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FULL PAPER

Responsive Fluorescence Probe for Selective and Sensitive Detection of Hypochlorous Acid in Live Cells and Animals

Huan Feng, [a] Qingtao Meng, *[a] Yue Wang, [a] Chengchen Duan, [b] Cuiping Wang, [a] Hongmin Jia, [a] Zhiqiang Zhang, [a] Run Zhang *[b]

Abstract: Development of effective bioanalytical method for rapid, sensitive and specific detection of HOCl *in vitro* and *in vivo* plays key role for better understanding the roles of this molecule in normal and diseased conditions, but remains challenging due to the highly reactive nature of HOCl and the complicated biological conditions. In this work, a new fluorescence probe, **PQI**, was developed for monitoring of HOCl level in biological samples. The **PQI** was easily synthesized by a one-step condensation reaction. Upon addition of HOCl, significant changes in absorption spectra and the colour of the solution were noticed, facilitating the "naked eye" detection of HOCl in PBS buffer. The fluorescence of **PQI** was found to be significantly increased within a few of seconds, leading to "OFF-ON" fluorescence response towards HOCl. The sensing mechanism, oxidation of thioether by HOCl, was confirmed by HRMS titration analysis. **PQI** features large Stokes shift, high sensitivity and selectivity, and rapid fluorescence response towards HOCl. Quantitative detection of HOCl in single live cells was demonstrated through fluorescence imaging and flow cytometry analysis. **PQI** was then successfully used in visualisation of HOCl in live zebrafish and nude mice.

Introduction

Reactive oxygen/nitrogen species (ROS/RNS) have been reported to be generated in a wide range of cellular process, and therefore being involved in various pathology of many conditions, including inflammation, cancer, cardiovascular and neurodegenerative diseases.[1] Therefore, investigation the bio-functions of ROS/RNS, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), nitric oxide (NO) and hypochlorous acid (HOCl) has attracted considerable attention in the past few decades.[2] Among ROS/RNS, HOCl is well known as one of the highly reactive oxygen species (hROS) that can react with various biomolecules, such as protein side chains, peptide bonds, and nucleic acid.[3] The endogenous HOCl in organisms is generated by the reaction of H_2O_2 with Cl^- ions under the catalysis of a heme enzyme, myeloperoxidase (MPO).[4] Recently studies revealed that this molecule is an important microbicidal agent during immune inflammation response, corresponding to the digestion of the bacterium engulfed by neutrophil phagosome.[5] However, overbalance of HOCl production during inflammation is implicated with many diseases, including Alzheimer disease, cardiovascular disease, atherosclerosis, ischemia-reperfusion injury in stroke, osteoarthritis, inflammatory bowel disease, myocardial infarction, organ transplant rejection, and even cancers.[1b, 6]

Therefore, development of rapid and effective methods for HOCl detection in biological systems has been emerged as a hot research topic in recently years, but remains a challenge due to the highly reactive nature of this molecule in live organisms [3a, 7]. In the past few decades, several methods have been reported for the detection of HOCl,[8] such as colorimetric, luminescent/fluorescent, electrochemical and chromatographic methods. Among the reported ROS detection methods, fluorescence-based analytical methods have been recognized as an indispensable technique for *in situ* analysis of the localization and dynamics of HOCl in biological systems, owing to their versatile advantages such as high sensitivity, simplicity for implementation, real-time detection, and good compatibility for bio-samples.[9] The responsive fluorescence chemosenosrs are thus the key for the development of fluorescence analytical methods for quantitative HOCl detection. Towards this end, a number of fluorescence probes have been developed based on the sensing mechanism of HOCl-mediated oxidation reactions with several moieties, such as group 16 elements,[10] oxime,[11] electron deficit C=C bond,[4b, 12] and *p*-methoxyphenol and *p*-alkoxyaniline.[13]

For sensitive detection of HOCl in biological samples, fluorescence probes with large Stokes shift are desirable to avoid self-quenching and measurement error from excitation light and scattered light.[14] Towards this end, our previous researches have contributed a series of phosphorescence probes for quantitative detection of HOCl in aqueous solution and live cells.[15] Considering the complicated synthesis procedure, we recently became interested in the development of fluorescence probe with large Stokes shift for quantitative detection of HOCl *in vitro* and *in vivo*.

In the present work, a new responsive fluorescence probe, **PQI**, was developed for the HOCl detection in aqueous solution, live cells and animals (Scheme 1A). The **PQI** was designed by incorporating HOCl-responsive thioether into a phenothiazine-quinolinium platform. **PQI** was easily synthesized by a one-step condensation reaction (Scheme 1B), and the chemical structure

