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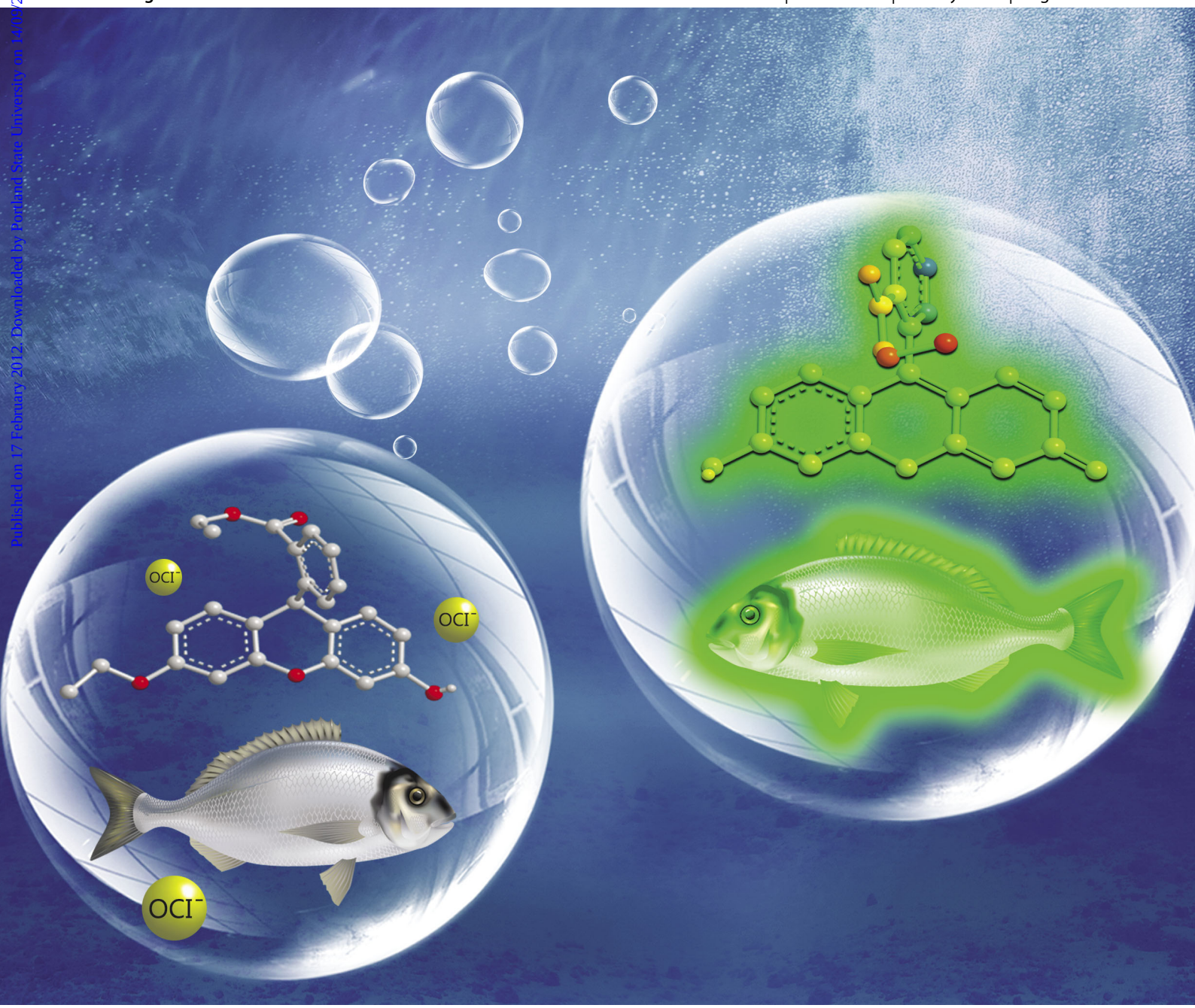

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COMMUNICATION

Fluorescence turn-on detection of hypochlorous acid *via* HOCl-promoted dihydrofluorescein-ether oxidation and its application *in vivo*†

