In-depth *in vivo* Crosslinking in Minutes by a Compact, Membrane-permeable and Alkynyl-enrichable Crosslinker

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Compound Synthesis Procedures

Reagents and materials

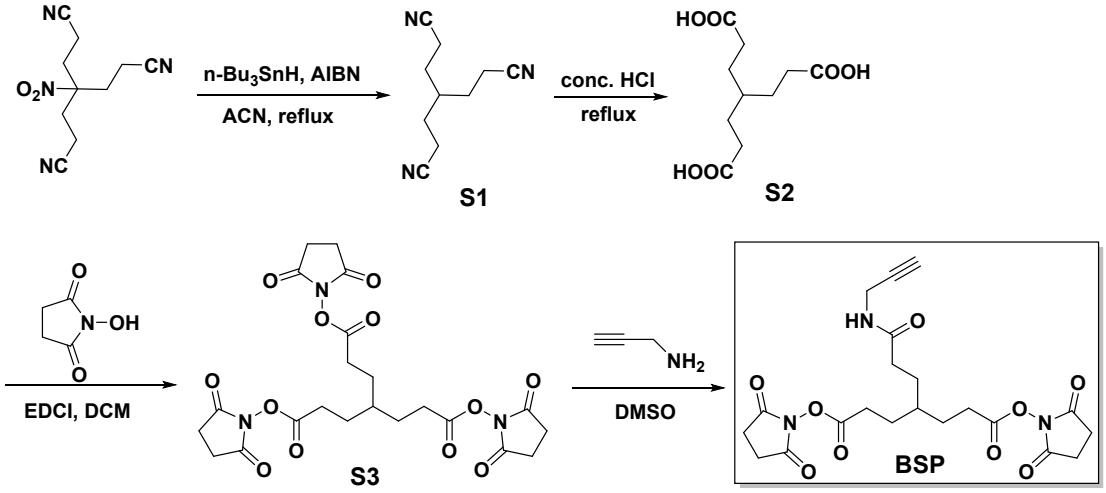
The chemical reagents were obtained commercially and used as received without further purification. All reactions were carried out in a dry three-neck flask under magnetic stirring.

Instruments and methods for the compound separation and structural characterization

The reaction process and purity of products were monitored by analytical HPLC from HITACHI chromaster (Japan) equipped with a DAD UV detector using C18 column (3 μm, 100 Å, 4.6 × 250 mm i.d.).

Semi-preparative RP-HPLC purifications were performed on DAD50 system from Hanbon (China) installed with a UV-visible 2000 detector using C18 column (10 μm, 100 Å, 50 × 250 mm i.d.). The freeze-drying of the crosslinker was carried out on a lyophilizer from Tianrey (China) at 30 ℃ for 24 h. $^{1}H/^{13}C$-NMR spectra were recorded on a Bruker AVANCE II 400 MHz spectrometer using $CDCl_3$ or DMSO-d6 as the solvent and TMS as the internal standard. Mass spectra were recorded on an LTQ Orbitrap Velos (Thermo Fisher Scientific).

Synthesis of BSP



Scheme S1. Synthesis route of BSP

4-(2-cyanoethyl)heptanedinitrile (trisnitrile, S1)[1]

A mixture of tris(2-cyanoethyl)nitromethane (9.2 g, 40 mmol), tri-n-butyltin hydride (14.0 g, 48 mmol) and AIBN (2.36 g, 14.4 mmol) in 300 mL HPLC-grade ACN was refluxed at 88 ℃ under N_2 atmosphere for 12 h. After being cooled to the room temperature, the organic solvent was removed under reduced pressure and the crude product was recrystallized twice with 50 ml methanol. The precipitate was washed with cool methanol and dried in vacuo for 24 h to afford a white solid compound S1 (6.31 g, yield 90.1%). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm) δ 2.45 (t, 6H, J = 7.2 Hz), 1.83-1.73 (m, 7H). Exact mass 175.1109 for $C_{10}H_{13}N_3$, found 198.1230 $[M+Na]^+$.