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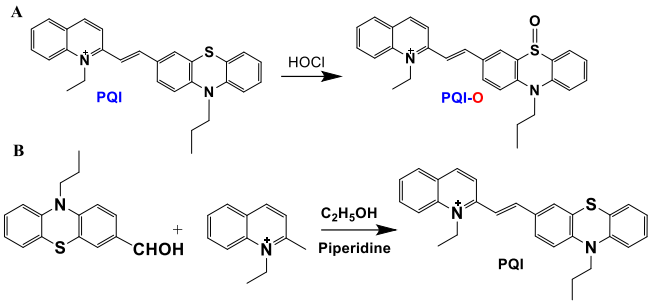
was well characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. **PQI** was found to be weakly fluorescent, while the fluorescence intensity can be significantly increased in the presence of HOCl. The "OFF-ON" fluorescence response of **PQI** towards HOCl was found to be completed within a few seconds, which allowed rapid detection of HOCl in biological systems. Notably, the new probe features large Stokes shift (128 nm), high sensitivity and selectivity, ensuring quantitative detection of HOCl in buffer and biological samples. Quantitative analysis of HOCl in live MCF-7 cells was then realized by confocal fluorescence imaging and flow cytometry analysis. Visualization of endogenous HOCl generation in zebrafish, and exogenous HOCl in nude mice were then demonstrated using **PQI** as the fluorescent probe.



Scheme 1. (A) The proposed sensing mechanism of PQI towards HOCl; (B) synthetic procedure of fluorescence probe, **PQI**.

Results and Discussion

UV-vis spectra response of PQI towards HOCl

The sensing performance of **PQI** towards HOCl among various ROS and biological related species was firstly evaluated by UV-vis titration in PBS buffer (pH 7.4, 20 mM, containing 10% DMSO). As shown in Figure 1, **PQI** displayed a strong absorption band centered at 490 nm. Upon addition of HOCl, this absorption band was gradually decreased and a new band centered at 440 nm was obtained. An isosbestic point was found to be at 478 nm upon the titration of **PQI** with HOCl. In agreement with the changes of absorption spectra, the color of the **PQI** solution was changed from brown to yellow (Figure 1, inset), indicating that **PQI** can serve as a potential "naked eye" indicator for HOCl detection in water samples.

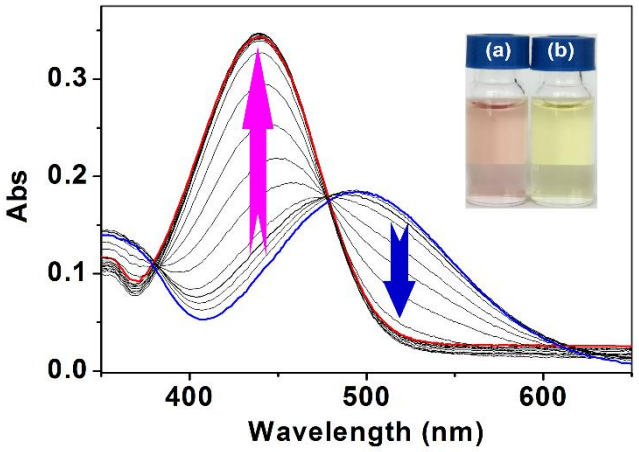


Figure 1. UV-vis absorption spectra of **PQI** (10 μM) in the presence of different amounts of HOCl (0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60 μM) in PBS aqueous buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4). Inset: Colorimetric changes of **PQI** in the absence (a) and presence (b) of HOCl.

To evaluate the selectivity of **PQI** toward HOCl, we then examined the changes of absorption spectra of **PQI** in the presence of various ROS, anions and biomolecules in PBS buffer. As shown in Figure 2, obvious changes in the absorption spectra of **PQI** were noticed upon addition of HOCl over other biological species including $^{1}O_2$, ·OH, O_2^{-}, $ONOO^{-}$, Br^{-}, AcO^{-}, Cl^{-}, F^{-}, I^{-}, HSO_3^{-}, HSO_4^{-}, S^{2-}, NO_2^{-}, NO_3^{-}, $P_2O_7^{4-}$, PO_4^{3-}, SO_3^{2-}, SO_4^{2-}, HCO_3^{-}, Pi, PPi, H_2O_2, Cys, Hcy, GSH. The result indicated that the **PQI** is highly selective toward HOCl, rather than other species. The specific UV-vis response of **PQI** towards HOCl was also confirmed by colorimetric assay (Figure S1), where the colour changes from brown to yellow was found in the presence of HOCl only.

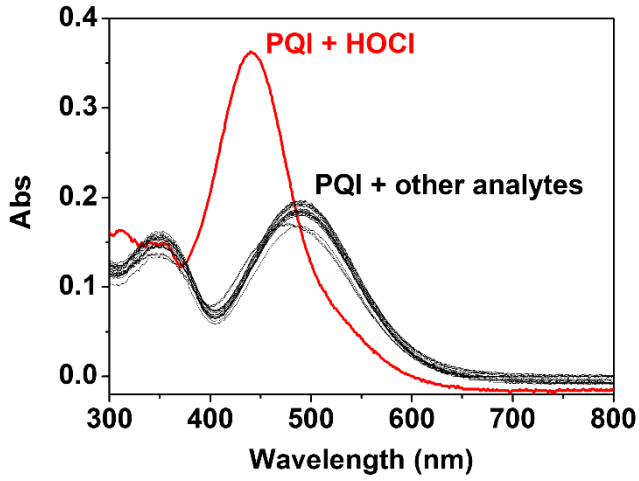


Figure 2. UV-vis absorption spectra of **PQI** (10 μM) in PBS aqueous buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4) upon addition of various analytes (20 μM): HOCl, $^{1}O_2$, O_2^{-}, •OH, $ONOO^{-}$, Br^{-}, AcO^{-}, Cl^{-}, F^{-}, HSO_3^{-}, HSO_4^{-}, S^{2-}, NO_2^{-}, NO_3^{-}, $P_2O_7^{4-}$, PO_4^{3-}, SO_3^{2-}, SO_4^{2-}, HCO_3^{-}, Pi, PPi, H_2O_2, Cys, Hcy, GSH.

