Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

# Tracking Pathogen Infections by Time-Resolved Chemical Proteomics

*Ying Zhang,* Der-Shyang Kao, Bing Gu, Rajdeep Bomjan, Mayank Srivastava, Haojie Lu, Daoguo Zhou, and W. Andy Tao**

anie_201911078_sm_miscellaneous_information.pdf

# Supporting Information

## 1. Materials

Hydroxybenzotriazole (HOBt), N-methylmorpholine (NMM), dichloromethane (DCM), Triisopropylsilane (TIS), Trifluoroacetic acid (TFA), N, N-diisopropylcarbodiimide (DIC), Tergitol (NP-40), piperidine, 2,2'-dithiodipyridine, Tris(2- carboxyethyl)phosphine hydrochloride (TCEP), 2-Chloroacetamide (CAA), Aniline, Ammonium acetate ($NH_4OAc$)Sodium periodate ($NaIO_4$) were purchased from Sigma- Aldrich (St. Louis, MO). NeutrAvidin Agarose beads and Sulfo-SBED (Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3′-dithiopropionate) are purchased from Thermo Fisher scientific Inc. (Rockford, IL, USA). Fmoc-Lys(Mtt)-Wang resin were from Kangbei Bio company (Ningbo, China). All antibodies including Anti-Cd11b antibody [EPR1344], Anti-Cd14 antibody [4B4F12] and Anti-Cd147 antibody [MEM-M6/1] were purchased from Abcam (USA). Materials were used directly without purification

## 2. Methods

2.1 Synthesis and characterization of the chemical proteomics probes

The multifuctional chemical proteomics probes were synthesized via standard solid phase peptide synthesis approach with the help from Kangbei Bio company (Ningbo, China). The synthetic route of $ONH_2$-functionalized probe is shown in Figure S5.

Briefly, 500 mg Fmoc-Lys(Mtt)-Wang resin was used as the starting material. 100 mL DCM (dichloromethane) was added to the resin and incubated for 3 hours, then DCM was removed by vacuum suction filtration. The resin was then treated with 100 mL 1% TFA and 2%TIS/DCM with the aid of $N_2$ for 30 min to deprotect the Mtt group. Then, the resin was washed with DMF for six times to remove the deprotection solution. After that, 183mg D-Biotin and appropriate DMF were added to the resin, agitate gently with $N_2$ for 10mins, then 101 mg HOBT and 118 μL DIC were added to the reactor in

order. Kaiser test was performed to check the coupling efficiency. After that, 100 mL 20% piperidine in DMF was added to the resin for deprotection of Fmoc (9-Fluorenylmethoxycarbonyl) and the resin was then washed with DMF for six times. 150 mg succinic anhydride and 330 µL NMM was added to the resin for 30 min to stop the reaction, and the resin was washed with DMF for six times again. The resin was activated by adding of 101 mg HOBT and 118 µL DICfor 10 mins and reacted with 332 mg Fmoc-NH-$PEG_3$-$NH_2$ for 2 hours to introduce the Fmoc protected amine group on the resin to obtain Cmpd 1 for the following coupling.

One hundred mL 20% piperidine solution in DMF was used to deprotect the Fmoc group from Cmpd 1. The excess reagents were removed by thorough washing of beads by DMF for six times. The resin was activated by adding of 101 mg HOBT and 118 µL DIC for 10 mins and reacted with ~450 mg Fmoc-Lys(Mtt)-OH for 2 hours to introduce the Fmoc protected amine group on the resin. The resin was then deprotected again with 100 mL 20% piperidine solution in DMF and then 150 mg succinic anhydride in NMM (N-methylmorpholine) was added to the resin and incubated for 30 min to stop the reaction. After washed with DMF for six times, the resin was activated by adding of 135.1 mg HOBT and 156 µL DIC for 10 mins and reacted with ~450 mg Fmoc-NH-$PEG_3$-$NH_2$ for 2 hours. The solution was removed by washing the beads with DMF. 100 mL 20% piperidine solution in DMF was used to deprotect the Fmoc group again and 120 mg (Boc-aminooxy)-acetic acid (cmpd 2) was added to the resin and reacted for 2 hours at room temperature to introduce the Boc (*tert*-Butoxycarbonyl)-protected aminoxy group. The resin was then treated with 1% TFA and 2% TIS in 20 mL DCM to remove the Mtt group and 120 mg of 6-Azidohex anoic acid (cmpd 3) was added and reacted for 2 hours at room temperature. The resin was washed with DMF and DCM sequentially, and the molecule was cleaved from the resin using 95% TFA for 1.5 hours. The cleavage step also deprotects the boc group, making a free aminoxy available in the product. The crude product was concentrated and purified using HPLC (Agilent 1100) using a gradient of 5-85% B (A: 0.1% TFA/$H_2O$, B: 0.1% TFA/$CH_3OH$) for 30 minutes on Waters XBridge Prep BEH130 C18 column 5 µm, 10 X 250 mm.

In order to add the diazirine functionality, the pure aminoxy-biotin product was

dissolved in DMF and reacted with 292 mg 2-(diphenylphosphanyl)phenyl 3-(3-methyl-3H-diazirin-3-yl)propanoate through staudinger ligation for 2 hours at room temperature. The product was purified by directly loading the mixture on the column and using similar HPLC conditions. The final product was characterized by LC-ESI-MS and $^{1}$H NMR.

2.2 Bacterial Strains and Culture Conditions

*Salmonella* strain used in this study was SL1344 (virulent wild-type strain of *Salmonella* Typhimurium). The frozen bacteria were routinely grown on LB plates with 1.5% agar and 150 μg/mL streptomycin at 37 °C. A single colony picked from the plate was inoculated into 3 mL of LB medium with 150 μg/mL streptomycin; then, the overnight culture was diluted 1:20 into 3 mL of LB broth. The bacteria were harvested for labeling and infection assays when they grew to the mid-exponential phase (OD600 = 0.9). Bacterial growth was monitored by measuring optical density at 600 nm.

