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COMMUNICATION

# A dual-function chemical probe for detecting erasers of lysine lipoylation

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**Abstract** Lysine lipoylation plays vital roles in cell metabolism and redox processes. For example, removal of lipoylation will decrease pyruvate dehydrogenase activity and affect the citric acid cycle. Despite the important functions of lysine lipoylation, the mechanisms for the addition and removal of this modification remain largely unexplored. Very few useful chemical tools are available to study the interactions of lysine lipoylation with its regulatory delipoylation proteins. For example, immunoaffinity purification-mass spectrometry is one of such tools, which highly relies on antibody efficiency and purification techniques. Single-step activity based fluorogenic probes developed by our groups and others is also an efficient method to study the deacylation activity. Affinity-based labeling probe using photo-cross-linker is a powerful platform to study the transient and dynamic interactions of peptide ligands with the interacting proteins. Herein, we have designed and synthesized a dual-function probe KTLlip for studying enzymatic delipoylation (eraser) activity and interaction of lysine lipoylation with the eraser at the same time. We show that KTLlip can be used as a useful tool to detect delipoylation as demonstrated by its ability to fluorescently label the eraser activity of recombinant Sirt2. We envision that the probe will help delineate the roles of delipoylation enzyme in biology.

**Keywords** dual-function, fluorescent probe, labeling, photo-cross-linker, lipoylation modification, eraser, sirtuin

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## 1 Introduction

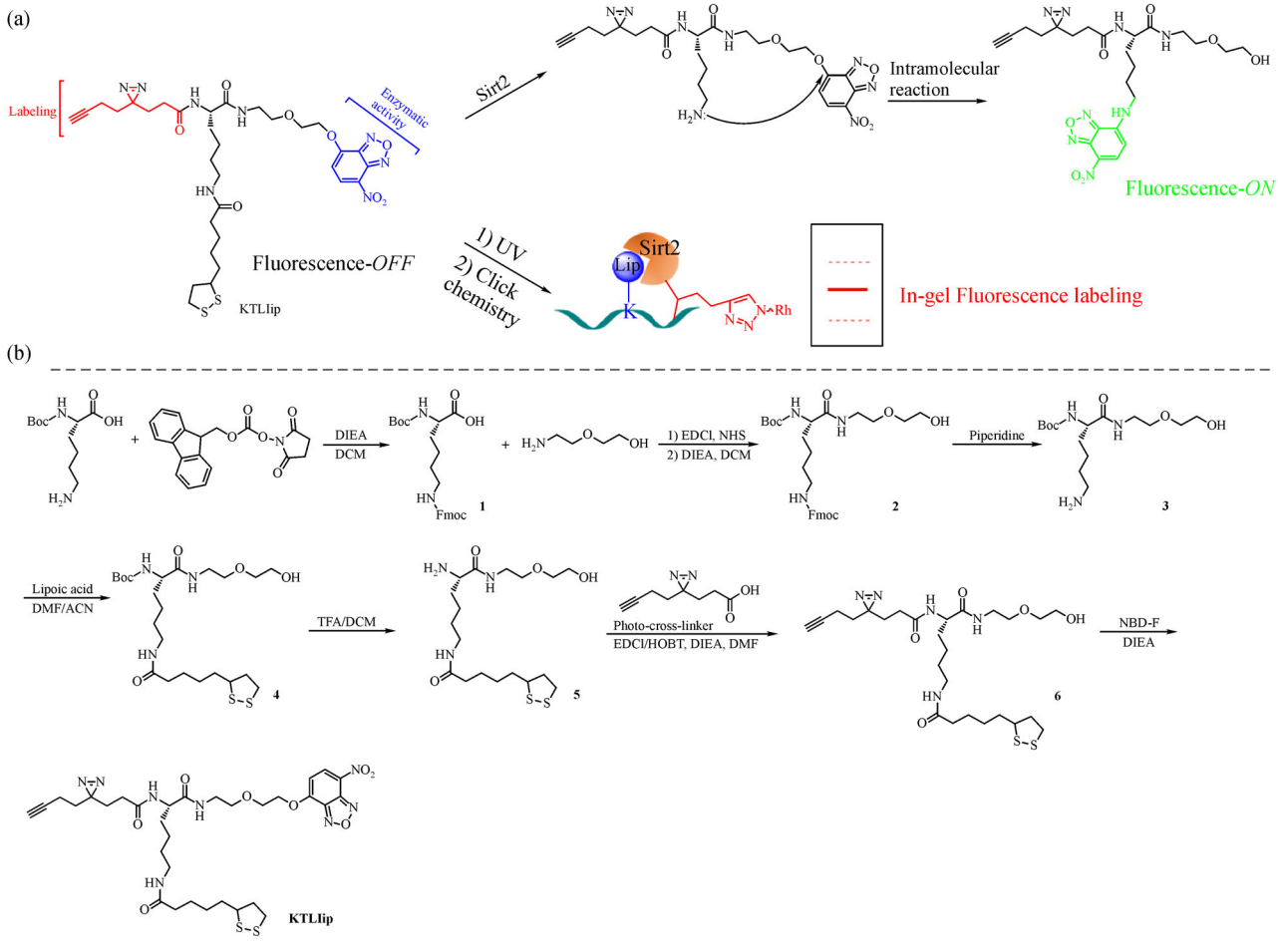
Lysine lipoylation (Klip) is a conserved modification in the dehydrogenase complexes, including the pyruvate dehydrogenase (PDH) complex, the branched-chain $\alpha$-ketoacid dehydrogenase (BCDH), the glycine cleavage complex and the $\alpha$-ketoglutarate dehydrogenase complex [1,2]. Klip is pivotal for the activity of these dehydrogenase complexes because of its cofactor role [3]. Aberrant activity of these metabolic complexes is associated with numerous diseases [4,5]. For example, lipoylation at K132 and K259 sites of the mammalian dihydrolipoyllysine acetyltransferase play an essential role in transferring the acetyl group from the PDH complex to coenzyme A. Removal of lipoylation at either K132 or K259 will result in loss of PDH activity [5,6]. In addition, lipoylation is reported to be involved in redox metabolism [7,8]. Thus, dissecting the molecular mechanism of Klip is highly important.

