Electronic Supplementary Information (ESI) for

A novel method for Tn antigen detection based on chemoenzymatic targeted labelling and CuAAC click chemistry

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MATERIALS AND METHODS

Materials and reagents. Tn-glycopeptide VGVT (α-GalNAc) ETP and T-glycopeptide T(α-GalNAc-Gal)VPAAVVVA were purchased from Susses Research. Beta-1,3-N-Acetylglucosaminyltransferase 6 (B3GNT6) was obtained from R & D systems. UDP-N-azidoacetylgalactosamine (UDP-GlcNAz) was sourced from Minghe Lvyuan. Mucin from bovine submaxillary gland (BSM), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), sodium dodecyl sulfate (SDS) and dodecyl-β-D-maltoside (DDM) were purchased from Aladdin. Bovine serum albumin (BSA), calcium chloride, magnesium chloride, sodium chloride, potassium chloride, urea, copper sulfate, 2,5-dihydroxybenzoic acid (DHB), dimethyl sulfoxide (DMSO) and sodium bicarbonate were procured from Sigma. NHS-C_2-N_3 ester, Biotin-PEG_4-Alkyne and tris [(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (BTTAA) were purchased from Confluore. PBS powder was purchased from Solarbio. Polystyrene 96-well plate was acquired from Corning. Tween-20 and tris were obtainted from Sangon. HRP-conjugated streptavidin was purchased from Abcam. Neutravidin resin was sourced from Thermo Scientific. VVA-Biotin was obtained from Vector Laboratories. Chemical substrate working solution was sourced from Epizyme. Nitrocellulose membrane was purchased from Bio-Rad. 3M Empore™ C18 Solid Phase Extraction (SPE) Disk was purchased from Beijing Yuwei.

Cell culture and protein sample preparation. The Jurkat cells were obtained from ATCC. The Jurkat cells were grown in RPMI-1640 medium (Roswell 5 Park Memorial Institute 1640) supplemented with 10% bovine serum and 100 U/mL of streptomycin and penicillin. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37 ℃. Then the cells were collected by centrifuge and gently homogenized in ice-cold lysis buffers of RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS), supplemented with 1% protease inhibitor cocktail. The generated protein lysate was ready for Tn detection with VVA-Biotin using Western-Blotting.

Preparation of azide labeled BSA. BSA was dissolved in PBS (pH 7.5) to 5 μg/μL, and NHS-C_2-N_3 ester was dissolved in DMSO to 20 mM. A total of 20 μg BSA was reacted with 3 mM NHS-C_2-N_3 ester in PBS (pH7.5). The mixture was incubated at room temperature for 30 min, followed by addition of 200 mM Tris-HCl (pH 7.4) to quench the reaction for an additional 10 min at room temperature.

Preparation of biotin labeled BSM & BSA. A click reaction mixture containing 2 mM $CuSO_4$, 4 mM BTTAA, 4 mM TCEP, and 1 mM Biotin-PEG_4-Alkyne. was pre-mixed and added to the concentrated protein solution. The reaction was performed at room temperature on an oscillator (1500 rpm) in the dark for 3 h^1.

Enzymatic labelling of Tn glycopeptide. 1 μg Tn glycopeptide was tagged with 8 mM UDP-

GlcNAz in 25 mM Tris-HCl, 150 mM NaCl, 5 mM Mn^{2+},10 mM Ca^{2+}, pH 7.5, by using 0.8 μg B3GNT6. The mixture was incubated at 37°C for 17 hours. To investigate the effects of different protein detergents on the glycan transfer reaction, three types of commonly used detergent, including urea (1 M), sodium dodecyl sulfate (SDS, 0.25%), and n-dodecyl-β-D-maltoside (DDM, 0.25%, 0.8%) were added individually to the reaction system, and the products were desalted by Sep-Pac C18 solid phase extraction column (Waters) before analyzed by MALDI-TOF or LC-MS/MS. To investigate the specificity of the enzymatic reaction, glycopeptide with T antigen, T(α-GalNAc-Gal)VPAAVVVA, was also subjected to UDP-GlcNAz labelling under the same condition. The product was desalted and analyzed by MALDI-TOF.

Enzymatic labelling of Tn glycoproteins. 20 μg Tn glycoprotein was tagged with 8 mM UDP-GlcNAz, 5 mM Mn^{2+},10 mM Ca^{2+}, 0.8% DDM, with 3.2 μg B3GNT6 in 25 mM Tris-HCl, 150 mM NaCl (pH 7.5). The mixture was incubated at 37°C for 17 hours.

For the enzyme catalyzed labelling of Tn glycoprotein from Jurkat cells, the cell pellets were washed with cold PBS for three times, and lysed in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1% cocktail and 1.5 mM $MgCl_2$. Then the cell lysate was homogenized using a Dounce homogenizer for 50 times and centrifuged at 1000 g for 10 min at 4 ℃. The supernatant were further centrifuged at 20000 g for 15 min. The derived pellets (corresponding to the membrane proportion of Jurkat cells) were dissolved in 25 mM Tris-HCl, 150 mM NaCl (pH 7.5), 0.8% DDM, and the protein concentration were determined by BCA assay. A certain amount of proteins were subjected to B3GNT6 mediated Tn labelling by adding 8 mM UDP-GlcNAz, 5 mM Mn^{2+},10 mM Ca^{2+}, and 3.2 μg B3GNT6 and incubated at 37°C for 17 hours. Subsequently, the derived proteins were buffer exchanged using a 10 kDa ultrafiltration tube, and incubated with a click reaction mixture containing 500 μM $CuSO_4$, 1 mM BTTAA, 1 mM TCEP and 500 μM Biotin-PEG_4-Alkyne in PBS for 3 h at 25°C. After that, the proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane for western blotting detection.

