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Detecting live bacteria instantly utilizing AIE strategies†

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A new class of biosensor molecules evoking fluorescent emission by rotation-restricted binding with bacteria was examined for its applicability in detecting live bacteria instantly. The fluorogens possessed multiple tetraphenylethene (TPE)-cored boronic acids to oligomerize through complexation with *cis*-diols on bacterial surfaces, resulting in aggregation-induced emission (AIE). The fluorogen having two boronic acid units discriminated between live and dead bacteria by showing AIE activity only with the latter. Live bacteria were instantly detected by consequent treatment with reagents of three and four di-boronates (which showed AIE activity with both live and dead bacteria). This phenomenon may lead to a practical method for live bacteria detection.

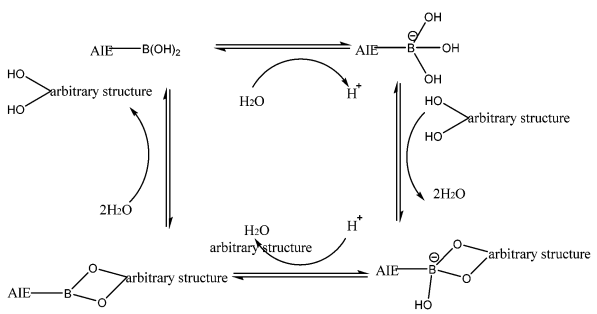
Introduction

Rapid detection of bacteria has attracted increasing research efforts in applications ranging from food processing, clinical diagnosis and operations, and pharmaceutical development to environment monitoring.[1–3] In these applications, bacterial viability is a vital inspection item for which rapid, simple and accurate detection offer significant improvement.

Bacterium growth involved in the abovementioned industries is currently identified by the plate count method[4,5] or high-resolution microscopy.[6] The counting method is a lengthy process typically over 24 h that grows bacteria with sufficient population to be examined on an agar medium.[7–10] For those bacteria that are viable but non-culturable, various high-resolution microscopic imaging methods become indispensable because their viabilities have to be recognized indirectly through changes in morphology and membrane potential deviation.[6] Compared to these laborious methods, detection of live bacteria using fluorescence-based methods may be direct and handy and would not require sophisticated instruments, thus being more adaptable to a variety of cases.[5,11,12] The most essential criterion for achieving rapid fluorescent detection is the creation of highly responsive and bacteria specific dyes.

To endow wild bacteria with fluorescent activity, electrostatic attachment of cationic dyes to anionic bacterial surface is commonly applied. The multiple charged fluorescent dyes, however, often result in severe alterations of bacterial states by inducing aggregation or metabolic changes in the bacteria. In addition, fluorescent labelling based on electrostatic adsorption is unable to distinguish between live and dead bacteria. To detect live bacteria, methods based on chemically specific mechanisms have to be developed.

The surfaces of most bacterial species are structured with abundant polysaccharides.[13] The major component of the bacterial cell wall is peptidoglycan. Peptidoglycan in Gram-positive bacterial cell wall accounts for 50–80% of the dry weight, and the peptidoglycan in the cell wall of Gram-negative bacteria accounts for 5–20% of the dry weight. Surface polysaccharides possess multiple hydroxyls in a variety of configurations[13] and are used as binding sites for fluorescent active reagents. Early studies of alcohol-affinitive molecules revealed that diols associated with



Scheme 1 Schematic of the binding process between phenylboronic acid and diol.

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phenylboronic acid rapidly and reversibly in aqueous media, as schematically described in Scheme 1.[14–17]

Phenylboronic acids are actively used as specific binding sites for biosensors to complex with diols of carbohydrates.[16–24] This mechanism inspired us to conjugate phenylboronic acids to fluorescently active dyes in order to detect polysaccharide surfaces of bacteria. In a previous study, a unique indicator molecule, 4,4′-(1,2-diphenylethene-1,2-diyl)bis(4,1-phenylene) diboronic acid (**TPE-2BA**), emitted fluorescence only when complexed with *cis*-diols of D-glucose to form aggregated states.[17] This phenomenon was named aggregation-induced emission (AIE).[25] This term represents the chemical mechanism where an indicator reagent becomes fluorescently inactive in free molecule form, but turns active in an aggregated state in which its aromatic planes are piled together and unable to rotate intramolecularly.[25–52] Phenyl rings function as peripheral aromatic rotors surrounding a central olefin stator, making the molecules prototype AIEgens, *i.e.*, the tetraphenylethene of AIE active reagents[25] (TPE). By conjugating selected binding heads (such as di-boronic groups) with peripheral phenyl rings, predetermined targets are complexed specifically and imaged fluorescently due to binding induced aggregation.

