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# Paper-based sensing of pancreatic-cancer biomarker $\alpha$ -chymotrypsin through turn-on lanthanide-luminescence†

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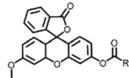
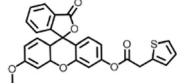
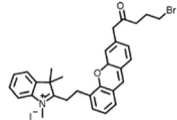
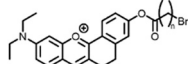
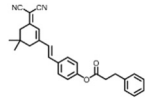
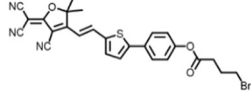
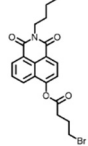
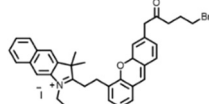
We report the facile detection of a pancreatic cancer biomarker  $\alpha$ -chymotrypsin (Chy) by turn-on, time-gated lanthanide luminescence for the first time. To the best of our knowledge, the non-peptide probe we designed is the simplest one currently available. The probe undergoes Chy-induced release of the sensitizing antenna (2,3-dihydroxynaphthalene), leading to enhanced lanthanide luminescence. The detection protocol was further modified to develop a paper-based sensor and was used to detect Chy in commercial tablets, and to rapidly screen Chy-inhibitors.

$\alpha$ -Chymotrypsin (Chy) is a well-known peptidase associated with protein digestion, cell proliferation, gene expression, cystic fibrosis, necrosis, inflammatory arthritis, and apoptosis of digestive proteins.<sup>1,2</sup> It preferentially cleaves peptide amide bonds or small ester substrates where the N-terminus to the scissile amide/ester bond is a large hydrophobic amino acid (Tyr, Trp, and Phe).<sup>3</sup> The enzyme has been used to treat rhinitis, pharyngitis, otorhinolaryngologic diseases, lung abscesses, tissue repair, sinusitis, redness, and for reduction of swelling in various situations like infection and surgery.<sup>4</sup> An abnormal expression of Chy, on the other hand, could lead to pancreatic fibrosis, diabetes mellitus,<sup>5</sup> hyperinsulinemia,<sup>6</sup> Crohn's Disease,<sup>7</sup> maldigestions, and pancreatic cancer.<sup>4,8</sup> It has been recognized as a potential biomarker for pancreatic function studies.<sup>4,8,9</sup> Therefore, developing a simple analytical protocol for detecting and quantifying chymotrypsin activity is important in clinical diagnostics, drug discovery, and therapy of associated disorders.<sup>10</sup>

Among the previously reported analytical protocols,<sup>6,11,12</sup> enzyme-linked immunosorbent assay (ELISA) and Western blot methods are less attractive because of their higher cost and time-consuming protocols. Peptide substrates<sup>13,14</sup> used in colorimetric and fluorimetric assays require special storage

to prevent rapid degradation. Non-peptide probe-based fluorescence-sensing of Chy has recently received much attention due to the greater intrinsic sensitivity of fluorescence technique and simple synthetic procedure of non-peptide molecules (Table 1).<sup>15–25</sup>

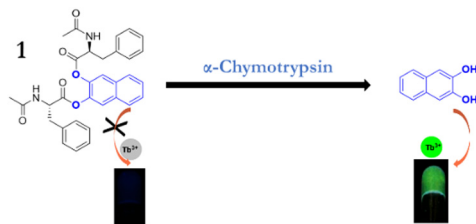
**Table 1** Literature reports of fluorometric & colorimetric probes

Sensing method	Probe structure	Ref.
Fluorescent & colorimetric		16
Fluorescent		17
Fluorescent & colorimetric		18
Fluorescent		19
Fluorescent		20
Fluorescent		21
Fluorescent		22
Fluorescent		23

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**Scheme 1** Non-peptide substrate **1** for Chy.

However, interference (autofluorescence) from endogenous components and background scattering in biological samples may obscure the output signal.