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Fluorescence response of PQI towards HOCl

The ability of **PQI** for the detection of HOCl in simulated physiological conditions was then evaluated by fluorescence titration in PBS buffer (DMSO: H_2O = 1:9, v/v; pH 7.4). As expected, free **PQI** displayed weak fluorescence in PBS buffer. The emission quantum yield was determined to be Φ_1=0.0029. Upon addition of HOCl, the fluorescence intensity was gradually increased. As shown in Figure 3, an approximate 75-fold fluorescence enhancement at 588 nm (λ_{ex} = 460 nm) of **PQI** was observed when 6.0 equiv. of HOCl was added. The product of **PQI** react with HOCl, **POI-O**, exhibited quantum yield of Φ_3 = 0.177. As shown in Figure S2, dose dependent enhancement in fluorescence intensity at λ_{em} = 588 nm showed a good linearity against the concentration of HOCl. According to the IUPAC criteria (LOD = 3σ/k), the detection limit (LOD) was calculated to be 15.6 nM. Such a low detection limit allows endogenous HOCl generation in live systems to be detected.

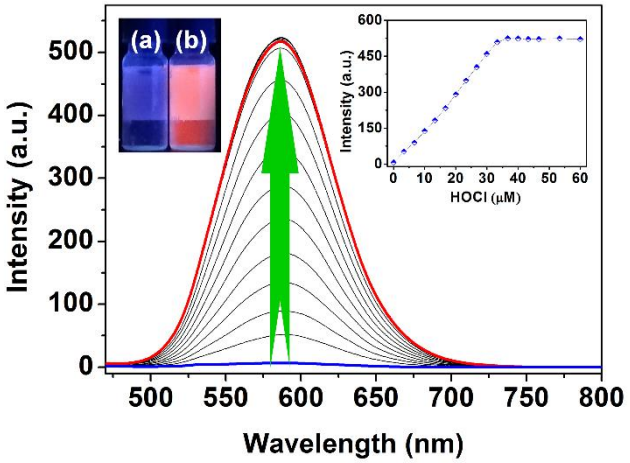

Figure 3. Fluorescence spectra of **PQI** (10 μM) in the presence of different amounts of HOCl (0-60 μM) in PBS aqueous buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4). Insert: fluorescence intensities of **PQI** at 588 nm as a function of HOCl (0-60 μM) and the changes of fluorescence colour in the absence (a) and presence (b) of HOCl. Excitation was performed at 460 nm.

In addition to the high sensitivity, **PQI** exhibited desirable selectivity towards HOCl detection over other competitive species. As depicted in Figure 4, a significant increase in fluorescence intensity of **PQI** was observed in the presence of HOCl. In contrast, negligible changes in fluorescence spectra can be observed when the addition of other competitive ROS and anions, including $^1O_2$, O_2^{-}, ·OH, $ONOO^{-}$, Br^{-}, AcO^{-}, Cl^{-}, F^{-}, I^{-}, HSO_3^{-}, HSO_4^{-}, S^{2-}, NO_2^{-}, NO_3^{-}, $P_2O_7^{4-}$, PO_4^{3-}, SO_3^{2-}, SO_4^{2-}, HCO_3^{-}, Pi, PPi, H_2O_2, Cys, Hcy, GSH. The specific fluorescence response of **PQI** towards HOCl was further confirmed by the changes of fluorescence colors, where red color fluorescence emission can only be observed in the presence of HOCl (Figure S3). Furthermore, it is notable that the fluorescence responses of **PQI** in a mixture containing all chosen competitive species were similar to that of HOCl only (Figure 4 inset). To further explore specificity of **PQI** toward HOCl, the influence of biological cations was also investigated. These cations include Al^{3+}, Fe^{3+}, Cr^{3+}, Ag^{+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, K^{+}, and Na^{+}. As expected, negligible changes of absorption spectra and fluorescence intensity were observed in the presence of co-existing competitive cations (Figure S4, S5). The results suggested that the **PQI** can be employed as selective probe for the detection of HOCl in biological systems.

Time-/pH-dependent fluorescence response of PQI towards HOCl

Rapid fluorescence response of the probe is the key that the probe to be used for the detection of analyte of interest in biological systems. Considering the highly reactivity nature of HOCl in live organisms, the probes that can respond the levels of HOCl within few seconds are demanded.[16] Therefore, time course fluorescence response of **PQI** towards the addition of HOCl was then evaluated by recording the changes of fluorescence intensity at 588 nm. As shown in Figure 5, pure **PQI** exhibited weak and stable fluorescence intensity in PBS buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4). Upon the addition of 1.5 equiv. of HOCl, the fluorescence intensity is rapidly increased and reaches to the maximum value within a few seconds, and then keeps at a steady level under the continuous excitation. When another amount of HOCl was added, the fluorescence intensity is rapid increased again and reached another maximum value. The results of rapid fluorescence response indicated that the probes have the potential for real-time monitoring of HOCl in biological systems.

