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Chemical proteomic profiling of protein *N*-homocysteinylation with a thioester probe†

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Hyperhomocysteinemia (HHcy) refers to a medical condition of abnormally high level of homocysteine (Hcy) in blood (>15 µmol L^{-1}) and has been clinically implicated with cardiovascular diseases and neurodegenerative disorders. Excessive Hcy can be converted to a reactive thioester intermediate, Hcy thiolactone (HTL), which selectively reacts with protein lysine residues ("*N*-homocysteinylation") and this non-enzymatic modification largely contributes to manifestations of HHcy. However, the proteome-wide detection of protein *N*-homocysteinylation remains a challenge to date. In this work, we report a chemoselective reaction to label and enrich *N*-homocysteinylation from complex proteome samples as inspired by native chemical ligation for protein synthesis. Alkynyl thioester probes are synthesized and the reaction is validated with small molecule and purified protein models successfully. We performed quantitative chemical proteomics to identify more than 800 *N*-homocysteinylated proteins as well as 304 *N*-homocysteinylated sites directly from HTL-treated HeLa cells. The chemical proteomics strategies will facilitate functional study of protein *N*-homocysteinylations in the HHcy-implicated diseases.

Introduction

Homocysteine (Hcy) is an intermediary amino acid involved in the metabolism of methionine and cysteine.[1] The normal concentration of Hcy in plasma is low (5–15 µmol L^{-1}) and increased accumulation (up to 500 µmol L^{-1}), namely, hyperhomocysteinemia (HHcy), is a contributing risk factor for cardiovascular diseases and neurodegenerative disorders.[2–4] Among various hypotheses, protein *N*-homocysteinylation represents a major mechanism of Hcy toxicity.[2,5–9] Protein *N*-homocysteinylation is a non-enzymatic post-translational modification of lysine residues mediated by homocysteine thiolactone (HTL), which is a reactive thioester intermediate generated from an error-editing reaction of Hcy with methionyl-tRNA synthetase (MetRS).[10] Several studies have shown that *N*-homocysteinylation on select proteins will cause protein damage,[11–13] aggregation[9] and auto-immune responses.[14,15]

Traditional methods for detecting *N*-homocysteinylation include high-performance liquid chromatography (HPLC)-based[16] and antibody-based assays.[17] They suffer from the limitations of poor sensitivity and lack of identity of modified proteins. Recently, mass spectrometry (MS)-based methods coupled with direct trypsin digestion[18–20] or aldehyde chemical labeling[21] have been developed to identify *N*-homocysteinylated proteins and sites. However, they were mostly applied on individual cases and about ten human proteins with *N*-homocysteinylated sites have been reported to date (Table S1†).

Chemical proteomics[22] is an emerging platform to understand the reactivity of amino acids,[23–26] targets of bioactive compounds,[27–30] protein–protein interactions[31,32] and post-translational modifications[33–36] in biological systems. The key component is specific conjugation with targets by chemical probes, followed by enrichment and MS-based identification. Considering low abundance and sub-stoichiometry of protein *N*-homocysteinylation, a specific chemical probe will be necessary for its large-scale profiling from complex proteomes of cells or tissues.

Herein, we report the development of a chemoselective reaction between thioester and *N*-homocysteinylation as inspired by native chemical ligation (NCL).[37] NCL is widely used for synthesizing native backbone proteins by ligating two peptides with an N-terminal cysteine and a C-terminal thioester, respectively.[38] To overcome the limitation of requiring a cysteine in the ligated sequence, many variant methodologies have been developed, including methionine ligation,[39] in which an N-terminal homocysteine is used for ligation, followed by *S*-methylation to form methionine (Scheme S1†). Given that the chemical structure of protein *N*-homocysteinylation is identical
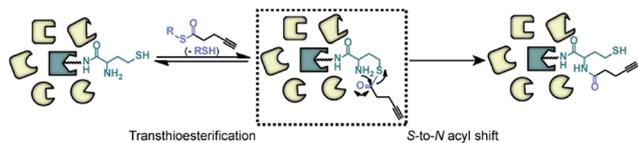
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Scheme 1 Chemoselective labeling of protein N-homocysteinylation by thioester probes. The labeling process involves the reversible transthioesterification. The resulting six-member intermediate subsequently rearranges through a rapid S-to-N acyl shift to yield the alkyne-labeled proteins.

