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# Ratiometric fluorogenic determination of endogenous hypochlorous acid in living cells

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**Abstract:**

Hypochlorous acid (HClO) is one of the most important ROS (reactive oxygen species) and common pollutant in tap-water. However, the determination of HClO with fast response and high sensitivity/selectivity is still an urgent demanding. Here we fabricated a ratiometric fluorescent probe **RC** based on TBET (through-bond energy transfer) on the platform of coumarin and rhodamine with the thiosemicarbazide group as the linker. This probe could display the characteristic fluorescence emission of coumarin. Upon addition of HClO, the linker was reacted into an oxadiazole, resulting in the opening of spiro-ring of rhodamine. The resultant then gives ratiometric fluorogenic changes. The probe exhibits fast response and high selectivity and sensitivity towards HClO with a low limit of detection (~140 nM). Eventually, **RC** is successfully applicated for determining spiked HClO in water samples and imaging endogenous HClO in living cells.

**Keywords**: Coumarin; Rhodamine; Hypochlorous acid; Ratiometric; Fluorescence.

## 1. Introduction

As one of major reactive oxygen species (ROS), hypochlorous acid (HClO) plays extremely important roles in the biological and pathological processes [1], such as the protection of immune system of animals [2]. Endogenous HClO generation is the resultant of peroxidation

of chloride ions ($Cl^-$) with catalyzed by mounting myeloperoxidase (MPO) in macrophages, monocytes and neutrophils [3-5]. However, many evidences have indicated that over production of HClO in organisms could be associated with various diseases, such as cardiovascular disorders [6] , inflammatory [7], lung injury [8], rheumatoid arthritis [9] and even cancer [10]. Therefore, to develop rapid and efficient techniques for determination of HClO in environmental water and biological samples has been becoming a quite significant purpose. [11]

Today, various methods are available for the determination of HClO, such as electrochemistry [12], spectrophotometry [13, 14], potentiometry [15, 16], fluorometry [17-20], and chemiluminescence [21, 22] and so on. Among them, spectrophotometry is a conventional and common approach. A colorimetric probe even could recognize the analytes by the naked eye, without the aid of the expensive instruments [23]. Meanwhile, the light-absorption ratiometric variation approach (LARVA) based on analyte-induced variations of dual absorption bands of a probe is able to achieve a wider linear detection scope and lower detection limitation for spectrophotometry [24]. But beside that, fluorometry based on fluorogenic probes might be the most attractive approach to determine HClO in both environmental and biological samples. It is because of its advantages on high sensitivity, excellent selectivity, the rapid response time, *in-site* detection capability and low cost [25]. Another key advantage is the higher spatial resolution and real-time monitoring capability, which are adapted to obtain the chemical information with higher precision from the complex biological system [26-32]. So far, "turn-on" fluorogenic probes have been commonly applied for the detection of HClO based on the oxidative characteristic of HClO to their specific functional groups, including p-methoxyphenol [33], ether [34], thioether [35, 36], hydrazone [37], oxime [38], hydroxamic acid [39] and hydrazide [40-42]. Although these "turn-on" probes are of practical utility for imaging and evaluation of HClO in living cells, external influences of variation of probe concentration can still complicate the quantification [43]. One solution is the ratiometric fluorogenic technique, by monitoring two emission bands, which provides a self-calibration to eliminate potential environmental distractions and allow quantitative measurement [44, 45]. Therefore, to fabricate the probes for ratiometric fluorogenic determinations of HClO with high selectivity and sensitivity is necessary and continuing to be of widespread interest.

There are two common types of ratiometric fluorogenic probes, mono-fluorophore-based [46, 47] and dual-fluorophore-based ones [48]. Fluorescence resonance energy transfer (FRET) and through-bond energy transfer (TBET) are two frequently-used principles in the design of dual-

fluorophore-based probes. In a FRET system, the distance between the donor and acceptor, orientation between the transition dipoles and spectra overlap integral of the donor fluorescence with the acceptor absorbance, are crucial factors to achieve efficient energy transfer. This certainly lacks flexibility owing to the limited options of the donor and acceptor fluorophore pairs [49-51]. Unlike the energy transfer through space pathway of FRET, energy transfer in TBET occurs through bonds in which the donor relays energy to the acceptor mostly through the twisted π-electron systems, which has no known constraint of a large overlap integral [51]. Actually, TBET could be regarded as a summation of several means of transferring energy. TBET process may operate simultaneously with FRET process but usually run much faster with more efficiency compared with the conventional FRET system [52, 53]. As a result, this strategy becomes more flexible and attractive to achieve an ideal ratiometric fluorescent sensing system [53]. Herein, a TBET-based probe (**RC**) was then fabricated for ratiometric fluorogenic determinations of HClO. The response mechanism is based on an HClO-triggered intramolecular desulfurization-cyclization cascade of thiol hydrazine, which resulted in the remarkable changes of dual emission and absorption bands. And **RC** exhibits high selectivity towards HClO over other ROS and several common analytes. Furthermore, **RC** could respond to HClO in a short response time (< 2 min). The fluorogenic detection has a low limit of detection of 140 nM and a wide pH range of 3 to 7.6. It is successfully applied in the quantitative detection of HClO under several spiked concentration with a good recovery. Finally, **RC** was successfully applied to detect endogenous and exogenous HClO in living cells with clearly separated signals.

