Long wavelength TCF-based fluorescence probe for the detection of Alkaline Phosphatase in live cells

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Abstract
A long wavelength TCF-based fluorescent probe (TCF-ALP) was developed for the detection of alkaline phosphatase (ALP). ALP-mediated hydrolysis of the phosphate group of TCF-ALP resulted in a significant fluorescence ‘turn on’ (58-fold), which was accompanied by a colorimetric response from yellow to purple. TCF-ALP was cell-permeable, which allowed it to be used to image ALP in HeLa cells. Upon addition of bone morphogenic protein 2, TCF-ALP proved capable of imaging endogenously stimulated ALP in myogenic murine C2C12 cells. Overall, TCF-ALP offers promise as an effective fluorescent/colorimetric probe for evaluating phosphatase activity in clinical assays or live cell systems.

1 Introduction
Alkaline phosphatase (ALP) is an ubiquitous enzyme found in the majority of human tissues, where it catalyses the dephosphorylation of various substrates such as nucleic acids, proteins and other small molecules (Millán, 2006, Coleman, 1992). ALP also plays an important role in signal transduction and regulation of intracellular processes (cell growth, apoptosis and signal transduction pathways) (Julien et al., 2011). Abnormal levels of ALP in serum are an indicator of several diseases including bone disease (Garnero and Delmas, 1993), liver dysfunction (Rosen et al., 2016), breast and prostatic cancer (Ritzke et al., 1998, Wymenga et al., 2001) and diabetes (Tibi et al., 1988). As a result, ALP is regarded as a key biomarker in medical diagnosis (Coleman, 1992, Ooi et al., 2007). Therefore, it is important to develop a fast, reliable and selective detection system for monitoring ALP activity that is amenable to clinical diagnostics.

There have been numerous approaches to determine ALP levels, including colorimetric (Yang et al., 2016, Hu et al., 2017), chemiluminescent (Jiang and Wang, 2012), electrochemical (Zhang et al., 2015b), surface-enhanced Raman methods (Ruan et al., 2006) and fluorescence (Cao et al., 2016, Fan...
et al., 2016). This work focused on the development of fluorescent probes for the detection of biologically relevant analytes (Sedgwick et al., 2017a, Sedgwick et al., 2018b, Wu et al., 2017, Sedgwick et al., 2017b, Sedgwick et al., 2018a, Zhang et al., 2019). Fluorescence has many advantages over other methods owing to its simplicity and high sensitivity/selectivity, providing rapid, non-invasive, real-time detection (Wu et al., 2017). Whilst there have been many fluorophores developed for assaying ALP activity such as organic dyes (Zhang et al., 2015a, Zhao et al., 2017), conjugated polymers (Li et al., 2014), inorganic semiconductor dots (Qian et al., 2015), and noble metal clusters (Sun et al., 2014), most require high probe concentrations and crucially rely on short wavelength emission, thus limiting their applicability in biological systems. Therefore, ALP probes that operate at long wavelengths are urgently required. Such probes should allow for deeper tissue penetration and be subjected to less cell-based autofluorescence (Liu et al., 2017, Zhang et al., 2017, Tan et al., 2017).

2 Results and Discussion

2.1 Chemistry

Here we report a TCF-based fluorescent probe that allows for the detection of ALP and/or ACP. As shown in Scheme 1, this probe (TCF-ALP) is based on the conjugation of 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) to an electron-donating moiety, a phosphorylated phenol; this affords an internal charge transfer (ICT) donor-π-acceptor (D-π-A) system whose fluorescence properties vary dramatically following ALP-mediated phosphate group cleavage (Gopalan et al., 2004, Liao et al., 2006, Lord et al., 2008, Bouffard et al., 2008, Jin et al., 2010, Teng et al., 2018, Sedgwick et al., 2017b).

