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Fluorescent Probes

A Ratiometric Fluorescent Probe Based on a Through-Bond Energy Transfer (TBET) System for Imaging HOCl in Living Cells

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Abstract: A simple ratiometric probe (Naph-Rh) has been designed and synthesized based on a through-bond energy transfer (TBET) system for sensing HOCl. In this probe, rhodamine thiohydrazide and naphthalene formyl were connected by simple synthesis methods to construct a structure of monothio-bishydrazide. Free probe Naph-Rh showed only the emission of naphthalene. When probe Naph-Rh reacted with HOCl, monothio-bishydrazide could be converted into 1,2,4-oxadiazole, which not only ensured that the donor and the acceptor were connected with electronically conjugated bonds, but also resulted in the spiro-ring opening and the emission of rhodamine. Therefore, a typical TBET process took place. The probe possessed high-energy transfer efficiency and large pseudo-Stokes shifts. As the first TBET probe for HOCl, Naph-Rh showed excellent selectivity and sensitivity toward HOCl over other reactive oxygen species (ROS)/reactive nitrogen species (RNS), and could respond fast to a low concentration of HOCl in the real sample. In addition, the probe was suitable for imaging HOCl in living cells due to its real-time response, excellent resolution, and reduced cytotoxicity.

Introduction

Hypochlorous acid (HOCl)/hypochlorite ($^{-}$OCl) is one of the most important reactive oxygen species (ROS) generated from hydrogen peroxide and chloride ions by the catalysis of myeloperoxidase (MPO) in living organisms.[1] Hypochlorite, as an endogenous microbicidal agent, plays a vital role in defending the invasion of pathogens.[2] On the other hand, uncontrolled generation of hypochlorite is closely associated with some diseases, such as arthritis, kidney disease, lung injury, atherosclerosis, osteoarthritis, and cancer.[3–8] However, the roles of HOCl/$^{-}$OCl in these diseases are not quite clear owing to the limitation of detection technology. Therefore, the development of imaging techniques for HOCl/$^{-}$OCl in vitro and in vivo is of significant interest. The fluorescent probes for HOCl/$^{-}$OCl have attracted much attention due to their natural advantages including excellent sensitivity, high selectivity, and rapid response time.[9–18]

In recent years, most turn-on fluorescent sensors based on BODIPY, cyanine, and rhodamine were reported for HOCl/$^{-}$OCl.[19–25] On the one hand, these probes have high fluorescence quantum yield and emission bands at the red region of the spectrum. On the other hand, they have high sensitivity owing to the lack of background signal. However, their applications were limited because of small Stokes shifts and interference from the environment, probe concentration, and excitation intensity.[26–34] Ratiometric probes based on fluorescence resonance energy transfer (FRET) can give a solution to the tissues, because they not only possess larger pseudo-Stokes shifts, but also have a recorded ratio signal of two emission intensities at different wavelength, which can afford a built-in correction. Normally, the donor and the acceptor in FRET sensors are usually linked with a nonconjugated spacer, and the energy transfers from the donor to the acceptor through space.[27,35–38] A substantial spectral overlap is necessary between the donor emission band and the acceptor absorption band, which sometimes limits the choice and the design of FRET sensors.[34] In contrast, for a TBET (through-bond energy transfer) system to be effective, the donor and the acceptor are linked with an electronically conjugated bond, through which the energy can transfer from the donor to acceptor without the need for a substantial spectral overlap between the donor emission and acceptor absorbance.[39] These can afford the desired flexibility in the choice of fluorophore and the large emission shifts between the two emission peaks. Thus, TBET-based probes exhibit high energy transfer efficiency, large pseudo-Stokes shifts,[40] and excellent resolution for fluorescence imaging due to two well-resolved emission peaks.[39] To the best of our knowledge, a fluorescent probe based on TBET for HOCl has not been reported so far, although

