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A succinyl lysine-based photo-cross-linking peptide probe for Sirtuin 5

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A succinylation-specific photo-cross-linking peptide probe has been developed for the NAD$^+$-dependent hydrolase Sirtuin 5. The probe, not only displayed robust labelling performance with purified Sirt5, but also enabled sensitive detection of the hydrolase in the presence of large excess of cellular proteins. It is anticipated that this probe, and future generations of it, will provide useful chemical tools for the functional analysis of Sirt5 and for the recently discovered PTM of lysine succinylation.

Silent information regulator 2 (Sir2), or sirtuins, are a family of proteins with NAD$^+$-dependent hydrolase activity.[1] Sirtuins have been implicated in many biological processes such as regulation of metabolism, caloric restriction and life span, ribosomal DNA transcription, genome stability, inflammation, cell cycle, fatty acid oxidation and urea cycle.[1,2a-c] Mammals have seven sirtuins (Sirt1 to Sirt7), which differ in their biochemical activities and sub-cellular localisation. Sirt1-3 exhibit robust NAD$^+$-dependent deacetylase activity and they complement the well-studied Zn^{2+}-dependent lysinedeacetylation activity of classes I, II and IV lysine deacetylases (KDACs, or historically, histone deacetylases, HDACs). Sirt4 and Sirt6 have been shown to facilitate ADP-ribosylation and tentatively classified as ADP-ribosyltransferases although the later also harbour very weak deacetylase activity and robust lysine side-chain fatty deacylation activity.[3a-c] Sirt7, although reported as a tumour suppressor p53 deacetylase,[4] indeed has only weak deacetylase activity. Sirt5, a mitochondrial sirtuin, also exhibits modest lysinedeacetylase activity. More importantly, this isoform has been recently shown to exhibit strong lysine side-chain desuccinylase and demalonylase activities,[5] revealing the reversible nature of the corresponding modifications.[6] Although the precise physiological roles of both this activity and the modifications are yet to be revealed, the fact that there is a dedicated enzyme to hydrolyse succinyl/malonyl lysine together with the recent identification of lysine succinylation as a frequently occurring modification in both prokaryotes and eukaryotes point to potentially significant functional roles.[7a-b] Although mass spectrometry provides a reliable platform for the detection and profiling of the modification, we currently lack chemical tools that enable modification-specific detection of the hydrolase. Such reagents are highly desirable as they may facilitate functional studies of this recently identified modification. Herein, we report synthesis and biochemical characterisation of a succinylation-selective photo-cross-linking peptide-based probe of Sirt5.

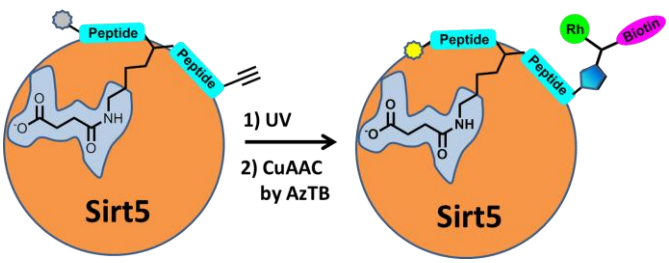

Fig. 1 Overall scheme for the labelling of Sirt5 by photo cross-linking clickable peptide probe **P1**.

We have chosen the succinylated mitochondrial protein glutamate dehydrogenase (GDH) for the peptide-probe design. Although succinylation was reported on nine different lysine residues in this protein, we chose succinyl-K503 as the basis for probe design as a corresponding peptide sequence has favourable kinetic parameters reported for Sirt5-mediated desuccinylation.[5] The design of the peptide probe was inspired by a photo-cross-linking strategy developed by Kapoor and co-workers for the identification of interaction partners of trimethylated lysine residues in histone tails.[8] The probe has three essential structural features. 1) A succinylated lysine residue for preferential interaction; 2) a photo-cross-linker unit, which enables conversion of the transient probe-protein interaction into co-valent binding by UV-light-mediated cross-linking; and 3) a clickable tag, which facilitates click chemistry-

mediated conjugation of the probe-protein complex to a reporter reagent bearing an orthogonal functionality to enable labelling (e.g. by in-gel fluorescence) or affinity enrichment (e.g. by biotin-avidin pull-down) for subsequent analysis (Fig. 1). The positioning of the photo-

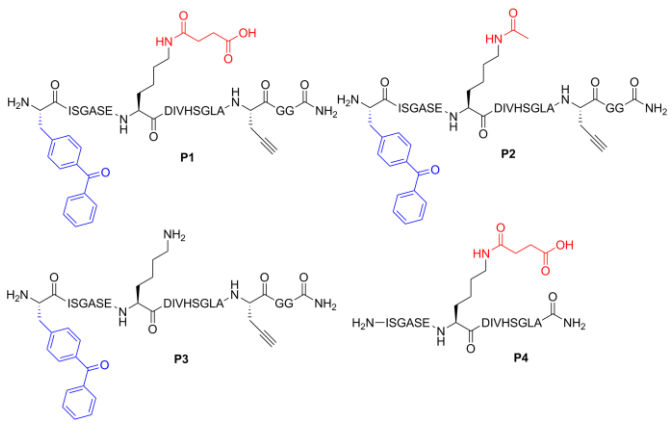

Fig. 2 Chemical structure of the peptide probes.

