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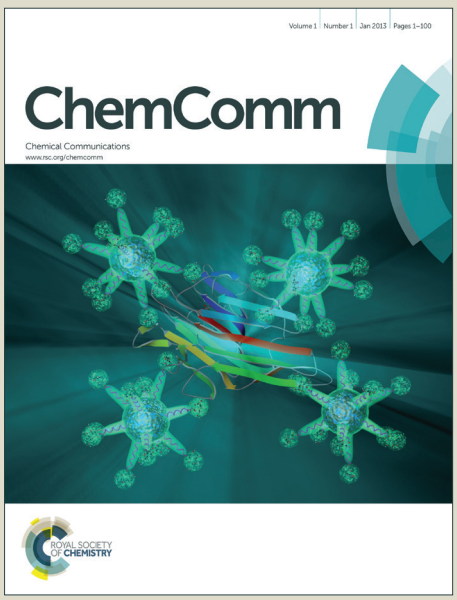

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ARTICLE TYPE

Mitochondria-targeted colorimetric and fluorescent probes for hypochlorite and their applications for *in vivo* imaging†

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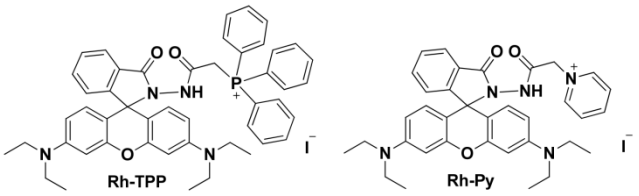
Two mitochondria-targeted real-time probes were presented, which could selectively respond to hypochlorite over other ROS. Meanwhile, the *"off-on"* probes could successfully apply in the *in vivo* imaging of hypochlorite in living mice.

Hypochlorous acid (HClO)/hypochlorite (ClO^-) is one of the ROS and is widely encountered in our daily life. For instance, sodium hypochlorite is frequently used as a bleaching agent. On the other hand, in living organism, hypochlorite is produced mainly in leukocytes from hydrogen peroxide and chloride ions in a myeloperoxidase (MPO)-catalyzed reaction,[1] which is linked to innate host defense and is of great importance in killing a wide range of pathogens.[2] Hence, the maintenance of normal level of ClO^- is fairly essential for numerous cellular functions. Whereas, abnormal hypochlorite level is considered to be associated with some diseases, like atherosclerosis, osteoarthritis, rheumatoid arthritis and lung injury.[3] Therefore, a rapid and sensitive detection of ClO^- *in vitro* and *in vivo* is of significant interest. To date, a number of fluorescent probes for ClO^- have been reported.[4] However, most of them are faced with some following drawbacks: (a) interference from other ROS, (b) poor water solubility, and (c) autoxidation and photo bleaching. Moreover, since mitochondria are the main source of intracellular ROS,[5] monitoring ClO^- in mitochondria is particularly meaningful and valuable. Nevertheless, quite few fluorescent probes for mitochondrial ClO^- have been developed,[6] of which the water solubility and selectivity still need to be improved.

In 2008, Ma group developed a highly selective fluorescent probe for ClO^- by oxidizing dibenzoylhydrazine into dibenzoyldiimide, which can further undergo decomposition in some nucleophilic solvents.[7] However, 30% THF was needed as co-solvent for this probe. Here, we reported two colorimetric and fluorescent probes **Rh-TPP** and **Rh-Py** for ClO^- with excellent water solubility, high selectivity and fast response, which utilize the oxidization-hydrolysis of benzoyl acetohydrazide (Scheme 1). **Rh-TPP** contains a triphenylphosphonium (TPP) moiety, which has been extensively utilized as a mitochondria-targeted functional group.[8] By contrast, a quaternarized pyridine moiety is appended to **Rh-Py**. Very recently, the pyridinium unit has been applied to target mitochondria secifically.[9] In these cases, the pyridinium moiety was conjugated to the fluorophore and became part of the whole luminophore. However, the cationic pyridinium moiety might interact with anionic species in mitochondria via electrostatic interaction, thus affecting the fluorescence properties of the probes. As for **Rh-Py**, the pyridinium moiety is attached to rhodamine via a flexible chain, which can avoid the negative effect mentioned above. Not surprisingly, both of **Rh-TPP** and **Rh-Py** can detect ClO^- in mitochondria and can be used for imaging ClO^- *in vivo*. Here, TPP moiety and pyridinium unit not only act as mitochondria-targeted carriers but also improve the water solubility of these two probes. These two compounds were prepared via simple process and characterized by ^1H, ^{13}C NMR and HRMS (see ESI).