In this context, lanthanide luminescence-based sensors with long radiative lifetimes and large Stokes shift are more advantageous since they allow time-gated luminescence measurement which eliminates short-lived fluorescence from biological systems.<sup>26</sup>

Our group has developed a non-covalent lanthanide sensitization approach without tedious synthesis.<sup>27–32</sup> An appropriate antenna (2,3-dihydroxynaphthalene for Tb<sup>3+</sup>) doped in lanthanide cholate hydrogels enhanced the corresponding lanthanide luminescence in the gel matrix. When covalently modified with enzyme-cleavable groups, the resulting masked-sensitizer (termed as ‘*pro-sensitizer*’) did not sensitize the lanthanide.<sup>33,34</sup> Only in the presence of the appropriate enzyme, the *pro-sensitizer* was cleaved, with the released sensitizer enhancing lanthanide emission.

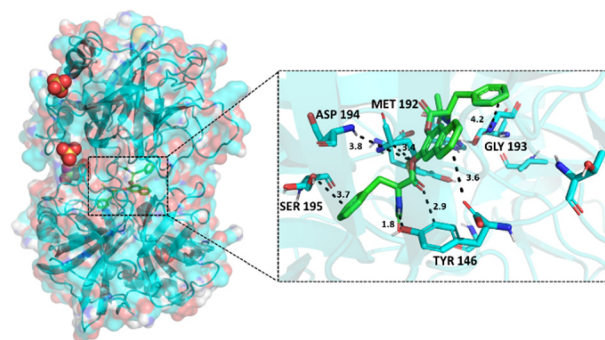
In the present work, we have combined the advantages of non-peptide probes and lanthanide-based *pro-sensitizer* strategies to develop a sensor for  $\alpha$ -chymotrypsin. We reasoned that a bis-phenylalanine ester derivative of 2,3-dihydroxy naphthalene (**1**) could be the simplest non-peptide substrate that would not sensitize Tb<sup>3+</sup>. Furthermore, if  $\alpha$ -chymotrypsin cleaves the substrate completely, the liberated 2,3-dihydroxynaphthalene will trigger Tb(III) sensitization (Scheme 1). This was indeed observed in practice. A paper-based protocol was subsequently explored to develop a low-cost, paper-based sensor. To the best of our knowledge, this work represents the first example of a time-gated detection of Chy using lanthanide luminescence and one of the rare examples of its paper-based sensing. To date, there is only one report of a paper-based flow sensor for detecting chymotrypsin and its inhibitors which utilized the viscosity change of gelatin.<sup>35</sup>

*Pro-sensitizer 1* was easily synthesized (Scheme S1†) by esterification of the two hydroxyl groups of 2,3-DHN with *N*-Ac-L-Phe. Tb-cholate gels were prepared by simply mixing equal volumes of Tb acetate (10 mM) and Na cholate (30 mM) solutions and sonicating the mixture for 3–5 s. When 2,3-DHN (37.5  $\mu$ M) was doped in the gel, it showed bright green emission under UV lamp (365 nm). On the other hand, Tb-cholate gel doped with probe **1** (37.5  $\mu$ M) was non-luminescent under the UV lamp. A time-dependent luminescence profile showed that the luminescence intensity of Tb(III) in the presence of *pro-sensitizer 1* remained

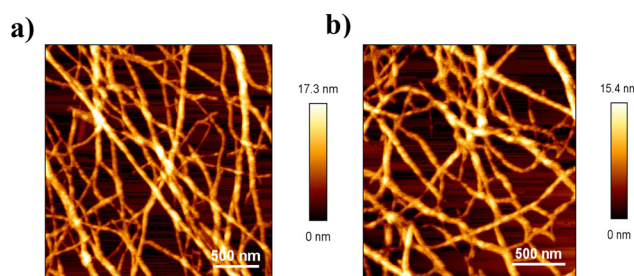
constant even after incubation at 25 °C for 60 min. The same gel, in the presence of 2.5  $\mu$ g mL<sup>−1</sup> of  $\alpha$ -chymotrypsin,<sup>36</sup> developed a bright green emission, with the intensity increasing with time (Fig. S4†). Control experiments performed with denatured  $\alpha$ -chymotrypsin (90 °C/20 min) showed no luminescence enhancement, indicating that luminescence enhancement was indeed due to  $\alpha$ -chymotrypsin action (Fig. S7†). Time-delayed excitation spectra showed a new band with  $\lambda_{\text{max}}$  at 335 nm (Fig. S3a†), like the absorption band of DHN (Fig. S1†), only in the presence of Chy, providing further evidence that the enzyme action released free DHN. Simulated binding modes of probe **1** in the active site of  $\alpha$ -chymotrypsin were analysed and the mode with the most interactions is shown in Fig. 1. The ester carbonyl of **1** interacts with Ser-195 (which is expected to facilitate the nucleophilic attack on the substrate) in the active site. The affinity calculated from the docking experiment was found to be −11.1 kcal mol<sup>−1</sup>.