TCF-ALP was synthesised in four steps with an overall yield of 27% (Scheme 2). In brief, 3-hydroxy-3-methyl-2-butanone, malononitrile and NaOEt were heated at reflux in EtOH for 1 h. The resultant precipitate TCF (1) was then treated with a mixture of piperidine (cat.) and 4-hydroxybenzaldehyde in EtOH to afford intermediate 2 (TCF-OH). Intermediate 2 was then treated with diethylchlorophosphate, DMAP (cat.) and NEt₃ in THF to give the phosphonate ester 3. Hydrolysis using trimethylsilyl iodide in dichloromethane (DCM) afforded TCF-ALP as a crystalline solid (Et₂O).

2.2 Spectroscopic studies of TCF-ALP

UV-Vis and fluorescence spectroscopic titrations of TCF-ALP were performed in 50 mM Tris-HCl buffer in the absence and presence of ALP from porcine kidney. In the absence of ALP, TCF-ALP was found to have no UV absorption features above ~550 nm; however, upon addition of ALP a bathochromic shift in the UV absorption maximum was observed (from 440 to 580 nm), which was accompanied by a change in colour from yellow to purple (Figure S1). ALP-mediated hydrolysis of TCF-ALP to form highly fluorescent phenol (2), was confirmed by 31P NMR studies and HRMS (See Figure S1 – S4). The effect of pH on the rate of ALP mediated hydrolysis of TCF-ALP was evaluated. It was found that incubation with 0.8 U/mL of ALP at pH 9.2 resulted in the largest fluorescence response (Figure S5). Consequently, all in vitro experiments to determine ALP activity were carried out in 50 mM Tris-HCl buffer at pH 9.2.

The kinetics of ALP towards TCF-ALP were determined via fluorescence spectroscopy (Figure S6 and S7), with the resultant fluorescence data analysed using the Michaelis-Menten equation (Figure S8). This revealed a $K_m$ of 35.81 ± 2.63 µM and a $V_{max}$ of 3029 ± 157.3 min⁻¹ for hydrolysis of TCF-ALP by ALP at pH 9.2 (see SI for details). TCF-ALP was then incubated with various concentrations of ALP (0.0 – 0.2 U/mL) for 15 minutes to evaluate its ability to monitor ALP activity. As shown in Figure 1, a significant fluorescence response was observed in the presence of ALP (58-fold) with a limit of detection (LOD) calculated as 0.12 mU/mL (Figure S9). This sensitivity is comparable to other
fluorescent probes found in literature (Table S3). Although serum alkaline phosphatase levels vary with age in normal individuals (Lowe et al., 2018), it is widely accepted that serum ALP levels in healthy adults lies between 39 – 117 U/mL (Sahran et al., 2018, Saif et al., 2005). This suggests that TCF-ALP is capable of detecting ALP in human serum, and therefore could be used in clinical assays.

Inhibition studies were carried out in the presence of sodium orthovanadate (Na$_3$VO$_4$), which is known to be a strong inhibitor of ALP activity. Addition of Na$_3$VO$_4$ resulted in a decrease in the fluorescence response in the TCF-ALP hydrolysis assay (see Figure S10) (Swarup et al., 1982). These inhibition studies enabled an IC$_{50}$ of 6.23 µM to be calculated (Figure S11), which is similar in value to other ALP substrates that have been reported in the literature (Tan et al., 2017, Zhang et al., 2015a).