Scheme 1 The structures of **Rh-TPP** and **Rh-Py**

Initially, to test whether **Rh-TPP** or **Rh-Py** can selectively detect ClO^- among ROS, we measured the absorption and emission spectra of these two compounds in the presence of ROS. To the solution of 5 μM **Rh-TPP** in PBS (pH 7.4, 10 mM, containing 0.1% DMSO) was added 50 μM NaClO and a typical absorption peak of rhodamine B at 560 nm appeared obviously,[10] accompanied by a colour change from colourless to light purple (Figure S1). While other ROS (i.e. 500 μM for ·OH, 100 μM for $O_2^{\bullet-}$, 100 μM for H_2O_2, 50 μM for $ONOO^-$, 100 μM for ^{t}BuOOH) were added to the solution of **Rh-TPP**, no change in the absorption spectra of **Rh-TPP** was observed. A similar phenomenon was discovered upon addition of ROS to the solution of 5 μM **Rh-Py** in PBS (pH 7.4, 10 mM, containing 0.1% DMSO), suggesting the excellent selectivity of **Rh-TPP** and **Rh-Py** toward ROS. Simultaneously, the addition of NaClO to the solution of **Rh-TPP** or **Rh-Py** also induced a dramatic fluorescence enhancement of these two compounds, ~200 fold for **Rh-TPP** and ~380 fold for **Rh-Py**, respectively, as shown in Fig. 1. However, the addition of other ROS caused negligible variation of the fluorescence of **Rh-TPP** and **Rh-Py**. Considering that cations might interact with benzoyl acetohydrazide unit and then lead to the spiroring-opening of rhodamine, the fluorescence responses of **Rh-TPP** and **Rh-Py** toward metal ions were explored (Figure S2). When 50 μM metal ions (Na^+, K^+, Mg^{2+}, Fe^{3+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Al^{3+}, Mn^{2+}, Ag^+, Cu^{2+}, Zn^{2+}) were added to the solution of 5 μM **Rh-TPP** or **Rh-Py** in

PBS, no obvious emission change was found. Accordingly, **Rh-TPP** and **Rh-Py** can act as highly selective and sensitive probes for ClO^- in water with dual modes.