2.3 Labeling of Bacteria by Probe

The bacteria was collected and washed 3 times with cold PBS and re-suspended in 25 mM $NH_4OAc$ buffer (pH 5.5). $NaIO_4$ dissolved in $NH_4OAc$ buffer was added to the bacteria at the final concentration of 100 μM $NaIO_4$ and incubated in dark at 4 °C for 30 min. Then the excess $NaIO_4$ was washed away by washing the bacteria 3 times with cold PBS and the bacteria were re-suspended in 25 mM $NH_4OAc$ buffer (pH 5.5). Then the $ONH_2$-functionalized probe was added to the bacteria at the final concentration of 1 mM and aniline was added at the final concentration of 10 mM. The mixture was incubated at 4 °C for 1 hour. Then the excess probe was washed away by washing the bacteria 3 times with cold PBS.

2.4 Infection and Intracellular Survival Assay

To check whether the labeling affected the infection rate and intracellular survival of *Salmonella*, labeled and unlabeled samples were subjected to gentamicin protection assay as outlined below.

Briefly, the bacterial cultures grown to an early stationary phase (overnight culture) were diluted to an OD600 of 0.1. The bacteria were then added to the macrophage cells at a multiplicity of infection (MOI) of 50 for 30 minutes. This was followed by washing the cells twice with PBS and incubating further for an hour in DMEM with 10% FBS and 100 μg/mL of gentamicin to kill extracellular bacteria. After one hour, cells were washed again three times with PBS and incubated for the required amount of time in 16 μg/mL of gentamicin containing DMEM. At 1 hour and 6 hours post infection, the infected macrophages were washed three times in PBS and lysed with 0.1% sodium deoxycholate. Samples were then serially diluted and plated onto LB plates for enumeration.

2.5 Macrophage Cell Infection

RAW 264.7 macrophage cells were grown in Dulbecco's modified Eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, US) under an atmosphere of 5% $CO_2$ at 37 °C. *Salmonella* infection of macrophage cells was performed when cell monolayers reached ~80% confluence. Infection was carried out for 15 min in Hanks' balanced salt solution (HBSS) with a MOI of 50. For 15 min infection, cells were washed and harvested immediately after UV treatment. For 1 hour and 6 hour samples, cell monolayers were washed with prewarmed HBSS (37 °C) after infection for 15 min and incubated for a further 30 min in prewarmed DMEM supplemented with 100 μg/mL gentamicin to kill extracellular bacteria. Subsequently, cell monolayers were washed again with prewarmed HBSS, and fresh DMEM supplemented with 10 μg/mL gentamicin was added. At the required time points, cells were washed extensively with PBS. The cells were then placed on the ice, and irradiated with UV for 10 min or without UV as control. The reason we chose the three time points (15 min, 1 hour and 6 hours) in this study is they are the key time points of SCV formation. *Salmonella* is a gram-negative facultative intracellular pathogen that can infect a broad range of mammalian hosts. Following invasion of host cells, the majority of *Salmonella* are known to reside in a membrane-bound compartment known as SCV. *Salmonella* actively remodels this compartment to

establish a protected niche where it can replicate. Bacterial replication is initiated approximately 6 hours post invasion (p.i.). SCV biogenesis can be separated into three stages; early (<30 min p.i.), intermediate (30 min to approx 5 hours p.i.) and the late (>5 hours p.i.). Therefore, we chose 15 min, 1 hour and 6 hour to investigate in this study. [The Salmonella-containing vacuole—Moving with the times Current Opinion in Microbiology 2008, 11, 38–45]. The cells were then collected and used for the following LC-MS/MS analysis.

2.6 Inside/Outside Assay

The ability of bacteria to infect host cells was evaluated with the inside/outside differential staining assay. Briefly, macrophage cells were infected with *Salmonella* at MOI of 50 for different time in a 24-well plate and incubated for 30 min at 37°C in 5% $CO_2$ for infection. The bacteria which remain outside the cells were identified using rabbit anti-*Salmonella* O-antigen and visualized with anti-rabbit Alexa Fluor 488. This was followed by permeabilization of cells with 0.2% Triton X-100 and staining all bacteria, including the ones inside, with Texas Red (Molecular Probes). Host cell nuclei and bacterial DNA were stained with 4, 6-diamidino-2-phenylindole (DAPI; Molecular Probes). The outside bacteria would be differentiated as they appear green or yellow and the ones inside as red when visualized under a double filter for both Alexa Fluor 488 and Texas Red.

2.7 Isolation of crosslinked proteins

After infection, cell pellets were collected and washed 3 times with cold PBS and lysed in 4% SDS with sonication. The sonication was kept on for 10 s and off for 20 s, and the cycle was repeated for 9 times. Then the protein mixture was separated from the cell debris by centrifuging the solution at 14,000 g for 10 min. BCA quantitation was used to measure the protein concentration in the supernatant. Before isolation, lysates for each sample were reduced and alkylated by boiling at 95 °C in 10 mM TCEP and 40 mM CAA respectively. The lysate was then diluted with 10 mM PBS (pH7.4) to ensure the concentration SDS at 0.1 %. Then Neutravidin beads (25 µL slurry) were

washed added to each of the samples. The mixtures were rotated overnight at 4 °C and the supernatant was removed afterwards. Then the beads were washed with 500 μL solutions of 1% SDS/PBS, 8 M urea, 10×PBS and 25 mM ABC sequentially. The beads were washed with each solution for 3 times.

2.8 SDS-PAGE, western blotting and pull-down assay.

After infection, cells were collected and washed 3 times with cold PBS and resuspended in 4% SDS to lyse cells with sonication. The sonication was kept on with a 20s interval for 9 cycles. The lysate was centrifuged for 10 min at 14,000 g at 4°C and the pallet was collected for the following SDS-PAGE separation, silver staining or western blot.

For SDS-PAGE analysis, the pellets for the SDS-PAGE analyses were resuspended in the SDS-PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 1.7% (w/v) SDS, 6% (v/v) glycerol, 100 mM dithiothreitol (DTT), and 0.002% (w/v) bromophenol blue, heated at 95 °C. Bacterial samples were prefractionated by 10% SDS-PAG and visualized by silver stain or used for western blot detected by antibody against biotin.

