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Rational design of a diaminomaleonitrile-based mitochondria – targeted two-photon fluorescent probe for hypochlorite *in vivo*: Solvent-independent and high selectivity over Cu^{2+}

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ABSTRACT

For the rational design of a solvent-independent diaminomaleonitrile (DAMN)-based fluorescent probe for monitoring ClO^- selectively without the interference from Cu^{2+}, a feasible strategy is to introduce the DAMN group into a sterically crowded π-conjugated framework. The addition of ClO^- will recover the planarity of the molecule, change the D-π-A structure, and influence intramolecular charge transfer (ICT) efficiency in the sensing system. Inspired by this strategy, a DAMN-based Schiff base derivative (**HCCN**) was designed and synthesized to detect ClO^- with a rapid response, a large Stokes shift and moderate two-photon excitation action cross-section. **HCCN** was highly selective for ClO^- detection with no analyte interference (especially from Cu^{2+}) in CH_3CN/PBS and other mixed solvent systems. Density functional theory (DFT)/TDDFT calculations supported that the enhanced fluorescence signal of **HCCN** to ClO^- was arisen mainly from the recovery of the planar structure and the accompanying enhancement of ICT efficiency in **4**. A co-staining experiment of **HCCN** showed its high affinity towards intracellular mitochondria; subsequently, **HCCN** was successfully applied for two-photon bio-imaging of exogenous ClO^- in fresh tissues and endogenous ClO^- *in vivo*.

1. Introduction

Mitochondria are the powerhouses of cells, producing adenosine triphosphate (ATP). A range of electronic transportation systems exist within the oxidation phosphorylation pathway related to this process, and the reactive oxygen species (ROSs) are also originated from it [1,2]. As one type of ROS, hypochlorite (ClO^-) is an effective antimicrobial agent and acts in a critical function for immunity [3,4]. Diseases, including Parkinson's disease (PD), Alzheimer's disease (AD) and a series of cancers, are linked to a ClO^- concentration under its abnormal level [5,6]. Therefore, monitoring ClO^- in a biological environment, especially in mitochondria, is a topic of great interest.

Small-molecule fluorescent probes are attractive for studying biological systems because of their several considerable merits, which include high specificity, simple operation and low cost, among others. Taking advantage of imaging techniques, researchers can utilize these probes as powerful tools to sense and visualize analytes *in vitro* and *in vivo* microscopically [7–12,]. To date, some probes have been used to detect and bioimage ClO^- [13–22]. However, the reported ones for monitoring and visualizing ClO^- in mitochondria are still limited [23–30], and most involve one-photon microscopy (OPM). In comparison with OPM, two-photon microscopy (TPM), offers several advantages for bioimaging [31–34]. To obtain a bright TPM image while avoiding the appreciable photodamage at laser powers commonly used in the imaging experiment, Kim and Cho suggested that the two-photon brightness or two-photon action cross-section ($\delta' = \delta \times \Phi$) of the probe should be greater than 50 GM [34]. However, two-photon fluorescent probes with $\delta' \geq 50$ GM, which can be used to track mitochondria [35–38] or to detect ClO^- in mitochondria [39,40] under TPM, have barely been reported. Probe **MITO-TP** was the

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first reported mitochondrial two-photon HClO probe with a large value of δ' for the oxidation product (δ' = 83 GM) [39]. However, a series of iridium (III) phosphorescent probes for detecting ClO^- in mitochondria or imaging mitochondria showed that their δ' values were much less than 50 GM in actuality [41,42]. Therefore, a novel mitochondrial two-photon probe for hypochlorite with a larger δ' for TPM application is extremely desirable.

In the past decade, diaminomaleonitrile (DAMN)-based Schiff base derivatives containing coumarin [43], naphthalimide [44], triphenylamine [45], 9-phenylcarbazole [45], aza-crown ether [46], naphthalene [47] and pyrene [48] units have been widely used as chemosensors to sense Cu^{2+} ions with a colorimetric/fluorometric response, where the DAMN group is used as the chelator to form a Cu^{2+}-complex (Route 1 in Scheme 1). Additionally, the DAMN moiety can be removed selectively by ClO^- to produce aldehyde or carboxylic product based on the de-diaminomaleonitrile oxidation reaction (Route 2 in Scheme 1) [24,41,49–51]. To determine whether the DAMN-containing chromophores can distinguish Cu^{2+} and ClO^-, Liu reported that a pyrene-DAMN derivative (**Py-DAMN-2**) that could respond to Cu^{2+} in a CH_3CN/H_2O (v/v = 1:1, 10 mM HEPES) system but showed almost no response to Cu^{2+} in a DMF/PBS (v/v = 2:8) system. However, **Py-DAMN-2** was used as a "turn-on" ClO^- chemosensor in DMF/PBS buffer system, by utilizing the strong coordination between the solvent DMF and **Py-DAMN-2** to eliminate the interference of Cu^{2+} [52]. Chao reported two DAMN-based iridium(III) complexes as phosphorescent probes for the selective detection of ClO^- [24,41], and Lin developed a coumarin-DAMN derivative for monitoring ClO^- with a ratiometric fluorescence response [49], both of which above were used DMF/PBS buffer system and were undisturbed by Cu^{2+} in ClO^- detection. However, Goswami reported the different results for two DAMN-based sensors for ClO^- using a CH_3CN/H_2O system. No selectivity of the probe **PDS** with a phenanthroline unit to Cu^{2+} was detected, and probe **CDH** with a carbazole unit had no interference of Cu^{2+} [50,51]. As before, the reports concerning on the selectivity of the DAMN-based fluorescent probe for ClO^- and Cu^{2+} remains elusive. Hence, the development of a solvent-independent DAMN-based fluorescence probe for the selective detection of ClO^- without the interference of Cu^{2+} would be advantageous.

