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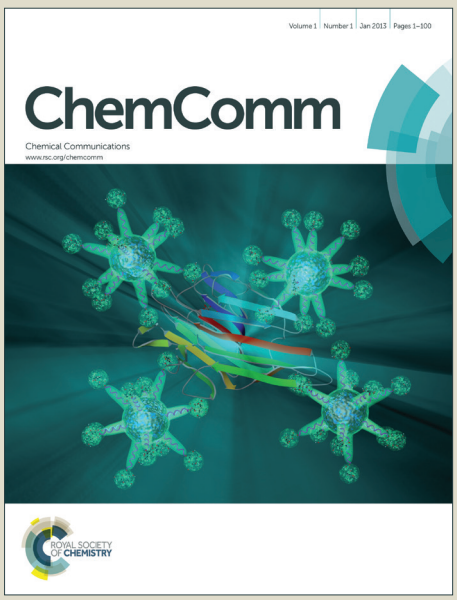

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ARTICLE

A novel aggregation-induced emission based fluorescent probe for angiotensin converting enzyme (ACE) assay and inhibitor screening

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A 'turn-on' fluorescent probe based on aggregation-induced emission (AIE) has been developed. It exhibits excellent selectivity and sensitivity to monitor angiotensin converting enzyme (ACE) activity both in solutions and in living cells as well as to screen ACE inhibitors *in vitro*.

Angiotensin converting enzyme (ACE), a zinc-dependent peptidase known for hydrolyzing angiotensin I into the vasoconstrictor angiotensin II, plays pivotal roles in the regulation of blood pressure, hematopoiesis[1], renal development[2], immune response[3], and other physiological processes[4]. ACE inhibitors such as captopril and enalapril have achieved dazzling successes in the treatment of cardiovascular disorders, primarily hypertension and congestive heart failure[5].To date, fluorescence resonance energy transfer (FRET) was the most common method for determination of ACE activity and screening ACE inhibitors[6,7]. The fluorescent probe and the quencher acceptor were linked by a specific peptide which can be recognized and cleaved by ACE. Peptide cleavage can result in the loss of FRET and the ACE activity can be evaluated via the changes in fluorescence intensity[8]. However, these fluorescent sensors often require more complicated synthetic steps and still suffer from photo-bleaching and background interference[9,10]. Moreover, conventional dyes tend to aggregate in aqueous medium, thus causing the ubiquitous aggregation-caused quenching (ACQ) effect[11,12].

To address these issues, development of fluorescent sensors with high fluorescent efficiency and high signal-to-noise ratio is still in urgent pursuit. Among the diverse candidates, a novel class of organic fluorophores has been developed to exhibit extraordinary aggregation-induced emission (AIE) behaviours, which are non-emissive fluorescent in solutions, but their fluorescent can be turned on in the aggregate state[13,14]. AIE-based fluorophores have aroused great attention due to their facile processability, high emission efficiency and contrast in the aggregated states[15,16]. More importantly, AIE-active fluorogens have overcome the notorious ACQ problem. Therefore, fluorescent probes with AIE characteristics have provided a new method for biosensors and cell imaging[17]. However, to our knowledge, little AIE-based probe has been exploited for inhibitor drug screening.

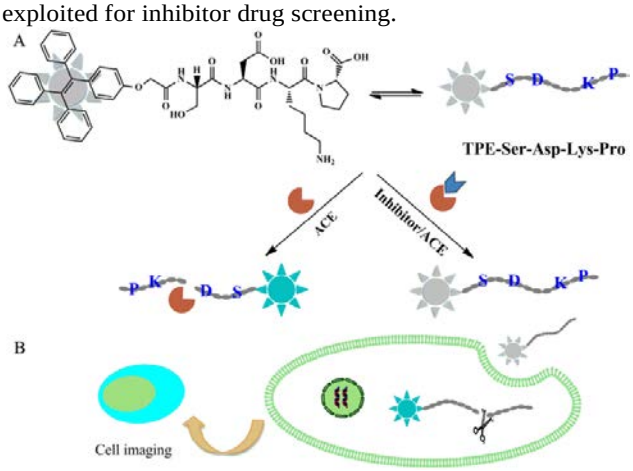

Fig. 1 Illustration of TPE-SDKP for ACE Activities Study (A) and cellular imaging (B).

