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Malononitrile as the ‘double-edged sword’ of passivation-activation regulating two ICT to highly sensitive and accurate ratiometric fluorescent detection for hypochlorous acid in biological system

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1. Introduction

Reactive oxygen species, keeping a certain concentration range under normal physiological conditions, had been proved to play an important role in maintaining cell morphology and maintaining basic cell function [1]. It was generally believed that HOCl can be obtained in organisms by hydrogen peroxide and chloride ions catalyzed by myeloperoxidase (MPO) [2]. As one of the representative substances in reactive oxygen species, HOCl played an irreplaceable role in daily life and vivo [3]. On the one hand, HOCl was used as bleach, disinfectant, deodorant, etc [4–7]. Especially, HOCl played a crucial role as the main disinfectant on the novel coronavirus (novel coronavirus (2019-nCoV)) incident. On the other hand, the excessive HOCl could cause various diseases such as cardiovascular disease [8], arthritis [9], arteriosclerosis [10] and cancer [11,12]. Therefore, it was of profound significance to realize accurate detection of HOCl in vivo [13–32].

Anisaldehyde, as an important intermediate in organic synthesis, was widely used in medicine, food and other fields [33–37]. To as we know, anisaldehyde could be as a spice in condiments in China, which fully demonstrated anisaldehyde owns particularly biocompatible and lower toxic.

Due to its attractive properties by controlling the emission wavelength of the probe molecules through the donor-acceptor system, dyes based on dicyanoisophorone (DCO) have received widespread attention [36,37]. Besides, the C=C bond of (DCO) was often used as the reaction site of HOCl, which could be oxidized to aldehydes or ketones by HOCl under mild conditions [38–41]. Due to the change of molecular structure, the molecular realized the ratiometric fluorescence detection to HOCl [42]. Therefore, it was a wise choice to design a ratiometric HOCl fluorescent probe combining anisaldehyde and DCO.

Considering the above, we synthesized a novel HOCl fluorescent probe (AI) based on anisaldehyde (donor) and DCO (acceptor) fluorescent dye (Scheme 1). Owing to the presence of electron-withdrawing CN group and electron-donating group —OCH3, the ICT-1 of probe AI was triggered to show obvious fluorescent signals. However, the electron-withdrawing CN group of probe AI attacked by HOCl, the ICT-1 process from —OCH3 moiety to CN group was disturbed. Simultaneously, the ICT-2 process was triggered from —OCH3 moiety to electron-withdrawing ketone group, which the fluorescence spectra displayed ratiometric manners to HOCl in solution.

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At the same time, the spectral test results were consistent with the above analysis, probe $AI$ showed good selectivity and low detection limits. Most importantly, the probe had low toxicity and high specificity for HOCl, which could achieve in vivo and in vitro monitoring of HOCl.

2. Material and methods

2.1. The preliminary of experiments

The details of the material, setting of the spectra and preparation of the imaging experiment were available in the supporting information.

2.2. The preparation and characterization of $AI$

The synthetic route of $AI$ was showed in Scheme 1. $AI$ was synthesized according to the literature report [43–45]. $AI$ was characterised by NMR and HR-MS: $^1$H NMR (600 MHz, DMSO-$d_6$): δ 7.67 (d, $J=7.6$ Hz, 2H), 7.28 (s, 2H), 6.99 (d, $J=7.6$ Hz, 2H), 6.84 (s, 1H), 3.80 (s, 3H), 2.61 (s, 2H), 2.54 (s, 2H), 1.02 (s, 6H) (Fig. S1); $^{13}$C NMR (151 MHz, DMSO-$d_6$): δ 170.83, 161.05, 156.96, 138.19, 130.94, 130.10, 129.11, 127.69, 123.16, 122.27, 114.92, 114.53, 113.71, 75.65, 55.59, 42.76, 38.62, 31.90, 27.91, 27.57(Fig. S2); HR-MS m/z: calcd for 304.15756; found: m/z: 304.15681(Fig. S3).

3. Results and discussion

3.1. Theoretical calculation

In our original design, the electron-withdrawing CN group of probe $AI$ was attacked by HOCl, and then weaker electron-withdrawing ketone group was formed. Due to the decrease of the ability of electron absorption, the wavelength of absorption and emission would be blue shifted. Therefore, we selected the Gaussian 09 program to predict the optical properties of $AI$. By the density functional theory (DFT) and B3LYP/6-31G(d) method, the optimized structure and energy levels of $AI$ and $AIO$ were obtained (Fig. 1). The LUMO (Lowest unoccupied molecular orbital) and HOMO (Highest occupied molecular orbital) of $AI$ and $AIO$ were uniformly distributed throughout the molecule. Compared with $AI$, the HOMO and LUMO of $AIO$ increased, and the gap enlarged, which was caused by the decreased electron-absorbing ability from -CN to carbonyl. Based on these results, we initially believed that $AI$ could be considered as an efficient ratiometric HOCl fluorescent probe.

