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Semi-synthesis of biotin-bearing activity-based ubiquitin probes through sequential enzymatic ligation, N-S acyl transfer and aminolysis reaction

Yu Wang[a,1], Qiong Xia[b,1], Xiao Hua[a], Yanyan Guo[a], Jing Shi[a,*], Yi-Ming Li[b,*]

[a] Department of Chemistry, University of Science and Technology of China, Hefei 230026, China
[b] School of Food and Biological Engineering, Key Laboratory of Metabolism and Regulation for Major Diseases of Anhui Higher Education Institutes, Hefei University of Technology, Hefei 230009, China

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ABSTRACT

Activity-based Ubiquitin probes (Ub-ABPs) carrying a reporter group have emerged as effective tools for the investigation of deubiquitinating enzymes (DUBs), such as studying the molecular mechanism of DUBs, profiling new DUBs. But so far, the synthesis of commonly used biotin-bearing Ub-ABPs is a technical challenge. Here, we report a one-pot semi-synthetic strategy for the acquiring of Ub-ABPs carrying a biotin tag through sequential enzymatic ligation, N-S acyl transfer and aminolysis reaction without any purification steps. These probes enable to capture the different family of DUBs for enrichment and immunoblotting using the attached biotin tag.

Ubiquitin (Ub), composed of 76 amino acids, is typically catalyzed by a cascade of E1/E2/E3 enzymes to form isopeptide bonds on the ε-amino group of substrate protein lysine, a process referred as ubiquitination [1,2]. Deubiquitinating enzymes (DUBs) remove Ub from substrate proteins to maintain the balance of ubiquitination *in vivo*, and therefore play key roles in variety of cell behaviors, such as chromatin remodeling, DNA damage repair and mitochondrial autophagy [3–6]. More recently, DUBs have emerged as new therapeutic targets for many diseases, especially cancer and neurodegeneration [7,8].

As effective tools to study DUBs, activity-based Ub probes (Ub-ABPs), have been widely used to analyze the molecular mechanism and activity of DUBs, screen new DUBs and develop DUB inhibitors [9–12]. These probes usually consist of three parts: a warhead, a reporting group, and a Ub conjugate motif, in which the reporting group such as human influenza hemagglutinin (HA) epitope [13–15] or biotin, is typically a tag for the enrichment of probe-labeled proteins [9,16]. Compared with HA tag, the biotin tag showed a higher affinity (see Table S1 in Supporting information for details) [17–19], but most of biotin-bearing Ub-ABPs need to be obtained by chemical total synthesis (Scheme 1a) [20–25], which is a technical challenge for most biochemical laboratories. Therefore, we wished to examine whether there is an easy-to-implement strategy for the preparation of Ub ABPs carrying biotin tags from recombinantly expressed proteins.

Herein, we developed a practical strategy for the semi-synthesis of biotin-bearing activity-based Ub probes through sequential bioorthogonal reactions. Starting from the recombinantly expressed Ub variant avi-Ub(G76C), biotin-Ub-ABPs, such as Ub-propargylamine (Ub-PA), Ub-vinyl methyl ester (Ub-VME) and Ub-chloroethyl (Ub-Cl), can be easily obtained by one-pot enzymatic biotinylation, N-S acylation transfer and oxidation induced aminolysis reaction (Scheme 1b). These probes enable to capture DUBs of different families, as well as to enrich or visualize probe-labeled proteins *via* biotin tags.

We first expressed an avi-tag (GLNDIFEAQKIEWHE) at the N-terminus of Ub unit, which can be recognized by *Escherichia coli* biotin holoenzyme synthetase (BirA) to load biotin on the ε-amino group of lysine [17]. Meanwhile, in order to prepare the probe carrying a warhead at the C-terminal, we mutated the 76-glycine at the C-terminus of Ub to cysteine, as previously reported, to obtain the Ub hydrazine intermediate through N-S acyl transfer [26,27]. Finally, combined with an oxidation induced aminolysis reaction, the warhead of probe was easily loaded to the C-terminus of Ub motif (Fig. 1a) [28,29].

* Corresponding authors.
E-mail addresses: shijing@ustc.edu.cn (J. Shi), ymli@hfut.edu.cn (Y.-M. Li).
[1] These authors contributed equally to this work.

