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A near-infrared fluorescent probe for selective detection of HClO based on Se-sensitized aggregation of heptamethine cyanine dye†

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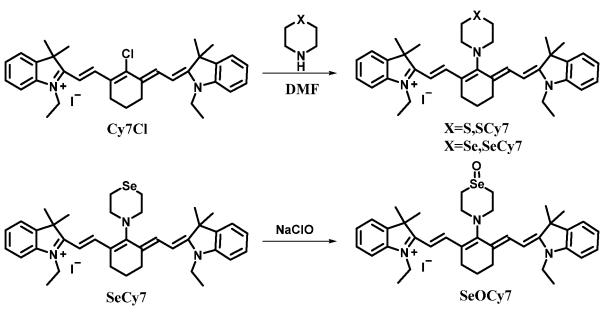
A Se-containing heptamethine cyanine dye based fluorescent probe was successfully developed and used for HClO detection with rapid response and high selectivity based on aggregation behavior. The probe could react with HClO with significant change in its fluorescence profile, which makes it practical for detecting HClO in fetal bovine serum and in living mice.

As a kind of reactive oxygen species (ROS), hypochlorite is widely employed in our daily life, such as in disinfection of drinking water, cooling-water treatment, household bleach, and as an antimicrobial.[1] However, once the used concentration of HClO cannot maintain the level within the range of 10^{-5}–10^{-2} M, high concentration of the hypochlorite solutions are a potential health hazard to plants, animals and even to human beings. On the other hand, endogenous hypochlorite is essential to life and has important antibacterial properties. Maintenance of appropriate concentrations of HClO is of great significance to the biosystems because excessive or misplaced generation of HClO can lead to severe tissue damage and several kinds of diseases (*e.g.* arthritis, lung injury, neuron degeneration and cancer[2]). These findings highlight the importance of HClO homeostasis. To determine the accurate concentration of HClO in environmental systems and monitor its *in vivo* site of action, fluorescent probes with high sensitivity, excellent selectivity and real-time capability to measure HClO are quite desirable. To develop satisfactory HClO probes, many research groups have come up with different frameworks to design selective probes for HClO determination.[3] Although these fluorescent molecular probes are able to detect HClO specifically, making HClO visible in living animals still arouses much attention.

Compared to light in the ultraviolet/visible (UV/vis) region, near-infrared (NIR) light is much more favorable for *in vivo* imaging due to its minimum photodamage to biological samples, good tissue penetration, and weak autofluorescence interference from the complicated living systems.[4] As an important kind of NIR dye, heptamethine cyanine dye, has been widely utilized to explore chemosensors for various inorganic and biologically related species.[5] The self-aggregation of dyes is a frequently encountered phenomenon, especially in terms of dyes used in aqueous solutions.[6] Though cyanine dyes belong to the best known self-aggregating dyes, this behavior, to the best of our knowledge, has seldom been applied in the design of HClO fluorescent probes. In addition, the selenium-containing structure which is reported to be selective to ROS is a good choice for developing a HClO probe.[7] It is noteworthy that selenium was introduced into the conjugated system of all such probes, which may contribute to the relatively long response time to some extent. Therefore, we hypothesized that a non-conjugated-selenium structure may be more sensitive to HClO, and will speed up the response time.[8]

Keeping all these ideas in mind, by introducing thiamorpholine and selenomorpholine into the structure of the heptamethine cyanine dye, we present two novel HClO probes **SCy7** and **SeCy7** based on the aggregation behavior of the heptamethine cyanine dye, using divalent sulfur and selenium as the redox-sensitive groups. After examining the properties of the two compounds, we found that **SeCy7** showed a much greater degree of aggregation, and was more sensitive to HClO. In this work, we report the synthesis of these two probes and the application of **SeCy7** in the measurement of HClO concentrations *in vitro* and its utility in HClO imaging in living mice.

The synthesis procedures of **SCy7** and **SeCy7** are shown in Scheme 1. **SCy7** and **SeCy7** were synthesized from the reaction of



Scheme 1 Synthesis of **SCy7**, **SeCy7** and the proposed recognition mechanism of **SeCy7** towards sodium hypochlorite.

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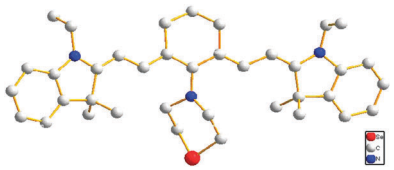

Fig. 1 Crystal structure of compound **SeCy7**.

compound **Cy7Cl** with thiamorpholine and selenomorpholine respectively in anhydrous DMF. The final compounds together with all the other intermediates (Fig. S13–S20, ESI†) were well characterized using ^{1}H NMR, ^{13}C NMR, ^{77}Se NMR (for **SeCy7**) and TOF-MS. And fortunately, we also obtained single crystals of **SeCy7** (CCDC number: 957815) by slowly evaporating its methanol solution at room temperature (Fig. 1). We then tested the aggregation behaviour of the two new compounds as well as the intermediate **Cy7Cl** in aqueous solution. As expected, upon increase of the concentration (5–30 μM), all the three dyes aggregated in PBS buffer (pH = 7.4, 20 mM), which could be easily illustrated by the changes of their absorption and emission spectra (Fig. S1–S3, ESI†). Surprisingly, **SeCy7** showed much weaker fluorescence intensity than that of **SCy7**. Such a low background enables **SeCy7** to be a better candidate for HClO detection.

