



# A ratiometric fluorescent probe for detecting hypochlorite in the endoplasmic reticulum†

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**A colorimetric and fluorescent probe ER-CIO was presented in this work to detect cellular hypochlorite with high selectivity and sensitivity. With an organelle targeting unit, ER-CIO was successfully applied in the bio-imaging of exogenous and endogenous hypochlorite in the endoplasmic reticulum in a ratiometric manner.**

The endoplasmic reticulum (ER), a membrane-rich structure found in all eukaryotes, is the largest cellular organelle. It is responsible for the synthesis, folding, modification, and delivery of proteins,<sup>1</sup> and most of the intracellular  $\text{Ca}^{2+}$  is stored in the ER.<sup>2</sup> The dysfunction of the ER, usually called ER stress, induces an unfolded protein response (UPR) in the ER,<sup>3</sup> and mediates complicated signaling pathways. Increased ER stress can even be associated with heart disease, stroke, neurodegenerative disorders, and cancer.<sup>4</sup> Hence, the ER has recently been recognized as an important target for cancer therapy.<sup>5</sup> Among the factors initiating ER stress, reactive oxygen species (ROS), including  $\cdot\text{OH}$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{ONOO}^-$ ,  $\text{H}_2\text{O}_2$ ,  $\text{ClO}^-$ , and  $^1\text{O}_2$ , have been identified to play vital roles in the UPR owing to their strong oxidability.<sup>6</sup> However, the exact association between ROS and ER activity has not been sufficiently revealed yet because of the lack of reliable techniques. Therefore, it is of extraordinary value to exploit novel tools to monitor ROS variation in the ER

for understanding the redox circumstance in the ER, thus assessing the ER status.

Nowadays, fluorometric analysis has attracted worldwide attention in bioimaging for its high sensitivity, excellent temporal and spatial resolution, and non-invasive imaging ability.<sup>7</sup> Several fluorescent probes have been reported to detect ROS variations in the ER, such as  $\text{O}_2^{\cdot-}$ ,<sup>8</sup>  $\text{H}_2\text{O}_2$ ,<sup>9</sup> and  $^1\text{O}_2$ .<sup>10</sup> These excellent works uncovered the ROS variation in certain ER processes. For instance,  $\text{O}_2^{\cdot-}$  was found to be generated in the ER during cisplatin-induced cell apoptosis.<sup>8</sup>  $\text{ClO}^-$  is one of the highly reactive oxygen species (hROS), and is mainly formed from hydrogen peroxide and chloride ions in a myeloperoxidase (MPO)-catalyzed process in living organisms.<sup>11a</sup> The physiological concentration of  $\text{ClO}^-$  could reach up to 200  $\mu\text{M}$ .<sup>11b</sup>  $\text{ClO}^-$  is involved in the maintenance of intracellular redox balance, and an abnormal intracellular  $\text{ClO}^-$  level is tightly relevant to many diseases, such as rheumatoid arthritis and cancers.<sup>12</sup> To this end, numerous fluorescent probes for intracellular  $\text{ClO}^-$  have been presented, including organelle-localized  $\text{ClO}^-$ .<sup>13</sup> However, these probes for organelle-localized  $\text{ClO}^-$  were mainly focused on detection of mitochondrial  $\text{ClO}^-$ , and the reports on ER-targeted  $\text{ClO}^-$  imaging are still quite limited, mainly owing to the shortage of dependable detection tools.<sup>14</sup> Additionally, the association between  $\text{ClO}^-$  variation and ER functions remains far from being understood, indicating the significance of devising new fluorescent probes for monitoring  $\text{ClO}^-$  variation in the ER region.

In the last few years, we have developed a series of fluorescent probes for mitochondrial  $\text{ClO}^-$  based on the oxidative hydrolysis of dibenzoylhydrazine.<sup>15</sup> The dibenzoylhydrazine moiety displayed rapid and sensitive response towards  $\text{ClO}^-$ , while  $\text{ONOO}^-$ , another hROS, might also react with this unit to produce unwanted signal interference.<sup>15c</sup> Therefore, more selective response sites should be considered when we set out to devise an ER-targeted fluorescent probe for  $\text{ClO}^-$  detection. In 2011, utilizing a  $\text{ClO}^-$ -triggered C=N cleavage reaction, the Lin group developed a probe for  $\text{ClO}^-$  using a diaminomaleonitrile-derived Schiff base as a reaction site,<sup>16</sup> and this was also applied in the design