cross-linker and the click tag in the native peptide sequence has to be carefully chosen such that these entities cause only minimal perturbations in the original interaction with the protein. Ideally, structural data of the original peptide sequence with the protein of interest should be available for the optimum positioning of these entities. Unfortunately, in the case of lysine succinylation such structural data are not available and therefore, we decided to place these entities in the peptide sequence in such a way that they are sufficiently far away (at least six amino acids) from the succinylated lysine residue. A total of four peptide probes (**P1** to **P4**) were designed (Fig. 2). We selected benzophenone-containing unnatural amino acid L-Bpa as the photo-cross-linking unit, whilst L-Propargyl glycine was used to introduce the clickable alkyne tag. Corresponding peptides with acetylated lysine and unmodified lysine were also designed as control probes. A competitor probe was also designed that carried succinylated lysine but lacked the clickable tag and photo-cross-linker. The peptide probes were synthesized by standard Fmoc solid-phase synthesis protocols using rink-amide resin on an automated synthesizer. The crude peptides obtained were purified by reverse-phase semi-preparative HPLC, and characterised by LC-MS (ESI†).

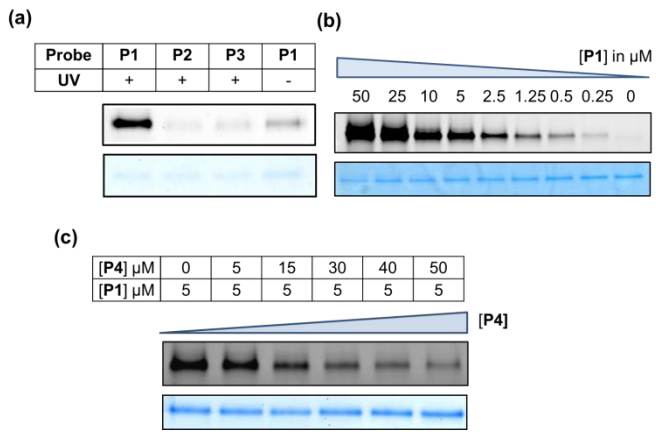

Fig. 3 (a) In-gel fluorescence scan showing the selectivity of **P1** vs. the control probes **P2** and **P3** on labelling of Sirt5 (0.4 μg). (b) Concentration-dependent labelling of Sirt5 (0.8 μg) by **P1**. (c) Effect of the competitor peptide **P4** on labelling of Sirt5 (0.8 μg) by **P1**.

We first examined labelling performances of the probes **P1** to **P3** with purified Sirt5 (Fig. 3a). Briefly, the probes were incubated at a fixed concentration of 5 μM with recombinant Sirt5 (0.4 μg protein per sample) for 10 min at room temperature (25°C) in Tris hydrochloride buffer at pH 7.5. The samples were then irradiated on ice with UV light (365 nm) for 30 min. After UV-irradiation, the samples were subjected to click chemistry using a trifunctional capture reagent azido-TAMRA-Biotin (AzTB) previously developed in our lab (see ESI†).[9] Labelling performance was evaluated by in-gel fluorescence imaging following SDS-PAGE. As shown in Fig. 3a, strong labelling of Sirt5 was observed only with the succinyllysine-containing probe **P1**. The labelling by **P1** was found to be UV-irradiation-dependent as evident from the very faint labelling observed in the absence of irradiation vs. strong labelling in the presence of irradiation (Fig. 3a, lane 4 vs. lane 1). Only very faint labelling was observed with the control probes **P2** and **P3**, - indicating the strong preference of Sirt5 for lysine succinylation over acetylation or the native peptide sequence. To determine the optimum concentration of the probe, we performed labelling experiments with varying amounts of **P1**. As shown in Fig. 3b, concentration-dependent labelling was observed with detection even at a low concentration of 0.25 μM of the probe. We then asked whether labelling could be competed with competitor peptide **P4**; as shown in Fig. 3c, **P4** was found to outcompete the labelling by **P1** in a concentration-dependent manner, indicating the on-target activity of the probe and its potential usefulness in complex proteomes.

