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Fluorescence-based chemical tools for monitoring ultrasound-induced hydroxyl radical production in aqueous solution and in cells†

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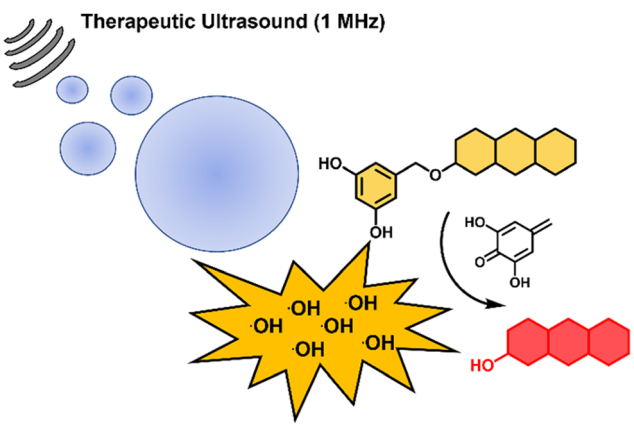
We report the synthesis of hydroxyl-radical (•OH) responsive fluorescent probes that utilise the 3,5-dihydroxybenzyl (DHB) functionality. 4-Methylumbeliferone-DHB (Umb-DHB) and resorufin-DHB (Res-DHB) in the presence of •OH radicals resulted in significant increases in their respective fluorescent emission intensities at 460 nm and 585 nm. The incubation of Res-DHB in HeLa cells followed by therapeutic ultrasound (1 MHz) resulted in a significant increase in fluorescence emission intensity thus permitting the ability to monitor ultrasound-induced •OH production in live cells.

The use of external stimuli to activate small molecules has shown tremendous potential in biological, biomedical and chemical research.[1–3] Recent efforts within the chemical community have utilized the production of •OH radicals derived from radiolysis to activate and/or release small molecules for both imaging and therapeutic applications.[4,5] Although a mainstay of cancer therapy, ionizing radiation suffers from several limitations, including radiation-associated toxicity and dose-limiting treatments.[6] An emerging alternative is the use of therapeutic ultrasound, which can be used for both localised ablation[7] and delivery/activation of anti-cancer agents.[8,9] The ability of ultrasound to propagate through soft tissues to much greater depths than light similarly offers advantages compared with photodynamic therapy and photocaging approaches.[10–12]

In a recent report by Peng and co-workers, ultrasound-induced cavitation effectively generated •OH radicals by sonolysis[13] to activate methylene blue-based urea conjugates for both imaging and therapeutic applications.[14] Inspired by this strategy, we rationalised the recently reported •OH radical responsive unit, 3,5-dihydroxylbenzyl (DHB)[4] may afford a general "sonocaging" unit that is selectively removed by •OH radicals generated from ultrasound irradiation. It is proposed •OH radical-mediated aromatic hydroxylation results in self-immolation releasing the active molecule (Scheme 1).

Owing to the simplicity and non-invasive nature of fluorescence probes,[15–20] in this study, we focused on the synthesis of DHB-functionalised 4-methylumbeliferone and resorufin fluorescent probes as shown in Fig. 1 (**Umb-DHB** and **Res-DHB**). We rationalised •OH radicals generated by ultrasound would selectively remove the DHB unit and lead to changes in their corresponding fluorescent emissions therefore allowing us to monitor the ultrasound-induced •OH production in solution and in mammalian cells.[21,22]



Scheme 1 Schematic of acoustic cavitation resulting in the •OH-mediated deprotection of the 3,5-dihydroxybenzyl unit and release of a highly fluorescent molecule. Grey lines depict sound waves.

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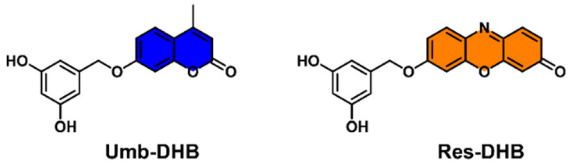

Fig. 1 Chemical structures of **Umb-DHB** and **Res-DHB**.

