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A colorimetric and ratiometric fluorescent probe with Meldrum’s acid as the recognition group for in vitro and in vivo imaging of hypochlorite

Lili Yang\textsuperscript{a,b}, Songsong Ruan\textsuperscript{a,b}, Anqi Zhang\textsuperscript{a,b}, Mengyan Hu\textsuperscript{a}, Jie Zhang\textsuperscript{c}, Kangjia Sheng\textsuperscript{a,b}, Jing Tian\textsuperscript{a}, Yongmin Zhang\textsuperscript{a,b,c}, Shaoping Wu\textsuperscript{a,b}, Jianli Li\textsuperscript{d}

\textsuperscript{a}School of Pharmacy; Key Laboratory of Resource Biology and Biotechnology in Western China (Northwest University), Ministry of Education; Biomedicine Key Laboratory of Shaanxi Province, Northwest University, Xi’an 710069, China.
\textsuperscript{b}Joint International Laboratory of Glycobiology and Medicinal Chemistry, Northwest University, Xi’an, Shaanxi 710069, China.
\textsuperscript{c}Hanbin District First Hospital, Ankang city, Shaanxi province 725000, China.
\textsuperscript{d}Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, College of Chemistry & Materials Science, Northwest University, Xi’an, Shaanxi 710127, P. R. China.
\textsuperscript{e}Sorbonne Université, CNRS, Institut Parisien de Chimie Moléculaire, UMR 8232, 75005 Paris, France.

* Tel.: +86 029 88304569; Fax: +86 029 88304569. E_mail: wushaoping@nwu.edu.cn

**Keywords:** Fluorescent probe, Ratiometric, Hypochlorite, Meldrum’s acid, Fast response, Bioimaging

**Abstract:**

The detection of ClO$^-$ is vital to comprehend the accurate effect of ClO$^-$ in various diseases and therapeutic interventions by fluorescent probe method. Herein, a special ratiometric probe DDD was designed and efficiently synthesized via a simple process for hypochlorite detection. The selectivity and sensitivity of probe DDD was excellent and the detection was not influenced by other reactive oxygen species. Probe DDD exhibited a fast response (< 1 s), wide Stokes Shift (> 100 nm), sensitive limit of detection (78 nM) and two well-separated emission (555 nm and 635 nm). The emission intensities ratio ($I_{555}/I_{635}$) was linearly enhanced ($I_{555}/I_{635} = 0.6273X - 1.9313$) with the ClO$^-$ concentration range from 4.0 to 20.0 μM. Furthermore, probe DDD has been successfully evaluated in monitoring exogenous HClO in SH-SY5Y neuroblastoma cells and zebrafish larvae with low cytotoxicity, good cell permeability and biocompatibility.
1. Introduction

Reactive Oxygen (ROS) are vital small signaling molecules that intercede many biological events in a serial of physiological processes, such as aging, autoimmunity, cancer, chronic inflammatory diseases and neurodegenerative disorders \([1-5]\). Hypochlorous acid, an important biologically important weakly acidic ROS which, is partly exists in the form of ClO\(^-\) in physiological environment \([6]\). In living organisms, HOCl is produced mainly by an oxidation reaction between H\(_2\)O\(_2\) and Cl\(^-\) using the catalysis of myeloperoxidase (MPO) within phagosomes \([7,8]\), and centrally linked to the immune defense systems \([9]\). However, uncontrolled production of HOCl could lead serious tissue damage and various kinds diseases, for instance hepatic injury \([10]\), atherosclerosis \([11]\), lung injury \([12]\), rheumatoid \([13,14]\), cardiovascular diseases \([15]\), neuron degeneration \([16]\), arthritis \([17]\), and cancer \([18,19]\). Therefore, the exploitation of reliable and accurate analytical tool for detecting HOCl is vital to illuminate its physiological functions in living systems.

Nowadays, detecting HClO in living systems has attracted extensive interest, especially taking advantage of on the fluorescence confocal imaging techniques because of its high sensitivity, selectivity, rapid response speed and ease of manipulation. Recently, many fluorescent probes have been constructed to detect HOCl in living systems \([20]\). Although some turn-off fluorescence probes were designed for the exploration and cell imaging of ClO\(^-\) based on rhodamine \([21,22,23]\), coumarin \([24,25]\), BODIPY \([26]\) and cyanine \([27]\), such probe applications have some defect due to single wavelength emission and the external environmental influence.

