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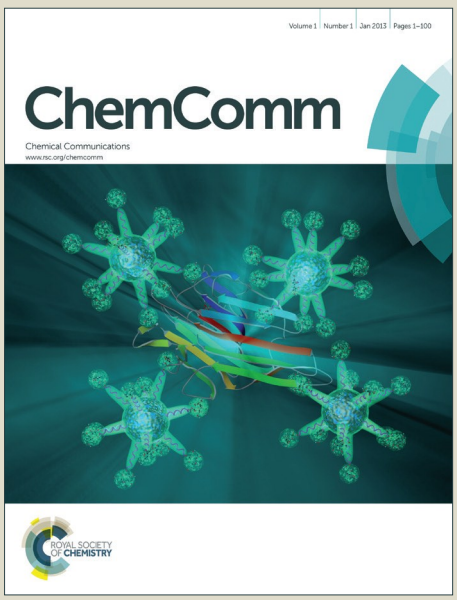

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HEPES is not suitable for fluorescence detection of HClO: a novel probe for HClO in absolute PBS

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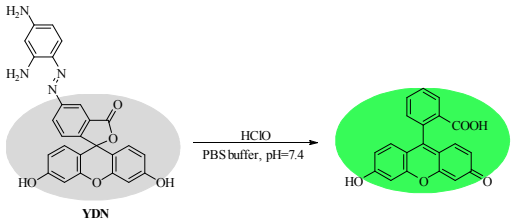
HEPES is not suitable for fluorescence detection of HClO because it can be oxidized by HClO. A novel probe for HClO was developed on the basis of an oxidation reaction with azo moiety, which can selectively and sensitively detect HClO in absolute PBS. Further, it works well in living mouse imaging.

Every year substantial morbidity and mortality occurred as caused by both chronic and acute inflammation.[1] To against inflammatory stimuli such as host defense against bacterial and fungal infections, organisms produce reactive oxygen species (ROS) in mitochondria of professional phagocytes.[2] Being one of the most important ROS, endogenous hypochlorous acid (HClO) is mainly generated by the catalysis of myeloperoxidase (MPO) with the presence of chloride and hydrogen peroxide in physiological conditions, which plays pivotal roles in the human immune-defense system. Nevertheless, the abnormal levels of HClO caused by MPO can lead to oxidative damage to biological proteins, lipids and nucleic acids.[3] Studies have shown that, the oxidative stress mediated by HClO is involved in a variety of physiological processes such as pathological atherosclerosis, cell apoptosis and cell senescence.[4] To better understand the HClO formation, efficiently methods are online in vivo monitoring.

Recent years, fluorescence sensing has been an advantageous imaging technique for real-time visual detection of analytes both in vitro and living organism.[5] In order to further clarify the function of HClO in organisms, extensive fluorescence probes have been carried out including coumarin dyes,[6] rhodamine and derivative dyes,[7] boron-dipyrromethene (BODIPY) dyes,[8] heptamethine cyanine dyes,[9] ruthenium(II) complex,[10] iridium(III) complex[11] and Lanthanide complex.[12] In 2011, Kim and Choi *et al.* synthesized a new probe for HClO based on the reaction with methylthioether group in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer containing 0.2% DMSO.[8c] Based on an oxidation reaction with diaminomaleonitrile, Goswami *et al.* detected HClO in a solution of CH_3CN-H_2O (4:6, v/v) in 2013.[13] In 2014, Peng *et al.* described a HClO probe based on the reaction with pyrrole group in 10 mM phosphate buffer saline (PBS) solution (ethanol/water = 1:9, v/v).[14] At the same year, Huo and Yin *et al.* designed a fluorescence chemosensor for HClO by oxidizing amino of the probe to form imine product in 10 mM HEPES.[15] Recently, Wang *et al.* developed a fluorescent probe for HClO in 10 mM PBS (containing 0.05% DMSO) via oxidizing thioether to sulfoxide or sulfone.[16] To sum up, most probes require organic reagent as a co-solvent due to their poor water-solubility, which might affect their application to some extent, especially in physiological environment. As for the buffer, HEPES is often employed. Unfortunately, HEPES and tris (hydroxymethyl) aminomethane (Tris) can be HClO scavengers according to the literature.[17] If HEPES or Tris is employed for HClO detection, HClO would definitely interact with the buffer, leading to inaccurate results.



Scheme 1 The proposed mechanism of **YDN** for the detection of HClO.

