A simple umbelliferone based fluorescent probe for the detection of nitroreductase

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Abstract

A simple nitrobenzyl-umbelliferone (**NCOU1**) was synthesised containing a nitroreductase (NTR) trigger moiety. The presence of NTR, resulted in the fragmentation of the parent molecule and release of the highly emissive fluorophore umbelliferone *via* an NTR-catalyzed reduction of the nitro group. In the presence of the NTR enzyme **NCOU1** gave rise to a 5-fold increase in fluorescence intensity at 455 nm and was selective for NTR over other reductive enzymes. These results indicate that **NCOU1** can be used as a simple assay for the detection of NTR.

Introduction

Nitroreductase (NTR), a member of the family of flavin-containing oxidoreductases, is one of a series of biomarkers that have been shown to be significantly upregulated in cells under hypoxic stress [1, 2]. Indeed, hypoxia, known to play a role in a number of diseases has garnered considerable attention in recent times owing to its role in tumour development and resistance to therapy [3, 4]. While a number of detection methods such as ^{19}F NMR, positron emission tomography (PET), single-photon emission computed tomography (SPECT) have been exploited to study hypoxia [5], the exact role played by NTR in tumour hypoxia remains elusive. Among the powerful tools available for NTR detection are synthetic small-molecular fluorescent probes owing to their high sensitivity, selectivity and high spatial and temporal resolutions [6-8]. Therefore, the selective fluorescent sensing of NTR has recently become a highly topical area of research [9-17].

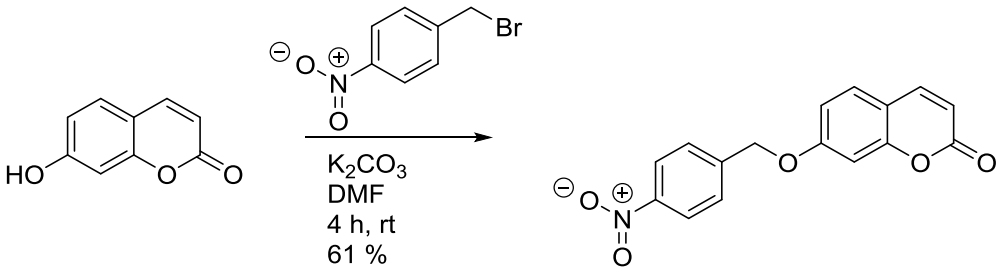
Qian and co-workers have developed a number of probes for the detection of hypoxia in HeLa cells [18, 19] and Hecht and co-workers recently confirmed the presence of NTR in the mitochondria of mammalian cells using a highly sensitive cyanine based probe [20]. Ma and co-workers have described a number of fluorescent probes to enable the sensitive detection and quantification of NTR in both mammalian [21, 22] and bacterial cells [23] while their most recent contribution described an example of an aggregation induced emission (AIE) based probe for NTR [24]. We have developed a ratiometric probe based on the 2-nitroimidazole moiety and demonstrated that such an approach can be used for the facile monitoring of NTR activity in mammalian cells under reductive stress both by confocal microscopy and flow cytometry [25]. With our interest in developing fluorescent probes to monitor markers of biological significance [26-31]. We were inspired to explore and develop more fluorescent

based systems for the detection of NTR and to gain a better understanding of the role played by NTR in hypoxia related cellular stress.

Our design centred around the coumarin moiety as a responsive fluorophore that could be 'triggered' upon reduction by NTR. We envisaged that our simple nitrobenzyl-umbelliferone probe (**NCOU1**), would give rise to a fragmentation of the parent molecule and release of the highly emissive fluorophore umbelliferone *via* the NTR-catalyzed reduction of the nitro group. Our design strategy is reinforced by the previously reported reduction of **NCOU1** using Zn/AcOH to produce a 'turn on' fluorescence response.[32] Our results clearly demonstrate the success of this approach, since treatment of **NCOU1** with NTR gave rise to a 5-fold increase in emission intensity at 455 nm and was shown to be selective for NTR over other reductive enzymes.

