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# A HClO-specific near-infrared fluorescent probe for determination of Myeloperoxidase activity and imaging mitochondrial HClO in living cells

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ABSTRACT

Hyperchlorous acid (HClO), produced from MPO, is recognized as a host defense that kills pathogens; a signaling molecule that initiates cell apoptosis; and a harmful agent when overproduced. Thus, measuring of endogenous HClO and MPO will always find its great importance in revealing biological roles under complex biological conditions. In this study, a turn-on near infrared (NIR) fluorescent probe Cy7-NphS has been designed and developed for highly selective and sensitive sensing of HClO and Myeloperoxidase (MPO) with fast response time. The newly developed probe has been successfully applied in real-time monitoring of HClO and MPO activity in PBS solutions and living HL-60 cells. When applied in MPO activity determination, the probe showed very high sensitivity with a detection limit of as low as $3.69 \times 10^{-3}$ U/mL. Furthermore, the living cell imaging study suggested that this probe could detect HClO in mitochondria.



## 1. Introduction

Hypochlorous acid (HOCl), a member of the ROS family, was a strong oxidative agent that played essential roles in many cellular processes. The most famous physiological function of HClO was to kill pathogens and thus contribute to host defense (Klebanoff, 2005; Weiss 1989). Recent evidence also suggested that endogenous HClO could serve as a signal to activate caspase and mediate cell apoptosis (Gloire et al., 2006; Sugiyama et al., 2004). However, overproduced HClO was believed to cause oxidative injuries to biomolecules such as nucleic acids, proteins and lipids (Eley et al., 1991; Schraufstatter et al., 1988; Tatsumi and Fliss, 1994). This chemical property of HClO made it a central pathogenic factor in a variety of diseases, such as cardiovascular diseases, atherosclerosis, osteoarthritis, rheumatoid arthritis and lung injury (Daugherty et al., 1994; Weitzman and Gordon, 1990; Winterbourn and Kettle 2000; Wu and Pizzo 2001). Elevated cellular HClO levels could be associated with increased MPO activity, because HClO was produced by MPO-catalyzed oxidation of $Cl^-$ by $H_2O_2$ in cells. It has been pointed out that MPO monitoring allowed identification of patients at risk of cardiac events even in the absence of myocardial necrosis (Asselbergs et al., 2004; Baldus et al., 2003; Meuwese et al., 2007). Therefore, the determination of MPO activity and cellular HClO levels has vast importance in understanding the biological roles of HClO as well as early diagnosis of HClO-related diseases.

Reaction-based small-molecule fluorescent probes represented a promising technology for measuring biological species due to its sensitivity and convenience (Chan et al., 2012; Cho and Sessler, 2009; Li et al., 2014; Yang et al., 2014; Yuan et al., 2013b). Recently, many efforts have been devoted to the development of fluorescent probes for measuring HClO (Cheng et al., 2013; Fan et al., 2015; Hou et al., 2015; Wang et al., 2013; Yue et al., 2015) and MPO activity (Goiffon et al., 2015; Gross et al., 2009). In this study, our goal was to develop a HClO-specific fluorescent probe that could monitor HClO levels and MPO activity in real-time without

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interference of bioautofluorescence (Ntziachristos et al., 2002; Yuan et al., 2013a) from the complex biological systems. Development of NIR fluorescent probes for HOCl represents a great challenge because cyanines, the most commonly used NIR dyes, are prone to oxidative cleavage and subsequent fluorescence quenching (Sun et al., 2014; Wang et al., 2009). As an attempt, we designed and developed probe Cy7-NphS, a new fluorescent probe that merit HClO selectivity, MPO specificity, high sensitivity, fast response time, and near-infrared emission at the same time. Herein we presented the application of Cy7-NphS in determination of MPO activity and imaging of HClO in biological samples. The results showed that Cy7-NphS was capable of monitoring MPO-derived HClO in real time in living cells. Through a linear relationship between the fluorescence intensity and MPO concentrations, the MPO activity could be quantitatively determined with a detection limit of $3.69 \times 10^{-3}$ U/mL. The results also showed that Cy7-NphS was mitochondria targeted and could image mitochondrial HClO in living cells.

## 2. Experimental section

### 2.1. Materials and instruments

Common reagents or materials were obtained from commercial source of analytical reagent grade, and used without further purification except as otherwise noted. MPO Peroxidase (MPO) and Salicyl hydroxamic acid (SHA) was purchased from Sigma Aldrich. Ultrapure water was used throughout the analytical experiments. A series of ROS is obtained or prepared as described in detail in supporting information. Steady-state UV/Vis was measured at room temperature on a Lambda 35 UV–visible Spectrophotometer (Perkin-Elmer) with 1.0-cm quartz cells. Fluorescence emission spectra were obtained at room temperature on a Fluoromax-4 Spectrofluorometer (Horiba-Jobin Yvon), with a Xenon lamp and 1.0-cm quartz cells.