To solve these problems, a probe **YDN** (Scheme 1) was developed here based on the fluorescein platform owing to its high fluorescence quantum yield as well as universal applicability.[18] **YDN** can be easily synthesized via a one-pot synthetic strategy, introducing an azo moiety into the 5-position of 5-amino fluorescein, which was fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS. As expected, **YDN** was almost non-fluorescent at first with the presence of electron-withdrawing azo moiety. Upon addition of trace amounts of

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HClO, **YDN** is transformed into fluorescein with a remarkable fluorescence enhancement in 2 min.

The oxidation reaction between azo moiety and HClO has not been previously employed in the design of fluorescence probe for HClO. Compared with the literature, **YDN** can detect HClO in absolute PBS solution without any organic solvent. The detection range is over 0-23 μM, and the detection limit is 8.7×10^{-9} M. Additionally, **YDN** can selectively and sensitively detect HClO over other ROS and reactive nitrogen species (RNS) in absolute PBS solution.

The pH-dependent response of **YDN** to HClO was investigated carefully. As shown in Fig S1, the probe showed a stable performance with HClO over the pH value ranged from 5 to 9. And the fluorescence intensity was significantly enhanced at physiological pH value (pH = 7.4), indicating that **YDN** can be suitable for biological applications. To make a comparison, **YDN** was tested in 10 mM HEPES and 10 mM PBS respectively. As depicted in Fig S2, **YDN** showed a quite different fluorescence response when treated with the same amount of HClO, more fluorescence enhancements can be observed in a solution of 10 mM PBS, demonstrating some HClO was consumed by HEPES. To provide some further evidence, differential pulse voltammetry (DPV) was performed in Fig S4. Obvious current increasing can be observed from -0.2 to -1.2 V (vs Ag/AgCl) for HEPES, Tris and **YDN**, however, not any response can be observed for PBS, demonstrating that HEPES and Tris are not suitable for HClO detection. All these are in good agreement with the literature.[17] Hence, PBS was employed for all the following measurements.

Fluorescence dynamics of the probe was recorded in PBS buffer (10 mM, pH 7.4) at room temperature. As displayed in Fig S5, a remarked fluorescence increase at 516 nm was observed and levelled off within 2 min, which suggested it can be an effective candidate for monitoring HClO in real time. Under the same condition, the UV-vis spectra of **YDN** exhibited maximum absorption at 492 nm. Upon addition of HClO (from 0 to 25 μM) to the solution of 5 μM **YDN**, the maximum absorption dropped noticeably and simultaneously a red-shifted absorption peak at 507 nm occurred, accompanied by a solution colour change from light orange to light pink (Fig S7a). Meanwhile, a marked fluorescence enhancement was observed at 516 nm owing to the oxidation reaction with azo moiety and leading to the formation of fluorescein when HClO was gradually added into the PBS solution of 5 μM **YDN** (Fig. 1a). And green fluorescence was observed upon 365 nm excitation under a hand-held UV lamp (Fig S7b). A good linear relationship was reached over the concentration range of HClO from 0 μM to 25 μM, $R^2 = 0.9988$ (Fig. 1b). According to the reported method,[11] the detection limit was determined to be as low as 8.7×10^{-9} M. Compared with the reported probes for HClO, **YDN** exhibited high sensitivity (Table S1).[14, 16, 19]

In order to evaluate the selectivity of **YDN** towards HClO over other analytes under simulated physiological conditions, representative species of ROS and RNS including ·OH, O_2^-, $^1O_2$, ROO^-, H_2O_2, t-BuOOH, NO_2^-, NO· and $ONOO^-$ were tested.[20] 200 μM ROS and RNS as well as 15 μM HClO were added respectively to a solution of 5 μM **YDN**. As shown in Fig. 2, almost no changes of fluorescence intensity was observed after the addition of excess ROS and RNS, while an intense fluorescence enhancement occurred with the addition of only 15 μM HClO. Additionally, common cations and anions such as Na^+, K^+, Zn^{2+}, Fe^{3+}, Mg^{2+}, Li^+, Mn^{2+}, Cu^{2+}, Al^{3+}, F^-, Cl^-, Br^-, I^-, CH_3COO^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, $H_2PO_4^-$ as well as GSH, Hcy and Cys (200 μM for each) didn't influence the detection (Fig S9), indicating that **YDN** can selectively measure HClO in complex biological environments.