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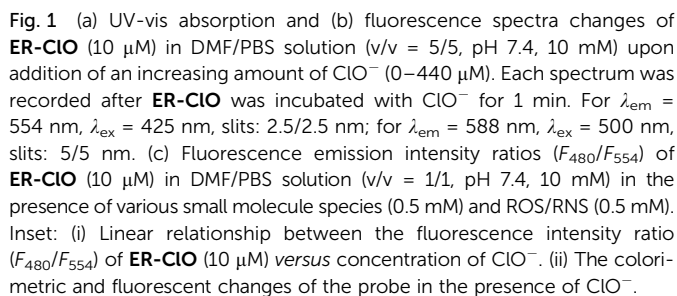
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After the demonstration of the optical response to  $\text{ClO}^-$ , the selectivity of **ER-CIO** toward  $\text{ClO}^-$  was checked. To the solution of 10  $\mu\text{M}$  **ER-CIO**, various common anions ( $\text{F}^-$ ,  $\text{HS}^-$ ,  $\text{HSO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{HPO}_4^{2-}$ ,  $\text{AcO}^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{I}^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{HCO}_3^-$ ), cations ( $\text{Al}^{3+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{3+}$ ), biothiols (GSH, Hcy, Cys), ROS ( $\bullet\text{OH}$ , *tert*-butyl hydroperoxide (TBHP), *tert*-butoxyl radical (TBO $\bullet$ ),  $\text{O}_2^-$ ,  $^1\text{O}_2$ ,  $\text{ONOO}^-$ ,  $\text{H}_2\text{O}_2$ , and  $\text{ClO}^-$ ), and NO was added respectively. As depicted in Fig. S2 (ESI $^\dagger$ ), no significant changes were observed in the absorption spectra when other tested species were added except  $\text{Hg}^{2+}$  and  $\text{ONOO}^-$ , which could induce a moderate decrement in the absorption band at 488 nm. On the other hand, the original absorbance at 488 nm thoroughly disappeared upon the addition of  $\text{ClO}^-$  and a new band at 425 nm was found, accompanied by a visible



To validate the reaction mechanism between **ER-ClO** and  $\text{ClO}^-$ , the mass analysis was performed. As seen in Fig. S5 (ESI<sup>†</sup>), after reaction with  $\text{ClO}^-$ , a strong peak at  $m/z$  534.1211 was found in the mass spectrum, which corresponded to the carboxylic product **5** ( $[\text{5} + \text{Na}]^+$ : 534.1418). In the previous reports,<sup>12a,16,17</sup>



Scheme 2 Proposed reaction mechanism of between **ER-CIO** and  $\text{ClO}^-$ .

diaminomaleonitrile-derived Schiff base reacted with  $\text{ClO}^-$  to form the corresponding aldehyde, thus resulting in the fluorescence changes. However, in this case, **ER-CIO** was not converted to coumarin aldehyde by  $\text{ClO}^-$ , probably owing to the different reactivities of diaminomaleonitrile-derived Schiff bases attached to different fluorophores. Accordingly, the reaction mechanism between **ER-CIO** and  $\text{ClO}^-$  was proposed in Scheme 2.

The desirable fluorescence properties of **ER-CIO** for  $\text{ClO}^-$  prompted us to exploit its utility for intracellular  $\text{ClO}^-$  detection. The cytotoxicity of the probe was first examined. As shown in Fig. S6 (ESI<sup>†</sup>), more than 80% of the cells stay alive even after incubation with 20  $\mu\text{M}$  **ER-CIO** for 24 h, indicating outstanding biocompatibility of the probe. Then, confocal fluorescent imaging tests were carried out on HeLa cells.

Cultured with 10  $\mu\text{M}$  **ER-CIO** for 30 min at 37 °C in PBS, an apparent fluorescence originating from the probe was observed in the red channel, while it was dim in the blue channel, suggesting the good cell membrane penetrability of the probe (Fig. 2). Upon the addition of exogenous  $\text{ClO}^-$ , the red fluorescence faded, and the blue emission gradually generated. The ratio of blue fluorescence and red fluorescence rose with an increasing



Fig. 2 Confocal fluorescence images of **ER-CIO** in HeLa cells incubated with different concentrations of  $\text{ClO}^-$ . HeLa cells were incubated with **ER-CIO** (10  $\mu\text{M}$ ) at 37 °C for 30 min, and then further treated with different amounts of  $\text{ClO}^-$  for 30 min. Fluorescence images of HeLa cells from the blue channel ( $\lambda_{\text{ex}} = 403 \text{ nm}$ ,  $\lambda_{\text{em}} = 425\text{--}475 \text{ nm}$ ) and red channel ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 552\text{--}637 \text{ nm}$ ). The ratiometric images ( $F_{\text{blue}}/F_{\text{red}}$ ) were obtained by mediating the yellow channel image with the related red channel image. Scale bar: 20  $\mu\text{m}$ .