Verification of the interaction between *Salmonella* and macrophage was carried out by pull-down assay and western blotting. In brief, the *Salmonella* were labeled by sulfo-SBED in PBS buffer at pH 7.0-8.0 for 1 h at 4°C and lysed. Then the lysate of *Salmonella* was incubated with macrophage lysate at 4°C overnight. After incubation, UV (365 nm) irradiation was performed for 10 min on the lysate mixture to induce crosslink among interacting proteins. Then NeutrAvidin beads (25 μL slurry for each sample) was added and incubated at 4°C with rotation overnight. The beads were sequentially washed with 500 μL of 1% SDS/PBS, 8 M urea, 10×PBS, and 25 mM ABC, then 15 μL of 2× SDS-PAGE loading buffer was added and boiled to elute the bound proteins. The eluates were separated by 12% SDS-PAGE and transferred on PVDF membrane. The membrane was blocked with 2% non-fat milk/TBST and probed with the indicated antibodies.

2.9 LC-MS analysis

The beads were suspended in 200 µL ABC buffer and proteins were digested on-bead at 37 °C using Lys-C for 3 hours and trypsin for 12 hours. The supernatant containing peptides was collected and desalted using in-house StageTips with SDB-XC (3M). The peptides were dried in SpeedVac before subjecting to LC-MS/MS analysis. The final peptide samples were reconstituted in HPLC-grade water for LC-MS/MS analyses, which were performed on a hybrid LTQ-Orbitrap Velos mass spectrometer equipped with nanoflow reversed phase liquid chromatography (EASY-nLC 1000, Thermo Scientific). The peptides were separated on a 45 cm in-house column (360 µm OD X 75 µm ID), packed with C18 resin (2.2 µm, 100Å, Bischoff Chromatography, Leonberg, Germany) and heated to 60 °C with a column heater (Analytical Sales and Services, Flanders, New Jersey). The mobile phase was comprised of 0.1% formic acid in ultra-pure water (solvent A) and 0.1% formic acid in 80% Acetonitrile (solvent B), and a 60 minutes gradient at a flow rate of 250 nL/min was performed using 5-100% B (0-5min), 10-32%B (5-45min), 32-50%B (45-48min), 50-95%B (48-52min), 95-95%B (52-57min), 95-6%B (57-58min) and 6-6%B (58-60). One full MS scan (m/z 350−1500) was acquired, and MS/MS analyses of 10 most intense ions were then performed. Dynamic exclusion was set with repeat duration of 24 s and exclusion duration of 12 s. For each time point, we analyzed two biological replicates for infected samples and control, and in total, we carried out 18 LC-MS/MS experiments.

2.10 MS data analysis and label-free quantification

Raw MS files were analyzed by MaxQuant version 1.5.2 (http://www.maxquant.org). MS/MS spectra were searched by the Andromeda search engine using default parameter against the decoy SwissProt mouse database (Version March 2018, 16,957 entries) or Salmonella typhimurium database (Version March 2018, 1916 entries) supplemented with 262 frequently observed contaminants and forward and forward and reverse sequences. In the main Andromeda search precursor mass and fragment mass were identified with an initial mass tolerance of 6 ppm and 20 ppm, respectively. The search included variable modifications of methionine oxidation and N-terminal acetylation, and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set

to 7 amino acids and a maximum of two mis-cleavages was allowed. The false discovery rate (FDR) was set to 0.01 for peptide and protein identifications. Each protein includes at least one unique peptide corresponding to one unique spectrum. MS runs use the 'match between runs' option. For matching, a retention time window of 20 sec was selected. In the case of identified peptides that are all shared between two proteins, these were combined and reported as one protein group. Proteins matching to the reverse database were filtered out. We used a strict criterion for removing control. At each time point, we have profiled two biological repeats and one control and each with three technique repeats. As long as this protein is identified in one technique repeat in the control sample, we consider it is a background protein. Potential crosslinked proteins are obtained after subtracting the control hits (host cell infected with probe-labeled *salmonella* but without UV irradiation) from the samples.

2.11 Hierarchical clustering

Protein abundance are quantified by iBAQ value calculated from Maxquant Software, and all the iBAQ quantification result were subjected into heatmap. Heatmap were constructed by hierarchy clustering using parameter of cosine distance in MATLAB "clustergram" function, and each row was normalized with standard deviation. Rows in heatmap denotes quantified proteins, and column denotes sample. Red color denotes highly expressed protein, whereas blue denotes lowly expressed protein. From Fig. 3c, based on the classification tree above the heatmap, we can see technique repeats for the same biological sample were clustered together, and samples from different time point were also clustered together, indicating the high reproducibility of both biological and technical replicates.

2.12 GO Analysis

Functional annotation of gene ontology and KEGG pathway for each protein were retrieved from UNIPROT database (http://www.uniprot.org). KEGG pathway and GO enrichment analysis of the differentially expressed proteins were conducted according

to the information from the KEGG Pathway and GO databases, respectively, using the following formula:

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

*N* is the number of all identified proteins that can be connected with GO or KEGG Pathway analysis information.

*n* is the number of differential proteins in *N*.

*M* is the number of proteins that can be connected with a certain GO term or pathway.

*m* is the number of differential proteins with certain GO term or KEGG pathway.

The ratio value was defined as the protein number of identified for each functional category normalized by protein number of genome background. The GO term or pathway is considered as a significant enrichment of differential proteins when p value is blow 0.05. Among them, several important biological process and pathway were particularly highlighted with red.

