Multiplying Identifiability of Clickable Peptides Using One-Pot Tagging of Homologous Biotinyl Azides

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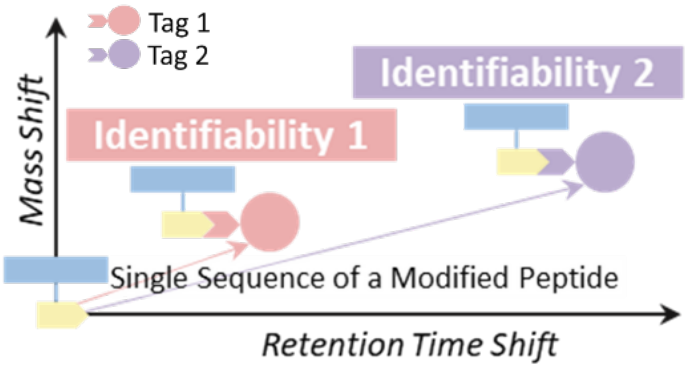
Abstract

Chemical proteomics plays a crucial role in understanding protein functions and developing covalent drugs but faces challenges in accurately identifying probe-modified peptides due to the generation of only a single modified peptide per probe reaction and potential ambiguities in proteomic identification. This work introduces a novel Single-Sequence Identification (SSI) principle, addressing these challenges by enhancing the detectability of modified peptides in proteomic experiments. Our innovative SSI approach involves creating multiple versions of a modified peptide within the proteomic identification space, thereby increasing the probability and confidence of peptide identification. We demonstrate the efficacy of this method using a one-pot triplex tagging technique that attaches three homologous biotinyl azides with varying polyethylene glycol (PEG) linker lengths to clickable proteins. This tagging-triplication method not only enables confident identification of peptides carrying two forms of tags, but also leverages tag diagnostic ions and the dependency of elution time on linker length to further boost identification accuracy. Additionally, we identified and addressed variability in CuAAC-tagged proteins by suggesting a split-and-pool strategy. The ease of integrating our tagging-triplication method into existing chemical proteomics workflows showcases its potential in enhancing peptide analysis reliability in chemical proteomics. These advancements highlight the significant implications of the SSI principle for future proteomics research. Data are available through ProteomeXchange: identifier PXD037770.

Table-of-Contents Graphic



Keywords

covalent modification, single-sequence identification, chemical proteomics, activity-based protein profiling, triplex tagging, peptide identifiability, one-pot preparation, clickable modification, biotin azide

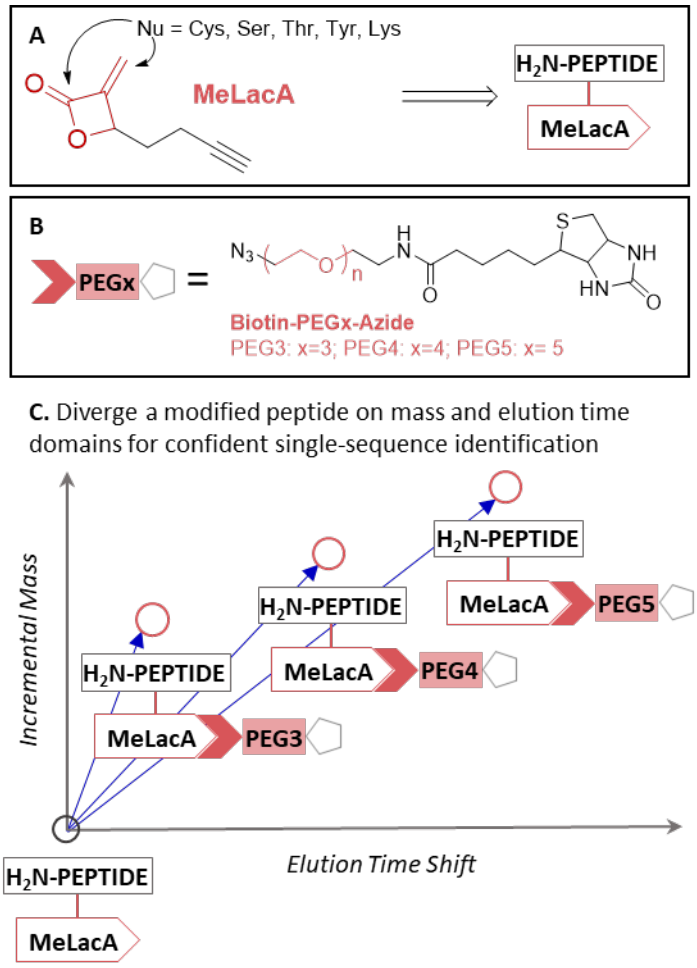
Introduction

Studying protein functions and developing covalent drugs are critically dependent on the precise analysis of protein nucleophiles and their covalent modifications.[1-5] While chemical proteomics, employing mass spectrometry (MS) and covalent probes,[6] has significantly advanced our ability to measure protein reactions with high structural specificity,[7-11] it faces a notable challenge that has remained under-addressed. This challenge arises in the MS analysis of probe-modified peptides, the central task in chemical proteomics. Typically, each protein nucleophile-probe reaction yields just one peptide sequence for analysis. The accuracy of analyzing these sequences is compromised by the inherent complexity of proteomic samples and the limitations of shotgun proteomics[12] methodologies. Mass spectra often lack complete sequence ions for these modified peptides, hampering the definitive identification of peptides and the precise localization of their modifications. This problem is particularly acute for modifications on abundant residues like serine or in low-abundance but biologically significant proteins, which are frequent targets of targeted covalent inhibitors (TCIs). Additionally, the complexity of proteomic samples can lead to the co-selection and fragmentation of unrelated ions, further complicating the interpretation of spectral data.

Addressing these analytical challenges, our study introduces the Single Sequence Identification (SSI) strategy, a novel approach that chemically enhances the detectability of individual modified peptides. Drawing inspiration from the proteomics convention of validating protein identities through multiple peptides of varying identification confidence,[13] the SSI method creates several distinct versions of each peptide sequence. These versions, each characterized by unique masses, elution times, and fragmentation patterns, allow the divergence of a single modified peptide sequence within the proteomics identification space. This strategy not only enables more robust cross-verification of peptide identities and modification sites but also significantly improves analytical certainty, especially for peptides yielding sparse MS/MS data.