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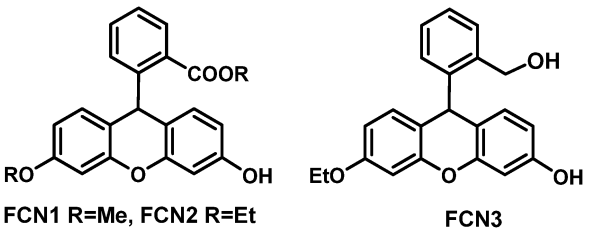
We report three new water-soluble dihydrofluorescein-ether probes FCN1, FCN2 and FCN3 for the detection of hypochlorous acid (HOCl), which were designed on the basis of a specific HOCl-promoted oxidation reaction. This work also provided a useful method to monitor the accumulated HOCl in specific organelles using a zebrafish model.

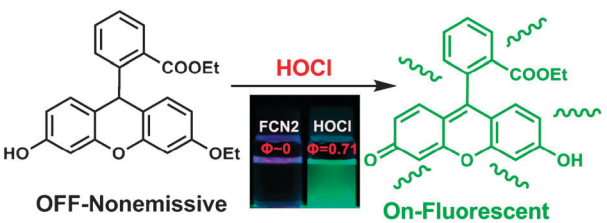
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) form as natural byproducts of the normal metabolism and play important roles in cell signaling and homeostasis.[1] ROS of particular interest is hypochlorous acid (HOCl, pK_a = 7.46), which is approximately half dissociated into the hypochlorite anion (OCl^-) under physiological conditions and is one of the most powerful natural oxidants.[2] Biologically, hypochlorous acid is produced by myeloperoxidase (MPO)-mediated peroxidation of chloride ions in activated neutrophils.[3] Although HOCl contributes to the destruction of bacteria in living organisms, the abnormal levels of HOCl caused by MPO have been implicated in several human diseases including neuron degeneration, cardiovascular diseases, osteoarthritis and renal disease.[4] Moreover, a specified amount of HOCl is used to treat food preparation surfaces and water supplies in daily life.[5] These biologically relevant findings motivate us to develop sensitive and specific probes for detecting HOCl in both water samples and living systems.

Compared with other detection methods, fluorescent probes have innate advantages including greater sensitivity, fast response time and simplicity of implementation, offering application methods not only for *in vitro* assays but also for *in vivo* imaging studies.[6] Recently, the known reported fluorescent probes for HOCl were designed based on the strong oxidation property of HOCl, thus *p*-methoxyphenol,[7] thiol,[8] alkoxyaniline,[2] dibenzoylhydrazine,[9] hydroxamic acid,[10] oxim derivatives[11] and oxazine conjugated nanoparticles[12] have been utilized as the components of HOCl-selective probes. Although those probes exhibit ideal properties in detecting HOCl quantitatively, fluorescent probes that show single-wavelength fluorescence enhancement (*turn-on*) response and work well in 100% *aqueous* physiological media are still a little scarce. In addition, although several examples have been focused on the studies of the HOCl level in living cells or endogenous HOCl in stimulated macrophage cells,[7,8] the rapid and specific methods for studies of the biological functions and deleterious effects of HOCl at the organ level *in vivo* have not yet been reported.



Scheme 1 Structures of probes **FCN1–FCN3**.

Probes based on the fluorescein platform possess several favorable properties such as excitation and emission wavelengths in the visible region as well as a high fluorescence quantum yield.[13] Therefore, we designed three probes (**FCN1–FCN3**, Scheme 1) with the dihydrofluorescein component that could selectively detect HOCl by a specific HOCl-promoted oxidation reaction, resulting in the photoswitch of the non-fluorescent deconjugated form to the strongly fluorescent conjugated form (Scheme 2). Moreover, we introduced a phenolic group and an ester group into **FCN1** and **FCN2** to increase the water solubility and modify the cell permeability. To the best of our knowledge, **FCN1–FCN3** are the first fluorescent probes for HOCl based on dihydrofluorescein in 100% *aqueous* media, and applied successfully in specific organelles using a zebrafish model.



Scheme 2 *Turn-on* sensing of HOCl *via* HOCl-promoted **FCN2** oxidation reaction (photos show fluorescence of **FCN2** in 100% *aqueous* media).