3.2. UV-vis and fluorescence spectra of $AI$ titrated with HOCl

In order to study the optical response of the probe $AI$ to HOCl, the detection methods of UV-vis and fluorescence spectra were carried out (Fig. 2a). Firstly we analyzed the UV-vis spectra of probe $AI$ upon increasing the concentration of HOCl in DMSO/PBS (1/1, v/v, pH = 7.4). As Fig. 2a showed, the absorption peak of 417 nm gradually disappeared, meanwhile the absorption peak of 345 nm gradually increased and an equal absorption point appeared at 360 nm with increasing of HOCl, which meant that $AI$ reacted with HOCl to generate a new substance ($AIO$). Subsequently, fluorescence property of $AI$ was also studied in DMSO/PBS (1/1, v/v, pH = 7.4). As Fig. 2b showed, $AI$ displayed maximum emission peak appeared at 570 nm under excitation at 370 nm in the system. When HOCl was added gradually, the emission

\[ \text{Scheme 1. The synthesis route of the probe } AI. \]

\[ \text{Fig. 1. Theoretical calculation of HOMO / LUMO energy gaps of } AI \text{ and } AIO. \]

\[ \text{Fig. 2. Absorption spectra of } AI (10 \mu M) \text{ with increasing HOCl (0–60} \mu \text{M}) (a) and (b) fluorescence spectra of } AI (10 \mu M) \text{ with increasing HOCl (0–60} \mu \text{M}) (\lambda_{ex} =370 \text{ nm}) \text{ in solution (DMSO:PBS = 1:1, pH = 7.4).} \]
peak at 570 nm disappeared, while the emission peak at 495 nm strengthened gradually, showing a good response to HOCl. The reasons for this phenomenon could be attributed to the ICT-1 process form $-\text{OCH}_3$ to DCO group was interrupted and that the ICT-2 process from $-\text{OCH}_3$ to ketone group was formed. Based on the above results, AI could be used as a ratiometric probe for identifying and detecting HOCl.

![Graph showing fluorescence intensity at 495 nm under different pH conditions and HOCl concentrations](image)

**Fig. 3.** (a) Fluorescence intensity of AI (10.0 μM) after response to 1 eq HOCl and 100 eq other analytes each at 495 nm ($\lambda_{ex} = 370$ nm). (b) The competitive test response of AI under the presence of various analytes.

![Graph showing fluorescence intensity at 495 nm under different pH conditions and HOCl concentrations](image)

**Fig. 4.** (a) the fluorescence intensity at 495 nm was obtained under different pH conditions in the presence and absence of HOCl. (b) The linearity between $I_{\text{probe}+\text{HOCl}} / I_{\text{probe}}$ with 10 μM probe and 0–50 μM HOCl in mixture system ($\lambda_{ex} = 370$ nm).

peak at 570 nm disappeared, while the emission peak at 495 nm strengthened gradually, showing a good response to HOCl. The reasons for this phenomenon could be attributed to the ICT-1 process form $-\text{OCH}_3$ to DCO group was interrupted and that the ICT-2 process from $-\text{OCH}_3$ to ketone group was formed. Based on the above results, AI could be used as a ratiometric probe for identifying and detecting HOCl.

![Graph showing 1H NMR titration spectra of AI in DMSO-d_6 upon addition of excess HOCl](image)

**Fig. 5.** $^1$H NMR titration spectra of AI in DMSO-d_6 upon addition of excess HOCl.
3.3. The selectivity of AI

In order to verify the selectivity of AI, some representative analytes, such as (H_2O_2, NO_2, ONOO^-, T-BuOO^-, 1.0 mM), other amino acids including (Cys, Hcy, GSH, L-Glu, L-Phe, L-Gly, L-Met, L-Ala, L-Thr, L-Try, 1.0 mM), and common interfering molecules (F, Cl, HPO_4^{2-}, H_2PO_4, CH_3COO^-, CO_3^{2-}, SCN^-, SO_3^{2-}, S_2O_3^{2-}, S_2^- 1.0 mM) were added into the system of DMSO: PBS(1:1, v/v, pH = 7.4) containing AI respectively [46]. As shown in Fig. 3 a, the fluorescence intensity at 495 nm showed negligible changes. But, the fluorescence intensity showed significantly changed responding with HOCl (100 μM), which fully confirmed that AI had good selectivity for HOCl. In addition, under the coexistence of other analytes, AI still showed an effective response to HOCl (100 μM), indicating that the presence of other analytes did not affect the detection of HOCl by AI (Fig. 3b).

