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The Evaluation of Ester Functionalised TCF-Based Fluorescent Probes for the Detection of Bacterial Species

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Abstract: The ester functionality is commonly seen in the areas of chemical biology and medicinal chemistry for the design of cell-permeable active molecules. Ester-based pro-drug/pro-sensor strategies are employed to mask polar functional groups (i.e. carboxylic acids) and improve the overall cell permeability of these functional molecules. However, their use as reactive units for sensing applications, including bacterial detection, has not been fully explored. Herein, we synthesised two TCF-based fluorescent probes, **TCF-OAc** and **TCF-OBu**. As expected, both **TCF-OAc** and **TCF-OBu** demonstrated a significant fluorescence (22- and 43-fold, respectively) and colorimetric response (yellow to purple) towards porcine liver esterase (PLE) with a limit of detection of 1.18 mU/mL and 0.45 mU/mL, respectively. With these results in hand, the ability of these probes to detect planktonic suspensions of gram-positive *Staphylococcus aureus* (*S. aureus*) and gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Escherichia coli* (*E. coli*) were evaluated. Different fluorescence responses for gram-positive and gram-negative bacteria were observed between **TCF-OAc** and **TCF-OBu**. After 1 h incubation, **TCF-OAc** proved more sensitive towards *S. aureus*, demonstrating a significant fluorescence "turn on" response (16-fold); whereas, **TCF-OBu** was more selective towards *P. aeruginosa*, with a 22-fold increase in the fluorescence response observed. These results demonstrate the influence of the ester chain length on the selectivity for bacterial species.

Keywords: Bacterial detection · Chemosensors · Colorimetric sensors · Diagnostics · Wound infection

Wound infections pose a significant risk to patients' health and are a financial burden to health care systems.[1] Routine microbiological analysis is needed for the accurate diagnosis of wound infections; however, these procedures are often slow and labour intensive.[2] Therefore, clinicians tend to diagnose wound infections through the observation of clinical indicators.[3] Unfortunately, this can lead to the misuse of antibiotics, which results in the development of antibiotic resistant bacteria.[4] To overcome these clinical challenges, the development of easy-to-use diagnostic devices for the accurate and rapid detection of pathogenic bacteria is highly desired.[5] Recent diagnostic methods include enzyme-linked immunosorbent assays (ELISA),[6] polymerase chain reaction (PCR)-based methods,[7] DNA arrays,[8] and mass spectrometric

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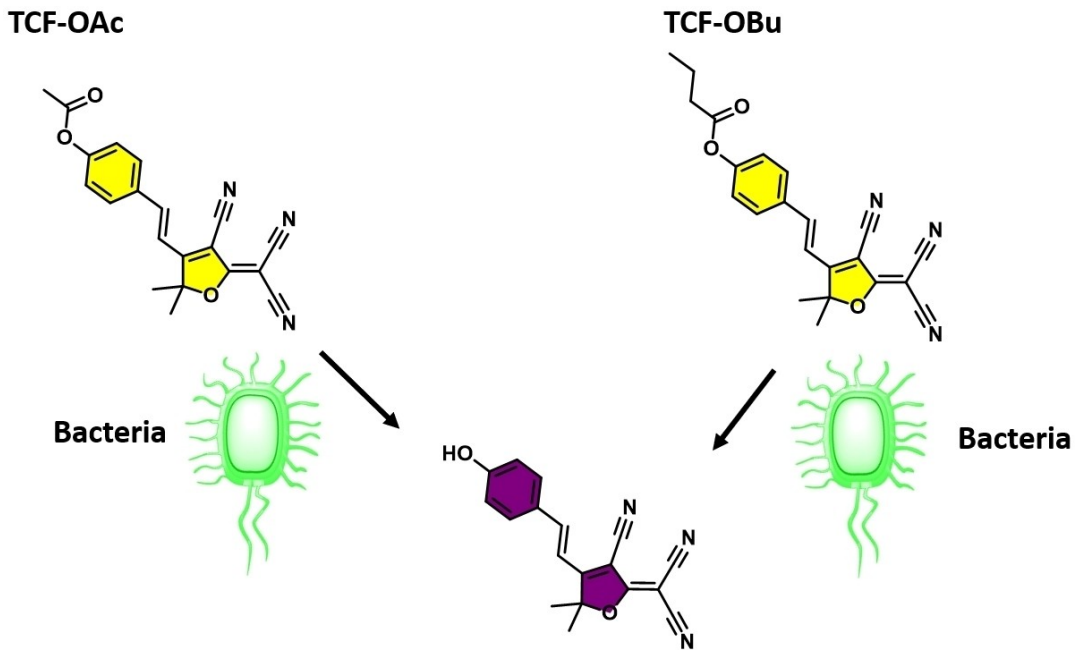
analysis.[9] However, these expensive and invasive methods require specialised and trained personnel.