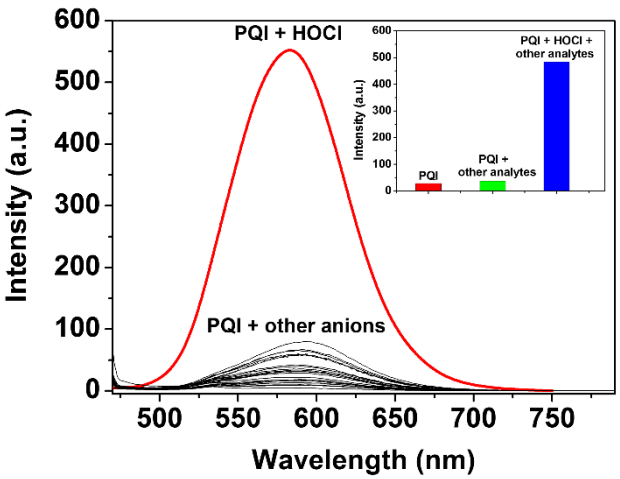

Figure 4. Fluorescence responses of **PQI** (10 μM) to various biological species (60 μM) in PBS aqueous buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4). The species include HOCl, $^1O_2$, O_2^{-}, ·OH, $ONOO^{-}$, Br^{-}, AcO^{-}, Cl^{-}, F^{-}, HSO_3^{-}, HSO_4^{-}, S^{2-}, NO_2^{-}, NO_3^{-}, $P_2O_7^{4-}$, PO_4^{3-}, SO_3^{2-}, SO_4^{2-}, HCO_3^{-}, Pi, PPi, H_2O_2, Cys, Hcy, GSH. Insert: Fluorescence responses of **PQI** (10 μM) towards HOCl in the presence of diverse coexisting competitive species. The intensities were recorded at 588 nm, excitation at 460 nm.

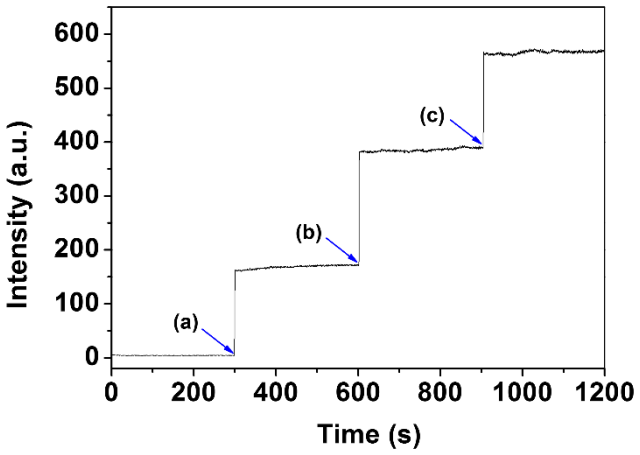

Figure 5. Time course of fluorescence response of **PQI** (10 μM) to the addition of HOCl at the concentration of (a) 15 μM, (b) 25 μM and (c) 40 μM in PBS buffer (DMSO: PBS = 1:9, 20 mM, pH 7.4). Excitation was performed at 460 nm and emission was recorded at 588 nm.

Effect of pH on the emission intensities of **PQI** solution was then investigated in the presence and absence of HOCl. As shown in Figure 6, weak and stable fluorescence emission of **PQI** was observed in the absence of HOCl in the pH 3.0 to 11.5. Upon the addition of 4 equiv. of HOCl, the emission intensities were significantly increased in the pH range being tested, suggested that the **PQI** can be employed as the probe for the detection of HOCl in acidic, neutral, and basic solution.

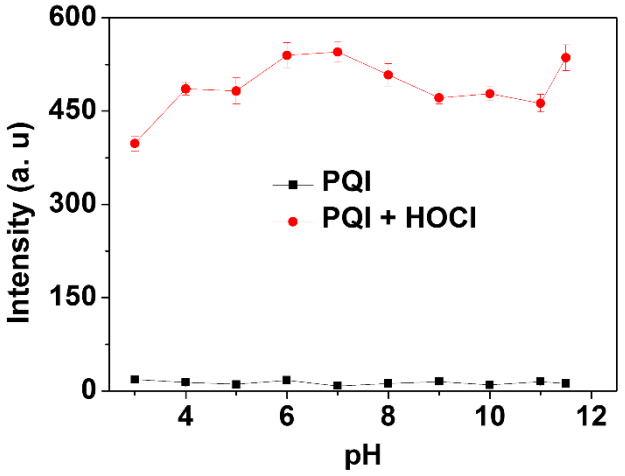

Figure 6. Effects of pH on the fluorescence intensities of **PQI** in the absence and presence of HOCl (60 μM). The intensities were recorded at 588 nm, excitation was performed at 460 nm.