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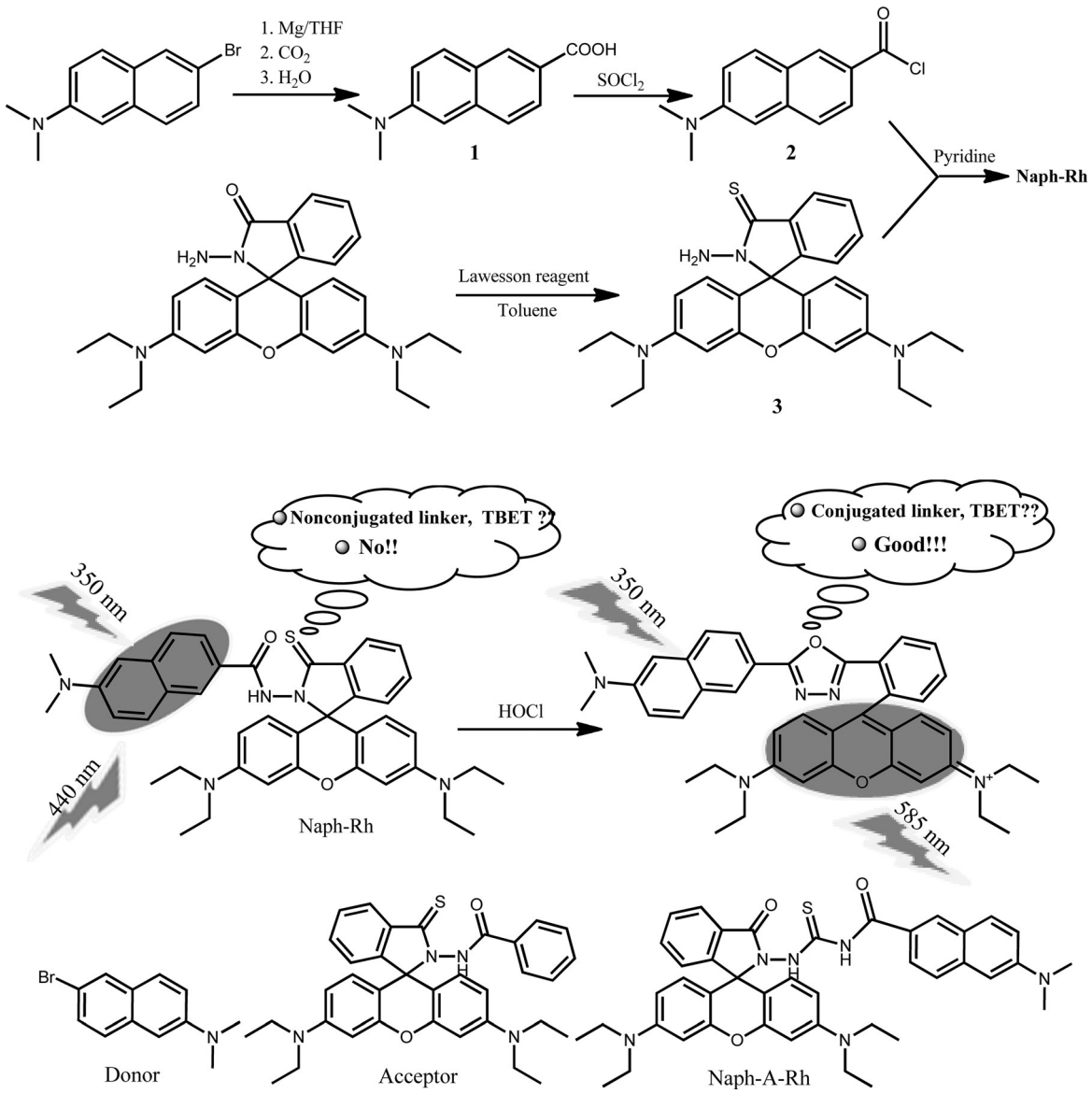

Scheme 1. Syntheses and proposed detection mechanism of Naph-Rh.

some TBET fluorescent probes were developed for the detection of other species.[39,41–43] Therefore, it is imperative to develop ratiometric probes on TBET for imaging HOCl in living cells.