AFM images of **1**-doped TbCh gel with or without enzyme incubation (30 min) indicated that the gel fibres were intact even upon enzyme action on the substrate. Clearly, Chy did not affect the gel morphology (Fig. 2) suggesting the gel's robustness and stability (Fig. 3).

To optimize the assay, two methods were tested. In method 1, the enzyme assay was carried out in the gel medium wherein both the stock solutions of Chy and substrate **1** were prepared in 30 mM sodium cholate solutions, and simple mixing of the two solutions with 10 mM aqueous Tb(OAc)<sub>3</sub> formed a gel that was incubated at 25 °C for 20 min. The gel turned luminescent with time,



**Fig. 1** Simulated binding model of probe **1** in the active site of Chy.



**Fig. 2** AFM images of (a) **1** doped TbCh gel (b) (**1** + enzyme incubated at 25 °C for 30 min) doped TbCh gel.



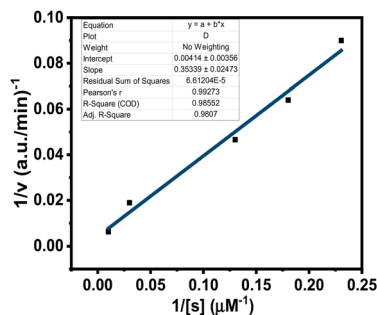


Fig. 3 Lineweaver-Burk plot for  $\alpha$ -chymotrypsin assay.

indicating the release of DHN upon the enzyme action. In method 2, both the stock solutions of Chy and **1** were prepared in 30 mM sodium cholate solution. This mixture was incubated at 25 °C, then mixed with 10 mM Tb(OAc)<sub>3</sub> to form the gel, which was luminescent indicating the formation of DHN.

Both methods involved incubation at 25 °C. Method 1 being more straightforward (enzyme incubation and gel processing in a single step) was followed for enzyme activity measurements. The  $K_M$  value was found to be 85  $\mu$ M, comparable with previous reports (Table S1†). The lower value of  $K_M$  denotes significant affinity of  $\alpha$ -chymotrypsin towards the synthetic non-peptide substrate.

To check the selectivity of the assay, compound **1** was incubated with  $\alpha$ -chymotrypsin and several other enzymes such as lipase, urease,  $\beta$ -glucuronidase, trypsin, and alkaline phosphatase that could co-exist with  $\alpha$ -chymotrypsin in biological samples. Tb(III)-luminescence enhancement was observed only with  $\alpha$ -chymotrypsin, and no luminescence changes could be detected with other enzymes even when the interfering enzyme concentration was 25-fold higher (Fig. 4).

The limit of detection (LOD) for Chy calculated using the formula  $\text{LOD} = 3\sigma/b$  ( $\sigma$  = standard deviation of the signal obtained from the blank and  $b$  = slope of the titration plot) were found to be 0.014  $\text{U mL}^{-1}$  and 0.037  $\text{U mL}^{-1}$ , for method 1 and method 2, respectively (Fig. 5). The response was generated within 15 min, indicating the assay's sensitivity, selectivity, rapidness, and practicality.