4-(2-carboxyethyl)heptanedioic acid (tris-carboxylic acid, S2)[1]

Compound S1 (6.1 g, 35 mmol) was refluxed in 40 ml conc. HCl for 1 h and then cooled to 0 ℃. The precipitate was filtered and washed with little cool water, dried in vacuo for 24 h to afford a white solid compound S2 (7.2 g, yield 88.7%). ^{1}H-NMR (400 MHz, DMSO-d6, ppm) δ 12.03 (m, 3H), 2.20 (t, 6H, J = 7.6 Hz), 1.48-1.43 (m, 6H), 1.35-1.31 (m, 1H). Exact mass 232.0947 for $C_{10}H_{16}N_6$, found 250.1298 $[M+H_2O]^+$.

Tris-succinimide ester (S3)

Compound S2 (1.86 g, 8 mmol), EDCI (4.14 g, 36 mmol), NHS (6.9 g, 36 mmol) were dissolved in 100 ml anhydrous CH_2Cl_2 and stirred at room temperature for 24 h. After being detected for completion by analytical HPLC, the solution was pooled into 100 ml deionized H_2O and washed with H_2O for 3 times. The organic layer was collected, dried over anhydrous Na_2SO_4 and removed under vacuum to afford a white solid compound S3 (3.98 g, yield 95.2%). Purity 99%, retention time at 28 min detected by analytical HPLC (A: H_2O, B: ACN, gradient method was set as follows: B from 5% to 50% over 40 min, 80% B over 10 min, 5% B over 10 min at a flow rate of 1 mL/min monitored by UV wavelength of 200 nm). ^{1}H-NMR (400 MHz, DMSO-d6, ppm) δ 2.81 (s, 12H), 2.71 (t, 6H, *J* = 7.6 Hz), 1.70-1.65 (m, 6H), 1.62-1.57 (m, 1H); ^{13}C-NMR (400 MHz, DMSO-d6, ppm) δ 170.7, 169.5, 35.5, 28.0, 27.3, 25.9. Exact mass 523.1438 for $C_{22}H_{25}N_3O_{12}$, found 524.0555 $[M+H]^+$.

Trifunctional crosslinker (BSP)

Compound S3 (1.05 g, 2 mmol) was dissolved in 30 ml DMSO and propargyl amine (110 mg, 2 mmol) in 1 ml DMSO was added dropwise to the solution. After stirred at room temperature for 5 min and detected for completion by analytical HPLC, the mixture was purified by semi-preparative RP-HPLC (A: 0.1% TFA/H2O, B: ACN, gradient method was set as follows: B from 20% to 50% over 30 min at a flow rate of 50 mL/min monitored by UV wavelength of 200 nm). The product-containing fraction (retention time, 22.0 - 26.0 min) was lyophilized at 30 ℃ for 24 h to afford the targeted crosslinker BSP as a light-yellow sticky oil (326 mg, yield 35.2%). Purity 99%, retention time at 26 min detected by analytical HPLC (A: 0.1% TFA/H_2O, B: ACN, gradient method was as follows: B from 5% to 50% over 40 min, 80% B over 10 min, 5% B over 10min at a flow rate of 1 mL/min monitored by UV wavelength of 200 nm). ^{1}H-NMR (400 MHz, DMSO-d6, ppm) δ 8.29-8.27 (m, 1H), 3.84 (t, 2H, *J* = 2.6 Hz), 3.07 (s, 1H), 2.81 (s, 8H), 2.69 (t, 4H, *J* = 7.8 Hz), 2.11 (t, 2H, *J* = 7.6 Hz), 1.63-1.60 (m, 4H), 155-1.49 (m, 3H); ^{13}C-NMR (400 MHz, DMSO-d6, ppm) δ 172.3, 170.7, 81.7, 73.3, 35.8, 32.5, 28.3, 28.1, 27.6, 25.9, 25.6. Exact mass 463.1591 for $C_{21}H_{25}N_3O_9$, found 464.2333 $[M+H]^+$.