In our previous work,[44] we reported two FRET fluorescent probes for HOCl/$^{-}$OCl. Although the probes showed excellent selectivity toward HOCl/$^{-}$OCl, the sensitivity and energy transfer efficiency are less than desirable. These limited their application in imaging cells. In order to overcome the above shortcomings, in this work, we designed and synthesized a simple TBET fluorescent probe (Naph-Rh, Scheme 1) for HOCl.

Rhodamine thiohydrazide and naphthalene formyl were connected by a simple synthesis method to construct a structure of monothio-bishydrazide. In the free probe Naph-Rh, the naphthalene fluorophore (donor) and rhodamine fluorophore (acceptor) were linked by a nonconjugated bond (monothio-bishydrazide), which does not seem to meet the requirement of a TBET system. But when probe Naph-Rh reacted with HOCl, monothio-bishydrazide could be converted into electronically conjugated bonds (1,2,4-oxadiazole confirmed by ESIMS, Figure S1, Supporting Information).[45] Thus, the spiro-ring of rhodamine was opened and the TBET system was setup simultaneously. The TBET system ensured that the energy could transfer successfully from the donor to the acceptor. Moreover, to avoid the spectral overlap between the donor emission and the acceptor absorbance, and enlarge the pseudo-Stokes shifts and emission shifts, we employed the naphthalene fluorophore (excitation, 350 nm; emission, 440 nm) as a donor to match with rhodamine fluorophore (absorbance, 570 nm; emission, 585 nm) as an acceptor. It can be seen from Figure 1 that the spectral overlap is insignificant, yet the energy transfer efficiency was calculated to be 83.4% (Figure S2, Supporting Information).[32,42] To prove the TBET, we synthesized a model compound Naph-A-Rh (Scheme 1) as a comparison. When Naph-A-Rh reacted with HOCl, the spiro-ring of rhodamine was opened

to give an 3-amino-1,2,4-oxadiazole derivative in which the linker retained the nonconjugated bond (see Scheme S1 and Figure S1, Supporting Information).[45] The energy-transfer efficiency was calculated to be 45.3% (Figure S2, Supporting Information). The results indicated that the energy transfer of Naph-Rh should mainly be based on TBET. The pseudo-Stokes shift is 235 nm and the emission shifts is 145 nm (Figure 1), which are larger than those of reported TBET probes.[32,46] The large emission shifts could improve imaging resolution in cells.

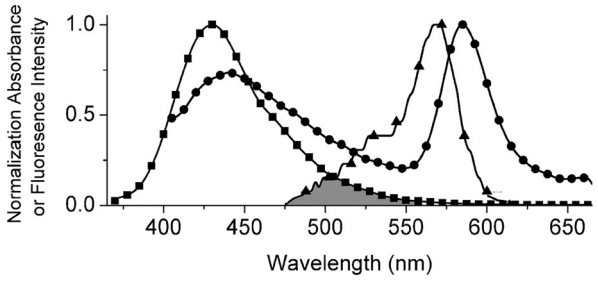

Figure 1. Normalized emission spectra of the donor (■), normalized absorption spectra of the acceptor after the addition of HOCl (5 μM; ▲), and normalized emission spectra of Naph-Rh after the addition of HOCl (5 μM; ●). Conditions: [Naph-Rh] = 5 μM, [donor] = 5 μM, [acceptor] = 5 μM, Na_2HPO_4 (0.05 M, pH 6)/EtOH (7:3, *v*/*v*), λ_{ex}: 350 nm (slit widths: 10/5 nm).