Western blotting analysis. Different amount of Jurkat proteins, Jurkat membrane proteins, and biotin-labeled Jurkat proteins derived by B3GNT6-mediated glycan transfer experiment were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and blocked with 5% BSA for 2 hours at room temperature. Then the membrane was incubated with VVA-biotin (2 μg/ml, 1:1000) at 4 ℃ overnight. The membrane was washed three times with TBS and incubated with HRP-conjugated streptavidin (1:10000) in 5% BSA for 2 hours at room temperature. After washed by TBS for three times, the membrane was reacted with ECL reagents for qualitative and quantitative analysis of target proteins.

ELISA analysis of azide labeled BSA. Azide-labeled BSA was immobilized onto a polystyrene 96-well microplate (Corning 96 well white flat bottom polystyrene high bind microplate) at the designated amount in 100 μL of carbonate buffer, pH 9.6, at 37 °C for 2 h. After the coating was completed, the wells were washed three times with 200 μL PBST (PBS, 0.05% Tween-20, pH 7.5) and blocked with 200 μL of blocking buffer of 5% skimmed milk in PBST for 1 h at 37 °C. Subsequently, each well was incubated with a click reaction mixture containing 500 μM $CuSO_4$, 1 mM BTTAA, 1 mM TCEP and 500 μM Biotin-PEG_4-Alkyne in PBS for 3 h at 25℃. After the click reaction was completed, each well was washed three times with 200 μL PBST, then 100 μL HRP-conjugated streptavidin was added, and incubated at 37 °C for 1 h. After further washing, 100 μL of chemiluminescence substrate working solution was added, and luminescence intensity was detected using a microplate reader[2].

Dot-Blot analysis of biotin labeled BSM & BSA. Dot-blot analysis was performed by spotting 5 μL of serial dilutions of biotin-labeled BSM onto nitrocellulose membranes (0.45 μm, Bio-Rad), followed by drying for 1–2 h at room temperature. The membrane was air-dried at room temperature prior to dot blot analysis. Membranes were sequentially washed three times each with 0.5 M KCl, 30% methanol, 5 M urea in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5), and TBST (TBS containing 0.1% Tween-20, pH 8.0). Membranes were blocked with 5% skimmed milk in TBST for 2 h at room temperature and incubated overnight at 4 °C with HRP-conjugated streptavidin diluted 1:10,000 in TBST. Detection was performed using a chemiluminescence substrate (WesternBright Peroxide, Advansta) and imaged with a Fusion FX5-XT Bioimaging system[2].

MALDI-TOF mass spectrometry analysis. After the enzymatic reaction, 1 μL of the reaction mixture was spotted onto a MALDI sample target and allowed to air-dry at room temperature. Subsequently, 1 μL of α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution was added. After solvent evaporation and crystal formation, mass spectrometry analysis was performed using a 5800 MALDI-TOF mass spectrometer (AB SCIEX) equipped with a 355 nm pulsed laser. Data were acquired in positive ion mode. The laser energy was adjusted between 6000 and 7000 to optimize resolution and signal-to-noise ratio. The scanning range for m/z was set from 500 to 2000.

Tryptic digestion of biotin labeled BSA & enrichment of biotinylated peptide. After the click reaction, TCEP was added to the BSA solution to a final concentration of 20 mM and incubated at 37 °C for 2 h. Subsequently, IAA was added to a final concentration of 40 mM and reacted at room temperature for 40 min in the dark. Samples were transferred to ultrafiltration tubes, washed once with 8 M urea in 0.1 M NH_4HCO_3, and twice with 0.1 M NH_4HCO_3. Add trypsin at an enzyme-to-protein ratio of 1:25 and incubate at 37°C for 16 hours. The resulting

peptides were lyophilized and stored at –20 °C for further use[3, 4].

Tryptic peptide was enriched with 100 μL of Neutravidin agarose resin (Thermo Scientific) for 4 h at RT, end-over-end rotation. Beads were washed three times with 5 M urea buffer (50 mM Tris, pH 7.4), 0.5 M KCl, and H_2O. Captured peptides were eluted with 8 M guanidine hydrochloride (pH 1.5) and further washed with 2% FA and 50% ACN. Eluates were combined, desalted using 3M Empore disk C18 material, dried, and stored at –20 °C.

Peptide desalting. Place a 3M Empore™ C18 solid phase extraction (SPE) disk into a pipette tip and activate it twice with 100 μL of methanol., equilibrated twice with 100 μL 50% ACN/0.1% TFA and 100 μL 0.1% TFA. The enriched peptides were acidified to pH 2–3 with 20% TFA, loaded onto desalting material, and allowed to flow through slowly. Subsequently, the material was washed three times with 100 μL 0.1% TFA. Finally, the peptides were eluted twice with 80 μL 50% ACN/0.1% TFA, and the eluates were lyophilized and stored at –20 °C[5].