In this contribution, we report a novel fluorescent probe containing a hydrophobic AIE-based fluorophore (tetraphenylethene (TPE)) and a hydrophilic Ser-Asp-Lys-Pro(SDKP)peptide sequence. The fluorescent probe was soluble in water completely and non-emissive fluorescent in aqueous medium. Because the circulating tetrapepetide N-acetyl-Ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of ACE[18,19], as shown in Fig.1, the

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specific peptide sequence could be recognized and cleaved by ACE. Then, the hydrophobic TPE residues could aggregate and the fluorescence was switched on in aqueous solution. This probe could be utilized for monitoring of ACE activity and determinating of the efficiencies of ACE inhibitors both in solution and in living cells. By monitoring the activity of ACE in living cells, we further demonstrated this biosensor can be applied in cellular imaging and *in situ* drug screening with high fluorescence contrast.

As one of typicalAIE-based fluorophore, TPE has a widely application in biosensor and cell imaging. The TPE-based fluorescent probe was synthesized through two processes as shown in Fig.1. Briefly, tetraphenylethylene with carboxylate group was synthesized by McMurry reactions. Then, TPE-SDKP was prepared by standard solid-phase synthesis.[1]H NMR and ESI-MS determined that we have obtained the fluorescent probe successfully. HPLC analysis indicated that the purity of TPE-SDKP was more than 98%(Fig. S1-S5).

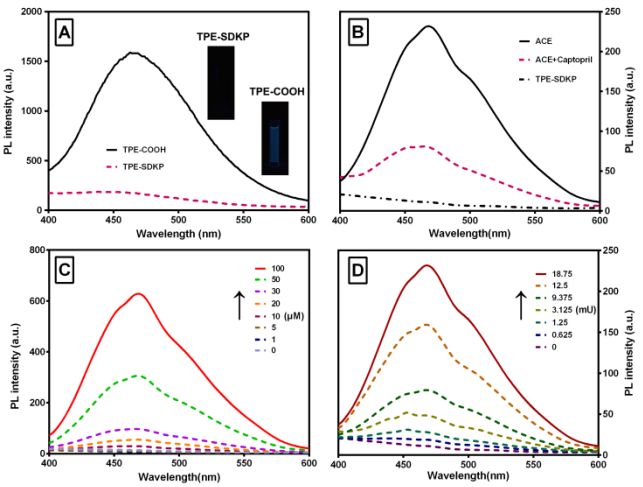

Fig. 2 (A) Photoluminescence (PL) spectra of TPE-COOH and TPE-SDKP in distilled water. Inset: photographs of TPE-COOH and TPE-SDKP in water taken under illumination by an UV lamp.(B) PL spectra of TPE-SDKP upon treatment with ACE in the presence and absence of inhibitor captopril.(C) PL spectra of varied concentrations of TPE-SDKP in the presence of ACE. (D) PL spectra of TPE-SDKP in the presence of varied concentrations of ACE. The incubation time is 3 h. [TPE-COOH] = [TPE-SDKP] = 50 μ M; ACE = 18.75 mM; [inhibitor] = 10 nM; λ_{ex} = 320 nm.

To explore whether the TPE-SDKP probe can be used in detecting ACE activity, the fluorescence emission blocking character of TPE-SDKP was compared with TPE (Fig.2A). It was found that TPE-COOH showed intense fluorescence in aqueous solution due to the amphiphilic property of TPE-COOH[20]. However, the TPE-SDKP was almost non-fluorescent in the same medium, indicated that TPE-SDKP has a good solubility in water. A following experiment has been performed *in vitro* for ACE activity assay (Fig.2B). Mixtures of TPE-SDKP and ACE were prepared and incubated with or without captopril. The results showed that the fluorescence increased sharply after TPE-SDKP treated with ACE, while impaired activity of ACE caused by ACE inhibitor captopril could be sensitively represented in fluorescence signal.

