Two 2-nitroimidazole-1,8-naphthalimide conjugates, 1 and 2, have been synthesised as fluorescence probes for the detection of reductive stress in HeLa cells. The 4-substituted derivative 1 was shown to act as a highly sensitive and selective substrate for nitroreductase where it exhibited a clear blue to green ratiometric fluorescence response visible to the naked eye. Moreover, biological studies demonstrated 1 could be activated in cellulo where the impact of reductive stress was easily monitored using confocal microscopy and flow cytometry.

Reactive oxygen species have long been known to play an important physiological role in numerous diseases, and when antioxidant defences are overwhelmed oxidative stress results. However, more recently, attention has been drawn to the opposite situation, reductive stress, where the cellular environment becomes more reduced than normal. Reductive stress, has also been shown to play a pivotal role in numerous disease states including, heart failure, Alzheimer’s disease and solid tumors which are characterised by an increase in reducing species such as glutathione, reduced NADPH or an increase in activation of oxidoreductase enzymes. Oxidoreductases such as nitroreductases (NTRs), are a particularly valuable marker for reductive stress being capable of reducing nitroaromatics to corresponding nitroso or amino derivatives thereby converting an electron-withdrawing substituent into an electron donating amino functional group.13 Oxidoreductases such as nitroreductases (NTRs), are a particularly valuable marker for reductive stress being capable of reducing nitroaromatics to corresponding nitroso or amino derivatives thereby converting an electron-withdrawing substituent into an electron donating amino functional group.

15 With the overall aim of developing more sensitive fluorescent probes for cellular reductive stress we set out to exploit the 2-nitroimidazole moiety as a bioreductively responsive ‘trigger’ and synthesise two new fluorescently responsive NTR sensors 1 and 2 based on the 1,8-naphthalimide fluorophore (Fig. 1). Given the rich photophysical properties displayed by 1,8 naphthalimides, their observed biocompatibility,23–26 and their dependence on the nature and location of any aryl substituents,27–29 we envisaged that both 1 and 2 would, upon reduction, give rise to a fragmentation of the parent molecule and release of the well-known amino-1,8-naphthalimide fluorophores (Fig. S8‡). It was anticipated that the conversion of the electron-withdrawing carbamate linker to the electron-donating amino functional group would lead to significant modulation of their optical properties and lead to a fluorescence shift visible to the naked eye.

Herein, we report the synthesis and spectroscopic evaluation of probes 1 and 2 (Fig. 1), varied in their attachment of fluorescent probes for NTR has only recently become a burgeoning area of research interest.10 Indeed, a number of research groups11–17 have recently published reports in this emerging field of research where their approaches have often taken advantage of various nitroaromatic ‘triggers’ such as nitrobenzene, nitrofuran or nitrothiophene due to their relative ease of reduction. However, despite being widely used in the development of bioreductive prodrugs, the 2-nitroimidazole functional group which is extremely sensitive to changes in reductive stress,21,22 has not been considered in this context.


COMMUNICATION

2-Nitroimidazole based fluorescent probes for nitroreductase; monitoring reductive stress in cellulo†

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Fig. 1 Chemical structures of the target NTR probes 1, 2 and NB.

†Electronic supplementary information (ESI) available: Synthesis and characterisation data, experimental details and figures relating to spectroscopic titrations as well as materials and methods involved in biological evaluation. See DOI: 10.1039/c7ob01406f

‡These authors contributed equally to this work.
position to the 2-nitroimidazole trigger, where we have shown the 4-substituted derivative 1 to be a highly selective probe for NTR. Moreover, we have also shown that, when compared to the 4-nitrobenzyl substituted analogue NB (Fig. 1) the 2-nitroimidazole based probe 1 shows considerably increased sensitivity to the NTR enzyme. We have also conducted biological profiling of 1 where we have shown that it enters HeLa cells rapidly by passive diffusion, is non-toxic and is amenable to monitoring NTR facilitated reductive stress in mammalian cells using both flow cytometry and confocal microscopy.

Sensors 1 and 2 were synthesized by the sequential reaction of the appropriate amino-1,8-naphthalimide, 4 or 5 with the 2-nitroimidazole 3 to afford 1 and 2 in 68% and 63% yield, respectively (Scheme S1†). The 2-nitroimidazole alcohol 3 was synthesised according to a recently improved synthesis reported by Conway et al.10 Briefly this consisted of formylation of sarcosine ethyl ester with subsequent treatment with cyanamide under reflux in acetate-buffered conditions to yield a 2-amino-imidazole derivative which was converted to the nitro functionalty via diazotisation and NaBH₄ mediated reduction of the ester functionality to produce the desired alcohol 3. Nitrobenzyl substituted analogue NB was synthesised in a similar manner to 1 and 2 using 4-nitrobenzyl alcohol (see ESI†).