The selectivity of TCF-ALP towards other biologically relevant enzymes (at their optimal pH values) was then determined (Figure 2 and S12), with TCF-ALP displaying high substrate selectivity for ALP over other common hydrolytic enzymes (e.g. trypsin, porcine liver esterase) or non-specific binding proteins (e.g. bovine serum albumin (BSA)). Interestingly, TCF-ALP produced a fluorescence response when treated with acid phosphatase (ACP). The detection of this enzyme is of significance since it is a tumour biomarker for metastatic prostate cancer (Makarov et al., 2009). Normal levels of ACP in serum range from 3.0 – 4.7 U/mL, and elevated ACP levels can be indicative of a variety of other diseases (Bull et al., 2002). Furthermore, TCF-ALP proved capable of detecting ACP (25-fold fluorescence enhancement) and ALP (38-fold enhancement) at a physiological pH of 7.1 (Figure S13 and S14). Kinetic determination of ALP and ACP towards TCF-ALP at pH 7.1 was conducted, and the resultant K$_m$ and V$_{max}$ were compared (see SI 2.1 and Figures S15-S18). It was found that ALP has a smaller K$_m$ value in comparison to ACP (0.38 ± 0.042 µM and 99.22 ± 13.16 µM respectively) and a lower V$_{max}$ (208 ± 3.81 min$^{-1}$ and 1962 ± 223.6 min$^{-1}$ respectively). Hence, ALP has higher affinity towards TCF-ALP compared to ACP, thus is more selective towards ALP at physiological pH.

According to current standards, determination of ALP and ACP is undertaken at the phosphatase’s optimum pH. For example, the Centers for Disease Control and Prevention (CDC) procedure for ALP determination is carried out in 2-amino-2-methyl-1-propanol (AMP) buffer at pH 10.3 ((CDC), 2012). This is in accordance with other literature sources (Guo et al., 2018, Di Lorenzo et al., 1991, Radio et al., 2006, Pandurangan and Kim, 2015). Likewise, ACP determination is carried out at pH 4-6 (Myers and Widlanski, 1993, Boivin and Galand, 1986, Li et al., 1984). Following these observations, further studies were conducted to determine selectivity at pH 5.0 and 9.2 (Figures S19 – S22). Results showed that TCF-ALP acts selectivity towards ACP at acidic pH, and ALP at alkaline pH. Therefore, TCF-ALP can be used to selectively detect ALP/ACP in clinical assays, or live cell systems (provided the buffer solution is optimal for the phosphatase under study).

### 2.3 Imaging of ALP in living cells

Prior to exploring whether TCF-ALP could be used to image ALP activity levels in live cells, the cytotoxicity of TCF-ALP was assessed using a MTT assay (Figure S23). Negligible cell toxicity was observed for TCF-ALP concentrations between 0 – 5 µM, and cell viability was only slightly reduced (91%) when incubated with 10 µM TCF-ALP, indicating good biocompatibility.

TCF-ALP proved cell permeable to HeLa cells that express ALP and provided a clear ‘turn on’ response (Figure 3). In contrast, pre-treatment of HeLa cells with 5 mM Na$_3$VO$_4$ prior to incubation with TCF-ALP resulted in minimal ‘turn on’. This was taken as evidence that the increase in TCF-ALP fluorescence levels seen for HeLa cells in the absence of Na$_3$VO$_4$ is due to ALP activity. We thus conclude TCF-ALP is a probe that allows for the selective cellular imaging of ALP activity.
Bone morphogenetic protein 2 (BMP-2) is capable of inducing osteoblast differentiation into a variety of cell types (Guo et al., 2014, Wang et al., 2015) via pathways that result in increased ALP mRNA expression, leading to increased ALP activity (Kim et al., 2004). Treatment of myogenic murine C2C12 cells with TCF-ALP resulted in a low fluorescence intensity (low ALP levels) being observed (Figure 4); however, pre-treatment of these cells with BMP-2 (300 ng/mL, 3 days) resulted in a significant increase in TCF-ALP-derived fluorescence intensity (high ALP levels). Once again, pre-incubation with 5 mM Na$_3$VO$_4$ led to no fluorescence response being observed in the cells treated with TCF-ALP (with or without BMP-2). This provided support for the notion that TCF-ALP is capable of imaging endogenous ALP activity induced by BMP-2.