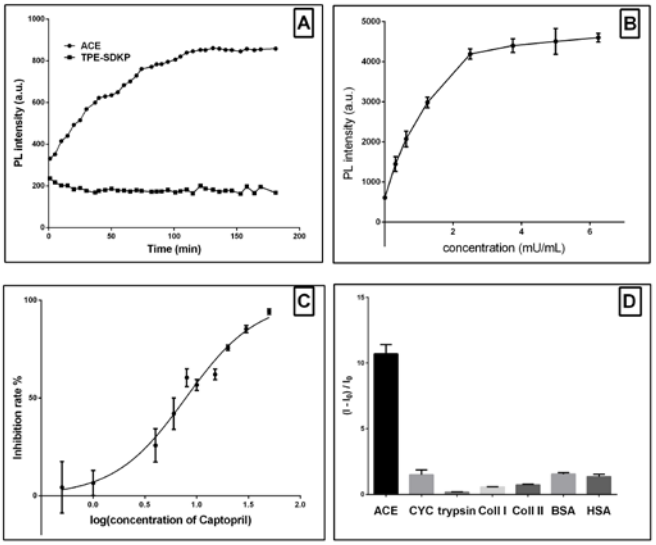

Fig.3(A)Time-dependent PL spectra of TPE-SDKP upon addition of ACE from 0 to 180 min. ACE = 18.75mM. (B) PL intensity of TPE-SDKP when incubated with concentration-dependent ACE, [TPE-SDKP]=200μM. (C) Concentration-dependent captopril inhibited ACE addition upon TPE-SDKP. (D)Plot of $(I-I_0)/I_0$ versus different enzyme/proteins, where I and I_0 represents the PL intensities of TPE-SDKP in Tris-buffer with or without the protein (0.5mg/mL).

Further exploration of the relationship between ACE and TPE-SDKP has been performed for optimizing the quantity of ACE and TPE-SDKP used in the assays. TPE-SDKP with varied concentrations from 0 to 100μM was incubated with ACE (18.75 mM) at 37℃. The PL spectra were recorded from 400 to 600nm. As shown in Fig.2C, the PL intensity gradually increased with the increasing concentration of TPE-SDKP. When TPE-SDKP was incubated with ACE in the concentration of 50μM, the PL intensity increased moderately. Therefore, 50μM of TPE-SDKP was used in following study. As shown in the Fig.2D, when the concentration of TPE-SDKP was fixed to 50μM, the PL intensity increased with the increasing concentration of ACE. Thus, the results suggested that TPE-SDKP was sensitive to the concentration changes of ACE, which could be an effective probe for detecting ACE.

The fluorescence detection of ACE hydrolyzing TPE-SDKP have been explored and gained more possibility to explore its kinetics by time. The increase of fluorescence could be attributed to the catalytic kinetics of the peptide. It has been proved Ser-Asp-Lys-Pro peptide sequence could be recognized and cleaved in the presence of ACE. As shown in Fig.3A, the substrate without ACE sustained a low and stable fluorescence.However, when TPE-SDKP incubated with ACE, the fluorescence rapidly increased within the first 100 min. After 100 min, most of the peptide had been hydrolyzed and the change rate of fluorescence became slower.Furthermore, when a constant amount of TPE-SDKP incubated with different concentration of ACE, the similar result was shown in Fig.3B. The PL intensity became stronger with the concentration increase of ACE. The intensity was rarely changed when the concentration of ACE was above 2.2 mU/mL, suggesting most of the peptides in TPE-SDKP have been enzymolyzed. To study the potential possibility of the probe for inhibitor screening, we tested the dose-dependent inhibition of captopril, a well-known ACE inhibitor. As shown in Fig.3C, the inhibitory activity was increased along with the concentration

of captopril. The result showed that the IC_{50} of captopril was 7.84nM, which exerted a good accordance with previous reported literature[21]. TPE-SKDP based assay was also applied to screen active components from Tongmaiyangxin Pills, a botanical drug for treating cardiovascular diseases and found two components with ACE inhibitory activity (Fig.S6).