An attractive alternative is the use of small molecule fluorescent and colorimetric probes because they are simple to use, highly sensitive, low in cost, easy to handle, can be used by a non-specialist and have fast detection times.[10] In addition, they offer a complementary strategy to smart wound technologies and point of care (PoC) devices.[11] Current small-molecule fluorescent probes utilise enzyme-based biomarkers to facilitate the detection of pathogenic bacteria,[12] which include elastases,[10e] phosphatases,[13] glycosidases,[14] proteases[15] and lipases.[16]

Ester functionalisation is commonly used in medicinal chemistry and chemical biology for the masking of polar alcohol and carboxylic acid functionalities on therapeutics or sensors to afford cell permeable pro-molecules.[17] Upon cellular uptake, these ester pro-molecules are expected to cleave by a range of cellular esterases and release the active molecule. However, recent studies have found various bacterial species exhibit significant substrate specificity for ester functionalities, which can influence the efficacy of a particular therapeutic.[17a] With this knowledge in hand, we expected that ester functionalised fluorescent probes differing in alkyl chain length may confer a level of selectivity for the detection of bacterial species. Here, we synthesised and evaluated two 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF)-based probe **TCF-OAc** (previously reported for hydrazine detection)[18] and the novel **TCF-OBu**) for the fluorescent and colorimetric detection of bacterial species. The ester deprotection (esterase-mediated and bacterial-mediated) of **TCF-OAc** and **TCF-OBu** results in the release of the donor-π-acceptor (D-π-A) system **TCF-OH**,[19] which affords an ideal long fluorescence emission wavelength (~600 nm) accompanied by a colorimetric change from yellow to purple (Scheme 1).[20]

In brief, **TCF-OAc** and **TCF-OBu** were synthesised through the simple acylation of **TCF-OH** using acetyl chloride and butyryl chloride, respectively - see supporting information for full details. With each probe in hand, UV-Vis and fluorescence titrations were carried out using porcine liver esterase (PLE). 10% DMSO was required to provide good aqueous solubility for **TCF-OAc** and **TCF-OBu**. As expected, the addition of PLE to both **TCF-OAc** and **TCF-OBu** resulted in a clear bathochromic shift from 450 nm to 570 nm, and a significant turn-on fluorescence response at 606 nm, which was indicative of the formation of **TCF-OH** (Figures 1 and S1–S4). This PLE mediated hydrolysis of **TCF-OAc** and **TCF-OBu** to **TCF-OH** was further confirmed by high resolution mass spectrometry (HRMS), Tables S2–S4, Figures S3 and S4. As shown in Figure 1, a dose-dependent increase in fluorescence intensity were observed for **TCF-OAc** and **TCF-OBu** with the addition of PLE (0–0.4 U/mL). Interestingly, **TCF-OBu** was found to have the greatest sensitivity with a limit of detection (LOD) of 0.45 mU/mL compared to **TCF-OAc** with a LOD of 1.18 mU/mL (Figures 1C and 1D, Tables S5 and S6). Kinetics of both **TCF-OAc** and **TCF-OBu** towards PLE were determined using the spectroscopic data and the Michaelis-Menten equation. This revealed a K_m of 7.21 ± 0.74 μM and a V_{max} of 1333 ± 73.64 min^{-1} for **TCF-OAc**, and a K_m of 27.51 ± 2.602 μM and a V_{max} of 15196 ± 1118 min^{-1} for **TCF-OBu**, indicating a greater affinity of PLE towards **TCF-OAc** over **TCF-OBu** (Figure S9–S12 and



Scheme 1. Colorimetric and fluorescent TCF-based probes, **TCF-OAc** and **TCF-OBu**, for the detection of bacteria.