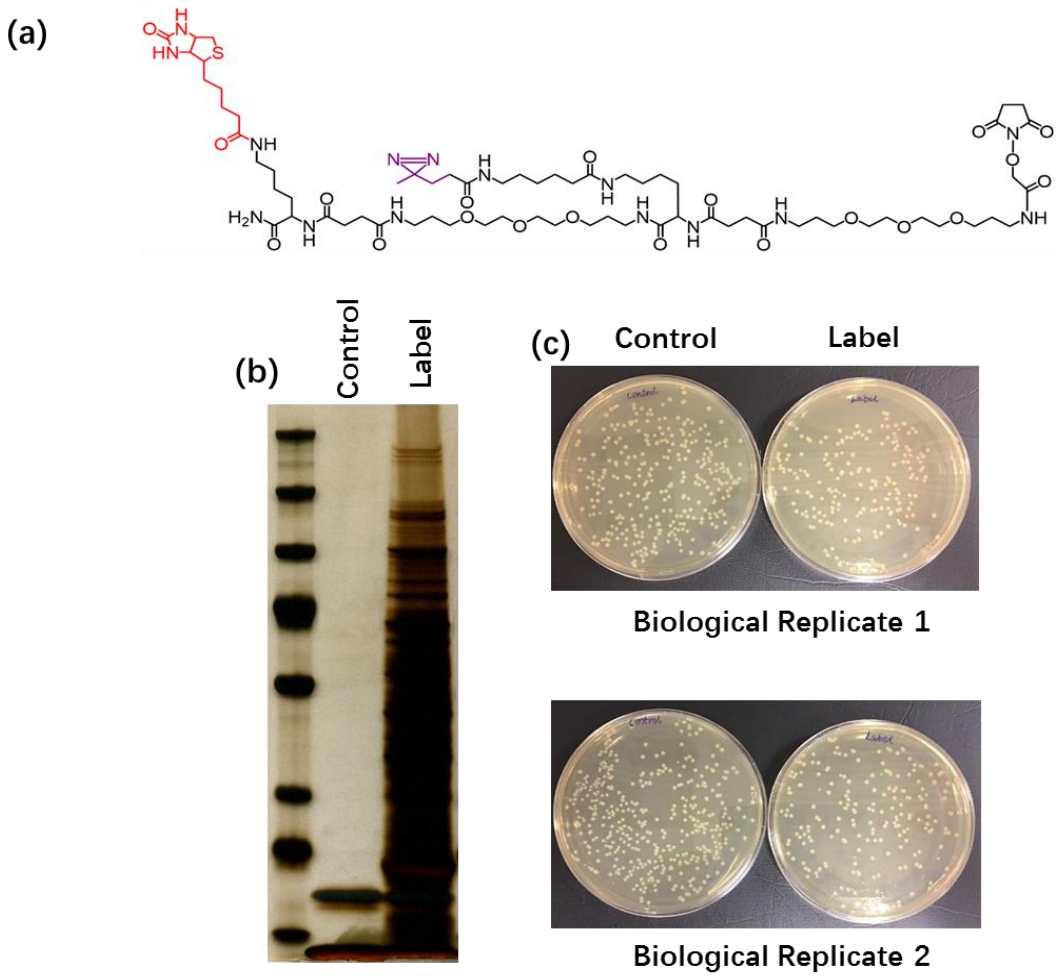



Figure S1 (a) Structure illustration of NHS-functionalized probe. (b) SDS-PAGE of *salmonella* lysate captured by NeutrAvidin beads. Before lysate, *salmonella* was labeled with NHS-functionalized probe. Unlabeled salmonella was also lysated and captured as control. (c) Comparison of the replication activity o*f salmonella* before and after labeling with NHS-functionalized probe. Enumeration of colony forming units (cfu) for labeled and unlabeled bacteria, left is without labeling the probe and overnight grown as the control; right is labeled with the probe and overnight grown.

