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A fluorescent probe for detection of histone deacetylase activity based on aggregation-induced emission†

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A tetraphenylethylene-derivative fluorescent probe for the one-step detection of histone deacetylases (HDAC) was developed. The deacetylation of the probe triggers electrostatic interaction between the molecules and automatically leads to fluorescence enhancement based on aggregation-induced emission (AIE).

Epigenetic regulation of gene expression plays an essential role in development, differentiation, and prevention of diseases.[1] The level of gene expression is modulated by the modification of DNA or histone proteins without any change of genetic information encoded in DNA sequences. The reversible acetylation of lysine residues near the N-terminus of nucleosomal histones by histone deacetylases (HDACs) and histone acetyltransferases (HATs) regulates chromatin structure and transcriptional activity.[1,2] Histone acetylation catalyzed by HATs generally leads to transcriptional activation, whereas deacetylation activity executed by HDACs results in transcriptional repression.[3] The activities of both HATs and HDACs affect various biological phenomena including angiogenesis,[4] apoptosis,[5] and the pathogenesis of malignant diseases.[6] In particular, HDACs are attracting attention, because these enzymes are the major targets of drug development for diseases such as cancer, neurological diseases, and metabolic diseases.[7] Therefore, the detection of HDAC activities is key to medical sciences as well as basic biology. Although tremendous efforts have been made to develop methods for investigating the deacetylation of HDAC substrates, all of these methods require multiple laborious processes for the detection of enzyme activity. Classical assays for the determination of HDAC activity were based on the incubation of the enzyme with [^{3}H]acetylated histones[8] and peptide substrates.[9] The radio-active assays, however, require the separation of the product from the substrate, and this process limits the assay throughput. A non-isotopic HDAC assay utilizing a fluorophore-conjugated peptide was reported in the literature and also requires a coupled two-step procedure.[10] In the first step of the assay, enzyme deacetylation of the peptides is initiated. In the second step, the HDAC reaction is detected *via* trypsin cleavage of the fluorophore from the peptides. The main limitation of this assay is its inability to permit continuous monitoring of the enzyme activity.

In this research, to overcome these problems, a new fluorescent probe, K(Ac)PS-TPE, was developed, which enables the detection of the deacetylation activity by simple mixing with HDAC. As a probe scaffold, tetraphenylethylene (TPE) was employed with a switching mechanism that is based on aggregation-induced emission (AIE). A recent breakthrough in the synthesis of fluorescent organic dyes, which induce AIE phenomena, has attracted special attention.[11] While TPE derivatives are AIE-active and weakly fluorescent in solution, these dyes become highly fluorescent upon aggregation.[12] In the dilute solution of TPE, fast rotation of the phenyl rings and partial twisting of the C=C bond quench its fluorescence.[13] On the other hand, in TPE aggregates, close intermolecular interactions obstruct the rotation of the phenyl groups resulting in fluorescence enhancement. The unique luminescence behavior of TPE has been harnessed for the development of biological sensors,[14] solid-state lighting materials,[15] and luminescent polymers.[16]

Considering this fluorescence property of TPE, the design rationale for the HDAC probe was devised, as illustrated in Scheme 1. K(Ac)PS-TPE contains *N*-α-*t*-butoxycarbonyl-*N*-ε-acetyl-L-lysine and propane sulphonic acid, which were attached to TPE. In this design strategy, the sulphonate moiety was chosen to increase the water solubility and to serve as a negative terminal charge. In this acetylated state, it was expected that the probe shows weak fluorescence due to the lack of aggregation of the probe. The deacetylation of the probe with HDAC generates KPS-TPE, which possesses primary aliphatic ε-amine of lysine. The amine was protonated under physiological conditions, as the pK_a value was close to 10.5. As a result, the newly generated cationic unit would trigger the aggregation of the probe (Scheme 1b) owing to the probable electrostatic interaction among the sulphonate unit and the cationic *N*-ε-ammonium ion. This phenomenon prevents the free rotor motions of the phenyl moiety because of the physical restraints in the aggregated state, and thus, KPS-TPE becomes highly fluorescent.

The synthetic route for K(Ac)PS-TPE is shown in Scheme S1 (ESI†). It involves cross McMurry coupling of benzophenone and 4,4′-diaminobenzophenone resulting in compound **1**.