In this study, we investigate the SSI principle using peptides modified at the cysteine residue with a clickable covalent probe. Cysteine-modified peptides were chosen for their reliably identifiable sequences and their importance in proteome-wide studies of protein function and covalent drug development. We treated HT-29 cells with a novel MeLac alkyne (MeLacA, racemic 4-(3-butynyl)-3-methyleneoxetan-2-one (α-methylene-β-lactone (MeLac) alkyne; **Scheme 1A**) probe,[14] and subsequent trypsin digestion and affinity enrichment yielded clickable cysteine-modified peptides. Employing a one-pot, multiplex tagging approach, we attached three different biotinylated azides, each with a varying polyethylene glycol (PEG) linker, to the modified peptides. This strategy effectively tripled the detectability of each clickable peptide, enhancing identification robustness. Compatible with existing chemical proteomics workflows, our multiplexed clicking method promises straightforward integration. Moreover, with various methods available for introducing clickable modifications into proteins,[15-21] this approach holds significant potential to advance MS analyses in studying protein reactivity and function and in developing innovative covalent therapeutics.

Scheme 1 *The Single-Sequence Identification (SSI) principle: Multiply the identifiability of peptides modified by the MeLacA probe. A: MeLacA reactions with protein nucleophiles lead to MeLac-modified peptides; B: biotinyl-PEGx-azides; C: Differential tagging diverge the identifiability of the single sequence of a MeLacA-modified peptide to three different identification locations (elution time, precursor mass).*



Experimental

Reactions of MeLacA with live HT-29 cells and protein extraction. HT-29 cells were cultured to a confluency of 90-95% in T75 flasks (about 8 million cells per flask) with Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (w/v) Pen/Strep antibiotics (FBS, Gibco) in a humidified, 5% CO_2 incubator at 37 °C. The cells were washed with 10 mL (for cells in each T75 flask) of phosphate-buffered saline (PBS, 1X, pre-heated at 37 °C) twice. The pre-aliquoted stock solution (100 mM, stored at -80 °C) of MeLacA in dimethylsulfoxide (DMSO) was thawed and diluted to 20 mM immediately before use. The reaction was initiated by adding 10 µL of 20 mM probe in DMSO to each T75 flask containing pre-washed cells in 10 mL of PBS, resulting in a working probe concentration of 20 µM. The flasks were placed on a rocking platform at 37 °C for 30 minutes. Ten milliliters of ice-cold tris-buffered saline (TBS, 1X) was added to each flask, and cells were then gently scraped off. The subsequent cell suspension was transferred to a 15-mL conical tube, centrifuged at 500 g, at 4 °C for 3 min. Each cell pellet was washed with 10 mL of PBS and either used immediately or stored at -80 °C.

To each fresh or frozen cell pellet, 1 mL of ice-cold PBS lysis buffer (prepared with 1X PBS and Pierce Protease and Phosphatase Inhibitor Mini Tablets) was added. The cell suspension was homogenized using an ultrasonic cell disruptor on ice. The homogenized lysate was centrifuged at 16,000 g at 4 °C for 30 min. Total protein concentration in the lysate supernatant was determined by the BCA assay (Pierce) and normalized to 1.5 mg/mL with PBS lysis buffer.

Preparation of enriched samples of probe-modified peptides. One milliliter of a cell lysate sample (1.5 mg mL^{-1}) was used for copper-catalyzed alkyne–azide cycloaddition (CuAAC) conjugation reactions. Cell lysate aliquots were processed for CuAAC attachment of four

different azide affinity tags: Biotin-PEG3-azide, Biotin-PEG4-azide, Biotin-PEG5-azide, and Desthiobiotin-PEG3-azide (Click Chemistry Tools). The tagging reaction for individual tag was performed in triplicates. The one-pot triplex tagging, denoted as *MixClick*, was performed in duplicates using a mixture of Biotin-PEG3-azide:Biotin-PEG4-azide:Biotin-PEG5-azide in a 1:1:1 ratio. Immediately before use, stock reagent solutions were prepared as follows: 1.7 mM tris(benzyltriazolylmethyl)amine (TBTA) in DMSO:*tert*-butanol at a 1:4(v/v) ratio, 50 mM copper(II) sulfate in water, 50 mM tris(2-carboxyethyl)phosphine (TCEP) in water, and 1.25 mM azide tag of choice for the simplex tagging reaction (or 1.25 mM Biotin-PEG3-azide, 1.25 mM Biotin-PEG4-azide and 1.25 mM Biotin-PEG5-azide in water for the triplex tagging reaction). For each reaction replicate, 136 µL of click reagent cocktail was prepared by mixing 68 µL of TBTA stock, 22.6 µL $CuSO_4$ stock, 22.6 µL of TCEP stock, and 22.6 µL of azide affinity tag stock of choice. To each cell lysate aliquot, 110 µL of the click reagent cocktail was added, resulting in working concentrations at 100 µM for TBTA, 1 mM for $CuSO_4$, 1 mM for TCEP, and 25 µM for the azide tag. The reaction mixtures were incubated at room temperature for 1 hour with constant agitation.

After the CuAAC conjugation, proteins in each reaction were precipitated in 10 mL of cold acetone at -20 °C overnight. The resulting protein precipitate was pelleted by centrifugation at 3000 g at -9 °C for 30 min. Each pellet was washed with 5 mL of cold methanol and redissolved in 200 µL of a reconstitution buffer (50 mM ammonium bicarbonate, 6 M guanidine HCl, and 10 mM 2-mercaptoethanol). The reconstituted protein samples were reduced with 10 mM dithioerythritol at 37 °C for 1 hour, followed by alkylation with 18.8 mM iodoacetamide at 25 °C in the dark for 30 min. Finally, 728 µL of 50 mM ammonium bicarbonate was added to each sample for digestion at a trypsin-to-protein ratio of 1:25 at 37 °C for 16 hours.

To each digested sample, 400 µL of pre-washed 50% (v/v) NeutrAvidin-agarose resin slurry (Pierce) was added, followed by 1-hour incubation at room temperature with constant

agitation. The resins were washed five times with 1 mL of Pierce IP Lysis Buffer, three times with 1 mL of PBS, and eight times with 1 mL of LC-MS grade water. The captured peptides were eluted in 600 µL of elution solvent (50% acetonitrile, 49% water, 1% trifluoroacetic acid, v/v) at 80 °C for 10 min. The subsequent eluate was concentrated in a vacuum concentrator, lyophilized, and reconstituted in 20 µL of 0.1% (v/v) formic acid and 2.5% (v/v) acetonitrile in LC-MS grade water for LC-MS/MS analyses.

LC-MS/MS and data analysis. Each sample of enriched peptides modified by MeLacA was analyzed in duplicates, both replicates coming from the same sample vial, on an Orbitrap Exploris 480 mass spectrometer equipped with the nanospray ionization source and an EASY-nLC 1200 system (Thermo Scientific). Peptides were separated on an Acclaim PepMap C18 column (15 cm x 75 µm I.D, 2 µm in particle size; Thermo Scientific). The autosampler temperature was 4.0 °C. The sample injection volume was 2.0 µL. The mobile phase flow rate was 300 nL/min. Solvent A was 0.1 % (v/v) formic acid in water, and solvent B was 0.1 % (v/v) formic acid in 80% (v/v) acetonitrile, 20% (v/v) water. A gradient of 3 to 100% (v/v) solvent B over 294 min was applied as follows: 3% B at 0 min, 38% B at 245 min, 56% B at 275 min, 100% B at 280 and 285 min, 3% B at 286 and 294 min.