Sirtuins are $NAD^+$-dependent histone deacetylases. The family of sirtuins consist of sirtuin 1–7 (Sirt1-7) [9–11]. Numerous reports have shown that sirtuins are involved in diverse biological processes such as metabolism and gene transcription [12,13]. Aberrant activity of sirtuins is associated with cancer [14]. Sirtuins exhibit different catalytic efficiency to remove lysine acetylation (Kac). Sirt1-3, in contrast to Sirt4-7, show robust deacetylase activity [15]. However, more and more evidences have shown that sirtuins can catalyse the removal of other alternative lysine modifications [15]. For example, Sirt5 was reported to catalyze the removal of acidic modifications on lysine, including succinylation, malonylation and glutarylation [16]. Sirt2/Sirt6 was identified as an eraser of lysine myristoylation [17,18]. Recent studies by Cristea et al. and our group have proved that Sirt4/Sirt2 can

remove Klip and decrease PDH activity in cellular environment [2,19].

To study the interaction of Klip with eraser regulatory proteins, several methods have already been developed. Immunoaffinity purification-mass spectrometry takes advantage of the affinity of lipoylated protein with the antibody. The interacting proteins of lipoylated proteins can be pulled down and analyzed further with mass spectrometry [2]. However, this method is highly dependent on antibody efficiency and purification techniques. Single-step fluorogenic probes, in contrast to two-step fluorescent method, developed by our group and others are efficient approaches to study the interaction of Kac with the eraser by detecting enzymatic deacylation activity [19–23]. Another strategy, termed affinity-based labeling probe using photo-cross-linker, is a powerful platform to study the transient and dynamic interactions of Klip and the interacting proteins. In this approach, a photo-cross-linker can convert noncovalent ligand-protein interaction into irreversible chemical linkage [19,23–26].