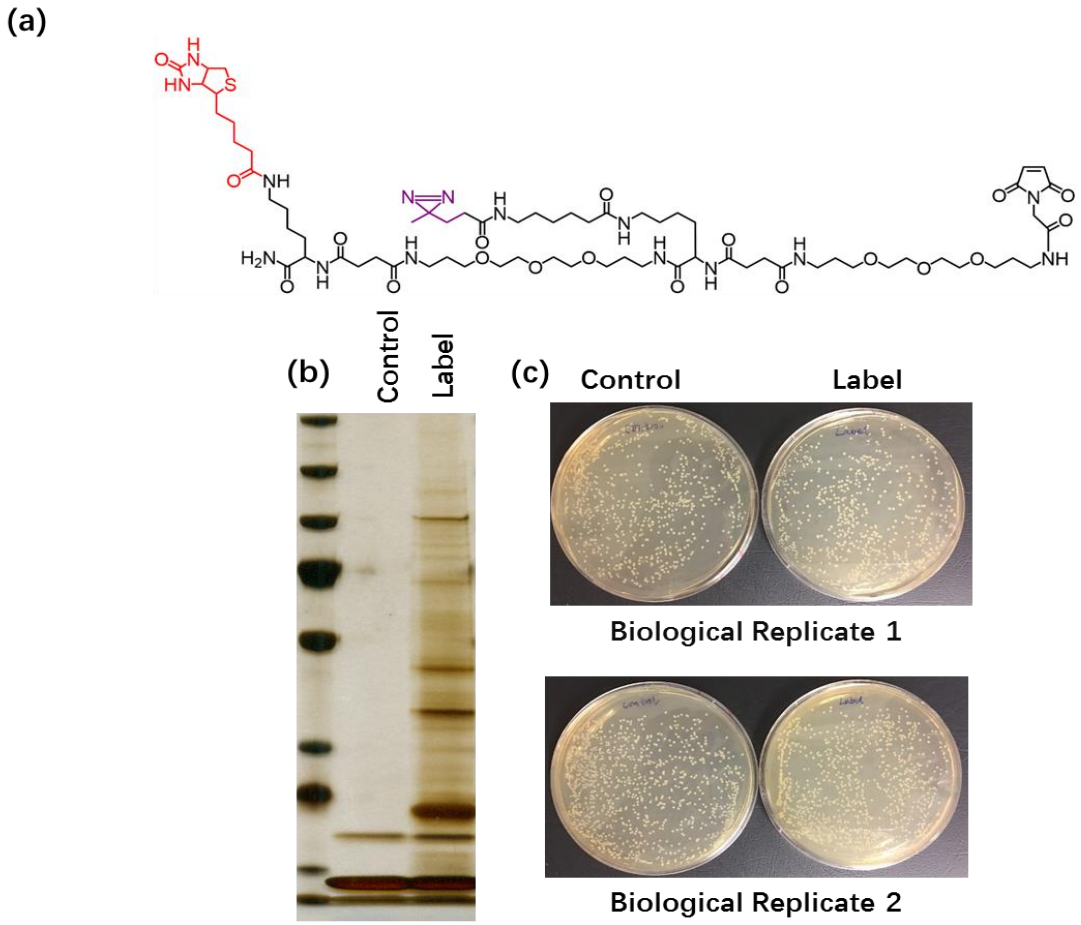



Figure S2 (a) Structure illustration of MAL-functionalized probe. (b) SDS-PAGE of *salmonella* lysate captured by NeutrAvidin beads. Before lysate, *salmonella* was labeled with MAL-functionalized probe. Unlabeled salmonella was also lysated and captured as control. (c) Comparison of the replication activity of salmonella before and after labeling with MAL-functionalized probe. Enumeration of colony forming units (cfu) for labeled and unlabeled bacteria, left is without labeling the probe and overnight grown as the control; right is labeled with the probe and overnight grown.

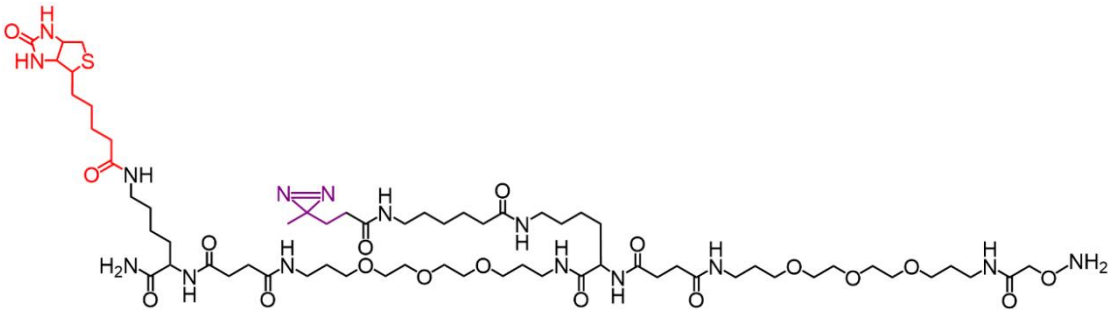


Figure S3 Structure illustration of $ONH_2$-functionalized probe.

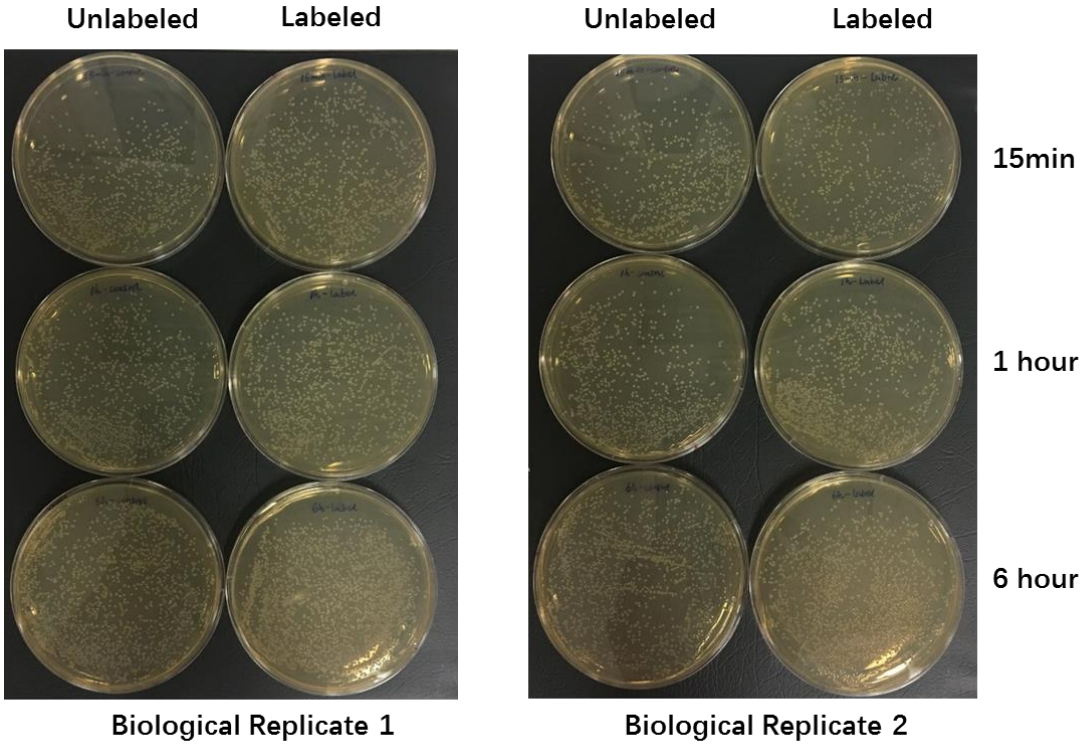



Figure S4 Comparison of the intracellular survival of salmonella before and after labeling with $ONH_2$-functionalized probe. Enumeration of colony forming units (cfu) for labeled and unlabeled bacteria, left is without labeling the probe, infected the macrophage and then overnight grown as the control; right is labeled with the probe, infected the macrophage and then overnight grown.