The Xcalibur software (v4.4.16.14) was used to control the instrument. The mass spectrometer operated in data-dependent acquisition (DDA) mode for monitoring positive ions at a spray voltage of 1900 V. Ion transfer tube temperature was 250 °C. The expected peak width was 45 s. The default charge state was 2. Advanced peak determination was set as true. Spectra were acquired for 0-280 min over the LC gradient. For MS1, the mass range was from 300 to 2000 *m/z*. The Orbitrap mass analyzer was set with a resolution of 60,000, a normalized AGC target of 100%, and a maximum ion time of 20 ms. For data-dependent MS2, the MIPS filter was applied in peptide mode. The intensity threshold was 5000. The quadrupole was set with an isolation window of 1.4 *m/z* and a fixed first mass of 140 *m/z*. The Orbitrap was set with

a resolution of 15,000, an AGC target of 1E5, and a maximum ion time of 60 ms. The DDA method was allowed up to 20 MS/MS scans per duty cycle and an NCE of 32. Precursors that triggered MS/MS scans were dynamically excluded from repetitive MS/MS scans for 45 s within ±5 ppm. Charge state exclusion was enabled to reject precursor ions with charge states outside the range of +2 to +6. MS/MS spectra were collected in the centroid data type. Acquired data are deposited in ProteomeXchange (PXD037770).

For the global analysis of MeLacA and MeLacA-related chemical modifications, open-search[22-24] was performed on all the samples using FragPipe (v17.1) with MSFragger[25,26] version 3.4 and Philosopher[27] version 4.1.0 (build 1635640020). Offset search with labile modification mode[28] was used to identify MeLacA-modified peptides with the desthiobiotin-PEG3-azide tag, and the modified peptides with biotin-PEG3-azide, biotin-PEG4-azide, or biotin-PEG5-azide. A set of default open-search parameters was used except for the items as follows: a precursor *m/z* tolerance (Delta mass) window of -150 to 900 Da, a peptide length range of 7 to 35 residues, a peptide mass range of 500 to 5000, a maximum missed peptide cleavage of 2, and a maximum of 4 variable modifications on a peptide. Fixed modification inclusion lists were disabled, and variable modifications were set for methionine oxidation, N-terminal acetylation, and cysteine carbamidomethylation. Finally, the DeltaMass[29] software tool (v1.2.2) visualized mass shifts in the open-search results. See parameter files for open-search and offset search in Supplemental Information (SI, Text S1-3). Post-search data analysis used Python with Jupiter Notebook. Spectrum visualization used PDV viewer.[30]

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[31] partner repository with the dataset identifier PXD037770.

Results and Discussion

Proteome-wide Michael addition of α-methylene-β-lactone (MeLac). We have recently demonstrated that the multi-electrophilic MeLac warhead can probe protein nucleophiles of different types in the live cell proteome.[14] MeLac labels reactive cysteine, serine, threonine, tyrosine, lysine residues, and the N-terminal amino group through the Michael acceptor or the acyl carbon atom on the warhead (**Scheme 1A**). When protein nucleophiles react at the Michael addition site of MeLac, a characteristic hydrolyzed product of the β-lactone ring can be observed to verify the Michael addition mechanism, resulting in a mass increase of 568.32206 Da.[14] Although nucleophilic thiols, amines, and hydroxyls can all react via the Michael addition, protein adducts of the addition reaction are dominantly attributed to MeLac reacting to nucleophilic cysteines.[14]

We used CuAAC to attach desthiobiotin-PEG3-azide to MeLacA-reacted proteins. Following protein digestion, desthiobiotin-avidin affinity enrichment produced samples of MeLacA-modified peptides for data-dependent acquisition (DDA) MS/MS. Triplicate preparations of the azide attachment and duplicate MS analysis for each click attachment summed to six LC-MS/MS runs. MSFragger open search[22-24] observed global MeLacA modifications on proteins, as previously reported.[14]

MSFragger[25,26] offset search was set for cysteine reacting with the Michael acceptor of MeLacA, which gave an incremental mass of 568.32206 Da (the hydrolyzed adduct of MeLacA with the attached desthiobiotin tag) for the probe-modified peptides. Labile modification search mode was also enabled, requiring the modified peptides to produce one of the two diagnostic ions at *m/z* 197.12845 and 240.17065; these are diagnostic fragment ions from the desthiobiotin moiety[14] (**Scheme 2**). The database search identified 520 unique probe-modified peptides with localized MeLac-cysteine reactions, after grouping overlapping peptides (peptides with and without missed cleavages that contain the same reacting cysteine) and verifying the output

peptides from the database search based on the numbers of cysteine residues and observed MeLac modification and iodoacetamide alkylation from sample preparation (**Table S1**). Sequence alignment of the identified peptides revealed enrichment of hydrophilic residues in the vicinity of the reacting cysteine (**Fig 1**). Sequences of MeLac-reacted peptides (487 peptides, **Table S1**) carrying a single cysteine residue were compared with a literature collection[8,32-36] of 9694 unique cysteine peptides which react with covalent probes carrying the iodoacetamide warhead, using the web-based pLogo software.[37] The sequence comparison observed the MeLac warhead reacting preferably with cysteines with serine at the +1 position or glycine at the +2 position. In comparison, the iodoacetamide warhead has preference for cysteines with arginine at the +1 position or lysine at the +2 or +3 position. The observed differences in nearby residues of the reacting cysteine might reflect differences in reaction mechanisms and the size and stereochemical arrangement of warhead atoms between MeLac and iodoacetamide.

Scheme 2. *Signature ions diagnostic to modified peptides with biotin and desthiobiotin-PEGylated tags.* S^{DB}*: signature ion for desthiobiotin;* S^{DB}_{PEG}*: signature ion for PEG-desthiobiotin;* S^{B}*: signature ion for biotin;* S^{B}_{PEG}*: signature ion for PEG-biotin.*