However, our previous study showed that TPE with only two phenyl groups equipped with boronic acid, abbreviated **TPE-2BA**, did not stain live bacteria.[3] It was quite likely on the basis of the AIE mechanism that the two phenyl rings without boronic acid were freely rotatable on the bacterial surface. We therefore hypothesized that AIEgens completely comprising phenylboronic acid should be able to image live bacteria. In this study, we synthesized three and four phenylboronic acid compounds, (named **TriPE-3BA** (4-(2,2-bis(4-boronophenyl)vinyl)phenyl)-boronic acid) and **TPE-4BA** (ethene-1,1,2,2-tetrayltetrakis-(benzene-4,1-diyl)tetraboronic acid), as fluorescent probes for live bacteria detection. The boronic acid-conjugated AIEgens could be used to achieve instant and reliable detection of live bacteria through a new mechanism, without causing aggregation and without altering the metabolism of live bacteria, which are associated with currently available indicators based on electrostatic interaction. AIE involving bacteria detection based on mild complexation between boronic acids and hydroxyls on bacteria surfaces may therefore offer improved detection of live bacteria.

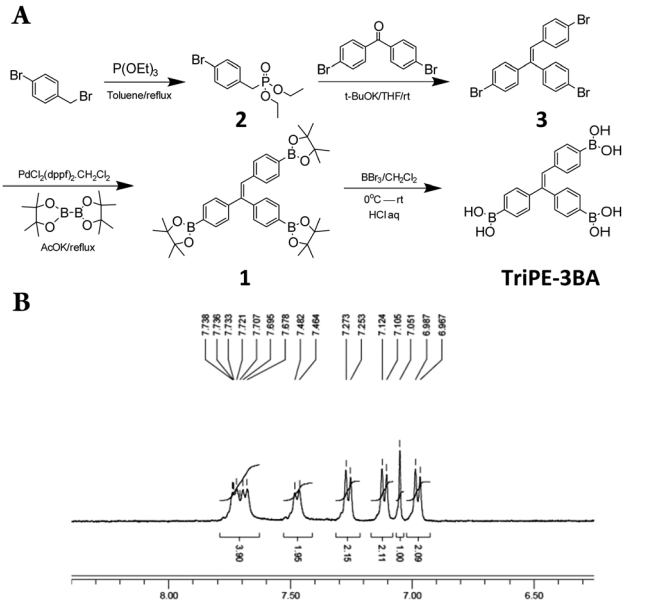

Fig. 1 (A) Synthetic route to **TriPE-3BA**. (B) ^{1}H NMR spectrum of **TriPE-3BA**.

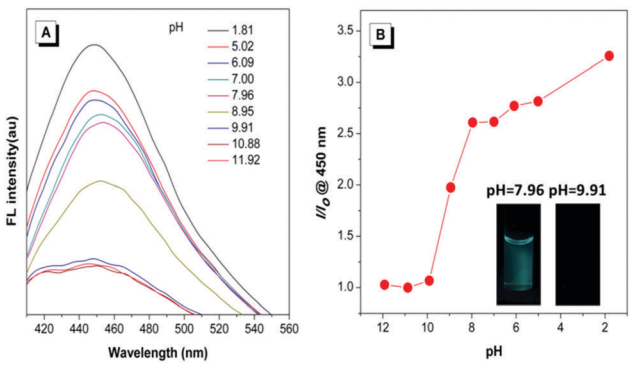

Fig. 2 (A) FL spectra of **TriPE-3BA** (10 μM) in Britton–Robinson buffers containing 2 vol% DMSO at different pH values. (B) Change in FL intensity (I) of **TriPE-3BA** at 450 nm with pH; I_0 is the intensity at pH 10.88. Inset: Photographs of mixtures of **TriPE-3BA** in aqueous buffers at pH 7.96 and 9.91 taken under illumination of a hand-held UV lamp (λ_{ex} = 365 nm).