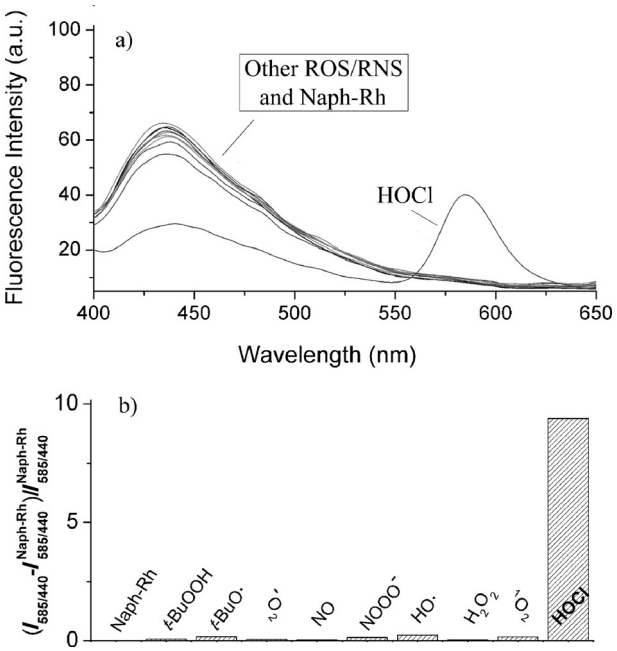

Figure 2. a) Fluorescence spectrum changes of Naph-Rh toward HOCl (5 μM) and other ROS/RNS (50 μM, $HO^{\bullet}$, $ONOO^-$, NO, H_2O_2, *t*BuOOH, *t*BuOO$^{\bullet}$, $^1O_2$, $^{-}O_2$). b) The relative ratio ($I_{585/440}-I^{Naph\text{-}Rh}{}_{585/440}$)/$I^{Naph\text{-}Rh}{}_{585/440}$) of fluorescence intensity ratio changes, $I_{585/440}$ is the ratio (I_{585}/I_{440}) of Naph-Rh with addition of ROS/RNS, $I^{Naph\text{-}Rh}{}_{585/440}$ the ratio (I_{585}/I_{440}) of Naph-Rh. Conditions: [Naph-Rh] = 5 μM, Na_2HPO_4 (0.05 M, pH 6)/EtOH (7:3, v/v), λ_{ex}: 350 nm (slit widths: 10/5 nm).

Results and Discussion

To evaluate the selectivity of Naph-Rh toward HOCl among ROS/reactive nitrogen species (RNS), the absorbance and emission spectra of Naph-Rh were measured in the presence of ROS/RNS at pH 6. When 5 μM HOCl was added to the solution of Naph-Rh (5 μM) in a mixture of Na_2HPO_4 (0.05 M, pH 6)/EOH (7:3, v/v), a characteristic absorption peak of rhodamine at 570 nm appeared and a typical absorption peak of naphthalene at 350 nm changed little (Figure S3, Supporting Information). Simultaneously, a typical emission peak of naphthalene (440 nm) decreased dramatically and a characteristic emission peak of rhodamine (585 nm) appeared with the addition of HOCl (Figure 2a). The results should be attributed to the TBET system between naphthalene and rhodamine. The free probe Naph-Rh was in a spiro ring-closing state and the TBET was off in the absence of HOCl, so only emission of naphthalene was observed in its fluorescent spectra. When the probe reacted with HOCl, the oxadiazole was generated resulting in the spiro ring-opening and the TBET. Therefore, the emission spectrum of rhodamine appeared and the fluorescent intensity of naphthalene weakened with excitation at 350 nm. Other ROS/RNS affected little the absorption and fluorescence spectra of Naph-Rh, although the concentration of other ROS/RNS reached up to 50 μM. Figure 2b showed that the ratio (I_{585}/I_{440}) of probe Nph-Rh in the presence of HOCl increased by about 10-fold compared to the ratio (I_{585}/I_{440}) of free probe Nph-Rh. The ratio (I_{585}/I_{440}) changed little with addition of other ROS/RNS into probe Nph-Rh. These results indicate that probe Naph-Rh has excellent selectivity toward HOCl over other ROS/RNS.

The titration experiments were implemented in a mixed solution of Na_2HPO_4 (0.05 M, pH 6)/EtOH (7:3, v/v). The fluorescence intensity at 440 nm diminished significantly with the addition of HOCl from 0 to 5 μM and a new emission peak at 585 nm enhanced gradually (Figure 3a). Meanwhile, the corresponding ratio (I_{585}/I_{440}) increased gradually and an excellent linearity was established between the ratio and the concentration of HOCl from 2.5 to 5 μM (Figure 3b) with the detection limit 0.1 μM ($S/N = 3$). Furthermore, it could also be seen from Figure 3a that the fluorescence intensity changed obviously at a low concentration of HOCl (0.5 μM). These results indicated that probe Naph-Rh showed excellent sensitivity toward HOCl and could detect a low concentration of HOCl in the real sample. The absorption spectra (Figure S4, Supporting Information) showed that the characteristic peak (570 nm) of rhodamine enhanced gradually with the addition of HOCl, which also illustrated that the rhodamine moiety of probe Naph-Rh could react with HOCl to induce spiro-ring opening.