Results and discussion

The synthesis of **NCOU1** was a simple one step alkylation of the commercially available umbelliferone using 4-nitrobenzyl bromide, K_2CO_3 and DMF (**Scheme 1**). The reaction proceeded cleanly and required minimal purification. **NCOU1** was fully characterised using ^{1}H NMR, ^{13}C NMR, IR, UV/vis and mass spectrometry. All spectroscopic data was consistent with the formation of the desired compound (See ESI).



Scheme 1 – Synthesis of NCOU1

With **NCOU1** in hand, the ability of the probe for the detection of NTR was evaluated. As illustrated in **Fig.1**, under a UV lamp **NCOU1** is non-fluorescent (a). The addition of NTR led to the reduction of the nitro functionality resulting in the elimination of the fluorescent umbelliferone (b). For comparative purposes, a solution containing umbelliferone was shown next to the two solutions (c). This observation was further confirmed using mass spectroscopic analysis (ESI Fig S7).

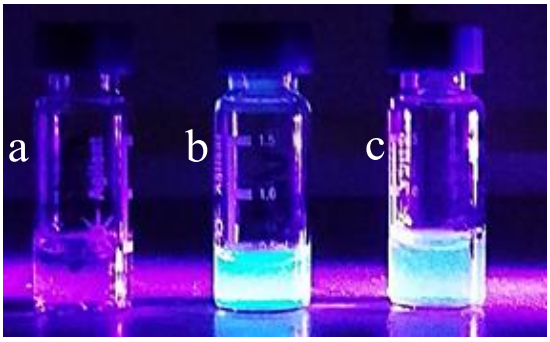


Fig. 1. Photograph of: (a) **NCOU1** (b) umbelliferone (c) **NCOU1** + NTR

Initially, **NCOU1** was treated with a larger concentration of the enzyme NTR (8 µg/mL) with the required co-factor NADH (500 µM). As shown in **Fig. 2** an increase in fluorescence intensity was observed over the course of 60 mins.

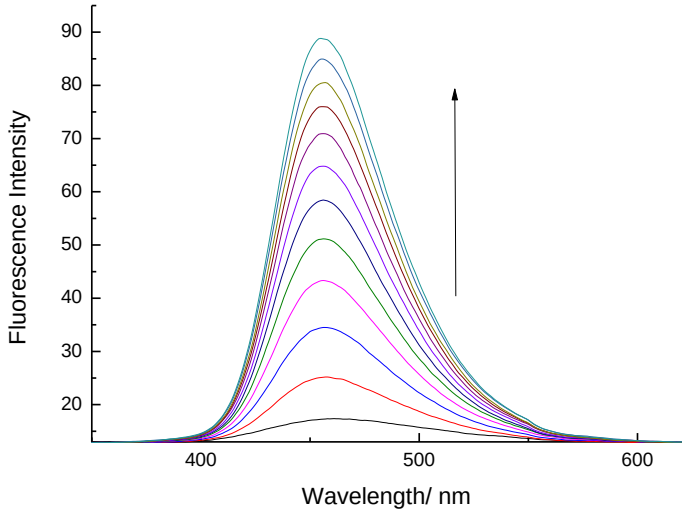


Fig. 2 – Fluorescence spectra of **NCOU1/** 10 µM with the addition of nitroreductase/ 8 µg/mL and NADH/ 500 µM and measured over 60 minutes in 10 mM PBS/ pH 7.4. λex = 315 nm

NCOU1 was shown to detect NTR in a dose-dependent manner. 8 µg/mL NTR resulted in the largest fluorescence response > 16-fold. **NCOU1** was also shown to detect NTR at concentrations as low as 1 µg/mL and at acidic, basic and neutral pH clearly demonstrating its ability to be used for biological applications shown in **Fig. 3** (See **Fig. S3** for pH experiment).