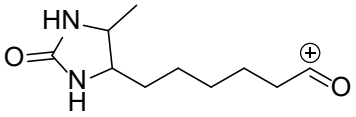
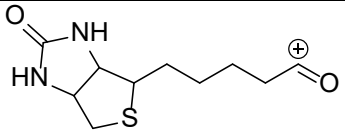
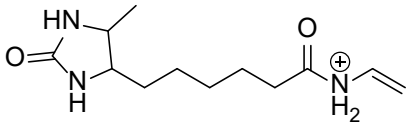
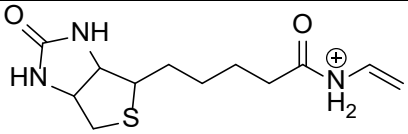
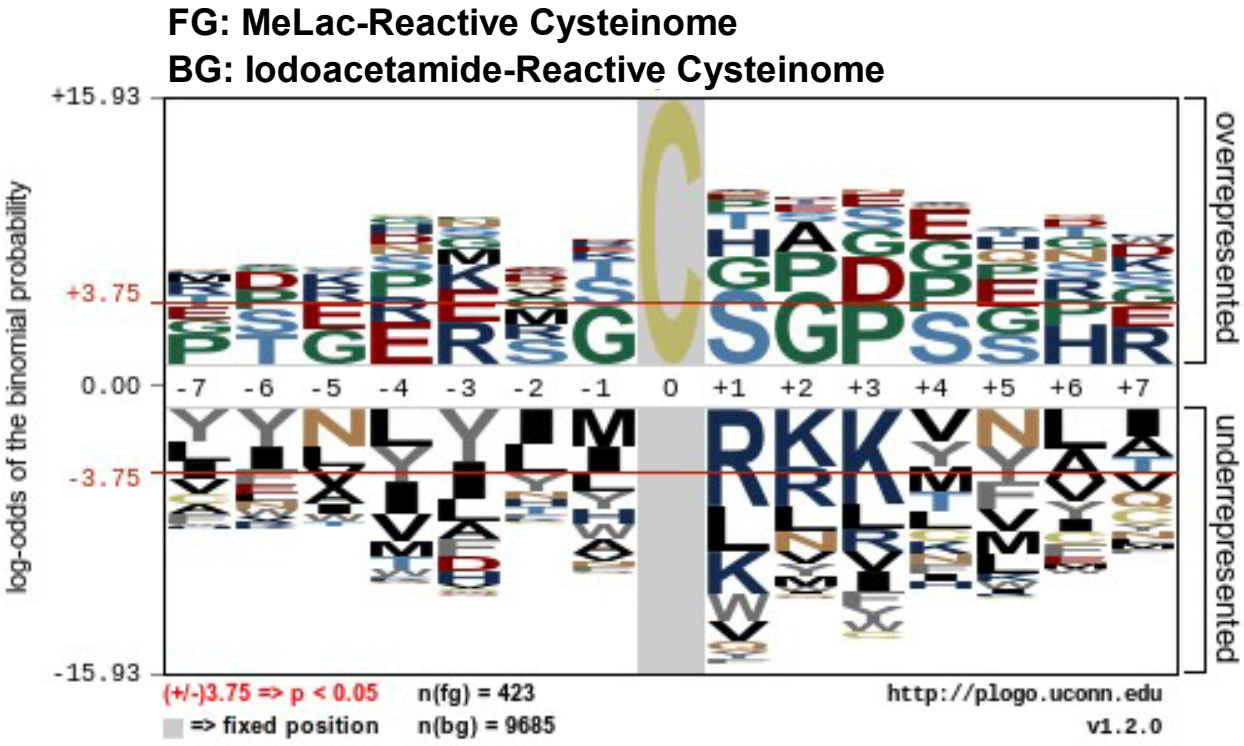
Desthiobiotin-PEGylated Tag	**Biotin-PEGylated Tag**
 Chemical Formula: $C_{10}H_{17}N_2O_2^+$ S^{DB} Exact Mass: 197.12845	 Chemical Formula: $C_{10}H_{15}N_2O_2S^+$ S^{B} Exact Mass: 227.08487
 Chemical Formula: $C_{12}H_{22}N_3O_2^+$ S^{DB}_{PEG} Exact Mass: 240.17065	 Chemical Formula: $C_{12}H_{20}N_3O_2S^+$ S^{B}_{PEG} Exact Mass: 270.12707

Figure 1. *MeLac-based chemical cysteinomics reveals different preferences of nearby residues from the iodoacetamide-based measurements. FG: foreground, MeLacA-modified cysteinyl peptide; BG: background, iodoacetamide-modified cysteinyl peptide.*



The MeLacA-modified peptides provide a compilation of probe-modified peptides for developing new methods to improve identification confidence. The identification of the cysteine-modified peptides can be supported by the existence of a cysteine residue that is not alkylated by iodoacetamide during sample preparation. For peptide-centric chemical proteomics, it is important to identify individual probe-modified peptides with high confidence due to the single-sequence limitation, especially peptides identified with low spectral counts. Notably, among the cumulative 520 identified peptides based on six DDA LC-MS/MS runs, 17 (3.3%) were identified with a single spectral count and 28 (5.4%) with two spectral counts, and 37 (7.1%) with three spectral counts, totaling 15.9% (**Table S1**).

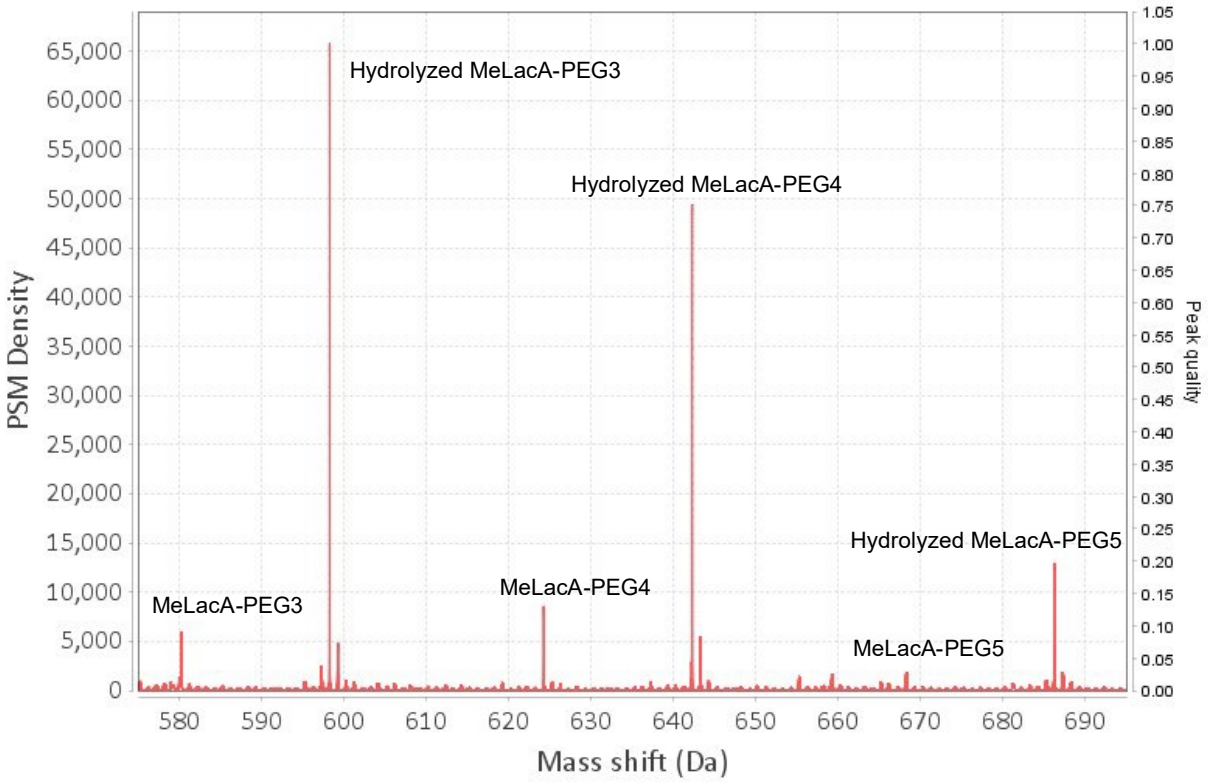
One-pot triplex tagging for multiplying identifiability of the single sequence of the probe-modified peptide. To increase the identification probability for the single sequence of the probe-modified peptide, we designed a principle of SSI through diverging individual modified peptides by their *m/z* values and LC elution times (**Scheme 1**). Thus, each sequence of modified peptide could be identified multiple times at separate locations in the proteomic identification space. Accordingly, a similar practice utilized to increase confidence in protein identification that requires two or more peptides can be implemented on the single sequence identification of a probe-modified peptide. We set a criterion that identification of a probe-modified peptide is reliable when the sequence of a particular modified peptide is identified at least twice with different sets of m/z values and elution times, analogous to the common practice for protein identification.[13]

