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Activatable fluorescence probes for hydrolase enzymes based on coumarin-hemicyanine hybrid fluorophore with large Stokes shift

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We show that the equilibrium of intramolecular spirocyclization of coumarin-hemicyanine hybrid fluorophores can be finely tuned by means of chemical modifications. We used this scaffold to develop activatable fluorescence probes with large Stokes shifts for γ-glutamyltranspeptidase and esterase.

Hydrolase enzymes play crucial roles in many biological processes and in diseases such as cancer development and metastasis[1-3]. Thus, visualizing these activities is important for understanding the functions of hydrolases in biological and pathophysiological contexts. Although various types of fluorescence probes for detecting hydrolase activities have been developed based on a series of fluorophores with emission in the visible to near-infrared (NIR) wavelengths,[4] the relatively small Stokes shifts of the fluorophores sometimes result in severe overlapping between the excitation and emission spectra, leading to poor signal-to-noise ratios. Thus, in general, a larger Stokes shift is advantageous to achieve sensitive detection.

The coumarin-hemicyanine (CHC) fluorophore is a hybrid structure of coumarin and hemicyanine[5, 6], which shows the distinct photophysical properties such as red-shifted fluorescence emission wavelength (> 620 nm), relatively large Stokes shift (>60 nm), and suitability for ratiometric measurement. Thus, CHC hybrid fluorophore has been utilized as the core fluorophore of probes targeting reactive oxygen species[7-9], cyanide[10, 11], hydrogen sulphide[12, 13], and sulfur dioxide[14, 15], as well as in a reagent inducing reductive stress[16]. In this work, we set out to develop new fluorescence probes with large Stokes shifts, targeting hydrolases, based on intramolecular spirocyclization reaction of CHC hybrid fluorophores.

Intramolecular spirocyclization reaction is a ground-state equilibrium between a spirocyclic form (closed form) and an intact fluorophore form (open form) which has been successfully utilized as a fluorescence switching mechanism of rhodamine-based fluorescence probes[17-20]. It has also been reported that CHC hybrid fluorophore bearing *p*-nitrophenol exists in spirocyclic form, and has been used as a photoactivatable fluorophore for super-resolution imaging[21], due to its ability to be converted to an open fluorescent form upon photoirradiation, followed by the thermal reversion to the original spirocyclic form. We speculated that the spirocyclic behavior of the CHC hybrid fluorophore might be altered by means of chemical

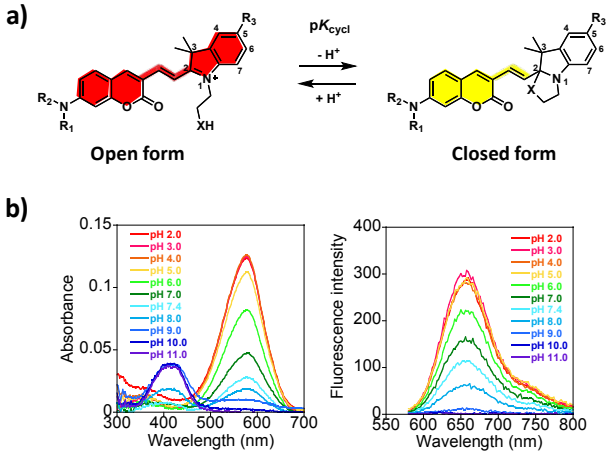

Fig. 1 (a) Chemical structures of the developed coumarin-hemicyanine (CHC) hybrid fluorophores bearing intramolecular nucleophiles, which undergo thermally equilibrating intramolecular spirocyclization in aqueous solution (equilibrium constant: pK_{cycl}). (b) Absorption and fluorescence spectra of 2 μM solutions of CHC-1 at various pH values in 0.2 M sodium phosphate buffer.

