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COMMUNICATION

Homogeneous probing lipase and α-amylase simultaneously by the AIEgens

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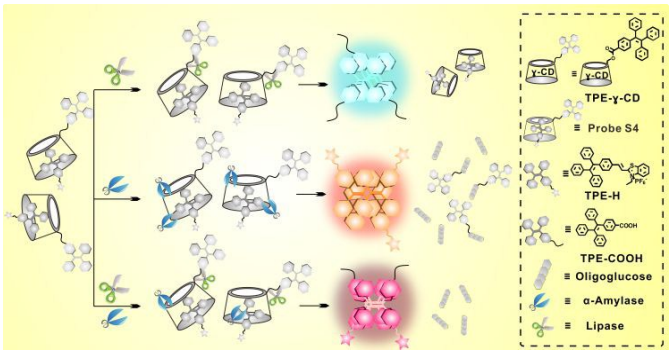
Jie Shi,[ab] Qianchun Deng,*[a] Ya Li,[a] Zhe Zheng,[c] Huijuan Shangguan,[a] Lu Li,[b] Fenghong Huang*[a] and Bo Tang*[b]

An AIE dual-reactive supermolecular probe has been firstly devised to simultaneously measure endogenous lipase and α-amylase activity in homogeneous system. Fluorescent quantitative analysis of lipase and α-amylase in real biological samples enables rapid and accurate diagnosis of diseases.

Lipase (**LPS**) and α-amylase (**AMY**) have a wide range of applications in the food and pharmaceutical industries, and they are also the core enzymes in the human digestive system.[1] In our body, α-amylase and lipase are mainly secreted by the pancreas and emerged in in digestive juices as well as other body fluids.[2] Under the conditions of pancreatic injury, pancreatic autolysis induces significant elevation of lipase and α-amylase levels in blood, which is the symptom of acute pancreatitis.[3] The mortality for severe acute pancreatitis is up to 30%, sufferers often endure pain and malnutrition and are most likely left with a higher risk of pancreatic cancer. Because the pancreas is relatively inaccessible, problems are often diagnosed by computed tomography (CT) and clinico-radiology and timely treatments are therefore delayed.[4] Thence, as the most pivotal indicators in the clinical diagnosis of acute pancreatitis, the rapid detection and sensitive monitoring the activity of lipase and α-amylase will be greatly desirable.[5]

Recently, some innovative strategies are being developed for lipase or α-amylase detection to replace the conventional turbidimetric and colorimetric methods,[6] but some specific substrates and tool enzymes have been used, possessing relatively complicated processes and suffer from lower sensitivity. Subsequently, our group synthesized some small-molecule fluorescent probes with aggregation-induced emission (AIE) properties for detecting sole lipase or α-amylase, which brings improved sensitivity and shortened testing time.[7] However, in clinical diagnosis of acute pancreatitis, depending on the cause and onset time, case of illness that solely elevated lipase or α-amylase levels could be found, so that the misjudgments may occur if we only measure one of them,[8] combining lipase and α-amylase determinations simultaneously is highly demanded for improvement of positive diagnosis rate of acute pancreatitis. [9] Unfortunately, the techniques for combining the two tests have been generally limited, for example, two substrates of enzymes should be selected for each test, lipase can only exert maximum activity at the two-phase interface,[10] making the test is conduct in two-phase medium and so on. Up to date, no breakthrough concept has emerged for design single small molecule probe test system to overcome these obstacles.



Scheme 1 Schematic illustration of fluorescent response mechanisms of probe **S4** to lipase, α-amylase, and lipase/α-amylase mixture.

