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Fluorescent probes are extensively used with ever-increasing functions for biological and medical applications. To further solve problems associated with tissue damage, long-wavelength fluorophores have been proposed for the construction of sensors with decreased energy requirements for activation and increased biocompatibility. As such we have repurposed an effective and biologically applicable probe **TCF-Bpin** combining boronate activating groups and a **TCF-OH** fluorophore, for monitoring the production of peroxynitrite ( $\text{ONOO}^-$ ) by bacteria upon treatment with antibiotics. In addition, externally added  $\text{ONOO}^-$  was intracellularly visualized, which makes not only the detection of oxidative stress in bacteria possible but also increases the understanding of the mechanisms.

Since 2015, 2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene) malononitrile (TCF) has been reported as a long-wavelength fluorophore which exhibits low-toxicity towards tissues and bright fluorescence.<sup>1</sup> Moreover, the internal charge transfer (ICT) donor- $\pi$ -acceptor (D- $\pi$ -A) system imparts several advantages including signal stability, deep tissue penetration, and detection sensitivity; which are superior to systems such as dicyanomethylene-4H-pyran (DCM), another red-emitting fluorophore.<sup>2-5</sup> This has made TCF-based fluorophores or imaging agents potentially clinically useful and brought them

into consideration for many fundamental research applications. It is well-known that **TCF-OH** is strongly fluorescent and can be significantly quenched upon locking the phenolic hydroxyl group with protecting groups, leading to the emergence of many “turn-on” fluorescence probes for different sensing targets based on the different locking groups.<sup>6-8</sup> Within the currently-developed TCF-based fluorescence probes, **TCF-Bpin**, is locked by a boronate group and can be released to **TCF-OH** under the action of reactive oxygen species (ROS) which are markers for many human diseases. This probe has been used for the solutional sensing of hypochlorite ( $\text{ClO}^-$ ),<sup>1</sup> while more recent research has indicated that it was more reactive and sensitive towards the more clinically indicative diseases (inflammation, cancer, ischemia-reperfusion, neurodegenerative, etc.) and peroxynitrite ( $\text{ONOO}^-$ ). Therefore, the sensitive fluorescence *in situ* imaging of intracellular  $\text{ONOO}^-$  was realized without toxicity towards cells.<sup>6</sup> These previous reports encouraged us to explore the use of the **TCF-Bpin** probe with bacteria.

Given that bacterial infections are a serious health concern, effective methods to track treatment process are in urgent demand (in particular methods to track oxidative stress which relates very closely with bacterial mortality), with this research we aimed to explore the possibility and potential of the probe to be repurposed for bacterial analysis

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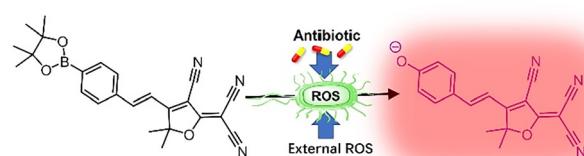
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**Scheme 1** Schematic illustration. Treatment of bacteria to enable ROS detection in cells: ROS and antibiotics were externally added to stress the cells to produce ROS. The ROS-containing bacterial cells activate **TCF-Bpin**, resulting in “turn-on” fluorescence.



(Scheme 1). ROS was monitored in bacterial cells, using **TCF-Bpin**, the cells had ROS externally added and stressed using chloramphenicol, tetracycline, and ciprofloxacin.<sup>9,10</sup> It is worth mentioning that  $\text{ClO}^-$  has not been reported as present in bacterial cells. While  $\text{ONOO}^-$  can readily exist under stress at therapeutic levels, as it is generally formed between superoxide and nitrile oxide (NO).<sup>11-18</sup> This aroused our interest and therefore we decided to explore if any sensor response can be observed from bacteria upon treatment with antibiotics.

In our previous work to detect  $\text{ONOO}^-$ , we found that **TCF-Bpin** could react with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), although the response is restricted. During initial solution tests, 10  $\mu\text{M}$  of  $\text{ONOO}^-$  could generate **TCF-Bpin** with a significant response (Fig. S1†), while the response towards both  $\text{ClO}^-$  and  $\text{H}_2\text{O}_2$  were minimal (Fig. S2 and S3†), even with concentrations 10-fold greater than  $\text{ONOO}^-$ . While it is known that DMSO might to some extent quench available ROS.<sup>19</sup> We observed similar responses in pure PBS solutions (data not shown). As such our results indicate that the probe could be used to monitor  $\text{ONOO}^-$  expression in microbial

pathogens. A sensitivity assay confirmed this observation, where **TCF-Bpin** was found to react with  $\text{ONOO}^-$  at low concentrations (Fig. S4†). While upon the addition of glutathione (GSH), a biological antioxidant, the signal partly rebounds (Fig. S5†).