Results and discussion

TriPE-3BA was synthesized according to the synthetic routes[53,54] outlined in Fig. 1. Successful synthesis of **1** was confirmed using ^{1}H-NMR, ^{13}C-NMR, HRMS (Fig. S1–S3, ESI†) and elemental analysis. Successful synthesis of **TriPE-3BA** was confirmed structure using ^{1}H-NMR, ^{13}C-NMR, HRMS (Fig. 1 and Fig. S4–S6, ESI†) and elemental analysis. **TPE-4BA** was synthesized according to the literature method.[55] HRMS (Fig. S7, ESI†) and elemental analysis are presented.

Prior to bacteria detection, the AIE activities of the synthesized molecules **TriPE-3BA** and **TPE-4BA** were confirmed *via* chemically switched aggregation and disaggregation states. For example, **TriPE-3BA** aggregated at neutral pH but disaggregated at pH > 9.9 since pK_a = 9 of its phenylboronic acid. The result in Fig. 2 confirmed from the AIE activity of **TriPE-3BA** that the fluorescent emission of its suspension was intense in its aggregated state (neutral pH) but dismissed in its soluble state (pH > 9). The AIE activity of **TPE-4BA** was also confirmed the same way (Fig. S8, ESI†).

To examine the practical feasibility of TPE-*x*BA class AIEgens in live bacteria detection, two well-studied and easy to handle ubiquitous bacteria – Gram-positive *S. epidermidis* and Gram-negative *E. coli* – were used as models. Dead and live cells were prepared by incubating *E. coli* and *S. epidermidis* in 75% ethanol and PBS buffer, respectively, for an optimized time. The concentrations of AIE dyes and pH of the staining solution

were also optimized. Since the fluorescence intensity varied significantly during the initial 0.5 h of incubation, all the samples were prepared by 0.5 h incubation prior to fluorescence imaging. **TriPE-3BA** or **TPE-4BA** 100×10^{-6} M in concentration was found to be appropriate for carrying out bacterial assays with sufficient signal intensity without any interruption from the background. For the pH of the assays, 7.4 was found to be optimal to avoid precipitation of AIE dyes and maintain viabilities of the bacteria. To confirm the capability of the TPE-*x*BA system to detect live bacteria, the well-used dead bacteria indicating dye PI (propidium iodide) was used for comparison. The bacteria were rinsed with fresh PBS buffer thrice to remove possible saccharides that might affect the results prior to the bacterial staining experiment.

The dead and live cells were first incubated with **TriPE-3BA** or **TPE-4BA** for 0.5 h prior to addition of PI, and then subjected to extended incubation for 20 min. As indicated in Fig. 3 and 4, **TPE-2BA** could not dye live bacteria (but only dead bacteria),[3] **TriPE-3BA** and **TPE-4BA** dyed live bacteria, and dead bacteria with blue color. However, the bacteria themselves emitted blue fluorescence upon the same laser radiation, and the intensity of this spontaneous fluorescence was nearly invisible compared to that emitted from **TriPE-3BA** and **TPE-4BA**; the fluorescent indicators bonded to the bacteria and were detected (Fig. S9, ESI†). Further treatment of bacteria samples with PI indicated dead bacteria with red colour but not live bacteria, confirming that live bacteria exists in the culture medium.

Considering that live and dead bacteria co-exist in samples under many real circumstances, they were co-cultivated with **TriPE-3BA** or **TPE-4BA**, followed by the same treatment with PI. Since live bacteria showed blue emission of AIEgens while dead bacteria emitted both blue and red fluorescence when treated with **TriPE-3BA**/**TPE-4BA** and PI, they could be discriminated by comparing the images of the sample treated with the two dyes.