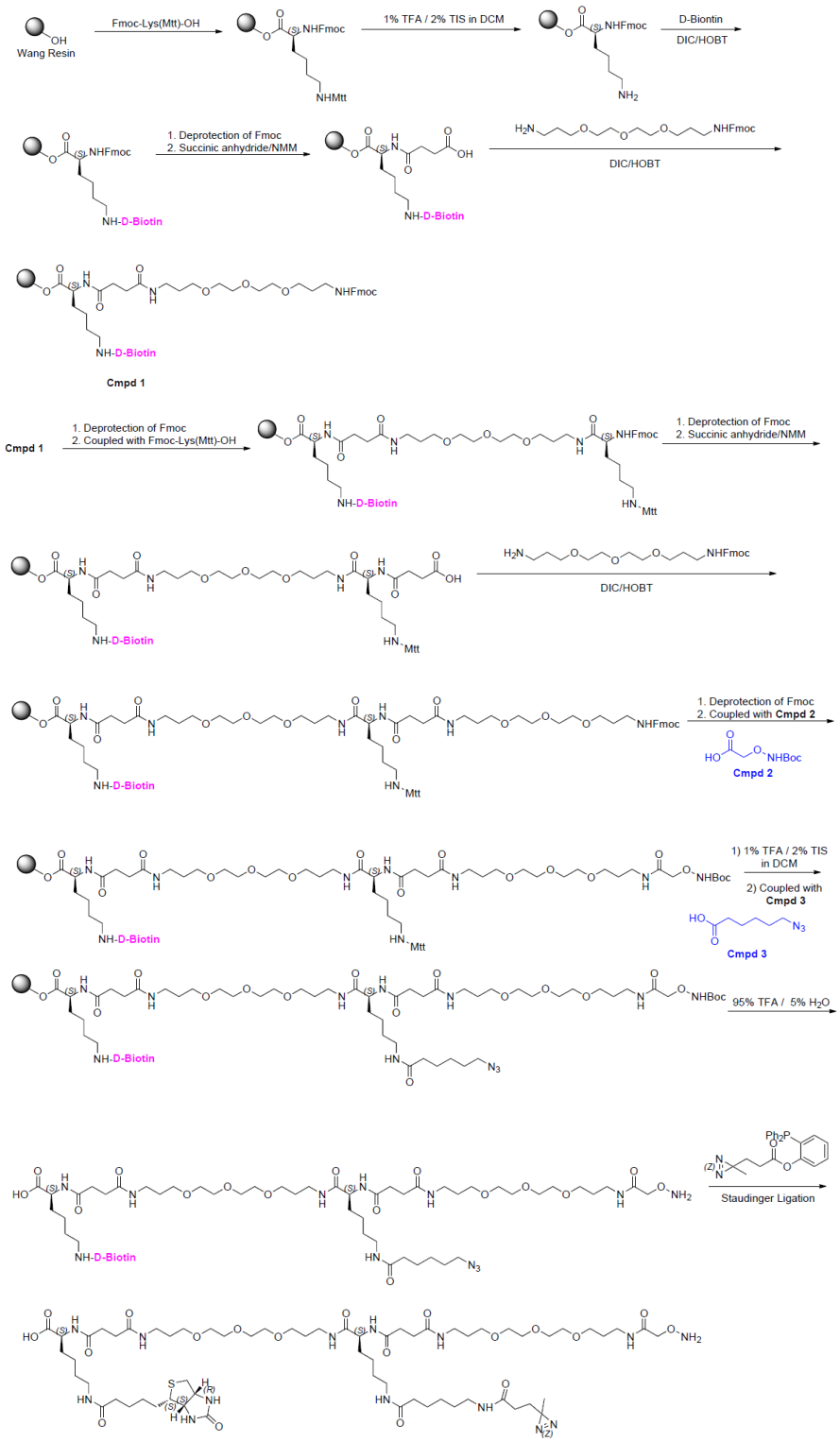
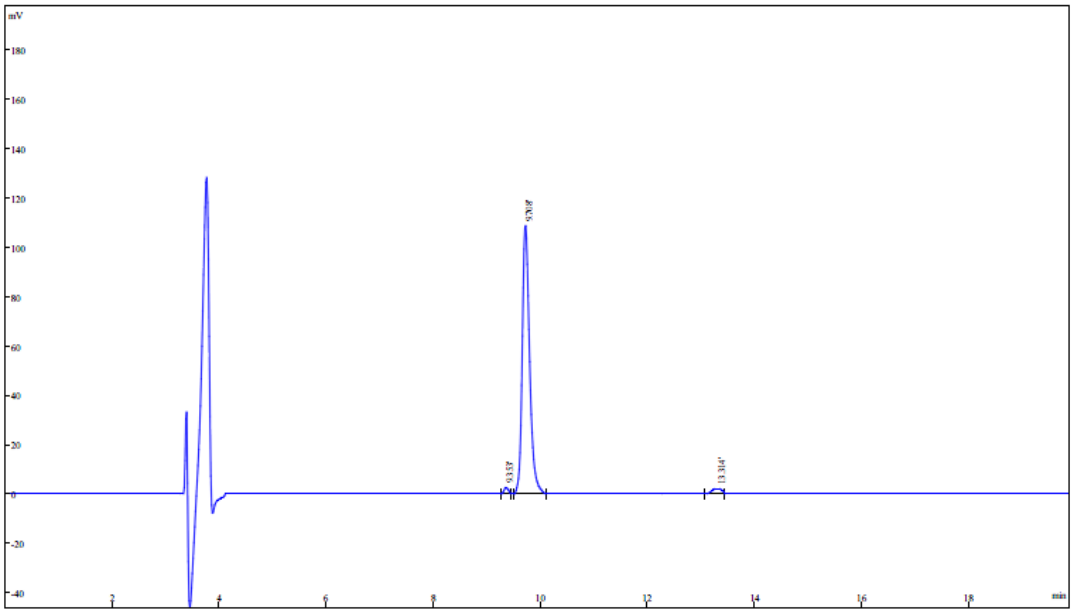



Figure S5 Synthetic route of the $ONH_2$-functionalized chemical proteomics probe.