LC-MS/MS analysis. To investigate the efficiency of the enzymatic labelling of the standard Tn glycopeptides, the labeled peptides were analyzed on a Q Exactive TM Plus mass spectrometer (Thermo Scientific, USA) coupled with a Dionex UltiMate 3000 RS LC nano system (Thermo Scientific, USA). The Peptides were dissolved in 0.1% FA, loaded onto a 3 cm trap column packed with C18-AQ beads (1.9 μm, 120 Å). The LC-MS/MS analysis used 0.1% FA in H_2O as mobile phase A and 0.1% FA in ACN as mobile phase B. The used gradient was described as follows: from 4% to 9% B for 1 min, from 9% to 30% B for 21 min, from 30% to 42% B for 4 min, from 42% to 90% B for 1 min, and held on 90% B for 3 min. For the analysis of biotinylated peptides from BSA, the gradient used were: from 12% to 35% B for 40 min, from 35% to 45% B for 4 min, from 45% to 95% B for 1 min, and held on 95% B for 5 min.

The Q Exactive mass spectrometer was operated in data-dependent mode. Full MS scans (m/z 350–1750) were acquired at a resolution of 60,000 with an AGC target of 3e6 and maximum injection time of 20 ms. The 20 most intense multiply charged ions (charge states ≥2) were fragmented by higher-energy collisional dissociation (HCD) at 27% normalized collision energy (NCE). MS/MS scans were also acquired by the Orbitrap mass analyzer with a 15,000 resolution, and the AGC target was set to 5e4 with a maximum injection time of 32 ms, scanning with a fixed first mass of m/z 120[4].

Database search and statistical analysis. The raw data were processed with pFind6 (version 3.1.5) using the following parameters: BSA was used as the protein database; the mass tolerances for precursor and fragment ions were set at 20 ppm. Enzyme specificity was set to trypsin with up to 3 missed cleavages allowed. Carbamidomethylation (Cys, +57.0215 Da), oxidation (Met, +15.9949 Da), and Biotin-PEG4-Alkyne modification (Cys, +457.2243 Da)

were set as variable modifications for all sample analyses. The false discovery rate (FDR) was controlled below 1%. Subsequently, identified peptides were quantified using Panda software, and statistical analysis was performed using OriginPro software.

Supplementary Table 1. Summary of Tn antigen detection methods

Detection Method	Detection Principle	References
Lectin affinity chromatography	uses lectins (VVA, PNA) that specifically recognize the Tn antigen to enrich Tn-containing peptides	7-10
Lectin-based electrochemical sensor for Tn detection	This method quantifies the Tn antigen by measuring changes in electrical signals generated by lectin-specific recognition events.	11, 12
Antibody-based method	This method uses two different antibodies that specifically recognize the Tn antigen, one as a capture antibody and the other as a detection antibody, to achieve quantitative analysis of the Tn antigen.	13, 14
Chemoenzymatic labelling-based method	This method utilizes enzymes such as galactose oxidase or glycosyltransferases that specifically recognize the Tn antigen for targeted labelling. The labeled species are subsequently captured by microspheres and analyzed by LC–MS/MS.	15,16, 17
COSMC knockdown mediated SimpleCell strategy followed by lectin-based or antibody-based affinity chromatography	by knocking out key O-glycosylation genes (such as C1GALT1 or COSMC) to generate cells that uniformly express the Tn antigen, enabling highly sensitive and systematic detection of Tn using specific lectins or antibodies at the proteome level.	18-20
O-glycoprotease (IMPa) based cleavage strategy	This method uses the O-glycoprotease IMPa to cleave at the C-terminal side of Tn-glycopeptides, thereby improving Tn detection.	21-23
Using T-synthase for the targeted labelling of Tn-peptide	This method utilizes T-synthase to modify Tn with isotopically labeled UDP-Gal($^{13}C_6$), thereby tagging and converting Tn to Gal($^{13}C_6$)-Tn. The modified Tn peptides are then released by OpeRATOR for detection.	24
One-step enzymatic method for Tn detection using T-synthase	This method utilizes T-synthase, which specifically recognizes the Tn antigen, to label the Tn-glycoepeptide with cleavable biotin tags, followed by streptavidin-based enrichment, for the highly efficient Tn detection.	25

Supporting Figures

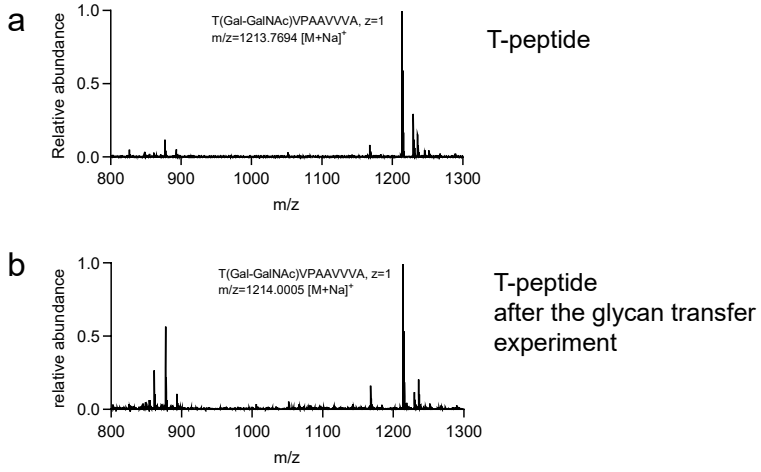


Figure S1. Specificity analysis of the B3GNT6-mediated glycan transfer reaction using a standard T-antigen–modified glycopeptide. A standard T-antigen–modified glycopeptide, T(GalNAc–Gal)VPAAVVVA, was used to examine the specificity of the B3GNT6-mediated glycan transfer reaction. The T glycopeptide before (a) and after (b) the reaction was analyzed by MALDI-TOF MS. The results indicate that the T glycopeptide cannot be labeled with GalNAz under the B3GNT6-mediated glycan transfer conditions.