Hence, to bypass these barriers, in this work, we engineered a host-guest inclusion **S4** (Scheme 1) for the simultaneously detection of lipase and α-amylase, which comprises one tetraphenylethene (**TPE**) conjugated with γ-cyclodextrin (**CD**) by esterification (**TPE-γ-CD**) and one orange emissive AIEgen (**TPE-H**). In the light of the AIE principle,[11] the **TPE-γ-CD** was soluble and very weakly emissive in aqueous media, for **TPE-H**, it's not soluble in water, and as a strong emitter in aqueous mixtures. When they are mixed with 1:1 molar ratio, **TPE-H** will drill into the hydrophobic cavity of the **TPE-γ-CD**, the

[a] Hubei Key Laboratory of Lipid Chemistry and Nutrition, Key Laboratory of Oilseeds Processing, Ministry of Agriculture, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, China. E-mail: shijie@caas.cn
[b] College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Normal University, Jinan 250014, China. E-mail: tangb@sdnu.edu.cn
[c] Department of Modern Physics, University of Science and Technology of China Hefei 230026, China.
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restriction of intramolecular rotation (RIR) effect is weakened, namely quenching the **TPE-H** fluorescence.[12] When the lipase is present, the ester bond linked insoluble **TPE-COOH** will be released and aggregated to produce blue fluorescence, if α-amylase was incubated, it will act on the α-1,4-glycosidic bonds of the **γ-CD**, resulting in this host molecule disintegrate and release the insoluble orange-emitting **TPE-H** again. And so forth, if probe **S4** encounters the lipase/α-amylase mixture, mixed color of fluorescence will be captured. Thus, the double emission turn-on results could be used for selective detection of lipase and α-amylase simultaneously. More importantly, first, the two emissive peaks were separated by roughly 150 nm upon the same excitation wavelength (390 nm) to avoid spectral interference and caused sensitive visual changes; Second, a micro hydrophilic-hydrophobic interface between the hydrophobic cyclodextrin cavity and the aqueous solution was constituted, which fascinates the interface activation effect of lipase and abandons the tedious two-phase test system. To our best knowledge, no reports to date have been published on the application of one fluorescent probe in testing lipase and α-amylase levels of real human serum samples, this is the first report on "one pot" assay for lipase and α-amylase simultaneously.

The preparation of supramolecular **S4** is quite simple, just need to mix **TPE-γ-CD** and **TPE-H** with a 1:1 molar ratio in DMSO. The supramolecular complex **S4** were characterized using NMR and mass spectrometry (Fig. S1, S2), satisfactory results were obtained corresponding to their molecular structures. As illustrate in Fig. S3, compared to free **TPE-H**, firstly, two groups of new peaks (7.55-7.51 and 7.37 ppm) that were ascribed to aromatic protons appeared, indicating that most of the phenyl in **TPE-H** is encapsulated in **TPE-γ-CD** while the former multiplets (7.15-7.11, 7.03-6.97 ppm) from aromatic protons of free **TPE-H** disappeared, the broader peaks told that their chemical environment has changed. Secondly, compared to merely **TPE-γ-CD**, the peaks in the range of 3.70-3.30 showed distinct changes, other new peaks were found in the range of 7.21-6.42 ppm, implying that the esterification linked **TPE** molecules on the **γ-CD** have been extruded to the outer cavity by **TPE-H**,[12] which is free from the constraint of **γ-CD**. Furthermore, the peaks in the range of 8.42-7.77 ppm were almost showed no changes, implying that the double bond and benzothiazole units were out of the cavity of **γ-CD**. The HRMS spectrum of **S4** can be seen in Fig. S2, the obvious molecular ion peak at m/z 2174.7642 was attributed to the $[M\text{-}PF_6]^+$ ion of **S4** (calculated, 2174.7677).

Afterwards, the supramolecular-based probe **S4** buffer solution (10 μM) was first incubated with different activity of lipase in a homogeneous buffer solution. Before the addition of lipase, the solution of **S4** was nearly no emission in the aqueous solution. However, as depicted in Fig. 1A, when incubated with a growing activity of lipase, the homogeneous test system does not affect the arousal of fluorescence, an apparent emission band at 460 nm appeared and increased, concurrently, the emission at 610 nm that ascribe to **TPE-H** is quiet. After 72 U L^{-1} lipase incubated, the intensity has reached to the maximum by about 24-fold compared to the initial one.

