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## Selective detection of peroxyxynitrite using an isatin receptor and a naphthalimide fluorophore†

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**Peroxyxynitrite is a reactive oxygen and nitrogen species that participates in various biological reactions. Therefore, it is important to readily detect and track peroxyxynitrite in biological systems. Here, a novel turn-on probe encapsulated in PEG DSPE-PEG/HN-I was used to fluorescently detect ONOO<sup>-</sup> rapidly. The encapsulation of HN-I using DSPE-PEG2000 optimizes the sensing performances of the naphthalimide probe and avoids ACQ. Using DSPE-PEG/HN-I to detect changes in the levels of exogenous ONOO<sup>-</sup> in HepG2 cells and endogenous ONOO<sup>-</sup> induced by LPS in RAW 267.4 cells was demonstrated.**

Peroxyxynitrite (ONOO<sup>-</sup>) is a short-lived bioactive agent, belonging to the category of reactive oxygen and nitrogen species (ROS/RNS). Peroxyxynitrite is an oxidant that generates free radicals, and acts as a Lewis base in living systems.<sup>1</sup> Upon exposure to peroxyxynitrite, biochemical cycles can be promoted while cellular function and viability may be adversely affected, depending on the concentrations of peroxyxynitrite.<sup>2</sup> Peroxyxynitrite at low

concentrations can contribute to programmed cell death while higher concentrations of peroxyxynitrite promote a disruption of cellular energy production resulting in necrotic cell death.<sup>3–5</sup> Peroxyxynitrite is involved in mediating numerous reactions, including the promotion of mitochondrial dysfunction, the regulation of cellular signalling pathways, the breaking of cellular redox status, and the induction of pain response under inflammatory conditions.<sup>6–10</sup> While, Hooper and Padalko have reported that peroxyxynitrite can mediate the immune response to virus infection.<sup>11,12</sup> However, due to a lack of rapid measurement tools suitable to detect peroxyxynitrite and quantify its concentrations *in vivo*, most of the biological reactions peroxyxynitrite participates in have only been investigated *in vitro*.<sup>2</sup> As such, in order to further explore the role of peroxyxynitrite in disease pathogenesis, it is necessary to investigate and develop effective methods for peroxyxynitrite detection.

Fluorescent probes can detect analytes based on changes in spectroscopic properties promoted by targeted reactions.<sup>13–15</sup> In general, a fluorescent probe consists of three units: a fluorophore that produces spectroscopic signals; a receptor that is able to react with a specific analyte; a linker that is suitable for connecting the fluorophore with the receptor.<sup>13,16,17</sup> According to the reaction between the receptor and the targeted analyte, a fluorescent probe can exhibit specified fluorescence responsiveness to its targeted analyte.<sup>18</sup> In addition, turn-on fluorescent probes can exhibit enhanced monitoring capabilities against dark backgrounds, which results in reduced background interference.<sup>18</sup>

Aryl boronate groups and  $\alpha$ -ketoamide groups have been widely applied in the design of peroxyxynitrite targeting probes due to their good sensitivity and rapid response.<sup>19</sup> However, these receptors can also react with other ROS species, such as hydrogen peroxide and hypochlorite.<sup>20–24</sup> Significantly, isatin exhibits a sensitive, selective and rapid response to peroxyxynitrite.<sup>25–28</sup>

Naphthalimide fluorophores are on the whole cell permeable and exhibit good photostabilities.<sup>29</sup> Due to the electron-withdrawing ability of its imide core, naphthalimide exhibits strong intramolecular charge transfer (ICT) in its solution state when the C-4 site of naphthalimide is modified by electron

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donors.<sup>30–32</sup> These properties make naphthalimide an excellent fluorophore candidate for the construction of chemiluminescent probes for analyte detection in biological systems.<sup>30,33–37</sup> However, one of the main drawbacks of naphthalimide-based systems is low solubility. Poor solubility and the planar structure make naphthalimide undergo  $\pi$ - $\pi$  stacking easily, which results in aggregation-caused quenching (ACQ).<sup>29,38,39</sup>