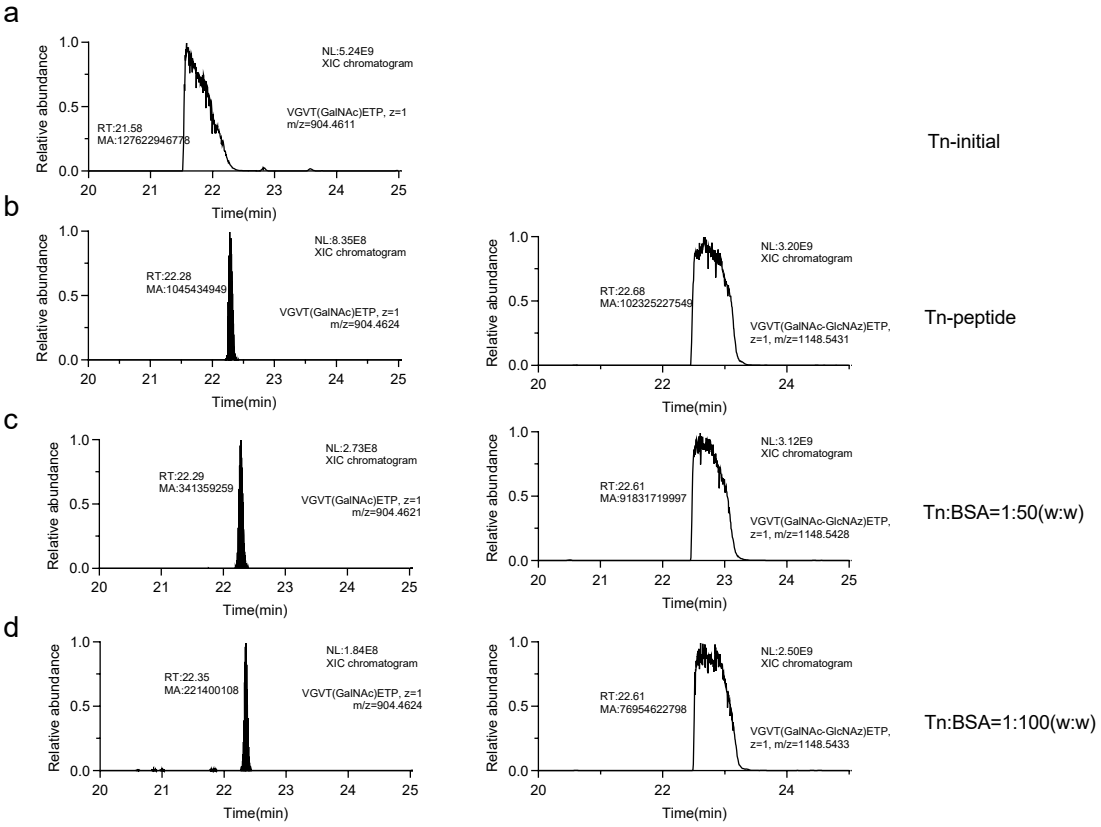


Figure S2. Evaluation of B3GNT6 enzymatic reaction efficiency and the effect of BSA on the reaction. (a) Extracted ion chromatograms (XICs) and corresponding peak areas of the Tn-glycopeptide precursor (VGVT(α-GalNAc)ETP) before the labelling reaction. (b) XICs of the precursor ions from the Tn-glycopeptide (left) and the GalNAz-labeled product peptide (right) after the B3GNT6-mediated labelling reaction without BSA. (c, d) Effects of BSA supplementation on the enzymatic reaction efficiency at Tn-glycopeptide–to–BSA ratios of 1:50 (w/w) and 1:100 (w/w), respectively.

