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A ratiometric fluorescent probe based on a coumarin–hemicyanine scaffold for sensitive and selective detection of endogenous peroxynitrite

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ABSTRACT

In the study described herein, the red emitting probe **CHCN**, which possesses a linked coumarin–hemicyanine scaffold, was developed for detection of peroxynitrite ($ONOO^-$) under physiological conditions. The studies show that **CHCN** displays a dual ratiometric and colorimetric response to $ONOO^-$ that is caused by an oxidation process. A possible mechanism of this oxidation process was proposed and confirmed by ESI-MS spectra for the first time. **CHCN** shown highly selective and sensitive towards $ONOO^-$ with a low limit of detection LOD (49.7 nM). Moreover, **CHCN** has appreciable cell permeability and, as a result, it is applicable to ratiometric detection of exogenous and endogenous $ONOO^-$ in living cells during phagocytic immune response. We anticipate that, owing to their ideal properties, probes of this type will find great use in explorations of the role played by $ONOO^-$ in biology.

1. Introduction

Reactive oxygen species (ROS) and nitrogen species (RNS) play important roles in physiology and pathology (Chen et al., 2011; Dickinson and Chang, 2011; Pacher et al., 2007; Winterbourn, 2008; Xu et al., 2013; Yang et al., 2010). Among ROSs and RNSs, peroxynitrite ($ONOO^-$) has received special attention owing to its unusually potent oxidizing ability and strongly nucleophilic character (Radi, 2013). As pointed out in recent reports, $ONOO^-$ is widely considered to be an important factor in the onset and progression of many cellular processes (Dickinson and Chang, 2011). For example, as a strong nucleophile, this species reacts rapidly with carbon dioxide in vivo to form the nitrosoperoxycarbonate ($ONOOCO_2^-$) intermediate. $ONOOCO_2^-$ then rapidly decomposes homolytically to generate carbonate radical ($CO_3^{-\bullet}$), which is believed to cause specific cellular damage (apoptotic or necrotic cell death) (Radi, 2013). As a potent oxidant, $ONOO^-$ reacts directly with electron-rich moieties. This process causes damage to a wide array of biomolecules (e.g., DNA and proteins) (Kryston et al., 2011; Soon et al., 2011), which leads to many human diseases, such as septic stroke, cancer, diabetes, inflammatory diseases, and neurodegenerative disorders (Szabo et al., 2007). In contrast, growing evidence suggests that the well-controlled generation of certain $ONOO^-$ serves as a signaling transduction factor for a wide range of normal cellular functions (Dickinson and Chang, 2011). Therefore, it is important to develop a clear understanding of the role played by $ONOO^-$ in cellular functions. For this purpose, it is essential to develop highly sensitive and selective methods for accurate and direct detection of $ONOO^-$.

However, as a consequence of its short half-life (<20 ms) under typical physiological conditions (Yang et al., 2006), direct and unambiguous detection of $ONOO^-$ in living cells can not be easily made by using traditional analytical methods (Chen et al., 2013b; Oushiki et al., 2010; Yu et al., 2012). In the past decade, a large number of fluorescent probes have been designed and constructed for specific detection of $ONOO^-$ (Chen et al., 2011; Dickinson et al., 2010; Kalyanaraman et al., 2012). These probes are based on various strategies that rely on direct oxidation reactions of boronates (Chen et al., 2013b; Sikora et al., 2009; Sun et al., 2014), active ketones (Peng and Yang, 2010; Sun et al., 2009; Yang et al., 2006), metal complexes (Rausaria et al., 2011), selenium (Xu

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et al., 2011), tellurium (Koide et al., 2012; Yu et al., 2013), and agents (Ueno et al., 2006; Zhang et al., 2012). Despite the encouraging progress that has been made, a great challenge still exists in developing fluorescent probes with high sensitivities and selectivities that are able to distinguish between $ONOO^-$ and other ROSs (e.g. ClO^- and H_2O_2) (Kalyanaraman et al., 2012; Zhang et al., 2012). Moreover, devising probes of this type, which selectively detect endogenous $ONOO^-$ in vivo without disturbance being offered by the presence of innate reducing biomolecules such as biothiols, is a particular demanding task (Chen et al., 2013b; Yu et al., 2013).

Recently, ratiometric type fluorescent probes, especially those that emit light in the near-infrared (NIR) region, have attracted increasing attention because of their intrinsic self-calibrating character and ability to be used for cell and body imaging (Bozdemir et al., 2011; Erbas-Cakmak et al., 2013; Guo et al., 2014; Kolemen et al., 2011; Wu et al., 2014; Yuan et al., 2010). However, compared to the numerous turn on/off fluorescence $ONOO^-$ probes that have been developed to date, only a few ratiometric probes of this type have been described.

In 2010, Nagano et al. reported that cyanine dyes (e.g. Cy5 and Cy7) are readily oxidized by various ROSs to form products that do not fluorescence. More importantly, the studies demonstrated that cyanine dyes display reactivities towards ROS that are governed by the lengths of their conjugated central polymethine chains. For example, Cy7 is more susceptible to ROS promoted oxidation than Cy5 (Oushiki et al., 2010). In spite of this phenomenon, the poor selectivities and the fluorescence quenching response of cyanine dye towards ROSs hinders their broad usage in biology. In order to overcome this limitation, we embarked on a program aimed at the development of a new kind of ratiometric florescence platform that relies on the utilization of a hybrid coumarin–hemicyanine dye. As has been reported recently, these types of hybrid dyes have been explored in the context of ratiometric fluorescent sensors for some common nucleophilic reagents, such as cyanide, sulfite and sulfide (Chen et al., 2013a; Lv et al., 2011; Sun et al., 2013).