Compound characterizations

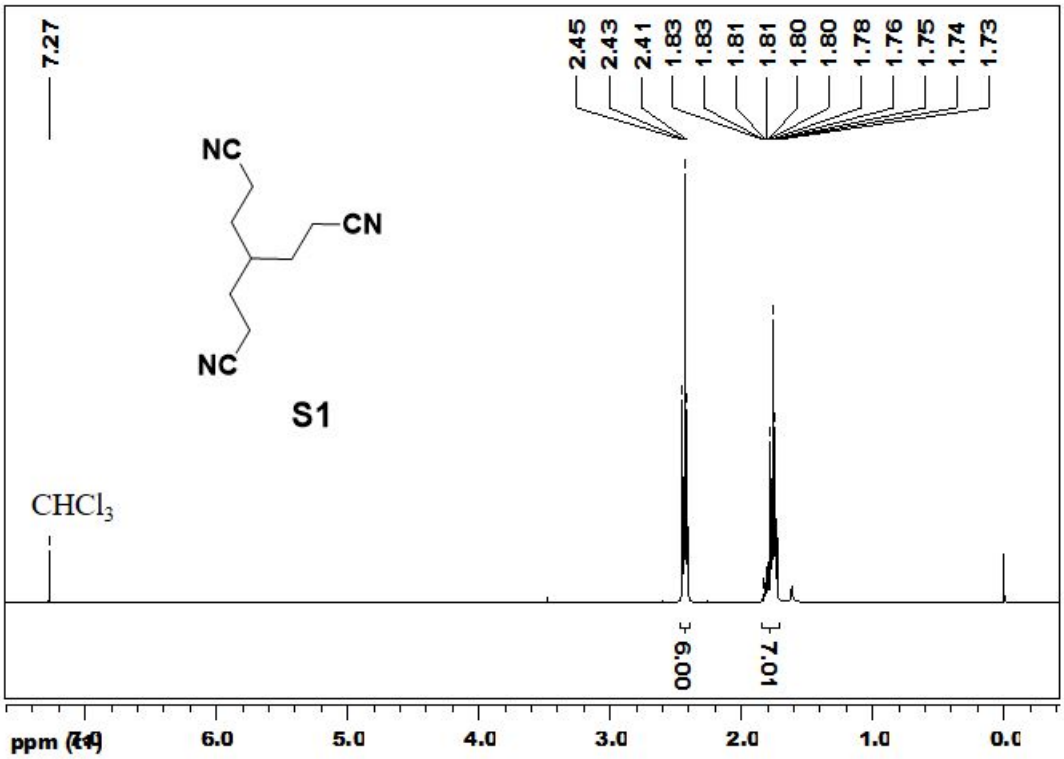


Figure S1. ^{1}H-NMR of compound S1

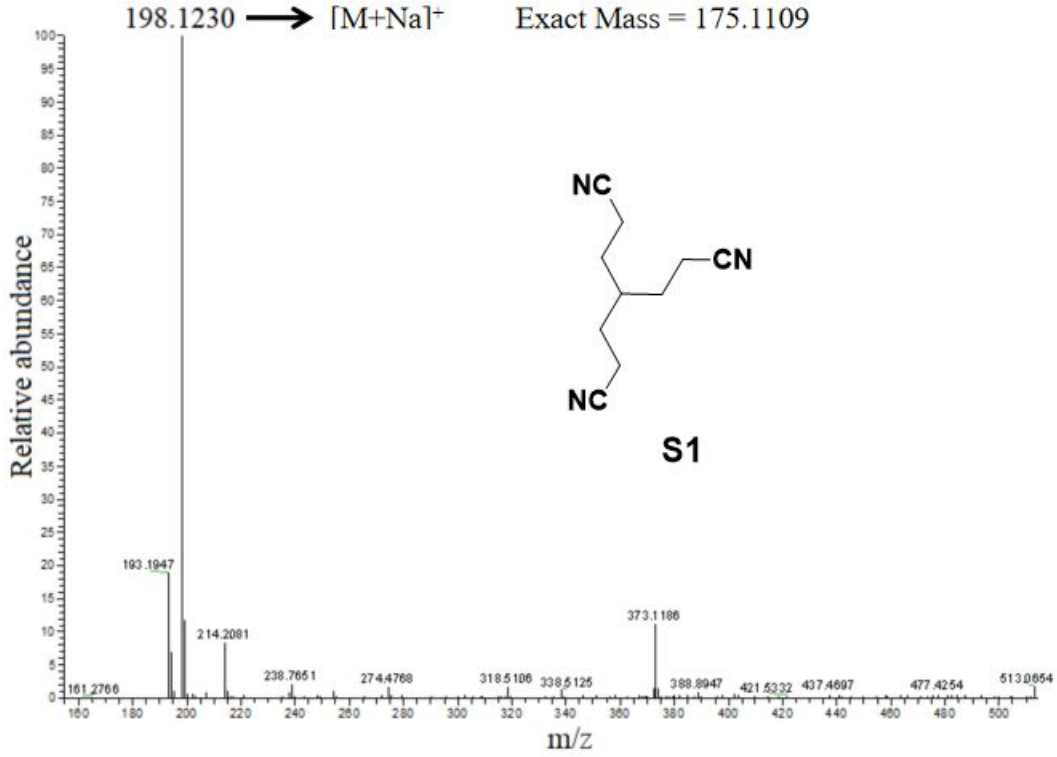