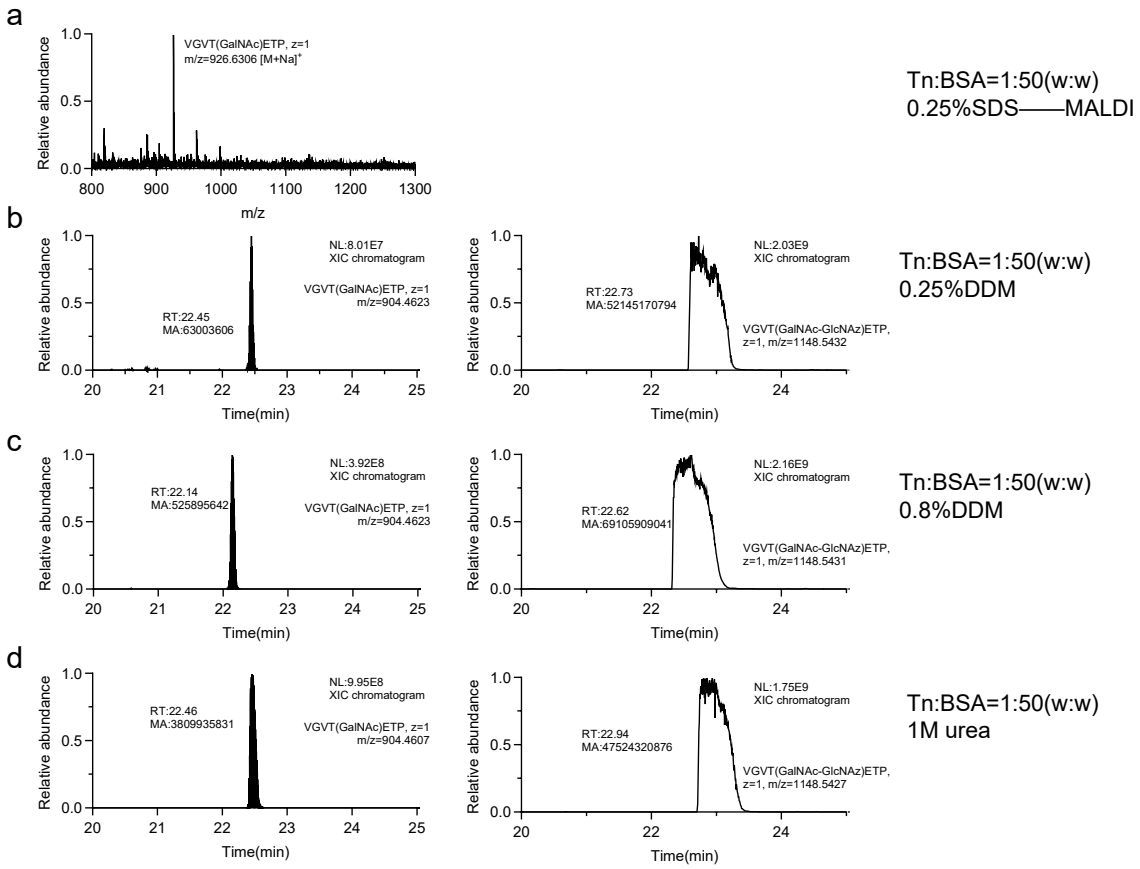


Figure S3. Effects of different protein detergents on the enzymatic reaction efficiency of B3GNT6. A standard Tn glycopeptide was mixed with BSA at a ratio of 1:50 (w/w) and subjected to the B3GNT6-mediated glycan transfer reaction in the presence of different detergents. Reaction products were analyzed by MALDI-TOF MS or nano-HPLC–MS/MS. (a) Enzymatic reaction efficiency of B3GNT6 in the presence of 0.25% SDS. The product was analyzed by MALDI-TOF MS because SDS is incompatible with nano-HPLC–MS/MS. The results indicated that SDS completely inhibited the reaction, with no detectable product. (b) Reaction performed with 0.25% DDM; (c) Reaction performed with 0.8% DDM; (d) Reaction performed with 1 M urea.