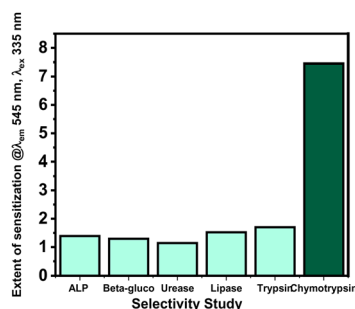


Fig. 4 Extent of emission enhancement at 545 nm ( $\lambda_{\text{ex}}$  335 nm) after incubating **1** with Chy (0.4  $\text{U mL}^{-1}$ ) and other enzymes (10  $\text{U mL}^{-1}$ ) for 30 min at 25 °C. Final incubation concentration of **1** was 37.5  $\mu$ M.

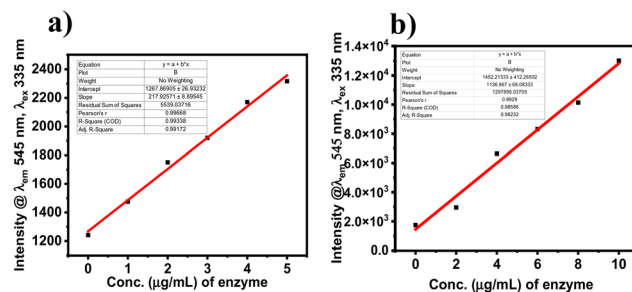


Fig. 5 Emission at 545 nm ( $\lambda_{\text{ex}}$  335 nm) for  $\alpha$ -chymotrypsin assay a) method 1 and b) method 2.

A rapid enzyme assay should be ideally suited for rapid screening of inhibitors, and we have explored this possibility too. 1-Naphthol, a known inhibitor<sup>37</sup> of  $\alpha$ -chymotrypsin is associated with reduced testosterone levels in adult men.<sup>35</sup> It is a metabolite of carbaryl and naphthalene that is an intermediate in the metabolism of xenobiotics by cytochrome P450. The  $\text{IC}_{50}$  value for 1-naphthol calculated using our methodology was 0.58 mM (Fig. S12†). Further refinement of our gel-based assay using **1** can lead to rapid screening of  $\alpha$ -chymotrypsin inhibitors and may greatly help drug discovery processes and detailed enzyme activities in real systems in the presence of potentially interfering metabolites.

To further improve the protocol, we have designed paper-based sensors.<sup>38–44</sup> TbCh hydrogel (5/15 mM) doped with the *pro*-sensitizer **1** (37.5  $\mu$ M) was stabilized at RT for 10 min, sonicated again (5–6 s) to reduce its viscosity. A 20  $\mu$ L aliquot of this weak gel was drop casted on 3.5 mm diameter discs cut from Whatman 3 paper using a standard one-hole punch. The paper absorbed the gel in 30 min and was transferred to a 96-well plate.  $\alpha$ -Chymotrypsin solution (10  $\mu$ L, prepared in NaCh) was added on each disc, and emission measurements were recorded using a plate reader after 15 min of air drying. The LOD using this paper-based strategy was found to be 330  $\text{ng mL}^{-1}$  or 0.012  $\text{U mL}^{-1}$  (Fig. 6a). This value is comparable to previous literature-reported values (Table S2†). The presence of Chy in a commercial tablet was readily detected using the paper-based method (Fig. 6b). One essential criterion for a sensor to smoothly reach end-users is its low

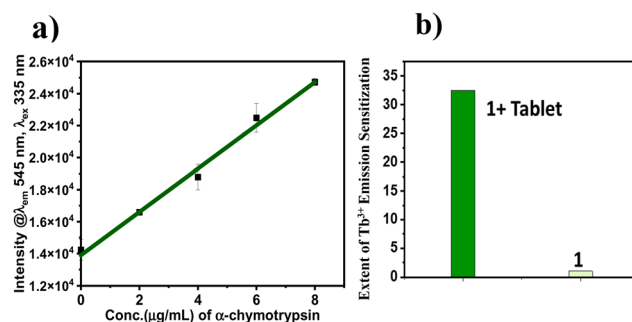


Fig. 6 (a) LOD measurement for paper-based  $\alpha$ -chymotrypsin assay, (b) detection of Chy in a commercial tablet.



cost which generally reflects its practicability in the real diagnostic field. In resource-limited areas, such a protocol can be very useful where fully equipped lab facilities are unavailable. Material cost for our developed paper-based sensor was calculated and was estimated to be less than GBP 0.01 for a single paper disc.