## 2. Experimental

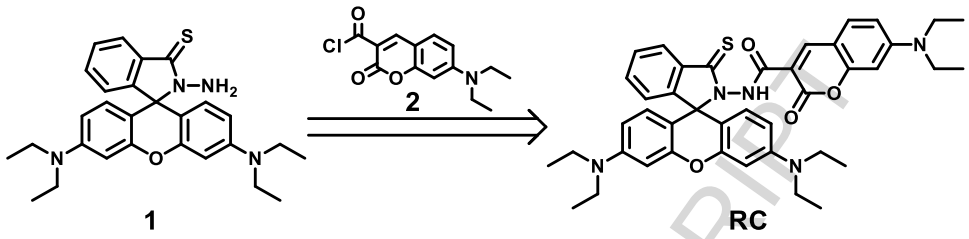
### *2.1. Materials and apparatus*

The common metal salts, anions salts and ROS chemicals were obtained from commercial company (Sigma). The relevant solvents ($CH_2Cl_2$, THF, DMSO) were used without further purification. The MTT (5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide), DMEM (Dulbecco's Modified Eagle's Medium) and FBS (Fetal Bovine Serum) were purchased from KeyGENBioTECH company.

The absorption data were collected by using a PerkinElmer Lambda 950 UV-Vis spectrometer. The fluorescence spectra were measured by using Hitachi F-4500 fluorescence spectrometer with the excitation wavelength at 420 nm. And the slit widths of both excitation and emission are 5 nm. The $^{1}H$ NMR and $^{13}C$ NMR (the proton and carbon nuclear magnetic resonance)

measurements were taken by using a Varian 300 MHz spectrometer. The high-performance liquid chromatography (HPLC) analysis were performed by using a Gilson Unipoint system with a Gemini C18 column under neutral condition. The pH measurements were taken by using the PHS-3C meter.

*2.2. Synthesis of **RC***




**Scheme 1** Synthesis of Probe **RC**

Compound **1** (235 mg, 0.5 mmol) and **2** (140 mg, 0.5 mmol) were dissolved in 5 and 15 mL $CH_2Cl_2$, respectively. The $Et_3N$ (0.15 mL, 1.1 mmol) and **2** solution was added to the solution of **1** with stirring under nitrogen atmosphere for 24 h. After the reaction completed, the organic layer was washed with water for three times, dried by $Na_2SO_4$, and then removed solvent under reduced pressure. The collected product was purified by column chromatography on alumina to afford 180 mg of **RC** (yellow solid, 50.4%). $^1$H NMR (300 MHz, $CDCl_3$-d) $\delta$ 8.80 (s, 1H), 8.24 (d, $J$ = 7.5 Hz, 1H), 7.41 (q, $J$ = 7.0, 6.2 Hz, 3H), 7.07 (d, $J$ = 7.6 Hz, 1H), 6.76 (s, 2H), 6.62 (d, $J$ = 7.9 Hz, 1H), 6.46 (s, 1H), 6.31 (d, $J$ = 7.6 Hz, 4H), 3.43 (q, $J$ = 7.1 Hz, 4H), 3.32 (d, $J$ = 7.1 Hz, 8H), 1.21 (t, $J$ = 7.1 Hz, 6H), 1.15 (t, $J$ = 7.0 Hz, 12H). $^{13}$C NMR (75 MHz, $CDCl_3$-d) $\delta$ 162.47, 159.74, 157.84, 152.81, 148.69, 136.11, 131.34, 130.47, 128.13, 122.81, 110.11, 109.45, 108.62, 108.41, 97.46, 96.75, 45.23, 44.49, 12.75, 12.56. ESI-MS [$M^+$]: 716.22.

*2.3. Preparation of **RC** and analytes*

The stock solution of **RC** (1 mM) were prepared by adding 0.01 mmol **RC** in 10 ml THF. 1 mM of each ROS was prepared in milli-Q water. And the stock solutions of metal ions (0.01 mM and 1 mM) and anions (0.01 mM) were prepared in milli-Q water.

Test solution of **RC** (5 μM) was prepared by placing 10 μL of **RC** (stock solution) in a 2 mL test tube, and then diluted to 2 mL with THF-PBS (20 mM, pH = 7.4, 5:5, v/v). The test solutions were prepared by adding 10 μL of **RC** (stock solution) and 100 μL of each testing species (stock solution) in 2 mL test tubes, respectively. Then, each mixture solution was diluted to 2 mL with THF-PBS (20 mM, pH = 7.4, 5:5, v/v) buffer solution.

The solution for titration measurement were prepared by adding the stock solution of **RC** (10 μL for the fluorescence measurement or 20 μL for the absorbance measurement) in 2 mL test tubes, then followed by adding various amount of the stock solution of HClO. Then, the mixture was diluted to 2 mL with the THF buffer solution.