One of the main limitations of single-wavelength emission fluorescent probes is the affect of the signal detection along with probe solution concentration, probe external environment and excitation intensity. However, ratiometric fluorescent probes could effectively assuage these shortcoming \([28]\), it could permit the measurement of fluorescence emission/excitation intensities at two wavelengths, the ratios of emission/excitation intensities would be independent of the external environmental influence \([29]\). Therefore, it is still great challenge to design and
synthesize novel fluorescent probes that could detect to HOCl in ratiometric type and real time image of the living biosystems.

2,2-Dimethyl-1,3-dioxane-4,6-dione, namely Meldrum’s acid, has proven its utility and multifunction in organic chemistry, which is applied in the numerous synthetic methodologies because of very multiple properties and unusual facets of reactivity [30]. To the best of our knowledge, there has been no study on the applications of Meldrum’s acid as the fluorescence probe recognition group. On the basis of our previous work [31], a novel colorimetric and ratiometric probe 5-((7-hydroxy-4,5-dihydronaphtho[1,2-b]thiophen-2-yl)methylene)-2,2-dimethyl-1,3-dioxane-4,6-dione(acronym: DDD) was reasonable designed and concise synthesized to detect hypochlorite in living cells and zebrafish larvae based on a naphthalenone scaffold and Meldrum’s acid as the specially recognition group. Additionally, a proposed recognition mechanism based on addition-ring-opening-elimination reaction was confirmed. Furthermore, probe DDD has a fast respond time to hypochlorite over a wide pH range, showing high selectivity over other ROS with excellent stability and good biocompatibility (Scheme 1).

![Scheme 1. Strategies to response of probe DDD.](image)

2. Materials and methods

2.1. Materials and instrumentation

All reagents with analytical grades were obtained from J&K Scientific Ltd. (Shanghai China). Hypochlorite stock solution was prepared from the 4 wt% industrial NaClO solution. Stock solutions (1.0 mM) of NO•, S2O32–, ClO4–, ONOO–, IO4–, NO2–, NO3–, F–, S2–, CN–, HSO3–, SO32–, Mg2+, Ca2+, N2H4•H2O, H2O2, Cys, GSH were prepared by proper amounts of salts. The E3 media was made from
NaCl, KCl, MgSO₄ and CaCl₂. The fluorescence quantum yield of probe DDD in different organic solvents was measured at 25°C.

The UV-vis spectra were measured on a Shimadzu UV-2550 and the fluorescence spectra were performed using a Hitachi F-7000 spectrophotometer. The cell imaging was collected by the Olympus FV1000 laser confocal fluorescence microscope. The zebrafish imaging was recorded by the Nikon SMZ25 laser confocal fluorescence microscope (Nikon Corporation, Tokyo, Japan).

2.2. Synthesis of compound 5-((7-hydroxy-4,5-dihydronaphtho[1,2-b]thiophen-2-yl) methylene)-2,2-dimethyl-1,3-dioxane-4,6-dione (DDD)

Compound 1 (50.0 mg, 0.22 mmol, 1.0 equiv.) was dissolved in boiling CH₃CN (5 mL), Meldrum’s acid (34.5 mg, 0.24 mmol, 1.1 equiv.) was added with 0.1 mL Et₃N. The mixture was refluxed at 85 °C for 2 h, then cooled down to 25 °C and stirred for 12 h. The formed precipitate was filtered with neutral filter paper and purified by recrystallization (Yield: 78.8%, Rf = 0.45, PE: EtOAc = 3:1). m.p. > 300 °C

1H NMR (600 MHz, DMSO-d₆) δ (ppm): 10.10 (s, 1H), 8.52 (s, 1H), 8.02 (s, 1H), 7.40 (t, J=3.8 Hz, 1H), 6.74 (d, J=4.1 Hz, 2H), 2.85 (d, J=3.6 Hz, 2H), 2.80 (d, J=3.9 Hz, 2H), 1.70 (s, 6H).

13C NMR (151 MHz, DMSO-d₆) δ (ppm): 163.05, 160.72, 159.75, 154.17, 147.98, 147.37, 147.98, 147.37, 139.08, 136.81, 131.94, 125.86, 121.19, 104.68, 102.96, 28.22, 26.83, 26.38, 22.46. FT-IR (KBr) ν: 3290 (Ar-OH), 2927 (C-H), 1735 (C=O), 1683 (C=O), 1573, 1421(C-H), 1270, 1150, 1023, 823 (Ar C-H) cm⁻¹. HRMS (C₁₉H₁₆O₅S): calcd. for [M-H]⁻ 355.0646; found: [M-H]⁻ 355.0648. (Fig. S6-S9).