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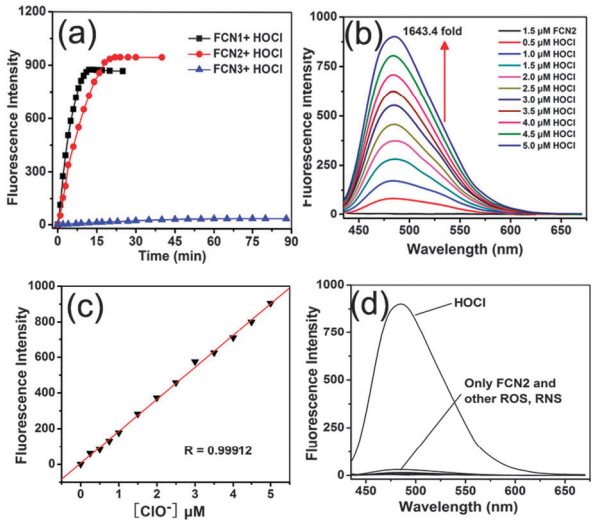

Fig. 1 (a) The time-dependent fluorescence changes acquired from reaction of **FCN1–FCN3** (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature. (b) Fluorescence spectra of **FCN2** (1.5 μM) in HEPES buffer recorded 30 min after the addition of HOCl (ranging from 0 to 5 μM). (c) The fluorescence enhancement at 485 nm as a function of HOCl concentration. (d) Fluorescence responses of **FCN2** (1.5 μM) with various ROS and RNS species (see ESI† for details): H_2O_2 (50 μM), $NaNO_2$ (200 μM), $ONOO^-$ (50 μM), $OH^\bullet$ (1 mM), $O_2^{\bullet-}$ (50 μM), NO (2 mM), HNO (100 μM) and $ROO^\bullet$ (200 μM).

The fluorescence intensity of **FCN1** and **FCN2** respectively, showed 6.5-fold and 2.4-fold enhancement upon exposure to 20 mM HEPES buffer in air for 24 h, indicating their auto-oxidation characteristics (Fig. S2, ESI†). **FCN3** did not show any auto-oxidation over more than 48 h due to its stability contributed by the benzenemethanol group. Upon interaction with HOCl, the fluorescence intensity of **FCN2** increased rapidly (Fig. 1b) and an approximately 1643.4-fold fluorescence enhancement after 30 min was observed (485 nm, $\Phi_f = 0.71$), which was extraordinarily sensitive compared to the previously reported probes (Table S3, ESI†). Similar fluorescence enhancements were observed for **FCN1** (1232.8-fold) and **FCN3** (31.6-fold) under the same conditions (Fig. 1a). Compared with **FCN1** and **FCN2**, **FCN3** had much longer time and less response increment to reach fluorescence intensity maximum. The auto-oxidation did not affect the HOCl detection because of the fast response time and the large fluorescence enhancement. In addition, the *pseudo*-first-order rate constants of **FCN1–FCN3** with HOCl were determined as $k' = 5.50 \times 10^{-3}$ s^{-1}, 2.54×10^{-3} s^{-1}, and 5.94×10^{-4} s^{-1}, respectively. Upon varying the concentrations of analyte, the second-order rate constant for the reaction of HOCl with **FCN2** (2.14×10^3 M^{-1} s^{-1}) was greater than that for the reaction of HOCl with **FCN1** (1.51×10^3 M^{-1} s^{-1}). Thus, among the three dihydrofluorescein-based probes, **FCN2** showed the best sensitivity for the detection of HOCl (order: **FCN2** > **FCN1** ≫ **FCN3**; Table S2, ESI†).

To further evaluate the specific nature of **FCN2** for HOCl, we examined the fluorescence enhancement of **FCN2** incubated with other biologically relevant ROS and RNS. Upon interaction with H_2O_2, NO_2^-, $ONOO^-$, $OH^\bullet$, $O_2^{\bullet-}$, NO, HNO and $ROO^\bullet$, the resulting solution only showed negligible fluorescent enhancement even when the amount of analyte is up to 1000 equiv. (Fig. 1d). Fluorescent titration experiments demonstrated that the fluorescence intensities of **FCN2** were linearly proportional to the concentration of HOCl in the range of 0.5–5 μM (Fig. 1c). The detection limit (S/N = 3) was also estimated to be 6.68 nM (0.71 ppb) on the basis of the signal-to-noise ratio (Fig. S3, ESI†), which is comparable to that of the fluorescence *turn-on* type probe for HOCl (Table S3, ESI†). Furthermore, the HOCl-promoted **FCN2** oxidation mechanism was supported by HPLC-MS in 50% CH_3CN/H_2O. In the presence of 10 equiv. of HOCl, a new peak appeared at 361.1 (*m*/*z*) in the spectrum, suggesting that the transformation from **FCN2** to FCN-Et was induced by HOCl.