First, to attach the DHB unit onto each fluorophore, we synthesised a DHB-based alkylating reagent (see ESI† for full synthetic procedures and Schemes S1–S3). In brief, literature reported protocols were followed to isolate bis-silyl protected 3,5-dihydroxybenzyl alcohol **2**.[4] The synthetic transformation of **2** to a suitable alkylating agent proved difficult. After extensive method screening, a tosyl-based alkylating agent **3** was successfully synthesized (see ESI† – Scheme S1). **Umb-DHB** was obtained in 16% yield *via* refluxing 4-methylumbeliferone, **3**, K_2CO_3 in THF followed by treating the crude mixture with TBAF (see ESI† – Scheme S2). Whereas **Res-DHB** was isolated in 27% yield by stirring resorufin, **3** and K_2CO_3 in DMF for 14 hours followed by the deprotection of the silyl ethers by treating the crude mixture with K_2CO_3 (5 eq.) and DMF (see ESI† – Scheme S3).[23]

Next, we tested the responsiveness of **Umb-DHB** and **Res-DHB** towards •OH radicals generated from the Fenton reaction. These measurements were needed to confirm the •OH radical responsiveness of the DHB functionality.[4] It is important to note that the Fenton reaction favours acidic pH.[24] However, most phenolic-based fluorophores are pH-sensitive. We therefore had to identify Fenton reaction conditions that reflected physiologically relevant pH values. Titrating Fe (II)–EDTA to solutions of either **Umb-DHB** (5 μM) or **Res-DHB** (5 μM) in PBS buffer, pH = 7.40 and hydrogen peroxide (H_2O_2, 10 mM) showed an Fe (II)-dependent (0–200 μM, 60 min incubation) increase in fluorescence emission intensity at 460 nm and 585 nm, respectively (Fig. 2a and b and Fig. S1 and S2, ESI†). A colour change from orange to pink was observed for **Res-DHB**, indicative of the release of resorufin (see ESI† – Fig. S3). Before evaluating the response of either probe to ultrasound irradiation, we tested their reactivity to other oxidants and biologically relevant species. Acoustic inertial cavitation produces several reactive molecules,[25] including the reactive oxygen species (ROS): H_2O_2 and peroxynitrite ($ONOO^-$).[26] Surprisingly, the addition of $ONOO^-$ (100 μM) led to an instant increase in the fluorescence emission intensities of both **Umb-DHB** and **Res-DHB** (Fig. 1c and d). We hypothesise this $ONOO^-$-mediated response is due to the rapid decomposition of peroxynitrous acid (ONOOH) to form •OH radicals.[27,28] $ONOO^-$ generating the largest signal is rationalised by differences in the rate of •OH radical production from each source. The Fenton reaction is slow (>60 min) and suboptimal at pH = 7.4.[24,29] This observation is supported by a similar response being seen using the traditional •OH radical fluorescent assay, terephthalic acid[30] (TA) (see ESI† – Fig. S4). The potential formation of $ONOO^-$ during sonication provides another means of deprotection for the DHB functionality.[26]

With the responsiveness of **Umb-DHB** (5 μM) and **Res-DHB** (5 μM) towards •OH radicals identified; we turned our attention