To implement the SSI principle, we developed a method of one-pot triplex tagging of homologous biotinyl azides. Peptides modified by covalent probes are analytes of interest in chemical proteomics. These peptides often contain a clickable alkyne handle, which provides a modular mechanism for the CuAAC attachment of a reporter for imaging or separation. The alkyne-azide click attachment of an affinity tag like biotin or desthiobiotin is an essential step in

peptide-centric chemical proteomics workflows. Thus, our method can be readily integrated into these workflows with a single reagent change. In contrast to the use of only one (desthio)biotinyl azide in standard workflows, we used a 1:1:1 mixture of three biotinyl azides to diverge the precursor mass and elution time of MeLacA-modified peptides. Although homologous desthiobiotinyl azides were preferred to implement the SSI principle, they are not commercially available. Desthiobiotin binding to (strept)avidin has a nearly equal specificity to biotin but with less affinity (K_d = 10^{11} vs. 10^{15} M, respectively).[38] Thus, desthiobiotin-tagged, probe-modified peptides can be eluted using mild, MS-friendly conditions, which improves the preparation of probe-modified peptides.[14]

MeLacA-reacted HT-29 cells were lyzed and subjected to one-pot triplex tagging, designated as MixClick, of three biotin-PEGx-azides. These biotinyl azides (PEG3, PEG4, or PEG5, respectively) differed in the PEG linker length (**Scheme 1B**). The CuAAC attachment of the biotinyl azides was replicated in two separate reactions for MixClick and three for the other workflows. Enriched MeLacA-modified peptides were analyzed three times for MixClick and two times for the others using the DDA MS/MS mode. MSFragger open search of the resulting spectra of all MS/MS runs observed global modifications of MeLacA modified peptides with biotin-PEGx-azide tags (x=3, 4, 5; **Fig 2**). Peptides with incremental masses due to modifications of hydrolyzed MeLacA-biotin-PEGx-azides were observed at 598.27848 Da (PEG3), 642.30470 Da (PEG4), and 686.33091 Da (PEG5), respectively, together with modifications attributed to the unhydrolyzed MeLacA warhead at 580.26792, 624.29413, and 668.32035 Da, respectively.

Figure 2. *Global MeLac modifications in the live HT-29 cell proteome. Major modification peaks in the DeltaMass plot are associated by MeLac modifications and hydrolyzed MeLac modifications with clicked biotin-PEG3, PEG4-, or PEG5-azide*.

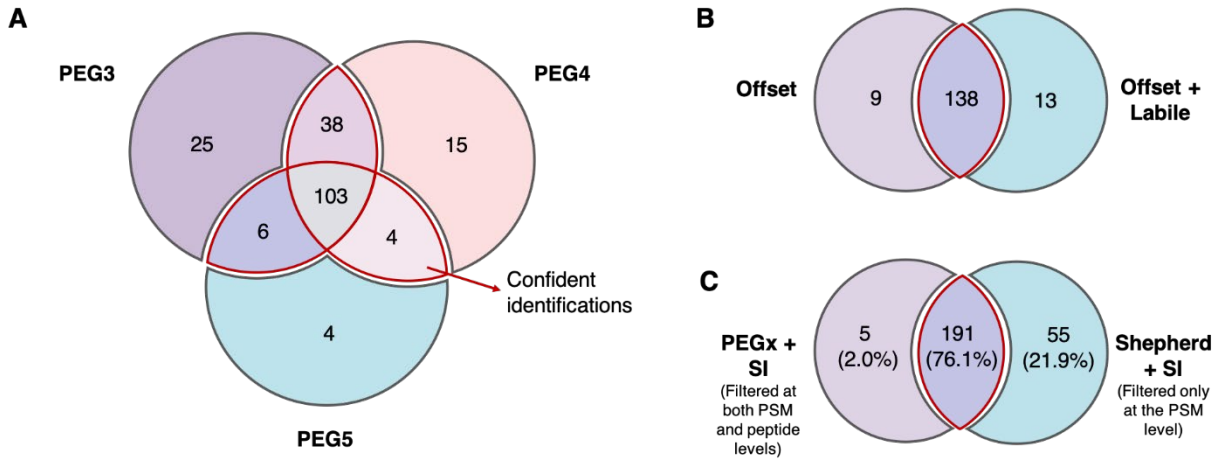


We performed a database search for peptides modified only at the cysteine residue by the Michael acceptor of the MeLac warhead. The offset MSFragger search used mass offsets of 598.27848, 642.30470, and 686.33091 Da, which corresponded to modifications of hydrolyzed MeLacA-biotin-PEG3/4/5-azides, respectively. Although acyl addition to the MeLac warhead by reduced cysteine residues is also possible, the probe-reacted proteins and related modified peptides are not stable to hydrolysis, and it is not possible to generate the incremental masses of hydrolyzed products used for the offset search. The acyl addition only leads to 580.26792, 624.29413, and 668.32035 Da, respectively. Additionally, an offset search with the labile mode[28] enabled was conducted, using two diagnostic ions of the biotin tag[39] at *m/z* 227.0849 (remainder mass 442.24275 Da) and *m/z* 270.1271 (remainder mass 399.20055 Da, see later in **Scheme 2**). These diagnostic ions were also used for PTM-Shepherd search[24] in the labile mode. Spectra from two replicates of one-pot MixClick (three DDA runs for each replicate) were used as a single dataset for the database search.