Investigation of the sensing mechanism

In the presence of HOCl, colorimetric and fluorometric dual responses were attributed to the oxidation of thioether to sulfoxide derivative of **PQI** (Scheme 1A).[17] To verify the recognition mechanism, the reaction mixture of **PQI** in the presence of HOCl was measured by high-resolution mass spectroscopy (HRMS). As shown in Figure S6, upon the addition of HOCl into the aqueous solution of **PQI**, the molecular ion peak of $[\mathbf{PQI}]^+$ at m/z = 423.1905 was disappeared. A new peak at m/z = 439.1848 was obtained, which can be assigned to the oxidized sulfoxide product, **PQI-O**.[18] The result indicated that the HOCl-induced oxidation of thiazine thioetherto sulfoxide is responsible for the HOCl sensing mechanism.

Analysis of HOCl in live cells

Encouraged by the superb sensing performance of **PQI**, we then evaluated its capacity for imaging of HOCl in live cells. The cell permeability of **PQI** was examined following the reported methods.[19] The partition coefficient ($\log P_{o/w}$) of **PQI** between 1-octanol and water was measured to be 1.78. Giving that the fact of good cellular membrane permeability of $\log P_{o/w}$ value located within the rage of 0-5, we conclude that **PQI** is able to be internalized easily by live cells. MCF-7 cells were stained with **PQI** for 30 min, followed by the treatment with HOCl for another 15 min. As shown in Figure 7, **PQI** deposited MCF-7 cells showed dark fluorescence, while the intracellular fluorescence was clearly enhanced after further treatment of cells with HOCl. The result suggested that the **PQI** is cell membrane permeable, and that can be used as the probe for visualisation of HOCl in live cells.

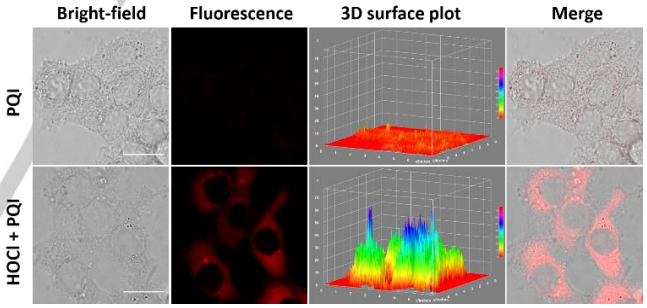

Figure 7. Bright-field, fluorescence, merged imaging, and Image J 3D surface plot analysis of HOCl in MCF-7 cells. MCF-7 cells were incubated with **PQI** (4 μM) for 30 min, followed by the treatment with HOCl (10 μM) for another 15 min. Scale bars are 20 μm.

We then evaluated the capability of **PQI** as the probe for quantitative detection of intracellular HOCl in single MCF-7 cells by flow cytometry analysis. The fluorescence intensities of 10,000 cells were recorded from each cell population. Figure 8 illustrated the shifts for the histogram and the changes of mean fluorescence intensity (MFI) of MCF-7 cells that stained with **PQI** and further treated with HOCl at different concentrations. Weak background fluorescence was obtained for the group of **PQI**-loaded MCF-7 cells. Upon treatment with HOCl for 15 min, clear shift of the histogram to the direction of strong fluorescence was observed, and such shifts were found HOCl concentration dependent (Figure 8A). In agreement with the shift of histogram,

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enhancement in the MFI was noticed, and the intensity is correlated with the concentration of HOCl administration. The results suggested that the **PQI** can be used as the probe for quantitative detection of HOCl at single cell level through flow cytometry analysis.

Fluorescence imaging of HOCl *in vivo*

The capability of **PQI** in imaging of HOCl *in vivo* was firstly evaluated using adult zebrafish as the animal model. It has been well documented that the HOCl is the key biomarker in inflammation response of zebrafish towards drug stimulation.[15b, 20] In this work, zebrafish was initially stimulated with LPS (2 μg /mL) for 3 h, followed incubation with 10 μM **PQI**. As shown in Figure 9, control groups (zebrafish only, and LPS-stimulated zebrafish) showed weak background fluorescence, while the fluorescence in zebrafish was gradually increased over the time when the LPS-stimulated zebrafish was further stained with **PQI**. The results indicated that the LPS-induced inflammatory response in zebrafish can be visualized using **PQI** as a fluorescence probe.

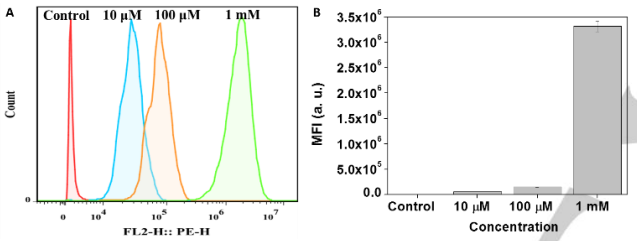

Figure 8. (A) The shifts of histograms of flow cytometry analysis; (B) mean fluorescence intensity per MCF-7 cell of different incubation conditions were examined by flow cytometry analysis. The MCF-7 cells were incubated with 4 μM **PQI** for 30 min, and then treated with different concentration of HOCl for 15 min.