Fig. 1 Testing reactivity of thioester probes in a small molecule reaction model. (A) Structures of three thioester probes, namely, AT-1, AT-2 and AT-3. (B) B-hcy, a small molecule mimic of protein N-homocysteinylation, was prepared to react with each of the probes. After reaction, NH_2OH and IAA were added to remove remaining thioester species (probes and reaction intermediates) and to block free thiols, respectively. (C) The product (a) was detected by LC-MS. The relative intensity of (a) was shown as the ratio of intensity of (a) versus that of an internal standard. Data represent mean values ± standard deviation; $n = 3$ per group.

to that of an N-terminal homocysteine, we hypothesize that thioester could serve as a specific probe to react with protein N-homocysteinylation and enable its global profiling in complex proteomes. As shown in Scheme 1, alkynyl thioester probes react with N-homocysteinylated proteins through a reversible transthioesterification to form a six-member intermediate, followed by a rapid intramolecular S-to-N acyl shift. Consequently, an N-homocysteinylated lysine can be selectively functionalized with an alkyne handle through an amide bond. A fluorescent or biotin reporter tag will be conjugated for either visualization by SDS-PAGE or enrichment for MS-based proteomic analysis. To test this hypothesis, we first synthesize a series of thioester probes and validate the reactions with small molecule model. Next, we chose the best probe to demonstrate its chemoselective labeling of N-homocysteinylation on purified proteins. Finally, we apply this probe in a chemoproteomic strategy to globally profile protein N-homocysteinylation in cellular proteomes. These studies highlight the novel application of NCL in the profiling of protein post-translational modifications and to our best knowledge, for the first time, report quantification of N-homocysteinylated proteins and sites in complex proteomes of mammalian cells.

Results and discussion

Validation of reaction between N-homocysteinylation and thioester probes with a small molecule model

To test our hypothesis, three thioester probes were designed and synthesized (Fig. 1A and Scheme S2A†). AT-1 and AT-2 employ alkyl thiol and benzyl thioalcohol as leaving groups, both of which were commonly used as C-terminal thioester reactive groups in NCL.[37,40] AT-3 was derived from mercapto-phenylacetic acid, MPAA, which is a non-malodorous and water-soluble thiol used as an excellent thiol additive to speed up peptide ligation.[41] In theory, AT-3 should have better solubility in aqueous buffer and better reactivity towards N-homo-cysteinylation. We first evaluated the reactivity of the three probes in a small molecule model. 2-Amino-N-benzyl-4-mercaptobutanamide (B-hcy) was synthesized as a mimic of N-homocysteinylation (Fig. 1B and Scheme S2B†) and reactions were carried out in a denaturing buffer condition commonly used for NCL. As shown in Fig. 1B and S1,† hydroxylamine (NH_2OH) was added to quench excessive thioester probes and reaction intermediates (b), followed by alkylation of thiol groups with iodoacetamide (IAA). The amount of desired
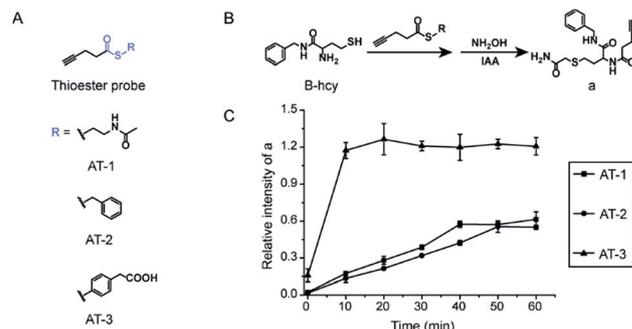
product (a) was monitored by LC-MS and plotted over time in Fig. 1C. As expected, AT-3 (1 mM) demonstrated the fastest reaction kinetic and B-hcy (100 μM) was consumed completely within 30 min at pH 7. Thus, AT-3 was chosen for labeling proteins next.

