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Development of ratiometric near-infrared fluorescent probes using analyte-specific cleavage of carbamate†

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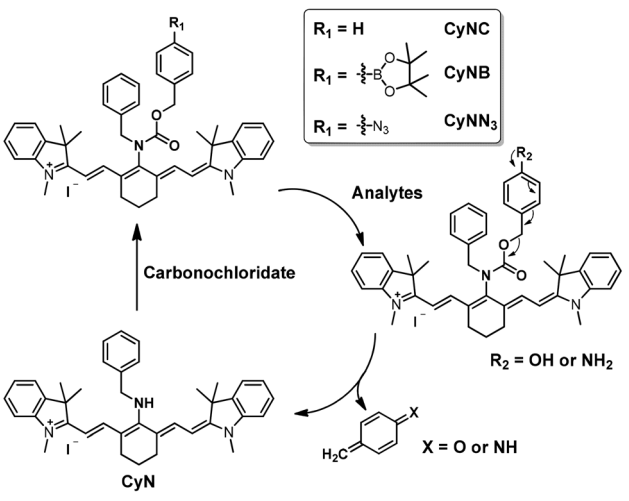
We report a facile method to design NIR ratiometric fluorescent probes in terms of analyte-induced carbamate cleavage. Two examples, CyNB and $CyNN_3$, exhibit a significant analyte-triggered response with ratiometric fluorescence change in the NIR range and dual-emission ratiometry in living cells.

Fluorescence imaging is a very powerful method for monitoring biomolecules in living systems because of its high sensitivity and selectivity, facility and fast response.[1] Recently, utilizing ratiometric fluorescent probes to analyze biological species has been drawing intensive attention. This type of probe, importing a built-in correction *via* the ratio of the emission intensity at two different wavelengths, can provide quantitative information on analytes.[2] Hence, a number of ratiometric fluorescence probes have been developed based on small molecules, biological molecules, conjugated polyelectrolytes or nanoparticles, *etc*.[3] However, most of them have absorption and emission in the ultraviolet or visible range, and are not adaptable to *in vivo* imaging and quantification. Near-infrared (NIR) dyes provide a feasible opportunity for *in vivo* imaging due to many distinct advantages, including deeper tissue penetration, minimum photodamage to biological samples, and minimum interference from background autofluorescence in the living systems.[4] Therefore, development of ratiometric fluorescent probes based on NIR dyes will contribute useful molecular tools for quantitative measurements *in vivo*.

Among NIR dyes, amine-substituted tricarbocyanine has been proved to be an excellent candidate for designing NIR probes through modifying the amino group with well-defined receptors.[5] The photophysical properties of this type of cyanine highly depend on the electron density of the *meso*-nitrogen atom due to the internal charge transfer (ICT). When such an ICT process was affected by a certain sensing event, sharp changes in absorption/fluorescence spectra were observed.[6] With these facts in mind, we envision that modifying the amine substituent of tricarbocyanine with an electron-withdrawing carbamate group would efficiently lower the electron density of the amine and could, in principle, give out an obvious bathochromic shift of fluorescence emission compared to that of normal amine-substituted tricarbocyanines. The concept will provide a rationale for molecular design of a generic model to construct NIR ratiometric probes by taking advantage of a chemospecific cleavage of the caged carbamate. Therefore, in this paper, we report the synthesis and photophysical properties of several carbamate-substituted tricarbocyanines and further evaluation of the performances of two NIR ratiometric probes utilizing this generic model (Scheme 1).

We initially synthesized benzylamine-substituted tricarbocyanine (CyN) and modified the *meso*-nitrogen atom with a carboxybenzyl group (CyNC) (Scheme 1). The photophysical properties of both CyN and CyNC are shown in Fig. 1 and



Scheme 1 Design strategy for NIR ratiometric fluorescent probes.

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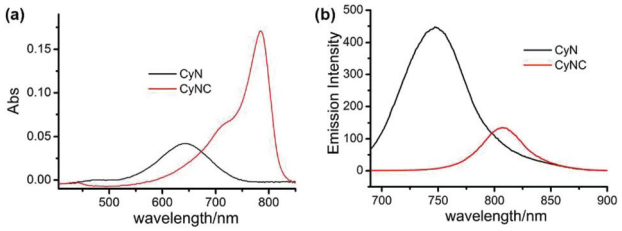

Fig. 1 (a) Absorption and (b) emission spectra of 1 μM CyN (black line) and 1 μM CyNC (red line) in a mixture of H_2O–DMSO (9 : 1, v/v, 10 mM HEPES, 100 mM NaCl, pH = 7.4) λ_{ex} = 671 nm.