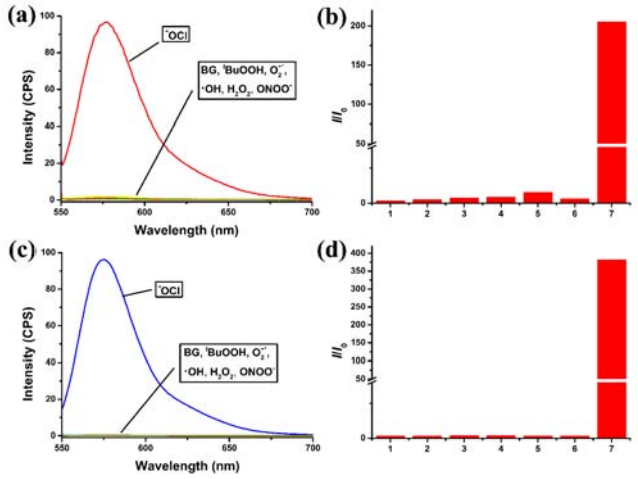


Fig. 1 Fluorescence spectra of (a) **Rh-TPP** and (c) **Rh-Py** and fluorescence emission enhancement (I/I_0) of (b) **Rh-TPP**(λ_{em} = 577 nm) and (d) **Rh-Py** (λ_{em} = 575 nm) before and after reaction with various ROS in PBS (pH 7.4, 10 mM, containg 0.1% DMSO) (1: free, 2: ·OH,3: $O_2^{\bullet-}$, 4: $ONOO^-$,5: H_2O_2,6: ^{t}BuOOH, 7: ClO^-). [**Rh-TPP**] = [**Rh-Py**] = 5 μM, ClO^-: NaClO (final 50 μM) was added and the mixture was stirred at 20 °C. ·OH: ferrous perchlorate (500 μM) and H_2O_2 (1 mM) were added at room temperature. $O_2^{\bullet-}$: KO_2 was dissovled in the anhydrous DMSO and then the appropriate aliquot was added (final 100 μM). H_2O_2: H_2O_2 (final 100 μM) was added and the mixture was stirred at 20 °C. $ONOO^-$: $ONOO^-$ (final 50 μM) was added and the mixture was stirred at 20 °C. ^{t}BuOOH: ^{t}BuOOH (final 100 μM) was added and the mixture was stirred at 20 °C. BG: background. Each spectrum was recorded at 30 min after reaction.λ_{ex}= 540 nm.

Fluorescence titration experiments demonstrated that the fluorescence of these two rhodamine derivatives could be enhanced apparently upon addition of NaClO (0-100 μM), as depicted in Fig.2, and a linearly proportional increment of emission intensity to the concentration of NaClO in the range of 0-10 μM was disclosed for either probe (Figure S3). The quantum yields of both **Rh-TPP** and **Rh-Py** were as low as 0.01 and reached to 0.29 and 0.51 in the presence of NaClO, respectively. According to the titration profiles, the detection limits (S/N = 3) toward NaClO were calculated to be 1.1×10^{-7} M for **Rh-TPP** and 2.4×10^{-8} M for **Rh-Py**. The effect of pH on the fluorescence response of **Rh-TPP** or **Rh-Py** to NaClO was investigated (Figure S4). The results indicated that both **Rh-TPP** and **Rh-Py** can effectively detect NaClO under neutral and basic conditions. As the pKa of HClO is 7.6, we reasoned that **Rh-TPP** and **Rh-Py** sense ClO^- instead of HClO. In view of the sligtly basic condition in mitochondria, we assumed that **Rh-TPP** and **Rh-Py** should be suitable for the detection of mitochondrial ClO^-.

Subsequently, the time dependent fluorescence changes of **Rh-TPP** and **Rh-Py** with NaClO were investigated (Figure S5). **Rh-TPP** or **Rh-Py** was non-emissive before the addition of NaClO. However, the emission intensities of these two probes increased profoundly and reached a plateau immediately once 10 equiv NaClO was added, indicating that the reaction between **Rh-TPP** or **Rh-Py** and NaClO can be accomplished within a few seconds, which is very important for the real-time imaging. The photostability of **Rh-TPP** and **Rh-Py** in the absence or presence of NaClO was also measured and the results showed that the fluorescence intensities of **Rh-TPP** and **Rh-Py** in the absence or presence of NaClO kept almost steady in 30 min (Figure S6), indicating elegant antioxidant abilities of both probes.