With 1 and 2 in hand, we evaluated their spectral characteristics in phosphate buffered saline (PBS) (pH 7.4, 10 mM) with 0.5% DMSO as co-solvent where both 1 and 2 demonstrated absorption and emission spectra characteristically different from the respective amino-1,8-naphthalimide starting materials 4 and 5. The absorption spectrum of 1 showed a λ max at ca. 360 nm while 2 showed a λ max at ca. 345 nm along with a broad shoulder centred at 390 nm. Upon excitation (λ exc = 345 nm) 1 gave rise to emission with λ max at 475 nm while 2 (λ exc = 345 nm) yielded emission with λ max ca. 460 nm, however, with a lower emission intensity; a known characteristic of 3-substituted-1,8-naphthalimides due to the unfavourable delocalisation of an internal charge transfer (ICT).27 The design of 1 and 2 relies upon their ‘unmasking’ at the hands of NTR to yield the corresponding amino-derivatives 4 and 5 thus causing significant shifting of spectral characteristics. With this in mind we also compared the photophysical characteristics of 1 and 2 with those of 4 and 5. The absorption spectrum of 4 showed a λ max at ca. 435 nm while that of 5 again showed slightly shifted maxima at ca. 345 nm and 410 nm. Emission from 4 (λ exc = 435 nm) showed a λ max at 550 nm (Φ F = 0.64)23 while that of 5 (λ exc = 345 nm) showed a λ max at 590 nm (Φ F = 0.46).31 again demonstrating a lower level of emission when compared against the 4-substituted derivative. Indeed, it was quickly noted when comparing the emission profiles of 1 and 4 that the shift in emission maxima was ca. 75 nm while that of 2 and 5 was 130 nm. Satisfied with the observed spectral variation between 1 and 4 and 2 and 5 we next investigated their spectroscopic behaviour when placed under mimicked reductive stress conditions by treatment with a type 1 NTR (oxygen insensitive NTR expressed in E. coli).

Emission spectra were measured in PBS (pH 7.4, 10 mM) with 0.5% DMSO as co-solvent in the presence of NADH (50 µM, as a cofactor of NTR) at 25° C. Upon treatment of 1 with NTR a drastic, time dependant spectral change was observed whereby a reduction in the emission maximum at 475 nm was accompanied by a concomitant increase in fluorescence at 550 nm, clearly visible to the naked eye as a blue to green colour change under UV irradiation (Fig. 2(a)). Over the course of the 20 minutes experiment the fluorescence intensity at 550 nm was increased by more than 8-fold while the emission at 475 nm had experienced a 5-fold decrease. The clear fluorescence colour change from blue to green suggested successful conversion of 1 to 4 as hypothesised in Fig. S8.† Moreover, ESI-LCMS studies of 1 treated with NTR under the same conditions confirmed the spectroscopic results above where the MS spectrum revealed peaks at m/z = 269.13 and 291.11 attributed to the [M + H]⁺ and [M + Na]⁺ ions of 4 respectively (see ESI†). Interestingly, the same result was not obtained for 2 which showed no fluorescence change when treated with NTR under the same conditions while ESI-LCMS only gave rise to peaks at m/z = 452.15 and 474.13 attributed to the [M + H]⁺ and [M + Na]⁺ ions of the parent sensor 2, respectively. Combined, these results suggest that, unlike 1, the reduction of 2 does not proceed under these experimental conditions. Similar experiments were carried out on the 4-nitrobenzyl substituted probe NB which showed a considerably weaker response to NTR undergoing approx. 30% decrease in emission at 450 nm and a negligible increase at 550 nm when

![Fig. 2](image_url)
treated with the enzyme (see ESI†). These results suggest that the 2-nitroimidazole moiety is considerably more prone to enzymatic reduction than its 4-nitrobenzyl analogue and points to a much more sensitive probe for NTR. This result is particularly relevant given the focus of recent literature on 4-nitrobenzyl substituted fluorescent probes.†