Table 1 Photophysical data of CyN and CyNC[a]

Dye	λ_{abs}/nm	ε_{max} [b]/×10^5 M^{-1} cm^{-1}	λ_{em}/nm	Φ[c]
CyN	645	0.42	747	0.062
CyNC	785	1.71	808	0.044

[a] 10 mM HEPES, 100 mM NaCl, 10% (v/v) DMSO, pH = 7.4. [b] Molar extinction coefficients are in the maximum of the highest peak. [c] Cardiogreen was used as the standard for quantum yield measurements (Φ = 0.13 in DMSO).[7]

Table 1. We found that benzylamine-substituted tricarbocyanine, CyN, exhibits a major absorption maximum at 645 nm and a characteristic NIR fluorescence emission maximum at 747 nm (Φ = 0.062) very similar to other amine-substituted tricarbocyanine analogues.[5] As expected, the electron-withdrawing carboxybenzyl group in CyNC induces remarkable bathochromic shifts in both absorption of 140 nm and emission of 61 nm, which is consistent with the ICT process involved in the *meso*-nitrogen atom of CyNC. Hence, these features afford an excellent opportunity to design a generic model or scaffold for single-excitation, dual-emission NIR ratiometric probes through specific chemoselective triggers.

To confirm the feasibility of our concept, we subsequently tried to introduce diverse caging groups into the cleavable carbamate group. It is well known that H_2O_2 and H_2S are small-molecule signal transducers and play vital roles in physiological and pathological processes in living organisms.[8] Developments of fluorescent probes for *in vivo* detection and visualization of H_2O_2/H_2S are still challenging research topics of chemistry and biochemistry. Recent disclosures show that reaction-based approaches are very efficient for designing H_2O_2/H_2S probes by taking advantage of caging groups, such as boronic acid/ester or azide, which can be specifically reacted with H_2O_2 or H_2S.[9,10] On the other hand, on the basis of the fact that the *p*-hydroxybenzyl or aminobenzyl moiety is able to self-immolate through an intramolecular 1,6-elimination,[11] we reason that importing an analyte-responsive group to the carbamate of CyNC could make the caged CyNC responsive to a particular molecule while not affecting the photophysical properties of the fluorophore. If the cleavage process was triggered by a specific analyte so as to release the NIR dye of CyN, remarkable ratiometric fluorescence signals would be obtained (Scheme 1 and Table 1). Herein, we demonstrate the

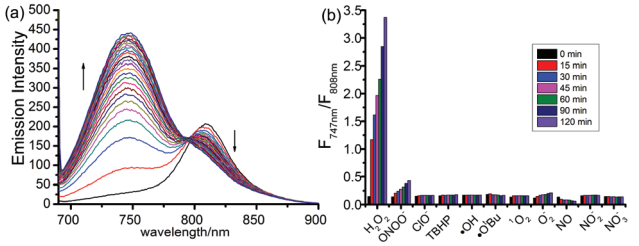

Fig. 2 (a) Fluorescence response of 2 μM CyNB to 300 μM H_2O_2. Spectra were acquired every 5 min after H_2O_2 was added. (b) Fluorescence responses of 2 μM CyNB to various ROS and RNS (300 μM). Bars represent emission intensity ratios $F_{747\ nm}/F_{808\ nm}$ at 0, 15, 30, 45, 60, 90, and 120 min after addition of each ROS or RNS. Data were acquired in a mixture of H_2O–DMSO (9 : 1, v/v, 10 mM HEPES, 100 mM NaCl, pH = 7.4), λ_{ex} = 676 nm.

evaluation of our design concept through two analyte-responsive NIR ratiometric probes CyNB and $CyNN_3$.