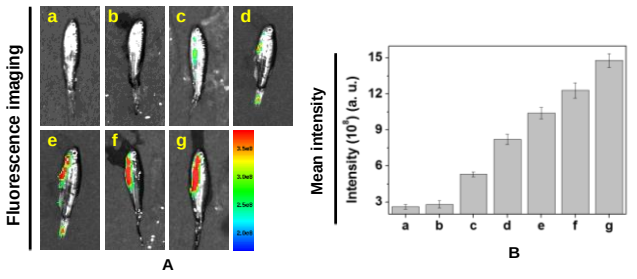

Figure 9. (A) Fluorescence imaging of endogenous HOCl production in zebrafish. (a) Zebrafish only; (b) zebrafish was stimulated with LPS (2 μg/mL) for 3 h; then stained with **PQI** (10 μM) for (c) 20 min; (d) 40 min; (e) 70 min; (f) 100 min and (g) 210 min, respectively. (B) The mean fluorescence intensities of areas of interest at different time showing in (a-g). The zebrafish were imaged with an excitation filter (465 nm) and an emission filter (610 nm).

We then evaluated the ability of **PQI** for visualizing of HOCl in live mice. **PQI** was subcutaneously injected into 6-8 week old nude mice, followed by the treatment of HOCl at the same area showing in Figure 10a (arrow). Fluorescence imaging was then performed at different time course after HOCl administering. As shown in Figure 10, the mice with **PQI** injection showed weak fluorescence, and the fluorescence intensity was gradually increased within 25 min. Then, the fluorescence intensity kept at a maximum value for at least 5 min. These results demonstrated that **PQI** can serve as a fluorescence probe for imaging of HOCl in live mice

Conclusions

In conclusion, a new fluorescence probe, **PQI**, has been successfully developed for the HOCl detection *in vitro* and *in vivo*. The probe was designed by incorporating HOCl-responsive thioether into a phenothiazine-quinolinium platform. The proposed sensing mechanism, oxidation of the thioether to sulfoxide was confirmed by HRMS titration. In PBS buffer solution (pH 7.4), the probe **PQI** displayed remarkable "OFF-ON" fluorescence response towards HOCl within a few seconds. The **PQI** featured large Stokes shift, high sensitivity and specificity, reliability at physiological pH, fast-responsive and red-emitting, enables its potential application in biological systems. Fluorescence imaging and flow cytometry analysis of HOCl in single cell level was demonstrated in MCF-7 cell line. *In vivo* sensing of endogenous HOCl generation in adult zebrafish and visualization HOCl in nude mice were then realized. The successful development of **PQI** is expected to contribute to the future studies of the physiological and pathological roles of HOCl in live organisms.

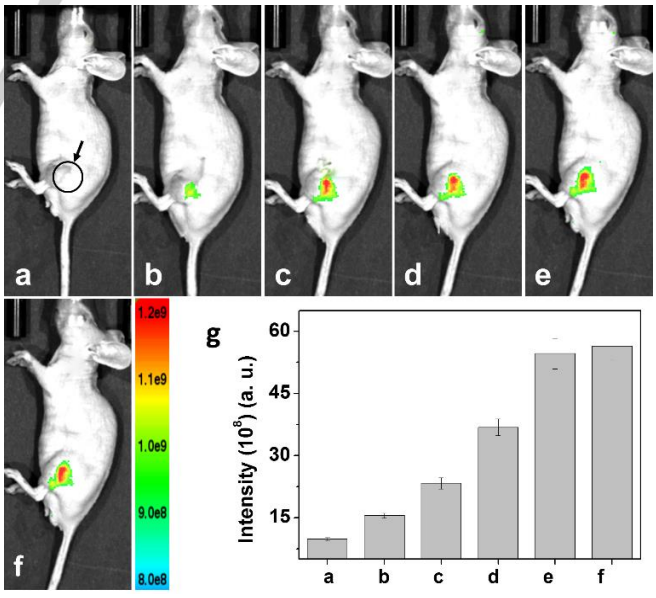

Figure 10. Fluorescence images and mean fluorescence intensity analysis of HOCl in mice. (a) **PQI** (100 μM, 125 μL) was subcutaneously injected into mice, followed by the injection of 20.0 μL HOCl (2 mM) to the area of interest (arrow in "a"). Images were recorded at different times, (b) 5 min; (c) 10 min; (d) 15 min; (e) 25 min; (f) and 30 min, respectively. The mean fluorescence intensities

of areas of interest at different time showing in (a-f). The mice were imaged with an excitation filter (465 nm) and an emission filter (610 nm).

Experimental Section

Materials and Instruments

Phenothiazine was purchased from Aladdin reagent Co. (Shanghai, China). 1-Bromopropane, phosphorus oxychloride, piperidine, iodoethane, metal ions (nitrate salts), and anions (sodium salts) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). 3-morpholinosydnonimine (SIN-1) ($ONOO^-$ donor) and sodium hypochlorite (NaOCl) were purchased from Sigma Aldrich. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin sulfate, and trypsin-EDTA were purchased from Life Technologies (Australia). Nude mice (6-8 weeks) and zebrafish were obtained from Experimental Animal Center of Dalian Medical University, China. All the experiments of living nude mice and zebrafish were performed in compliance with the relevant local laws and institute guidelines, and also the institution committee of Dalian Medical University has approved the experiments. Unless otherwise stated, solvents and reagents were of analytical grade from commercial suppliers and were used without further purification. Deionized water was used throughout.

