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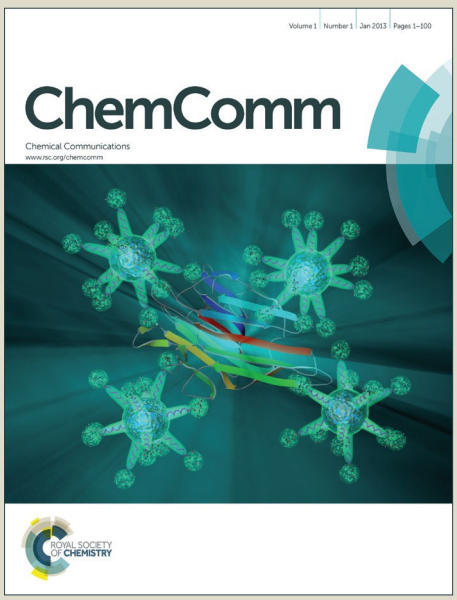

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ARTICLE

Fluorescent switchable AIE probe for selective imaging of dipeptidyl peptidase-4 *in vitro* and *in vivo* and its application in screening DPP-4 inhibitors

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A novel fluorescent probe for *in vitro* and *in vivo* imaging of dipeptidyl peptidase-4 (DPP-4) was designed and synthesized. This probe is successfully utilized to screen DPP-4 inhibitors in living 3T3-L1 cells and zebrafish, which provides a novel approach for the discovery of anti-diabetes drugs.

Type 2 diabetes mellitus (T2DM) is a common metabolic disorder with growing morbidity worldwide. However, previous therapies for T2DM including PPARγ agonists and glucosidase inhibitors have limited efficacy and significant mechanism-based side effects.[1] In the past decade, dipeptidyl peptidase-4 (DPP-4) has become one of the most promising and remarkable targets for treating T2DM.[2] DPP-4 is a type II transmembrane glycoprotein widely distributed in tissues and circulates. DPP-4 can cleave N-terminal amino acids from glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which play important roles in regulating glucose-induced insulin secretion.[3] Oral DPP-4 inhibitors such as sitagliptin and alogliptin have exhibited satisfied clinical outcomes.[4] Thus, screening small-molecule inhibitors of DPP-4 become a crucial issue in T2DM drug discovery. Moreover, circulating level of DPP-4 is considered as a potential biomarker for the prognosis of lymphoblastic leukaemia and hyperglycemia,[5] which also raise the demand of sensitive assay to detect DPP-4 in biological samples.

Recently, florescent probes with aggregation-induced emission (AIE) characteristics have attracted increasing interesting since Tang et al. found the AIE phenomenon.[6] Taking advantage of the distinct feature of AIE molecules that they are non-emissive in solution but become highly emissive in the aggregate state, various AIE molecules such as tetraphenylethene (TPE), 9,10-distyrylanthracene (DSA), and silacyclopentadiene (silole) have been utilized as basic scaffolds for the design of novel probes.[7,8,9] A few probes with high selectivity have been applied in sensing Cu^{2+}, Al^{3+}, and other ions,[10,11,12] monitoring physiopathological events,[13,14,15] and screening drug candidates.[16,17,18]

Herein, we designed and synthesized a novel fluorescent probe with AIE characteristics for DPP-4 assay and imaging (Scheme 1). TPE was employed as the AIE fluorophore to conjugate with a hydrophilic peptide Lys-Phe-Pro-Glu (KFPE) that can be specifically cleaved by DPP-4. The probe is non-emissive in solution, whilst after incubate with DPP-4, fluorescent signal will be switched on due to the aggregation of TPE-residue. As shown in Scheme 1, this probe can easily penetrate the phospholipid layer of cell membrane, which endows it with high sensitivity for in situ imaging of DPP-4 activity in living cells and organisms. Moreover, this probe can be utilized to measure the reduction of fluorescent emission in the presence of DPP-4 inhibitors, which facilitates screening anti-diabetes drugs *in vitro* and *in vivo*.