Moreover, cell system could be much complicated compared with the simple buffer solution, which promoted us to inspect whether the characters of TPE-SDKP could be affected by different enzymes or proteins. TPE-SDKP was incubated with ACE, bovine serum albumin (BSA), trypsin, collagenase I/II, human serum albumin (HSA), and cytochrome C (CYC). Fig.3D showed that compared to ACE, other enzymes/proteins presented relatively weaker changes in $(I-I_0)/I_0$, which proved the proposed biosensor could be used as a specific probe for track ACE related cellular events *in vitro*. Slight increase of fluorescence signal in the presence of BSA and HSA might be attributed to the electrostatic induced assembly between ε-amino-group of lysine in the SDKP peptide and carboxyl anion groups of BSA, which was in accordance to the reports that BSA–TPE derivative conjugates has AIE characteristics[22].

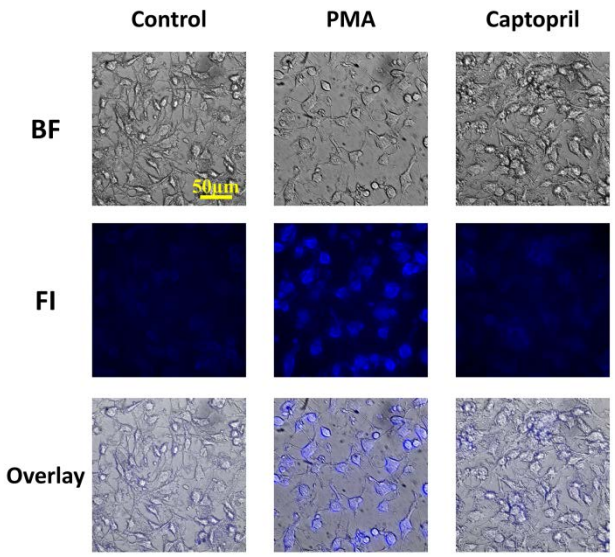

Fig. 4 Bright-field (BF), fluorescence (FL), and overlay images of normal HUVEC,HUVEC treated with PMA, and HUVEC treated with PMA plus captopril. The images were acquired using a fluorescence microscope (ZEISS) equipped with DAPI filter. All images share the same scale bar (50μm).

We further explored the characters of TPE-SDKP for live-cell imaging of human umbilical vein endothelial cell (HUVEC). After cultivated for 24 h, HUVEC cells were separately incubated with normal medium, 250nM phorbolmyristate acetate (PMA) which is a strong stimulus for up-regulation of ACE expression, and PMA plus captopril (100nM). After 36 h incubation, TPE-SDKP (50μM) was added into each sample. The bright field and fluorescent images were captured after 3 hours incubation. As shown in Fig.4, normal HUVEC show a relatively low fluorescent signal, as previous literature has reported that normal expression of enzymatically active ACE in the HUVEC[23], especially located in the membrane (Fig.S7). In comparison of normal cells, PMA stimulated HUVEC show a strong fluorescent signal with approximate 3.7-fold (Fig.S8). Meanwhile, treatment of captopril in PMA-stimulated HUVEC induced a significant decrease in fluorescent signal. Additionally, we also performed real-time imaging experiments in HT-29 human colorectal cancer cells and human mesenchymal stem cells (hMSC). As shown in Fig.S7, HT-29 showed rare ACE expression, while hMSC showed a relatively high expression of ACE, which was in accordance to previous report that local rennin angiotension expression participated in the differentiation of hMSC to adipocytes[24]. This result suggested that our probe might be used in track the phenotype of hMSC during its differentiation to adipocytes.

In summary, we have developed a fluorescent probe that contained a hydrophobic AIE-based fluorophore (TPE) and a hydrophilic peptide sequence. The fluorescent probe was soluble in water and non-emissive fluorescent in aqueous medium. However, when the specific peptide sequence could be recognized and cleaved by ACE, the hydrophobic TPE residues could aggregate and the fluorescence was switched on in aqueous solution. This probe could be utilized for monitoring ACE activity and evaluating the activities of ACE inhibitors in solution. The outstanding properties of this probe also highlight its potential use as a novel living cell imaging probe for determinating of ACE activity and ACE inhibitors screening.