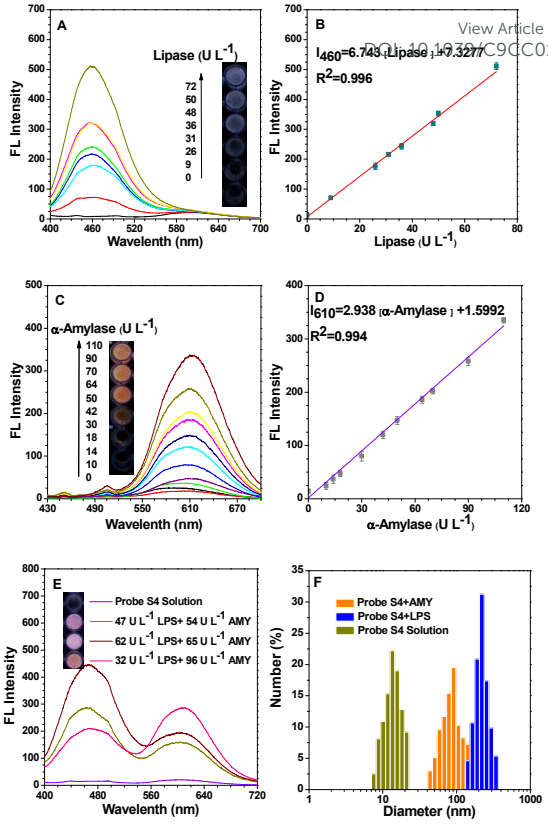

Fig. 2 Fluorescence spectra and of 10 μm **S4** with the incubation of lipase (A) and α-amylase (C) in PBS buffer (pH 6.6, 0.1 M) for 30 min; B) Region of the calibration curve of lipase (B) and α-amylase (D); E) Fluorescence spectra upon mixing 10 μm **S4** and lipase and α-amylase; F) Dynamic light scattering (DLS) results for the solution of probe **S4** (10 μM) in PBS buffer in the presence of lipase (100 U L^{-1}) or α-amylase (150 U L^{-1}) for 30 min. Excitation: 390 nm; the insets are the corresponding photographs under 365 nm UV lamp.

Next, we try to quantify the α-amylase activity with a pure **S4** test system. As seen in Fig. 1C, the intensity centred at about 610 nm increased correspondingly, and the fluorescence depicted no enhancement when the enzyme reached 110 U L^{-1}. From the calibration curve (Fig. 1B, D), the relative fluouorescence intensity at about 460 nm and 610 nm gave a linear increase with the concentration of lipase as well as α-amylase in the range of 0-72 U L^{-1} (R^2=0.996) and 0-110 U L^{-1} (R^2=0.994), respectively. The limit of detection (LOD) is calculated to be as low as 0.11 U L^{-1} for lipase, 0.17 U L^{-1} for α-amylase, which are the best ones compared with previous reports. On the other hand, we conducted HRMS to analyze the product of each enzymatic reaction. The results in Fig. S4 and S5 suggest that compound **TPE-COOH** and **TPE-H** appeared after hydrolysis. The inserted photograph in Fig. 1A and Fig. 1C reflect the significant changes in fluorescence under various enzyme activities, which can be easily distinguished by the naked eye under UV light. To verify the possibility of determination of lipase and α- amylase activity in chorus by the **S4** probe, we incubated it by the mixed lipase and α-amylase solution with a defined activity. The results in Fig. 1E revealed that the emission at 460 nm and 610 nm is relatively independent and not affected by each other, the activity of the two enzymes can be simultaneously quantitatively analysed