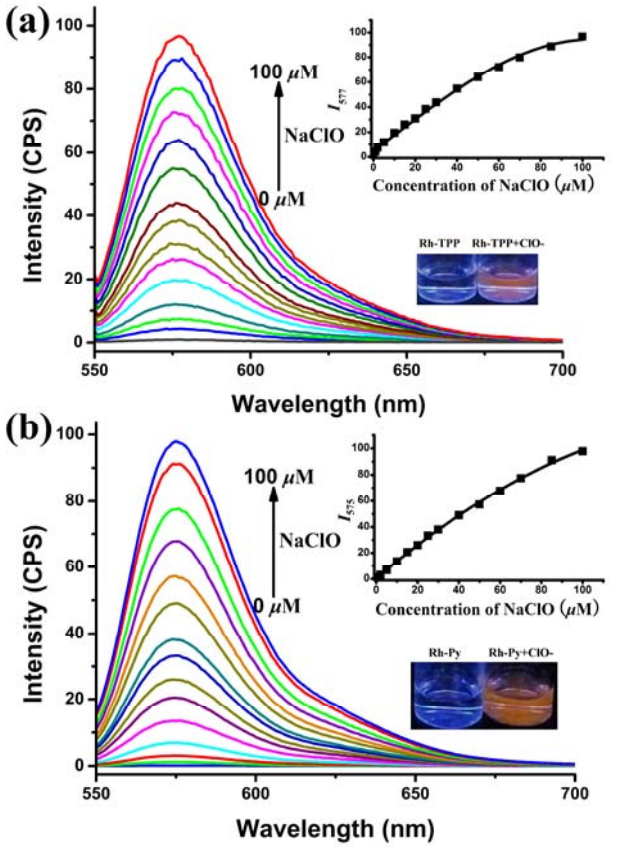


Fig. 2 Fluorescence titration spectra of (a) **Rh-TPP** and (b) **Rh-Py** upon the addition of NaClO (0 - 100 μM) in PBS (pH 7.4, 10 mM, containg 0.1% DMSO). [**Rh-TPP**] = [**Rh-Py**] = 5 μM, λ_{ex} = 540 nm, slit: 3 nm/3 nm. Inset:the titration curve plotted with the fluorescnece intensity of (a) **Rh-TPP**at 577 nm and (b) **Rh-Py**at 575 nm as a function of NaClO concentration, respectively.

To confirm that the reaction mechanism was as same as reported,[7] the reaction solution of **Rh-TPP** or **Rh-Py** with NaClO also underwent ESI-MS analysis (Figure S7-S8). The peak at m/z ~443.2 corresponding to rhodamine B was found in both MS spectra, which demonstrated the mechanism of the oxidization-hydrolysis of benzoyl acetohydrazide. The detailed reaction process was illustrated in Scheme S2.

The desirable fluorescence properties of **Rh-TPP** and **Rh-Py** for ClO^- prompted us to its utility for the detection of intracellular ClO^-. A standard MTT assay showed that cell viability was rarely changed even though 10 μM **Rh-TPP** or **Rh-Py** was added for 24 h (Figure S9). However, the addition of 20 μM **Rh-TPP** led to the death of more than 85% of HeLa cells, while more than 95% of cells still kept alive after 20 μM **Rh-Py** was added, indicating that a pyridinium unit might show lower toxicity than a triphenylphosphonium moiety did. Then, confocal microscopy experiments were carried out on HeLa cells. HeLa cells were incubated with 5 μM **Rh-TPP** or **Rh-Py** for 30 min at 37 °C in PBS. Upon excitation at 543 nm, there was no intracellular orange fluorescence between 555-600 nm (Figure S10 b and f). When 100 μM NaClO was added then incubated for another 10 min, intense fluorescence emerged in **Rh-TPP** or **Rh-Py**-loaded

cells (Figure S10 c and g), showing that **Rh-TPP** and **Rh-Py** are well cell-permeated and can react with intracellular ClO^- rapidly. To further examine the subcellular localization of **Rh-TPP** and **Rh-Py**, commercially available mitochondrial dye, Mito Tracker Green, was employed for a co-localization study. As displayed in Fig. 3, the changes in the intensity profile of linear regions of interest (ROIs) (synthetic probes and MitoTracker Green costaining) tended toward synchronization, rhodamine signals in the **Rh-TPP** or **Rh-Py**-loaded cells overlayed well with the fluorescence of Mito Tracker Green, demonstrating that **Rh-TPP** and **Rh-Py** were site-specifically internalized in mitochondria in living cells.

