Reversible two-photon fluorescent probe for imaging of hypochlorous acid in live cells and in vivo

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Herein, we have developed a novel reversible two-photon fluorescent probe that is well suited for monitoring HOCl levels selectively and instantaneously. Results showed the reversible and instantaneous responses of the probe towards intracellular HOCl. Moreover, the probe was successfully applied to the imaging of the HOCl levels in zebrafish and mice via two-photon imaging.

Hypochlorous acid (HOCl), as one of the biologically important reactive oxygen species (ROS), is produced by the myeloperoxidase- \( \text{H}_2\text{O}_2-\text{Cl}^- \) system, mainly in leukocytes, and plays a significant role in many biologically vital processes.\(^1\)–\(^9\) However, the excessive generation of HOCl is associated with many inflammation-related diseases, including lung injury, rheumatoid arthritis, and renal disease.\(^10\)–\(^14\) Therefore, exploring HOCl fluctuations in cells and in vivo is of great significance. Fluorescence sensing is advantageous because of its high sensitivity and selectivity. More importantly, it is able to achieve the visualization analysis of HOCl fluctuations in cells and in vivo through fluorescence imaging.\(^15\)–\(^28\) For the ideal monitoring of HOCl levels in cells and in vivo, the fluorescence probe must possess the ability of fast response, reversibility and good penetration depth, as well as it should cause very less damage to cells or in vivo. Therefore, the fabrication of novel fluorescent probes based on the abovementioned merits for the imaging of HOCl is highly demanded.

As is well known, two-photon (TP) imaging methods have advantages over their one-photon (OP) counterparts by offering the ability of increased penetration depth and reduced specimen photodamage owing to the excitation employing two lower energy photons.\(^29\)–\(^30\) Currently, several efforts have been made to develop a TP-based fluorescent probe and have been applied to the imaging \( \text{H}_2\text{O}_2 \) and thiols;\(^31\)–\(^33\) however, no reversible and instantaneous TP fluorescent probes for imaging HOCl in live cells and in vivo have been reported to date. Considering the dynamic change of HOCl in cells or in vivo, the reversible and instantaneous properties of TP fluorescent probes are necessary to visualize the changes in HOCl concentration. Based on the abovementioned considerations, a novel turn-on probe (FO-PSe) for HOCl was designed based on the 9-fluorenone covalently conjugated with two selenium-containing compounds (Scheme 1). The reversible recognition ability of the probe can be achieved through the oxidation–reduction cycles of the selenium center. 9-Fluorenone was selected as a TP fluorophore because of its excellent TP fluorescent performance. In the presence of HOCl, the selenium of the probe was oxidized specifically and the \( \pi \) electron of the conjugated system redistributed accordingly. Results showed that the OP and TP fluorescence of FO-PSe enhanced markedly in the presence of HOCl. Importantly, the probe FO-PSe was successfully applied to cells, zebrafish and mice to dynamically visualize the changes of the HOCl levels under various stimulations. Compared with previous fluorescent probes for HOCl, the probe combined the merits of deep penetration, less photodamage, reversibility and instantaneity, which makes it well suited for the dynamic and reversible fluorescence imaging of HOCl fluctuations in live cells and in vivo.

The optical properties of FO-PSe were investigated in an aqueous medium buffered to physiological pH (0.015 M HEPES, DMSO/water = 1:9 v/v, pH 7.4). The FO-PSe features a prominent absorption band centered at 415 nm (Fig. S1, ESI†). A corresponding emission maximum appears at around 520 nm, and the excitation maximum is around 415 nm for OP and 800 nm for TP. After the addition of HOCl, TP fluorescence emission was enhanced significantly (Fig. 1b) and the structure of the products (FO-PSeO) was confirmed by mass spectrometry (\( m/z \): 524.9556 (M + H))

![Scheme 1](image_url) Proposed reaction mechanism, structures of FO-PSe and its oxidized product FO-PSeO.
The reversibility implied that FO-PSe can be used for dynamic detection and imaging of HOCl levels in cells and in vivo. In addition, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in RAW 264.7 macrophages was performed (Fig. S5, ESI†) and the results showed less cytotoxicity of FO-PSe.

