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Taking advantage of the aromatisation of 7-diethylamino-4-methyl-3, 4-dihydrocoumarin to fluorescently sense superoxide anion

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The aromatisation of 7-diethylamino-3, 4-dihydrocoumarin provides an alternative fluorescent probe to selectively detect the concentration of superoxide anion in solution. In addition, we reported the advantage to evaluate $O_2^{\bullet-}$ sensing probes in anhydrous DMSO instead of in aqueous buffers when using KO_2 as the surrogate of $O_2^{\bullet-}$.

Reactive oxygen species (ROS) refers to a group of reactive oxygen metabolites, including the superoxide anion ($O_2^{\bullet-}$), ozone (O_3), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), singlet oxygen ($^1O_2$), hydroxyl radical (•OH) and several others.[1-6] They are involved in many important physiological and pathological events in living organisms.[7-9] Among these ROS, $O_2^{\bullet-}$ is a highly reactive oxidising agent. Within physiological conditions, $O_2^{\bullet-}$ is typically found in very low concentrations and is consumed quickly by various surrounding reductants.[1, 2] Thus it is very difficult to detect the concentration of $O_2^{\bullet-}$ within biological settings.

The measurement of $O_2^{\bullet-}$ relies on various electrochemical, spectrophotometric, luminescent, and vibrational methods.[1] Fluorescence is a popular type of luminescent method that has been well recognised for its high sensitivity.[10-12] Fluorescent methods to sense $O_2^{\bullet-}$ often utilise a fluorescent-quenched probe, which is oxidised by $O_2^{\bullet-}$ to "switch on" the fluorescence.[1] Several types of fluorescent $O_2^{\bullet-}$ sensing probes have been reported, most notably probes based on the dehydrogenation reaction with benzothiazoline[13] and the sole interconversion of phenol and quinone.[14] However, due to their various roles in biology, there is a constant demand for novel and sensitive $O_2^{\bullet-}$ sensing probes.[2]

Our group has a burgeoning interest in the development of selective and accurate fluorescent probes for a range of analytes. The fluorophore of coumarin has been widely used in various fluorescent detections owing to its excellent photo-physical properties.[12] We synthesized the potential probe molecules containing the key scaffold 7-amino-4-methyl-3, 4-dihydrocoumarin and their oxidised fluorescent coumarin counterparts (Figure 1, ESI). Probe **1** is an oil like compound, and it has good water-solubility. Probe **1** can be dissolved into various aqueous buffers to the concentration of 500 µM without any precipitation (ESI, Fig. S1-3). In addition, both probe **1** and its' fluorogenic compound **2** remain stable under various pH conditions (pH 3, 7 and 11, ESI, Fig. S4). We reasoned that we could take advantage of the facile oxidation of probe **1** to the fluorogenic coumarin **2**, to provide the community with a sensitive ROS probe. In addition, we also synthesized probe **3** (7-diethylamino-4-hydroxymethyl-3, 4-dihydrocoumarin 4-naphthylacetate, ESI) with enhanced cell permeability, which will be used in cellular level of ROS examination.

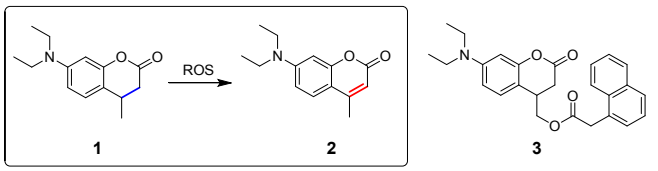

Fig. 1 The structures of probe **1** (7-diethylamino-4-methyl-3, 4-dihydrocoumarin) and the oxidised fluorogenic compound **2** (7-diethylamino-4-methyl-coumarin). Probe **3** (7-diethylamino-4-hydroxymethyl-3, 4-dihydrocoumarin 4-naphthylacetate) has improved cell permeability.