The probes can be easily obtained through the reaction between CyN and the corresponding carbonochloridate (see the ESI†). As shown in Fig. 2, we assessed the photophysical properties of CyNB in a mixture of H_2O–DMSO (9 : 1, v/v, 10 mM HEPES, 100 mM NaCl, pH = 7.4). CyNB exhibits a maximal absorbance at 785 nm ($\varepsilon = 1.49 \times 10^5$ M^{-1} cm^{-1}) and a characteristic emission maximum at 808 nm (Φ = 0.036), which are almost the same as those of CyNC. Treatment of CyNB with H_2O_2 in a time period of 2 h resulted in remarkable blue shifts of 140 nm in absorption and 61 nm in fluorescence spectra (Fig. 2a and Fig. S1a†). These spectral changes clearly demonstrate that the self-immolative process was triggered by H_2O_2 and subsequently liberated the NIR dye, CyN with a characteristic of much shorter wavelengths in both absorption and emission spectra (Table 1). The ratio of emission intensities ($F_{747\ nm}/F_{808\ nm}$) varied from 0.15 to 3.4 in the reaction with H_2O_2, indicating that CyNB is an excellent NIR dual-emission ratiometric fluorescent probe for H_2O_2 (R/R_0 up to 22). Kinetic measurement of CyNB (1 μM) with H_2O_2 (1 mM) under the pseudo-first-order conditions gave an observed rate constant of $k_{obs} = 1.26 \times 10^{-3}$ s^{-1} (Fig. S3a†). At low concentration of H_2O_2 (30 μM), CyNB also exhibited distinct fluorescence ratiometric response, but with a slower reaction rate (Fig. S4†). Further, the selectivity of CyNB for H_2O_2 was also examined in the presence of other biologically relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as $ONOO^-$ (peroxynitrite), OCl^-, TBHP (*tert*-butylhydroperoxide), $^{\bullet}OH$, $^{\bullet}O^tBu$, $^1O_2$, $O_2^{\bullet-}$, NO, NO_2^- and NO_3^- under the identical conditions. Due to the high H_2O_2-specificity of boronate groups, the ratiometric emission response of CyNB to H_2O_2 is highly selective. However, other ROS/RNS exerted no obvious fluorescence ratio response (Fig. 2b). These results suggest that the sensing scaffold of CyN/CyNB is practicable for NIR ratiometric H_2O_2-selective probes in aqueous media.

We further test the feasibility of our concept using another NIR probe, $CyNN_3$, containing an azide group which is highly specific to H_2S.[10] The spectroscopic properties of $CyNN_3$ were measured in a mixture of H_2O–CH_3CN (8 : 2, v/v, 40 mM

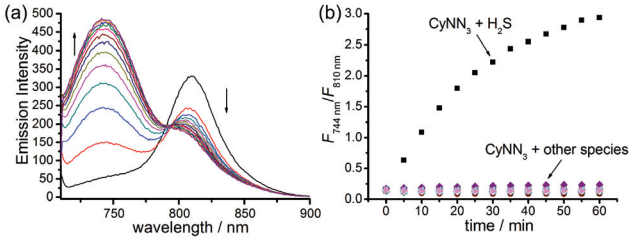

Fig. 3 (a) Fluorescence response of 2 μM $CyNN_3$ to 4 mM H_2S. Spectra were acquired every 5 min after H_2S was added. (b) Fluorescence responses of 2 μM $CyNN_3$ to various relevant species for 1 h. Data shown: H_2S, Cl^-, Br^-, I^-, AcO^-, N_3^-, SCN^-, HCO_3^-, HPO_4^{2-}, H_2O_2, TBHP, O_2^-, NO, NO_2^-, NO_3^-, HSO_3^-, SO_3^{2-}, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, Cys, GSH, and Hcy at 4 mM. Data were acquired in a mixture of H_2O–CH_3CN (8 : 2, v/v, 40 mM HEPES, pH = 7.4), λ_{ex} = 698 nm.

HEPES, pH = 7.4). Again, we found the tuning of the caging site in $CyNN_3$ did not apparently affect the photophysical properties of the fluorophore of CyNC. $CyNN_3$ shows a maximal emission at 810 nm (Φ = 0.033) in the absence of H_2S upon excitation at 698 nm. By treating with 4 mM H_2S (Na_2S was used as a hydrogen sulfide source in all experiments),[10b] the fluorescence spectrum shows a gradual decrease at 810 nm with a concomitant increase at 744 nm (Fig. 3a). These spectroscopic changes are similar to those of CyNB. The ratio of emission intensities ($F_{744\ nm}/F_{810\ nm}$) varied from 0.16 to 2.9 in response to H_2S during 1 h, and *ca.* 18-fold emission ratio change was thereby obtained, indicating that a self-immolative process can also be triggered by H_2S. Under the pseudo-first-order conditions (2 μM $CyNN_3$, 4 mM H_2S), the observed rate constant was measured to be 9.91×10^{-4} s^{-1} (Fig. S3b†). When $CyNN_3$ was treated with a low concentration of H_2S (0.4 mM), a distinct fluorescence ratiometric response but with a slower reaction rate was observed (Fig. S5†). The fluorescence responses of $CyNN_3$ to other biological species were also investigated in parallel under the identical conditions. As shown in Fig. 3b, the ratiometric emission response of $CyNN_3$ is highly selective for H_2S over other anions, ROS, RNS, reactive sulfide species (RSS), and, in particular, different thiols. The results indicate that $CyNN_3$ is a good candidate to selectively detect H_2S in aqueous solution.

Moreover, in order to verify the practicability of our tricarbocyanine scaffold for intracellular imaging, we also measured the fluorescence spectra of CyNB, $CyNN_3$, and CyN at different pH values. Emission intensities and ratio calibrations remain almost constant in the range of pH 6–8 (Fig. S6†). This evidence indicates that both CyNB and $CyNN_3$ are pH-independent in the physiological pH range and are suitable for ratiometric detection of H_2O_2 and H_2S in living cells, respectively.