The synthesis of TPE-based probe was conveniently achieved in four steps as illustrated in Scheme S1. The design of peptide sequence was optimized to ensure that the probe dissolved in aqueous solution is non-emissive and subsequently can be recognized by DPP-4. It is clear that DPP-4 preferentially cleaves peptides with the amino acid proline in position 2 of the N-terminus of the peptide chain to switch on strong fluorescent signal due to the aggregation of TPE-based residue (Fig.S1, ESI†). DPP-4 is unable to recognize peptides KFPG and GPD connected to TPE-core due to the specificity of substrate sequence.[23] Thus, TPE-KFPE was finally selected as the appropriate probe and characterized with HPLC, ^{1}H NMR, and ^{13}C NMR spectroscopy (Fig.S2-S4, ESI†).

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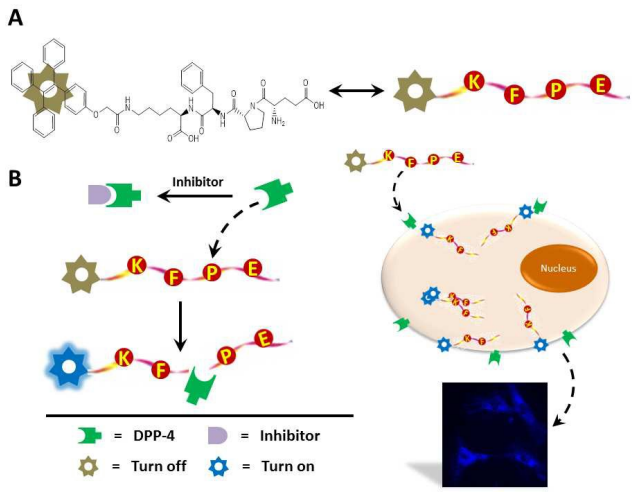
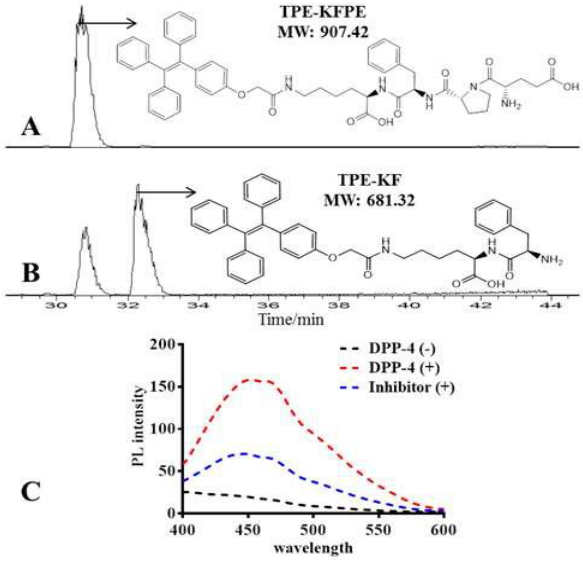


Scheme 1 Schematic illustration of TPE-KFPE (A) for DPP-4 assay and inhibitor screening and living cell imaging (B).

The assay system for detecting DPP-4 activity in solution was optimized. The acidified or alkalized solution may lead to invalid assay, but solution with the pH range of 6.5 to 8.2 can produce satisfied fluorescence enhancement (Fig.S5, ESI†). High ionic strength of HEPES buffer solution may reduce the signal (Fig.S6, ESI†). We further used liquid chromatography coupled with mass spectrometery (LC-MS) to analyze our probe and its hydrolysis products after incubation with DPP-4. After incubated with DPP-4 (5 mU/mL) for 30 min at 37℃, a new peak TPE-KF appeared in the LC-MS chromatogram (Fig.1B), which is the hydrolysis product of our probe and leads to the boost of fluorescent signal. By comparison of ^{1}H NMR and MS data of TPE-KFPE with the product of enzymatic reaction after incubation with DPP-4, it clearly showed that two amino acids were cleavaged from the probe (Fig.S2, S7, ESI†). When incubated the probe with DPP-4 and diprotin A, a known DPP-4 inhibitor, the probe is enough sensitive to monitor the suppression of DPP-4 activity (Fig.1C).