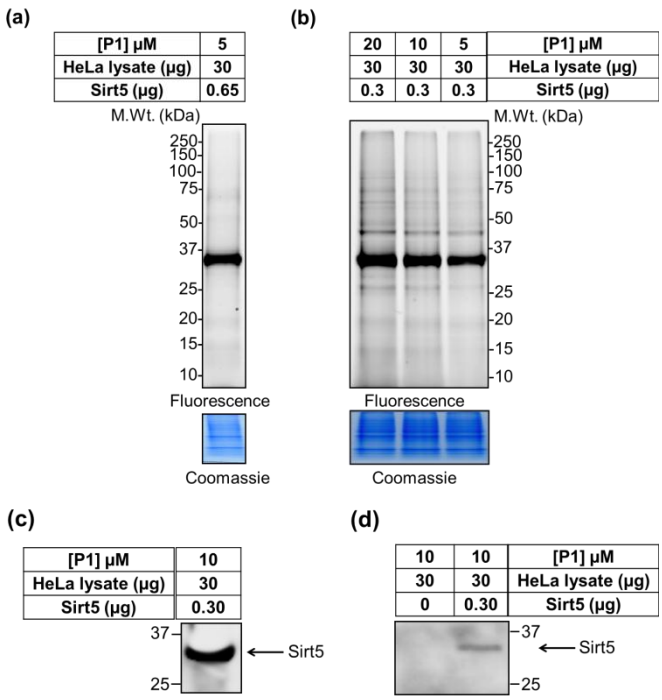

Fig. 4 (a & b) In-gel fluorescence showing the labelling of HeLa lysate (30 μg total proteins) spiked with Sirt5 (0.65 μg and 0.3 μg respectively) by **P1**. (c) & (d) Anti-Sirt5 blot and anti-biotin blot, respectively, of Sirt5-spiked HeLa lysates after labelling with **P1**.

The robust labelling performance of **P1** with purified Sirt5 encouraged us to evaluate its ability to label the enzyme in a complex proteome. **P1** was treated with HeLa whole cell lysate with spiked Sirt5. As shown in Fig. 4a, a clean selective labelling of the spiked Sirt5 was observed in the presence of excess of cellular proteins using 5 µM concentration of the probe. Upon increasing the probe concentration and decreasing the added Sirt5, labelling of other cellular proteins were observed (Fig. 4b), however, without any reduction in the labelling efficiency of Sirt5. It is possible that some of the labelled proteins may represent binding partners of the succinyl lysine modification. Labelling of Sirt5 was also visualised by Western-blotting using anti-Sirt5 antibody (Fig. 4c). Western-blotting using anti-biotin antibody further confirmed the labelling of Sirt5 (Fig. 4d).

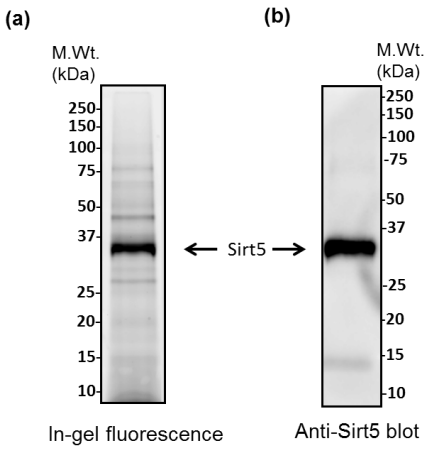

Fig. 5 (a) In-gel fluorescence after pull-down of **P1**-treated (20 µM) Sirt5 spiked (0.3 µg) HeLa lysate using NeutrAvidin-Agarose resin. (b) Western-blot of the same gel after electrotransfer of proteins from the gel to a PVDF membrane detecting the Sirt5 by anti-Sirt5 antibody.

In order to further validate the labelling efficiency, we performed pull-down of the labelled proteome using NeutrAvidin-Agarose resin (Fig. 5). Following pull-down, the bound proteins were eluted by boiling the beads at 95°C in PBS with 2% SDS and SDS loading dye. The eluted proteins were then resolved by SDS-PAGE and visualised by in-gel fluorescence. Robust labelling of Sirt5 was observed along with faint non-specific detection of few other cellular proteins (Fig. 5a). Proteins from the same gel were electrotransferred to a PVDF membrane and probed by Western-blotting using anti-Sirt5 antibody, which detected labelled and pull-down Sirt5 (Fig. 5b), confirming the robust labelling performance of the probe.

Conclusions

In conclusion, we have developed the first photo-cross-linking affinity-based probe for Sirt5. **P1** showed promising results for Sirt5 profiling in the presence of large excess of cellular proteins, and further improvements in the probe's sensitivity might be achieved by altering the positioning of the photo-cross-linker and the clickable tag in the peptide sequence via combinatorial probe-library synthesis and testing, or by the incorporation of longer peptide sequences. Use of alternative photo-cross-linking entities (e.g. diazirine-based reagents and aryl azides) in a combinatorial probe-library could also be investigated as several recent reports show significantly system-dependent effects on photo-cross-linking efficiencies across chemically distinct cross-linkers.[10] These probe design principles could be extended for the profiling of interaction partners of side-chain succinylation/malonylation modification of lysine residues in histones and other non-histone proteins. Ultimately, succinylation-selective chemical probes such as **P1** reported herein and its future structural variants will help us to unravel the functional roles of Sirt5, and the recently discovered post-translational modification of lysine side chain succinylation.

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Notes and references

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