Recently, our group has developed a dual-function probe for studying Kac, which combined one-step fluorescent method for detecting enzymatic activity and affinity-based labeling probe for probe/protein interaction analysis [23]. This strategy can enable the dual detection of the lysine deacetylation process. Specifically, it can detect deacetylation activity in real time and label the eraser of Kac simultaneously. In the current study, we extended this strategy to design and synthesize a dual function probe KTLlip (lysine lipoylation fluorescence turn-on and labeling probe, Scheme 1(a)). We expect the probe to be capable of detecting delipoylation activity in a continuous manner and the simultaneous selective labeling of the Klip eraser.




**Scheme 1** (a) Mechanism of our strategy to detect enzymatic delipoylation activity and label the eraser by dual-functional probe KTLlip; (b) Synthetic route of probe KTLlip (DIEA: *N*,*N*-Diisopropylethylamine; DCM: dichloromethane; EDCI: *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride; NHS: *N*-hydroxysuccinimide; DMF: *N*,*N*-dimethylformamide; ACN: acetonitrile; TFA: trifluoroacetic acid; HOBT: 1-hydroxybenzotriazole hydrate; NBD-F: 4-fluor-7-nitro-benzofurazan).

## 2 Experimental

### 2.1 Reagents and materials

Starting materials were analytical grade from commercial vendors and used directly. Thin layer chromatography (TLC) with pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness) was applied to monitor reaction. Flash column chromatography using silica gel (Merck 60 F254 nm, 70–200 mesh) was carried out to purify compounds. $^{1}$H nuclear magnetic resonance ($^{1}$H-NMR), $^{13}$C nuclear magnetic resonance ($^{13}$C NMR) analysis was performed with Bruker 300 MHz NMR spectrometers. The data were reported with the $CD_3OD$ (3.31 ppm) peaks as reference. Multiplicities are indicated as follows: m (multiplet), br (broad singlet), t (triplet), d (doublet), s (singlet). Mass spectra were obtained on PC Sciex API 150 EX ESI-mass spectrometers.

pH value was measured using a HANNA HI 2211 pH/ORP meter. Fluorescence spectra analysis was performed with a FluoroMax-4 fluorescence photometer. Ultraviolet-visible (UV) absorption measurement was recorded with UV-VS shimadzu 1700. Sirt1-3 in this study were recombinantly expressed and purified as previously reported [16].

### 2.2 Enzymatic reaction

The probe KTLlip was incubated with sirtuin and cofactor $NAD^+$ for specified time at 37 °C in 20 mmol/L HEPES buffer (pH 8.0) containing 1 mmol/L $MgCl_2$, 2.7 mmol/L KCl, 150 mmol/L NaCl.

### 2.3 Measurement of absorption and fluorescence spectra of KTLlip probe

The reaction volume was 50 μL. After reaction, the reacted mixtures were subjected to absorption and fluorescence measurements. The absorbance spectra were collected between 300–550 nm. The fluorescence spectra were collected between 510–600 nm with $\lambda_{ex}$ = 480 nm and slit width = 5 nm.

### 2.4 Cu(I)-catalyzed cycloaddition/click Chemistry

To the samples labelled by KTLlip, 20 μmol/L of rhodamine azide, 40 μmol/L tris(3-hydroxypropyltriazolylmethyl)amine, 0.4 mmol/L tris(2-carboxyethyl)phosphine and 0.4 mmol/L $CuSO_4$ was added. The reaction was incubated at room temperature for 2 h.