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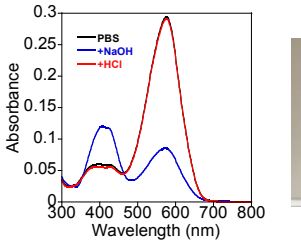
modification. Firstly, we prepared **CHC-1**, which has a hydroxyethyl (HE) group at the indolenium N1 atom as a potential intramolecular nucleophile (Figure 1a, Scheme S1). **CHC-1** exhibited absorption/emission maxima at 576/660 nm with a Stokes shift of 84 nm in aqueous buffer at pH 2.0. Ratiometric changes in absorption spectra were observed as the pH changed from acidic to alkaline: the absorbance at 576 nm decreases while that at around 400 nm increases. The appearance of blue-shifted absorption at around 400 nm at alkaline pH strongly suggests the generation of an intramolecularly spirocyclized structure with a divided π-conjugation of the fluorophore (Figure 1b, Table 1). According to previous reports, nucleophiles such as cyanide or phosphine can intermolecularly attack the CHC hybrid fluorophore at the indolenium C2 atom or at the carbon-carbon double bond of the hemicyanine unit[10, 16], and a *p*-nitrophenyl group intramolecularly attacks the indolenium C2 atom[21]; both reactions result in a significant blue-shift of the optical properties. In order to identify the structure of the spirocyclic form of **CHC-1**, ^{1}H-NMR spectrum of **CHC-1** was measured after washing with NaOH aq. to accelerate spirocycle formation. We observed two distinct proton peaks with a large coupling constant (15.9 Hz) which derives from the carbon-carbon double bond of the hemicyanine unit, providing direct evidence of nucleophilic attack of HE group at the indolenium C2 atom of **CHC-1** (Figure S1).

In order to test the reversibility of the pH-dependent transition between the π-extended open form and the spirocyclic form, changes in the absorbance of **CHC-1** were monitored (Figure 2). The distinctive absorbance at 576 nm derived from the π-conjugated CHC hybrid decreased after addition of NaOH aq, and subsequent addition of HCl aq. resulted in complete recovery of the absorbance, confirming the reversibility of the spirocyclization. Next, we determined the equilibrium constant of intramolecular spirocyclization, pK_{cycl}, which is defined as the pH at which the absorbance of the compound decreases to a half of the maximum absorbance as a result of spirocyclization[22]. The pK_{cycl} value of **CHC-1** was calculated to be 6.5 from the pH titration curve, indicating that **CHC-1** exists predominantly in its spirocyclic form at the physiological pH of 7.4 (Figure S2c).

Further, we examined whether the pK_{cycl} values of CHC hybrids can be modulated by changing the nucleophilicity of the intramolecular nucleophile; for this purpose, we prepared **CHC-2** and **CHC-3** bearing an aminoethyl (AE) and a mercaptoethyl (ME) group, respectively (Table 1, Schemes S2, S3). **CHC-2** showed pH-dependent optical properties similar to those of **CHC-1**, but its pK_{cycl} value was shifted to the more acidic side (pK_{cycl} = 5.4, Figure S2a,c). In contrast, **CHC-3** showed little absorbance at around 600 nm and almost constant absorbance at 400 nm at all pH values examined, suggesting that it mainly exists in its spirocyclic form throughout this pH range (Figure S2b,c). These results are in accordance with our previous findings with rhodamine scaffolds that the spirocyclic structure is increasingly stabilized as the nucleophilicity of the intramolecular nucleophile increases, shifting the pK_{cycl} value to the acidic side[23, 24].