The offset search in the labile mode gave 103 MeLacA-modified peptides identified with all three biotin tags and 147 modified peptides identified with at least two tags (**Fig 3A**, **Table S2**). A comparison of the identified peptides with 2 or 3 biotin tags showed that the identification sensitivity increased slightly with the inclusion of diagnostic ions (9.5% unique identifications) as a searching constraint (**Fig 3B**, **Table S3**). Thus, when diagnostic ions are unavailable or the search engine does not support diagnostic ions in an automated database search, modified peptides could still be confidently identified with our method of multiplexed tagging, which changes the precursor mass and elution time of a single modified peptide sequence, multiplying the identifiability of the modified peptide in the search space. To explore the potential of probe diagnostic ions for improving search sensitivity, the PTM-Shepherd output was compared with peptide identified using Offset search with labile mode for observing diagnostic ions (**Fig 3C**, **Table S4**). Although most unique PTM-Shepherd peptides were identified with only one PEG

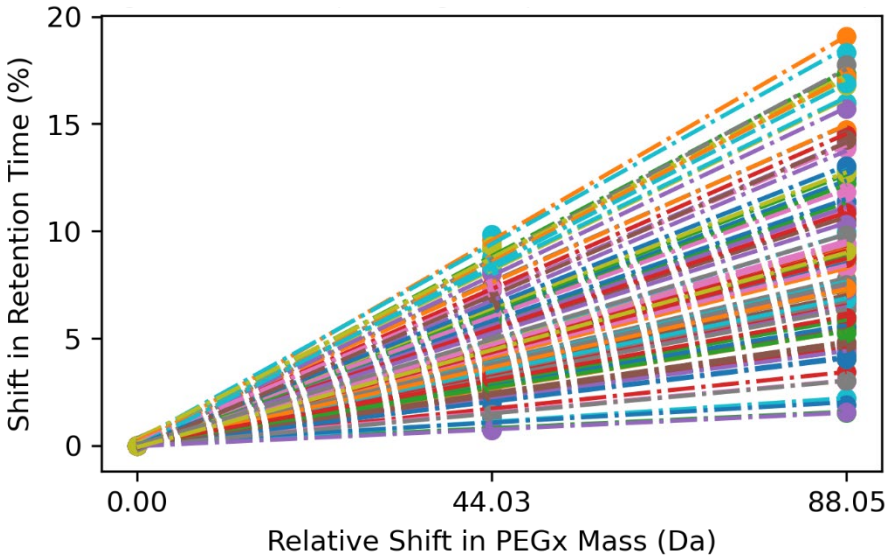
tag but two diagnostic ions, as expected, six peptides were reported with both PEG3 and PEG4 tags. 102 out of 191 shared identifications contained all three PEG tags; 46 contained two tags; 43 contained one tag. There were also six unique identifications from Offset search only; one had 3 tags, two had two tags and two had one tags. Considering that the Offset search results were FDR filtered at both PSM and peptide levels, but the PTM-Shepherd results were only filtered at the PSM level, it is tempting to postulate that FDR filtering at the peptide level could be adjusted to increase the search sensitivity of probe-modified peptides that can produce highly selective diagnostic ions.

Figure 3. *MixClick, one-pot tagging of triplex biotinyl azides, to improve the identification confidence for MeLacA-modified peptides. A: identified peptides based on individual PEG tags. Probe-modified peptides were prepared by the MixClick workflow (n=2 for click attachment of triplex biotinyl azides, n=3 for LC-MS/MS runs). B: numbers of identified peptides that carry 2 or 3 PEG tags compared between Offset searches without and with signature ions. C: Offset search with diagnostic ions enabled compared with PTM-Shepherd output; peptide identification required at least 1 PEG tag but two diagnostic ions.*



Triplicate DDA runs for replicate preparations of MixClick were also analyzed to investigate the relationship between the mass increase and the corresponding elution time shift. Elution times of probe-modified peptides, identified with all three biotin tags, increased linearly with the PEG linker length. The relationship for Dataset R2_3 (click reaction replicate 2 and MS run replicate 3), which had the highest number of identified probe-modified peptides, is shown as an example in **Fig 4**. While the linear correlation was tight (R^2=0.996±0.006), the sensitivity (slope) of the elution time for differentially tagged peptides varied. No correlation between the slope and peptide hydrophobicity was found. The linear correlation can provide a constraint to confirm database search results for MeLacA-modified peptides. For instance, when two biotin tags identify a modified peptide (e.g., PEG3 and PEG4), the longer tag has to elute later for the peptide identification to be valid. Integration of elution time constraint into database search is needed in future work to utilize the observed characteristic of differentially tagged peptides.

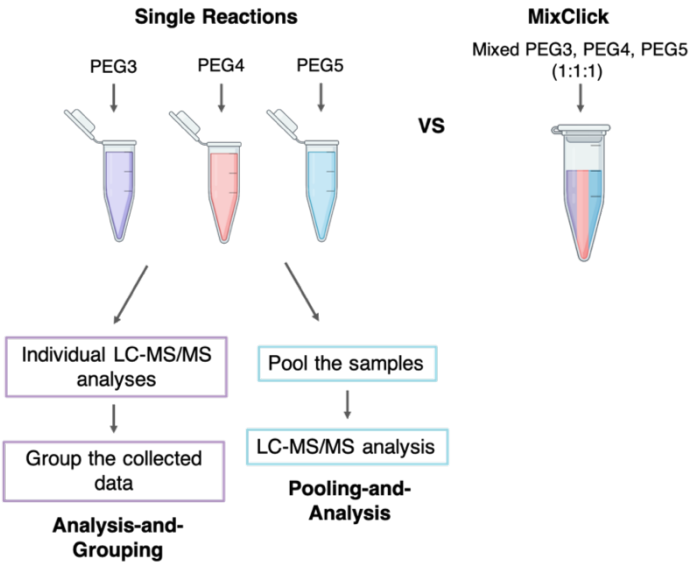
Figure 4. *Diverged identifiability of a single sequence of the modified peptide. Linear correlations exist in differentially tagged MeLacA-modified peptide between changes in the peptide mass and elution time. Elution time shifts and incremental masses are normalized to peptides carrying the PEG-3 tag*.



Workflow comparison. The MixClick workflow for one-pot tagging of probe-modified peptides with a mixture of all the biotin-PEG-azide tags (1:1:1) was compared with two other workflows: Analysis-and-Grouping and Pooling-and-Analysis (**Fig 5**). The efficiency of each workflow was evaluated based on the average number of identifications per DDA run (lower table in **Fig 5**).