Figure S2. LTQ-mass spectra of compound S1

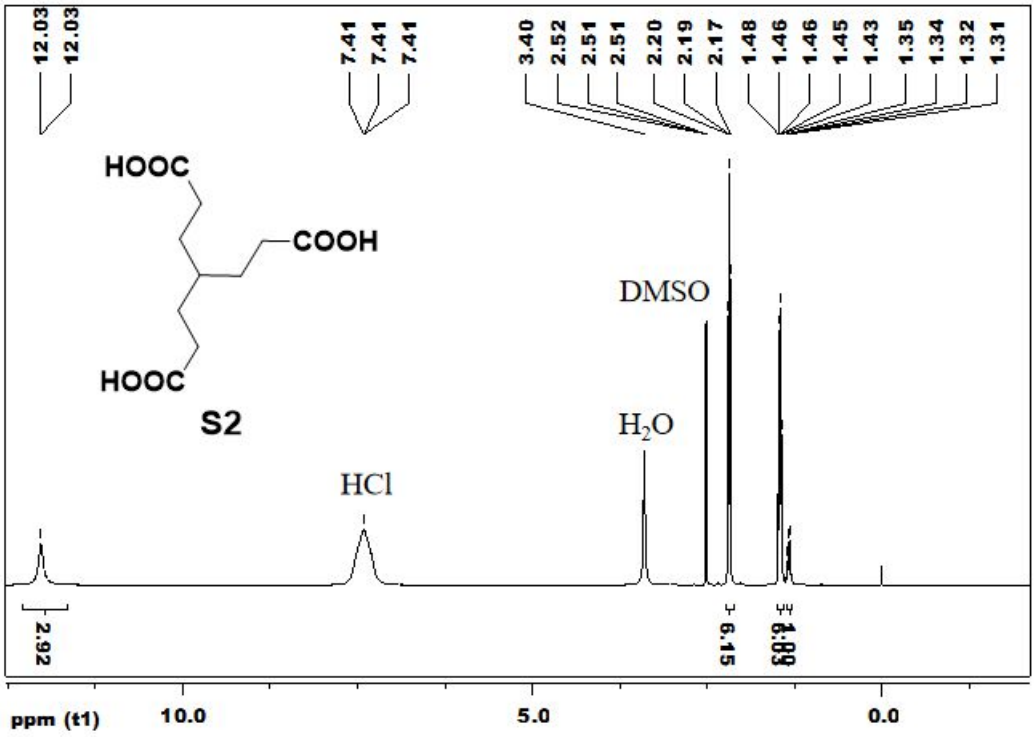


Figure S3. ^{1}H-NMR of compound S2

