Front. Chem. Sci. Eng. 2020, 14(1): 117–121
https://doi.org/10.1007/s11705-018-1785-9

COMMUNICATION

Dual enzyme activated fluorescein based fluorescent probe

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Abstract A simple dual analyte fluorescein-based probe (**PF3-Glc**) was synthesised containing *β*-glucosidase (*β*-glc) and hydrogen peroxide (H_2O_2) trigger units. The presence of *β*-glc, resulted in fragmentation of the parent molecule releasing glucose and the slightly fluorescent mono-boronate fluorescein (**PF3**). Subsequently, in the presence of glucose oxidase (GOx), the released glucose was catalytically converted to D-glucono-*δ*-lactone, which produced H_2O_2 as a by-product. The GOx-produced H_2O_2, resulted in classic H_2O_2-mediated boronate oxidation and the release of the highly emissive fluorophore, fluorescein. This unique cascade reaction lead to an 80-fold increase in fluorescence intensity.

Keywords chemosensors, dual-activation, GOx, fluorescence, *β*-glucosidase, molecular logic

1 Introduction

Glucose is an essential source of fuel for all cells in the body for use in metabolic pathways. Despite its necessity in everyday life, it is implicated in a wide range of diseases, including diabetes, Alzheimer's and cancer. The role of glucose in cancer and Alzheimer's disease development has only recently started to be understood. In all cancer cells there is an increased need for glycolysis and glucose uptake for ATP production; corresponding to over-expression of glucose transporter protein (GLUT and SGLT) which promote cancer cell proliferation [1]. Recent research has also indicated that lower rates of glycolysis and downregulated expression of the same glucose transport proteins combined with higher brain glucose levels correlate to enhanced *β*-amyloid plaque formation in Alzheimer's patients. This causes further neurodegeneration and increased expression of disease symptoms, including memory loss and impaired cognitive function [2,3].

β-Glucosidases (*β*-glc) catalyse the hydrolysis of glycosidic bonds with the release of glucose. The enzyme is a powerful tool for degredation of cellulose in plant cell walls [4]. In addition, *β*-glc has been identified as a target critically involved with breast cancer growth and chemoreistance. Lui et al. have shown that *β*-glc inhibition suppressed growth of breast cancer cells and significantly sensitised breast cancer cells to chemotherapy [5]. Targeting *β*-glc is emerging as a possible therapeutic stategy in the treatment of breast cancers which are resistant to a single chemotherapeutic agent alone.

Glucose oxidase (GOx) is known to catalyse the oxidation of glucose to D-glucono-*δ*-lactone, producing hydrogen peroxide (H_2O_2) as a by-product. The primary function of GOx is as a defence mechanism in fungi and insects, where the H_2O_2 produced is used to kill bacteria [6]. In this work, we set out to develop a unique reaction-based fluorescent probe capable of monitoring the activity of *β*-glc through exploiting the close relationship between GOx, glucose and the generation of H_2O_2. GOx is widely used in biosensors as a molecular diagnostic tool to detect glucose levels in biological fluids [7].

Quantitative measurement of H_2O_2, correlates with the level of GOx present in the system. As H_2O_2 cannot be visualised directly *in vitro*, it is often used as an analyte towards reaction-based fluorescence probes producing a fluorescent product. Many fluorescent sensors have been developed for the detection of H_2O_2; see reviews by Chang et al. and James et al. [8,9]. H_2O_2 is a highly reactive oxygen species (ROS), generated through controlled physiological processes. H_2O_2 is a by-product of catalytic

Received August 15, 2018; accepted October 17, 2018

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activity in the mitochondria and protein folding in the endoplasmic reticulum. However, H_2O_2 can become deleterious when cells are under oxidative stress, as levels are elevated and can cause irreversible cellular damage via oxidation of biomolecules [10,11].