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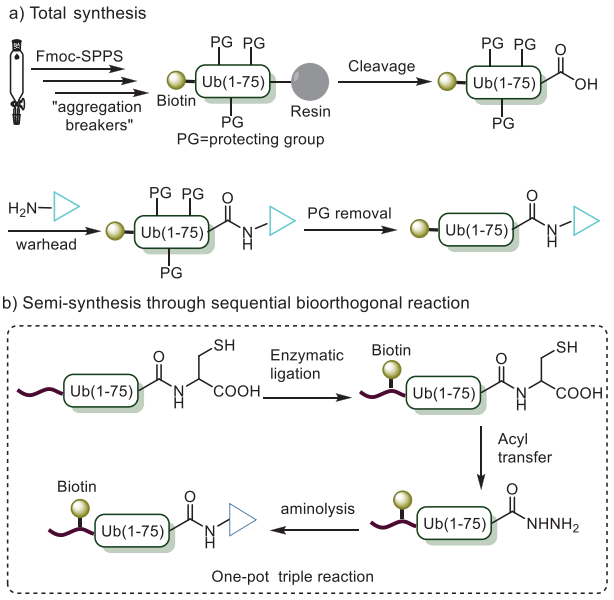

Scheme 1. Strategies for the synthesis of activity-based Ub probes carrying a biotin tag. (a) Total chemical synthesis strategy. (b) One-pot semi-synthesis strategy through a sequential bioorthogonal reaction.

Specifically, the Ub mutant named avi-Ub(G76C) (**1**) which carried an avi-tag was expressed in *E. coli*. Then, the 0.7% perchloric acid was slowly added to cell lysate for precipitating unwanted protein (Fig. 1c, Figs. S1 and S2 in Supporting information). After centrifuging the suspension, the supernatant was dialyzed into MES buffer (20 mmol/L MES, pH 7.0), and concentrated to a protein concentration of 10 mg/mL. In order to load the biotin tag onto **1**, $MgCl_2$ (5 mmol/L), ATP (2 mmol/L), D-biotin (0.45 mmol/L) and BirA (1 μmol/L) were added to the supernatant and placed at 37 °C for enzymatic biotinylation [17,30]. Through monitoring the reaction using RP-HPLC, we found that **1** was completely disappeared after 1 h, and a new peak was emerged at the same time (Fig. 1b). Electrospray ionization (ESI)-MS analysis further confirmed that the appeared product **2** was biotin-loaded avi-Ub(G76C) (Fig. 1d). To convert the C-terminus of intermediate **2** to a hydrazide group, biotin-loaded avi-Ub (G76C) was reacted with hydrazinolysis reagent (MESNA 100 mg/mL, NH_2NH_2-HCl 50 mg/mL, tris(2-carboxyethyl)phosphine (TCEP) 5 mg/mL, pH 7.0) for 48 h at 50 °C as we previously developed protocol [26]. As shown in Figs. 1b and e, the C-terminal of **2** was almost converted to hydrazide to afford **3** followed by **3** dialyzing into MES buffer (20 mmol/L MES, pH 3.0) and added $NaNO_2$ (7 equiv.) for 25 min at −15 °C [31–39]. Then, propargylamine (200 equiv.) was added to the above reaction (pH value was adjusted to 8.0), and the new aminolysis product was generated by stirring at room temperature for 1 h [28,29]. The identity and purity of avi-biotin-Ub-PA (probe **1**) were confirmed by HPLC (Fig. 1b, isolated yield 70%) and ESI-MS (Fig. 1f and Fig. S3 in Supporting information). In this context, starting from 1 L of LB broth medium, about 25 mg of probe **1** was obtained within 5 days. Similarly, we were able to obtain avi-biotin-Ub-VME (probe **2**) (Fig. 1g and Fig. S4 in Supporting information) and avi-biotin-Ub-Cl (probe **3**) (Fig. 1h and Fig. S5 in Supporting information) through this sequential triple reaction without any purification steps. Next, we renatured probe **1** using the method previously reported [14,15,25]. In addition, we measured the circular dichroism spectra (CD) of the probe **1**, and used Ub-PA as comparison. As shown in Fig. S6 (Supporting information), the CD spectra of probe **1** and Ub-PA were nearly identical, indicating that synthesized probe **1** has the correct secondary structure. We also measured the circular dichroism spectra of probe **2** and probe **3**, and the results were similar to those of probe **1** and Ub-PA (Fig. S6).

With probe **1** in hand, we investigated whether the inclusion of an additional avi-tag would have an impact on the efficiency of probe **1** in capturing DUBs. We selected three different families of DUBs as examples, namely, UCHL1, OTUB1 and USP7-CD. Probe **1** (10 μmol/L) was incubated with three DUBs (UCHL1, OTUB1 and USP7-CD in 5 μmol/L) at 37 °C for 1 h respectively. Then, the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 2, we observed the cross-linked band corresponding to the conjugate of probe **1** and