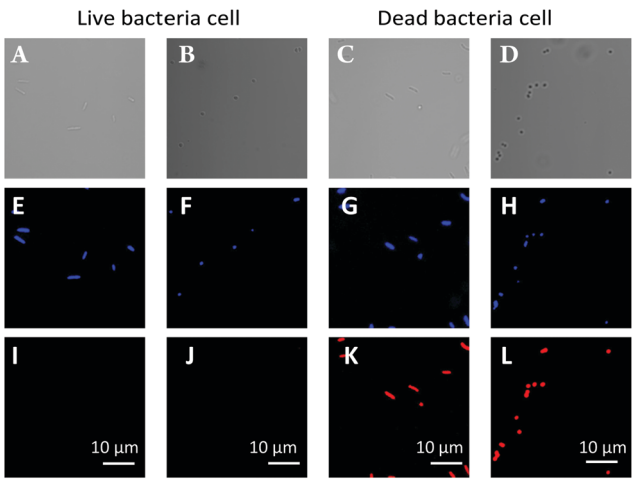

Fig. 3 (A–D) Bright-field and (E–L) fluorescence images of live and dead (A, E, I, C, G and K) *E. coli*, (B, F, J, D, H and L) *S. epidermidis* stained first with 100×10^{-6} M **TriPE-3BA** for 0.5 h and then 50×10^{-6} M PI for 20 min. The micrographs were taken under irradiation of (E–H) UV light and (I–L) green light.

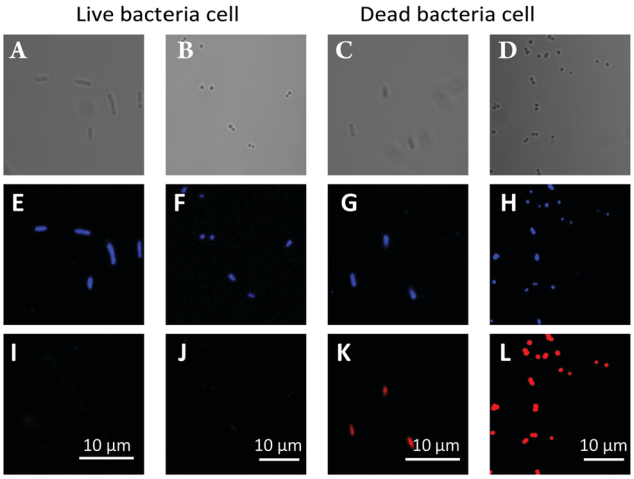

Fig. 4 (A–D) Bright-field and (E–L) fluorescence images of live and dead (A, E, I, C, G and K) *E. coli*, (B, F, J, D, H and L) *S. epidermidis* stained first with 100×10^{-6} M **TPE-4BA** for 0.5 h and then 50×10^{-6} M PI for 20 min. The micrographs were taken under irradiation of (E–H) UV light and (I–L) green light.

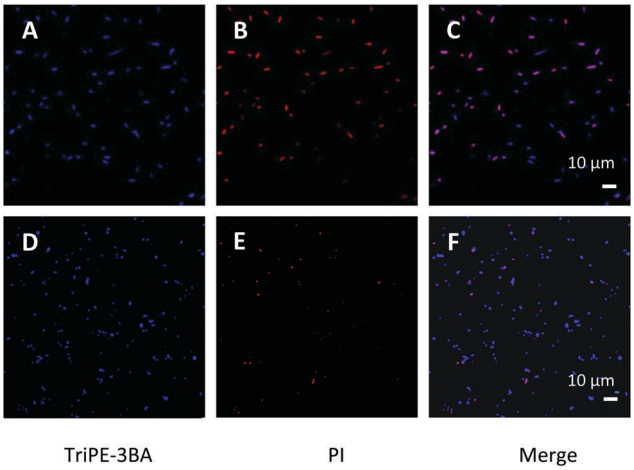

Fig. 5 (A–F) Fluorescence images of live and dead (A–C) *E. coli*, (D–F) *S. epidermidis* stained (A and D) first with 100×10^{-6} M **TriPE-3BA** for 0.5 h and then (B and E) 50×10^{-6} M PI for 20 min. (C) The merge of A and B. (F) The merge of D and E.

The images shown in Fig. 5 and 6 confirmed the rationale that on placing the image obtained by PI treatments on top of the image of **TriPE-3BA**/**TPE-4BA** treatment, we observe some un-overlapped blue dots, which were obtained from live bacteria. The boronic acid conjugated AIEgens may serve as a detection agent to indicate live and dead bacteria with a new mechanism.