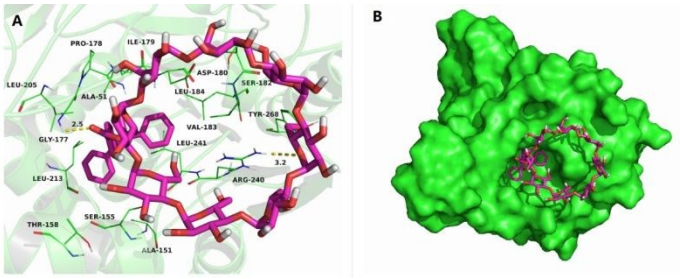

Fig. 2 Compound **TPE-γ-CD** was docked to the binding hydrophobic pocket of the lipase: A) Detailed view; B) Total view.

through the fluorescence emission intensity and the curves. The photograph clarified that, compared to another photographs in Fig. 1A and Fig. 1C, the emission of two enzymes system shows obvious distinction to the sole enzyme. Moreover, dynamic light scattering (DLS) measurements was used to determine the change of particle size after incubation (Fig. 1F). The average particle size of the **S4** aggregates was distinctly larger after incubation with lipase or α-amylase than that of **S4** only.

To explain the high activity of **TPE-γ-CD** against the lipase, the theoretical binding mode between them was performed. In Fig. 2A, the four phenyl groups of the **TPE-γ-CD** located at the big hydrophobic pocket, surrounded by the residues Ala-51, Ala-151, Pro-178, Ile-179, Val-183, Leu-184, Leu-205, Leu-213 and Leu-241, forming a strong hydrophobic binding. Detailed analysis showed that one of the phenyl group of TPE-**γ-CD** formed a anion-π interaction with the residue Asp-180. Importantly, two key hydrogen bonds were observed between the residues Gly-177 (bond length: 2.5 Å) and Arg-240 (bond length: 3.2 Å) and the **TPE-γ-CD**, which was the main interaction between them. All these interactions helped **TPE-γ-CD** to anchor in the binding site of the lipase. Meanwhile, to reveal that **S4** can analyze lipase activity in a homogeneous solution, molecule **S1**,[7] a heterogeneously reactive lipase probe we reported before, was then docked into the binding pocket of the lipase (Fig. S6). We can infer that, from Fig. 2B and Fig. S6B, for **TPE-γ-CD**, the hydrophobic cavity of **γ-CD** can drive the "lid" structure above the lipase active cavity to open,[10] making two hydrophobic regions connected, so that the ester bond enters, but **S1** can't do this and still needs a hydrophobic medium to open the "lid", which rational explains why **S4** can react with lipase only in homogeneous media. In addition, the estimated binding energies were -7.6 kcal mol^{-1} for **S1** and -8.4 kcal mol^{-1} for **TPE-γ-CD**, respectively, suggesting **TPE-γ-CD** was more active to bind the lipase than that of **S1**, which explained that **TPE-γ-CD** shows slight higher sensitivity than **S1**.

The time-course analysis of enzymatic catalytic reaction was addressed. In Fig. 3A, upon incubation with various activities of lipase (0-65 U L^{-1}), the intensity of fluorescence gradually increased by prolonging the incubation time, and the rate of fluorescence enhancement was directly corresponded to the levels of lipase. At last, it reaches the plateau after about 7 minutes. For α-amylase in Fig. 3B, the activity gradient was 0-100 U L^{-1}, the fluorescence intensities were stop to increase after 25 minutes, under each activity. Since the α-amylase