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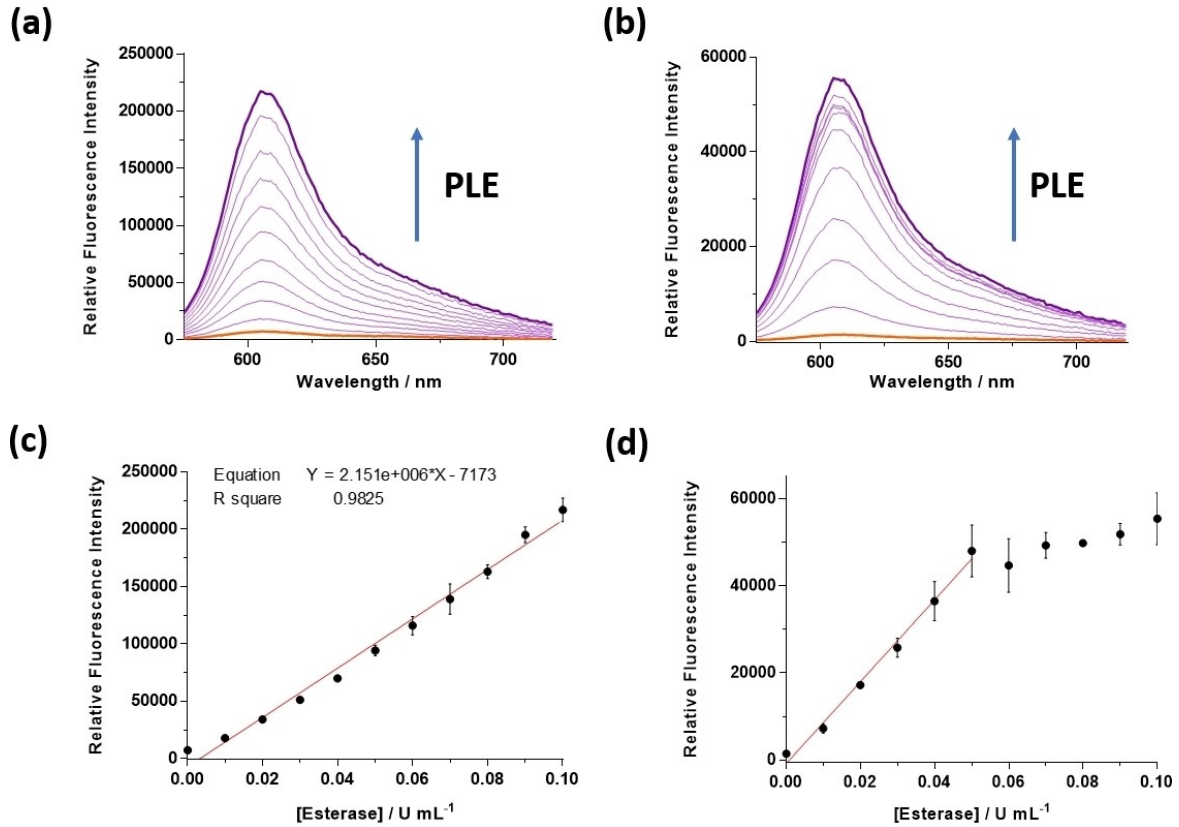

Figure 1. Fluorescence spectra of a) **TCF-OAc** (10 μM) and b) **TCF-OBu** (10 μM) with the addition of PLE (0–0.4 U/mL). Relative fluorescence intensity of c) **TCF-OAc** and d) **TCF-OBu** with the addition of PLE (0–0.4 U/mL) at 606 nm, error bars indicate the standard deviation. The measurements were made 15 min after PLE addition in PBS buffer, 10% DMSO pH = 7.4 at 25 °C, λ_{ex} = 542 (bandwidth 15) nm.

Tables S5–S9). This is reflected in the selectivity when both probes were screened against other enzymes and biological analytes (Figure S13–S15).

Upon determining the enzyme-responsive nature of both **TCF-OAc** and **TCF-OBu**, their ability to detect pathogenic bacteria was evaluated (Figure 2). In this study, three common bacterial pathogens: *S. aureus*, *E. coli*, and *P. aeruginosa* were used, which include clinical and commercial strains (Table S1). Each bacterial isolate was grown in tryptic soy broth (TSB) for 24 h at 37 °C, standardised to c. 10^8 CFU/mL, centrifuged, and resuspended in phosphate buffered saline (PBS with 10% DMSO, pH 7.4) containing **TCF-OAc/TCF-OBu** (10 μM). After 1 h incubation of **TCF-OAc** with gram-positive *S. aureus* MRSA252 and NCTC 10788, significant increases in fluorescence intensity was observed (One-way ANOVA, $p < 0.0001$ for both; 11- and 16-fold, respectively). This increase in fluorescence intensity was approximately 2-fold higher than **TCF-OBu** when incubated with the same bacterial isolates (6- and 9-fold, respectively). Conversely, **TCF-OBu** was found to be more selective towards gram-negative *P. aeruginosa* PAO1 and P885 with a 22-fold increase in fluorescence intensity seen for both strains (One-way ANOVA, $p < 0.0001$ for both). This response was approximately 4-fold higher than **TCF-OAc** when incubated with the same strains (6- and 4-fold, respectively). Interestingly, *E. coli* DH5α and NSM59 elicited a response comparable to the negative control for both **TCF-OAc** and **TCF-OBu**. To ensure that each probe had no influence on the viability of the bacteria, toxicity studies were performed. As shown in Figures S16 and S17, the bacterial cell density remained stable upon incubation and no clinically significant decrease in bacterial cell counts were observed for both **TCF-OAc** and **TCF-OBu** (t-test; $p < 0.05$); demonstrating the suitability of these probes for diagnostic applications. We believe the selectivity observed could be due to a number of factors including slow cellular uptake, difference in enzyme/bacteria recognition, and local environmental conditions (i.e. pH and PBS), although more research is needed to identify the exact reason for these selectivity differences. However, the current results illustrate that subtle changes in the ester chain length has a significant impact on the bacterial selectivity of the fluorescent probes under these conditions. This finding is of particular significance as developing a fluorescent probe that is selective for a particular bacterial species could aid diagnosis and enable the rapid provision of appropriate antibiotic treatments, which should minimalize the potential for the development of drug resistant bacteria.