Although there was a possibility that the fluorescence images resulted from aggregated AIEgens at pH 7.4, our previous results using **TPE-2BA** to treat live and dead bacteria showed that only the dead bacteria were stained with blue color,[3] suggesting that the dye did not aggregate under our experimental concentration. The difference between **TPE-2BA** and **TriPE-3BA** (or **TPE-4BA**) in binding live bacteria represented the

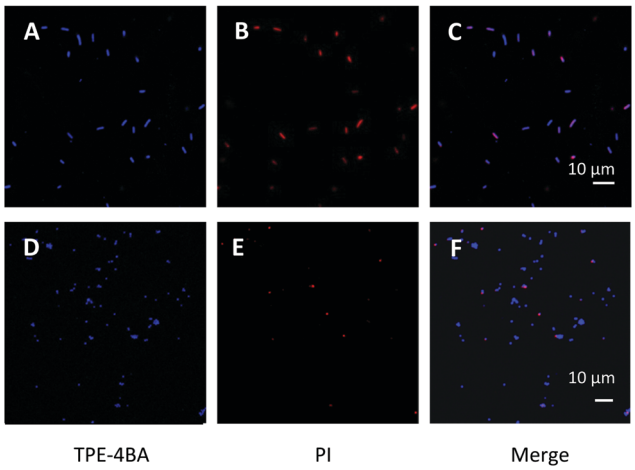

Fig. 6 (A–F) Fluorescence images of live and dead (A–C) *E. coli*, (D–F) *S. epidermidis* stained (A and D) first with 100×10^{-6} M **TPE-4BA** for 0.5 h and then (B and E) 50×10^{-6} M PI for 20 min. (C) The merge of A and B. (F) The merge of D and E.

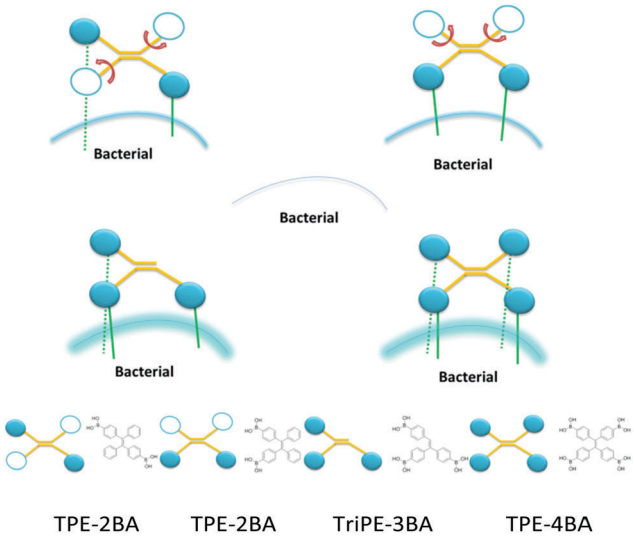

Fig. 7 Proposed mechanism for bacterial sensing by AIE-active bioprobes.

typical properties of AIEgens. We speculated that the boronic acid of **TPE-2BA** bonded with hydroxyls on bacterial surfaces, leaving the other two unbonded aromatic rings free for rotation. With **TriPE-3BA** and **TPE-4BA**, each of the phenyl rings was conjugated with boronic acid and complexed with the bacterial surface hydroxyl so that rotation of all the phenyl rings were fully restricted (Fig. 7).

Conclusion

We demonstrated the feasibility and mechanistic rationales of using AIEgens to detect live bacteria. Since the binding of dyes with bacteria was achieved through boronic acid–diol interaction, the drawbacks of the currently-used detecting reagent with multi-ionic binding such as aggregation and altered metabolism of live bacteria could be avoided. The newly demonstrated boronic acid complexing mechanism for bacteria detection offers additional choices for assaying biologic markers.

Experimental section

Materials and instrumentation

Luria broth agar, Luria broth, and sodium phosphate were purchased from USB Co., Ltd. Potassium phosphate monobasic was purchased from ReideldeHaën. PI, potassium chloride, sodium chloride, boron tribromide, acetic acid, phosphoric acid, hydrochloric acid, sodium hydroxide, boric acid, toluene, bis(pinacolato)diboron, bis(4-bromophenyl)methanone, 4-bromobenzyl bromide, [1,1′-bis(diphenylphosphino)ferrocene] dichloropalladium(II), potassium *tert*-butoxide, and potassium acetate were purchased from Sigma-Aldrich. Triethyl phosphite was purchased from Alfa Aesar. Dichloromethane, purchased from Sinopharm Chemical Reagent Co., Ltd, was of analytical grade. DMSO was purchased from Alfa Aesar in HPLC grade (packaged under argon in a resealable ChemSeal™ bottle). THF was purchased from Aldrich and distilled under normal pressure from sodium benzophenone ketyl under argon immediately before use.