Chemoselective labeling of N-homocysteinylation on purified proteins

Unlike the small molecule model, proteins are composed of various amino acids such as nucleophilic lysine residues that have been reported to react with thioester under physiological conditions.[42] Notably, thioester probes have been implemented to profile non-enzymatic acylation in proteomes.[43] We therefore tried to optimize the condition in order to achieve chemoselective labeling of N-homocysteinylation on purified proteins. As depicted in Fig. 2A, N-homocysteinylated proteins were firstly generated by incubating purified proteins with HTL at room temperature and the modified proteins were further verified by ESI-MS analysis (Fig. S2†). N-homocysteinylated proteins were dissolved in denaturing buffer and labeled with AT-3 (1 mM). After removal of excessive probes, the labeled proteins were conjugated with rhodamine-azide via copper(ı)-catalyzed azide-alkyne cycloaddition (CuAAC)[44] and separated by SDS-PAGE for direct visualization of in-gel fluorescence. As shown in Fig. 2B, lysozyme was labeled at various pH conditions for different amounts of time. The overall labeling intensity rose with increasing pH and time regardless of HTL modifications, indicating potential off-target reaction with lysine residues. We quantified the labeling selectivity (+HTL versus −HTL) for four selected conditions (marked with red asterisks, Fig. 2B) and the results showed that the selectivity (+HTL versus −HTL) dropped sharply at higher pH or with increased time of labeling. We then subjected the non-HTL-induced samples to trypsin digestion and analysis by liquid chromatography-tandem MS (LC-MS/MS), which did reveal nonspecific labeling of lysine residues
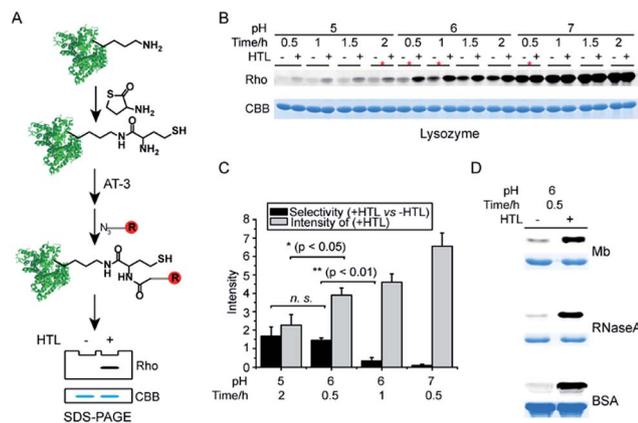
Fig. 2 Chemoselective labeling of purified proteins with AT-3. (A) Purified proteins were induced with HTL to generate N-homocysteinylation. The modified proteins were labeled with AT-3, conjugated with rhodamine-azide *via* CuAAC and visualized by in-gel fluorescence (Rho). The Coomassie staining is used as a loading control (CBB). (B) and (C) lysozyme was labeled with AT-3 at varying pH conditions and for different lengths of time. The band intensities were quantified with ImageJ and data represent mean values $\pm$ standard deviation; $n = 3$ per group. (D) Other proteins (Mb, RNaseA and BSA) were subject to the above workflow and the labeling signals were also HTL-dependent.

(Table S2†). Therefore, to balance between the labeling selectivity and overall intensity, we chose pH 6 and 0.5 h for selective labeling of N-homocysteinylated proteins (Fig. 2C). Under this condition, other HTL-induced proteins (Mb, RNaseA and BSA) were also labeled with good selectivity (Fig. 2D). We further identified the AT-3 labeled adducts on sites of N-homocysteinylation in these proteins by LC-MS/MS (Fig. S3†).

Profiling of N-homocysteinylated proteins and sites with a quantitative, MS-based chemical proteomic platform