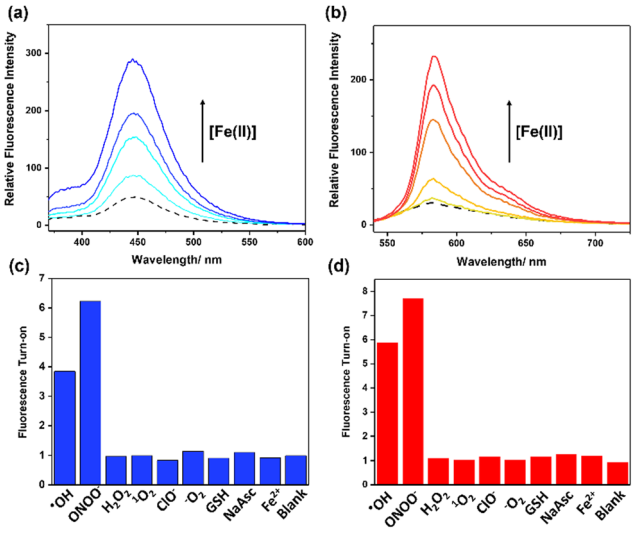

Fig. 2 (a) Fluorescent spectra of **Umb-DHB** (5 μM) with increasing concentrations of Fe(II)-EDTA (0–200 μM, λ_{ex} = 350 nm, 60 min incubation, Slit widths: 10 and 2.5). (b) Fluorescent spectra of **Res-DHB** (5 μM) with increasing concentrations of Fe(II)-EDTA (0–200 μM, λ_{ex} = 500 nm, 60 min incubation, Slit widths: ex 10 nm and em 3.5 nm). These measurements were performed in PBS buffer solution pH = 7.40 and H_2O_2, (10 mM) (c) Changes in relative fluorescence intensity of **Umb-DHB** (5 μM) at 460 nm with other ROS and biologically relevant species (100 μM each species) at λ_{ex} = 350 nm, Slit widths: ex 10 nm and em 2.5 nm. (d) Changes in relative fluorescence intensity of **Res-DHB** (5 μM) at 585 nm with other ROS and biologically relevant species (100 μM each species) at λ_{ex} = 500 nm, Slit widths: ex 10 nm and em 3.5 nm. These measurements were performed in PBS buffer solution pH = 7.40.

to monitoring changes in their respective fluorescence emissions when exposed to ultrasound (1 MHz) produced from a bespoke sonoreactor (see ESI† – methods). The bespoke sonoreactor was designed to create a region of high intensity ultrasound and hence acoustic cavitation in a water filled vessel. As seen in Fig. 3a and b, an increase in fluorescence emission was seen with increasing sonication time (0–6 min) at 1.2 W cm^{-2} time averaged power per unit area. The release of each fluorophore was confirmed by LC-MS analysis (see ESI† – Fig. S5–S8) and the TA assay confirmed the ultrasound-induced production of •OH radicals (see ESI† – Fig. S9).[30] It is important to note that the ultrasound parameters need further optimisation in order to fully convert each probe to the corresponding fluorophores.

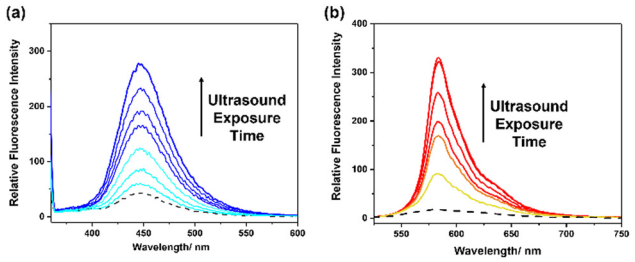

Fig. 3 (a) Fluorescence spectra of **Umb-DHB** (5 μM) with increasing exposure time to ultrasound (1.083 MHz, 1.2 W cm^{-2}) in PBS, pH = 7.40 (b) Fluorescence spectra of **Res-DHB** (5 μM) with increasing exposure time to ultrasound (1.083 MHz, 1.2 W cm^{-2}) in PBS, pH = 7.40.

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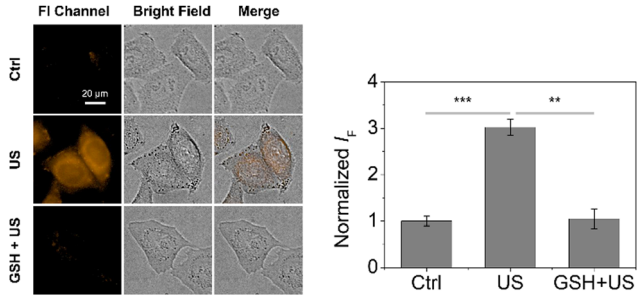

Fig. 4 Fluorescence imaging and quantification of HeLa cells incubated with **Res-DHB** (10 μM) with and without the addition of GSH (10 mM) upon sonication. Ultrasound: 1 MHz, 1.5 W cm^{-2}, 50% duty cycle, 6 ms pulse length. Time: 5 min. Mean values ± standard deviation, $N = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$.