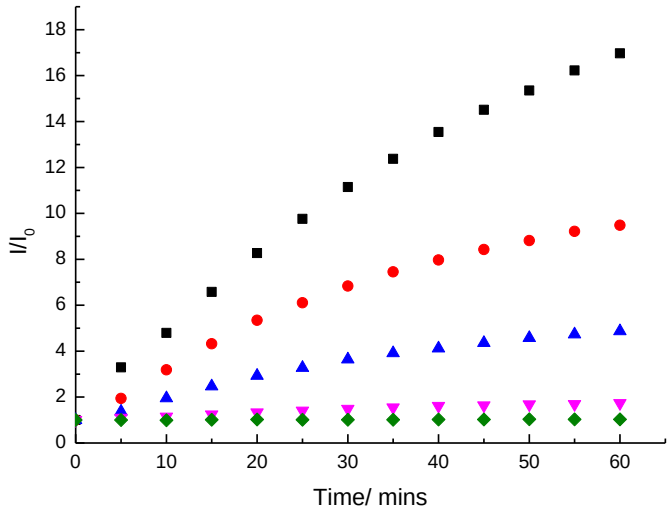


Fig. 3 – Dose-response time curve of **NCOU1**/ 10 µM with additions of nitroreductase/ 0, 0.5, 1, 4 and 8 µg/mL and NADH/ 500 µM in 10 mM PBS/ pH 7.4. λex = 315 nm

Due to the complexity of biological samples, the selectivity of **NCOU1** towards another reductive enzyme, DT-Diaphorase (hNQO1), was performed. DT-Diaphorase is a cellular reductase that is widely distributed in all cellular environments including the cytosol, golgi complex, nucleus, mitochondrial membranes and endoplasmic reticulum, as well as in extracellular components [33] thus the ability to differenciate between such reductive enzymes is of the utmost importance. **NCOU1** was shown to be highly selective towards NTR over DT-Diaphorase as shown in **Fig. 4**. Moreover, in the presence of the known NTR inhibitor dicoumarol **NCOU1** displayed a very minor fluorescence response to NTR clearly demonstrating its selectivity for the active NTR enzyme.

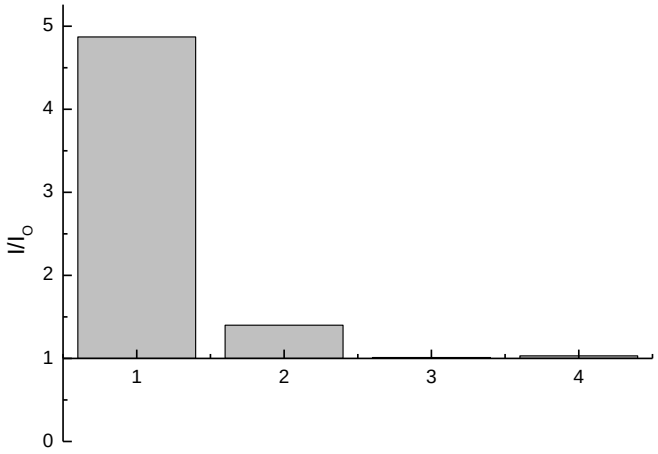


Fig. 4 - Selectivity bar chart of **NCOU1**/ 10 µM with addition of **1** – nitroreductase/ 1 µg/mL, **2** - nitroreductase and dicoumarol/ 1 µg/mL, 3 - DT Diaphorase/ 1 µg/mL and a blank. All measurements contained NADH/ 500 µM in 10 mM PBS/ pH 7.4 λex = 315 nm/ λ_{em} = 455 nm

Conclusion

Nitrobenzyl-umbelliferone (**NCOU1**) was synthesised by a simple one step alkylation of umbelliferone with 4-nitrobenzyl bromide. **NCOU1** was shown to detect NTR at a low concentration (1 µg/mL*)*. Fragmentation of the parent molecule and release of the highly emissive fluorophore umbelliferone *via* the NTR-catalyzed reduction of the nitro group. **NCOU1** gave rise to a 5-fold increase in emission intensity at 455 nm and was shown to have an excellent selectivity for NTR over other reductive enzymes. These results demonstrate the ability of **NCOU1** to be used as a simple and cheap assay for the detection of NTR.

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