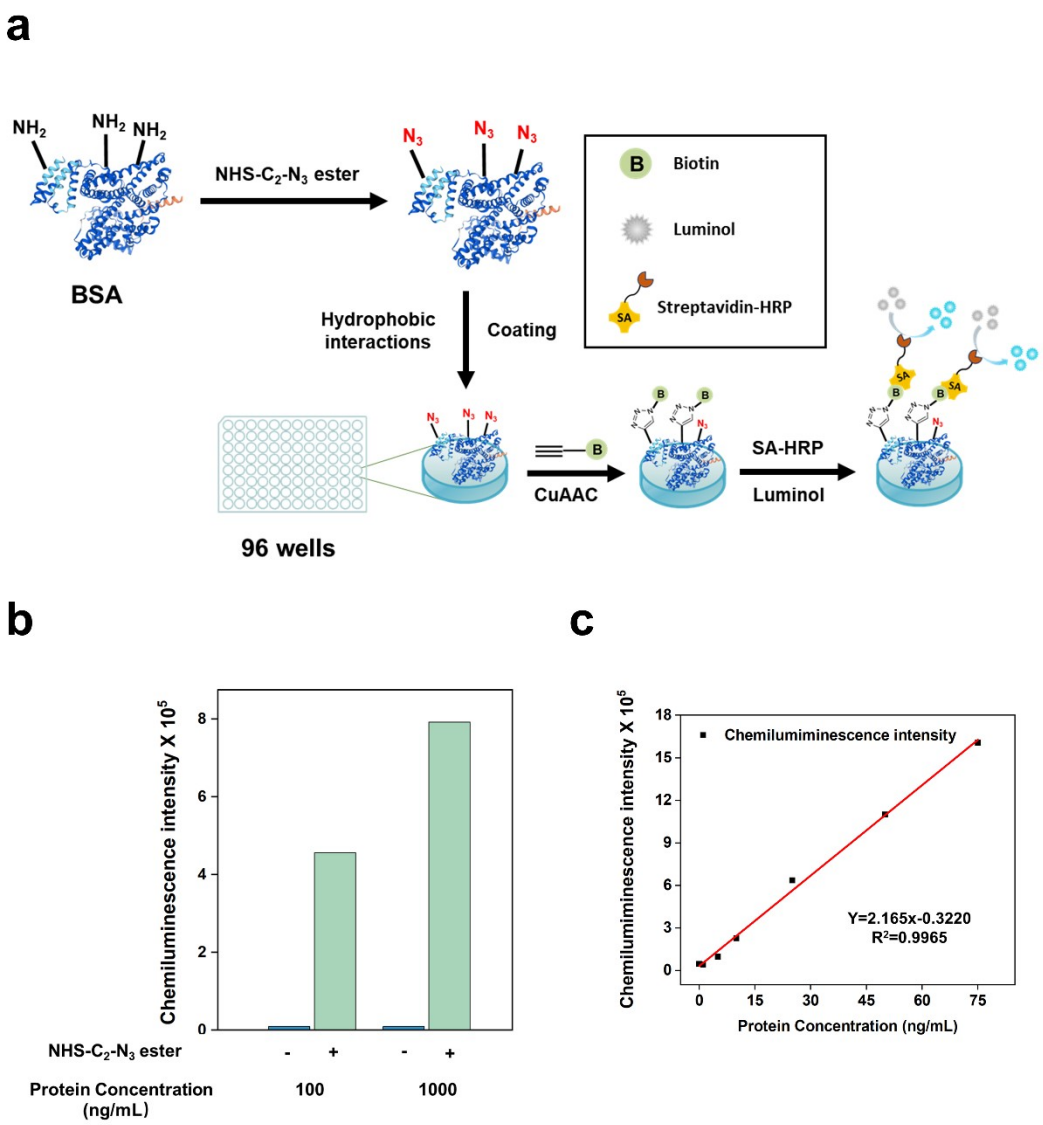
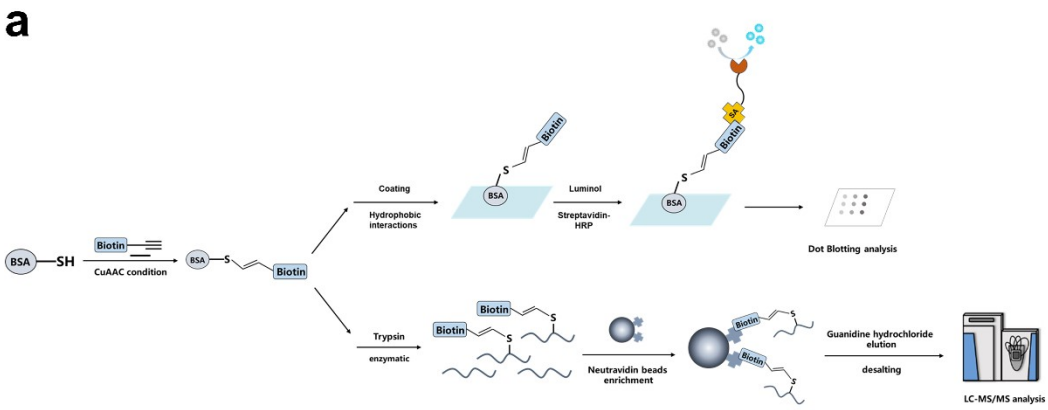
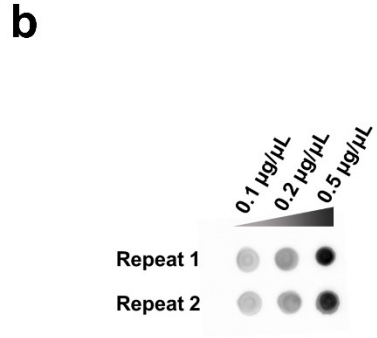


Figure S4. Performance evaluation of the CuAAC-mediated chemiluminescence detection strategy using azide-labeled BSA. (a) Experimental workflow; (b) Comparison of chemiluminescent signals under different conditions; (c) Standard curve of chemiluminescent detection for protein quantification.


a
BSA
SH
Biotin
CuAAC condition
Coating
Hydrophobic interactions
Luminol
Streptavidin-HRP
Dot Blotting analysis
Trypsin
enzymatic
Neutravidin beads enrichment
Guanidine hydrochloride elution
desalting
LC-MS/MS analysis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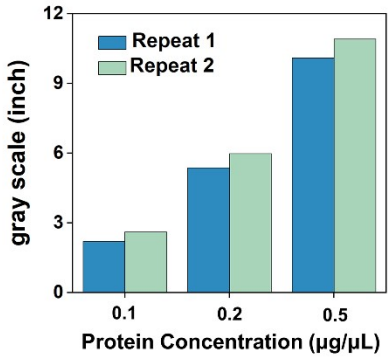
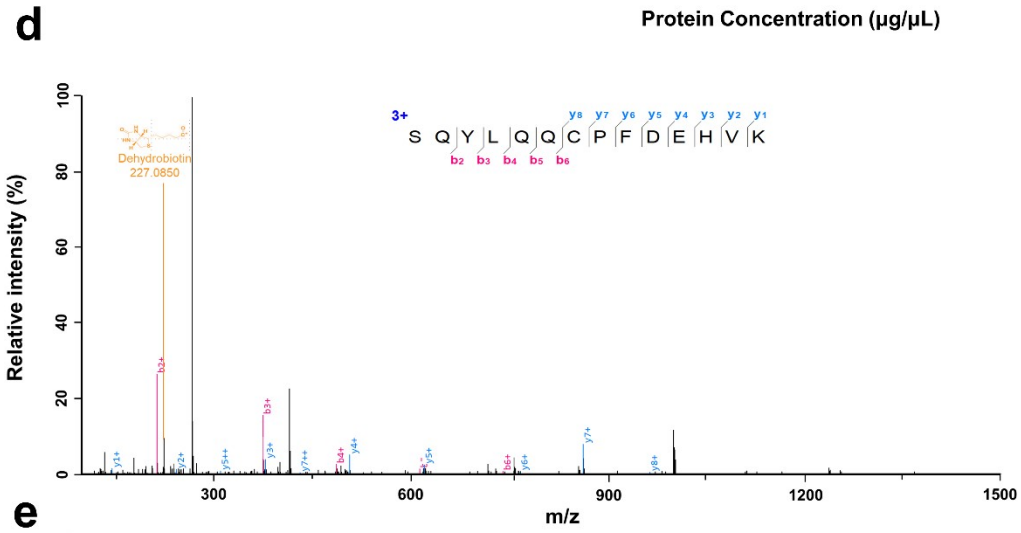
0.1 μg/μL
0.2 μg/μL
0.5 μg/μL
Repeat 1
Repeat 2