We next set out to apply the AT-3 probe to characterize N-homocysteinylated proteins and sites in complex cellular proteomes using a quantitative, MS-based chemical proteomic platform (Fig. 3A). Soluble proteomes of HeLa cells treated with H_2O or HTL (0.2 mM) were labeled by AT-3 (1 mM), conjugated with biotin-azide *via* CuAAC, enriched by streptavidin beads and proteolytically digested by trypsin. The tryptic peptides from H_2O- and HTL-treated samples were then isotopically differentiated by reductive dimethylation (ReDiMe),[45] combined pairwise and fractionated by high-pH HPLC for LC-MS/MS analysis. The selective labeling of HTL-modified proteomes were confirmed by western blotting (Fig. S4A†) and the proteomic results were summarized in Fig. S4B and C.† A total of 1519 candidate N-homocysteinylated targets were quantified across three biological replicates ($R_{H/L} \geq 2$, HTL-treated proteome *versus* the untreated control) (Table S3†). In addition to the *in vitro* profiling, we also applied the AT-3 probe to identify N-homocysteinylation from HeLa cells treated with HTL *in situ*. The results of cell viability assays indicated no measurable cytotoxicity for HeLa cells after incubation with HTL (0.5 mM) for 24 h at 37 °C (Fig. S5A†). Under this condition, HTL-
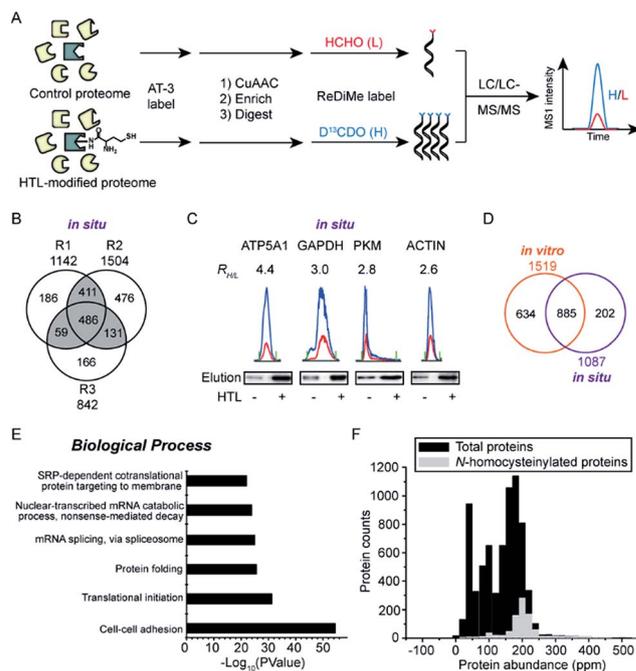

Fig. 3 Quantitative MS-based profiling of N-homocysteinylated proteins in HeLa proteomes induced with HTL *in vitro* and *in situ*. (A) Schematic workflow for MS-based profiling of N-homocysteinylated proteins in combination with stable isotope dimethyl labeling method. (B) Venn diagram showing the number of identified potential targets across three biological replicates (*in situ*). (C) Verification of the enrichment of N-homocysteinylation on ATP5A1, GAPDH, PKM and ACTIN. Extracted MS1 chromatograms of representative tryptic peptides with enrichment ratios ($R_{H/L}$) were shown and western blots indicated that selected proteins are enriched by AT-3 in HTL-treated samples. (D) Venn diagram showing the overlap of proteins with N-homocysteinylation quantified from *in vitro* and *in situ* profiling. (E) Bioinformatic analysis of N-homocysteinylated proteins based on involvement in specific biological processes. (F) Abundance distribution of N-homocysteinylated proteins according to the whole proteome abundance data of HeLa cells.

dependent labeling signals were again detected with AT-3 (Fig. S5B†) and quantitative chemoproteomic experiments identified more than 1000 potential targets of N-homocysteinylation (Fig. 3B and S5C, Table S4†). We further verified the enrichment of N-homocysteinylation by AT-3 on four candidate proteins, ATP5A1, GAPDH, PKM and ACTIN, by affinity purification and immunoblotting (Fig. 3C and S6†). Finally, among the 1087 potential targets identified in three biological replicates, 885 targets were overlapped with the *in vitro* data (Fig. 3D, Table S4†). Gene ontology analysis by DAVID (Database for Annotation, Visualization and Integrated Discovery)[46] revealed that the N-homocysteinylated protein targets were enriched in important biological processes such as cell–cell adhesion (annexin A2; filamin B), translational initiation (eukaryotic translation initiation factors; ribosomal proteins) as well as protein folding (peptidylprolyl isomerases; heat shock proteins) (Fig. 3E and Table S4†). Comparison with the whole proteome data of HeLa cells[47] reveals that the majority of N-homocysteinylated targets were of high abundance (Fig. 3F). The results were consistent with a previous

report that human plasma proteins were *N*-homocysteinylated proportionally to its abundance.[48]