Next, we examined whether the pK_{cycl} values of CHC hybrids can also be modulated by the electron-withdrawing/donating properties of substituents on the hybrid structure, in order to identify a suitable scaffold for developing fluorescence probes for hydrolases. As target hydrolases, we first focused on aminopeptidases, a family of proteases that cleave the N-terminal amino acid of peptides or proteins. Thus, we prepared **CHC-4** with an amino group at the indolenium C5 atom and **CHC-6** with an amino group in the coumarin unit (Table 1, Scheme S4, S5). We also



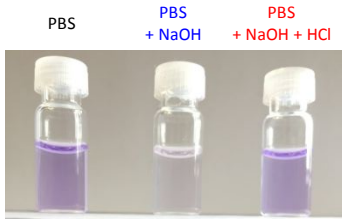
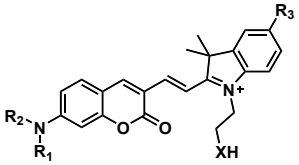


Fig. 2 Reversibility of the pH-dependent intramolecular spirocyclization in aqueous solution. 1 N NaOH aq (3 μL) was added to 2 μM CHC-1 solution in PBS. Then 1 N HCl aq (3 μL) was added.

Table 1 Photophysical properties of the CHC hybrid fluorophore [a]



	XH	NR_1R_2	R_3	λ_{abs}[nm][b]	λ_{em}[nm][b]	Δλ [nm]	pK_{cycl}	Φ_{fl} [b]
CHC-1	OH	NEt_2	H	576	660	84	6.5	0.040[c]
CHC-2	NH_2	NEt_2	H	596	662	66	5.4	0.021[c]
CHC-3	SH	NEt_2	H	-	-	-	< 2	-
CHC-4	OH	NEt_2	NH_2	590	664	74	7.6	0.018[c]
CHC-5	OH	NEt_2	NHCOMe	586	668	82	7.3	0.037[c]
CHC-6	OH	NH_2	H	520	616	96	7.5	0.026[d]
CHC-7	OH	NHCOMe	H	385	585	200	6.8	0.010[e]
CHC-8	OH	NEt_2	CO_2H	600	666	66	8.1	0.024[c]
CHC-9	OH	NEt_2	CONHMe	596	668	72	6.4	0.025[c]

[a]Absorption maximum (λ_{abs}), fluorescence emission maximum (λ_{em}), Stokes shift (Δλ), equilibrium constant for intramolecular spirocyclization (pK_{cycl}) and absolute quantum yield (Φ_{f1}). [b] Φ_{f1} of the open form measured in 0.2 M sodium phosphate buffer (pH 2.0). [c-e]Excitation wavelength was [c]570 nm, [d]520nm, and [e]400 nm.

prepared their acetylated derivatives (**CHC-5** and **CHC-7**) as models of aminopeptidase substrates. We observed little difference in pK_{cycl} values between **CHC-4**/**CHC-5** (7.6 and 7.3, respectively, Figure S3), and the fluorescence of the open form of **CHC-4** is quenched in the physiological pH range, suggesting that **CHC-4** is not a suitable candidate scaffold for fluorogenic probes for aminopeptidases. In contrast, we observed a modest difference in pK_{cycl} values between **CHC-6**/**CHC-7** (7.5 and 6.8, respectively, Figure S4); this implies that 20% of **CHC-7** and 56% of **CHC-6** exist in the open form at pH 7.4 giving an expected fold change due to the spirocyclization of 2.8. This is not sufficiently large for obtaining highly fluorogenic probes for aminopeptidases. However, we noticed that significant fluorescence activation at pH 7.4 could be obtained by utilizing the blue-shift in the absorption of the open form upon amidation (from **CHC-6** to **CHC-7**), together with some contribution from a change in the fluorescence quantum yield of the open forms (Φ_{fl} = 0.026 for **CHC-6** and 0.010 for **CHC-7**). Thus, we next prepared **gGlu-CHC** by replacing the acetyl group of **CHC-7** with a γ-glutamyl moiety as a probe for γ-glutamyltranspeptidase (GGT), which is known to be overexpressed in various types of cancer and so is of interest as a biomarker enzyme for fluorescence imaging of cancer (Figure S5, Scheme S6)[25, 26]. **gGlu-CHC** showed similar optical properties to those of **CHC-7** (pK_{cycl} = 6.9), and the fluorescence intensity of **gGlu-CHC** increased dramatically upon reaction with GGT (Figure S5c). We also confirmed that **gGlu-CHC** was hydrolysed by GGT to **CHC-6** as a fluorescent hydrolysis product, which shows a large Stokes shift of 96 nm (Figure S5d). Further, when we applied **gGlu-CHC** to human lung carcinoma cell line A549, which highly expresses GGT[27], we observed strong fluorescence activation, which was suppressed by co-incubation with a GGT inhibitor (Figure S6). Further, we saw no marked fluorescence activation in H226 cells, which have low GGT expression. These results indicate that **gGlu-CHC** works as a fluorogenic probe with a large Stokes shift to visualize GGT activity in live cells.