*2.4. Cell cytotoxic assays and imaging*

For evaluation of the biotoxicity of **RC** and HClO, MTT assay were performed according to the reference reported [54]. HeLa cells were passed and plated in 96-well plates, and then incubated for one night. On the second day morning, the different amount of **RC** (final concentration of 0, 4, 8, 10, 15 and 20 μM) and HClO (final concentration of 0, 5, 10, 25, 50, 75 and 100 μM) were dissolved in the fresh DMEM. Then this fresh DMEM were used to replace the previous DMEM in the 96-well plate. After exposure in **RC** for 24 h (or HClO for 1 h, 3 h and 24 h), the medium was removed and 50 μL of MTT solution (5 mg/mL) was added for incubation for another 4 h. Then 150 μL DMSO was added after the MTT solution was removed. The plated were placed on the orbital shaker for agitating 5 min. The absorbance of each well at 570 nm was measured and the cell viability (%) was calculated according to the reference [55]. Confocal fluorescence imaging studies were performed on a Leica confocal laser-scanning microscope. Cell imaging photographs were taken after incubation with the probe and analyte. All the bio-samples were excited with 405 nm.

## 3. Results and discussion

*3.1. Molecular design and sensing mechanism of **RC** towards HClO*

The coumarin and rhodamine fluorophores are respectively chosen as donor and acceptor due to their well-separated emission bands with a wavelength difference of 110 nm, which could provide a higher resolution for dual-channel signal in buffer solution and cell imagining. Refer to the already synthetic approach [52], rhodamine thiohydrazide (**1**) and 7-diethylamino coumarin formyl (**2**) were covalently joint to give **RC**, where a structure of non-conjugated monothiol-bishydrazide was formed (Scheme 1). Monothiol-bishydrazide is reactive towards HClO, which is able to promote a cyclization reaction to form a conjugated 1,2,4-oxadiazole and simultaneously induces spirolactam-opening of rhodamine [36, 51]. As a result, TBET system is set up between the newly formed 3-susbstituted 7-diethylamino ocoumarin and xanthene derivative of rhodamine. Therefore, upon addition of HClO, the resulted solution gives ratiometric fluorescent signals when excited at the excitation wavelength of coumarin.

The HPLC analysis was performed to demonstrate the proposed process. As shown in Fig. 1A, **RC** ($m/z$ = 716.22 $[M + H]^+$) exhibits RT (retention time) at 4.31 min. Upon addition of 10 equiv. of HClO, **RC** were partly consumed and converted to the new compound **RCO** ($m/z$ = 682.97 $[M + H]^+$) with RT at 3.19 min (Fig. 1B). The results strongly supported that the proposed sensing mechanism of this system.

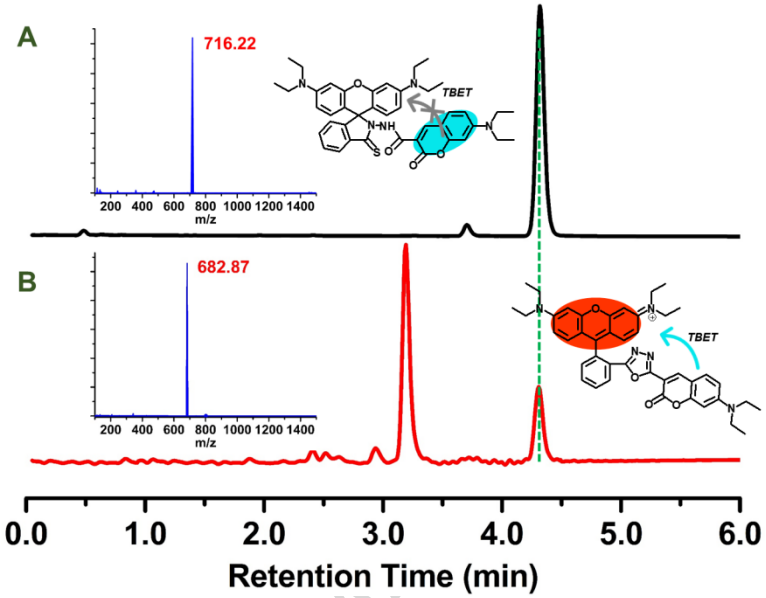



**Fig. 1.** HPLC profiles of (A) isolated **RC**, (B) **RC** treated with HClO (20 equiv.) buffered with 10 mM $NH_4OAc$) over 6 min.

As mentioned above, TBET may operate simultaneously with FRET. Generally, the effective spectral overlap between the donor emission and the acceptor absorption is one necessary requirement for the FRET system. In this system, a slight spectral overlap may induce a little FRET process in **RC** (Fig. S1A). Therefore, to get clear evidence, a simple evaluation was performed to study whether the TBET dominates energy transfer in the proposed sensing system. As shown in Fig. S1B, we measured the ratio ($F_{590}/F_{480}$) change of **RC** in the individual presence of HClO (25 μM) and $H^+$ (20 mM). As well known, strong acid could induce the ring-opened response of rhodamine spirolactams [56]. In the presence of $H^+$, the non-conjugated bond between the coumarin and rhodamine were generated, which only lead to the FRET process (Fig. S1C). Obviously, the introduction of acid only promoted a weak increase of the ratio ($F_{590}/F_{480}$), as shown in Fig. S1B, which also demonstrated a very low energy transfer efficiency for FRET process in this system. However, the introduction of HClO promoted the opening of spiro-ring of rhodamine and the generation of electronically conjugated bonds (Fig. S1C). Then the TBET process enabled the energy transfer from donor to acceptor through the

conjugated bonds, the emission ratio ($F_{590}/F_{480}$) increase of **RC** induced by HClO is much bigger than that by $H^+$ (Fig. S1B), which verified much more transfer efficiencies donated by TBET than FRET.