The MixClick workflow identified a total of 263 peptides from 6 DDA runs or 44 identifications per DDA run, having the highest efficiency among three workflows compared. In the Analysis-and-Grouping workflow, the MeLacA-reacted proteins were individually tagged with biotin-PEG3-azide, biotin-PEG4-azide, or biotin-PEG5-azide. The enriched samples of tagged peptides were then analyzed separately via DDA-MS/MS (**Fig 5**). A total of 17 DDA runs gave 225 peptides identified by two or three PEG tags, i.e., 13 identifications per DDA run. Pooling individually tagged samples for simultaneous DDA analysis, the Pool-and-Analysis workflow (**Fig 5**) had lowest efficiency. Pooling separately tagged samples to reduce the number of DDA runs resulted in significantly diminished identifications of probe-modified peptides; a total of 6 DDA runs for the pooled samples (the same number of runs as the triplex tagged samples) only identified 44 peptides, or 7 identifications per DDA run. This was presumably partly due to the increased sample complexity caused by the additive introduction of non-probe-carrying peptides nonspecifically carried through enrichment preparations of probe-modified peptides.

Figure 5. *Comparison of workflows for the implementation of SSI and the number of peptide identifications associated in each.*

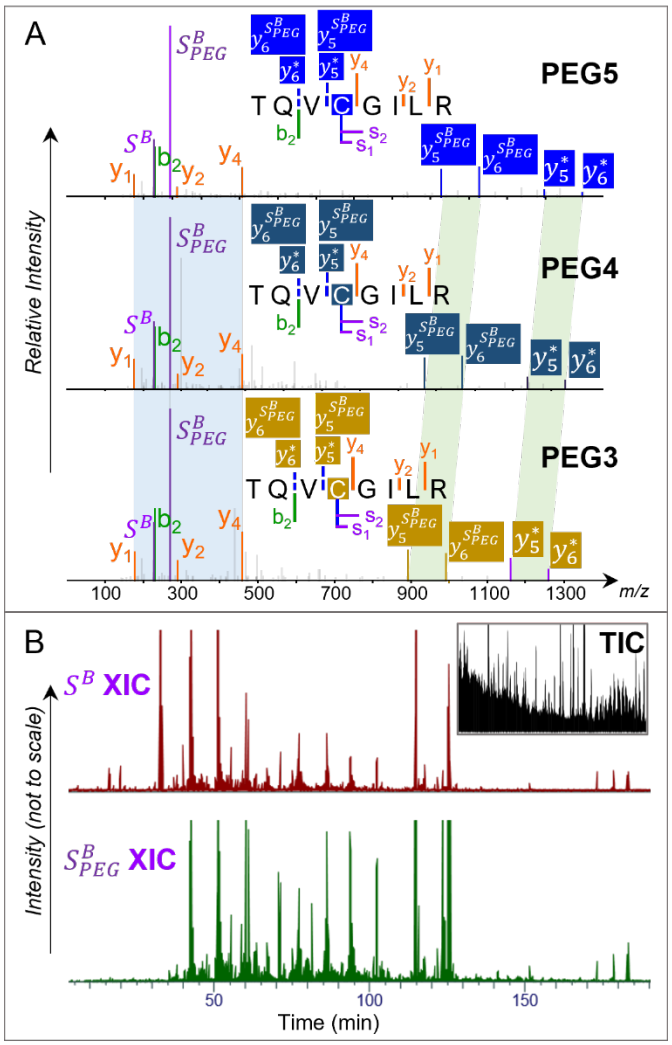


	Total 2PEGx Identifications	Number of DDA Runs	Average Identifications per DDA run
MixClick	263	6	44
Analysis-and-Grouping	225	17	13
Pooling-and-Analysis	44	6	7

Spectral characteristics and selective signature ions of triplex-tagged MeLacA-modified peptides. Comparison among differentially tagged peptides revealed two groups of sequence ions: the modification-carrying and the unmodified, as well as diagnostic signature ions common to all forms of tags (**Fig 6A**). MS/MS spectra from the modified peptide TQVCGILR of glucose-6-phosphate 1-dehydrogenase (P11413, *G6PD*) illustrated these three groups of ions. Unmodified sequence ions included y_1, y_2, y_3, and b_2. Modification-carrying ions included differentially tagged ions y_5 and y_6, denoted as y_5^* and y_6^*, with the shifting incremental mass for a PEG unit. Comparison among the MS/MS spectra also showed another group of intense ions (denoted as $y_5^{S_{PEG}^B}$ and $y_6^{S_{PEG}^B}$) with the same mass shifts attributed to the neutral loss of a biotin-PEG fragment. The neutral loss complemented the generation of an intense signature ion for the biotin-PEG moiety for all forms of tags at *m/z* 270.1271 (S_{PEG}^B). Another signature ion was attributed to the biotin moiety of the tags at *m/z* 227.0849 (S^B).

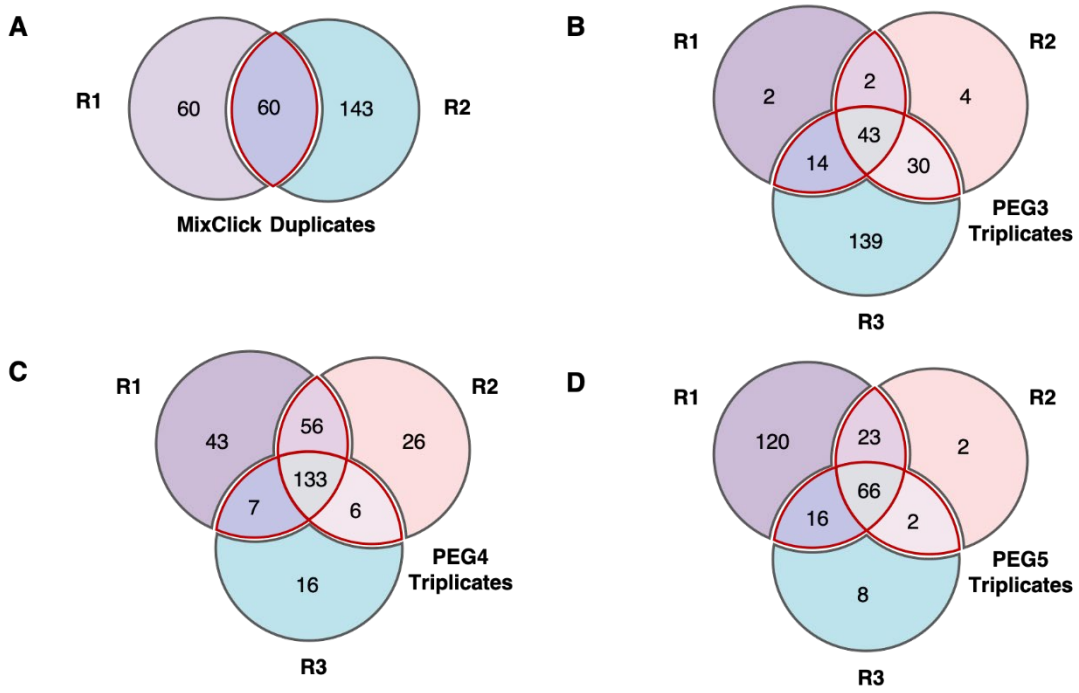
The existence of fragment ions in MS/MS spectra that are selective signatures of peptide modifications (**Fig 6B**) can facilitate and confirm the identification of modified peptides. Predictable fragment ions in the MS/MS spectra of inhibitor-modified peptides have improved peptide identification.[40] Diagnostic ions can be reasoned based on gas-phase fragmentation of the common structural moiety of modified peptides. Diagnostic signature ions for desthiobiotinyl (*m/z* 197.12845)[14] and desthiobiotin-PEGylated (*m/z* 240.17065),[14] biotinyl (*m/z* 227.0849),[39,41] and biotin-PEGylated (*m/z* 270.1271) peptides (**Scheme 2**) improved the identification of the MeLacA-modified peptides. Comparison among MS/MS spectra of peptides differentially tagged with biotin-PEGx-azides revealed additional fragment ions common to the MeLacA modified peptides at *m/z* 312.13764, 633.27348, 677.29970, 721.32591, 141.054, 159.065, 419.232, and 509.278. These fragment ions were not evident from the tag (modification) structure. However, they had much lower intensities than the diagnostic ions at *m/z* 227.0849 and 270.1271 (**Fig 6**). Thus, these fragments may be useful in supporting an identification rather than being diagnostic.