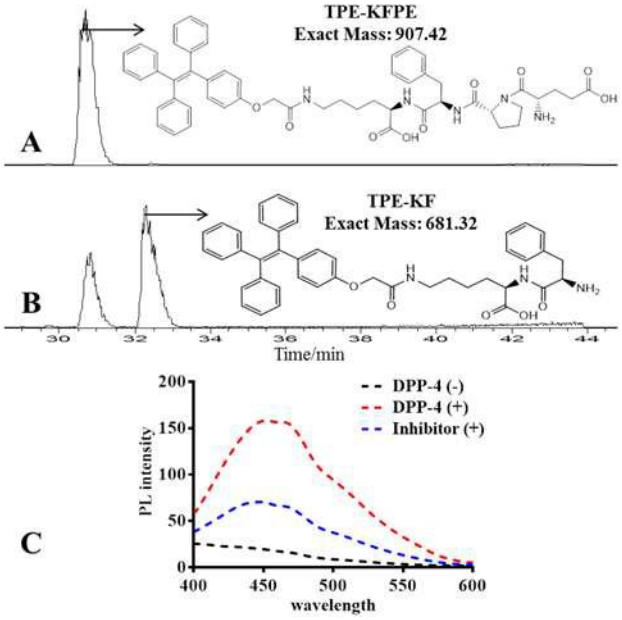


Fig. 1 LC-MS chromatograms of TPE-KFPE before (A) and after incubated with DPP-4 (B). Corresponding fluorescent spectra of TPE-KFPE before and after incubated with DPP-4 in the presence or absence of diprotin A (C). λex = 320 nm.

Kinetic experiments showed that the enzymatic reaction was almost completed within 30 minutes, while none fluorescence enhancement was observed in the absence of DPP-4 (Fig.2A). The linear range of our probe for determining DPP-4 activity was measured using a fluorescence microplate reader. There is a good linear correlation (R = 0.9608) between the relative fluorescence intensity and the amount of DPP-4 in the range from 0.1 to 0.5 mU/mL with 10 μM probe (Fig. 2B). We determined the inhibitory rates of diprotin A with different concentrations on DPP-4 activity by the proposed assay system. As shown in Fig.2C, a dose-dependent curve was obtained with a IC_{50} value of 4.15 μM, which is in accordance to previous reference.[19] The probe was also applied in screening potential DPP-4 inhibitors from natural products. An abundantly existed catechin in green tea, (-)-epigallocatechin-3-gallate (EGCG), was found to dose-dependently inhibit DPP-4 activity in the concentration of 5 μM to 25 μM (Fig.S8, ESI†), which is similar to a recent report.[19] These results indicated that the TPE-KFPE probe can be utilized as sensitive assay to measure DPP-4 activity as well as to screen DPP-4 inhibitors.

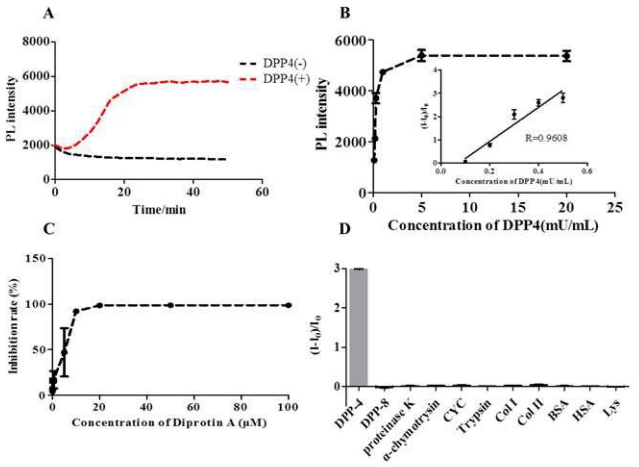


Fig. 2 (A) Kinetic study of TPE-KFPE reacted with DPP-4. (B) Dose-dependent of TPE-KFPE in the presence of DPP-4. (C) Dose-dependent inhibition of DPP-4 by diprotin A. (D) Plot of $(I-I_0)/I_0$ versus different enzymes/proteins. TPE-KFPE = 10 μM, DPP-4 = 5 mU/mL, λex = 320 nm, λem = 450 nm.