We investigated the pH effect on the ratiometric response of Naph-Rh toward HOCl (Figure S5, Supporting Information). The results showed that probe Naph-Rh could effectively detect HOCl at pH 3–7. Thus, we deduced that Naph-Rh is suitable for imaging in vivo. Notably, the changes of time-dependent fluorescence intensity ratio (I_{585}/I_{440}) indicated that probe Naph-Rh could rapidly respond to HOCl and reached a plateau within 50 s (operation time), which was very important for real-time imaging of HOCl in situ (Figure S6, Supporting Information).

Murine RAW264.7 can produce HOCl in the immune system by stimulation with lipopolysaccharide (LPS),[19,47] So we em-

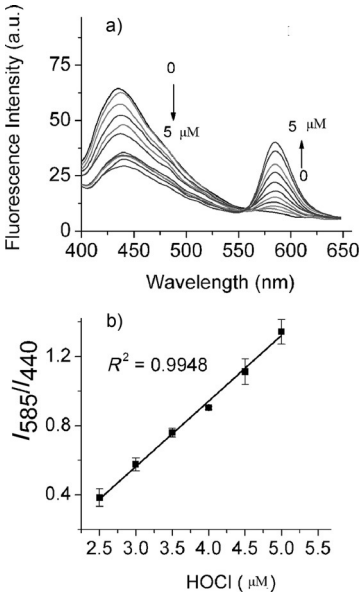

Figure 3. a) Fluorescence spectra and b) fluorescence intensity ratio (I_{585}/I_{440}) changes of Naph-Rh with the addition of HOCl (0–5 μM). Conditions: [Naph-Rh] = 5 μM, Na_2HPO_4 (0.05 M, pH 6)/EtOH (7:3, v/v), λ_{ex}: 350 nm (slit widths: 10/5 nm).

ployed RAW264.7 cells to investigate probe Naph-Rh for imaging endogenous HOCl in living cells. In the control group, after RAW264.7 cells had been incubated with Naph-Rh (10 μM) for 4 h, strong blue fluorescence (Figure 4a) and very faint red fluorescence (Figure 4b) were observed. When RAW264.7 had been incubated with probe Naph-Rh for 4 h after stimulating with LPSs for 12 h, the strong blue fluorescence darkened (Figure 4c) and the faint red fluorescence brightened (Figure 4d). The corresponding ratio (red to blue) of fluorescence intensity increased obviously (Figure 4e). The results indicated that probe Naph-Rh was suitable for imaging endogenous HOCl in the living cells. In addition, the cytotoxicity of Naph-Rh was evaluated in RAW264.74 cells. Little toxic effect on cell viability was observed after incubation with Naph-Rh (10 μM) for 4 h (Figure 4f), which demonstrated that Naph-Rh could be used for clinical applications in living organisms.

Conclusions

In summary, a TBET strategy is employed to design a ratiometric probe Naph-Rh for imaging endogenous HOCl in living cells. As we know, this probe is the first TBET-based fluorescent probe for HOCl. It shows two well-resolved emission peaks with high energy transfer efficiency and large pseudo-Stokes shifts. This probe exhibits high selectivity toward HOCl over other ROS/RNS and can respond fast to a low concentration of HOCl in the real sample. Little cytotoxicity is crucial for Naph-Rh to be used for clinical applications in living organisms. We hope this strategy might promote the development of TBET-based probes for HOCl.