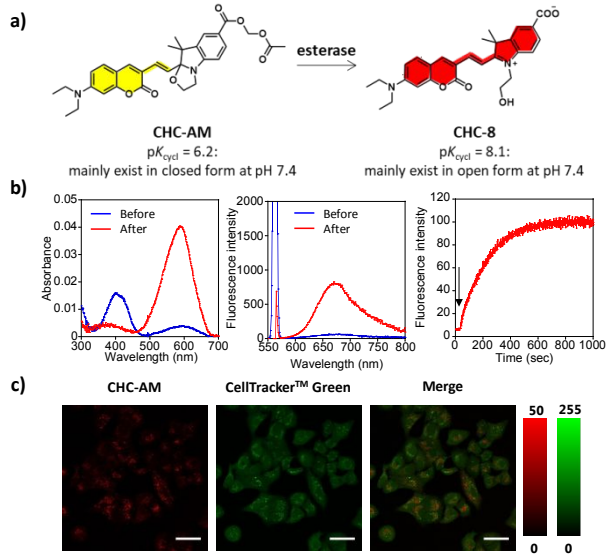


Fig. 3 (a) A newly developed fluorescence probe for esterase based on the coumarin-hemicyanine hybrid fluorophore, CHC-AM. (b) Absorption (left) and fluorescence (middle) spectra of 1 μM CHC-AM before (blue) and after (red) reaction with 10 units of esterase. (right) Time-dependent changes in the fluorescence intensity of CHC-AM upon addition of esterase. Spectra were measured in 0.2 M sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent. The excitation and emission wavelengths were 560 nm and 680 nm, respectively. (c) Fluorescence confocal microscopy imaging of live A549 cells. A549 cells were incubated with 1 μM CHC-AM and 1 μM CellTracker™ Green CMFDA containing 0.2% DMSO as a cosolvent for 1 h. Excitation and detection wavelengths were 552 nm and 650-700 nm for CHC-AM, and 495 nm and 505-540 nm for CellTracker™ Green CMFDA. Scale bar: 50 μm.

Next, we examined whether a larger change in the pK_{cycl} value of the CHC hybrid fluorophore could be obtained by targeting other kinds of hydrolase. Specifically, we focused on carboxypeptidase and esterase, which hydrolyse an electron-withdrawing amide or ester moiety to carboxylate. We prepared **CHC-8** with a carboxylic group at the indolenium C5 atom, and examined how the spirocyclic behaviour changes upon amidation of this carboxylic group (**CHC-9**) (Table 1, Scheme S7). The pK_{cycl} values of **CHC-8** and **CHC-9** were calculated to be 8.1 and 6.4, respectively (Figure S7), meaning that 83% of **CHC-8** and 9% of **CHC-9** exist in the open form at pH 7.4 and so the expected fold change due to the spirocyclization is more than 9. These results indicate that changes in the electron-withdrawing ability of the substituent at the indolenium C5 atom (from amide to carboxylate) successfully induced a sufficient shift of the pK_{cycl} value (ΔpK_{cycl} = 1.7). Based on this finding, we designed and synthesized a probe targeted for esterase, **CHC-AM**, by converting the carboxy group of **CHC-8** to acetoxymethyl (AM) ester (Figure 3a, Scheme S8). Since the ester moiety has a strong electron-withdrawing ability, comparable to that of the amide moiety according to the Hammett equation, we expected that the AM ester derivative would show a sufficiently low pK_{cycl} value. As expected, **CHC-AM** showed similar optical properties to those of **CHC-9**, and its pK_{cycl} value was calculated to be 6.2, meaning that only 6% of **CHC-AM** exists in its open form at pH 7.4, and the expected fold change due to the spirocyclization is as large as 13 (Figure S8a). We confirmed that **CHC-AM** was hydrolysed by esterase to produce **CHC-8** as a fluorescent hydrolysis product with a large Stokes shift of 66 nm, resulting in fluorescence activation of up to 14-fold (Figure 3b, Figure S8b). We also examined whether we could detect esterase activity in a ratiometric manner by using two excitation and emission wavelengths (Figure S9), but the fluorescence of the coumarin moiety was also increased by the hydrolysis of the AM group, probably due to quenching of the coumarin units of CHC-AM by the indoline moiety, and this might limit the sensitivity in ratiometric application of this probe.