2.3. Titration experiments

1.0 mM ClO⁻ standard solution was prepared from 84.5 μL of 4% NaClO solution in 50 mL of ultrapure water for titrations experiments. Probe DDD (8.9 mg) was dissolved in 25 mL of absolute ethanol to prepare probe stock solution (1.0 mM). 50 μL of the DDD stock solution was diluted to 5 mL (20% EtOH, 10 mM PBS buffer, pH = 7.4) in a glass tube. The other analyte (Cys, GSH, NO•, S₂O₅²⁻, ClO₄⁻, ONOO⁻, IO₄⁻, NO₂⁻, NO₃⁻, F⁻, S²⁻, CN⁻, HSO₃⁻, SO₃²⁻, Mg²⁺, Ca²⁺, N₂H₄•H₂O, H₂O₂)
was dissolved in water and added to DDD stock solution (50 µL) under the same condition. The fluorescence method was used to record the spectra of these solutions.

2.4. Cytotoxicity experiments

The cytotoxicity of probe DDD was detected by cell counting kit-8 (CCK-8). SH-SY5Y neuroblastoma cells were came from American Type Culture Collection. Cells were added to 96-well plates with a density of 1×10^5 cells per well and cultured in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in an incubator (37 °C, 5% CO2) for 24 h. The different concentrations of probe DDD (0, 5, 10, 15, 20 and 25 µM) were incubated with neuroblastoma cells for 24 h, and then 10 µL CCK-8 was put into each hole of the 96-well plate for 1 h at 37 °C. Finally, we used a microplate reader to measure the absorbance at 450 nm.

2.5. Living Cell imaging

For cell imaging experiments, SH-SY5Y neuroblastoma cells were placed on the laser confocal culture dishes and incubated for 24 h. SH-SY5Y neuroblastoma cells were incubated with probe DDD (10 µM) at 36 °C for 10.0 min, washed with PBS buffer solution to remove excess DDD, and then the different concentrations of ClO⁻ solution (0, 5, 10, 15, 20, 25 µM, respectively) was added for another 10.0 min. the laser scanning confocal microscope was used to visualize cell.

2.6. Zebrafish larvae imaging

Zebrafishes were provided by institute of Hydrobiology (China Zebrafish Resource Center, Chinese Academy of Sciences). To image the exogenous ClO⁻, zebrafish larvae (three-days) were put into a 24 well cell culture cluster and incubated in E3 media containing 3 µM probe DDD at 28 °C for 15 min, the residual probe was washed by E3 media three times, then treated with different concentrations of ClO⁻ solution (0, 3, 5, 10 µM).

3. Results and discussion

3.1. Design and synthesis of fluorogenic substrates DDD
The synthesis of probe DDD was simply achieved in one facile condensation as displayed in Scheme 2. Treatment of 6-methoxy-1-tetralone with POCl₃ in anhydrous DMF at 90 °C gave the intermediate product. The intermediate product reacted with chloroacetaldehyde, Na₂S, K₂CO₃ in anhydrous DMF to give the aldehyde 1' [32,33]. Compound 1 (see Scheme S1) was obtained by demethylation of aldehyde 1' with BBr₃ [34], then reacted with Meldrum’s acid using Et₃N as catalyst in anhydrous CH₃CN to obtain probe DDD [35].

Scheme 2. Reagent and conditions: a. Meldrum’s acid, Et₃N, CH₃CN, 78.8%.