However, to our knowledge, hybrid coumarin–hemicyanine dyes have not been explored as sensing platforms for ROSs. Taking these factors into account, we have developed the ROS probe **CHCN**, a conjugated hybrid dye composed by a coumarin group and a hemi-cyanine moiety linked through a carbon-carbon double bond. The results of this effort show that **CHCN** can be employed to detect $ONOO^-$ in a dual ratiometric and colorimetric manner with high sensitivity and selectivity. In addition, we have demonstrated an application of **CHCN** for ratiometric detection of exogenous and endogenous $ONOO^-$ in living cells.

2. Materials and methods

2.1. General information of materials and methods

Unless otherwise noted, all materials were obtained from commercial sources and were used without further purification. Solvents were dried according to standard procedures. ^1H NMR and ^{13}C NMR in CDCl3 were measured on a Bruker AM-300 spectrometer with tetramethylsilane (TMS) as internal standard. Mass spectra were obtained using a JMS-HX 110A/110A tandem mass spectrometer (JEOL). UV–vis spectra were obtained using a Scinco 3000 spectrophotometer (1 cm quartz cell) at 25 °C. Fluorescence spectra were recorded on RF-5301/PC (Shimada) fluorescence spectrophotometer (1 cm quartz cell) at 25 °C. Deionized water was used to prepare all aqueous solutions.

2.2. Generation of ROS/RNS

H_2O_2 was from dilution of 28% solution in water. Tert-butyl hydroperoxide was from dilution of 70% solution in water. $ROO^{\bullet}$ was generated from 2,2′-Azobis (2-amidinopropane) dihydrochloride. $NO^{\bullet}$ was generated from SNP (Sodium Nitroferricyanide (III) Dihydrate). $^{\bullet}O^{2-}$ was generated by mixing xanthine (25 μM) and xanthine oxidase (3.2 mU/mL). $^{\bullet}OH$ was from the reaction of Ammonium iron (II) sulfate (100 μM) and H_2O_2 (100 μM). NaClO was from dilution of 12% solution in water. $ONOO^-$ was prepared according to previous literature and the concentrated was determined by absorbance at 302 nm (Xu et al., 2013). The above ROS or RNS were incubated with FBS in 50 mM KH_2PO_4 (pH 7.4) for 30 min respectively.

2.3. Cell-culture and confocal microscopy experiments

The Human normal lung cell (WI38 VA13) cells and a macrophage cell line murine (RAW 264.7) were cultured on the surface of a glass slide in SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA ferric sodium salt) at 37 °C in 5% CO_2. The cells were subcultured by scraping and seeding on six-well plates according to the instructions from the manufacturer. Cells were grown to confluence prior to experiment. For confocal microscopy experiments, WI38 VA13 cells were stained with **CHCN** (5 μM) for 30 min and washed with DPBS, and then treated with various concentration of exogenous $ONOO^-$. Then the cells were investigated by using a dual emission imaging mode. The green fluorescence channel images were collected at 490–540 nm with an excitation wavelength at 473 nm, and the red fluorescence channel images were collected at 575–675 nm with an excitation wavelength at 559 nm, respectively. Murine RAW 264.7 macrophage cells were then stimulated with lipopolysaccharide (LPS, 1 μg/mL) for 16 h, and interferon-γ (IFN-γ, 50 ng/mL) for 4 h, followed by phorbol 12-myristate 13-acetate (PMA, 10 nM) for further 30 min for production of endogens $ONOO^-$, or pretreated the cells with either a scavenger of superoxide, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, 100 μM), or an NO synthase inhibitor, aminoguanidine (AG, 1 mM), to reduce cellular endogenous $ONOO^-$. After that, the cells were stained with **CHCN** (5 μM) for 30 min, and then washed with DPBS for three times before imaging. The fluorescence images were obtained from a dual emission imaging mode as abovementioned.

2.4. Synthesis of *CHCN*

Diethylaminocoumarin-3-aldehyde (50 mg, 2 mmol) and 1,3,3-trimethyl-2-methyl-eneindoline (60 mg, 2 mmol) were dissolved in absolute ethanol (20 mL) and refluxed for 10 h. After being cooled to room temperature, remove the ethanol under reduce pressure. The corresponding solid was further purified by silica gel column chromatography using DCM/Methanol (20/1, v/v) as eluent to afford **CHCN** as a deep violet solid (0.312 g, yield: 68%). ^1H NMR (300 MHz, $CDCl_3$-d_3) δ 10.05 (s, 1H), 8.60 (d, J=16.2 Hz, 1H), 8.13 (d, J=9.3 Hz, 1H), 8.10 (d, J=16.2 Hz, 1H), 7.52–7.63 (m, 4H), 6.72 (dd, J=9.3 Hz, 1H), 6.47 (s, 1H), 4.31 (s, 3H), 3.54 (q, J=7.1 Hz, 4H), 1.85 (s, 6H), 1.34 (t, J=7.1 Hz, 6H). ^{13}C NMR (75 MHz, MeOD-d_3): δ 158.1, 154.7, 150.3, 143.1, 140.3, 132.3, 128.7, 122.7, 113.8, 112.4, 110.0, 96.4, 78.1, 51.7, 45.1, 32.6, 25.4, 11.4. MS: [M^+] at 401.23.