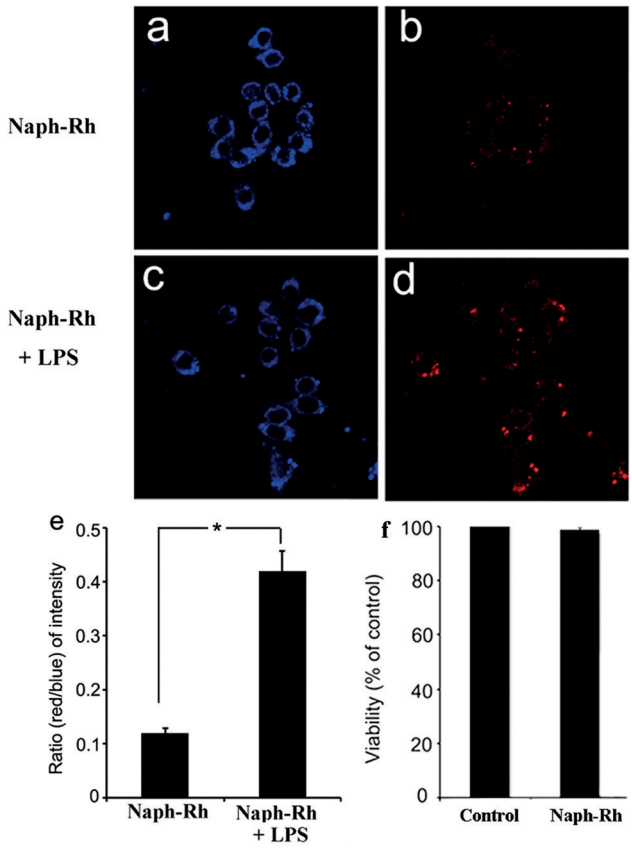

Figure 4. Fluorescence images of RAW264.7 cells incubated with a 10 μM probe for 4 h from a) blue channel and b) red channel. Fluorescence images of RAW264.7 cells pretreated with 1 μg mL^{-1} lipopolysaccharide (LPS) for 12 h, then incubated with 10 μM probe for 4 h from c) blue channel and d) red channel. e) The ratio (red to blue) of fluorescence intensity, $*p < 0.05$. The results are presented as means ± SE with replicates $n = 5$. f) Viability of RAW264.7 cells after treatment with the probe for 4 h at a concentration of 10 μM. Excitation: 405 nm; blue channel: 405–550 nm and red channel: 550–650 nm.

Experimental Section

Preparation of the test solutions

Naoh-Rh was dissolved in EtOH for a stock solution (1 mM). Test solutions were prepared by displacing 50 μL of the stock solution into a 10 mL volumetric flask. The solution was diluted to 10 mL in a mixture of phosphate buffer (0.05 M, pH 6) and EtOH (7:3, *v/v*). Small aliquots of each testing species solution were added. The resulting solutions were shaken well and incubated for 1 h at room temperature before recording spectra.

Cell culture and imaging

RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured as routine in DMEM medium containing 10% fetal bovine serum. All cells were maintained at 37 °C under humidified conditions and 5% CO_2. RAW264.7 cells were passed on small glasses and incubated for 24 h, and then incubated with LPS 1 μg mL^{-1} for 12 h. Before the staining experiment, cells were washed 3 times with PBS, incubated with 10 μM probe for 4 h, and then washed 3 times with PBS and underwent imaging measurements with a confocal micro-

scope (Zeiss LSM780, Carl Zeiss Canada) at 405 nm excitation. The emission of the blue channel was 405–550 nm and the red channel 550–650 nm.

Synthesis of compound 1: Dissolve 6-bromo-*N*,*N*-dimethylnaphthalen-2-amine (5.0 g, 20 mmol) in dry THF (100 mL) and transfer the solution into a pressure-equalizing dropping funnel. First, about 5 mL of the above solution was dropped into the reaction flask containing 480 mg magnesium under a N_2 atmosphere, and a little iodine was added to initiate the reaction. Then, the remainder of 6-bromo-*N*,*N*-dimethylnaphthalen-2-amine solution was slowly dropped into the reaction flask under a boiling state; the mixture was kept refluxing for 2 h after addition.