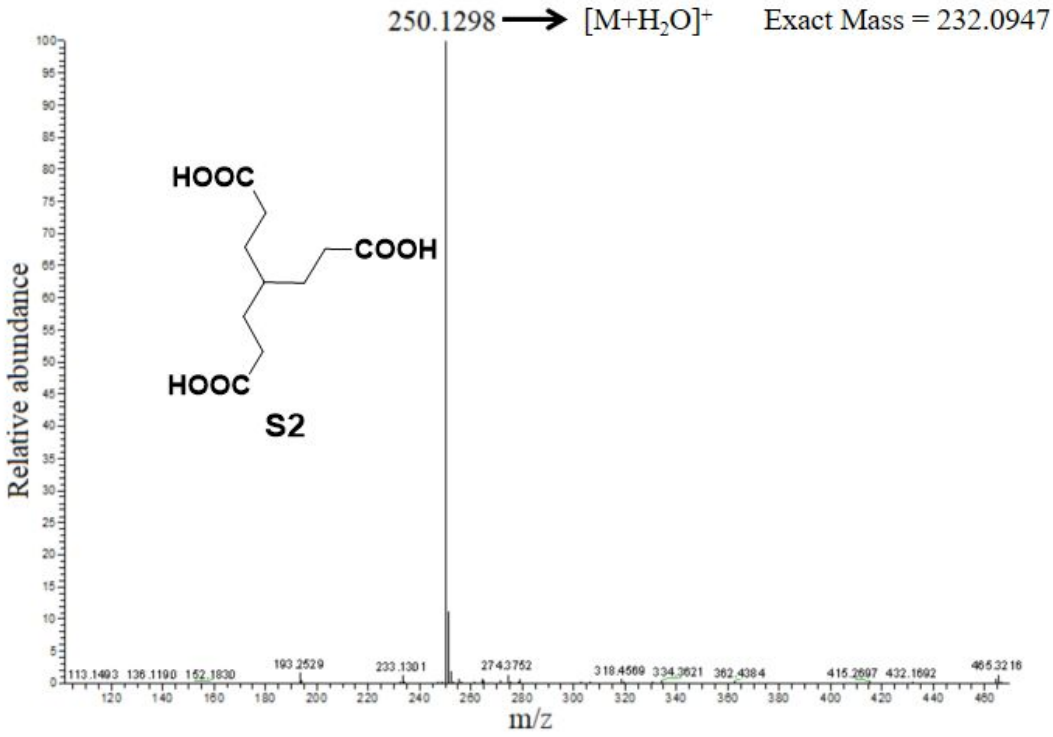


Figure S4. LTQ-mass spectra of compound S2

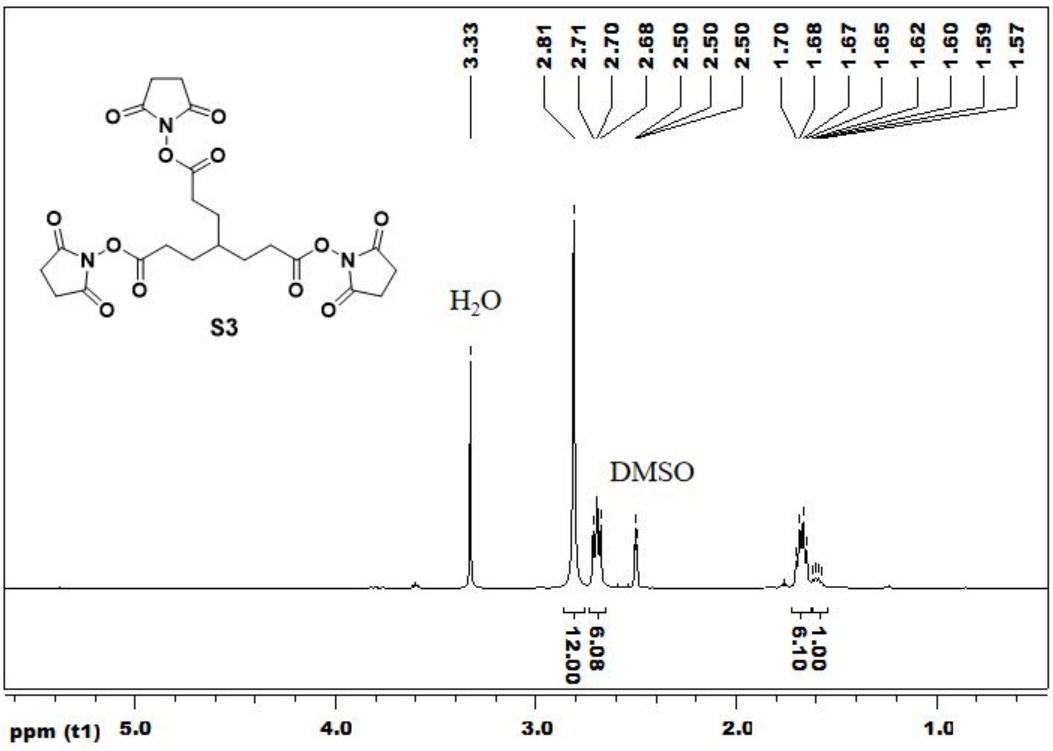