Finally, we applied **CHC-AM** to A549 cells, and observed fluorescence activation in cells upon hydrolysis of AM ester by the ubiquitous intracellular esterase (Figure 3c). Thus, we successfully designed **CHC-AM** as a cell-permeable esterase probe based on the CHC hybrid fluorophore by using intramolecular spirocyclization as a fluorescence

switching mechanism. This design strategy with CHC hybrid fluorophore should also be applicable to design probes targeted to other hydrolases such as carboxypeptidases, thus having the potential to greatly expand the range of available fluorescence probes.

Conclusions

We have prepared a series of coumarin-hemicyanine (CHC) hybrid fluorophores, and demonstrated that their optical properties can be controlled by means of intramolecular spirocyclization. By making use of the shift in spirocyclization behaviour upon esterification of the carboxy group at the indolenium C5 atom, we developed an activatable fluorescence probe for esterase, which shows strong fluorescence activation upon reaction with esterase. We also developed an activatable fluorescence probe for GGT by utilizing the significant blue-shift in absorption upon amidation of the amino group of the coumarin unit, together with a contribution from the spirocyclization. Importantly, the fluorescent hydrolysis products of the developed probes exhibit large Stokes shifts, enabling sensitive detection of the target enzymes due to a minimum overlapping between the excitation and emission spectra. Thus, we believe that this design strategy with CHC hybrid scaffold will be applicable to develop a range of activatable fluorescence probes, providing useful tools for probing the functions of hydrolases in biological and pathophysiological contexts.