3 Conclusions

In summary, a long wavelength TCF-based fluorescent probe (TCF-ALP) has been prepared with the goal of detecting ALP activity. ALP hydrolysates the phosphate group of TCF-ALP resulting in a significant ‘turn on’ fluorescence response (58-fold) within 15 minutes. These spectroscopic changes were accompanied by a colorimetric change from yellow to purple. This enables TCF-ALP to be used as a simple assay for the evaluation of ALP activity. Further analysis revealed that TCF-ALP could also be used as a probe for detecting ACP activity. TCF-ALP was shown to be cell permeable, enabling its use as a fluorescent probe for monitoring ALP levels in HeLa cells. TCF-ALP also proved capable of imaging endogenously stimulated ALP produced in myogenic murine C2C12 cells through the addition of bone morphogenetic protein 2. We thus suggest that TCF-ALP offers promise as a tool for measuring ALP and ACP activity levels in clinical assays or in live cell systems.

4 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 Author Contributions

LG and ACS carried out synthetic and spectroscopic experiments and co-wrote the manuscript with TDJ and JLS. JEG and GTW carried out background experiments. GK carried out cellular imaging experiments. JPL carried out the $^{31}$P NMR titrations. J-YM and ATAJ are supervisors of LG and GTW. SDB, JY, JLS and TDJ both conceived the idea and helped with the manuscript.

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7 Acknowledgments

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8 Supplementary Material

Data supporting this study are provided as supplementary information accompanying this paper, which is available free of charge.

9 References


## 10 Captions for Figures and Schemes

**Scheme 1** – A TCF-based fluorescence probe (TCF-ALP) for the detection of alkaline phosphatase.

**Scheme 2** – Synthesis of TCF-ALP.

**Figure 1** - Fluorescence spectra of TCF-ALP (10 µM) produced via the addition of alkaline phosphatase (ALP; 0 – 0.2 U/mL) in 50 mM Tris-HCl buffer, pH = 9.2 at 25 °C. λ<sub>ex</sub> = 542-15 nm. All measurements were made 15 min after the addition of ALP.

**Figure 2** - Fluorescence spectra of TCF-ALP (10 µM) recorded in the presence of trypsin (0.8 BAEE U/mL), porcine liver esterase, protease from *Streptomyces griseus*, proteinase K, bovine serum albumin (0.1 mg/mL), acid phosphatase (50 mM Tris-HCl, pH = 5.0) and alkaline phosphatase (50 mM Tris-HCl, pH = 9.2). All enzymes were standardised to 0.8 U/mL in Tris-HCl buffer pH 7.1 unless otherwise stated. λ<sub>ex</sub> = 542-15 nm/ λ<sub>em</sub> = 606 nm. Fluorescence measurements were made 30 min after adding the enzyme in question.

**Figure 3** - HeLa cells incubated under the following conditions: (a) No treatment. (b) TCF-ALP (10 µM, 30 min). (c) Pre-treated with Na<sub>3</sub>VO<sub>4</sub> (5 mM, 30 min), followed by the addition of TCF-ALP (10 µM, 30 min). (d) Pretreated with Na<sub>3</sub>VO<sub>4</sub> (0.5 mM, 30 min) and TCF-ALP (10 µM, 30 min). Cells were washed with DPBS before their fluorescence images were acquired using a confocal microscope. Top half: fluorescence images, bottom half: fluorescence images merged with its corresponding DIC image. Ex. 559 nm/ em. 575-675 nm. Scale bar : 20 µm. DIC - differential interference contrast.

**Figure 4** - TCF-ALP in C2C12 cell. C2C12 cells were treated with 300 ng/mL BMP-2 for 3 days and then pretreated with 5 mM levamisole for 30 min and stained with 10 µM probe for 30 min. After washing with DPBS, fluorescence images were acquired by confocal microscopy. (a) only probe, (b) levamisole + probe, (c) BMP-2 + probe (d) BMP-2 + levamisole + probe. Top : fluorescence images, bottom : merged with DIC image. Ex. 559 nm/ em. 575-675 nm. Scale bar : 20 µm. DIC - differential interference contrast.
Figure 2.TIF

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