3.2 Photophysical properties study

The emission characteristics of probe DDD were studied in EtOH-PBS solution (pH 7.4). Then the spectroscopic propertie of DDD was evaluated in various polarity solvents including EtOH, MeOH, DMF, DMSO. On the other hand, the fluorescence quantum yield of DDD-CIO⁻ adduct in different organic solvents was measured at 25 °C using the 0.1 M fluorescein (Φ₁ = 0.79) as a standard. The calculated fluorescence quantum yield for DDD-CIO⁻ adduct in different organic solvents was depicted in Table 1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>λ_{abs} (nm)</th>
<th>ε_{max} (M⁻¹cm⁻¹)</th>
<th>λ_{em} (nm)</th>
<th>Φ_{F,X}</th>
<th>Stokes shift (nm)</th>
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</thead>
<tbody>
<tr>
<td>DMF</td>
<td>487</td>
<td>25600</td>
<td>565</td>
<td>0.41</td>
<td>125</td>
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<tr>
<td>DMS</td>
<td>477</td>
<td>32000</td>
<td>563</td>
<td>0.56</td>
<td>123</td>
</tr>
<tr>
<td>MeOH</td>
<td>499</td>
<td>35200</td>
<td>561</td>
<td>0.57</td>
<td>121</td>
</tr>
<tr>
<td>THF</td>
<td>483</td>
<td>45700</td>
<td>561</td>
<td>0.78</td>
<td>121</td>
</tr>
<tr>
<td>EtOH</td>
<td>499</td>
<td>30500</td>
<td>556</td>
<td>0.75</td>
<td>116</td>
</tr>
<tr>
<td>EtOAc</td>
<td>478</td>
<td>67700</td>
<td>544</td>
<td>0.12</td>
<td>104</td>
</tr>
<tr>
<td>DCM</td>
<td>482</td>
<td>60800</td>
<td>544</td>
<td>0.21</td>
<td>104</td>
</tr>
<tr>
<td>H₂O</td>
<td>424</td>
<td>27400</td>
<td>542</td>
<td>0.37</td>
<td>102</td>
</tr>
<tr>
<td>TCM</td>
<td>483</td>
<td>74900</td>
<td>540</td>
<td>0.23</td>
<td>100</td>
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<tr>
<td>ACN</td>
<td>473</td>
<td>21500</td>
<td>530</td>
<td>0.70</td>
<td>90</td>
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<tr>
<td>DMSO</td>
<td>492</td>
<td>20100</td>
<td>520</td>
<td>0.48</td>
<td>80</td>
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</tbody>
</table>
UV-vis and emission spectra of probe DDD in the absence and presence of hypochlorite are shown in Fig.1. Probe DDD (10 μM) alone showed an absorption band with a peak at 495 nm in EtOH-PBS solution (pH 7.4) at 25 °C with the unadjusted inner filter effect [36]. With the addition of hypochlorite (20 μM), a red shift of 80 nm was observed, a new band centered at 575 nm (Fig. 1a). As shown in Fig. 1b, the free probe DDD exhibited two emission peaks at 555 nm and 635 nm when excited at 440 nm. Stimulation of probe DDD (10 μM) with hypochlorite (20 μM) generated a strong fluorescence peak at 555 nm and accompanied by the peak decrease at 635 nm.

Fig. 1 UV-vis absorption (a) and emission spectra (b) of probe DDD (10 μM) in the absence and presence of ClO⁻ (20 μM) with 20% EtOH solution (10 mM PBS buffer, pH 7.4). λex= 440 nm, slits: 5.0 nm/5.0 nm, volt: 700 v.

3.3. UV-vis and fluorescence spectrum of probe DDD react with hypochlorite

The UV-vis spectra of probe DDD (10 μM) upon titration with hypochlorite were measured in EtOH-PBS solution (pH 7.4) (Fig. 2) for further insight into the interaction between probe DDD and hypochlorite. When hypochlorite was continuously added in probe DDD solution, the absorbance value at 495 nm decreased sharply and the peak at 575 nm increased significantly, which induced a colour change of the test solution from rose red to purple under simulated daylight irradiation (Fig. 3a). UV-visible titration experiments between the ratios of absorbance value at 575 nm and 495 nm (A575 nm/A495 nm) and the concentration of hypochlorite anions showed high coefficient (R² = 0.9915) in 0 ~ 12 μM with good linearity. These data revealed that probe DDD could be used for colorimetric detection hypochlorite under visible light.
Fig. 2 a) UV-vis spectra of probe DDD (10 μM) in the presence of various concentrations of ClO⁻ (0-12 μM) with 20% EtOH solution (10 mM PBS buffer, pH 7.4). Each spectrum was recorded after 1 min at 25 °C; b) Plot of absorbance ratios of probe DDD as a function of ClO⁻ concentration at 575 nm and 495 nm (A_{575nm}/A_{495nm}).

Fig. 3 Image of color change of probe DDD (10 μM) after the addition of different ions (10 μM) in 20% EtOH solution (10 mM PBS buffer, pH 7.4) at 25 °C. (a) Color of DDD in the absence/presence of ClO⁻ under daylight lamp. (b) Fluorescence images of DDD in the absence/presence of ClO⁻ under fluorescent lamp.