Considering the above results, we chose to concentrate our efforts on 1 where the release of 4 was also investigated in the presence of different concentrations of NTR (0–5 µg mL⁻¹) in order to evaluate its sensitivity to the enzyme. As shown in Fig. 2(b), higher concentrations of NTR resulted in a more rapid increase in fluorescence intensity whereby treatment with 5 µg mL⁻¹ NTR resulted in almost 90% conversion to 4 after just 5 minutes at 25°C. Moreover, good linearity was obtained over a wide concentration range of 0.02–5 µg mL⁻¹ NTR, demonstrating a true ratiometric response of probe 1. The emission measured from 1 in the absence of NTR remained constant over the course of the experiment demonstrating the stability of the probe under these mimicked biological conditions. Experiments over a range of pH values (pH = 5–9) further demonstrated the applicability of probe 1 to a biological environment whereby it was shown to perform best at pH values consistent with those found in biological media (7–8) (see ESI†). Tests on the specificity of the fluorescence response were also evaluated in the presence of a number of common biological interferents (NaCl, CaCl₂, MgCl₂, glucose, H₂O₂, aspartic acid, lysine, serine, tyrosine, lysozyme) where again probe 1 displayed a high level of both stability and selectivity towards NTR over all of the other species tested. These results taken collectively clearly establish that 1 can respond to NTR in a ratiometric fashion, selectively and sensitively, yielding a clear fluorescence change from blue to green, visible to the naked eye. Having evaluated the spectroscopic properties of 1 and its susceptibility to bioreduction we next wished to evaluate 1 as a biological probe for reductive stress in cellulo. The ability of 1 to be internalised in target cells was first assessed via fluorescence confocal microscopy in cervical cancer HeLa cells. Populations of live cells (0.5 × 10⁵) were incubated with 1 (10 µM) at 37°C for 4 h. The results obtained are exemplified in Fig. 3(a), which shows 1, upon successful uptake into cells residing in a cytoplasmic location with a strong emission of blue fluorescence. In contrast, reference compound 4, under the same conditions, clearly exhibits strong green emission also emanating from the cytoplasm. The rate of uptake of compounds 1 and 4 into cells was also investigated using flow cytometry where, again, both compounds were shown to be rapidly taken up into cells, reaching their maximum uptake within 15 min (Fig. 3(b)). 1 and 4 were also taken up into cells when incubated at 4°C for the duration of compound treatment (Fig. 3(c) and (d)) suggesting passive diffusion to be the mechanism of uptake as endocytosis, which requires ATP, is inhibited at this temperature. In agreement with this, when cells were pre-treated with the general endocytosis inhibitor dynasore, there was also no effect on the uptake of 1 or 4 (Fig. 3(d)). Cellular viability in the presence of 1 and 4 was also measured using an AlamarBlue assay (Fig. 4(c)). Results showed that an IC50 value could not be determined for 1, showing no observed toxicity and similarly a modest IC50 value of 46 µM was observed for 4 when cells were treated for 24 h. Interestingly, compounds 2 and 5 were shown to have increased toxicity with IC50 values of 7 µM and 35 µM, respectively, presumably due to their structural similarities to the known anti-proliferative agent amonafide.† With the biological behaviour of 1 characterised we next wished to evaluate its ability to monitor reductive stress in cells. In order to mimic reductive stress on the cell population, HeLa cells were treated with NTR (1 µg mL⁻¹) and its co-factor NADH (0.5 M) before addition of 1 or 2 (10 µM). Modulations of fluorescence were then assessed using confocal microscopy. Gratifyingly, after being subjected to such reduction conditions, the emission from 1 was seen to rapidly change to emission consistent with compound 4, suggesting successful reduction of 1 to release 4 under these cell culture conditions (Fig. 4(a)). The emission spectra of the compounds recorded within cells demonstrated the λmax of 1 was 465 nm (range 410 to 595 nm), whilst the λmax of 4 was 512 nm (range 456 to 661 nm). Incubation of 1 under reducing conditions revealed a cellular λmax of 512 nm (range 428 to 659 nm), again suggesting successful reduction of 1 under these cell culture conditions (Fig. 4(b)). The corresponding images and emission spectra for 2 and reference compound 5 (see ESI) show separate λmax values of 446 nm and 521 nm respectively, with no
modulations upon subjection to reducing conditions; this supports the results of the enzymatic spectroscopic studies described above which showed that treatment of 2 with NTR did not release 5.

In summary, we have reported the synthesis of two 2-nitroimidazole-1,8-naphthalimide conjugates 1 and 2. Compound 1, through a reduction–fragmentation mechanism, displays a rapid, sensitive and selective ratiometric response to NTR, clearly visible to the naked eye. In addition, we have demonstrated the applicability of 1 to cellular detection of reductive stress using both confocal microscopy and flow cytometry where 1 allows facile discrimination of cells under normal conditions and those under reductive stress. Considering the recent observation of Hecht and co-workers that NTRs are indeed prevalent in the mitochondria of A549 human lung adenocarcinoma cells,\(^\text{33}\) the use of ratiometric probes for NTR such as 1 will provide new perspectives on the role of reductive stress in various diseases. We are actively pursuing this line of research within our lab.

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