Buffer A:0.1% TFA in 100% water(v/v)
Buffer B:0.1% TFA in 80% acetonitrile+ 20% water(v/v)
Gradient:29-49% Buffer B in 20min
Flow:1ml/min Wavelength:220nm
Column:Venusil C18 5um4.6*250mm

Chromatogram




| Signal | RT(min) | Area% | Area | Height |
|---|---|---|---|---|
| 1 | 9.353 | 1.574 | 17246 | 2756 |
| 2 | 9.708 | 95.44 | 1045408 | 109770 |
| 3 | 13.314 | 2.99 | 32749 | 2476 |
| Total | | 100 | 1095403 | 115002 |

Figure S6 HPLC analysis of the $ONH_2$-functionalized chemical proteomics probe

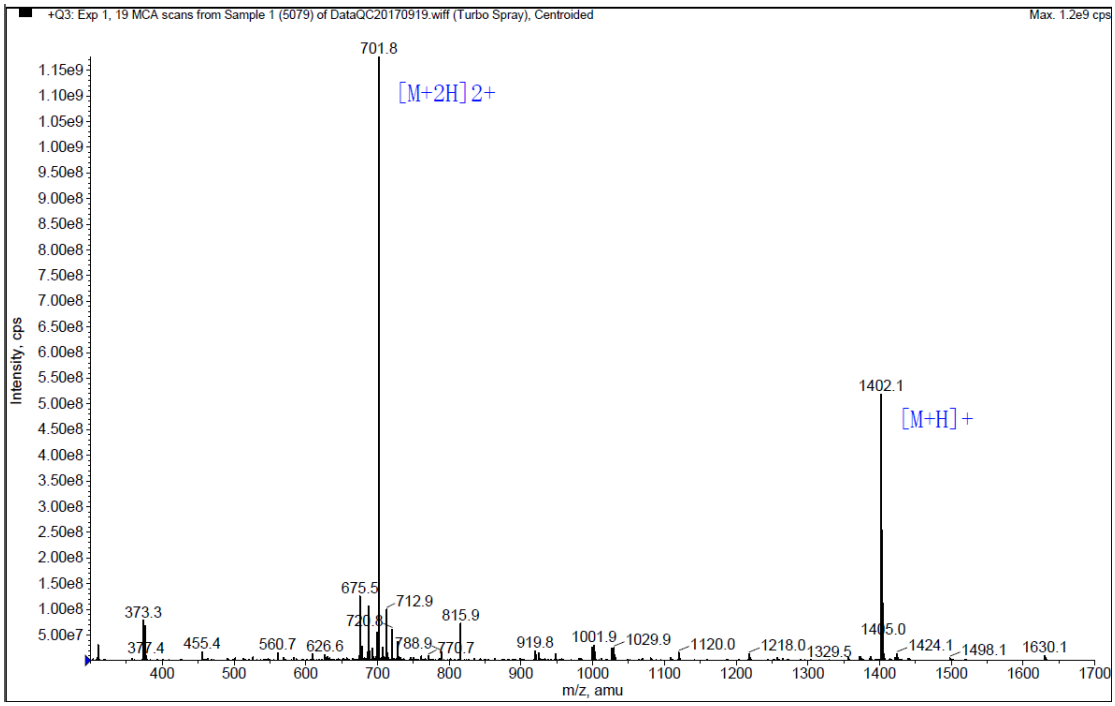



Figure S7 ESI-MS of the $ONH_2$-functionalized chemical proteomics probe

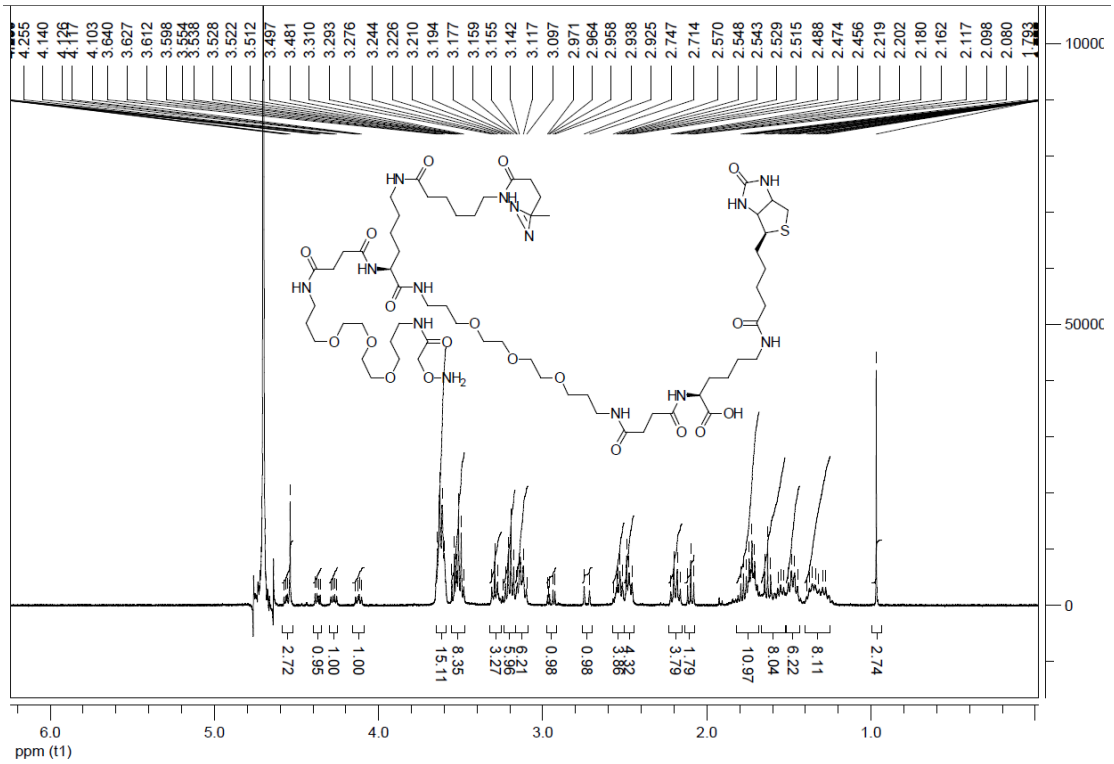