When it comes to the detection of antibiotic-induced stress, we sought to use the probe with drug-stressed bacterial cells to see if signals can be detected. Then, specific inhibitors were used to confirm that the ROS species responsible for the response was  $\text{ONOO}^-$ . Using standard minimum inhibitory concentration (MIC) assays which determine the minimum concentration of an antibiotic required to completely inhibit bacterial growth (Fig. S6–S8†), we selected *Pseudomonas aeruginosa* (*P. aeruginosa* PAO1, Gram negative) and *Staphylococcus aureus* (*S. aureus* NCTC 10788, Gram positive) as model bacteria and screened three common broad-spectrum antibiotics, chloramphenicol, tetracycline, and ciprofloxacin. Then, the probe was added to bacteria incubated with sub-MIC levels of antibiotics (with antibiotic concentrations just below the full-inhibitory concentration). It was found that *S. aureus* generated

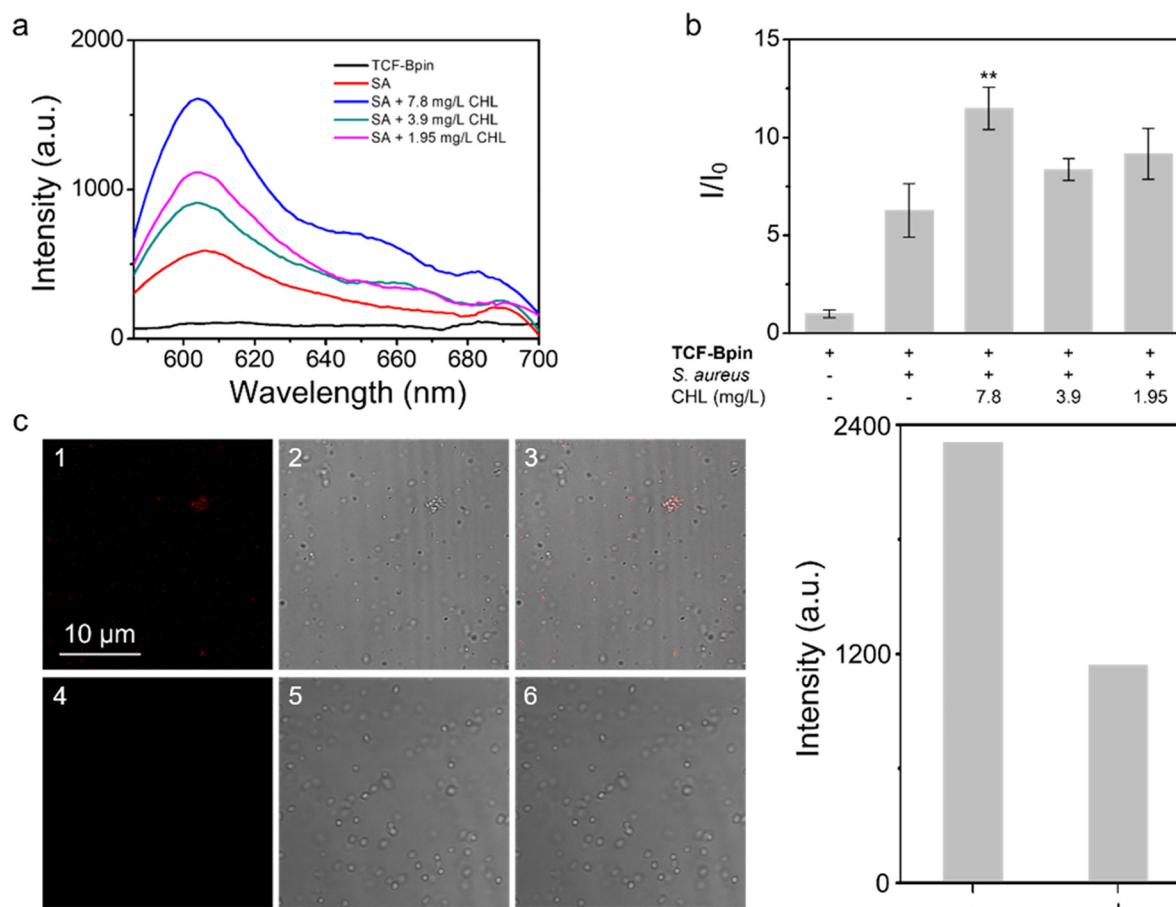


Fig. 1 (a) Fluorescence spectra of **TCF-Bpin** with *S. aureus* (SA) without antibiotic treatment (control) and *S. aureus* picked out from the MIC plate wells with treatment of sub-MIC concentrations of chloramphenicol (CHL). (b) Corresponding change in fluorescence. (c) Confocal laser-scanning microscopic images and intensity changes of **TCF-Bpin** incubated CHL-treated *S. aureus* without (1–3) or with (4–6)  $\text{ONOO}^-$  inhibitor.  $\lambda_{\text{ex}} = 560$  nm ( $\lambda_{\text{ex}} = 561$  nm laser source),  $\lambda_{\text{em}} = 606$  nm.



detectable signals upon treatment with chloramphenicol (Fig. 1a and b), while the response for tetracycline and ciprofloxacin, and that among *P. aeruginosa* were generally negligible (Fig. S9–S13†). Further, we used two multidrug-resistant (MDR) strains, *S. aureus* Mu50 and *P. aeruginosa* ATCC BAA-2110, as controls. However, we still observed similar responses (Fig. S14–S19†), indicating that oxidative stress was not completely associated with drug resistance for these strains. Since, the drugs are still causing stress, suggestive of enhanced antioxidant levels existing in MDR strains to support survival. Using an ONOO<sup>−</sup> inhibitor uric acid, the signal decreased (Fig. S20†), thus confirming