In most cases, structural modification is used to solve solubility problems and overcome the ACQ of naphthalimide-based fluorophores.<sup>40–42</sup> However, this approach requires additional time-consuming synthesis. Therefore, we decided to explore a much simpler approach and use PEG encapsulation to improve solubility.<sup>43,44</sup> Polyethylene glycol (PEG) is widely used in the field of drug delivery as an excipient to improve molecular solubility.<sup>45–47</sup> In addition, DSPE-PEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-poly (ethylene glycol)) can reduce aggregation, improve the stability of nanoparticles and increase the circulation times of molecules *in vivo*.<sup>47–49</sup>

Here, we selected 4-hydroxy-1,8-naphthalimide (HN) as the fluorophore and a 1-methylindoline-2,3-dione moiety as the receptor to construct a novel fluorescent probe HN-I for the detection of ONOO<sup>-</sup>. According to the specific redox reaction between ONOO<sup>-</sup> and 1-methylindoline-2,3-dione, the fluorophore HN is released (Scheme 1).<sup>32,50</sup> In order to ensure the improvement of water solubility and the reduction of ACQ effect, we used DSPE-PEG2000 to encapsulate the fluorescent probe HN-I (Scheme 1).

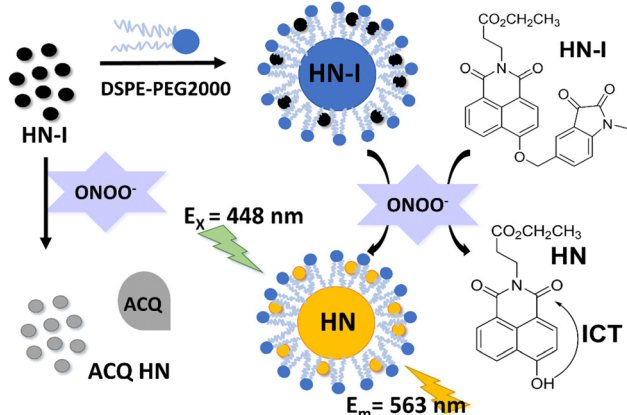
We were inspired by the group of Bruemmer who found that isatin-based groups could detect ONOO<sup>-</sup> with high selectivities without any interference from other ROS/RNS.<sup>25</sup> As such, we prepared HN where the C-5 position of a 1-methylindoline-2,3-dione moiety was linked to a naphthalimide (Fig. S1, ESI†). Upon the addition of ONOO<sup>-</sup>, the fluorescence of HN-I can be turned on (Fig. S2 and S3, ESI†). In addition, by using DSPE-PEG2000, the aqueous solubility of DSPE-PEG/HN-I can be improved. As we mentioned above, the improvement in solubility of HN-I when using DSPE-PEG2000 decreases the  $\pi$ - $\pi$  stacking of HN-I, which reduces ACQ and contributes to the recovery of the fluorescence (Fig. S4 and S5, ESI†). In other words, the sensing performances

of DSPE-PEG/HN-I were optimized by the encapsulation of DSPE-PEG2000 (Fig. S4 and S5, ESI† and Fig. 1a).