The specificity and cytotoxicity of TPE-KFPE probe were further exploited to ensure its capability for assessing DPP-4 in physiological environment. As shown in Fig.2D, the probe incubated with DPP-4 produced a much higher change in $(I-I_0)/I_0$ compared to the signals induced by cytochrome C (CYC), trypsin, collagenase I/II (Coll I/II), bovine serum albumin (BSA), human serum albumin (HSA), proteinase K, α-chymotrysin and lysozyme. Interestingly, DPP-8, another dipeptidyl peptidase, also can not recognize the probe which suggested satisfied specificity of TPE-KFPE on DPP-4. Incubation of the probe (10, 30, 50 μM) with 3T3-L1 preadipocyte cells for 24 h showed no significant effects on cell survival (Fig.S9, ESI†). Because TPE-KFPE contains both hydrophilic and hydrophobic regions that minimizes free energy when interacted with the biological membrane, the probe can easily penetrate the lipid bilayer of cells, Moreover, the size of the probe is very small before aggregation, which is also helpful for facile and minimally invasive cell penetration. This feature is of great significance for the subsequent *in vitro* and *in vivo* applications in molecular imaging.

DPP-4 widely exists in human body as an intracellular and serum-soluble form, and is considered as an adipokine produced by adipocytes.[20] Previous method for measurement of intracellular DPP-4 content needs tedious steps like cell collection, sample preparation, and enzyme-linked immunosorbent assay.[21] Our probe exhibited its ability to tracking DPP-4 activity in living cells by fluorescence microscope. As shown in Fig.3, owing to the AIE character of TPE-KFPE, high-contrast staining of intercellular region of cells was achieved after incubated 50 μM TPE-KFPE with 3T3-L1 preadipocyte cells for 1 h. The dose-dependent relationship of DPP-4 concentrations in 3T3-L1 cells with fluorescent signals can be sensitively measured by the probe (Fig.S10, ESI†). The probe was also successfully utilized to screen DPP-4 inhibitors *in vitro*. Fluorescent images of 3T3-L1 cells pretreated by DPP-4 inhibitor diprotin A (100 μM) and above mentioned active compound EGCG (100μM) showed that only weak fluorescent was detected. Quantitative analysis of those images reveals that the relative fluorescent signal is significantly decreased to approximately 65% compared to the control (Fig.S11 , ESI†), which suggests that the probe can also be used for *in vitro* screening of DPP-4 inhibitors.

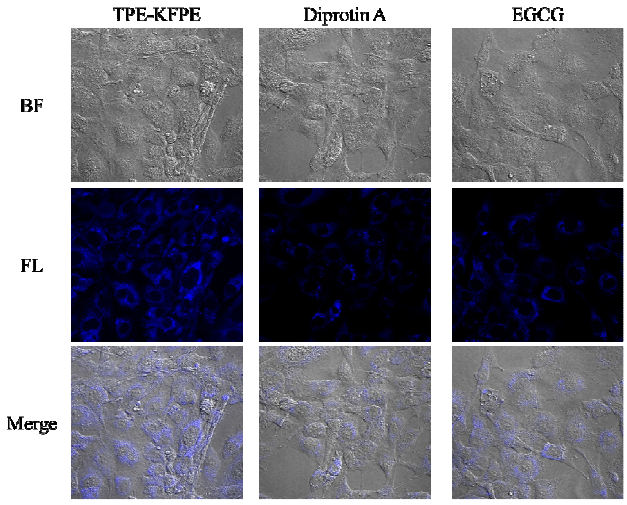


Fig. 3 Bright-field (BF), fluorescence (FL), and overlay images of 3T3-L1 cells stained by TPE-KFPE in the absence or presence of diprotin A and EGCG. The images were captured using Nikon A1R laser scanning confocal microscope equipped with 405 nm laser (60 × lens). Excitation and emission wavelength: 405 nm and 425-475 nm.