Probe **1** was prepared 10 mM stock solution in acetonitrile. Firstly, we incubated 10 µM probe **1** with 500 µM of various analytes (H_2O_2, *tert*-butyl hydroperoxide, ClO^-, $ONOO^-$, $^1O_2$, •OH) in 10 mM PBS buffer, pH7.4 at 37°C for 5 min. The results (Figure 2A) showed that the measured fluorescence intensity of all six reactions was similar to that of the blank solution, which means that probe **1** cannot be oxidised by any of these ROS at such conditions. Then, we started to examine the reaction between probe **1** and $O_2^{\bullet-}$. We chose the Xanthine oxidase (XO)/ hypoxanthine (HPX) system as it is known to reproducibly produce $O_2^{\bullet-}$.[2, 15] Thus, we incubated 10 µM and 50 µM of probe **1** with a XO/HPX system (~1.4 U/ml) and analysed its fluorescence. To our delight we observed as strong and time dependent fluorescence change (Figure 2B). As shown, the oxidation of probe **1** by $O_2^{\bullet-}$ proved rapid in nature. We

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

observed that the fluorescence produced in this system is both time and substrate concentration dependent. As the time progresses, the fluorescence gradually reaches peak level. Thus, $O_2^{\bullet-}$ can specifically turn on the fluorescence of probe **1**.

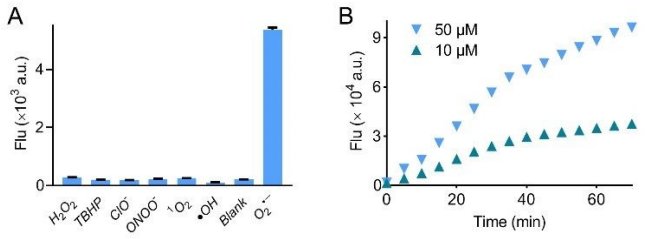

Fig. 2 Fluorescence responses of probe **1** to various ROS, fluorescence intensity was measured with E_x = 371 nm/E_m = 468 nm. (A), 10 μM probe **1** was mixed with 500 μM various ROS (H_2O_2, TBHP, ClO^-, $ONOO^-$, $^1O_2$, •OH) in 0.01 M PBS buffer, pH 7.4 and $O_2^{\bullet-}$ (XO/HPX) in 0.1 M HEPES buffer, pH 7.4 for 5 min at 37°C; (B), Time course for the change in fluorescence intensity observed with various concentrations of probe **1** incubated with XO/HPX in HEPES buffer.

Next, instead of using aqueous buffer, we selected anhydrous DMSO as the solvent for *in vitro* $O_2^{\bullet-}$ detection. We examined the oxidation of probe **1** by $O_2^{\bullet-}$ in DMSO. Both probe **1** (final concentration 2.5 mM) and KO_2 (final concentration 7.5 mM) were mixed in anhydrous DMSO, and the fluorescence (E_x = 371 nm/E_m = 468 nm) was measured at 37°C for 10 min (Figure 3A). As is shown in Figure 3A, in DMSO probe **1** was quickly oxidised by $O_2^{\bullet-}$ to form the fluorogenic product. The fluorescence reaches to peak level after 5 minutes of reaction. We also measured the LOD of probe **1** to detect $O_2^{\bullet-}$ in DMSO as 1.1 μM (Figure 3B), which is reasonable sensitive among these reaction based fluorescent probes.[2, 16] In order to confirm the fluorogenic product, the reaction mixture in DMSO was scanned the fluorescence spectra, which was overlapped with the fluorescence spectra of compound **2** in DMSO (Figure 3C, 3D). Then the reaction mixture was also analysed by LC-MS. Both compound **1** and **2** were found in the reaction mixture as the major products with retention times of 2.82 min and 4.52 min respectively (ESI, Fig. S5). Thus, we confirmed that $O_2^{\bullet-}$ can oxidise probe **1** to fluorogenic compound **2**.