Considering the positive effects of aggregation, we tested the optical properties of **SeCy7** in PBS buffer (pH = 7.4, 20 mM). UV/vis spectra of **SeCy7** (30 μM) exhibited two absorption peaks at around 695 nm and 752 nm, respectively. After being treated with NaClO (a commonly used HClO source, 0–2.0 equiv.), both of the two absorption peaks decreased drastically (Fig. S4, ESI†), showing that **SeCy7** can react with HClO efficiently. The changes of the emission spectra of **SeCy7** in the presence of HClO were also studied. The probe exhibited very weak fluorescence in aqueous solution. Upon addition of NaClO, the emission spectra at about 786 nm enhanced gradually and reached a plateau when 2.0 equiv. of NaClO was added (Fig. 2). At this point, a 19.4-fold fluorescence enhancement was observed. The whole recognition process finished within just dozens of seconds (Fig. S5, ESI†). Such a short response time will enable the real-time detection of HClO. Moreover, a good linear relationship (FL at 786 nm *vs.* HClO concentration) was obtained with the detection limit of 0.31 μM for HClO ($3\sigma/k$), indicating that **SeCy7** is suitable for quantitative detection of HClO (Fig. S6, ESI†).

The unique sensing performance of **SeCy7** to HClO drives us to clarify the detection mechanism. We deduced that **SeCy7** encountered serious aggregation in aqueous solution, thus showing very weak fluorescence. However, after the reaction with HClO, **SeCy7** was converted into **SeOCy7**. Meanwhile, the aggregated probe was gradually released and the fluorescence intensity boosted. To confirm the formation of quadrivalent selenium after addition of HClO to **SeCy7** solution, the ^{77}Se NMR spectra of **SeCy7** and the product after being treated with HClO are shown in Fig. S7 and S8 (ESI†). The resonance signal at 149 ppm was assigned to the bivalent selenium of **SeCy7** (Fig. S7, ESI†). After reaction with HClO, the signal corresponding to the quadrivalent selenium of **SeOCy7** at 783 ppm apparently emerged (Fig. S8, ESI†). TOF-MS has also been used to prove our hypothesis. Fig. S9 (ESI†) indicated that the formula of the main product was $C_{38}H_{43}N_3OSe$ (*m*/*z* calc. 642.2963, found 642.2991), which could be corresponded to **SeOCy7**.

Next, we examined the selectivity of **SeCy7** towards HClO and other reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}$OH), nitric oxide (NO$^{\bullet}$), *tert*-butyl hydroperoxide (TBHP), *tert*-butoxy radical (TBO$^{\bullet}$), superoxide (O_2^{-}), and singlet oxygen ($^1O_2$). In 20 mM PBS buffer, 2.0 equiv. NaClO as well as 20 equiv. other ROS were added respectively to a 30 μM solution of **SeCy7**. As shown in Fig. 3, **SeCy7** features a robust fluorescence turn-on response that is selective for HClO over other ROS. Other ROS including hydrogen peroxide, nitric oxide, *tert*-butyl hydroperoxide, *tert*-butoxy radical and superoxide did not lead to fluorescence turn-on responses. The excellent selectivity for HClO over other ROS shows that **SeCy7** has the potential to detect HClO in a complex biological environment. The pH titration curves (Fig. S10, ESI†) also reveal that the fluorescence intensity of **SeCy7** and the corresponding product after the treatment of NaClO remain almost stable at pH 4–10, demonstrating that **SeCy7** can work in the biological pH range without being influenced.

The favourable fluorescence properties of **SeCy7** for HClO prompted us to further establish its utility in the determination of HClO in biological samples. **SeCy7** was first evaluated to detect HClO in commercially available fetal bovine serum. Bovine serum containing NaClO with different concentrations

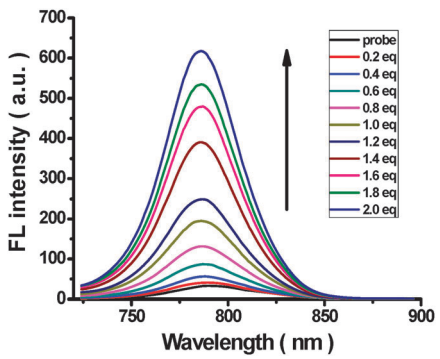

Fig. 2 Fluorescence emission spectra of **SeCy7** (30 μM) upon the addition of increasing concentrations of sodium hypochlorite (0–2.0 equiv.) in PBS buffer (pH 7.4, 20 mM). The arrows indicate the changes in the emission intensities with the increased sodium hypochlorite concentrations; λ_{ex} = 690 nm.