^{1}H-NMR and ^{13}C-NMR spectra were recorded with an AVANCE600MHZ spectrometer (BRUKER) with chemical shifts reported as *ppm* (in DMSO, TMS as internal standard). API mass spectra were recorded on an Agilent 6530 QTOF spectrometer. Fluorescence spectra were measured with Perkin Elmer LS55 luminescence spectrometer (USA). Absorption spectra were measured with a Perkin Elmer Lambda 900 UV/VIS/NIR spectrophotometer (USA). Fluorescent images were obtained using Leica SP8 laser-scanning microscope. The images were analysis by Image J software version 1.44p. Flow cytometry analysis was recorded on an Accuri C6 flow cytometer, BD Biosciences with a 488 nm laser excitation and emission 565 ± 20 nm. The data were analyzed with Flowjo software. HOCl in mice were imaged by a SPECTRAL Ami Imaging Systems (Spectral Instruments Imaging, LLC, Tucson, AZ) with an excitation filter 465 nm and an emission filter 610 nm. All the data were calculated using the region of interest (ROI) function of Amiview Analysis software (Version 1.7.06), and values are presented as the mean ± SD for each group of three experiments.

Synthesis of PQI

The procedure for the synthesis of **PQI** is illustrated in Scheme 1. Specifically, 10-ethyl-10H-phenothiazine-3-carbaldehyde (269.1 mg, 1 mmol) [18, 21], 1-ethyl-2-methylquinolinium iodide (0.172 g, 1 mmol) were dissolved in 20 mL ethanol, and then catalytic amount of piperidine was added dropwise into the solution. The reaction mixture was refluxed for 6 hours under argon atmosphere. The precipitate was filtrated, washed with cold ethanol and dried under vacuum. **PQI** was obtained as purple-black powder in 83% yield. ^{1}H NMR (DMSO-d_6, 600 MHz), δ (ppm) 9.00 (s, *J* = 10.74 Hz, 1H), 8.53-8.58 (m, 2H), 8.33 (d, *J* = 9.18 Hz, 1H), 8.23 (t, *J* = 18.6 Hz, 1H), 8.16 (t, *J* = 8.85 Hz, 1H), 7.92 (m, 2H), 7.75 (d, *J* = 8.94 Hz, 1H), 7.68 (d, *J* = 18.72 Hz, 1H), 7.24 (t, *J* = 8.46 Hz, 1H), 7.19 (d, *J* = 9.0 Hz, 1H), 7.13 (d, *J* = 10.26 Hz, 1H), 7.09 (d, *J* = 9.6 Hz, 1H), 7.01 (t, *J* = 8.67 Hz, 1H), 5.15 (m, 2H), 3.92 (t, *J* = 7.77 Hz, 2H), 1.74 (dd, *J* = 8.18 Hz, 2H), 1.56 (t, *J* = 8.07 Hz, 3H), 0.97 (t, *J* = 4.28 Hz, 3H), ^{13}C NMR (DMSO-d_6, 150Hz), δ (ppm) 155.8, 148.1, 147.4, 144.1, 143.7, 138.6, 135.5, 131.2, 130.8, 129.2, 128.4, 127.7, 127.4, 124.2, 123.8, 122.8, 121.4, 119.3, 116.9, 116.2, 116.1, 49.1, 46.9, 20.0, 14.6, 11.4. HRMS-API (positive mode, m/z) for [**PQI**]$^+$: calcd 423.1889, found: 423.1905. Mp: 234.6-236.2 °C.

General procedures of spectra detection

A stock solution of **PQI** was prepared in dimethyl sulfoxide (DMSO) at the concentration of 0.5 mM. Prior spectroscopic measurements, the solution was freshly prepared by diluting the stock solution to corresponding PBS buffer solution at the concentration of 10 μM (DMSO: H_2O = 1:9, 20 mM, pH = 7.4). Solutions of a series of anions and biomolecules (20 mM) were prepared in deionized water. A stock solution of HOCl was prepared by dilution of the commercial hypochlorous acid solution and stored according to the previous literatures.[3c] The concentration of HOCl was determined by using its molar extinction coefficient of 391 $M^{-1}\cdot cm^{-1}$ at 292 nm before use. Hydroxylradical (·OH) was generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide. Superoxide anion radical (O_2^-) was generated from the xanthine-xanthine oxidase system. Singlet oxygen ($^1O_2$) was generated from the Na_2MoO_4-H_2O_2 system in 0.05 M carbonate buffer of pH 10.5. $ONOO^-$ was obtained by using SIN-1 as a donor. Hydrogen peroxide (H_2O_2) was diluted immediately from a stabilized 30% solution, and was assayed using its molar absorption coefficient of 43.6 $M^{-1}\cdot cm^{-1}$ at 240 nm. For spectroscopic analysis of HOCl, HOCl at different concentration was added into the **PQI** solution (total volume 3 mL), followed by the spectroscopic measurements immediately. Excitation and emission slits are 5 nm.