First, the absorption spectra of DSPE-PEG/HN-I were explored (Fig. 1a). Initially, the ICT effect of the probe was inhibited so that the probe was nonfluorescent. However, on the addition of ONOO<sup>-</sup>, the naphthalimide fluorophore of DSPE-PEG/HN was released, and the recovery of the ICT effect resulted in fluorescence enhancement. As such, when the solution was excited at 448 nm, the fluorescence emission of DSPE-PEG/HN at 563 nm was enhanced. The fluorescence intensity of DSPE-PEG/HN-I increased with increasing concentrations of ONOO<sup>-</sup> from 0  $\mu$ M to 30  $\mu$ M (Fig. 1b). For ONOO<sup>-</sup> from 0–15  $\mu$ M, the fluorescence response was linear (Fig. 1c). The limit of the detection of DSPE-PEG/HN-I for ONOO<sup>-</sup> was calculated to be 22 nM (Fig. 1c). The reaction rate between DSPE-PEG/HN-I and ONOO<sup>-</sup> was rapid. From Fig. 1f, the fluorescence intensities reached the highest level at around 240 s. In addition, the selectivity of DSPE-PEG/HN-I was evaluated. Based on the graph of Fig. 1e, DSPE-PEG/HN-I exhibited no fluorescent responses upon the addition of other ROS including ROO<sup>•</sup>, H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>•-</sup>, <sup>•</sup>OH, <sup>1</sup>O<sub>2</sub> and ClO<sup>-</sup>. The pH sensitivity of DSPE-PEG/HN-I was then evaluated from 4.5 to 9.5 (Fig. 1d).<sup>9,51,52</sup> Over a pH range from 6.5 to 9.5, DSPE-PEG/HN-I exhibited low pH sensitivity, which confirms that DSPE-PEG/HN-I can be used for monitoring ONOO<sup>-</sup> in biological systems.

Based on excellent sensing performances of DSPE-PEG/HN-I in solution, we evaluated DSPE-PEG/HN-I for imaging ONOO<sup>-</sup> in a human liver cancer cell line (HepG2). Before cell fluorescence imaging tests, the cell viability of DSPE-PEG/HN-I was investigated in live HepG2 cells by a cell counting kit-8 (CCK-8) assay. The results suggested that DSPE-PEG/HN-I showed almost no cytotoxicity (cell viability  $\approx$  100% treated with a 40/40  $\mu$ M DSPE-PEG/HN-I) (Fig. S7, ESI†). DSPE-PEG/HN-I was evaluated with and without SIN-1 (a typical ONOO<sup>-</sup> exogenous donor). As expected, without SIN-1, there was no fluorescence observed (Fig. 2a). However, with a concentration increase of SIN-1, the fluorescence intensity enhanced 5-fold (Fig. 2a–c). Then, DSPE-PEG/HN-I was also shown to detect exogenous ONOO<sup>-</sup> in SIN-1-treated human cervical cancer cell line (HeLa). However, after pretreatment of cells with *N*-acetylcysteine (NAC, an ONOO<sup>-</sup> scavenger), it attenuated the increase in the fluorescence of DSPE-PEG/HN-I induced by SIN-1 (Fig. S8, ESI†). Furthermore, the possibility of using DSPE-PEG/HN-I for endogenous ONOO<sup>-</sup> detection was also investigated in a macrophage cell line (RAW 264.7). RAW 264.7 cells were incubated with lipopolysaccharide (LPS) which can promote inflammatory response and upregulate the concentration of ONOO<sup>-</sup>.<sup>53–56</sup> As shown in Fig. 2D, the fluorescence intensity of DSPE-PEG/HN-I exhibited a 1.5-fold increase for LPS-loaded RAW 264.7 cells. After RAW 264.7 cells were incubated with both LPS and NAC, weak fluorescence was observed. All these results indicated that DSPE-PEG/HN-I can be applied for the detection of both exogenous and endogenous ONOO<sup>-</sup>.

In summary, a novel turn-on probe was designed for the highly selective detection of ONOO<sup>-</sup>. To avoid the ACQ effect, a disadvantage of naphthalimide fluorophores, DSPE-PEG2000 was used to encapsulate HN-I to improve the sensing performances.

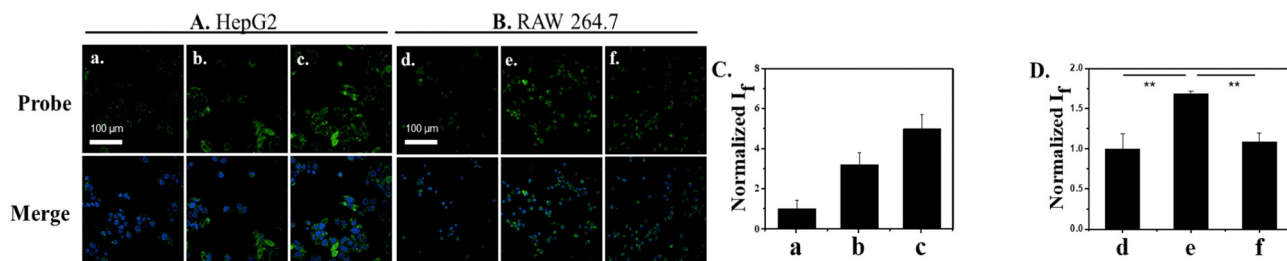


Scheme 1 Schematic for use of DSPE-PEG/HN-I in the detection of peroxynitrite reaction mechanism of HN-I.





