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A naphthalimide–naphthyridine derivative has been synthesized for the detection of hydroxyl radicals. It can distinguish hydroxyl radicals from other reactive oxygen species with high selectivity and short response time. Moreover, it has no cellular toxicity, and can be effectively used for intracellular detection of hydroxyl radicals.

Reactive oxygen species (ROS) are associated with a wide range of physiological and pathological processes, such as signal transduction, inflammation, ischemic or traumatic brain injury, carcinogenesis, and neurodegenerative diseases. 1,2 The primary ROS include hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl) and hypochlorite (ClO⁻), singlet oxygen (¹O₂), superoxide anion (O₂⁻), peroxynitrite (ONOO⁻), and hydroxyl radical (·OH). 1 Among those, the hydroxyl radical possesses high reactivity, and numerous biomacromolecules are vulnerable to oxidative damage from hydroxyl radicals, including carbohydrates, nucleic acids, lipids, and amino acids. 3 Methods to detect hydroxyl radicals have been widely explored, such as electron spin resonance spectroscopy and fluorescent probes. 4 Fluorescent probes are more advantageous than other methods because they have high sensitivity and accuracy, and can monitor hydroxyl radicals for real-time visualization in living cells. 5

Over the past decade, several types of fluorescent probes have been developed for the detection of hydroxyl radicals. Spin trapping hydroxyl radical probes, which can react with free radicals, causing a significant fluorescence enhancement, are the most common. 6–9 Aromatic hydroxylation probes were reported based on coumarin 10, 11 and a special Tb⁶⁺ complex. 12 In addition, rhodamine dyes have been applied to the design of a hydroxyl radical probe by oxidative hydrogen abstraction. 13 These probes show high sensitivity, but have low spatial resolution and are easily affected by environmental factors. By contrast, ratiometric fluorescent probes are not affected by factors such as probe concentration and environmental conditions, which makes them the ideal fluorescent probe. To date, only a few examples of hydroxyl radical ratiometric probes have been reported, including an inorganic–organic hybrid nanoprobe 14–17 and pure organic probe. 18, 19 However, those probes are rarely applied in the detection of hydroxyl radicals in living cells. Hence, designing a ratiometric probe for intracellular detection of hydroxyl radicals would be very useful.

1,8-Naphthalimide derivatives possess excellent spectroscopic properties, such as good photostability, high fluorescent quantum yield, and tuneable fluorescence emission. 20–23 The fluorescence spectra of 1,8-naphthalimide derivatives mainly depend on the electron donating ability of the 4-position substituent group based on the ‘push–pull’ internal charge transfer from the 4-position group to the electron withdrawing imide. Therefore, the 4-position is an important target for the design of fluorescent probes using 1,8-naphthalimide derivatives. Similarly, the fluorescence spectra of 7-methyl-naphthyridine derivatives can be tuned by a 2-position substituent group. Herein, we designed a ratiometric fluorescent probe 1, by connecting the 4-position of the naphthyridine group and the 2-position of naphthyridine groups using ethylenediamine (Scheme 1). The probe has high selectivity and sensitivity towards hydroxyl radicals over other reactive oxygen species, and can be used for intracellular detection of hydroxyl radicals.

Scheme 1 Chemical structure and synthetic routine of probe 1

The details of synthesis and characteristics of 1 are in the ESI†. The absorption spectrum of 1 (10 μM) shows two typical absorption bands at 371 and 461 nm in H₂O–DMF (98:2, v/v) (Fig. 1), which are characteristic of the naphthyridine and naphthalimide moieties, respectively, by comparison to the contrastive compounds S1 and S2.
High-level selectivity is of paramount importance for an excellent chemosensor. The selectivity of 1 was checked among seven species related to ROS. Fluorescence changes of 1 upon additions of hydroxyl radicals (50 eq) and other ROS (50 eq of H$_2$O$_2$, tert-butyl-hydroperoxide, ClO$^-$, ONOO$^-$, O$_2$, O$_2^-$, F and Fe$^{2+}$) in H$_2$O–DMF (98:2, v/v) are shown in Fig. 2a. Upon treatment with 50 eq of hydroxyl radicals, a marked strong blue emission at 418 nm was observed in less than 2 min, which indicated that hydroxyl radicals reacted with 1 rapidly at room temperature. In addition to the fluorescence changes, a large ratiometric fluorescence response at 418 to 552 nm was observed (F$_{418}$/F$_{552}$ = 53.2) with addition of 50 eq of hydroxyl radicals. By contrast, hypochlorite only induced a very weak ratiometric response with F$_{418}$/F$_{552}$ = 1.2, and negligible changes in the emission ratio (F$_{418}$/F$_{552}$ < 0.5) were noted upon addition of H$_2$O$_2$, tert-butyl-hydroperoxide, ONOO$^-$, O$_2$, O$_2^-$, F and Fe$^{2+}$ (Fig. 2b). The fluorescence images also display the sharp contrast response between hydroxyl radicals and other ROS by 1 (Fig. 2c). Moreover, both the fluorescence wavelength and intensity of 1 showed very tiny difference with the change of pH value from 3.0 to 8.0 (Fig. S3 in ESI†), which meant that pH has almost no influence on the detection of hydroxyl radicals by probe 1.