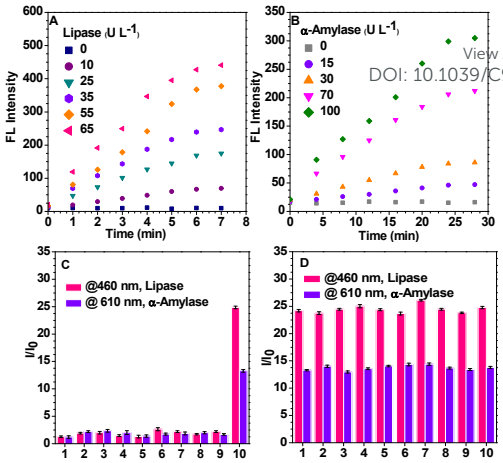

Fig. 3 Time-dependent fluorescence intensity of probe **S4** (10 μM) at 460 nm and 610 nm in the presence of different activities of lipase (A) and α-amylase (B) in PBS (pH 6.6, 0.1 M). Excitation wavelength: 390 nm. C) Fluorescence response of 10 μM probe **S4** for 30 minutes in the presence of different species (PBS buffer, pH 6.6, 0.1 M); (1) alkaline phosphatase; (2) α-chymotrypsin; (3) typsin; (4) pepsin; (5) lactatedehydrogenase; (6) thrombin; (7) alcohol dehydrogenase; (8) lysozyme; (9) AChE; (10) lipase or α-amylase; D) fluorescence response of 10 μM probe **S4** for 30 minutes in the presence of lipase or α-amylase and with the addition of different species respectively (PBS buffer, pH 6.6, 0.1 M); No. 1-No. 9 are the same as those of Fig. 3A; (10) all species; The concentration of lipase and α-amylase are 75 U L^{-1}, other protein enzymes are greater than or equal to 100 U L^{-1}. Excitation wavelength: 390 nm.

involved hydrolysis of γ-CD is slower than ester bond. Therefore, all the assay procedure could be finished in about 25 minutes, considering whether the aggregated product of lipase is stable after 25 minutes, the stability of enzymatic aggregates was also investigated and the results in Fig. S7 tell us they are very stable. Again, we confirmed the photostability of S4 system under continuous irradiation with a UV light (Fig. S8). The influences of common proteins in the blood or human body were all studied. From Fig. 3C, only lipase and α-amylase can arouse two fluorescence emissive responses while the nearly no reply were appeared for other enzymes, which exhibits about 24-fold and 12-fold fluorescence enhancement compared to the assay system with nothing added. We tested the interference of these proteins in the probe **S4** assay system later, as shown in Fig. 3D, in the presence of other interfering proteins, no more changes in fluorescence intensity can be seen, and demonstrating that probe **S4** is highly selective to lipase and α-amylase.

The kinetics of lipase and α-amylase on the probe **S4** are summarized in Fig. S9. For lipase, herein, hydrophobic cavity of **γ-CD** were used to build micro interfaces. Firstly, the substrate dependence of the initial degradation velocities for lipase were measured (Fig. S9A, B). The data of **S4** for the enzyme kinetics follow the Michaelis-Menten equation, and the kinetic parameters have been determined as V_{max} = 2.23 μM min^{-1} and K_m = 4.29 μM, which is similar to probe **S1** reported previously (K_m = 4.23 μM),[7] validating the good affinity between the probe and lipase. Soon after, the kinetics of α-amylase on the probe **S4** were also evaluated (Fig. S9 C, D), the related kinetic parameters have been determined as V_{max} = 4.14 μM min^{-1} and K_m = 4.82 μM, compared to the α-amylase probe **S2**,[7] the K_m value of **S4** is slightly larger, which also stats that the hydrolysis of **γ-CD** is a relatively slow process.

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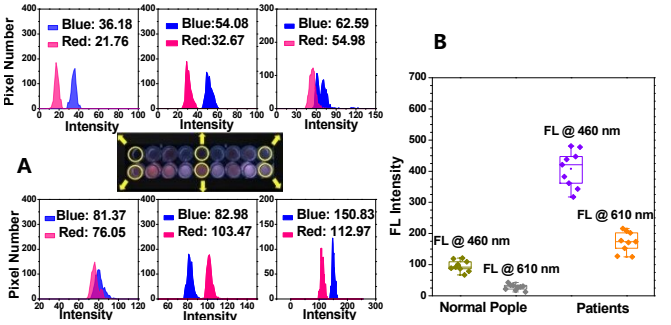

Fig. 4 A) Picture of probe **S4** after incubation with 10-fold diluted real human serum samples under UV light illumination at 365 nm. Upper row: normal people; lower row: patients. The histograms show the intensity distributions of pixels in the red and blue channels. The numbers in the figures represent the average intensity per pixel in the blue and red color channel. B) Box plot representation of fluorescence intensity at 460 nm and 610 nm of detection system in response to lipase and α-amylase from diluted serum specimens of normal people and acute pancreatitis patients.