c
Repeat 1
Repeat 2
gray scale (inch)
Protein Concentration (μg/μL)


d
Relative intensity (%)
m/z
Dehydrobiotin
227.0850
3+
S Q Y L Q Q C P F D E H V K

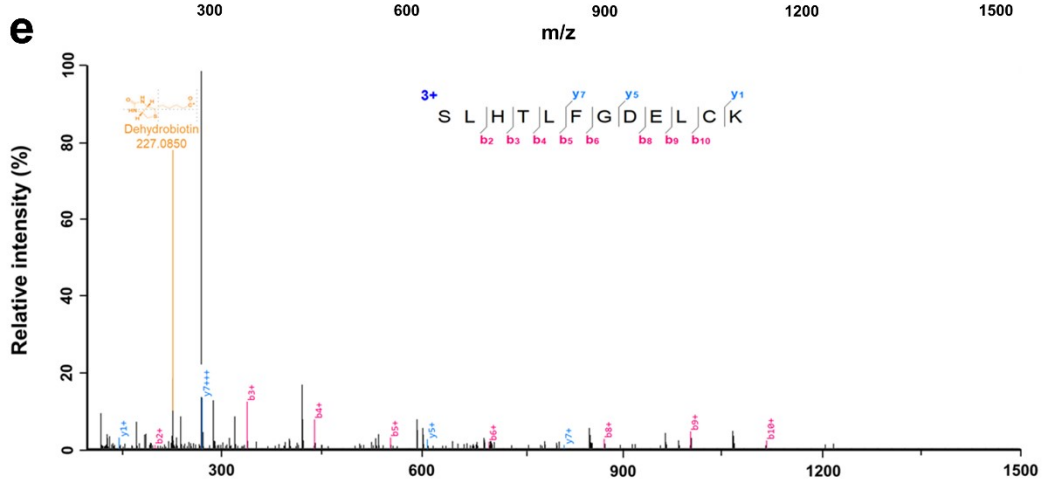

e
Relative intensity (%)
Dehydrobiotin
227.0850
3+
S L H T L F G D E L C K

Figure S5. Dot blotting analysis of CuAAC byproduct thiolated alkyne and mass spectrometric analysis of the modified peptide segment. (a). Experimental workflow; (b) Dot blotting analysis of the thiolated alkyne byproduct; (c) Quantitative grayscale analysis of the dot signals shown in panel a; (d)Mass spectrum of the peptide SQYLQQCPFDEHVK, which contains a free cysteine residue. The monoisotopic mass of the characteristic biotin fragment ion is 227.0854; (e)Mass spectrum of the peptide SLHTLFGDELCK, which contains a cysteine forming disulfide bonds. The monoisotopic mass of the characteristic biotin fragment ion is 227.0854.