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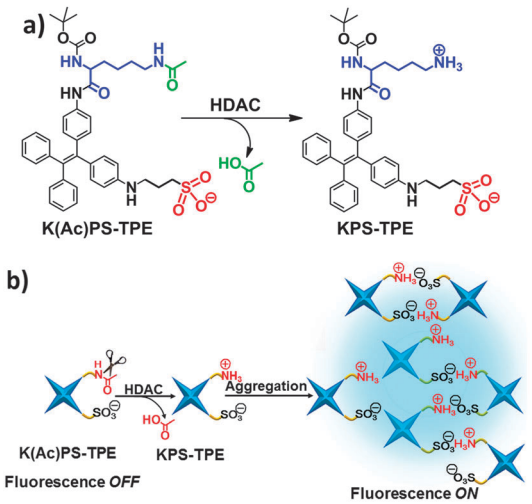

Scheme 1 (a) Proposed enzymatic reaction of K(Ac)PS-TPE with HDAC. (b) Schematic representation of the aggregation-induced fluorescence enhancement of K(Ac)PS-TPE by HDAC reaction.

N-α-*t*-Butoxycarbonyl-*N*-ε-acetyl-L-lysine was attached to one of the amino groups of **1** and 1,3-propanesultone was reacted with the other amino group to form K(Ac)PS-TPE. First, the stability of the probe in buffer (pH 8.0) at 37 °C was examined. The reversed-phase HPLC analyses showed that the probe displayed sufficient stability in the reaction buffer (pH 8.0) up to 24 h (Fig. S2, ESI†). The progress of the enzymatic reaction was also monitored by using reversed-phase HPLC (Fig. 1 and Fig. S3, ESI†). Sirt1 was used as a HDAC for the characterization of the probe in this research. Sirt1 is a NAD^+-dependent enzyme, which deacetylates various substrates such as histone,[17] p53[18] Ku70,[19] NF-κB,[20] and forkhead proteins.[21] For the deacetylation, NAD^+ is consumed as a co-substrate to afford nicotinamide, the deacetylated peptide, and the metabolite

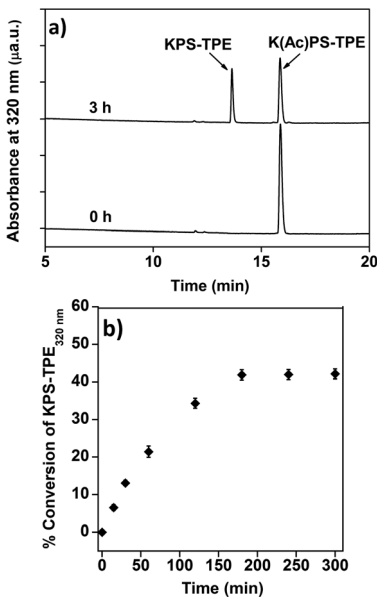

Fig. 1 (a) Reversed-phase HPLC analyses of the reaction progress of K(Ac)PS-TPE with Sirt1 (500 nM) in reaction buffer (pH 8.0) at 37 °C. (b) The conversion of K(Ac)PS-TPE to KPS-TPE was plotted against the progress over time.

2′-*O*-acetyl-ADP-ribose. This protein is implicated in diverse biological phenomena, including chromatin silencing,[22] axonal degeneration, and cell death.[23] After incubation of the probe with Sirt1 for 3 h, a new peak appeared in the chromatogram (Fig. 1a). Time-course experiments showed that the enzymatic reaction was almost completed within 3 h and the reaction yield was 42% (Fig. 1 and Fig. S3, ESI†). The elutant corresponding to the peak at the retention time of 13.9 min was collected and examined by ESI mass spectrometry. The presence of the expected deacetylated compound KPS-TPE (m/z, $[M + H^+]$: found 713.28; calculated 713.33) was confirmed. These results clearly indicated that the probe was deacetylated by Sirt1. The influence of acetate on the enzyme reaction was investigated to check whether the back reaction of KPS-TPE occurred in the presence of acetate, since the reaction yield did not increase after 3 h. Enzyme reaction was conducted in the presence of acetate and the reversed-phase HPLC showed that the acetate had no significant inhibitory effect on the enzyme activity (Fig. S4, ESI†). The possibility of enzyme inactivation during the assay was also examined. Fresh Sirt1 was added to the enzyme reaction mixture, which has been already incubated for 3 h, and then the enzyme reaction was checked with reversed-phase HPLC (Fig. S5, ESI†). No significant progress of the enzyme reaction was observed after the addition of fresh enzyme. A possible reason for the imperfect reaction is that the probe, K(Ac)PS-TPE, interacted with the product, KPS-TPE, and participated in the aggregate formation and thereby hindered further interaction of the probe with the enzyme.