To investigate the reactivity and spectral details of 1 (10 μM) with hydroxyl radicals, spectroscopic titration was carried out upon addition of hydroxyl radicals (from 0 to 500 μM) in H$_2$O–DMF (98:2, v/v) at room temperature (Fig. S4 in ESI†). As shown in Fig. 3, the absorbance of 1 at both 371 and 461 nm increased with the addition of less than 2 eq of hydroxyl radical. Correspondingly, the intensity of the emission band at 552 nm of 1, which is characteristic of the naphthalimide moiety, increased at first and then decreased. As the amount of hydroxyl radicals increased to 10 eq, the absorbance at 461 nm, a characteristic of the naphthalimide moiety, gradually weakened, while the absorbance at 371 nm, a characteristic of the naphthyridine moiety, enhanced and blue shifted to 355 nm. Simultaneously, the intensity of the emerging fluorescence at 418 nm was drastically enhanced during the titration process. In addition, at least 10 eq of hydroxyl radical, an excellent linear correlation between the ratio of two emission intensities (F$_{418}$/F$_{552}$) and the hydroxyl radical concentration was obtained with R$^2$ = 0.99946, which indicated the ratio of fluorescence intensity at 418 and 552 nm increased as a linear function of the hydroxyl radical concentration. The detection limit of 1 to hydroxyl radical is 2.0 × 10$^{-7}$ M, which is superior to that in previous reports (7.3 × 10$^{-7}$ M). Thus, probe 1 has a high selectivity, quick reaction time and low detection limit for hydroxyl radicals, and can likely be used to detect intracellular hydroxyl radicals.

The mechanism of the reaction between 1 and hydroxyl radical was studied. The main product with blue fluorescent emission was isolated and purified by column chromatography as a yellow oil. The $^1$H NMR and mass spectra suggest that the product is a hydroxyl modified naphthyridine moiety without naphthalimide (Fig. S5 and S6). This means that probe 1 dissociates into a blue emissive naphthyridine moiety and a nonfluorescence naphthalimide part after the treatment of hydroxyl radical.
radicals, the original strong fluorescence in the green channel was entirely quenched with the fluorescence enhancement in the blue channel ($\lambda_{ex} = 405$ nm, Fig. 4b). The sensing capability of 1 for hydroxyl radicals produced by physiological stimulation was further investigated by the introduction of phorbol 12-myristate-13-acetate (PMA), which stimulates production of intracellular ROS. 1 RAW264.7 cells were incubated with 1 (5 $\mu$M) for 30 min, then cells were treated with PMA (50 ng mL$^{-1}$) for 1 h. Confocal fluorescence images very similar to that following stimulation with Fenton’s reagent were observed by PMA stimulation (Fig. 4c), which revealed that probe 1 responds to intracellular hydroxyl radicals produced by physiological stimulation. To confirm that the fluorescence changes ascribed to the generated hydroxyl radicals, cells were treated with TEMPOL (5 mM, a radical scavenger) for 1 h before stimulation with PMA. The fluorescence in the green channel was still present (Fig. 4d), which indicated that 1 could effectively detect intracellular hydroxyl radicals in living cells.

**Notes and references**


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**Fig. 4** CLMS images of RAW264.7 cells (a) incubated with 1 (5 $\mu$M) for 30 min and (b) followed by incubating with Fenton’s reagent (5 $\mu$M) for 1 h, (c) cells incubated with 1 (5 $\mu$M) for 30 min, and then with PMA (50 ng mL$^{-1}$) for 1 h and (d) incubated with PMA for 1 h, TEMPOL (5 mM) for another 1 h and finally with 1 for 30 min; 1-4 are bright field images, fluorescence images in blue channel (420–450 nm), green channel (520–570 nm) and overlay images, respectively.

Cytotoxicity is an important indicator for applicability of 1 as a probe in biological systems. Therefore, the effect of 1 on cell proliferation was determined by means of a MTT assay in Hela cells. 1 had no cellular toxicity in 36 h at a concentration of 5 $\mu$M. Even at a higher concentration (20 $\mu$M), the cellular viabilities were estimated to be greater than 85% in 36 h (Fig. S8). The low cytotoxicity and high biocompatibility make 1 a suitable candidate for intracellular detection of hydroxyl radicals.

In summary, a novel naphthalimide–naphthyridine derivative has been designed and synthesized for utility as a ratiometric fluorescence probe for the detection of hydroxyl radicals. The fluorescent probe exhibits high selectivity and can clearly distinguish hydroxyl radicals from other reactive oxygen species. It also has good sensitivity to hydroxyl radicals and responds rapidly in less than 2 min in H$_2$O–DMF solution. In addition, the detection limit for hydroxyl radicals can reach as low as $2.0 \times 10^{-7}$ M. Moreover, the fluorescent probe shows excellent photostability, low cytotoxicity and high biocompatibility, and can be used for intracellular detection of hydroxyl radicals, which is very significant to follow the tracks of hydroxyl radicals in physiological and pathological processes.

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