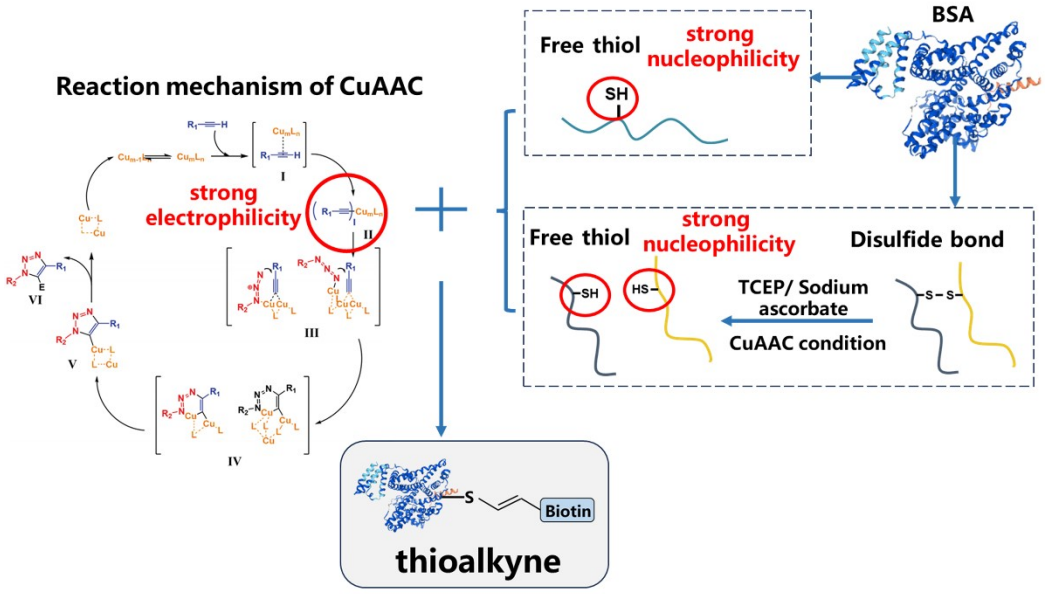


Figure S6. Side-reaction between sulfhydryl and alkynyl. The natural free thiol groups in proteins or those generated by the reduction of disulfide bond during CuAAC reaction with the addition of Tris(2-carboxyethyl)phosphine or other reductant, can react directly with alkynes via nucleophilic addition. In this study, the side reaction resulted in the formation of biotinylated BSA and compromise the specificity of Tn detection.

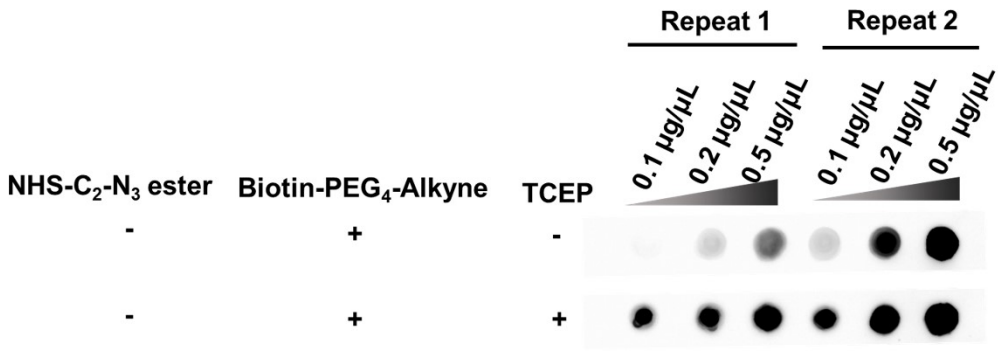


Figure S7. Investigation of the side reaction between sulfhydryl and alkynyl by adding a larger amount of TCEP (20 mM) during the CuAAC reaction. BSA protein without azide-labelling was used as sample. The results indicated that increasing the concentration of TCEP to 20 mM, compared to the commonly used 2 mM, led to the formation of more byproducts. This may be attributed to the generation of free thiols resulting from the reduction of disulfide bonds in BSA by TCEP.

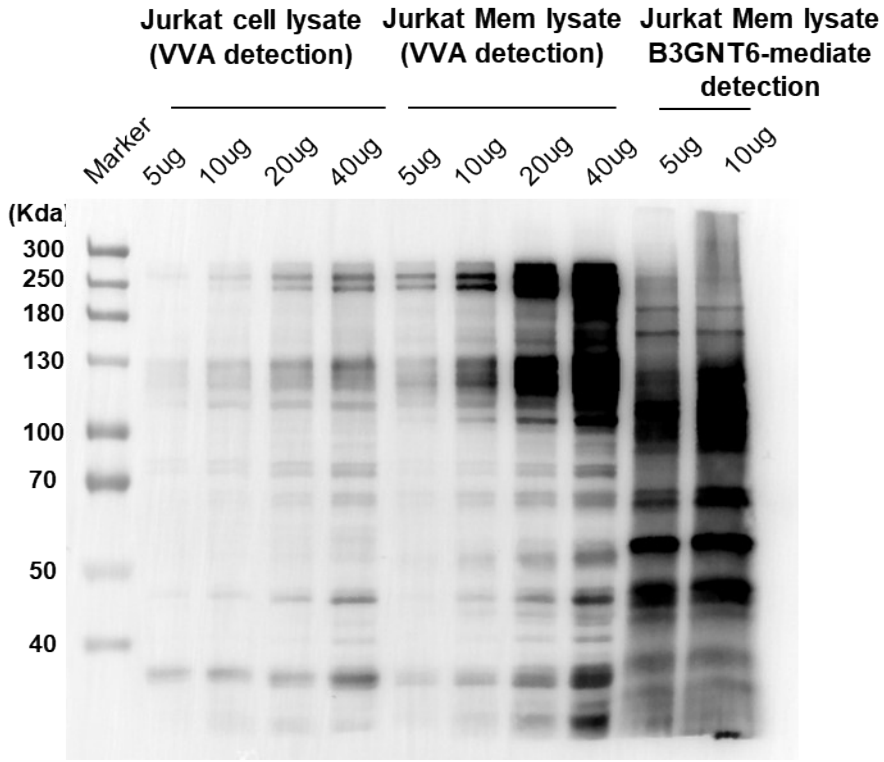


Figure S8. Comparison of the B3GNT6-mediated method with the traditional VVA method for detecting Tn in Jurkat cells by Western blotting. Different amounts of proteins from Jurkat whole-cell lysates were detected using VVA–biotin by Western blotting. To ensure compatibility with the B3GNT6-mediated labelling reaction, Jurkat membrane pellets obtained by hypotonic lysis and Dounce homogenization were dissolved in 25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.8% DDM. After the labelling and click-chemistry reactions, the resulting proteins were analyzed by Western blotting. For comparison, different amounts of proteins from Jurkat membranes were also analyzed using the VVA–biotin strategy.

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