### 2.5 In-gel fluorescence scanning

Chilled acetone was added to the mixture which was obtained from click chemistry reaction to precipitate the proteins. The proteins were then washed with cold methanol. The air-dried pellet was re-dissolved in 1 × SDS loading buffer by heating at 95 °C for 10 min, and resolved by SDS-PAGE. The labeled proteins were visualized using a FLA-9000 Fujifilm system (excitation 532 nm).

### 2.6 Chemical synthesis and characterisation

To obtain KTLlip, we designed a synthetic scheme, which was illustrated in Scheme 1(b). The photo-cross-linker was synthesized as described before [23,24]. The synthesis commenced with a commercially available material, Boc-Lys-OH. Selective protection at ε-amino group of lysine with the fluorenylmethoxycarbonyl protecting group (Fmoc) was conducted to yield **1**. Following coupling reaction using an aminoethoxyl linker and further deprotection of Fmoc, **3** was obtained with 75% yield. By using lipoic acid for functionalization, **4** was generated. The intermediate **5** underwent coupling with a photo-cross-linker and further nucleophilic substitution of NBD-F to afford KTLlip. The chemical structure of final probe was determined unambiguously using NMR and mass spectrometry.

(2S)-6-(5-(1,2-Dithiolan-3-yl)pentanamido)-2-(3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanamido)-N-(2-(2-hydroxyethoxy)ethyl)hexanamide (**6**). Firstly, the photo-crosslinker was synthesized as reported before [19]. A solution of **4** (648 mg, 1.2 mmol) in TFA/DCM (1:1) was reacted at room temperature and monitored with TLC. After completion of the reaction, cold ethyl ether was added to wash the reaction residues two times to get the pellet product **5** for synthesis of **6** directly.

EDCI (150 mg, 0.78 mmol) and HOBt (97 mg, 0.72 mmol) were added to a solution of photo-crosslinker (100 mg, 0.6 mmol) in anhydrous DCM (6 mL), and the mixture was stirred for 1.5 h at room temperature. After activation, a solution of compound **5** (303 mg, 0.72 mmol) with DIEA (233 mg, 1.8 mmol) in dry DMF (2 mL) were then added, and the residues were stirred overnight furtherly. After completion of the reaction and solvent removal under reduced pressure, the residue was purified by flash chromatography (DCM/MeOH, 100/1-15/1) to afford the product **6** (89 mg, 26% yield). $^{1}$H-NMR (300 MHz, $CD_3OD$, $\delta_{ppm}$): 4.29-4.23 (m, 1H), 3.66 (t, $J$ = 4.2 Hz, 2H), 3.61-3.51 (m, 5H), 3.43-3.36 (m, 2H), 3.21-2.97 (m, 4H), 2.50-2.40 (m, 1H), 2.35-1.98 (m, 7H), 1.93-1.82 (m, 1H), 1.77-1.24 (m, 16H). $^{13}$C NMR ($CD_3OD$, 75 MHz, $\delta_{ppm}$): 176.08, 174.54, 174.48, 83.76, 73.52, 70.53, 70.49, 62.32, 58.90, 57.67, 54.91, 41.43, 40.53, 40.10, 39.46, 37.08, 35.84, 33.45, 32.85, 30.89, 30.20, 29.99, 29.78, 26.90, 24.31, 13.96. ESI-MS calcd for $[M + H]^+$ 570.27; Found 570.1.

(2S)-6-(5-(1,2-Dithiolan-3-yl)pentanamido)-2-(3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanamido)-N-(2-(2-