that the existence of ONOO<sup>−</sup> was due to antibiotic stresses. Moreover, no stress was detected among the original strains without external drug treatments (Fig. S21†), showing that the initial level of ROS in the bacteria can be ignored. Such changes in ROS are also occurring intracellularly. TCF-Bpin-stained chloramphenicol sub-MIC *S. aureus* exhibited fluorescence, which could be quenched by uric acid (Fig. 1c). For other antibiotics, although the intensity changed with or without inhibition, no clear images could be obtained (Fig. S22 and S23†).

Subsequently, we explored the feasibility of the probe to detect ROS by external treatment of ROS. However, highly oxidative ROS are very toxic to bacteria, especially H<sub>2</sub>O<sub>2</sub> and HClO which are used as household disinfectant sprays.

Consequently, significant mortality was observed among the cells when treating them with ROS directly (Fig. S24†), where H<sub>2</sub>O<sub>2</sub> and ClO<sup>−</sup> could completely kill the bacteria at 15 μM (Fig. S24†). As a result, it is not possible to allow the concentration to exceed 10 μM for detecting intracellular ROS, and hence signals from both H<sub>2</sub>O<sub>2</sub> and ClO<sup>−</sup> can be ignored (since only a minimal signal can be seen when the concentration reaches 100 μM as shown in Fig. S2 and S3†). This hypothesis was verified under imaging conditions, where clear fluorescence staining was observed among ONOO<sup>−</sup>-treated cells (Fig. 2), while H<sub>2</sub>O<sub>2</sub> and ClO<sup>−</sup> treated cells remained dark (Fig. S25 and S26†).

Moreover, SIN-1 (3-morpholi-nosyndnonimine), a commercially available ONOO<sup>−</sup> generator was also used with *S. aureus* under imaging conditions. This resulted in detectable staining comparable to direct treatment with ONOO<sup>−</sup> (Fig. 2). In our previous study, we confirmed that the probe TCF-Bpin exhibits excellent biocompatibility, showing no damage to a range of cell lines including A549, HeLa, Hep-G2, and RAW264.7.<sup>6</sup> Herein, the low-toxicity towards bacterial cell lines has further been verified, demonstrating that the probe would not induce any damage to bacteria by interrupting cellular processes (Fig. S27†).