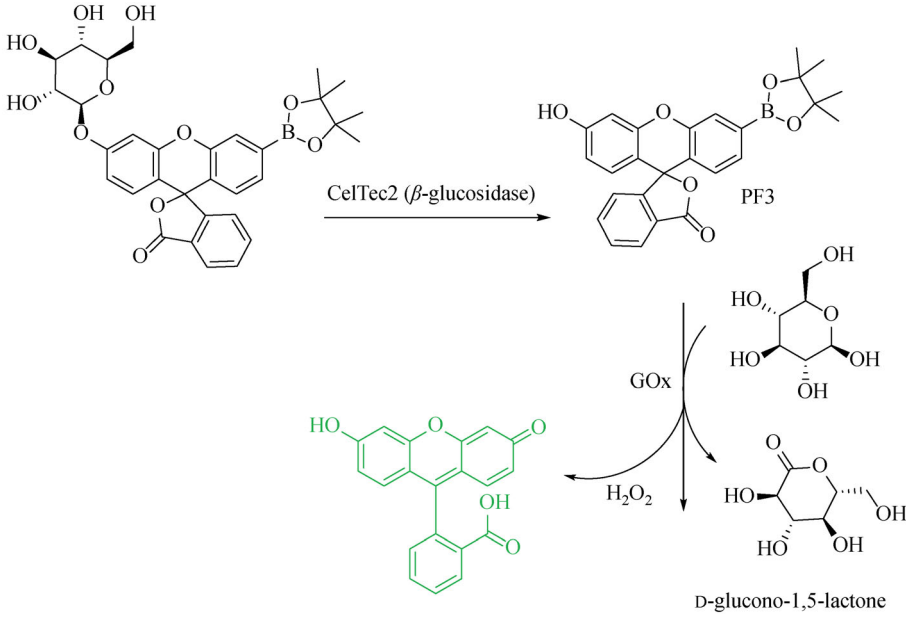
There are limited examples of fluorescence-based probes which utilise *in situ* generation of H_2O_2 by GOx to produce a fluorescence response. Most notable is the Amplex Red enzyme assay (Available from ThermoFisher.com), which requires the peroxidase enzyme to produce a fluorescent adduct [12,13]. While previous probes designed to detect GOx activity in the literature, have glucose in the system as an external additive [14].

Fluorescein derivatives are some of the most common fluorescent reagents for biological research, because of the excellent fluorescence quantum yield of the fluorescein and good water solubility. The fluorescein motif allows for dual-activated sensor systems as it has two free phenolic alcohols which can be derivatised independently, as illustrated by James et al. [15]. Therefore, in this work, we have used a similar strategy by incorporating glucose onto the mono-boronate fluorescein, **PF3** [16] to form **PF3-Glc**. **PF3-Glc** was able to undergo a unique reaction cascade, in which glucose is generated as a product of a reaction between **PF3-Glc** and β-glc. In turn, the glucose reacts with GOx to produce H_2O_2, which results in the formation of highly fluorescent fluorescein through H_2O_2-mediated oxidation of the boronate ester (Scheme 1).