((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)ethoxy)ethyl) hexanamide (**KTLlip**). NBD-F (11 mg, 0.06 mmol) and *N*, *N*-Diisopropylethylamine (16 mg, 0.12 mmol) were added to a solution of **6** (15 mg, 0.03 mmol) in anhydrous DCM/DMF (3:1). The mixture was stirred and monitored with TLC at room temperature. After completion of the reaction and solvent removal under reduced pressure, the residue was purified by flash column chromatography (DCM/MeOH, 50/1-15/1), followed by preparative TLC, to afford the final product KTLlip as a yellow solid (2.5 mg, 13% yield). $^{1}$H-NMR (300 MHz, $CD_3$ OD, $\delta_{ppm}$): 8.63 (d, $J$ = 8.4 Hz, 1H), 6.92 (m, $J$ = 8.4 Hz, 1H), 6.78-6.70 (m, 2H), 6.38 (br, 1H), 4.53 (t, $J$ = 4.5 Hz, 2H), 4.16-4.08 (m, 1H), 3.91 (t, $J$ = 4.5 Hz, 2H), 3.63-3.55 (m, 3H), 3.36-3.30 (m, 2H), 3.22-3.02 (m, 4H), 2.49-2.39 (m, 1H), 2.15-1.97 (m, 7H), 1.89-1.82 (m, 1H), 1.74-1.36 (m, 16H). $^{13}$C NMR ($CD_3OD$, 75 MHz, $\delta_{ppm}$): 174.43, 173.31, 172.86, 156.01, 147.11, 145.82, 136.78, 131.87, 107.21, 84.40, 72.06, 71.75, 70.75, 70.41, 69.57, 57.77, 54.68, 41.45, 40.07, 39.63, 39.50, 37.07, 35.71, 33.18, 32.46, 30.91, 30.19, 29.91, 29.44, 26.65, 23.72, 14.08. ESI-MS calcd for $[M + H]^+$ 733.27; Found 733.40.

## 3 Results and discussion

### 3.1 Detection of enzymatic delipoylaiton activity of Sirt2 with KTLlip

We first tested whether the probe can be used for detection of enzymatic delipoylation activity. A series of enzymatic reactions with recombinant Sirt2 were conducted (Fig. 1). As shown in Fig. 1, probe only showed no fluorescence. After addition of cofactor $NAD^+$ and Sirt2, a significant fluorescence increase was readily observed (Fig. 1(a)). In contrast, only negligible fluorescence increase was observed when the enzyme Sirt2 or the cofactor $NAD^+$ was absent (Fig. 1(a)). These experiments indicated that Sirt2 recognizes KTLlip as a lipoyl-modified substrate and the fluorescence turn-on reaction required functional enzymatic activity.

### 3.2 Selectivity studies of enzymatic delipoylation activity

We moved on to investigate the response of KTLlip towards other sirtuins, including Sirt1 and Sirt3 (Fig. 1(b)). The results suggested that the probe can react with Sirt2 preferably. The activity of Sirt1 toward KTLlip was also observed, however the activity is much lower than that of Sirt2. The delipoylation activity of Sirt3 was negligible, which was consistent with our and Cristea's previous works [2,19].

### 3.3 Reaction time

We then performed time-dependent experiment with Sirt2 (Fig. 2). The fluorescence signal was weak at the beginning. It increased quickly after incubation with Sirt2 and finally plateaued after 3 h. Those data proved that KTLlip can be used as a platform to detect enzymatic delipoylation activity continuously.

### 3.4 Labeling of recombinant target enzyme of lipoylation with probe KTLlip

Next, we evaluated the potential of KTLlip to label recombinant enzymes with in-gel fluorescence detection (Fig. 3). Sirt2 has been identified as eraser of Klip [19]. It was chosen for our concept study. Concentration-dependent experiments with probe KTLlip were first performed (Fig. 3(a)). Different concentrations of KTLlip was incubated with Sirt2 with the same UV irradiation time (15 min) in HEPES buffer respectively. The reaction mixtures were then subjected to click chemistry. The