To prove the selectivity of FO-PSe for HOCl, various ROS and reactive nitrogen species (RNS) were selected to test the fluorescence responses with HOCl, and the results are shown in Fig. 2. It can be seen from Fig. 2 that FO-PSe exhibited high fluorescence enhancement for HOCl, while other ROS and RNS, such as singlet oxygen, hydroxyl radical, superoxide anion, nitric oxide and peroxynitrite (ONOO⁻), did not lead to significant fluorescence response with FO-PSe, even at higher concentrations than HOCl, under the same conditions. This demonstrates the high selectivity of FO-PSe towards HOCl. Thus, the properties of selectivity, reversibility and instantaneous, endow the probe with the ability of detection and imaging of HOCl in cells and in vivo.

Because the probe FO-PSe exhibited high sensitivity, selectivity, and reversibility, we next tested the performance of FO-PSe for monitoring HOCl in living cells under physiological conditions. The mouse macrophage cell line RAW264.7 was chosen as a bioassay model because macrophage cells activate the generation of ROS after exposure to PMA. As shown in Fig. 3, there was almost no fluorescence in the absence of stimulant. In contrast, strong fluorescence was observed after treatment with PMA (1.0 μg ml⁻¹). When the cells were treated with 4-aminobenzoic acid hydrazide (ABAH), a potent inhibitor of MPO,¹⁷ the fluorescence was significantly reduced in the stimulated cells. These results indicate that FO-PSe can be used to selectively visualize HOCl in cells. In addition, TP (Fig. 3d–f) fluorescence imaging analysis of living mice macrophages was investigated, and the results were consistent with that of OP fluorescence images. However, from the point of imaging, the TP imaging more clearly showed the distribution of HOCl in the cell, which further indicated the merits of the as-prepared TP-based probe. Moreover, identical results from OP and TP imaging indicated that FO-PSe could visualize the changes of HOCl levels in cells. More importantly, identical results from the two fluorescence imaging methods make the results more convincing.

To exhibit the applications of FO-PSe in intravital imaging, the OP and TP fluorescent imaging of reversible cycles in zebrafish was
then performed (Fig. 4 and Fig. S6 and S7, ESI†). As shown in Fig. 4, a much stronger response was observed when the probe-loaded zebrafish were incubated with zymosan (Zym) (Fig. 4b) and the fluorescence of these zebrafish decreased markedly after adding vitamin c (Vc) (Fig. 4c). The same results were obtained by adding HOCl followed by treatment with Vc (Fig. S4, ESI†), which showed the good performance of the probe for imaging HOCl levels in vivo.

Then, the probe was applied to the imaging of HOCl in the abdomen of mice (Fig. S7, ESI†). As shown in Fig. S7 (ESI†), the fluorescence was enhanced obviously by zymosan stimulation and reduced treatment with Vc. These results strongly suggested that FO-PSe was able to image HOCl levels in vivo. At the same time, the probe FO-PSe was used to monitor the changes of HOCl levels deep inside mice and zebrafish (Fig. 5 and Fig. S8, ESI†). It can be seen from Fig. 5 and Fig. S8 (ESI†) that FO-PSe was able to probe HOCl in deep tissues. Using the advantage of TP deep-tissue imaging (288 μm), the broader application of FO-PSe can be achieved in bioimaging.

According to the features of HOCl in cells or in vivo, we have designed and synthesized a novel HOCl probe with TP fluorescence properties, which has been used for monitoring intracellular HOCl levels selectively and reversibly. Moreover, the probe as a fluorescent imaging tool was well suited for tracking HOCl levels in zebrafish and mice via the high resolution and deep tissue penetration of TP imaging. The reversible and TP features of the probe were efficiently applied in various depth imaging of HOCl, making it an ideal tool to further investigate the HOCl levels in live cells and in vivo.

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Notes and references