In conclusion, we developed an innovative luminogenic supramolecular framework for highly selective and sensitive sensing of  $\alpha$ -chymotrypsin. A non-peptide-based small-molecule probe was designed, which released the sensitizer molecule upon the action of Chy. As the sensing strategy involved time-gated luminescence of sensitized Tb(III), it has a competitive advantage over the prevalent methodologies that commonly employ fluorogenic probes. The technique was subsequently utilized in the testing an inhibitor of  $\alpha$ -chymotrypsin. Furthermore, the gel's soft, solid-like characteristics facilitated enhanced immobilization on a passive substrate like paper, thus leading to the development of a simple, inexpensive paper-based sensor. This marks the first demonstration of merging the benefits of both paper-based sensing technologies and a delayed luminescence output for  $\alpha$ -chymotrypsin assay. Considering these attributes, this probe framework emerges as an appealing prospect for forthcoming analytical applications in drug exploration and the clinical diagnosis of ailments related to the pancreas in pharmaceutical and pathological investigations.<sup>45</sup>

## Data availability

The data supporting this article have been included as part of the ESI.†

## Conflicts of interest

There are no conflicts to declare.

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## Notes and references

- H. Tsukada and D. M. Blow, *J. Mol. Biol.*, 1985, **184**, 703–711.
- A. Kumar and P. Venkatesu, *Chem. Rev.*, 2012, **112**, 4283–4307.
- H. U. Bergmeyer, *Methods of enzymatic analysis*, Academic Press, London, 1974.
- S. Yamashita, M. Sakabe, T. Ishizawa, K. Hasegawa, Y. Urano and N. Kokudo, *Br. J. Surg.*, 2013, **100**, 1220–1228.
- G. Montalto, A. Carroccio, G. Marino, M. Soresi, C. Di Marco and A. Notarbartolo, *Acta Diabetol. Lat.*, 1990, **27**, 157–163.
- S. H. Bharmal, S. A. Pendharkar, R. G. Singh, M. O. Goodarzi, S. J. Pandol and M. S. Petrov, *Pancreatology*, 2017, **17**, 876–883.
- J. P. Van De Merwe and G. J. J. Mol, *Digestion*, 1982, **24**, 1–4.
- J. Zhou and M. Sahin-Tóth, *J. Gastroenterol. Hepatol.*, 2011, **26**, 1238–1246.
- Y. Chen, J. Cao, X. Jiang, Z. Pan and N. Fu, *Sens. Actuators, B*, 2018, **273**, 204–210.
- W. Appel, *Clin. Biochem.*, 1986, **19**, 317–322.
- G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol. Chem.*, 1961, **236**, 2930–2935.
- K. Xu, F. Liu, J. Ma and B. Tang, *Analyst*, 2011, **136**, 1199–1203.
- L. Liu, C. Liu and L. Gao, *Biosensors*, 2023, **13**(2), 263–272.
- D. Milićević and J. Hlaváč, *ACS Omega*, 2024, **9**, 17481–17490.
- P. J. Brynes, P. Bevilacqua and A. Green, *Anal. Biochem.*, 1981, **116**, 408–413.
- L. Wu, S. H. Yang, H. Xiong, J. Q. Yang, J. Guo, W. C. Yang and G. F. Yang, *Anal. Chem.*, 2017, **89**, 3687–3693.
- H. Xiong, R. R. Li, S. Y. Liu, F. S. Wu, W. C. Yang and G. F. Yang, *ACS Appl. Bio Mater.*, 2018, **1**, 310–317.
- S. Mu, Y. Xu, Y. Zhang, X. Guo, J. Li, Y. Wang, X. Liu and H. Zhang, *J. Mater. Chem. B*, 2019, **7**, 2974–2980.
- Y. Qu, Z. Xu, J. Wang, W. Liu, A. Iqbal, K. Iqbal, Y. Su, Y. Cao, J. Yang, W. Qin and Y. Liu, *Sens. Actuators, B*, 2023, **382**, 133552.
- X. Zou, Y. Zhao, C. Lai, Y. Liang and W. Lin, *J. Mater. Chem. B*, 2021, **9**, 8417–8423.
- C. Fan, J. Gao, Y. Gao, X. Yang, G. Li, X. Wang, F. Li, J. Zhou, H. Yu, Y. Huang, J. Chen, Y. Shan, L. Chen, C. Fan, J. Gao, Y. Gao, X. Yang and G. Li, *Chin. Chem. Lett.*, 2024, 109838.
- Y. Chen, J. Cao, X. Jiang, Z. Pan and N. Fu, *Sens. Actuators, B*, 2018, **273**, 204–210.
- Y. Zhao, X. Zou, X. Liang, L. Huang and W. Lin, *Sens. Actuators, B*, 2023, **382**, 133553.
- H. Shi, C. Liu, J. Cui, J. Cheng, Y. Lin, L. Gao and R. Luo, *New J. Chem.*, 2020, **44**, 20921–20929.
- K. Kuromizu, Y. Shimokawa, O. Abe and N. Izumiya, *Anal. Biochem.*, 1985, **151**, 534–539.
- M. C. Heffern, L. M. Matosziuk and T. J. Meade, *Chem. Rev.*, 2014, **114**, 4496–4539.
- S. Bhowmik and U. Maitra, *Chem. Commun.*, 2012, **48**, 4624–4626.
- A. Biswas and U. Maitra, *RSC Adv.*, 2022, **12**, 26106–26110.
- S. Bhowmik, S. Banerjee and U. Maitra, *Chem. Commun.*, 2010, **46**, 8642–8644.
- A. Biswas and U. Maitra, *Chem. Commun.*, 2024, **60**, 6765–6768.
- R. Laishram, S. Bhowmik and U. Maitra, *J. Mater. Chem. C*, 2015, **3**, 5885–5889.
- T. Gorai, S. Sakthivel and U. Maitra, *Chem. – Asian J.*, 2020, **15**, 4023–4027.
- T. Gorai and U. Maitra, *J. Mater. Chem. B*, 2018, **6**, 2143–2150.
- T. Gorai and U. Maitra, *ACS Sens.*, 2016, **1**, 934–940.