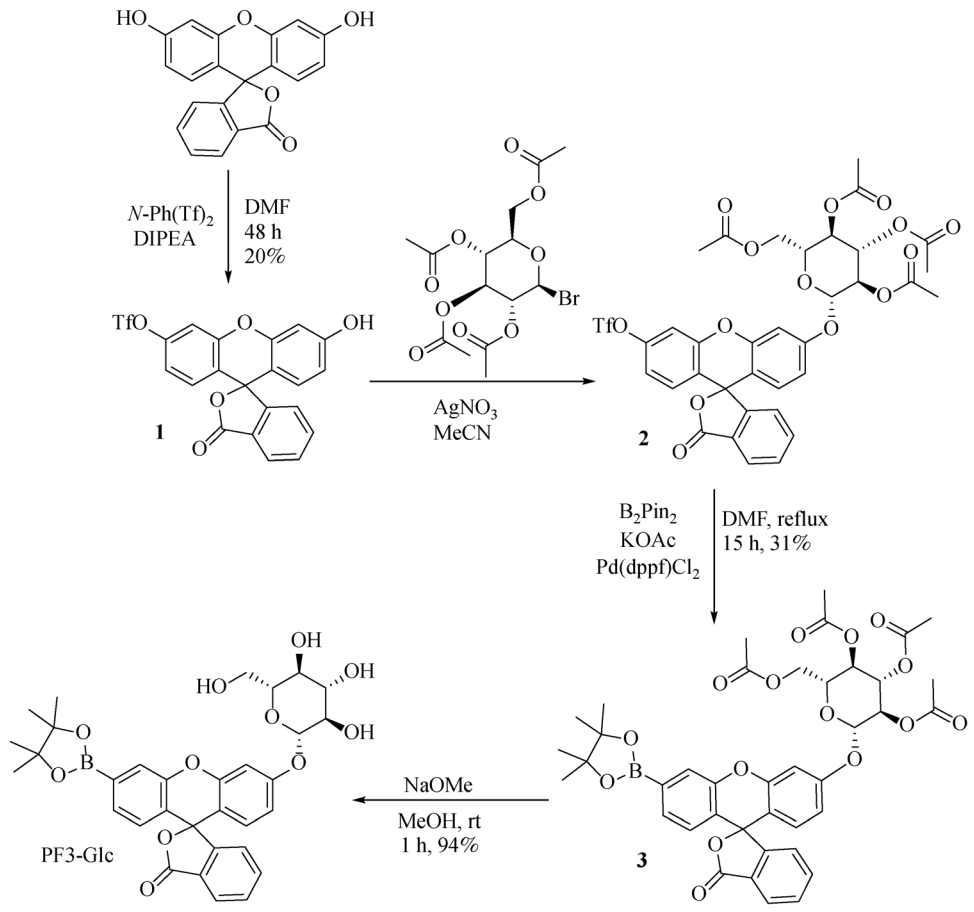
2 Results and discussion

PF3-Glc was readily synthesised in four steps (Scheme 2). Fluorescein was triflated using *N*-phenyl bis(trifluoromethanesulfonamide), to afford fluorescein mono-triflate [17]. A Koenigs-Knorr glycosylation reaction was then carried out to selectively form the β-glycosidic bond with the per-acetylated sugar. Followed by a Suzuki-Miyaura borylation cross coupling, to introduce the boronate ester group. The final step was an acetate deprotection using catalytic sodium methoxide. Under these conditions **PF3-Glc** was prepared in an overall yield of 10%**.**

With **PF3-Glc** in hand, fluorescence studies were undertaken. **PF3-Glc** is initially non-fluorescent; the probes spectral properties were investigated in different solvent systems (see ESI Figs. S1 and S2). We find that there is a small fluorescence response in dimethyl sulfoxide, methanol and water, with little change in the ultra-violet-visible spectra. We chose to undertake all further experiments in phosphate buffered saline (PBS) to mimic a biological system. On the addition of CelTec2 (0.5 U), a commercially available enzyme blend known to contain β-glc, there was a small increase in fluorescence intensity after one-hour incubation (Fig. 1). This fluorescence increase was consistent with the generation of mono-functionalised fluorescein probes [15−17]. Incremental additions of GOx resulted in a much larger increase in fluorescence intensity. The fluorescent spectra were collected after 1.5 h incubation with both enzymes. When the sensor was incubated with GOx only, no turn on fluorescence response was observed after one hour. This clearly demonstrated that the β-glc enzyme cleaves a molecule of glucose to act as a substrate for GOx, which in turn produces H_2O_2 *in situ* to oxidise the boronate ester to the corresponding phenol. Both enzymes show no autofluoresence in the buffered system (PBS buffer, pH =



Scheme 1 Structure of PF3-Glc/PF3 and the proposed sensing mechanism for sequential detection of β-glc and H_2O_2



Scheme 2 Synthesis of **PF3-Glc**

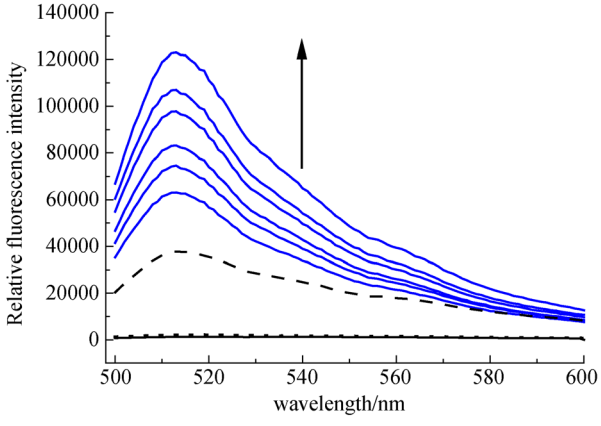


Fig. 1 Fluorescence spectra of PF3-Glc (500 nmol/L) with titration of GOx (1, 2, 4, 6, 8, 10 U, blue lines) in the presence of CelTec2 (0.5 U). Spectra of sensor with GOx (10 U, dotted line) only and CelTec2 (0.5 U, dashed) only are also shown. The black solid line represents the sensor only. The spectra were obtained after 1.5 h of incubation with both enzymes. The data was taken in PBS buffer pH = 7.4 (100% H_2O) at 25°C where λ_{ex} = 472 (bandwidth 16) nm

7.3 (100% H_2O), see ESI, Fig. S2).

To demonstrate that H_2O_2 is required for a complete turn on response, a fluorescence experiment was undertaken where CelTec2 (0.5 U) was added to **PF3-Glc** and the probe was incubated for 60 min at 25°C. This led to a small increase in fluorescence intensity (two-fold). Addition of H_2O_2 (500 µmol/L) resulted in a large fluorescence increase (Fig. 2). The fluorescence spectra were taken one hour after H_2O_2 addition.