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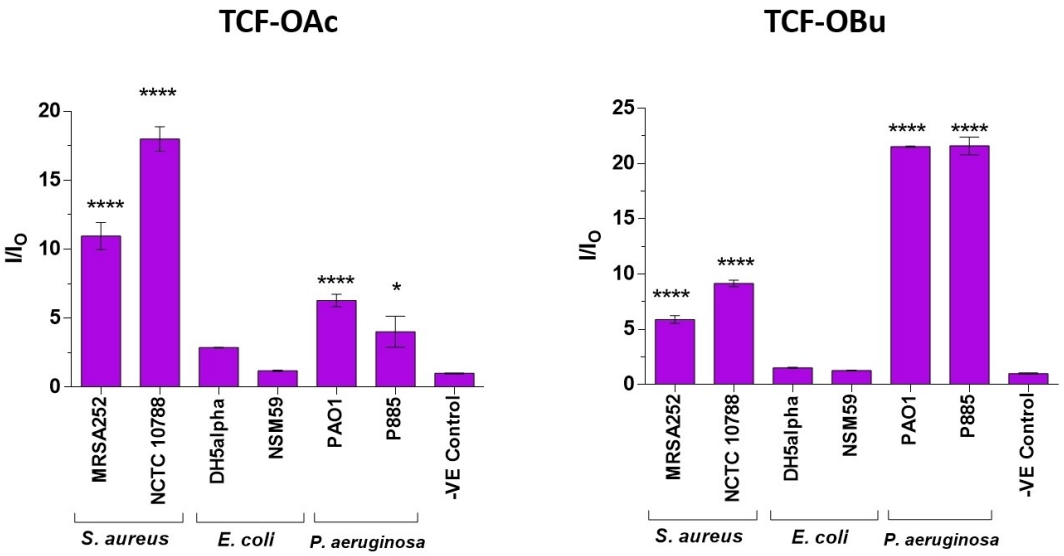

Figure 2. Selectivity bar chart of **TCF-OAc** (10 μM) and **TCF-OBu** (10 μM) in PBS buffer, 10% DMSO, pH 7.4 after 1 h incubation with various bacterial strains (10^8 CFU/mL) at 25 °C. λ_{ex} = 542 (bandwidth 15) nm. λ_{em} = 606 nm. Error bars indicate standard deviation (n = 3). Statistical analysis conducted using a One-way ANOVA with Bonferroni *post-hoc* multiple comparison test (compared to the negative control). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

In conclusion, we have synthesised and evaluated two fluorescent probes, **TCF-OAc** and the novel **TCF-OBu** for evaluation against bacterial pathogens. Both **TCF-OAc** and **TCF-OBu** were shown to have a clear concentration-dependent fluorescence increase and an obvious colour change from yellow to purple in the presence of PLE model. **TCF-OBu** demonstrated a lower limit of detection compared to **TCF-OAc** (1.13 and 0.45 mU/mL, respectively), whereas, **TCF-OAc** displayed an enhanced selectivity towards esterases. **TCF-OAc** displayed the greatest selectivity towards *S. aureus*, while **TCF-OBu** displayed an enhanced selectivity towards *P. aeruginosa.* Interestingly, no effect was observed upon incubation with *E. coli.* These results illustrate that subtle changes to the ester chain length of ester-functionalised fluorescent probes have a significant influence on their ability to detect and distinguish pathogenic bacteria. We are currently exploring these probes in hydrogel systems for the development of smart wound dressings.

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