Figure S8 $^{1}H$ NMR of the $ONH_2$-functionalized chemical proteomics probe

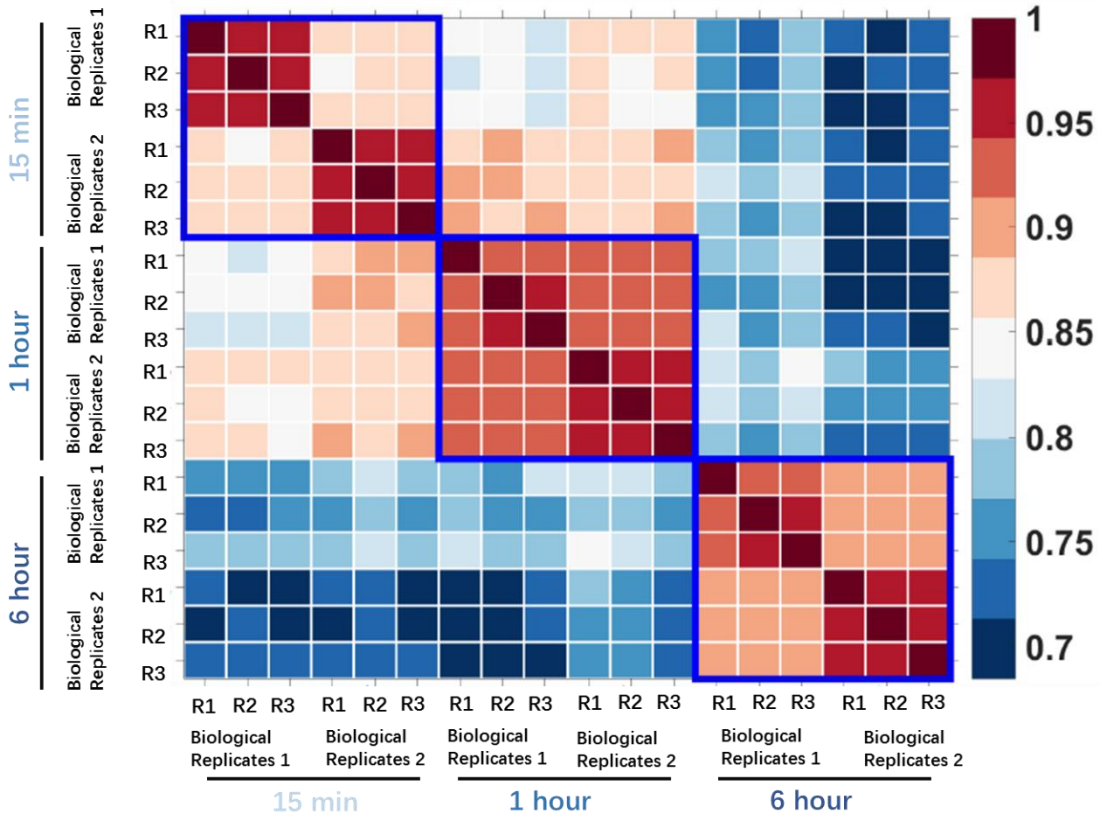



Figure S9 Pearson correlations between the biological replicates and the technical replicates.

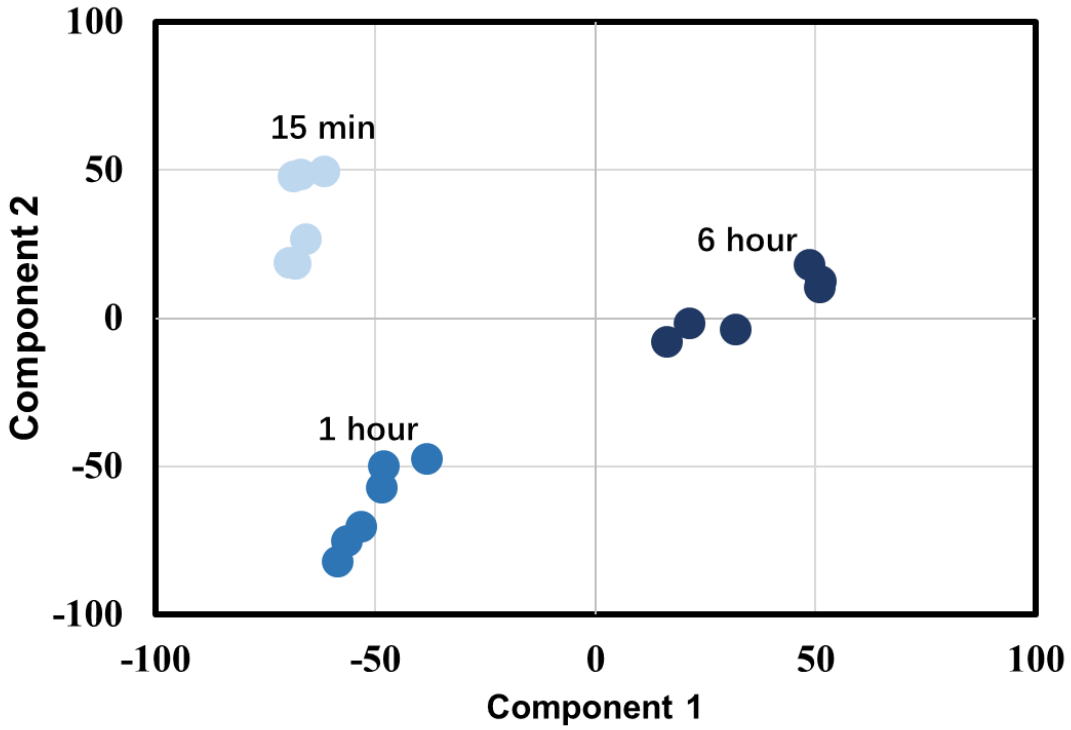



Figure S10 Principal Component Analysis (PCA) of proteins identified in different time points.

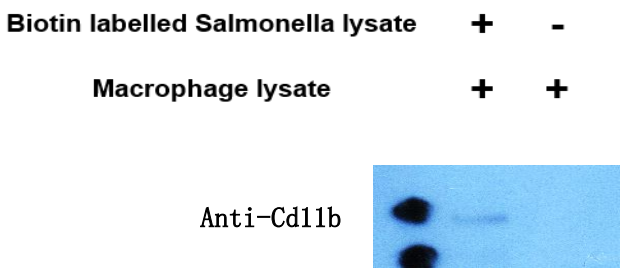



Figure S11. Detection of Cd11b by western blotting. Proteins from macrophage lysate were pulled down by biotin-labeled or unlabeled *Salmonella* using NeutrAvidin beads and analyzed by western blotting using antibodies against Cd11b.