### 2.2. Synthesis and structural characterization of Cy7-NphS

2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]−3,5-(propane-1,3-diyl)−1,3,5-heptatrien-1-yl)−1-ethyl-3,3-dimethyl-3H-indolium (Cy7-Cl) was synthesized according to a slight modification of the literature procedure. After that, the synthesis of Cy7-NphS was described as below. Under the nitrogen atmosphere, a mixture containing Cy7-Cl and 5 equiv of 4-Amino thioanisole, (C7H9NS) in anhydrous N, N′-dimethylformamide (DMF) was stirred at 90 °C. The reaction was monitored by TLC analysis and was stopped when there was no more Cy7-Cl. Then, the DMF was removed under reduced pressure. The resulting residue was purified on a short-column chromatography (silica gel, ethyl acetate: methanol=5:1).1HNMR(400 MHz, d6-DMSO) δ (ppm): 8.70(s, 1 h), 7.93(d, 2 h), 7.44(d, J=7.3 Hz, 2 h), 7.26(m, 6 h), 7.12(t, J=7.5 Hz, 2 h), 6.89(d, J=8.6 Hz, 2 h), 6.04(d, J=14.0 Hz, 2 h), 4.08(m, J=7.0 Hz, 4 h), 2.6(s, 4 h), 2.30(s, 3 h), 1.85(s, 2 h), 1.30 (s, 10 h), 1.22(s, 8 h) 13CNMR (d6-DMSO, 500 MHz) δ(ppm): 170.45, 157.97, 145.98, 142.74, 142.39, 141.28, 130.66, 128.91, 127.53, 124.82, 122.86, 117.33, 110.83, 98.76, 48.70, 38.84, 28.02, 24.70, 21.82, 17.68, 12.42 HRMS: m/z $C_{41}H_{50}N_3S^+$ Calcd 614.36, found [M]+(614.36).

### 2.3. General procedure for monitoring ROS

On the basis of the procedure following, all the measurements of the activity of different ROS were carried out in 100 mM PBS (pH7.40). The probe Cy7-NphS (Ethanol, 15 μL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 3.0 μM with 1000 μL Acetonitrile and 3985 μL PBS buffers (pH=7.40), several ROS including NaClO was added. The mixture was equilibrated for 30 s before measurement. The fluorescence intensity was measured at λex=750 nm. The excitation and emission slits were set to 5 nm, respectively.

### 2.4. General procedure for monitoring Myeloperoxidase

Measurement of MPO activity was carried out in 100 mM PBS (pH 7.40). With a total volume of 3000 μL, culture mixtures that contained PBS, Cy7-NphS and specified amount of MPO were mixed gently. Then reactions were started by addition of proper amount of $H_2O_2$. To ensure that metabolites formation was enzyme and $H_2O_2$ dependent, control incubations were carried out without enzyme sources/$H_2O_2$ at the same time. The solution was incubated at 37 °C over 20 min while determined the fluorescent intensity of $\lambda_{em}$=790 nm ($\lambda_{ex}$=750 nm ) at 1 min interval.

### 2.5. Cell culture and confocal fluorescence imaging

HL-60 cells lines were obtained from the CAS (Chinese Academy of Sciences) Cell Bank. and cultured in IMDM (Hyclone) medium supplemented with 20% (v/v) fetal bovine serum (FBS) in a humidified incubator containing 5% $CO_2$ at 37 °C. The cells in 35 mm × 12 mm glass bottom cell culture dishes were set at a density of $2.5 \times 10^5$/mL. The stock solution of Cy7-NphS in DMSO (1 mM) was diluted with phosphate buffered saline solution (100 mM, pH 7.4, 138 mM NaCl) with final concentration of 10 μM. Confocal fluorescence images (Ex. 635 nm, Em. 700–800 nm) were observed with Olympus FV1000 confocal laser-scanning microscope with an objective lens ( × 100). Hoechst and mitotracker green were used to co-localize in cell imaging study. (Ex. 405 nm, Em. 450–550 nm) was set for Hoechst and (Ex. 488 nm, Em. 500–600 nm) was set for mitotracker green.

## 3. Results and discussion

### 3.1. Design and synthesis of Cy7-NphS

The development of NIR fluorescent probes for HClO with turned-on fluorescence represented a great challenge because cyanines, the most commonly used NIR dyes, were vulnerable to HClO attack. As a result, the NIR fluorescence of cyanine-based probes for HClO was usually quenched due to the oxidative cleavage of the cyanine backbone when they responded to this species. Our Strategy for designing a “turn-on” NIR fluorescent probe for HClO relied on the facilitate functionalizaion of a heptacyanine dye with 4-(methylthio)-benzenamine group, a sulfur-containing moiety that reacted with HClO more kinetically superior than the cyanine backbone (Scheme 1). The mechanism of the fluorescence enhancement of CyNPhS in response to HClO can be explained by photoinduced electron transfer (PET) process (detailed information provided in supporting information). The advantage of this design could be rationalized as (1) the sulfur-containing moiety as the HClO reaction center could rule out the concerns of HClO-induced NIR fluorescence quenching; (2) the oxidation of the sulfur center could be expected as an effective means for modulating the fluorescence of the cyanine; (3) the designed probe could be synthesized in a simple one-step reaction. The details of the synthetic procedure of Cy7-NphS are described in the Supporting information (Scheme S1). The chemical structures of Cy7-NphS were verified by HRMS, $^1$H NMR, and $^{13}$C NMR (see Figs. S1 – 3, ESI†).

After getting this compound, we then tested its fluorescence response to HClO in the NIR region. As shown in Fig. S4 (ESI†), Cy7-