Since HOCl in living cells or organisms is synthesized predominantly from hydrogen peroxide and chloride ions catalyzed by the MPO enzymatic system,[7] we employed NIH3T3 cells (mouse embryonic fibroblast cells) to examine the capacity of **FCN2** to detect the production of HOCl in a biological environment. Incubation of NIH3T3 cells with 5 μM **FCN2** in an MPO enzymatic system (MPO, 1.5 U per 100 mL; NaCl, 250 mM) for 30 min at 37 °C yielded negligible intracellular background fluorescence (Fig. 2b). Conversely, addition of 10 μM H_2O_2 to probe-loaded cells for further 30 min incubation elicited a significant enhancement in intracellular fluorescence (Fig. 2d). Also, **FCN2** exhibited rapid fluorescence response to HOCl in cells, and over 40% of the total fluorescence intensity increase was observed after 5 min (Fig. 2f). The overlay of fluorescence and bright-field images revealed that the fluorescence signals were localized in the whole cells (Fig. 2a and e). In addition, the MTT assays suggested that **FCN2** had almost no change in cell viability even when the amount of probe is up to 100 μM (Fig. S13, ESI†). Thus, all results indicated that **FCN2** was cell membrane permeable, nontoxic, and capable of detecting HOCl produced by MPO-mediated peroxidation of Cl^-.

Zebrafish has proved to be a highly valuable vertebrate model for *in vivo* imaging due to its transparent nature during all stages of embryonic development.[14] Therefore, the external HOCl in the embryo was tracked by **FCN2** at various time points during development. At 19 h post-fertilization (hpf),

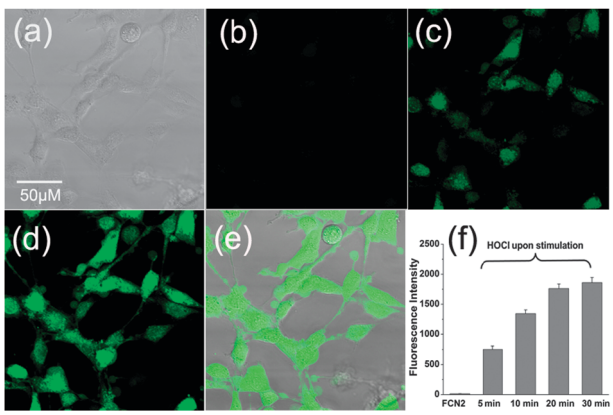

Fig. 2 DIC (a) and confocal fluorescence (b) images of NIH3T3 cells preincubated with NaCl (250 mM) and 5 μM **FCN2** in MPO (1.5 U per 100 mL); fluorescence images in the course of the production of HOCl stimulated with H_2O_2: incubated with H_2O_2 (10 μM) for 5 min (c) and 30 min (d); (e) overlay image of (a) and (d); (f) the average emission intensities of cells were analyzed by using IPP software.

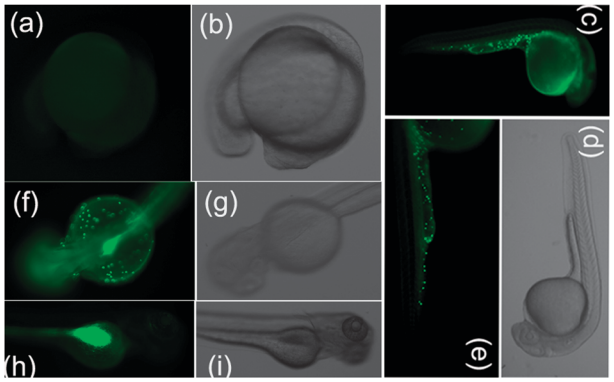

Fig. 3 Fluorescence microscopy images of AB/Tubingen larvae zebrafish incubated with 10 μM HOCl (E3 embryo media, 28.5 °C) during the development. 1-Phenyl-2-thiourea (PTU, 0.003%) was added to depress the development of pigment after 8 h of incubation. Fluorescence and DIC images of (a, b) 18 h-old, (c, d, e) 28 h-old, (f, g) 54 h-old, and (h, i) 78 h-old zebrafish further incubated with 5 μM **FCN2** for 1 h.