Fig. 3 Confocal fluorescence images of three cell lines stained with (a, e and i) 0.5  $\mu\text{M}$  ERTB (blue channel:  $\lambda_{\text{ex}} = 403 \text{ nm}$ ,  $\lambda_{\text{em}} = 425\text{--}475 \text{ nm}$ ) and (b, f and j) 10  $\mu\text{M}$  **ER-CIO** (red channel:  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 552\text{--}637 \text{ nm}$ ) at 37 °C for 30 min. (c, g and k) Overlay images. (d, h and l) Intensity profiles of regions of interest (ROI) across cells. Scale bar: 10  $\mu\text{m}$ .

amount of  $\text{ClO}^-$ , signifying that **ER-CIO** could image intracellular  $\text{ClO}^-$  in a ratiometric manner.

Thereafter, **ER-CIO** was co-cultured with commercial ER trackers to identify its organelle localization in three cell lines (L929, HeLa and SMMC-7721). As illustrated in Fig. 3, the fluorescence of **ER-CIO** was well matched with that of ER-Tracker Blue-White DPX (ERTB) with Pearson's coefficients of 0.93, 0.96, and 0.95 in the three cell lines, respectively, strongly confirming the primary accumulation of the probe in the ER with certain cellular universality. Moreover, the blue fluorescence of the probe generated after the reaction with  $\text{ClO}^-$  was also demonstrated to root in the ER (Fig. S7, ESI<sup>†</sup>) using ER-Tracker Red (ERTR) as a control. Additionally, the co-staining experiments of the probe with other commercial organelle dyes, *i.e.* LysoBrite Blue 22642 (LB) for lysosomes and MitoLite Blue FX490 (MB), were performed in L929 cells. As illustrated in Fig. S8 (ESI<sup>†</sup>), the fluorescence of **ER-CIO** was partially overlapped with that of LTB and MTB with Pearson's coefficients as 0.54 and 0.49, respectively. Combined together, **ER-CIO** possessed prominent ER targetability, which was the cornerstone for detecting the  $\text{ClO}^-$  in the ER.

Ultimately, **ER-CIO** was utilized to sense endogenous  $\text{ClO}^-$  in HeLa cells. HeLa cells were pretreated with phorbol 12-myristate 13-acetate (PMA, a ROS stimulant) for 30 min, and then treated with **ER-CIO** for another 30 min. As seen in Fig. 4, compared with the cells treated with the probe only, moderate fluorescence appeared from the blue channel in PMA-stimulated cells with a remarkable ratio change. This result indicated that **ER-CIO** was successfully applied in monitoring the fluctuation of endogenous  $\text{ClO}^-$  in the ER.

In short, we presented a colorimetric and fluorescent probe **ER-CIO** for the detection of ER  $\text{ClO}^-$  in a ratiometric manner. This probe rapidly responded to  $\text{ClO}^-$  with good sensitivity and excellent selectivity. By virtue of the methyl sulfonamide group, the probe was able to accumulate in the ER and was





Fig. 4 Confocal fluorescence images of endogenous  $\text{ClO}^-$  by ER-CIO. HeLa cells were incubated with or without PMA at 37 °C for 30 min, and then further treated with ER-CIO (10  $\mu\text{M}$ ) for another 30 min. For the blue channel:  $\lambda_{\text{ex}} = 403 \text{ nm}$ ,  $\lambda_{\text{em}} = 425\text{--}475 \text{ nm}$ ; for the red channel:  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 552\text{--}637 \text{ nm}$ . The ratiometric images ( $F_{\text{blue}}/F_{\text{red}}$ ) were obtained by merging the yellow channel image with the related red channel image. Scale bar: 20  $\mu\text{m}$ .

successfully applied in the fluorescence imaging of the exogenous and endogenous  $\text{ClO}^-$  in the ER. Owing to its desirable properties, ER-CIO could be used as an efficient tool in the bioanalysis of disease-associated  $\text{ClO}^-$  variation in the ER, which could be useful in various biological and clinical applications, and further research is ongoing.

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## Conflicts of interest

There are no conflicts to declare.

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