The fluorescence spectra of K(Ac)PS-TPE (10 μM) were measured at 37 °C in the presence and absence of Sirt1 (500 nM) in HEPES buffer (pH 8.0) containing 500 μM NAD^+ (Fig. 2a). The fluorescence intensity of the probe was significantly increased in the presence of Sirt1, while only slight fluorescence enhancement was observed in the absence of the enzyme. The fluorescence intensity increased after 3 h of enzyme reaction, though the HPLC indicated the completion of the reaction. This discrepancy can be explained by the kinetic difference between the aggregate formation and the enzyme reaction. It is considered that the fluorescence enhancement was delayed because the aggregation process was slower than the enzyme reaction. The influence of NAD^+ on the fluorescence intensity of the probe was also examined. The intensity was slightly increased after addition of 500 μM of NAD^+ due to the possible non-covalent interaction between the probe and NAD^+ (Fig. S6, ESI†). Basal fluorescence intensity of the probe in the absence of enzyme was observed due to the interaction with NAD^+. Although NAD^+ caused the slight increase of background fluorescence, the fluorescence intensity amplified by the enzyme reaction was significantly higher than the background intensity. Furthermore, the enzymatic reaction was examined by using inactivated Sirt1 which was achieved by heating to 100 °C for 5 min. The increase in the fluorescence intensity of the probe was restrained and almost coincided with that of the background level (Fig. 2b). These results indicated that non-specific interaction between the probe and the enzyme was not observed and that the fluorescence increase in the enzymatic reaction resulted solely from the formation of the deacetylated product. From the absorption spectrum

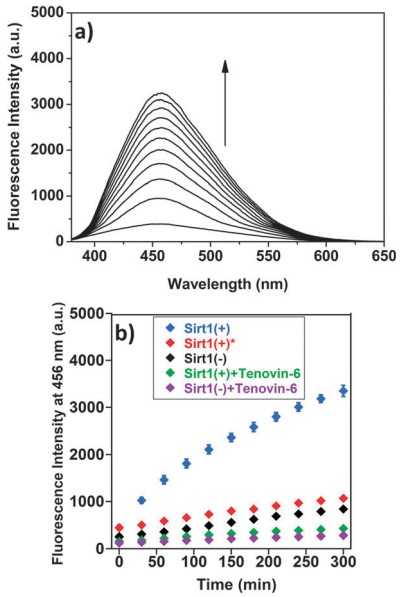

Fig. 2 (a) Time-dependent fluorescent spectra of the probe (10 μM) with Sirt1 (500 nM) in the presence of 500 μM of NAD^+ in the reaction buffer (pH 8.0) at 37 °C. The spectra were measured every 30 min after the addition of the enzyme up to 5 h. Excitation wavelength: 345 nm. (b) Fluorescent intensity of the probe (10 μM) at 456 nm was plotted against the incubation time. K(Ac)PS-TPE was incubated in the reaction buffer (pH 8.0) at 37 °C in the presence and absence of various additives; * corresponds to the Sirt1 inactivated by heat at 100 °C for 5 min.

(Fig. S7, ESI†) of the probe after the addition of Sirt1 (500 nM), it was found that the absorbance at 343 nm was increased. In contrast, the absorbance remained unchanged in the absence of Sirt1. The increased absorbance of the enzymatic reaction was expected to be due to the aggregated state. The fluorescence excitation maximum (Fig. S8, ESI†) was found at 345 nm, which coincided with the absorption maximum (343 nm) in this aggregated state.

The enzymatic reaction in the presence of a potent Sirt1 inhibitor, tenovin-6, was investigated.[24] The fluorescent intensity of the probe with tenovin-6 (1 mM) in the absence and the presence of Sirt1 (500 nM) was measured. The data showed that there was no significant difference in the fluorescence intensity of the probe in both cases (Fig. 2b). Reversed-phase HPLC analyses (Fig. S9, ESI†) after 5 h of reaction showed no formation of the deacetylated product. Thus, enzyme inhibition by tenovin-6 was confirmed. This finding demonstrated that the probe can be utilized to investigate the inhibitor activity.

In conclusion, a fluorescent probe, K(Ac)PS-TPE, for the detection of Sirt1 activity was designed and synthesized. The deacetylation of the probe triggers the electrostatic interaction between the anionic sulphonate and cationic lysine and automatically leads to fluorescence enhancement based on AIE. The advantage of this probe is that the enzymatic activity was distinctly detected by using a one-step procedure, in which the probe was simply mixed with the enzyme. Since the fluorescence increase of the probe was restrained in the presence of an HDAC inhibitor, the probe can also be utilized in inhibitor assays. Thus, this new probe should be valuable to the field of epigenetics and drug discovery.

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