We next applied CyNB and $CyNN_3$ to confocal fluorescence ratiometric imaging of H_2O_2 and H_2S in living cells, respectively. The fibroblast NIH 3T3 cells were incubated with 5 μM CyNB in serum-free DMEM (0.5%, v/v, DMSO) for 30 min at 37 °C, and then washed with PBS three times before imaging. Upon excitation at 635 nm, both emission channels of 700–770 nm and 780–800 nm displayed evident fluorescence

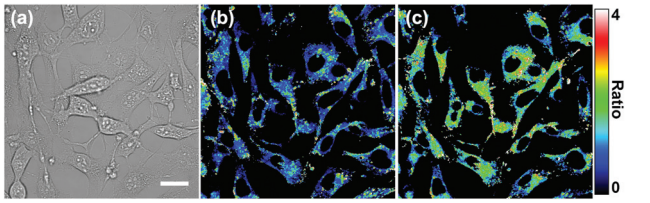

Fig. 4 Fluorescent imaging of H_2O_2 in CyNB-labeled NIH 3T3 cells. (a) Bright-field image; (b) cells were stained with 5 μM CyNB at 37 °C for 30 min; (c) CyNB-labeled cells were treated with 100 μM H_2O_2 for 30 min at 25 °C. λ_{ex} = 635 nm, scale bar: 20 μm, ratio bar: 0–4.

signals (Fig. S7†). Accordingly, the pseudocolor ratio image ($Em_{700–770\ nm}/Em_{780–800\ nm}$) gave an average value of 0.87 ± 0.12 (Fig. 4b) by using the FV10-ASW software (Olympus, Ver. 2.1c). When the cells were treated with 100 μM H_2O_2 at 25 °C for 30 min, significant enhancements in the ratio were obtained with an average value of 1.63 ± 0.13 (Fig. 4c). These are consistent with the observations in aqueous buffer, indicating that CyNB can visualize H_2O_2 in living cells by dual-emission ratiometry under biological conditions. We then tested the ability of $CyNN_3$ to detect intracellular H_2S in living cells (Fig. 5 and Fig. S8†). Similarly, $CyNN_3$ is also cell-permeable and can stain the NIH 3T3 cells in the same optical windows. The ratio imaging ($Em_{700–770\ nm}/Em_{780–800\ nm}$) gave an average value of 1.20 ± 0.14 (Fig. 5a). Treatment of $CyNN_3$-loaded cells with 1 mM H_2S for 30 min at 37 °C led to a sharp increase in the ratio to 2.05 ± 0.28 (Fig. 5b). Moreover, in the presence of $ZnCl_2$ (an efficient eliminator of H_2S),[10b,g] addition of H_2S did not obviously induce the increase of the fluorescence ratio (1.16 ± 0.13, Fig. 5c). The data establish that $CyNN_3$ is capable of imaging intracellular H_2S in living cells.

In summary, we have developed a new tricarbocyanine-based scaffold for constructing NIR ratiometric fluorescent probes by specific analyte-induced cleavage of carbamate. Accordingly, we prepared two NIR ratiometric probes: CyNB for H_2O_2 and $CyNN_3$ for H_2S. By highlighting the significant ratiometric fluorescence signal output in aqueous solution and dual-emission ratiometry in living cells of both probes, we expect that this design concept can contribute an effective method for development of NIR probes through a simple modulation of the caging site on the carbamate moiety.

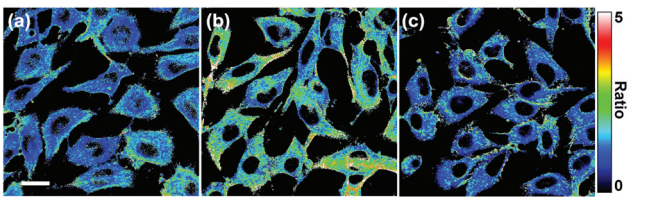

Fig. 5 Fluorescent imaging of H_2S in $CyNN_3$-labeled NIH 3T3 cells. (a) Cells were stained with 5 μM $CyNN_3$ at 37 °C for 30 min and further incubated in serum-free DMEM for 30 min; (b) $CyNN_3$-labeled cells were treated with 1 mM H_2S for 30 min at 37 °C; (c) cells were pretreated with 2 mM $ZnCl_2$ for 30 min, then incubated with 5 μM $CyNN_3$ for 30 min, and further treated with 1 mM H_2S for 30 min at 37 °C. λ_{ex} = 635 nm, scale bar: 20 μm, ratio bar: 0–5.

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