### *3.2. Time-dependent and pH-dependent studies of **RC** towards HClO*

To acquire an optimal experimental condition of **RC** towards HClO, the time-dependent and pH-dependent fluorescence response of **RC** towards HClO were investigated. As shown in Fig. 2A and 2a), the constant intensities at 480 nm and 590 nm as well as the constant ratio ($F_{590}/F_{480}$) of free **RC** (5 μM) show the probe is extremely stable in the assay condition. However, upon addition of HClO (20 μM), the intensity at 480 nm and 590 nm sharply decreased and increased respectively, which caused a remarkable enhancement of the ratio ($F_{590}/F_{480}$). And the ratio reached the maximum value in about 1.5 min and remained stable in the next 1 min. It indicated that **RC** showed a fast response towards HClO in the buffer solution. Then the responses of **RC** towards HClO at different pH conditions were also investigated. In the absence of HClO, **RC** kept almost stable over a wide range of pH from 3.0 to 10.0 (Fig. 2B). However, in the presence of HClO, the probe gave a remarkable increase ratio ($F_{590}/F_{480}$) in the range of 3.0-7.6. Therefore, considering the complete reaction of **RC** with HClO as well as a better incubation of **RC** and HClO in living cells, the further experiments were performed exposed to analytes for 5 min in the neutral pH of 7.4.

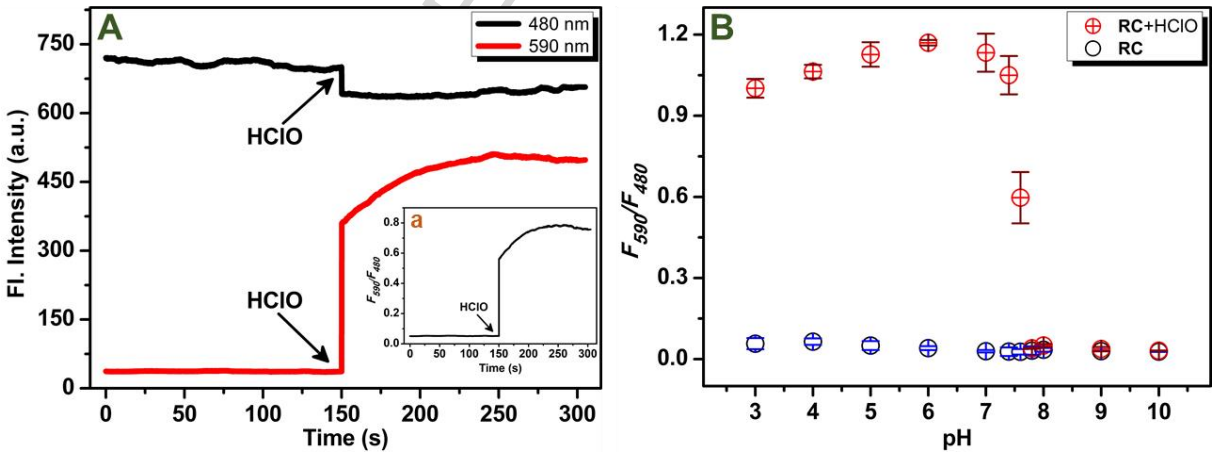



**Fig. 2.** (A) Real-time fluorescence intensity at 480 nm and 590 nm as well as (inset: a) the ratio ($F_{590}/F_{480}$) of **RC** (5 μM) in the absence and presence of HClO (20 μM); (B) The ratios ($F_{590}/F_{480}$) of the **RC** (5 μM) in the absence and presence of HClO (25 μM) at various pH values.

### *3.3. Fluorescence and UV-vis spectra of **RC** towards HClO*

The UV-Vis absorption spectra of the probe **RC** (10 μM) in the absence and presence of HClO in the buffer solution were exhibited in Fig. S2A. The introduction of HClO results in an obvious change in the color from yellow to orange-red, which shows **RC** could conveniently detect HClO by the "naked eye" (Fig. S2A inset). With the increase of HClO concentration (0-160 μM), the absorbance at 440 nm gradually dropped with a slight redshift, which attributes to the fact that this reaction induced the conversion of 3-amide to 3-1,3,4-oxadiazole substitute in the 7-diethylamino coumarin. Because these two substituent units have different electronic structures that affects the absorption profile of 3-substituted 7-diethylamino coumarin. This is different from the case that no substituent group of coumarin is involved in the analyte-induced reaction, where the absorption profile of coumarin does not change much [36, 57]. Meanwhile, a new absorption band centered at 568 nm and gradually increased, which clearly ascribes to the substituted xanthene resulted from ring-opening spirolactam of rhodamine. Hence, the gradual enhancement of the absorbance ratio ($A_{568}/A_{440}$) and a good linearity ($R^2$=0.9874) between the ratios ($A_{568}/A_{440}$) and the concentration of HClO (0-30 μM) were observed (Fig. S2B and Fig. S2C), allowing the ratiometric absorbance determination of HClO level in the buffer solution.