the embryo displayed nonspecific faint green fluorescence, conceivably because the protective chorion resisted **FCN2** from penetrating (Fig. 3a). During development, the fluorescence signal was brighter and a few bright green dots distributed in two zygomorphic areas around its yolk extension after 25 h of incubation (Fig. 3c and e). It was notable that the bright green dots moved to the top of the yolk sac at 54 hpf (Fig. 3f), which may be attributed to the accumulated HOCl by excess ingestion of some cells around yolk sac and yolk extension. At 78 h hpf, the bright green dots were no longer observed (Fig. 3h), and a significant green area was distributed in the yolk sac. There were no evident developmental defects in fish exposed to 10 μM HOCl (E3 embryo media) during fluorescence imaging.

HOCl is established in a clean digestion system associated with the decrease of sensitivity to airborne pathogens and pollutants, and an increased ability to modulate infections and pathogenic challenges.[15] However, chemosensor-based methodology for monitoring the accumulated HOCl in living organisms has never been demonstrated, which makes it difficult to assess the deleterious effects of HOCl. We explored further whether **FCN2** could be used to monitor the accumulated HOCl. An adult zebrafish (3 months old with identifiable organs) was exposed to 100 μM HOCl in E3 embryo media for 24 h for accumulating HOCl in organs, and then incubated with 5 μM **FCN2** for 30 min. Intriguingly, the fluorescence signals were detected in the eye, eggs, intestine, and liver of the zebrafish (the highest intensity was observed in the gall bladder), but not in the brain and heart after a 24 h accumulation period (Fig. 4a). These preliminary studies are informative for the accumulated HOCl level in the order: gall bladder > intestine > eye > liver > eggs (Fig. 4b).

In summary, we have developed three water-soluble dihydrofluorescein-ether probes **FCN1–FCN3** on the basis of a specific HOCl-promoted oxidation reaction. Significantly, **FCN2** showed a fast response time (~20 min), meanwhile it displayed the larger fluorescence enhancement (1643.4-fold) and an extraordinarily lower detection limit (0.71 ppb) compared to the previously reported probes. **FCN2** can not only visualize HOCl produced by MPO-mediated peroxidation of chloride ions

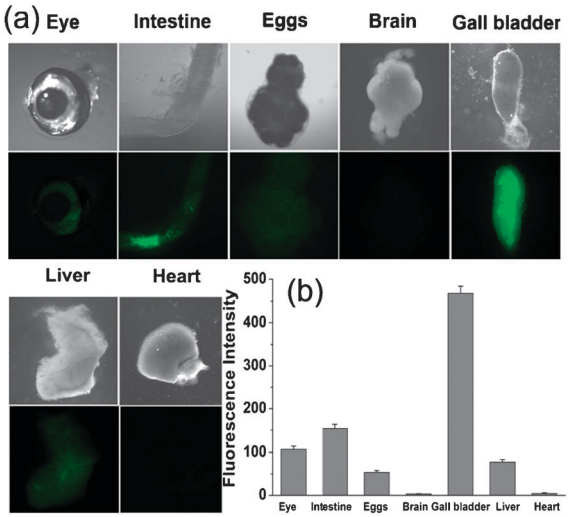

Fig. 4 (a) Adult zebrafish were exposed to 100 μM HOCl in E3 embryo media for 24 h at 28.5 °C and then incubated with 5 μM **FCN2** for 30 min. (b) The average emission intensities of isolated organs were analyzed by using IPP software.

in living cells, but also estimate the accumulated HOCl level in zebrafish organs. This methodology may also be used as an alternative tool for assessing the deleterious effects of HOCl.

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