**Fig. 1** (a) UV-vis of **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) without (the orange line) or with (the blue line) the addition of  $\text{ONOO}^-$  (30  $\mu\text{M}$ ). (b) Emission spectra for **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) in the presence of  $\text{ONOO}^-$  (0–30  $\mu\text{M}$ ). (c) Dose dependence curve at  $\lambda_{\text{max}} = 563 \text{ nm}$ . Inset: Linear fluorescence signals of **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) towards  $\text{ONOO}^-$  (0–15  $\mu\text{M}$ ). (d) Effects pH left on the fluorescence intensities of **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) without (red bars) or with (blue bars)  $\text{ONOO}^-$  (30  $\mu\text{M}$ ). (e) Selectivity data for Emission spectra for **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) upon the addition of  $\text{ONOO}^-$  (30  $\mu\text{M}$ ),  $\cdot\text{OH}$  (500  $\mu\text{M}$ ),  $\text{O}_2^{\cdot-}$  (500  $\mu\text{M}$ ),  $^1\text{O}_2$  (500  $\mu\text{M}$ ), after 5 min.  $\text{H}_2\text{O}_2$  (1 mM),  $\text{ROO}^{\cdot}$  (500  $\mu\text{M}$ ), and  $\text{ClO}^-$  (500  $\mu\text{M}$ ) were measured after 30 min. (f) The graph of time driver of **DSPE-PEG/HN-I** (10/10  $\mu\text{M}$ ) with the concentrations of  $\text{ONOO}^-$  at 0, 5, 15  $\mu\text{M}$ . The data was obtained in PBS buffer (5.5 mM, containing 1% DMSO), pH = 7.4 at 25  $^\circ\text{C}$ ,  $\lambda_{\text{ex}} = 448 \text{ nm}$ ,  $\lambda_{\text{em}} = 563 \text{ nm}$ .



**Fig. 2** (A) Imaging of exogenous  $\text{ONOO}^-$  in HepG2 cells. **DSPE-PEG/HN-I** (20/20  $\mu\text{M}$ , 1 h)-loaded HepG2 cells incubated with various concentration (a–c: 0, 2, 4 mM) of SIN-1 for 4 h, and then imaged. (B) Imaging of endogenous  $\text{ONOO}^-$  in RAW 264.7 cells. (d) RAW 264.7 cells incubated with 20/20  $\mu\text{M}$  **DSPE-PEG/HN-I** for 4 h and imaged. (e) RAW 264.7 cells were treated with  $1.0 \mu\text{g mL}^{-1}$  LPS for 24 h and incubated with 20/20  $\mu\text{M}$  **DSPE-PEG/HN-I** for 1 h and imaged. (f) RAW 264.7 cells were treated with  $1.0 \mu\text{g mL}^{-1}$  LPS for 24 h in the presence of 1 mM NAC and incubated with 20/20  $\mu\text{M}$  **DSPE-PEG/HN-I** for 1 h, and then imaged. Normalized intensities in a–c (C) and image d–f (D).  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$ . In these cellular experiments, DMSO was used at the concentration of 0.2%.

We determined that **DSPE-PEG/HN-I** can detect  $\text{ONOO}^-$  rapidly in solution. In addition, **DSPE-PEG/HN-I** can be used to image exogenous and endogenous  $\text{ONOO}^-$ . These results confirm the potential of the **DSPE-PEG/HN-I** for the monitoring of  $\text{ONOO}^-$  in biological systems.

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## Conflicts of interest

There are no conflicts to declare.

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