The fluorogenic response of **RC** (5 μM) towards HClO (0-50 μM) were investigated in the same buffer solution. As shown in Fig. 3A, with the increase of HClO concentration (0-50 μM), the emission at 480 nm of the coumarin gradually decreased and a new emission at 590 nm of the rhodamine appeared and gradually increased, indicating HClO triggered the formation of conjugated bonds and induced the "switch on" of TBET process. Meanwhile, the ratio ($F_{590}/F_{480}$) of fluorescence intensities at the two wavelengths increased gradually (Fig. 3B). Consistently, the fluorescence of **RC** showed a remarkable change from cyan to orange-red upon addition of HClO. In addition, the fitting curve illustrated that the logarithmic function of ratios ($F_{590}/F_{480}$) showed a good linear ($R^2$ = 0.9901) response to the HClO level in the range of 0-30 μM (Fig. 3C), and the limit of detection were calculated to be 140 nM (3δ/k), which is much lower than the residual chlorine concentration (10 μM or less) in tap water [58]. It indicated **RC** showed a good sensitivity towards HClO and could be employed in quantitative determination of HClO in environmental water samples.

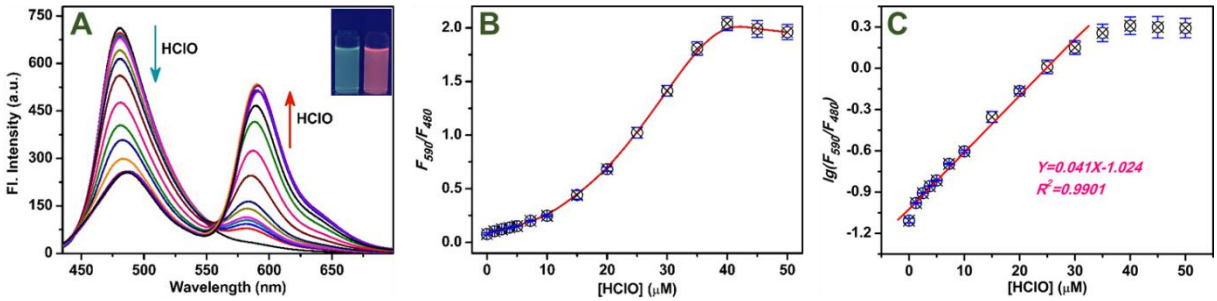



**Fig. 3.** (A) The fluorescence spectra, (B) the emission ratios ($F_{590}/F_{480}$) and (C) log plot of emission ratios ($F_{590}/F_{480}$) of **RC** (5 μM) in the presence of various concentration of HClO.

### *3.4. The selective studies of **RC** towards HClO*

Selectivity is another parameter which is as important as sensitivity of the fluorogenic probes. So, the evaluation of the selectivity of **RC** towards HClO over other relevant ROS, several metal ions and common anions were investigated. As shown in Fig. 4, S3 and S4, the relevant ROS (125 μM), the common metal ions (125 μM) and anions did not cause obvious changes in the emission spectra (Fig. 4A, S3A and S4A) and ratios (Fig. 4B, S3B and S4B). However, remarkable red shift of fluorescence spectra and obvious enhancement of fluorescence ratio ($F_{590}/F_{480}$) occurred as **RC** was only treated with HClO (125 μM). The results indicated the probe **RC** displayed a high selectivity towards HClO over some common relevant analytes.

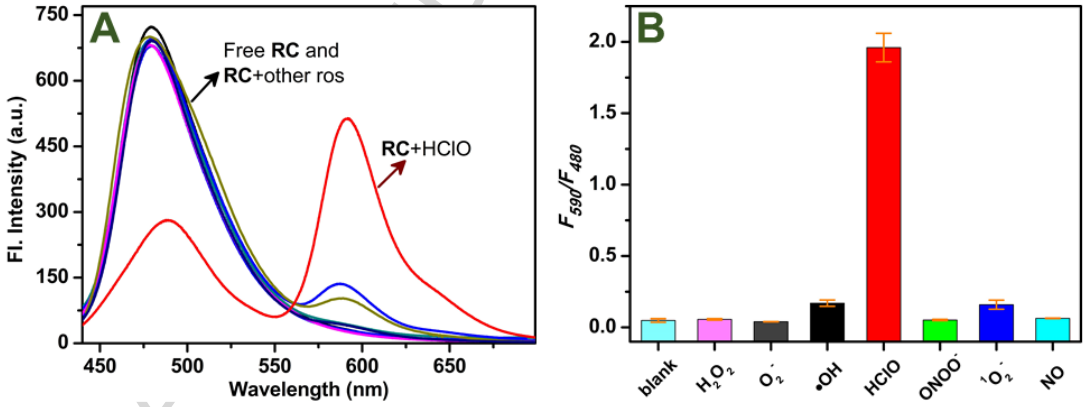



**Fig. 4.** (A) The fluorescence spectra and (B) the emisson ratios ($F_{590}/F_{480}$) of **RC** (5 μM) in the presence of the relevant ROS (125 μM).

### *3.5. Comparison with other probes*

For evaluation of the performance of the molecular fluorometric sensor, we listed some common tradition methods and compared with the fluorometry method. As shown in Table 1, various methods are available for the determination of HClO, such as electrochemistry, UV

spectrophotometry, potentiometry, luminescence and fluorescence[12, 13, 15] [17, 18, 21] [42]. As well known, each method have its advantages and disadvantages. Take fluorometry as the example, the optimal advantages is the *in-site* application in the *vivo* biological samples with high resolution and fast response. However, some molecular sensor also have limitation of measuring in the water medium. Usually, the organic solvent is necessary for some molecular fluorescent sensor during the sensing process. Hence, scientists developed many ways, including linked with water-soluble chain [59] or assembled with surfactant [60], for increasing the water solubility of sensors. Now this study on better modification of fluorogenic probes for increasing water solubility and biocompatibility is more and more concerned by the researchers.