Next, the fluorescence changes of hypochlorite titration with probe DDD were investigated in EtOH-PBS solution (pH 7.4). Fig. 4 illustrated variations of fluorescence spectra of probe DDD upon addition of different concentrations hypochlorite. As we can see from Fig. 4a, the emission maximum of probe DDD underwent a blue-shift from 635 to 555 nm upon an increasing amount of hypochlorite. The intensity ratios of fluorescence at 555 nm and 635 nm (I_{555nm}/I_{635nm}) as a function of the concentration of hypochlorite increased linearly in the scope of 4 ~ 20 μM hypochlorite anions (R² = 0.9911) (Fig. 4b). Additionally, a color of the test solution change from lavender to blue was observed under fluorescent lamp. The minimum detection limit of a signal-to-noise ratio was then calculated to be 78 nM from eleven blank solutions (Table S2). These outcomes illustrated that probe DDD have excellent potential for qualitative and quantitative analysis of hypochlorite level.
along with luxuriously sensitivity. Compared with UV-vis method, the fluorescence method of the detection hypochlorite has a wider linear range and lower detection limit.

**Fig. 4** a) Fluorescence spectra of probe **DDD** (10 μM) in the presence of various concentrations of ClO⁻ (0-20 μM) in 20% EtOH solution (10 mM PBS buffer, pH 7.4) at 25 °C; b). Plot of emission ratios at 555 nm and 635 nm (I$_{555 \text{ nm}}$/I$_{635 \text{ nm}}$) of probe **DDD** as a function of ClO⁻ concentration. λ$_{ex}$ = 440 nm, slits: 5.0 nm/5.0 nm, volt: 700 v.

3.4. Selectivity experiment

The selectivity of probe **DDD** toward ClO⁻ was evaluated by investigating a variety of biological relevant species, including ROS, RSS, cations and anions. As diagrammed in **Fig. 5a**, when ClO⁻ was added, the fluorescence intensity of probe **DDD** at 555 nm significantly increased. On the other hand, the fluorescence intensity exhibited no obvious change upon addition of other analytes at considerable concentrations.

As shown in **Fig. 5b**, the anti-interference ability of **DDD** was examined to other analytes. The detection of ClO⁻ did not interfere under all the competing analytes, these analytical results indicated that probe **DDD** has excellent selectivity for ClO⁻ over a various interfering species that can be exist in complex biological systems.
3.5. Time influences and pH-dependent

We also investigated the fluorescence intensity alterations of probe DDD toward hypochlorite with a time-dependent by kinetic study. As showed in Fig. 6a, the intensity ratios of fluorescence at 555 nm and 635 nm (I_{555nm}/I_{635nm}) increased significantly and reached a plateau within one second. Additionally, the fluorescence intensity of DDD-ClO\(^{-}\) adduct almost remained constant within 30 min, indicating that probe DDD was a “fast response” fluorescent probe for real time monitoring hypochlorite levels in vivo and in vitro.

On the other hand, The pH tolerance of the free probe DDD and DDD-ClO\(^{-}\) adduct was researched by adding PBS buffers, which were prepared in the pH range from 3.0 to 14.0 in 20% EtOH mixed solution (Fig. 6b). The notable changes fluorescence intensity of the free probe DDD could not cause in the pH range from 3.0 to 10.0. At the same time, Probe DDD itself was very stable in the pH range of 3.0 to 10.0. When hypochlorite was added in the DDD solution, fluorescence intensity increased significantly in the pH range from 7.0 to 13.0. These results demonstrated that probe DDD could tolerate a wide range of pH change and could be used in biological systems.
3.6 Recognition mechanism study

The reaction of DDD with hypochlorite of the mechanism was shown in Scheme 3. ClO$^-\$ as a hard nucleophile attack to carbon-carbon double bond and the addition product 3 is observed. Next water reacted as nucleophilic reagent on the carbonyl to give intermediate 4 which, after proton transfer and hydrolysis, gave malonic acid derivative 5. To support our speculation, the reaction of DDD (50 μM) and ClO$^-\$ (100 μM) was carried out in MeOH solution at 0 °C for 3 min, after that the reaction mixture was diluted appropriately and checked to high resolution mass spectroscopy, where two anticipated molecular ion peaks at m/z 317.0760 and 355.0630, representing malonic acid derivative 5 (C$_{16}$H$_{14}$O$_5$S, Exact Mass: [M-H]$^-\$ 317.0484) and DDD (C$_{19}$H$_{16}$O$_5$S, [M-H]$^-\$ 355.0640) were obtained, respectively (Fig. S10).