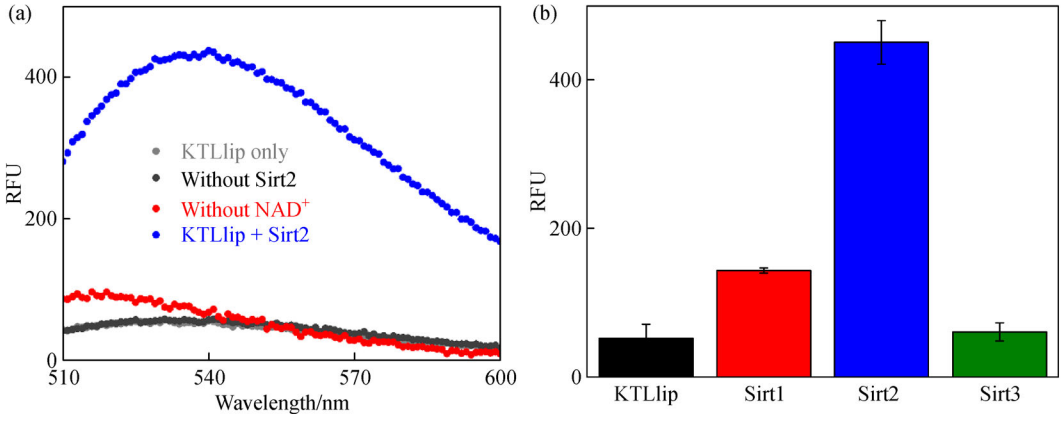



**Fig. 1** (a) Fluorescence spectra of KTLlip (10 μmol/L) with Sirt2 (40 ng/μL) under various conditions ($\lambda_{ex}$ = 480 nm); (b) fluorescence detection of KTLlip with different sirtuins.

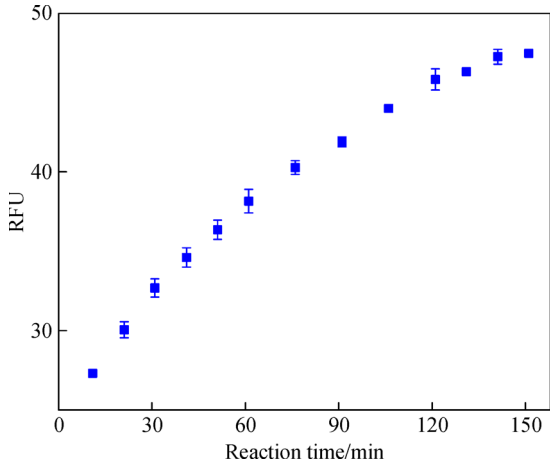



**Fig. 2** Time-dependent study of KTLlip (10 μmol/L) incubated with Sirt2 (40 ng/μL) in HEPES buffer (20 mmol/L, pH = 8.0) with 200 μmol/L $NAD^+$ at 37 °C ($\lambda_{ex}$ = 480 nm).

obtained protein samples were separated by SDS-PAGE. Finally, the in-gel fluorescence scanning experiments were conducted. The results showed that recombinant Sirt2 can be readily labelled at a probe concentration of 2.5 μmol/L. No fluorescence signal was detected in the absence of KTLlip. The labeling of recombinant Sirt2 increased with higher concentrations of probe, suggesting that the labeling follows a concentration-dependent manner.

We then performed UV irradiation time-dependent experiments (Fig. 3(b)). The results suggested that the labeling efficiency increased with increasing time. The fluoresce intensity of recombinant Sirt2 was readily observed at 5 min (Fig. 3(b)). These results indicated that probe KTLlip can efficiently label recombinant Sirt2.