The above reaction solution was cooled to room temperature. Subsequently, CO_2 was bubbled into the reaction solution for 10 h, and then the mixture was poured into ice-water, extracted with dichloromethane, and purified by column chromatography to give a pale-yellow solid (2.3 g). Yield: 53.5%; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 12.60 (s, 1H), 8.38 (s, 1H), 7.89 (d, J = 9.2 Hz, 1H), 7.80 (dd, J = 8.6, 1.7 Hz, 1H), 7.68 (d, J = 8.7 Hz, 1H), 7.27 (dd, J = 9.1, 2.5 Hz, 1H), 6.96 (d, J = 2.2 Hz, 1H), 3.06 ppm (s, 6H); ^{13}C NMR (75 MHz, DMSO): δ = 167.67, 149.90, 137.00, 130.38, 130.13, 125.73, 125.47, 124.66, 123.18, 116.46, 104.78, 39.95 ppm; HRMS (ESI): *m*/*z*: calcd for $C_{13}H_{14}NO_2$: 216.1025 $[M+H]^+$; found: 216.1022.

Synthesis of compound 2: Compound **1** was added into thionyl chloride (3 mL) at 0 °C and kept stirring for 2 h. After the reaction completed, thionyl chloride was removed under reduced pressure to give a gray/white solid.

Synthesis of compound 3: Rhodamine B hydrazide (1.0 g, 2.2 mmol) and Lawesson reagent (890 mg, 2.2 mmol) were dissolved in dry toluene (40 mL). The reaction solution was heated and kept at reflux for 8 h under a N_2 atmosphere. Toluene was removed under reduced pressure and the crude compound was purified by column chromatography (petroleum ether/ethyl acetate = 4:1) to give a white solid (263 mg). Yield: 25.3%; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.94–7.83 (m, 1H), 7.62–7.46 (m, 2H), 7.05 (dd, J = 5.6, 2.7 Hz, 1H), 6.40 (d, J = 2.4 Hz, 2H), 6.37 (d, J = 2.5 Hz, 1H), 6.34 (d, J = 2.5 Hz, 1H), 6.17 (d, J = 8.8 Hz, 2H), 5.36 (s, 2H), 3.38–3.27 (m, 8H), 1.09 ppm (t, J = 6.9 Hz, 12H); ^{13}C NMR (100 MHz, DMSO): δ = 181.02, 153.33, 149.71, 149.13, 136.35, 132.43, 129.12, 128.20, 124.11, 123.40, 108.49, 103.76, 97.88, 73.13, 44.17, 12.89 ppm; HRMS (ESI): *m*/*z* calcd for $C_{28}H_{33}N_4OS$: 473.2375 $[M+H]^+$; found: 473.2402; *m*/*z*: calcd for $C_{28}H_{34}N_4OS$: 237.1227 $[M+2H]^{2+}$/2; found: 237.1237.

Synthesis of Naph-Rh: To the solution of compound **3** (118 mg, 0.25 mmol) in dry pyridine (5 mL), compound **2** (58.3 mg, 0.25 mmol) was added at RT under a N_2 atmosphere. The reaction solution was stirred at RT for 2 h, and was then heated and kept at reflux for 6 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (petroleum ether/ethyl acetate = 3:1) to give a pale-yellow solid (50.6 mg). Yield: 30.2%; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.60 (s, 1H), 8.09 (s, 1H), 8.01 (d, J = 6.6 Hz, 1H), 7.74 (d, J = 9.1 Hz, 1H), 7.68–7.48 (m, 4H), 7.24 (dd, J = 9.1, 2.3 Hz, 1H), 7.13 (d, J = 6.5 Hz, 1H), 6.91 (d, J = 1.9 Hz, 1H), 6.49 (d, J = 8.7 Hz, 2H), 6.34 (m, 4H), 3.33 (s, 8H), 3.03 (s, 6H), 1.07 ppm (t, J = 6.9 Hz, 12H); ^{13}C NMR (75 MHz, DMSO): δ = 164.53, 153.15, 149.51, 148.55, 136.31, 135.86, 132.92, 129.73, 129.65, 128.74, 128.20, 125.34, 125.11, 124.74, 124.35, 123.74, 116.50, 107.60, 104.81, 103.33, 96.99, 63.45, 59.71, 43.60, 12.40 ppm; HRMS (ESI): *m*/*z*: calcd for $C_{41}H_{44}N_5O_2S$: 670.3216 $[M+H]^+$; found: 670.3261; *m*/*z* $[M+2H]^{2+}$/2 calcd for $C_{41}H_{45}N_5O_2S$: 335.6647; found: 335.6663.