The selectivity of **PF3-Glc** was then evaluated against other ROS, ClO^-, peroxyl radical (ROO•), hydroxyl radical (•OH), superoxide ($O_2^{-\cdot}$) and singlet oxygen ($^1O_2$) with and without CelTec2 (Fig. 3). In the absence of CelTec2 there is little observable response from all ROS (See ESI, Fig. S3). In the presence of CelTec2 the probe was highly selective towards H_2O_2 over all other ROS evaluated (See ESI, Fig. S4).

3 Conclusions

We have developed a dual enzyme activated fluorescent probe **PF3-Glc** for *β*-glc and GOx (H_2O_2). The system can be used to monitor *β*-glc activity by the *in-situ* generation

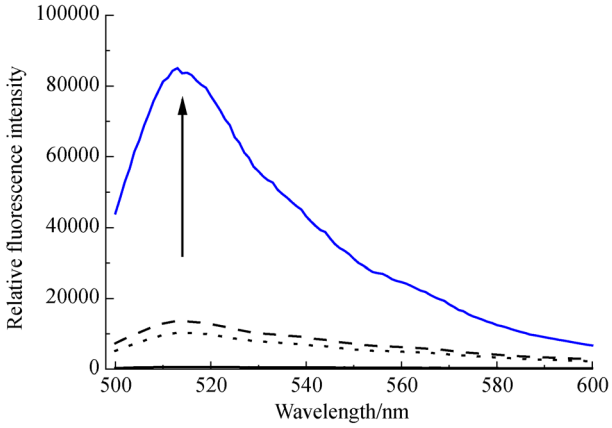


Fig. 2 Fluorescence emission spectra for PF3-Glc (250 nmol/L) in the presence of CelTec2 (0.5 U) incubated for 30 min at 25°C, prior to addition of H_2O_2 (0.5 mmol/L) which was left to react for a further 60 min. The data was obtained in PBS buffer, pH = 7.3 (100% H_2O w/w) at 25°C, λ_{ex} = 472 (bandwidth 16) nm. The black solid line represents the sensor only. The dotted line represents CelTec2 (0.5 U). The dashed line represents H_2O_2 (0.5 mmol/L)

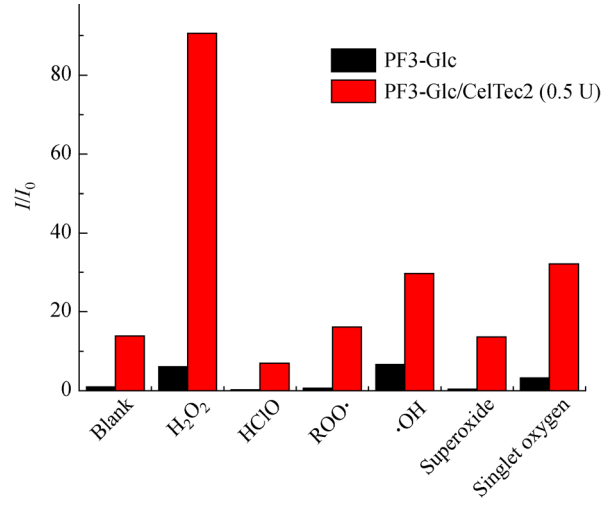


Fig. 3 Selectivity data for PF3-Glc (250 nmol/L). The sensor is incubated with CelTec2 (0.5 U) for 1 h, followed by the addition of ROS. Hydrogen peroxide (0.5 mmol/L) was incubated for 1 h before measurement. $HClO^-$ (0.5 mmol/L) and ROO^- (0.5 mmol/L) were incubated for 30 min before measurement was taken. Singlet oxygen (0.5 mmol/L), superoxide (0.5 mmol/L) and –OH (0.5 mmol/L) were measured immediately after addition. Data shows difference in fluorescence intensity at λ = 510 nm after 1 h. The data was taken at pH = 7.3 and 25°C

of glucose, which is subsequently transformed into H_2O_2 by GOx and detected by **PF3**. **PF3-Glc** is an easy to prepare dual enzyme activated fluorescein based fluorescent probe. This is a simple proof of concept system and we are currently exploring how dual enzyme activation can be used to develop fluorescent sensors with enhanced selectivity and incorporating therapeutic units [18].

Acknowledgements We would like to thank the EPSRC and the University of Bath for funding. TDJ wishes to thank the Royal Society for a Wolfson Research Merit Award. MLO, JEG and ACS thank the EPSRC for their studentships. NMR and MS Characterisation facilities were provided through the Material and Chemical Characterisation Facility (MC^2) at the University of Bath. The EPSRC UK National Mass Spectrometry Facility at Swansea University is thanked for analyses. All data supporting this study are provided as supplementary information accompanying this paper.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11705-018-1785-9 and is accessible for authorized users.

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