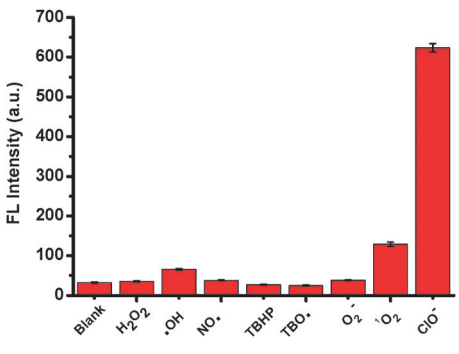

Fig. 3 Fluorescence responses of **SeCy7** (30 μM) to NaClO (2.0 equiv.) and other ROS (20.0 equiv. for H_2O_2, $^{\bullet}$OH, NO$^{\bullet}$, TBHP, TBO$^{\bullet}$, O_2^{-}, $^1O_2$) at 786 nm. Conditions: PBS buffer (pH 7.4, 20 mM). λ_{ex} = 690 nm (three times for each experiment).

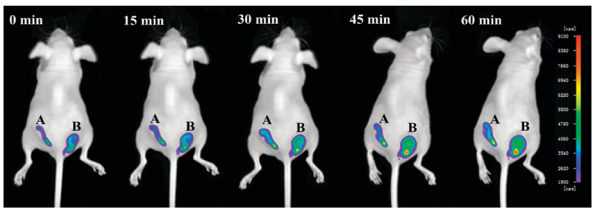

Fig. 4 Representative fluorescence images (pseudocolor) of a nude mouse given a skin-pop injection of **SeCy7** (25 μL, 60 μM, region A). Representative fluorescence images (pseudocolor) of a nude mouse given a skin-pop injection of LPS and PMA and a subsequent skin-pop injection of **SeCy7** (25 μL, 60 μM, region B). Images were taken after incubation for 0, 15, 30, 45 and 60 min, respectively. Images were taken using an excitation laser of 690 nm and an emission filter of 820 ± 20 nm.

(0, 5, 10, 15, 20, 25, 30, 35 and 40 equiv.) was prepared. These serum solutions were then incubated with 60 μM **SeCy7** at room temperature. Fig. S11 (ESI†) shows that the fluorescence intensity enhanced with the increased concentration of HClO, indicating that **SeCy7** is suitable for HClO detection in serum without addition of any co-solvents or detergents.

We then evaluated the suitability of the probe for visualizing HClO in living animals. Institute of cancer research (ICR) mice were selected. The mouse was given an s.p. (skin-pop) injection of **SeCy7** (60 μM, 25 μL DMSO) on the right leg, and 15 minutes later, the mouse was given an s.p. injection of 2.0 equiv. of NaClO in the same region. The mouse was imaged by using a NightOWL II LB983 small animal *in vivo* imaging system with a 690 nm excitation laser and an 820 ± 20 nm emission filter. Fig. S12 (ESI†) shows representative fluorescence images of an ICR mouse at different times after the injection of NaClO. It was demonstrated that the fluorescence intensity enhanced gradually within 40 minutes, proving that **SeCy7** can detect NaClO *in vivo* without the interference of background signals. More importantly, all these images were obtained from normal mice without unhairing because of the NIR absorption and emission properties of the probe.

Finally, we investigated the possibility of **SeCy7** detecting the endogenous HClO in living mice. In this case, nude mice were selected as our model. According to the previous literature, the synergistic effect of lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) can stimulate cells to produce HClO.[9] A solution of LPS (1 μg mL^{-1}) was injected into the back (region B) of the mouse, and 12 hours later, PMA was then injected into the same region. The probe (60 μM, 25 μL DMSO) was injected into the back of the mouse (region A and B) after 30 min of the above disposal. The pictures were taken under the imaging system after the mice were incubated for 0, 15, 30, 45 and 60 min, respectively. As shown in Fig. 4, the fluorescence intensities of region A were stable, while the signals at region B became stronger gradually within 60 min. The result established that **SeCy7** was a desired imaging agent for visualizing endogenous HClO *in vivo*. Taking these results together, it is established that **SeCy7** is suitable for detecting HClO in living animals.

In summary, we have developed a selective NIR fluorescent probe **SeCy7** for HClO based on the aggregation behavior of a heptamethine cyanine dye derivative. Upon addition of HClO, the fluorescence emission profile of **SeCy7** changed significantly. **SeCy7** showed a rapid fluorescence response that was selective for HClO over other related species. We have also compared the aggregation behavior of **SCy7** and **SeCy7**, and found that a Se-containing compound (**SeCy7**) showed a much greater aggregation degree and was more sensitive to HClO. Moreover, **SeCy7** could be used to detect HClO in commercial fetal bovine serum and make HClO visible in living mice.

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