Due to the high activity and oxidability of ROS, there is a chance that ROS might be consumed by other ingredients in the solution instead of the desired probe. This will cause problem for the detection. Firstly, there is a controversy regarding of the feasible surrogate of $O_2^{\bullet-}$ used in *in vitro* detection.[2, 16-18] Four types of $O_2^{\bullet-}$ surrogate were often used in previous literatures. Type (1), $O_2^{\bullet-}$ was produced in enzymatic systems, such as, XO/HPX;[18] Type (2), Solid KO_2 was dissolved in aqueous solution as the stock solution;[15] Type (3), Solid KO_2 was dissolved in anhydrous DMSO as the stock solution;[16] Type (4), Solid KO_2 was directly mixed with the aqueous solution containing the probes.[19]

For type (1), this is a classic enzymatic method to produce $O_2^{\bullet-}$. We speculated that the amino acid residues at the active centre of enzyme form a hydrophobic environment to temperately protect $O_2^{\bullet-}$, which also limit the excessive H_2O molecules, and favour the reaction between $O_2^{\bullet-}$ and probe **1**. Indeed, in this study probe **1** was successfully oxidised by $O_2^{\bullet-}$ produced in XO/HPX system. For type (2), it is clear now that in the KO_2 aqueous solution there is no superoxide anion instead HO^- and H_2O_2. Tampieri *et al* proved that 'when KO_2 is dissolved in water, superoxide cannot survive for a time long enough to be transferred and used in other processes'.[16, 18]

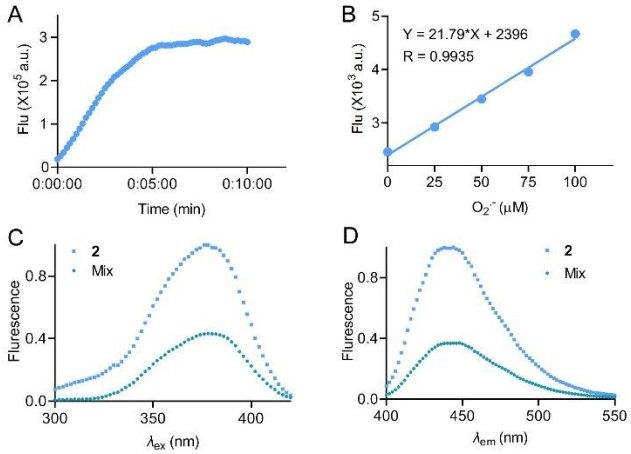

Fig. 3 Fluorescence responses of probe **1** to $O_2^{\bullet-}$ in anhydrous DMSO, fluorescence intensity was measured with E_x = 371 nm/E_m = 468 nm. (A), Probe **1** (2.5 mM) and KO_2 (7.5 mM) were reacted in DMSO, the fluorescence was measured. (B), A linear correlation was observed between the fluorescence intensity and $O_2^{\bullet-}$ concentrations in DMSO. (C, D), The fluorescence excitation and emission spectra (normalized fluorescence intensity) of reaction mixture or compound **2** in DMSO.

However, for type (3) and (4), when the 'KO_2 in DMSO' or 'solid KO_2' was added into the aqueous solution, the superoxide anion was immediately surrounded and competed by H_2O and the probes. We designed a study to shed a light on this circumstance. We mixed weighed solid KO_2 (10 mM final concentration of $O_2^{\bullet-}$) with 1 mM probe **1** in 10 mM PBS buffer (pH 7.4). A big amount of gases were released immediately and lasted for 3-5 seconds. Interestingly, no increased fluorescence was detected in the reaction mixture compared to the blank solution without KO_2 (ESI, Fig. S6). This means almost all superoxide anion reacted with H_2O, and no superoxide anion reacted with probe **1** in 10 mM PBS buffer, pH 7.4. Taken together, in the condition of type (3) and (4), in aqueous solution the number of H_2O is far more than the number of the probes. H_2O also reacts vigorously with $O_2^{\bullet-}$ with the k_{obs} of 9.7×10^7 $mol^{-1}S^{-1}$,[1, 17] and it is quite hard for the probe **1** to compete with H_2O to react with $O_2^{\bullet-}$. On contrary, stable $O_2^{\bullet-}$ can be detected in DMSO solution, which means the controllable concentration of $O_2^{\bullet-}$ in anhydrous DMSO. Furthermore, our probe is stable under various pH condition, the aromatised **2** can release fluorescence in DMSO. Thus, in