3.4. The detection limit and pH dependent of the AI for HOCl

In addition, we also studied the limits of AI, and observed a good linear relationship I_{495} in the range of 0–50 μM. The LOD of AI for HOCl (3σ/slope) [25-34] was calculated to be 0.84 μM (Fig. 4A).

We studied the change of fluorescence intensity (I_{495}) in different pH ranges (2.0–12.0) to further explore the optical properties of AI (Fig. 4B). After a series of tests, we found that the probe showed a good response to HOCl under pH 5–7, indicating that the probe could be used in the physiological environment.

3.5. Proposed mechanism

The original intention of our design was that the probe AI with combining with anisaldehyde and DCO owned a large conjugated system, owing to the presence of electron-withdrawing CN group and electron-donating group –OCH_3, the ICT-1 of probe AI was triggered to show obvious fluorescent signals. Then the electron-withdrawing CN group of probe AI attacked by HOCl, the ICT-1 process from –OCH_3 moiety to CN group was disturbed. Meanwhile, the ICT-2 process was triggered from –OCH_3 moiety to electron-withdrawing ketone group. Therefore, the fluorescence spectrum showed ratiometric change after...
responding to HOCl. In order to confirm the theoretical calculation and spectra experimental results, we adopted HR-MS and $^1$H-NMR to study the reaction mechanism between $AI$ and HOCl (Fig S4). The m/z of the oxide with reacting with HOCl was 256.14633 by calculated, the data that we obtained was 257.1384[$M+H]^+$, which preliminarily confirmed our conjecture. The $^1$H NMR data of $AI$ in DMSO-$d_6$ showed that characteristic peaks in the spectrum did not increase or decrease, but some chemical shift occurred (Fig. 5), which was ascribed to the stronger electron-withdrawing CN group had formed. As shown in Scheme 2, the possible recognition mechanism between $AI$ and HOCl was proposed.

3.6. Imaging in HeLa cells

To test the application of biological experiments, we first carried out cytotoxicity experiments according to literature methods [46-49]. The results showed that the cells survival rate was more than 85 % (CCK-8 method), indicating that the probe $AI$ had the future to be applied into cells (Fig. S5). The cell imaging of $AI$ was obtained by fluorescence confocal microscope. Reference was made to above spectral experiments, we selected yellow channels ($\lambda_{em} = 550-590$ nm) and blue channels ($\lambda_{em} = 470-530$ nm) for observation. $AI$ was incubated to the HeLa cells and cultured at 37°C for 20 min. Fluorescence signal was observed in the yellow channel. After adding HOCl to the medium, the culture continued at the same temperature for 20 min, it could be observed that the fluorescence signal was enhanced of the blue channel and the yellow channel was weakened (as shown Fig.6b, 6c), which showed that $AI$ could detect exogenous HOCl at the cell level.

According to previous reports, cells produce endogenous HOCl under the stimulation of LPS (a lipopolysaccharide that stimulates cells to produce HOCl) [50]. Therefore, LPS and HeLa cells were incubated for 12 h and then adding $AI$ for incubation for 20 min, as shown in the Fig. 5d, obvious fluorescence signal could be observed in the yellow channel and the blue channel overtly. Comprehensive consideration with cell imaging experiments, $AI$ could be used as a ratiometric probe to detect endogenous and exogenous HOCl.

3.7. Imaging of mice

Based on all the research results of the cells experiments, we further tested the practicality of $AI$ in vivo imaging. Due to the background fluorescence of the mice, we selected the fluorescence signal of 500–590 nm to detect HOCl in the mice (Fig. 7). $AI$ was injected into the subcutaneous of the mouse, and strong fluorescence signal was observed. When HOCl was injected into the same area, the fluorescence signal gradually enhanced with the time delay. The above results indicated that probe $AI$ could identify and detect exogenous HOCl in mice.

4. Conclusions

In summary, we synthesized a novel ratiometric fluorescent probe ($AI$) based on anisaldehyde (donor) and DCO (acceptor) fluorescent dye to detected HOCl. A series of experimental results showed that probe $AI$ had better fluorescence response, highly selectivity and lower detection limit to HOCl. In addition, $AI$ could be used to detect endogenous and exogenous HOCl in HeLa cells and also successfully applied into mice imaging. Based on the above experimental results, we believed $AI$ provides a powerful tool for better understanding the contributions of HOCl in physiological and pathological processes.

CRediT authorship contribution statement

**Yan Shi:** Investigation, Writing - original draft. **Fangjun Huo:** Funding acquisition. **Caixia Yin:** Conceptualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.snb.2020.128793.
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