We finally sought to identify exact sites of *N*-homocysteinylation using a tandem orthogonal proteolysis strategy.[49] As illustrated in Fig. 4A, proteomes of HTL-induced HeLa cells (0.5 mM) were labeled with AT-3, conjugated with a photocleavable biotin-azide tag *via* CuAAC, and subjected to streptavidin enrichment and on-beads trypsin digestion. The AT-3 adducted peptides were released from streptavidin beads upon irradiation with ultraviolet (UV) light (365 nm, 0.5 J cm^{-2}, 60 min) and analyzed by LC-MS/MS for site identification. In total, 304 unique sites of *N*-homocysteinylation were identified across two biological replicates (Fig. 4B and Table S5†). For example, the MS/MS spectrum generated by higher-energy collisional dissociation (HCD) fragmentation unambiguously supports the adduct of *N*-homocysteinylation on Lys54 of Profilin-1 (TFVNITPAEVGVLVGK*DR) (Fig. 4C and S7†). We also identified two sites of *N*-homocysteinylation, K57 (Fig. S8†) and K80 (Fig. S9†), from Histone H3, the former of which has been previously identified from purified histones of HEK293T cells.[50] The identified sites of *N*-homocysteinylation belong to 168 proteins, most of which (≥97%) were quantified from HeLa cells with *in situ* treatment of HTL (Fig. 4D and Table S5†). Among them, 25 proteins were with the corresponding ratios, $R_{H/L}$, less than 2, suggesting possible non-specific labeling of lysine residues within the same protein. The sequence analysis did not reveal any obvious conserved motifs surrounding *N*-homocysteinylated lysine residues (Fig. S10A†). In addition, most of the *N*-homocysteinylated lysines do not have heightened intrinsic reactivity according to a recent ABPP profiling study[23] (Fig. S10B and Table S5†). Interestingly, 10 proteins

reported with thioester-reactive lysine residues by Meier and colleagues[43] are also found in our list (Fig. S10C and Table S5†), including GAPDH that has three lysine residues (K66, K84 and K219) modified by both malonyl-CoA and HTL due to similar reactivity. Finally, 46 sites of *N*-homocysteinylation have been annotated as sites of acetylation according to the Uniprot database, suggesting a potential crosstalk between *N*-homocysteinylation and acetylation as previously revealed on purified histones[50] (Fig. S10D and Table S5†).

Conclusions

In summary, we have developed a chemoselective strategy inspired by NCL to label protein *N*-homocysteinylation. Although NCL was developed more than two decades ago, its applications have been mainly limited to protein synthesis. Our method exploited the unique structure of *N*-homocysteinylation and extended, for the first time, the usage of thioester probes to the profiling of this disease-related protein post-translational modification. We further applied the thioester probe in combination with MS-based quantitative proteomics to generate a global portrait of protein *N*-homocysteinylation in mammalian cells. Particularly, over 800 *N*-homocysteinylated proteins and 300 sites of *N*-homocysteinylation were identified from HeLa cells induced under HHcy-mimicking conditions. Our data suggested that the non-enzymatic process is tightly associated with physical accessibility of lysine residues towards the reactive intermediate and might be functionally implicated in crosstalk with other lysine modification. This powerful chemoproteomic platform will provide valuable information to help decipher molecular mechanisms of HHcy-implicated diseases.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.
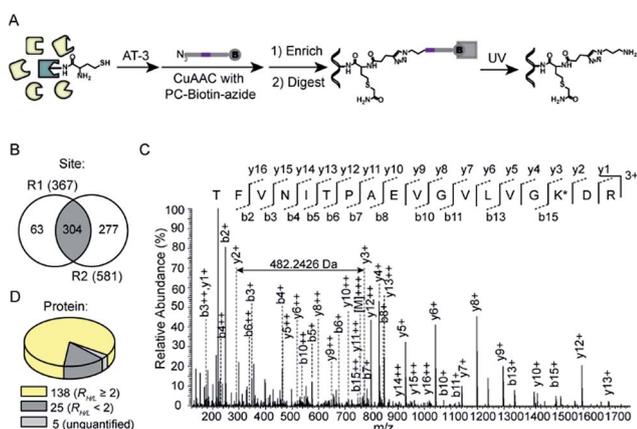
Acknowledgements

Fig. 4 Identification of sites of *N*-homocysteinylation from HeLa cells induced with HTL *in situ*. (A) Schematic workflow for MS-based identification of residue sites of *N*-homocysteinylation using a tandem orthogonal proteolysis strategy. (B) Venn diagram showing the number of sites of *N*-homocysteinylation identified across two biological replicates. (C) MS/MS spectrum of TFVNITPAEVGVLVGK*DR, a peptide from Profilin-1. All b and y ions are labeled. The *m/z* difference of 482.2426 between y2$^+$ and y3$^+$ supports the expected modification on the lysine residue (see Fig. S7† for the exact structure) as denoted by K*. (D) Categorization of 168 proteins with identified sites of *N*-homocysteinylation based on distribution of their quantification ratios, $R_{H/L}$.

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