Synthesis of acceptor: To the solution of compound **3** (94 mg, 0.20 mmol) in dry pyridine (4 mL), benzoyl chloride (0.3 mL) was added at RT under a N_2 atmosphere. The reaction solution was stirred at RT for 2 h, and was then heated and kept at reflux for 6 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (petroleum ether/ethyl acetate = 4:1) to give a pale-red solid (31.7 mg). Yield: 28.2%; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 10.69 (s, 1H), 8.02 (d, J = 6.8 Hz, 1H), 7.95 (d, J = 7.4 Hz, 2H), 7.61 (d, J = 7.5 Hz, 3H), 7.50 (t, J = 7.4 Hz, 3H), 7.40 (d, J = 7.3 Hz, 1H), 7.15 (d, J = 6.6 Hz, 1H), 6.45 (d, J = 8.3 Hz, 1H), 6.35 (d, J = 8.3 Hz, 3H), 3.31 (d, J = 6.8 Hz, 8H), 1.07 ppm (t, J = 6.7 Hz, 12H); ^{13}C NMR (75 MHz, DMSO): δ = 167.23, 164.32, 153.20, 148.57, 135.97, 132.93, 132.78, 131.52, 130.71, 129.60, 129.19, 128.78, 128.49, 128.03, 127.70, 124.31, 107.63, 97.01, 43.60, 39.96, 12.36 ppm; HRMS: *m*/*z*: calcd for $C_{35}H_{37}N_4O_2S$: 577.2637 $[M+H]^+$; found: 557.2728; *m*/*z*: calcd for $C_{35}H_{38}N_4O_2S$: 289.1358 $[M+2H]^{2+}$/2; found: 289.1394.

Synthesis of Naph-A-Rh: Naph-A-Rh was synthesized according to the reported method.[48] Briefly, to the solution of potassium thiocyanate (97.2 mg, 1 mmol) in acetonitrile (20 mL), compound **2** (233 mg, 1 mmol) was added slowly at RT. After addition, the reaction solution was heated and kept at reflux for 45 min, and then filtered to remove KCl. Rhodamine B hydrazide (456.6 mg, 1 mmol) was added into the above filtrate, and kept at reflux for 2 h. Acetonitrile was removed under reduced pressure and the crude compound was purified by column chromatography (petroleum ether/ethyl acetate/triethylamine = 10:10:1) pale-yellow solid (357 mg). Yield: 50.1%; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 11.48 (s, 1H), 8.40 (s, 1H), 7.91 (d, J = 6.3 Hz, 1H), 7.82 (d, J = 9.2 Hz, 1H), 7.76 (dd, J = 5.8, 2.6 Hz, 2H), 7.66–7.57 (m, 3H), 7.27 (dd, J = 9.2, 2.4 Hz, 1H), 7.11 (d, J = 6.5 Hz, 1H), 6.93 (d, J = 2.1 Hz, 1H), 6.40 (d, J = 14.7 Hz, 6H), 3.34 (d, J = 4.1 Hz, 8H), 3.05 (s, 6H), 1.10 ppm (s, 12H); ^{13}C NMR (75 MHz, DMSO): δ = 167.95, 165.16, 153.02, 152.94, 151.77, 150.20, 148.58, 148.03, 137.13, 132.27, 129.53, 128.01, 127.59, 123.40, 122.04, 116.49, 107.71, 105.38, 103.21, 97.34, 64.66, 59.64, 43.58, 12.40 ppm; HRMS (ESI): *m*/*z*: calcd for $C_{42}H_{45}N_6O_3S$: 713.3274 $[M+H]^+$; found: 713.3264.

Acknowledgements

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Keywords: fluorescent probes · hypochlorous acid · naphthalene · rhodamine · through-bond energy transfer

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