Next, we evaluated the *in vivo* tracking ability of TPE-KFPE in zebrafish, a well-established model system for developmental biology and high throughput screening. The zebrafish larvae are transparent, which offers great convenience for optical and fluorescence microscopic detection. Fig.4A and 4B show the images of whole bodies of normal zebrafish larvae and larvae microinjected with TPE-KFPE (2.5 mM, 10 nL) for 2 h. The zebrafish larvae exposed to the probe exhibits significant fluorescence in yolk sac, liver, pancreas, and other organs connected to gastrointestinal tract. This can be explained that DPP-4 is highly expressed in liver, exocrine pancreas, and small intestine.[22] The accumulated fluorescence in the yolk sac and in the intestine indicated that TPE-KFPE entered into the digestive system and may be eliminated from the body. A representative series of optical sections for *in vivo* imaging of DPP-4 by our probe showed that distribution pattern of DPP-4 chiefly located in yolk sac and blood vessel of zebrafish (Fig.S12, ESI†). Further, we also stained the zebrafish pretreated with diprotin A with the probe. As shown in Fig.4C, relative low fluorescent signals can be observed. An approximate 50% decrease of fluorescence intensity is observed in DPP-4 inhibitor treated zebrafish (n=6, Fig.S13, ESI†). This suggests that TPE-KFPE can be used for *in vivo* sensing of DPP-4 and screening the potential DPP-4 inhibitor in zebrafish.

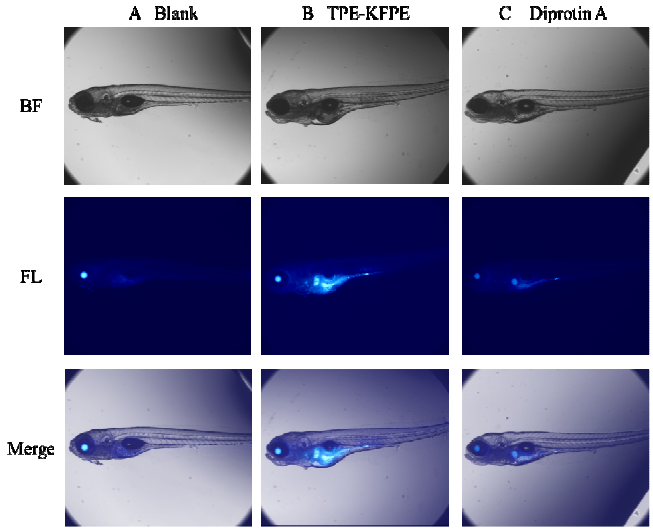


Fig. 4 Bright-field (BF), fluorescence (FL), and overlay images of zebrafish. (A) Zebrafish without probe. (B) Zebrafish stained by TPE-KFPE. (C) Zebrafish pre-incubated with diprotin A stained by TPE-KFPE. The images were captured using a fluorescent microscope equipped with Andor Zyla cMOS camera and 5 × lens.

In summary, we herein for the first time reported a probe with AIE characteristics that exhibits excellent fluorescent switchable property in the present of DPP-4. By the introduction of a specific peptide to TPE core, the probe shows excellent sensitivity and linear range in the detection of DPP-4 activity both in vitro and in vivo. The probe is successfully applied to screen DPP-4 inhibitors in living cells and zebrafish. The assay system demonstrated here provides a new approach to enabling *in vivo* high-throughput screening of T2DM drugs in zebrafish.

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