Next, the LOD, solution system, response time and biological application of probe **RC** were compared with several early publications. As shown in Table 1, Yang [18] reported one off-on fluorescent sensor with a low LOD of 8.2 nM but a slow reaction time. Li [45] synthesized two ratiometric fluorescent sensors which could detect HClO within 1 min but with a higher LOD. In addition, other reported chemosensors, such as coumarin-based [20], iridium(III) complex-based [42] and naphthalimide-based probe [61], display excellent properties for the detection of HClO with fast response and low LODs. And our probe **RC** possessed low LOD and short response time, which are comparable with most of the reported literature.

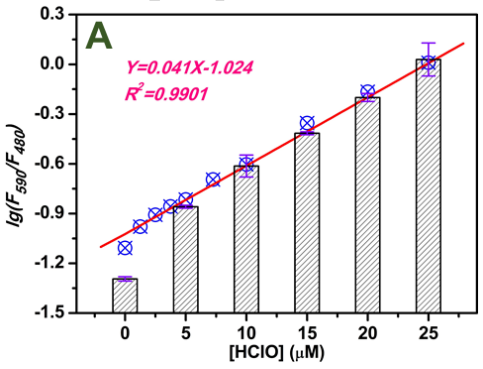
Table 1. The comparison of fluorogenic probes with other methods for HClO in the published literatures.

| Ref. | Method | LOD(M) | Buffer solution | Response time | Bio-imaging |
|---|---|---|---|---|---|
| [12] | electrochemistry | 1.33 μM | PBS (pH = 7) | Several hours | No |
| [13] | spectrophotometry | 22.8 mM | Water | 24 h | No |
| [15] | potentiometry | 10 μM | PBS (pH = 7.2) | 20 min | No |
| [21] | chemiluminescence | 7.62 μM | test strip (pH = 7.0 or 9.5) | 5 min | No |
| [42] | Photoluminescence/ Electrogenerated chemiluminescence | 93.3 nM/ 280 nM | EtOH-PBS (1:1, pH = 7.4) | within 1 s | Yes |
| [17] | Fluorescence | 340 nM, | Tris-HCl (50 mM, pH = 7.4). | 60 min | Yes |
| [18] | Fluorescence | 8.2 nM | Deionized water | 35 min | Yes |
| [19] | Fluorescence | 120 nM/ 26 nM | $CH_3CN$-PBS (pH = 7.4, 1:1) | 4 min/7 min | Yes |
| [20] | Fluorescence | 8.3 nM | PBS (0.01 M, pH 7.4) | 20 s | Yes |

| | | | | | |
|---|---|---|---|---|---|
| [36] | Fluorescence | 660 nM. | EtOH -PBS (8 : 2, pH = 5) | Within 1 min | Yes |
| [37] | Fluorescence | 0.2 μM | DMF-PBS (8 : 2, pH 7.4) | — | Yes |
| [45] | Fluorescence | 530 nM /760 nM | $CH_3OH$-HEPES (1:1, pH = 7.0) | within one minute | Yes |
| [51] | Fluorescence | 0.1 μM | EtOH -PBS (3 : 7, pH = 6) | within 50 s | Yes |
| [61] | Fluorescence | 208.9 nM/ 17.3 nM | DMSO-PBS (3:7, pH =7.4) | 3 s/4 s | Yes |
| This work | Fluorescence | 140 nM | THF-PBS (5:5, pH = 7.4) | 1.5 min | Yes |

### *3.6. Application in tap water samples*

As mention above, the probe **RC** could sensitively detect HClO in the buffer solution. HClO is commonly utilized as the disinfectant [62] for the tap water, which causes a certain concentration of hypochlorous acid remaining. Hence, whether this probe could be applied in the determination of HClO in tap water samples is of interest. The tap water samples in this fluorogenic determination experiment were taken from Linköping University. As shown in Fig. 5, HClO in tap water sample was not detected, this is because that the tap water in Sweden is directly drinkable water. Then the water samples were spiked with different concentrations of standard HClO solution. And the results demonstrated **RC** was able to measure the concentration of spiked HClO with good recovery (Fig. 5B). The good results indicated that **RC** could be potentially employed for the determination of HClO in tap water samples that treated with chlorine for disinfection.




B

| Tap Water samples | HClO Spiked (μM) | Found (μM) | Recovery (%) |
|---|---|---|---|
| 1 | 0 | No | - |
| 2 | 5 | 4.1 | 82.0 |
| 3 | 10 | 10.02 | 100.2 |
| 4 | 15 | 14.83 | 98.9 |
| 5 | 20 | 20.1 | 100.5 |
| 6 | 25 | 25.68 | 102.7 |

**Fig. 5.** (A) The log plot of emission ratios ($F_{590}/F_{480}$); (B) the testing results of HClO concentration of **RC** (5 μM) in the presence of various concentration of HClO in tap water.