![Scheme 3. Proposed mechanism for the reaction of probe DDD with ClO$^-\$.](image-url)
3.7. Cytotoxicity of living cells and fluorescence imaging

CCK-8 method was used to evaluate the cytotoxicity of probe DDD. As displayed in Fig. S11, the cell viability was up to 95% when 10 μM probes were incubated with the SH-SY5Y neuroblastoma cells for 24 h. These cytotoxicity results showed that probe DDD possess low cytotoxicity and excellent biocompatibility at range of 0 ~ 25 μM in living cells.

On the basis of these above experiments, the imaging exogenous ClO$^-$ of probe DDD in living cells was explored (Fig. 7). After the SH-SY5Y neuroblastoma cells were mixed with probe DDD (10 μM) for 10 min, the bright red fluorescence and weak green fluorescence was observed in living cells. Then, an apparent increase was checked in green channel fluorescence when cells treated with various concentration of ClO$^-$ (10 μM, 20 μM and 30 μM) for 10 min, but the red channel fluorescence was made a dramatic decline. The phenomenon displayed that probe DDD can detect hypochlorite in living cells, possessing great cell-membrane permeableness and good ratio imaging in vivo.

We also used Image J software to calculate the fluorescence signal ratio of SH-SY5Y neuroblastoma cells in two channels ($F_{\text{green}}/F_{\text{red}}$), and the consequences appearance that the fluorescence intensity ratio of cells increased with the increase of ClO$^-$ concentration (Fig. 7b). Therefore, these data indicated that probe DDD not only has good membrane permeability in biological applications, but also provides a ratio response to changes in ClO$^-$ levels in living cells.
**3.8 Imaging of exogenous ClO⁻ in vitro**

Mostly, the zebrafish larvae experimental results were also suitable for human body [37] because of 87% homologous genes with human [38]. To evaluate the potential application of probe DDD for imaging exogenous ClO⁻ *in vivo*, we used zebrafish larvae as the animal mode in this work. As could be seen from **Fig. 8**, the zebrafish larvae showed no fluorescence without probe DDD and ClO⁻. After the zebrafish larvae was incubated with probe DDD (3 μM) for 15 min, next the residual probe was rinsed by E3 media three times, the zebrafish larvae displayed bright red fluorescence and weak green fluorescence. Then zebrafish larvae caused an evident fluorescence increase in the green channel treated with 3, 5 and 10 μM ClO⁻ for 15 min, along with a distinct fluorescence weakening in red channel. The imaging results indicated that probe DDD could exhibit the ratio fluorescence response of hypochlorite in living bodies and provided the new avenue of whole-organism investigations.
**Fig. 8** Fluorescence images of exogenous ClO\(^{-}\) in zebrafish larvae incubated with probe DDD for 15 min. The control was without probe DDD and ClO\(^{-}\) (a-c); the zebrafish larvae was fed with the probe DDD (3 μM) for 15 min (d-f); the zebrafish larva was pretreated with probe in E3 media for 15 min and was then incubated with 3 μM ClO\(^{-}\) (g-i), 5 μM ClO\(^{-}\) (j-l) and 10 μM ClO\(^{-}\) (m-o) for 15 min. red channel (λ\(_{ex}\)= 488 nm, λ\(_{em}\)= 570-670 nm), green channel (λ\(_{ex}\)= 405 nm, λ\(_{em}\)= 470-570 nm).

### 4. Conclusion

In summary, probe DDD with Meldrum’s acid reporting moiety was designed and synthesized to detect ClO\(^{-}\). Probe DDD demonstrated excellent sensitivity and selectivity toward hypochlorite and exhibited a fast response, a large Stokes Shift, sensitive limit of detection, physiologically pH range and two well-separated emission (555 nm and 635 nm) which was prepared via a simple process. Additionally, the probe had low cytotoxicity, good cell permeability and biocompatibility, and has been used in fluorescence confocal imaging in living cells and zebrafish larvae. Moreover, we anticipate that the novel fluorescence probe DDD could be used to advance reveal basic physiology information about ClO\(^{-}\) in living organization.

### Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://

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