Finally, the probe was applied to simultaneously sensing lipase and α-amylase in human serum at the point of care. We use 10-fold diluted serum specimens from 9 acute pancreatitis patients and 9 normal people. After the incubation, we took a picture of the mixtures in 96-well plate with a digital camera, selected the designated area, and quantified the signal using a software script, determines the average intensity per pixel (AIP) in the blue and red color channels.[13] Fig. 4A and inset photograph indicted that the patients' AIP values of blue and red color channels were significant higher than the normal ones. Therefore, the AIP of normal people can be used to build the threshold values for the point of care testing and diagnosis of acute pancreatitis. Subsequently, the using of calibration curve (Fig. 1B, D), as a standard, to the simultaneous quantitative analysis of lipase and α-amylase levels in human serum, the results in Fig. 4B and Table S1 indicate that the lipase and α-amylase levels of normal people was in the range of 88.49-168.57 and 37.23-141.25 U L^{-1}, which were within the normal range of enzymes. For patients, their lipase and α-amylase levels were significantly higher, being in the range of 460.13-702.16 and 420.01-729.75 U L^{-1}. The photograph in Fig. 4A, on one hand, it is clear that very weak fluorescence is observed from normal ones' samples and that for patients' samples, significant double fluorescence emission "light-up" can be noticed, demonstrating the possibility of rapid eyeball diagnosis. On the other hand, if there are very few cases in which lipase or α-amylase is elevated independently, our probe system will exhibit monochromatic emission, affording the early warning of pancreatic injury either. In addition, the determined results were confirmed with those obtained by the complex commercial lipase assay kit and α-amylase assay kit methods (Table S2).

In summary, we have successfully designed and fabricated a dual-reactive AIE probe that can respond to both lipase and α-amylase with two differentiable fluorescent signals under the same excitation wavelength. The combination with the enzymatic hydrolysis function and decomposition of host-guest inclusion triggered the AIE affect, and there is a 150 nm way between two emission peaks, making the signal distinguished quite easily by the naked eye. Importantly, this simple test system can simultaneously perform quantitative analysis of lipase and amylase in a homogeneous system within 25 minutes, showing selectivity and not interfered by other biological components, depending on the color of "lighting up", bedside testing can be realized. On the basis of the advantages above, this system can be popularized and applied in sensing of lipase and α-amylase during clinical diagnosis.

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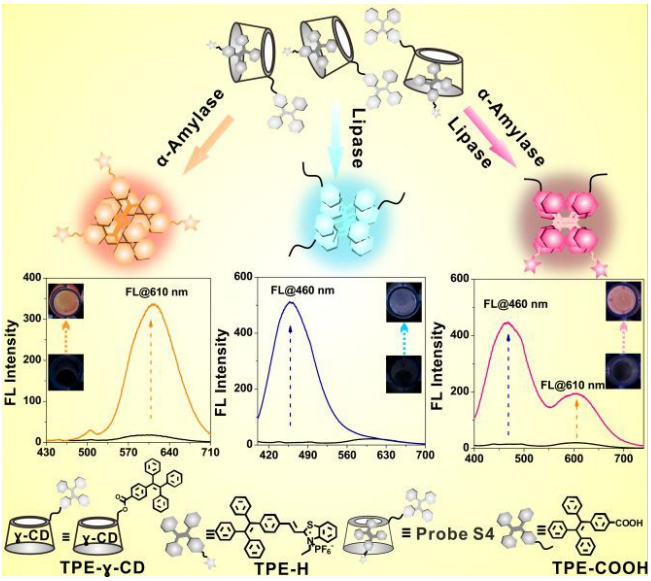

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