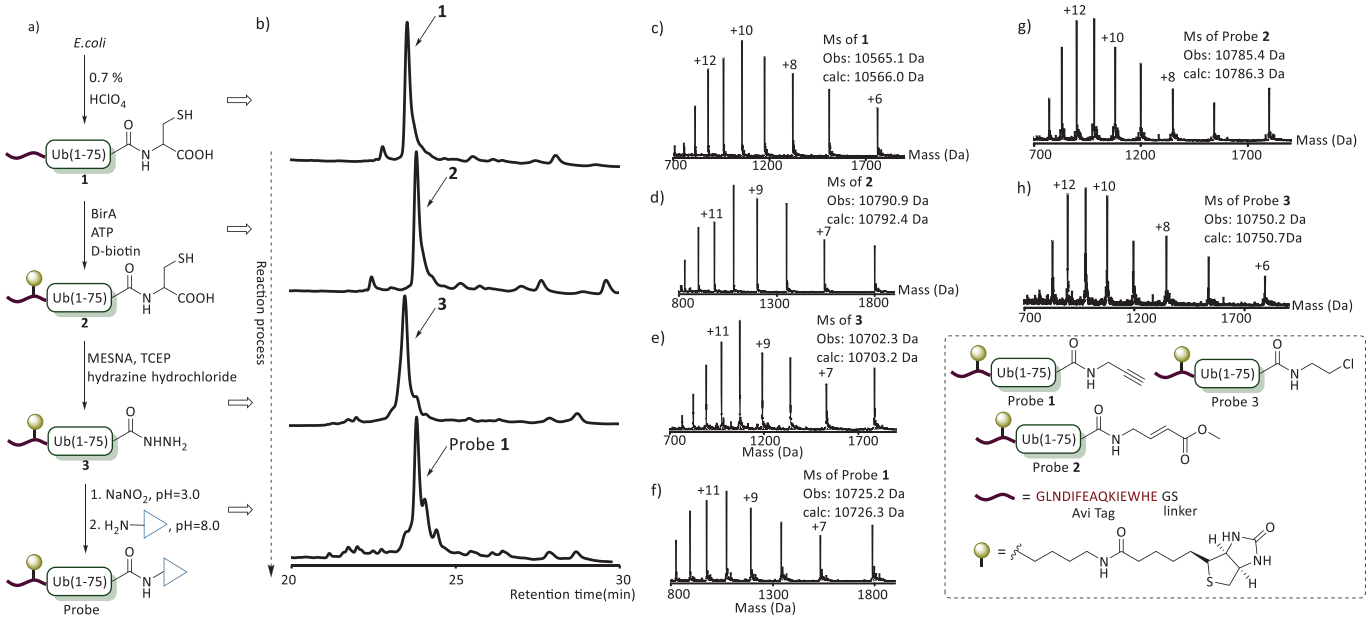

Fig. 1. Chemical synthesis and characterization of activity-based ubiquitin probes. (a) The synthesis route of activity-based ubiquitin probes with the biotin tag. (b) Analytical RP-HPLC analysis of the one-pot reaction. ESI-MS analysis of **1** (c), **2** (d) and **3** (e). ESI-MS analysis of probe **1** (f), probe **2** (g) and probe **3** (h).

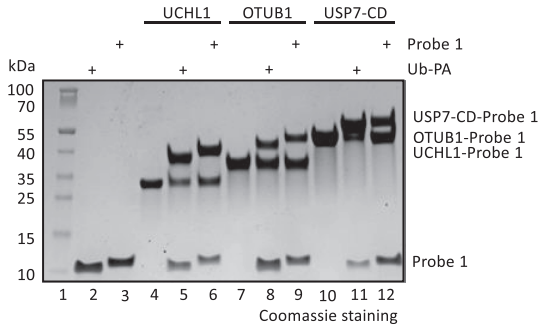

Fig. 2. SDS-PAGE analysis of labeling of UCHL1, OTUB1 and USP7-CD by probe **1**. Ub-PA was used as a control. Lane 2: Ub-PA = 8.5 kDa; Lane 3: probe **1** = 11 kDa; Lane 4: UCHL1 = 26 kDa; Lane 5: Ub-PA, UCHL1, UCHL1-Ub-PA = 35 kDa; Lane 6: probe **1**, UCHL1, UCHL1-probe **1** = 37 kDa; Lane 7: OTUB1 = 32 kDa; Lane 8: Ub-PA, OTUB1, OTUB1-Ub-PA=41 kDa; Lane 9: probe **1**, OTUB1, OTUB1-probe **1** = 43 kDa; Lane 10: USP7-CD = 42 kDa; Lane 11: Ub-PA, USP7-CD, USP7-CD-Ub-PA = 51 kDa; Lane 12: probe **1**, USP7-CD, USP7-CD-probe **1** = 53 kDa.

three DUBs (lane 6, lane 9 and lane 12), suggesting that the synthetic probe **1** could label DUBs. Compared with native Ub-PA, probe **1** possessed similar efficiency to UCHL1 and OTUB1(lane 5, lane 6, lane 8 and lane 9). But we also found that the efficiency of labeling USP7-CD was slightly lower than Ub-PA (lane 11 and lane 12), which possibly affected its interaction with USP7-CD due to the avi-tag (15 aa) at the N-terminus of Ub [40].