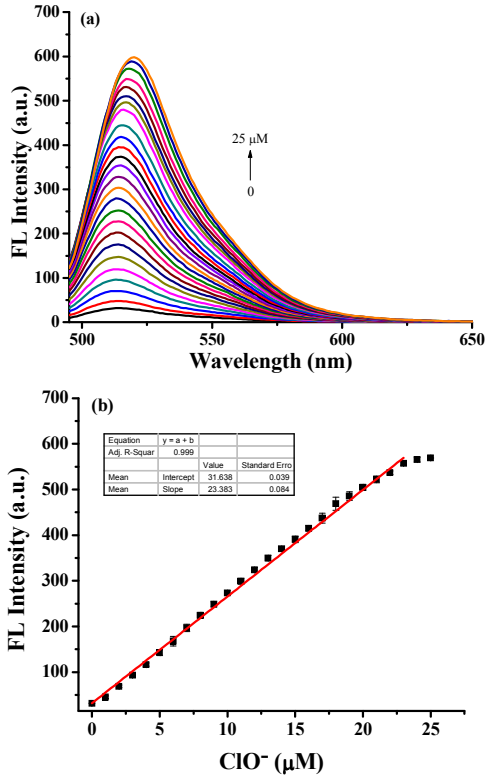

Fig. 1 Fluorescence titration studies of **YDN** upon addition of HClO. (a) Fluorescence spectra of **YDN** (5 μM) upon addition of HClO (0-25 μM) in PBS buffer (10 mM, pH 7.4) at room temperature. (b) The linear relationship between the fluorescent intensity and HClO concentration. All data were collected at 2 min after the addition of HClO. λ_{ex} = 485 nm, λ_{em} = 516 nm. Error bars standed for the mean value of three experiments.

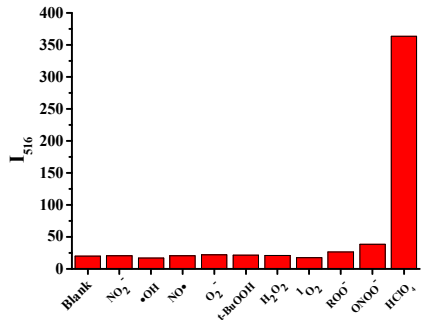

Fig. 2 Competitive experiments for HClO selectivity. Fluorescence responses of **YDN** (5 μM) to HClO (15 μM) and other ROS/RNS (200 μM for each) in PBS buffer (10 mM, pH 7.4) at room temperature. All data were collected at 2 min after each addition. λ_{ex} = 485 nm, λ_{em} = 516 nm.

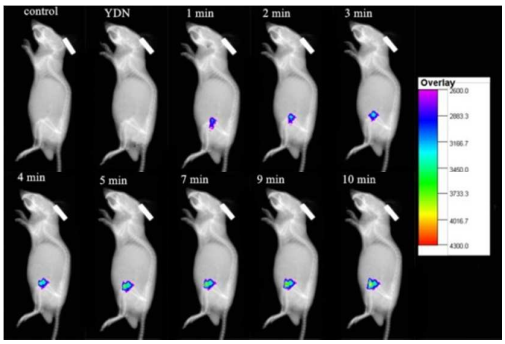

Fig. 3 Fluorescence images of living nude mice. Subcutaneous injection of the solution of PBS buffer (100 μL, control), subcutaneous injection of the solution of **YDN** (100 μM, in 100 μL PBS buffer), and then a solution of HClO (50 equiv., in 100 μL PBS buffer) was injected. Images were taken from 1 min to 10 min.

Then the potential of **YDN** for imaging HClO was evaluated in living cells. The Hela cells incubated with **YDN** (5 μM) exhibited almost no fluorescence in green channel (Fig S10b). By contrast, the cells stained with HClO (20 μM) showed bright fluorescence (Fig S10e), in good agreement with the fluorescence turn-on profile in the solution. These results indicated that **YDN** was capable of sensing HClO in the living cells. MTT assay show that 5 μM **YDN** has no obvious cytotoxicity to the cells (Fig S11). Finally, the possibility of **YDN** detecting HClO was evaluated in living mice. In this case, a solution of **YDN** (100 μM, in 100 μL PBS buffer) was subcutaneously injected into the nude mouse. And 5 minutes later, 50 equiv. HClO (in 100 μL PBS buffer) was injected into the same region. The pictures were recorded under the fluorescence imaging system from 1 min to 10 min after the above disposal. As the control group, a solution of 100 μL PBS buffer was injected into the nude mouse. As shown in Fig. 3, no fluorescence intensity was detected in the control and in the mouse injected probe **YDN** alone. While, strong signals were observed after the injection of HClO. The strongest fluorescence signals were collected in 10 min, which provided the possibility for real-time imaging HClO in vivo.

In conclusion, a novel probe for HClO has been developed by oxygenolysis of azo moiety for the first time. Compared with the reported probes for HClO, **YDN** showed excellent water solubility as well as high selectivity and sensitivity. Its application in mouse imaging will contribute to insight into the HClO formation and inflammation treatment in the future. Convincing evidences have shown that reductive buffer like HEPES and Tris etc. is not suitable for HClO detection, providing valuable reference for the solution selection in other ROS detection later.

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