### 3.5 Labeling of target enzyme of lipoylation in lysate with probe KTLlip

We next asked whether Sirt2 can be captured selectively by KTLlip in complex lysate environment. HEK 293 lysates spiked with Sirt2 were incubated with KTLlip. The mixtures were then treated with UV irradiation and subjected to click chemistry reaction. A subsequent in-gel fluorescence scanning experiments were then carried out. As shown in Fig. 3(c), a faint fluorescence band of Sirt2 could be observed, suggesting that Sirt2 can be detected in complex cellular environment. However, the labeling was not selective. To figure out the plausible reasons for KTLlip's mediocre labeling efficiency of target enzyme in lysate environment, we compared the labeling efficiency of KTLlip with another affinity-based probe KPlip (Fig. 3(d)) [19]. KPlip was previously reported by us, which can label Sirt2 efficiently and selectively. Compared with KTLlip, KPlip contains longer peptide sequence with several hydrophilic residues such as Ser, Thr and Asp. As shown in Fig. 3(e), the labeling efficiency of KPlip was much higher than KTLlip. We reckoned that the mediocre performance of KTLlip might be caused by its solubility and peptide recognition efficiency.

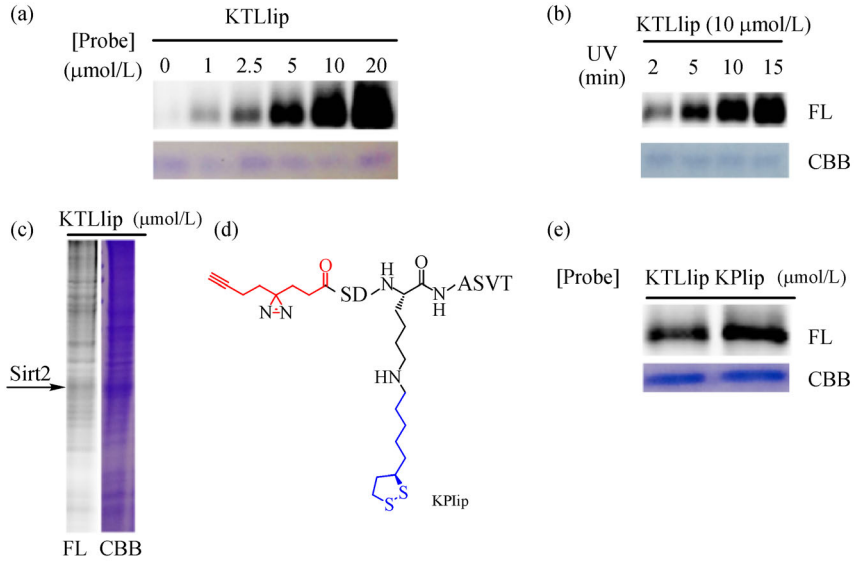



**Fig. 3** (a) Concentration-dependent labeling of KTLlip (0, 1, 2.5, 5, 10 and 20 μmol/L) with recombinant Sirt2 (0.3 μg/lane) under 15-min of UV irradiation; (b) UV irradiation-time-dependent (2, 5, 10, 15 min) labeling of KTLlip (10 μmol/L) with recombinant Sirt2 (0.3 μg/lane); (c) labeling of Sirt2-spiked (2 μg) HEK293 lysate (20 μg) by KTLlip (10 μmol/L); (d) molecular structure of affinity-based probe KPlip for Klip; (e) comparison of labeling of Sirt2 (0.3 μg/lane) using KTLlip and KPlip probe.

## 4 Conclusions

We have developed a dual-function probe KTLlip with fluorescence turn-on and labeling readout for studying lysine delipoylation. Specifically, KTLlip acts as a substrate for recombinant Sirt1/Sirt2 and undergo a tandem delipoylation/intramolecular nucleophilic exchange reaction to produce fluorescence signal. As such, it will prove extremely valuable for performing activity measurements on recombinant Sirt1/Sirt2, as an aid to inhibitor screening studies for the identification of drug lead candidates. In addition, the probe can fluorescently label recombinant Sirt2 efficiently. However, KTLlip showed low selectivity in labeling Sirt2 in complex lysate environment. This might be caused by mediocre solubility of the probe and recognition capacity of target enzymes. It underscores the importance of introducing an appropriate peptide sequence for enzyme recognition. We envision that the study will provide new insight for further investigating the roles of delipoylation enzyme in biology.

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