- 35 S. Liu, X. Wang, Q. Hu, Y. Geng and H. Dong, *J. Food Compos. Anal.*, 2023, **116**, 105064–105070.
- 36 Chy did not act on the bis-D-phenylalanine ester derivative of DHN.
- 37 R. A. Wallace, A. N. Kurtz and C. Niemann, *Biochemistry*, 1963, **2**, 824–836.
- 38 Q. Ju, M. O. Noor and U. J. Krull, *Analyst*, 2016, **141**, 2838–2860.
- 39 K. Tenda, B. van Gerven, R. Arts, Y. Hiruta, M. Merckx and D. Citterio, *Angew. Chem., Int. Ed.*, 2018, **57**, 15369–15373.
- 40 S. K. Mahadeva, K. Walus and B. Stoeber, *ACS Appl. Mater. Interfaces*, 2015, **7**, 8345–8362.
- 41 F. Güder, A. Ainla, J. Redston, B. Mosadegh, A. Glavan, T. J. Martin and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 2016, **55**, 5727–5732.
- 42 B. Liu, D. Du, X. Hua, X. Y. Yu and Y. Lin, *Electroanalysis*, 2014, **26**, 1214–1223.
- 43 C. Parolo and A. Merkoçi, *Chem. Soc. Rev.*, 2013, **42**, 450–457.
- 44 A. M. López-Marzo and A. Merkoçi, *Lab Chip*, 2016, **16**, 3150–3176.
- 45 A detailed validation with clinical samples will be published after completing the assay-development of other relevant enzymes for whole pancreatic function studies.