Quantum yield measurement

The relative fluorescence quantum yields were determined using optically matching solutions of fluorescein (Φ_f = 0.85 in 0.1 M NaOH aqueous solutions) as the reference. The excitation was performed at an excitation wavelength of 470 nm and the quantum yield is calculated using the following equation.[22]

$$\Phi_{unk} = \Phi_{std}\frac{(F_{unk}/A_{unk})}{(F_{std}/A_{std})}\left(\frac{\eta_{unk}}{\eta_{std}}\right)^2 \quad (1)$$

Where Φ_{unk} and Φ_{std} are the radiative quantum yields of the sample and standard, F_{unk} and F_{std} are the integrated emission intensities of the corrected spectra for the sample and standard, A_{unk} and A_{std} are the absorbances of the sample and standard at the excitation wavelength, and η_{unk} and η_{std} are the indices of refraction of the sample and standard solutions, respectively. Excitation and emission slit widths were modified to adjust the luminescent intensity in a suitable range. All the spectroscopic measurements were performed in triplicate and averaged.

Cell line and cell culture

Human breast cancer cell, MCF-7 (ATCC®HTB-22™) was obtained from American Type Cell Collection. MCF-7 cells were cultured in DMEM, supplemented with 10% FBS, 1% penicillin, 1% streptomycin sulphate in a humidified 5% CO_2/95% air incubator at 37 °C. The growth medium was changed every two days. The cells were routinely subcultured by trypsin-EDTA solution and growth to 80% confluence prior to experiments.

Confocal fluorescence imaging of HOCl in MCF-7 cells

For imaging of HOCl in live cells, MCF-7 cells were seeded at a density of 1.5 × 10^5 cells/mL in a 22 mm coverglass bottom culture dishes (ProSciTech, Australia). The cells were then incubated at 37 °C in a 5% CO_2/95% air incubator for 24 h. The culture medium was replaced with the freshly prepared medium containing **PQI** (4 μM, 0.1% DMSO as the co-solvent), followed by the further incubation at 37 °C in a 5% CO_2/95% air incubator for 0.5 h. The cells were washed with PBS (3×2 mL/dish), and then treated with 10 μM HOCl for another 15 min. After washing with PBS for three times to remove excess HOCl, the cells were subjected to confocal microscope fluorescence imaging measurements.

Flow cytometry analysis of HOCl in MCF-7 cells

Flow cytometry analysis was employed for evaluating the fluorescence response of **PQI** towards HOCl. In a six-chamber culture plate, the MCF-7 cells were seeded into each well at the density of 2.5×10^5 cells/mL. After 24 h, the cells were washed with PBS for three times, and then incubated with 4 μM **PQI** for 0.5 h. Then, the cells were further incubated with HOCl at the concentration of 10 μM, 100 μM, and 1000 μM for another 15 min. The cells were washed with PBS (3×2 mL/dish), detached from the well using trypsin-EDTA and then subjected to flow cytometry analysis.

Fluorescence imaging of HOCl in zebrafish

Zebrafish pre-stimulated with LPS (2 μg/mL) for 3 h was incubated with 10 μM **PQI**, and then the fluorescence images were recorded over the times. The untreated zebrafish, and the LPS-stimulated zebrafish were employed as the control group.

Fluorescence imaging of HOCl in nude mice

The nude mice (6-8 week old mice) were anesthetized by isoflurane in a flow of oxygen in during all of the experiments. For imaging of exogenous HOCl in live mice, **PQI** (100 μM, 125 μL) was injected into mice, followed by the administering of HOCl (2 mM, 20.0 μL) in the same area. Imaging for the injection area were performed each 5 min within 30 min. Excitation filter, 465 nm and emission filter, 61 nm.

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Keywords: Hypochlorous acid • Fluorescence probe • Fluorescence imaging • *In vitro* • *In vivo*

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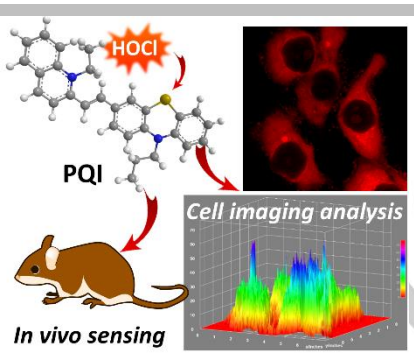
FULL PAPER

Sensing and Imaging of hypochlorous acid (HOCl): A new responsive fluorescence probe, **PQI**, is reported for quantitative fluorescence detection of HOCl in aqueous solution and live organisms. Visualisation of HOCl in live cells and *in vivo* sensing and imaging of HOCl in zebrafish and nude mice were achieved by using **PQI** as the fluorescence probe.



Huan Feng, Qingtao Meng, Yue Wang, Chengchen Duan, Cuiping Wang, Hongmin Jia, Zhiqiang Zhang, Run Zhang**

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Responsive Fluorescence Probe for Selective and Sensitive Detection of Hypochlorous Acid in Live Cells and Animals