*3.7. Cell imaging*

To further employ the probe **RC** for tracking HClO in the biological system, the fluorescence imaging experiments were carried out in Raw 264.7 and HeLa cells. Firstly, we investigated the cell viability of **RC** within HeLa cells. The living cells were treated with various concentrations of **RC** for 24 h, and the cells viability shows the probe has negligible cytotoxicity at the concentration of 10 μM during imaging experiments for 30 min (Fig. S5). In addition, we also carried out the MTT experiments for evaluation of the cytotoxicity of various concentration of HClO. As shown in Fig. S6, after Raw cells were exposed under different concentrations of HClO (maximum: 100 μm) for 1 h or 3 h, the cell viability changed little compared to control group. However, the cell viability decreased to almost 80% when the Raw cells were treated by 100 μM HClO for 24 H. Hence, our experiments had evidenced the almost nonbiotoxicity of 100 μm HClO to the cell under the bio-imaging experimental conditions. Afterwards, we carried out the exogenous and endogenous HClO detection by using probe **RC** in living cells and the results are depicted in Fig. 6 and 7. As shown in Fig. 6, HeLa cells were incubated with **RC** (10 μM) for 30 min, and obvious fluorescence in the cyan channel was observed, but the fluorescence in the red channel was absent. Then the cells were washed with PBS three times and treated with HClO (100 μM) for another 30 min. It was noticed that a district fluorescence decreases in the cyan channel, accompanied by a remarkable fluorescence enhancement in the red channel. This is kept consistent with HClO induced TBET-ON state and resulted ratiometric fluorescence response in the buffer solution. In addition, co-incubation of HClO scavenger, N-acetyl-L-cysteine (NAC), was the control experiment. As shown in Fig. 6L), after the presence of NAC, HClO was mostly consumed by the reaction with NAC, thus the promoting reaction cascades were inhibited and only cyan fluorescence appeared.

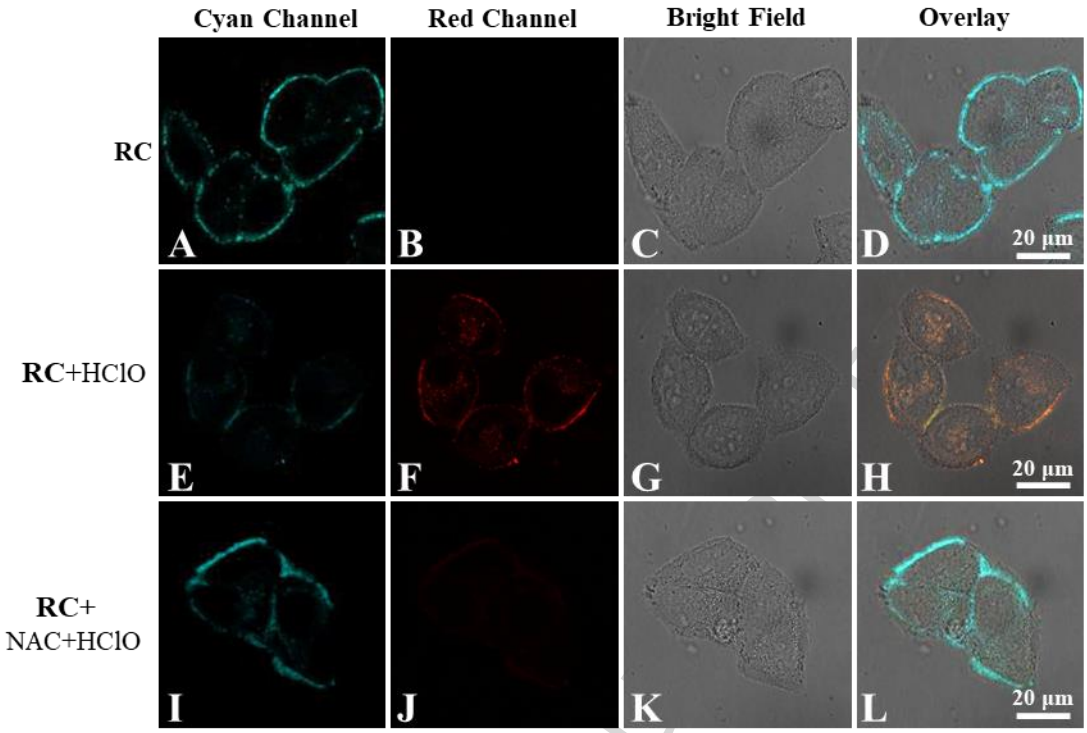



**Fig. 6.** Confocal fluorescent images of HeLa cells. A, B, C, D) Cells incubated with **RC** (10 μM) for 30 min (control); E, F, G, H) **RC**-stained cells treated with HClO (100 μM) for another 30 min; I, J, K, L) **RC**-stained cells treated with NAC (10 mM) and HClO (100 μM) for another 30 min. The Cyan-channel and the Red-channel are collected at 480 ± 10 nm and 580 ± 10 nm, respectively.