Acknowledgements

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Notes and references

1 X. Z. Shen and K. E. Bernstein, *Cell Cycle*, 2011, **10**, 1363.
2 J. F. Giani, T. Janjoulia, S. Fuchs, J. Peti-Peterdi, K. E. Bernstein and R. A. Gonzalez-Villalobos, *Faseb J*, 2013, **27**.
3 K. E. Bernstein, R. A. Gonzalez-Villalobos, J. F. Giani, K. Shah, E. Bernstein, T. Janjulia, Y. Koronyo, P. D. Shi, M. Koronyo-Hamaoui, S. Fuchs and X. Z. Shen, *Biol Chem*, 2014.
4 K. E. Bernstein, F. S. Ong, W. L. B. Blackwell, K. H. Shah, J. F. Giani, R. A. Gonzalez-Villalobos, X. Z. Shen and S. Fuchs, *Pharmacol Rev*, 2013, **65**, 1.
5 C. G. Smith and J. R. Vane, *Faseb J*, 2003, **17**, 788.
6 M. C. Araujo, R. L. Melo, M. H. Cesari, M. A. Juliano, L. Juliano and A. K. Carmona, *Biochemistry*, 2000, **39**, 8519.
7 A. K. Carmona, S. L. Schwager, M. A. Juliano, L. Juliano and E. D. Sturrock, *Nat Protoc*, 2006, **1**, 1971.
8 M. A. Sentandreu and F. Toldra, *Nat Protoc*, 2006, **1**, 2423.
9 K. Li, J. Pan, S. S. Feng, A. W. Wu, K. Y. Pu, Y. T. Liu and B. Liu, *Adv Funct Mater*, 2009, **19**, 3535.
10 R. N. Day and M. W. Davidson, *Chem Soc Rev*, 2009, **38**, 2887.
11 Y. Hong, J. W. Lam and B. Z. Tang, *Chem Commun (Camb)*, 2009, 4332.
12 Z. J. Ning, Z. Chen, Q. Zhang, Y. L. Yan, S. X. Qian, Y. Cao and H. Tian, *Adv Funct Mater*, 2007, **17**, 3799.
13 W. Z. Yuan, P. Lu, S. Chen, J. W. Lam, Z. Wang, Y. Liu, H. S. Kwok, Y. Ma and B. Z. Tang, *Adv Mater*, 2010, **22**, 2159.
14 Y. Hong, J. W. Lam and B. Z. Tang, *Chem Soc Rev*, 2011, **40**, 5361.
15 A. Qin, J. W. Y. Lam and B. Z. Tang, *Prog Polym Sci*, 2012, **37**, 182.
16 X. Zhang, X. Zhang, L. Tao, Z. Chi, J. Xu and Y. Wei, *J Mater Chem B*, 2014, **2**, 4398.

17 K. Dhara, Y. Hori, R. Baba and K. Kikuchi, *Chem Commun (Camb)*, 2012, **48**, 11534.
18 A. Rousseau, A. Michaud, M. T. Chauvet, M. Lenfant and P. Corvol, *J Biol Chem*, 1995, **270**, 3656.
19 M. Azizi, A. Rousseau, E. Ezan, T. T. Guyene, S. Michelet, J. M. Grognet, M. Lenfant, P. Corvol and J. Menard, *J Clin Invest*, 1996, **97**, 839.
20 X. Xue, Y. Zhao, L. Dai, X. Zhang, X. Hao, C. Zhang, S. Huo, J. Liu, C. Liu, A. Kumar, W. Q. Chen, G. Zou and X. J. Liang, *Adv Mater*, 2014, **26**, 712.
21 R. Kapel, A. Chabeau, J. Lesage, G. Riviere, R. Ravallec-Ple, D. Lecouturier, M. Wartelle, D. Guillochon and P. Dhulster, *Food Chem*, 2006, **98**, 120.
22 J. P. Xu, Y. Fang, Z. G. Song, J. Mei, L. Jia, A. J. Qin, J. Z. Sun, J. Ji and B. Z. Tang, *Analyst*, 2011, **136**, 2315.
23 I. V. Balyasnikova, S. M. Danilov, V. R. Muzykantov and A. B. Fisher, *In Vitro Cell Dev Biol Anim*, 1998, **34**, 545.
24 K. Matsushita, Y. Wu, Y. Okamoto, R. E. Pratt and V. J. Dzau, *Hypertension*, 2006, **48**, 1095.