Subsequently, we examined the enrichment efficiency of the probe **1** for labeled proteins using the biotin tag. To this end, mixture of the probe **1** and DUBs were incubated with streptavidin agarose beads at 4 °C for 2 h to enrich the probe **1**-DUB complex. To remove unreacted DUBs, streptavidin agarose beads were thoroughly washed, which might remove part of the complex formed by probe **1** and DUBs [25,41,42]. Then cross-linked proteins were eluted from beads with 2 × loading buffer at 95 °C for 10 min. As shown in Fig. 3a, we observed the cross-linked band formed by probe **1** with three DUBs respectively (lane 4, lane 5 and lane 6), showing that probe **1** with the biotin tag could enrich its labeled proteins effectively.

Next, DUBs labeling assays using probe **1** were carried out in the complex environment of cell lysate, which was detected by immunoblotting. Probe **1** (10 μmol/L) was incubated with HeLa cell lysate at 37 °C for 1 h. The anti-biotin immunoblotting showed that lane 3 had more discrete bands, compared with lane 2 (Fig. 3b). To determine whether these discrete bands contained the conjugate of probe **1** with DUBs, we performed immunoblotting using typical DUB antibodies. As shown in Fig. S7 (Supporting information), the anti-OTUB1 immunoblotting results showed that the new band corresponding to the complex of OTUB1-probe **1** (lane 2) was clearly formed. Similar results were also observed in anti-USP15 immunoblotting (Fig. S8 in Supporting information, lane 2). This indicates that the probe **1** carrying the biotin tag can effectively detect the probe-labeled protein in a complex cell lysate, and is expected to perform proteome-wide DUB profiling combined with label-free quantitative (LFQ) mass spectrometry in the future.

In summary, we report a new approach combining sequential enzymatic biotinylation, N-S acyl transfer and oxidation induced aminolysis reaction for the generation of biotin-tag-bearing activity-based Ub probes. In this protocol, whole proteins mentioned above could be expressed in *E. coli*, and all warheads are commercial obtained. In addition, the probe can be obtained one-pot without multiple purification steps during the reaction. The conjugate of avi-biotin-Ub-PA with diverse DUBs can be enriched and purified by streptavidin agarose beads. Anti-biotin immunoblotting in cell lysate showed that probe could monitor its labeled protein in complex environment fast and sensitively. We expect this feasible synthesis protocol could provide more convenient strategies for the synthesis of activity-based ubiquitin-like probes.

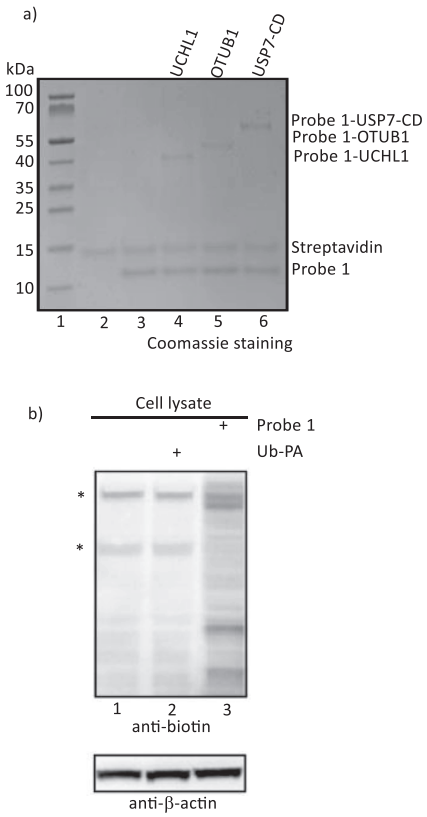

Fig 3. (a) Characterization of the labeling results by SDS-PAGE. Lane 1 protein marker; Lane 2 streptavidin agarose beads; Lane 3 streptavidin agarose beads and probe 1; Lane 4 streptavidin agarose beads, UCHL1 and probe 1; Lane 5 streptavidin agarose beads, OTUB1 and probe 1; Lane 6 streptavidin agarose beads, USP7-CD and probe 1. (b) Cell lysate profiling using probe 1. Labeled proteins were analyzed by immunoblotting using an anti-biotin antibody. Ub-PA was used as a control. Lane 1: HeLa cell lysate; Lane 2: HeLa cell lysate and Ub-PA; Lane 3: HeLa cell lysate and probe 1. The asterisk indicates endogenous biotinylated conjugate proteins.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.108010.

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