Considering that the electronic characteristics and steric hindrance can affect the optical performance of the probe, we envisioned that if the DAMN group was introduced into a sterically demanding framework, such as the carbazole moiety, it would be forced to twist out of the carbazole plane. If the carbazole unit can block the affinity of Cu^{2+} to the DAMN group through steric hindrance, the colorimetric and fluorometric responses would be restrained. Encouraged by this strategy, along with the related work of two-photon fluorescent probes with a carbazole skeleton in our group [53–57], we here report a DAMN-containing, mitochondria-targeted two-photon fluorescent probe (**HCCN**) for ClO^-; this probe utilizes the de-diaminomaleonitrile reaction to the corresponding aldehyde derivative by ClO^-. As expected, **HCCN** was highly selective for ClO^- detection without the interference of other analytes (especially for Cu^{2+}) in CH_3CN/PBS and other mixed solvent systems. As shown in Scheme 2, the carbazole moiety was shared as a donor (D) with two accepting units (A1 and A2) at its end. The DAMN unit as an electron-withdrawing group (A2) was non-planar with the carbazole plane and converted into another electron-withdrawing aldehyde group (A3) with a planar structure upon the reaction of ClO^-. This conversion from A1-π-D-π-A2 in **HCCN** to A1-π-D-π-A3 in **4** could lead to the changes of the intramolecular charge transfer (ICT) efficiency in the testing system, and subsequently alter the fluorescence and absorption properties. We speculated that **HCCN** might be a "turn-on" fluorescence probe for ClO^- because of the enhanced ICT efficiency, which could be clearly elucidated by DFT/TDDFT calculations. The high selectivity of **HCCN** for ClO^- detection without the interference of other metal ions (especially for Cu^{2+}) might also be supported by calculation results. We believe that introducing DAMN group into a sterically demanding framework is a promising strategy for the rational design of a ClO^- fluorescent probe to eliminate the interference of Cu^{2+}.

2. Experimental section

2.1. General information

All reagents and solvents were commercially purchased. Mitotracker Deep-Red (MTDR) was purchased from Invitrogen (USA). Lipopolysaccharides (LPS) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (USA). *N*-Hydroxybenzenesulfonamide (Piloty's acid, PA) was purchased from Cayman Chemical (USA). ^1H NMR spectra were collected using Bruker–400 MHz spectrometers and ^{13}C NMR spectra were obtained on 100 MHz spectrometers. MS spectra were recorded on an AB Sciex MALDI-TOF 5800 mass spectrometer. ESI-TOF spectra were collected using a Waters Xevo G2 QT mass spectrometer. Elemental analysis was performed using an Elementar Vario EL elemental analyzer. The fluorescence quantum yields were tested using a HORIB FluoroMax-4P. Fluorescence spectra were recorded on a Hitachi F-7000 spectrometer. UV–vis absorption spectra were obtained using a Tech-comp UV 1000 spectrophotometer. The synthesis route of probe **HCCN** is shown in Scheme S1 and the detailed description and characterization of **HCCN** and other intermediates are available in the supporting information.

3. Results and discussion

3.1. Photophysical properties of *HCCN* response to ClO^-

Initially, we tested the influence of different mixed solvent systems on **HCCN** response to ClO^-; **HCCN** diluted with five common organic solvent/PBS mixed media (including DMF/PBS and CH_3CN/PBS, among others) showed high selectivity for ClO^- without the interference of Cu^{2+} (Fig. S1†). The fluorescence and absorption titration experiments of **HCCN** with the addition of ClO^- were carried out in CH_3CN/PBS buffer (2/8, v/v, 20 mM, pH 7.4) by experimental optimization. As shown in Fig. 1a, the emission of **HCCN** was weak with a small quantum yield of 0.5%. The emission intensity at 541 nm was enhanced substantially with increasing concentrations of ClO^- (0-0.8 mM), and the fluorescence intensity maximum was obtained upon the addition of 40 equivalents of ClO^- to the **HCCN** solution, accompanied by a 17.5-fold fluorescence enhancement with an increased fluorescent quantum yield of 8.9%. As seen in Fig. 1b, when adding ClO^- continuously to **HCCN**, the absorption peaks located at 388 nm and 351 nm gradually disappeared. Simultaneously, the absorbance at 436 nm was decreased along with a 10 nm of **hypochromic shift**. An isosbestic point at 318 nm is clearly observed, followed by an increase at 292 nm. In addition, the large Stokes shift (115 nm) of **HCCN** upon the addition of ClO^- would be a good photophysical property of **HCCN** as a small-molecule fluorescent probe.

A linearly increasing fluorescence intensity versus ClO^- molar concentration (0–5 μM) was displayed for **HCCN**, and the detection limit ($3\sigma/k$) was calculated to be 0.44 μM according to the titration profile (Fig. S2a†). The reaction rate of **HCCN** to ClO^- was studied (Fig. S3), and the result showed that the reaction can be completed within 1 min, indicating a fast response of the probe to ClO^-. The influence of pH on **HCCN**'s response to ClO^- is shown in Fig. S4. **HCCN** showed almost no fluorescence and remained steady in a certain pH range ($5 \leq pH \leq 10$). Compared with **HCCN** alone, the enhanced fluorescence signals of **HCCN** to ClO^- slightly increased