this study we decided to perform *in vitro* detection of superoxide anion in DMSO. Probe **1** can detect $O_2^{\bullet-}$ sensitively in DMSO with the LOD of 1.1 μM.

Secondly, special attention should be given to the solvents and reaction buffers used in the ROS detection. For the detection of ClO^-, phosphate buffer is preferred because both Tris-HCl and HEPES buffers can consume ClO^-.[9, 20, 21] In addition, DMSO is also reported as a good ClO^- scavenger, which can inhibit the oxidation ability of ClO^- at concentration as low as 0.00005% (v/v).[22] Thus, in the detection of ClO^-, DMSO cannot be used to prepare the stock solution of the probes. An interesting work by Sando's group described the oxidation between 7-amino-4-methyl-3, 4-dihydrocoumarin and the ClO^- in phosphate buffer (pH 7.4) with 0.1% DMF.[19] In this study, we also observed a weak fluorescence turn-on between probe **1** (7-diethylamino-4-methyl-3, 4-dihydrocoumarin) and excessive ClO^- in phosphate buffer (pH 7.4) with 0.1% DMF. However, if we used other solvents (Ethanol, Acetonitrile, Isopropanol, Acetone, Tetrahydrofuran, Dioxane) to prepare the 10 mM stock solution of probe **1**, we cannot observe any obvious fluorescence turn-on between probe **1** and excessive ClO^- in phosphate buffer (pH 7.4) (ESI, Fig. S7). Thus, without the presence of the catalyst, probe **1** does not react with ClO^- in phosphate buffer.

Moreover, we also used these six stock solutions of probe **1** to re-evaluate the reactions between probe **1** and several other ROS. Our results showed that generally all six solvents (final concentration 0.1%) did not affect the reaction between probe **1** and these ROS (H_2O_2, *tert*-butyl hydroperoxide, $^1O_2$, •OH, $ONOO^-$) in phosphate buffer (pH 7.4) (ESI, Fig. S8). We also found that probe **1** in all six solvents can be oxidized by $O_2^{\bullet-}$ in DMSO, and the reaction mixture exhibited the fluorescence when diluted in various buffers (ESI, Fig. S9-11). This proved the general application of probe **1** to detect superoxide anion.

Lastly, we also synthesized probe **3** with enhanced hydrophobic properties. Probe **3** was planned to be used in cellular level of ROS examination. Unfortunately, we were unable to use either probe **1** or its' derivative **3** (ESI) to sense the $O_2^{\bullet-}$ produced in PMA treated mammalian cells.[14, 16] Considering $O_2^{\bullet-}$ is placed the most top position for its oxidability in all ROS,[1, 2] selective $O_2^{\bullet-}$ probes usually exhibit low reactivity, which will not react with other ROS. In this study, probe **1** and its derivative **3** can selectively recognize $O_2^{\bullet-}$ but not other ROS, thus the overall reactivity of probe **1** and **3** should be low, and we speculated only a marginal amount of probe **3** was oxidised by relatively low level of $O_2^{\bullet-}$ in the complex cellular environment.

In conclusion, we report that the use of 7-diethylamino-4-methyl-3, 4-dihydrocoumarin as a reaction based fluorescent probe to selectively detect $O_2^{\bullet-}$ in solution. In addition, we also proposed and proved the advantages of *in vitro* detect $O_2^{\bullet-}$ in anhydrous DMSO instead of aqueous buffers when used KO_2 as the source of superoxide anion.

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Conflicts of interest

There are no conflicts to declare.

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