^{1}H and ^{13}C NMR spectra of **1** and **TriPE-3BA** were measured on a Mercury plus 400 MHz NMR spectrometer in $CDCl_3$ and CD_4O, respectively, using tetramethylsilane (TMS; $\delta = 0$ ppm) as internal standard. High-resolution mass spectra (HRMS) were taken on a GCT Premier CAB 048 mass spectrometer operating in MALDI-TOF mode. Elemental analysis for O was performed on a Thermo Scientific FlashSmart elemental analyzer. Elemental analyses for C and H were performed on a vario EL cube elemental analyzer. Elemental analysis for B was obtained by Thermo Scientific iCAP 7600 ICP-OES. UV-vis absorption spectra were obtained on a Milton Ray Spectronic 3000 array spectrophotometer. FL spectra were recorded on a Hitachi 4500 spectrofluorometer. Laser confocal scanning microscopy images were collected on a Zeiss laser scanning confocal microscope (LSM7 DUO) and analyzed using ZEN 2009 software (Carl Zeiss).

Synthesis and characterization

TriPE-3BA was prepared according to the synthetic route shown in Fig. 1. Detailed experimental procedures and characterization results are given below.

Preparation of dixethyl(4-bromobenzyl)phosphonate (2) and 4,4′,4″-(ethane-1,1,2-triyl)tris(bromobenzene) (3). The two products were prepared according to previously published experimental procedures.[53,54] A white solid was obtained in 65% yield.

Preparation of 2,2′,2″-(ethane-1,1,2-triyltris(benzene-4,1-diyl))-tris(4,4,5,5-tetramethyl-1,3,2-dioxaborolane) (1). Ar was bubbled for 30 minutes through a mixture of anhydrous dioxane (120 mL) and 5.7 g KOAc (0.06 mol) in a dried 300 mL two-neck round bottle flask. TriPE-3Br (3.92 g, 0.008 mol), bis(pinacolato)diboron (6.7 g, 0.026 mol), and [1,1′-bis(diphenyl-phosphino)-ferrocene]-palladium(II) dichloride dichloromethane adduct (227 mg, 0.28 mmol) were added under Ar. The mixture was degassed through three freeze–pump–thaw cycles and heated to reflux at

110 °C for 72 h and monitored using thin-layer chromatography. The reaction mixture was cooled to room temperature and then, the obtained dioxane was removed under reduced pressure and poured to ice-cooled water. The solution was stirred for 10 minutes and filtered; the residue was washed with water and dried under vacuum. The residue was dissolved in chloroform and purified using flash column chromatography with ethyl acetate/chloroform (1/1 v/v) as eluent. The solvent was removed under reduced pressure and then, the product was collected through suspension in MeOH (200 mL) and filtered. The obtained residue was washed with excess MeOH and dried under vacuum to yield TriPE-Bpin$_3$ as pure product. A white solid was obtained in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ (ppm)): 7.74 (d, 4H), 7.56 (d, 2H), 7.30 (t, 2H), 7.15 (d, 2H), 7.01 (d, 3H), 1.27–1.4 (m, 36H). ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 145.86, 143.23, 139.94, 134.7, 129.79, 129.96, 127.05, 83.79, 67.09, 24.96. HRMS (MALDI-TOF, *m*/*z*): ($[M + H]^+$) calcd for $C_{38}H_{49}B_3O_6$, 634.3808: found 634.3802. Anal. calcd (%) for $C_{38}H_{49}B_3O_6$: C, 71.96; H, 7.79; B, 5.11; O, 15.14; found C, 71.93; H, 7.75; B, 5.12; O, 15.17.