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Conflicts of interest

There are no conflicts to declare

Notes and references

1. M. Egeblad and Z. Werb, *Nature Reviews Cancer*, 2002, **2**, 161.
2. C. López-Otín and L. M. Matrisian, *Nature Reviews Cancer*, 2007, **7**, 800.
3. B. N. Vajaria and P. S. Patel, *Glycoconjugate Journal*, 2017, **34**, 147-156.
4. W. Chyan and R. T. Raines, *ACS Chemical Biology*, 2018, **13**, 1810-1823.
5. J.-A. Richard, M. Massonneau, P.-Y. Renard and A. Romieu, *Organic Letters*, 2008, **10**, 4175-4178.
6. W. Sun, S. Guo, C. Hu, J. Fan and X. Peng, *Chemical Reviews*, 2016, **116**, 7768-7817.
7. L. Yuan, W. Lin and J. Song, *Chemical Communications*, 2010, **46**, 7930-7932.
8. J. Zha, B. Fu, C. Qin, L. Zeng and X. Hu, *RSC Advances*, 2014, **4**, 43110-43113.
9. X. Zhou, Y. Kwon, G. Kim, J.-H. Ryu and J. Yoon, *Biosensors and Bioelectronics*, 2015, **64**, 285-291.
10. X. Lv, J. Liu, Y. Liu, Y. Zhao, Y.-Q. Sun, P. Wang and W. Guo, *Chemical Communications*, 2011, **47**, 12843-12845.
11. Y. Shiraishi, M. Nakamura, K. Yamamoto and T. Hirai, *Chemical Communications*, 2014, **50**, 11583-11586.
12. Y. Chen, C. Zhu, Z. Yang, J. Chen, Y. He, Y. Jiao, W. He, L. Qiu, J. Cen and Z. Guo, *Angewandte Chemie International Edition*, 2013, **52**, 1688-1691.
13. Y. Yang, C. Yin, F. Huo, Y. Zhang and J. Chao, *Sensors and Actuators B: Chemical*, 2014, **203**, 596-601.
14. W. Xu, C. L. Teoh, J. Peng, D. Su, L. Yuan and Y.-T. Chang, *Biomaterials*, 2015, **56**, 1-9.
15. J. Yang, K. Li, J.-T. Hou, L.-L. Li, C.-Y. Lu, Y.-M. Xie, X. Wang and X.-Q. Yu, *ACS Sensors*, 2016, **1**, 166-172.
16. A. Tirla and P. Rivera-Fuentes, *Angewandte Chemie International Edition*, 2016, **55**, 14709-14712.
17. S. Ando and K. Koide, *Journal of the American Chemical Society*, 2011, **133**, 2556-2566.
18. M. Sakabe, D. Asanuma, M. Kamiya, R. J. Iwatate, K. Hanaoka, T. Terai, T. Nagano and Y. Urano, *Journal of the American Chemical Society*, 2013, **135**, 409-414.
19. J. B. Grimm, B. P. English, J. Chen, J. P. Slaughter, Z. Zhang, A. Revyakin, R. Patel, J. J. Macklin, D. Normanno, R. H. Singer, T. Lionnet and L. D. Lavis, *Nat Meth*, 2015, **12**, 244-250.
20. L. Wang, M. Tran, E. D'Este, J. Roberti, B. Koch, L. Xue and K. Johnsson, *Nature Chemistry*, 2019, DOI: 10.1038/s41557-019-0371-1.
21. J. Cusido, S. S. Ragab, E. R. Thapaliya, S. Swaminathan, J. Garcia-Amorós, M. J. Roberti, B. Araoz, M. M. A. Mazza, S. Yamazaki, A. M. Scott, F. M. Raymo and M. L. Bossi, *The Journal of Physical Chemistry C*, 2016, **120**, 12860-12870.
22. M. Kamiya, D. Asanuma, E. Kuranaga, A. Takeishi, M. Sakabe, M. Miura, T. Nagano and Y. Urano, *Journal of the American Chemical Society*, 2011, **133**, 12960-12963.
23. S. Kenmoku, Y. Urano, H. Kojima and T. Nagano, *Journal of the American Chemical Society*, 2007, **129**, 7313-7318.
24. S.-n. Uno, M. Kamiya, T. Yoshihara, K. Sugawara, K. Okabe, M. C. Tarhan, H. Fujita, T. Funatsu, Y. Okada, S. Tobita and Y. Urano, *Nat Chem*, 2014, **6**, 681-689.
25. A. Pompella, V. De Tata, A. Paolicchi and F. Zunino, *Biochemical Pharmacology*, 2006, **71**, 231-238.
26. Y. Urano, M. Sakabe, N. Kosaka, M. Ogawa, M. Mitsunaga, D. Asanuma, M. Kamiya, M. R. Young, T. Nagano, P. L. Choyke and H. Kobayashi, *Science Translational Medicine*, 2011, **3**, 110ra119.
27. H. Hino, M. Kamiya, K. Kitano, K. Mizuno, S. Tanaka, N. Nishiyama, K. Kataoka, Y. Urano and J. Nakajima, *Translational Oncology*, 2016, **9**, 203-210.