Resorufin was chosen in this study due to its extensive use in fluorescence probe design for imaging key chemical biomarkers *in vitro* and *in vivo*.[31,32] We hypothesized that **Res-DHB** may have the potential to image ultrasound-induced production of •OH radicals in live cells. However, to demonstrate the potential of this strategy in biological settings, we had to first evaluate the fluorescence response of **Res-DHB** to ultrasound generated from a clinically used commercial instrument – WED-100 (1 MHz). The fluorescence response of **Res-DHB** correlated with intensity (0, 0.5, 1.5, 2 W cm^{-2} for 5 min) and increasing duration of ultrasound irradiation (0, 5, 10, 15, 20, 25, 30 min at 1.5 W cm^{-2}) (see ESI† – Fig. S10). The release of resorufin was confirmed by mass spectrometry (see ESI† – Fig. S11). In addition, we synthesised other bis-functionalised benzyl resorufin derivatives (**R1–R9**) including 3,5-dimethoxylbenzyl[33]-functionalised resorufin in an effort to identify additional •OH radical responsive units. To our surprise and despite literature precedent with ionising radiation,[4,33] only **Res-DHB** afforded noticeable changes in fluorescence emission intensity when sonicated for 5 min and 10 min (see ESI† – Fig. S12). This suggests that ionizing radiation may deprotect these types of protecting groups by additional mechanisms.

With the promise shown by **Res-DHB** using WED-100, we next turned to evaluating the responsiveness of **Res-DHB** to ultrasound-induced •OH radical production in live cells. CCK-8 assay was first performed to evaluate the cell viability of HeLa cells when incubated with different concentrations of **Res-DHB**. Both **Res-DHB** and the parent fluorophore resorufin were found non-toxic at the relevant fluorescence imaging concentrations (0–10 μM) and imaging timeframe (>2 h) (see ESI† – Fig. S13). No toxicity was seen for the duration of the ultrasound irradiation (5 min, 1.5 W cm^{-2}) – see ESI† – Fig. S14. We next incubated **Res-DHB** (10 μM) for 120 min followed by sonication (1.5 W cm^{-2}) for 5 minutes. As seen in Fig. 4, this treatment resulted in a significant increase (3-fold) in fluorescence emission compared to the HeLa cells not exposed to ultrasound irradiation. This increase in fluorescence emission was dependent on exposure time (0–10 min) and power (0–2 W cm^{-2}) (see ESI† – Fig. S15 and S16). In addition, pork tissue (2 cm) placed between the cells followed by ultrasound treatment (1.5 W cm^{-2}, 5 min) showed that distance did not impact the ultrasound induced fluorescence response (see ESI† – Fig. S17). However, due to the ROS-dependent nature of this strategy and the complexity of cellular environments, we subsequently tested the fluorescence response in the presence of the antioxidant GSH.[34] As seen in Fig. 4, GSH had a clear inhibitory effect on the ultrasound-induced fluorescence response (Fig. 4). This data suggests that this ultrasound-induced activation could be limited to cell types due to GSH concentrations ranging vastly in cancers.[35]

In summary, we have synthesised two hydroxyl-radical (•OH) responsive fluorescent probes (**Umb-DHB** and **Res-DHB**) that utilise the DHB functionality. Fluorescence changes were observed in solution and in cells when irradiated with therapeutic ultrasound frequency. However, GSH concentration was found to impact the ultrasound-induced fluorescence response in HeLa cells. These observations highlight the potential of ultrasound activation of small molecules, however further work is needed to identify the correct ultrasound parameters and cell types for this strategy to be appropriate. Further work is ongoing in our laboratories.

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Conflicts of interest

There are no conflicts to declare.

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