Preparation of (4-(1,2-bis(4-boronophenyl)vinyl)phenyl)-boronic acid (TriPE-3BA). To a 50 mL round bottom flask TriPE-Bpin$_3$ (300 mg, 0.48 mmol) in dichloromethane (20 mL) was added. The solution was cooled to 0 °C and then, a solution of boron tribromide in dichloromethane (3 mL, 2.0 M) was added under Ar. The ice bath was removed and the solution was stirred for 3 h. The reaction was quenched with ice water (20 mL). The resultant precipitate was washed with dilute hydrochloric acid (0.1 M) and water, and then dried under vacuum. A white solid was obtained in 84% yield. ^{1}H NMR (400 MHz, CD_4O, δ (ppm)): 7.68–7.74 (m, 4H), 7.47 (d, 2H), 7.26 (d, 2H), 7.11 (d, 2H), 7.05 (s, 1H), 6.97 (d, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ (ppm)): 133.83, 133.45, 133.06, 129.21, 128.44, 128.11, 126.28. HRMS (MALDI-TOF, *m*/*z*): ($[M + H]^+$) calcd for $C_{20}H_{19}B_3O_6$, 388.1461: found, 388.1454. Anal. calcd (%) for $C_{20}H_{19}B_3O_6$: C, 61.94; H, 4.94; B, 8.36; O, 24.75; found C, 61.93; H, 4.91; B, 8.38; O, 24.75.

Preparation of (ethane-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl)tetraboronic acid) (TPE-4BA). The synthetic routes and characterization data are presented on pages S5, S6, S11 and S13 in the ESI† (ref. 55, S. Dalapati *et al.*, *J. Am. Chem. Soc.* 2016, **138**, 5797–5800). HRMS (MALDI-TOF, *m*/*z*): ($[M + H]^+$) calcd for $C_{26}H_{24}B_4O_8$, 508.1843: found, 508.1847. Anal. calcd (%) for $C_{26}H_{24}B_4O_8$: C, 61.51; H, 4.76; B, 8.52; O, 25.21; found C, 61.46; H, 4.80; B, 8.50; O, 25.19.

Sample preparation

Detailed experimental procedures for the preparation of solutions, bacteria staining and fluorescence imaging used in this investigation are given below.

Preparation of stock solutions of TriPE-3BA and TPE-4BA in DMSO. A stock solution of **TriPE-3BA** with concentration 50 mM was prepared by dissolving 97 mg (250 μmol) **TriPE-3BA** in 5 mL DMSO. A stock solution of **TPE-4BA** with concentration 50 mM was prepared by dissolving 127 mg (250 μmol) **TPE-4BA** in 5 mL DMSO. The solution was stored in the dark before use.

Preparation of stock solution of PBS. PBS was prepared by dissolving NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) in 800 mL distilled water, adjusting the pH to 7.4 with HCl, and calibrating to 1 L by adding H_2O. PBS was sterilized by autoclaving for 20 min at 15 psi (1.05 kg cm^{-2}) in liquid cycle and stored at room temperature.

Bacteria staining. A single colony of *E. coli* and *S. epidermidis* on solid culture medium Luria broth (LB) was transferred to 5 mL liquid culture medium and grown at 37 °C for 10 h. The concentrations of bacteria were determined by measuring optical density at 600 nm (OD600) and then, 10^9 CFU bacteria was transferred to a 1.5 mL microcentrifuge tube. Bacteria were harvested by centrifuging at 11 700*g* for 3 min. After removal of supernatant, the bacteria were either killed with 75% ethanol (dead bacteria) or dealt with 200 μL PBS (living bacteria), followed by washing with PBS. Dye solution (1 mL) of **TriPE-3BA** or **TPE-4BA** in PBS (100 μM) was added to the microcentrifuge tube. After dispersing with a vortex, the bacteria were incubated in a shaking incubator at 30 °C for 0.5 h and then washed with 2.7 mL buffer solution (pH 10) to dissolve the free dye in order to lower the background. Then, the bacteria were stained with 50×10^{-6} M PI for 20 min.

Fluorescence imaging of bacteria. To take fluorescence images, about 2 μL stained bacteria solution was transferred to a glass slide and then covered with a coverslip. The bacteria were imaged under a laser confocal scanning microscope using different combinations of excitation and emission filters for each dye: for **TriPE-3BA** and **TPE-4BA**, excitation filters = 405 nm and emission filter = 440–480 nm; for PI, excitation filter = 535 nm and emission filter = 615 nm.

Conflicts of interest

There are no conflicts to declare.

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