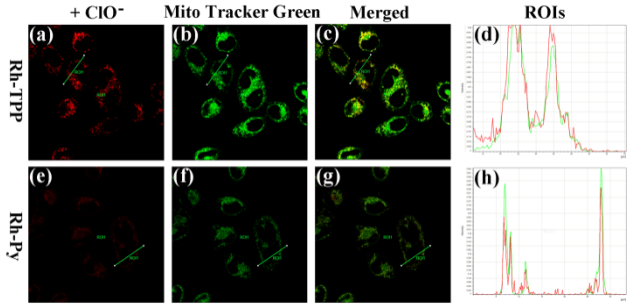


Fig. 3 HeLa cells were stained with **Rh-TPP** or **Rh-Py** for 30 min, and then incubated with NaClO (100 μM) for 10 min; finally, Mito Tracker Green (1 μM) was added to the cells and the cells were incubated for another 20 min. (a) and (e): the fluorescence images of **Rh-TPP** and **Rh-Py**in presence of NaClO; (b) and (f): the fluorescence images of Mito Tracker Green; (c) and (g): merged images;(d) and (h): intensity profile of ROIs across HeLa cells. Red lines represent the intensity of synthetic probes and green lines represent the intensity of Mito Tracker Green. Bars: 25 μm.

We finally evaluated the suitability of the probes for visualizing ClO^- in living animals. Nude mice were selected as our model. The mice were given a skin-pop injection of **Rh-TPP** or **Rh-Py** (50μL, 100 μM in PBS (pH 7.4, 10 mM, containing 0.1% DMSO)) on the right side, and 15 minutes later, themice were given an injection of 10 equiv of NaClO in the same region. The mice were then imaged at different time. As shown in Fig. 4, both of **Rh-TPP** and **Rh-Py** could respond to the NaClO injected into the mice, and the fluorescence intensity hardly changed after 20 min, proving that **Rh-TPP** and **Rh-Py** can detect NaClO *in vivo* without the interference of background signals.

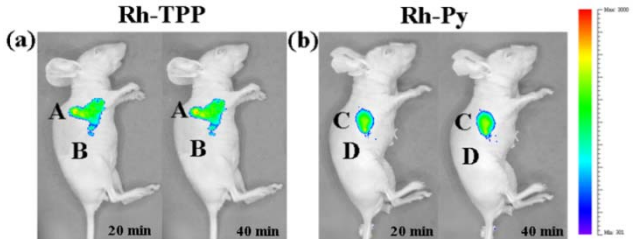


Fig. 4 Representative fluorescence images (pseudocolor) of nudemice given a skin-pop injection of (a) **Rh-TPP** and (b) **Rh-Py** (50 μL, 100 μM in PBS (pH 7.4, 10 mM, containing 0.1% DMSO), region A and C, respectively) and a subsequent skin-pop injection of NaClO (50 μL, 1mM in PBS (pH 7.4, 10 mM)). Representative fluorescence images (pseudocolor) of nude mice given a skin-pop injection of (a) **Rh-TPP** and (b)**Rh-Py**(50 μL, 100 μM in PBS (pH 7.4, 10 mM, containing 0.1% DMSO), region B and D, respectively). Images were taken after incubation for 20, and 40 min, respectively. Images were taken using anexcitation laser of 534 nm and an emission filter of 586 ± 20 nm.

Conclusions

In summary, two colorimetric and fluorescent probes **Rh-TPP** and **Rh-Py** for ClO^- were prepared based on the oxidization-hydrolysis of benzoyl acetohydrazide. Both of the probes exhibited high sensitivity and excellent selectivity toward ClO^- among ROS and cations with a rapid response time. The imaging of intracellular ClO^- by utilizing these two probes was also examined and it was demonstrated that **Rh-TPP** and **Rh-Py** could detect ClO^- in mitochondria with the help of a triphenylphosphonium (TPP) and pyridinium unit, respectively. Ultimately, **Rh-TPP** and **Rh-Py** were applied in the *in vivo* imaging of ClO^- in living mice. We anticipate that these two compounds can be utilized in a variety of chemical and biological applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Synrhesis and characterization of the probes; Absorption spectra and the time dependent fluorescence of probes with hypochlorite]. See DOI: 10.1039/b000000x/

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