 1c. A twodimensional map with plots depending on both the changes in fluorescence intensity  $(\Delta S/S_0)$  and the emission ratios  $(I_{482}/$  $I_{375}$ ) was then established. As shown in Figure 2b, all these bacteria were distinguished well from visual observation. Note that the sensor can also discriminate between Gram-negative bacteria and Gram-positive bacteria (Figure 2b). By compared Gram-negative bacteria with Gram-positive bacteria with a similar fluorescence increase (for example, C. violaceum vs B. subtilis, or E. coli vs B. Cereus), we noted that Gram-negative bacteria had a stronger tendency to induce pyrene monomer emissions. A simple interpretation is that, due to the higher isoelectric points than Gram-positive species, Gram-negative bacteria have a tendency to disperse pyrenes from each other more effectively, like what zwitterionic surfactant micelle did. Correspondingly, Gram-positive bacteria and anionic surfactant micelle have a tendency to promote PI-BactD to form pyrene excimers. However, we must note that reasons behind the different responses of Gram-negative and Gram-positive bacteria are likely more complicated. Indeed, there are significant differences of the cell walls between Gram-negative and Gram-positive bacteria. Generally, Gram-negative bacteria have two layers of membranes, outer and inner (plasma) membrane, separated by a thin layer of peptidoglycan. The outer membrane is heavily populated with lipopolysaccharides (LPS) and negatively charged by phosphate groups and carboxylate groups present in sugar acids. Gram-positive bacteria contain a single cell membrane surrounded by a thick layer of peptidoglycan. Cell surfaces are negatively charged largely by phosphate groups in the glycerolphosphate repeat units. These structural differences should also play an important role in the interactions between PI-BactD and bacteria. In summary, different emission intensity of pyrene monomer (375 nm) and excimer (482 nm) for different bacteria demonstrated the high sensitivity and selectivity of PI-BactD for bacteria identification.

To further understand the mechanism of the selective binding of **PI-BactD** to different bacteria surfaces, we measured the zeta potentials ( $\zeta$ ), which reflect the surface charges of the bacteria to investigate the interactions between bacteria and **PI-BactD** in PBS (20 mM). As shown in Figure 3a, the zeta potentials of all bacteria exhibited positive potential shift changes with the addition of **PI-BactD**, but the extents of the change were different.

The fluorescence ratiometric responses enable our sensor to quantitatively detect bacteria. First, we monitored the growth of *E. coli* and *B. subtilis*, employing both optical density (OD<sub>600</sub>) and fluorescent emission ratios ( $I_{375}/I_{482}$  (see Figure S7 in the Supporting Information) at the same time. The growth curves almost completely coincided, demonstrating the capability of the sensor for quantitative analysis of bacteria (Figure 3b). To ensure the accuracy of quantitative detection, the titration experiments were then tested and displayed the sensitivity of the probe to various concentrations of bacteria ( $10^3-10^8$  CFU/mL; see Figures S8 and S9 in the Supporting Information). Furthermore, the ratiometric signals of PI-



**Figure 3.** (a) Zeta potentials of *S. aureus, E. coli* and *B. subtilis* with or without **PI-BactD** in PBS (20 mM, pH 7.4). (b) Growth curves of *E. coli* DH5 $\alpha$  and *B. subtilis* monitored by both OD<sub>600</sub> and fluorescent emission ratio ( $I_{375}/I_{482}$ ). The inside of the ordinate is for *B. subtilis*, and the outside is for *E. coli* DH5 $\alpha$ .

**BactD**  $(I_{482}/I_{375})$  adopt a linear relationship with different concentrations of both *E. coli* and *B. subtilis* bacteria in low concentrations (Figure S10 in the Supporting Information). In addition, the detection limits were calculated to be  $1.2 \times 10^5$  CFU/mL for *B. subtilis* and  $3.8 \times 10^5$  CFU/mL for *E. coli*, respectively.

We note that the other two signal channels,  $\Delta I_{482}/I_{482}^{\circ}$  and  $\Delta I_{375}/I_{375}^{\circ}$  can also be used collectively to construct six more

2D analysis map (Figure S11 in the Supporting Information). Except the group of  $\Delta S/S_0$  and  $\Delta I_{482}/I_{482}^{\circ}$  (see Figure S11f), each of these two channels can identify the 10 bacterial species well. In addition, to validate the efficiency of our sensor, 32 unknown bacterial samples randomly collected from the 10 species of bacteria grown in different batches were tested using the procedures developed above. Twenty-eight out of 32 bacteria were correctly determined through measurement of the Mahalanobis distance, and the detection accuracy is 87.5% (Table S2 in the Supporting Information).

Since the sensor can identify various species of bacteria, depending on its transformability as a function of different bacterial surfaces, we felt curious whether it was also sensitive to subtle differences in the same species of bacteria, such as MDR bacteria strains. We first detected artificial ampicillinresistant strains of *E. coli* DH5 $\alpha$  and *E. coli* BL21 (DE3) through transferred plasmid with the antiampicillin gene. All of them can be successfully discriminated (see Figure 4a). Compared to normal bacteria, artificial strains expressed a type of  $\beta$ -lactamase in periplasm to deactivate ampicillin properties by breaking the  $\beta$ -lactam ring open. The subtle differences among these bacteria still induced significant fluorescence changes, indicating that the changes. It is known that MDR E. coli and MDR S. aureus strains are major causes in Gram-negative and Gram-positive bacterial infections. Thus, 14 strains of MDR E. coli or MDR S. aureus isolated from clinical specimens (see Tables S3 and S4 in the Supporting Information) were used to further examine the sensitivity and selectivity of our sensor. The 2D map in Figures 4b and 4c showed that most of the strains were discriminated successfully. Moreover, Gram-positive and Gram-negative bacteria also can be well-distinguished. These results demonstrate the rapid, efficient, sensitive, and selective features of our sensor for bacteria identification. Particularly, the identification of MDR E. coli or MDR S. aureus may have potential application in clinical diagnosis. In addition, the ability of discriminating bacterial surface differences makes it possible for our one-molecule sensor to detect even more bacterial species than our current report. We are currently using this sensor to study more bacterial species and track bacterial resistance.

In conclusion, we have reported a simple sensor for identifying bacterial species. This imidazolium-derived pyrene aggregate bound the anionic bacteria surface and disassembled



**Figure 4.** Two-dimensional (2D) map identifying antibiotics-resistant bacteria: (a) *E. coli* DH5 $\alpha$ , *E. coli* BL21 (DE3), and their artificial ampicillinresistant bacteria strains (+); (b) MDR *E. coli* strains isolated from clinical specimens; (c) MDR *S. aureus* clinical isolates. Each strain has five replicates. OD<sub>600</sub> = 0.5. Dotted lines are present to distinguish between Gram-negative and Gram-positive bacteria; they are defined as presented in Figure 2b.

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to form various combinations of pyrene monomer and excimer binding modes, as controlled by electrostatic and hydrophobic interaction with various bacteria surfaces. Depending on this ability, our sensor can rapidly identify 10 species of bacteria and 14 clinical isolated multidrug-resistant bacteria and determine their staining properties (Gram-positive or Gramnegative).

### ASSOCIATED CONTENT

#### **S** Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.8b01466.

Synthesis, characterization, experimental details (PDF)

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#### Notes

The authors declare no competing financial interest.

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