Figure 6. *MS/MS spectral characteristics for triplex-tagged MeLacA-modified peptides. Ions* y_n^* *carry the conjugate modification of the reacted MeLacA probe and biotinyl-PEGx azido tags (**A**). Ions* $y_n^{S^B_{PEG}}$ *are neutral loss (269.11980 Da) of* y_n^* *ions; protonated ions of the neutral loss are the signature ion* S_2*. An intense biotin signature ion at m/z 227.08487 is denoted as* S^B*. An intense biotinyl-PEG signature ion at m/z 270.12707 is denoted as* S^B_{PEG}*. The signature ions are highly selective (**B**) among fragment ions in MS/MS spectra (insert: TIC of all ions in MS1 and MS2 spectra).*



A caveat in the CuAAC step for preparing the clickable proteome for proteomic analysis. The CuAAC attachment of a clickable (desthio)biotin tag is a common step in chemical proteomics workflows. Significant variabilities were observed in the identification of probe-modified peptides when replicating reactions of CuAAC attachment of biotinyl azides were compared. One-pot CuAAC click reaction of triplex biotinyl azides, MixClick, can only solve the variability issue of MeLacA-reacted proteins modified with different tags when they are done in separate reactions, but not the variability from different click preparations. Unique identifications from replicate click reactions attributed to 77% of the total identification (**Fig 7A**, **Table S5**). When click reactions were performed in triplicate, as shown by results for samples of Analysis-and-Grouping (**Table S5**), variations in unique identifications were still high: 62% for biotin-PEG3-azide (**Fig 7B**), 30% for biotin-PEG4-azide (**Fig 7C**), and 55% for biotin-PEG5-azide (**Fig 7D**). Given the substantial variation observed in the number of probe-modified peptides identified across different CuAAC reactions, we propose the implementation of a split-and-pool practice during the click reaction step of chemical proteomics workflows to enhance both the number and reproducibility of peptide identifications. The split-and-pool practice entails dividing a sample of clickable probe-reacted proteome into multiple aliquots, subjecting each aliquot to an individual click reaction, and subsequently combining the separate aliquots for downstream sample preparations. This practice should improve quantitative chemical proteomics platforms that involve separate steps to use non-isotopic clickable probes for capturing reactive protein nucleophiles and isotopic or isobaric peptide derivatization for reactivity quantitation.[21,32,33]

Figure 7. *Variability of CuAAC attachment of biotinyl azides to MeLacA-reacted proteins. A: replicates (R1 and R2) of MixClick (3 DDA runs each); B: triplicates (R1, R2, and R3) of the biotin-PEG3-azide attachment (2 DDA runs each); C: triplicates (R1, R2, and R3) of the biotin-PEG4-azide attachment (2 DDA runs each); D: triplicates (R1, R2, and R3) of the biotin-PEG5-azide attachment (2 DDA runs each). Peptide identifications are based on two or three PEG tags; thus, unique identifications for individual replicates are confident.*



Conclusion

In conclusion, this work introduces an innovative strategy within the realm of chemical proteomics, designed to enhance the accuracy and reliability of analyzing modified peptides. Anchored in the Single Sequence Identification (SSI) principle, our approach successfully multiplies the identifiability of each modified peptide. By generating multiple distinct versions of the same peptide sequence, each uniquely characterized within the proteomic identification space, we have markedly increased the likelihood of accurate peptide identification. This advancement was realized through a one-pot triplex tagging method that attaches diverse biotinylated azides to clickable proteomes. Reliable identifications stemmed from peptides carrying two forms of tags, mirroring the conventional proteomics method of using multiple peptides for robust protein identification. Diagnostic ions from the tags and the PEG linker length's impact on elution time have further enhanced identification confidence. Additionally, our study addressed the variability issue in tagged proteins resulting from CuAAC reactions, proposing a split-and-pool technique as a viable solution. These results highlight the SSI principle's potential to significantly refine the identification of modified peptides, especially in proteomes containing chemical and post-translational modifications. An open question remains: Is it feasible to combine spectral data from differentially tagged peptides during data processing and database search to counteract the signal dilution caused by divergently multiplying single sequences of modified peptides? Moving forward, our goal is to explore alternative reagent technologies to expand the SSI principle's applications, particularly for reactivity analysis of non-cysteine nucleophiles, and to apply our one-pot triplex tagging method to specific biological systems. This line of inquiry may open new avenues in proteomics research, potentially revolutionizing our understanding of protein interactions and functions.

Supplemental Information

Text S1: Offset search parameters for identifying MeLacA-modified peptides with the desthiobiotin-PEG3-azide tag.

Text S2: Open search parameters for identifying MeLacA-modified peptides with biotin-PEGx-azide tags.

Text S3: Offset and Labile search parameters for identifying MeLacA-modified peptides with biotin-PEGx-azide tags.

Table S1: List of MeLacA-desthiobiotin-modified peptides.

Table S2. List of probe-modified peptides identified using triple tagging (PEGx sequence) for Figure 3A.

Table S3. Comparison of probe-modified peptides identified with two or three PEGx tags using Offset search with and without labile mode for Figure 3B.

Table S4. Comparison of FDR filtering at the PSM level and both PSM and peptide levels for Figure 3C.

Table S5. List of probe-modified peptides identified for each click reaction replicate of MixClick and Analysis-and-Group workflows.

Data Availability

Data are available through ProteomeXchange with the identifier PXD037770.

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