In summary, a TCF-based long-wavelength boronate probe TCF-Bpin has been repurposed for bacterial applications and used to detect the possible production of ONOO<sup>−</sup> due to

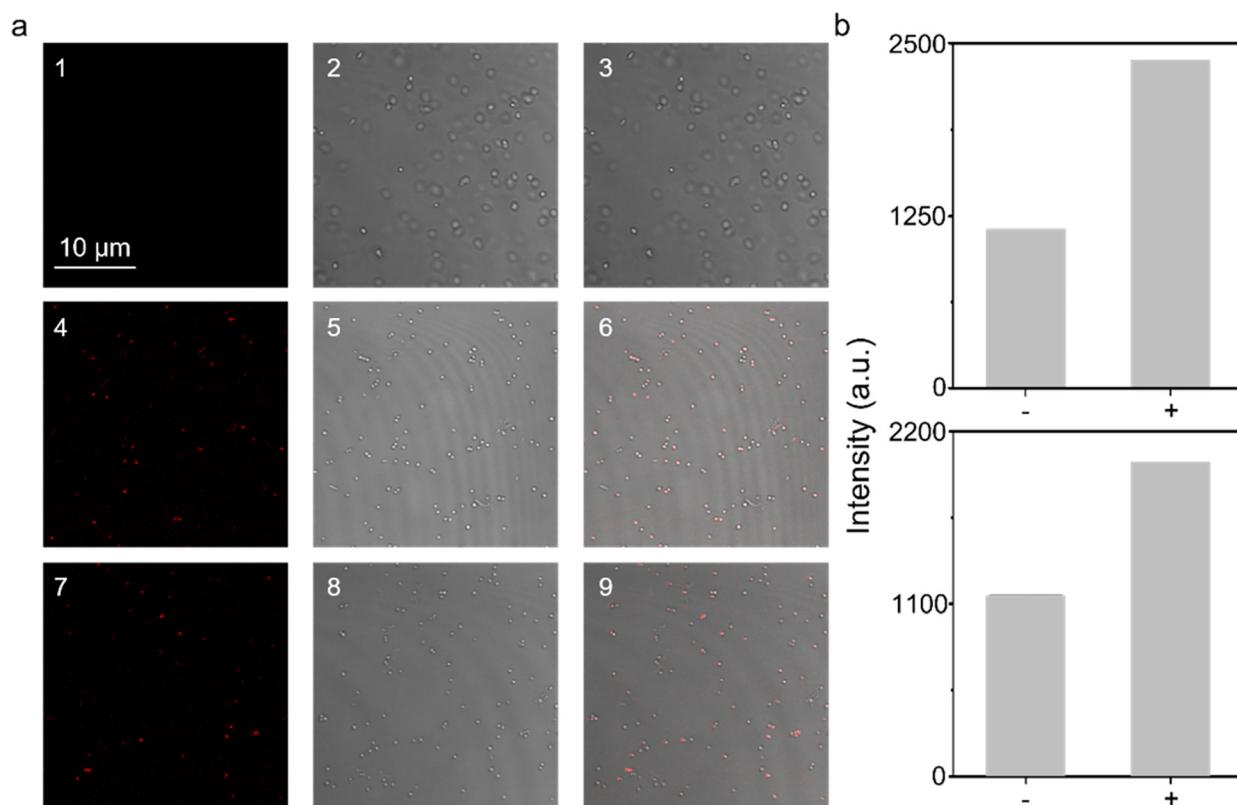


Fig. 2 (a) Confocal laser-scanning microscopic images of TCF-Bpin incubated *S. aureus* without (1–3) or with (4–6) incubation of ONOO<sup>−</sup> (10 μM), or with ONOO<sup>−</sup> inducer SIN-1 (7–9). (b) Intensity changes of TCF-Bpin incubated *S. aureus* without or with incubation of ONOO<sup>−</sup> (above), and of TCF-Bpin incubated *S. aureus* without or with incubation of SIN-1 (below).  $\lambda_{\text{ex}} = 561$  nm laser source,  $\lambda_{\text{em}} = 606$  nm.



oxidative stress caused by antibiotic treatment, which has not been previously reported. Meanwhile, externally added  $\text{ONOO}^-$  has been visualized intracellularly using the probe. The inhibition-based “double-check” assay confirmed the production of  $\text{ONOO}^-$  upon bacterial treatment with the antibiotic chloramphenicol, emphasizing not only the potential use of **TCF-Bpin** for detecting oxidative stress in bacteria but also the possibility of understanding the mechanism of oxidative stress.

## Conflicts of interest

There are no conflicts to declare.

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