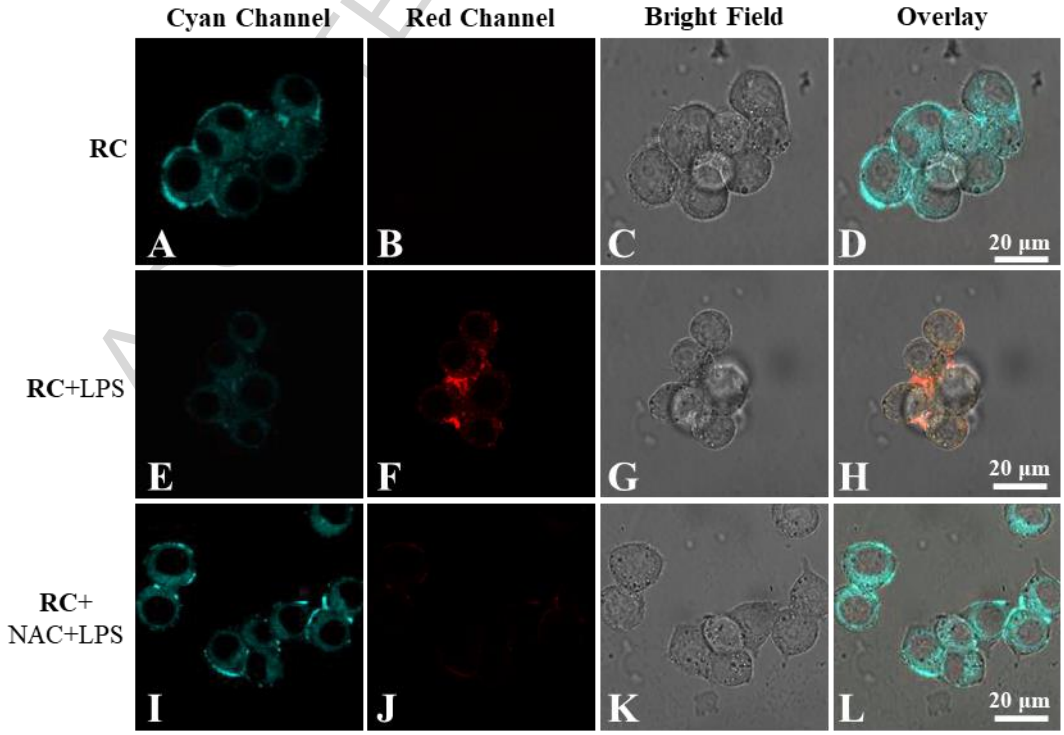


**Fig. 7.** Confocal fluorescent image of raw 264.7 cells. A, B, C, D) Cells incubated with **RC** (10 μM) for 30 min (control); E, F, G, H) **RC**-stained cells treated with **LPS** (100 μM) for another 30 min; I, J, K, L) **RC**-stained cells treated with NAC (10 mM) and LPS (100 μM) for another 30 min. The Cyan-channel and the Red-channel are collected at 480 ± 10 nm and 580 ± 10 nm, respectively.

In order to further verify the practical detection and imaging HClO in the living cells, we tried to track the endogenous HClO *via* the stimulation of drug in cells by suing **RC**. One typical inflammation-induced drug, lipopolysaccharides (LPS), which could prompt the cells to generate the ROS, were introduced into RAW cells, and the cells were treated with **RC** for 30 min. Obviously, the cells showed the fluorescence change from cyan to red (Fig. 7A and 7F). Meanwhile, no HClO-induced fluorescence increasing in red channel was observed in the RAW cells treated with NAC. These results demonstrated that **RC** is capable of the ratiometric fluorogenic imaging endogenously HClO.

## 4. Conclusion

In summary, we fabricated a probe **RC** composed of coumarin-rhodamine fluorophore for ratiometric absorbance and fluorescence determination of HClO in buffer solution and living cells. The sensing mechanism of **RC** towards HClO and TBET-based energy transfer for giving ratiometric fluorescence changes have been determined experimentally. **RC** possessed a high selective and sensitive colormetric and fluorescent response toward HClO. Ratiometric absorbance and fluorogenic determinations of HClO was then established. Importantly, logarithms of fluorescence ratios were well fit by a linear regression in a wide range of 0 to 30 μM with a low limit of detection (3δ/k, 140 nM). **RC** was then successfully applied in the detection of several concentrations of spiked HClO in tap water samples. Confocal laser fluorescence microscopic experiments demonstrated that **RC** can visualized and detect the endogenous and exogenous HClO in living cells.

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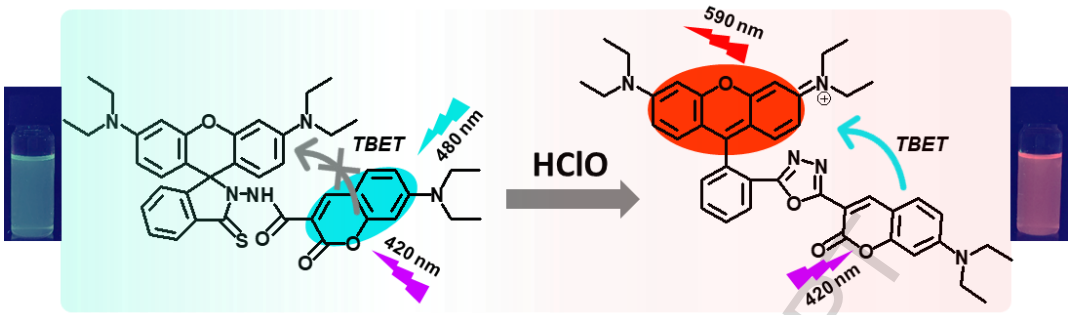
## Graphical Abstract



Highlights

- A TBET-based ratiometric fluorescent probe for hypochlorous acid.
- High selectivity and rapid response (within 90 s) for hypochlorous acid over other ROS in a broad pH range.
- Detection of exogenous and endogenous hypochlorous acid in living cells.