Figure S5. ^{1}H-NMR of compound S3

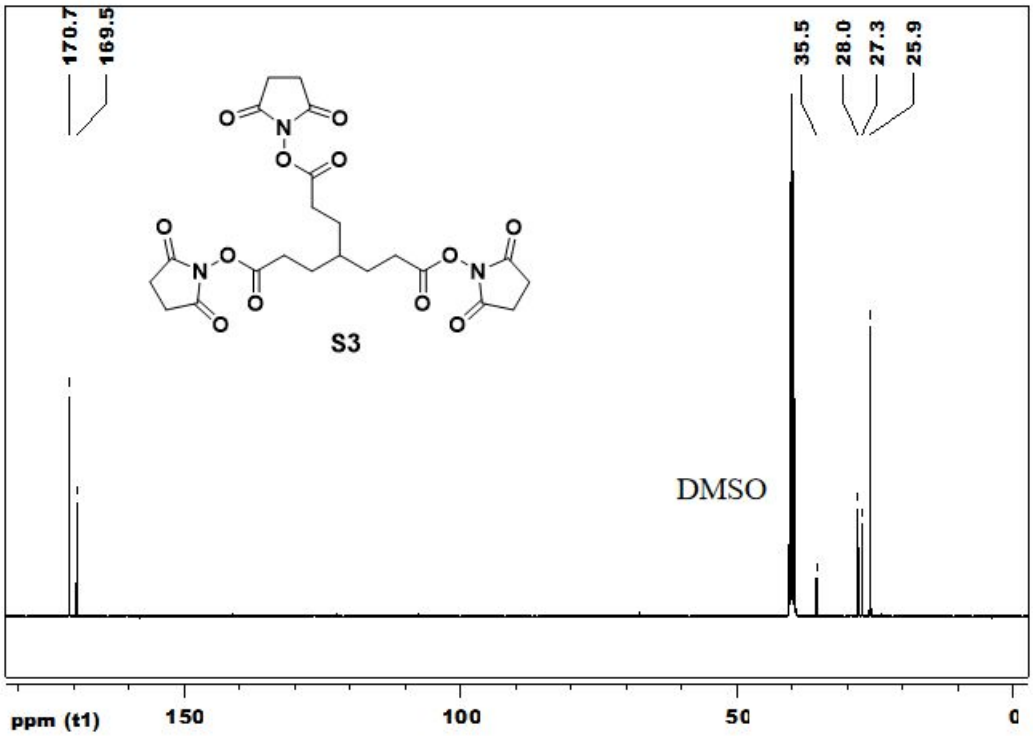


Figure S6. ^{13}C-NMR of compound S3

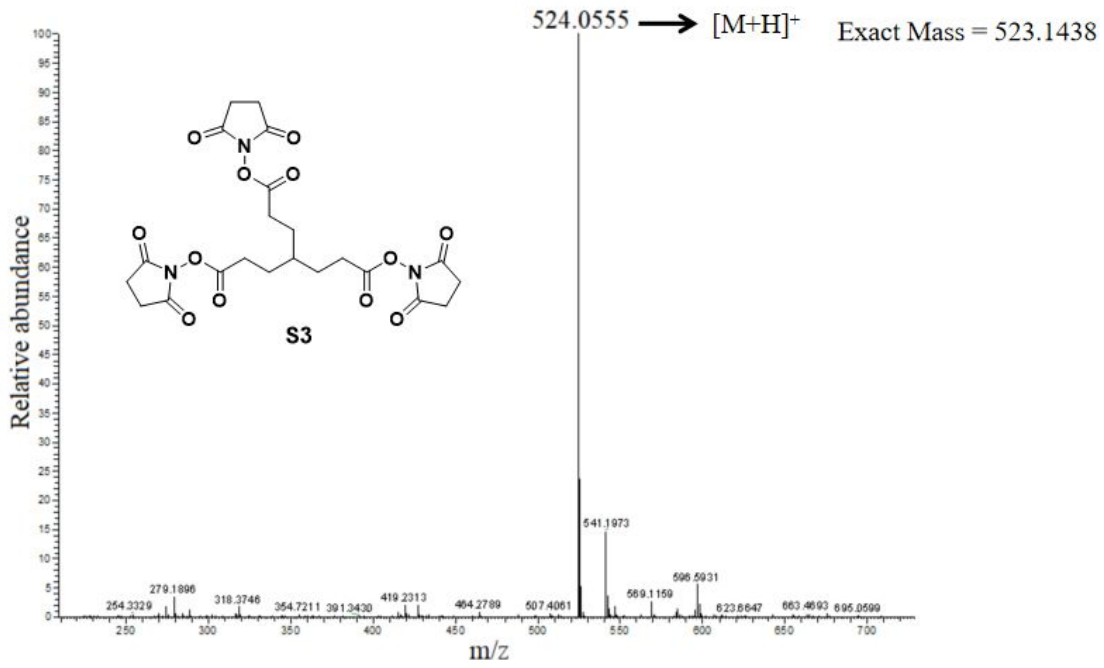


Figure S7. LTQ-mass spectra of compound S3

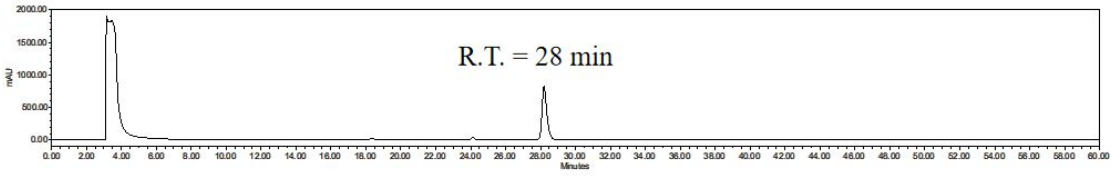


Figure S8. HPLC analysis of compound S3

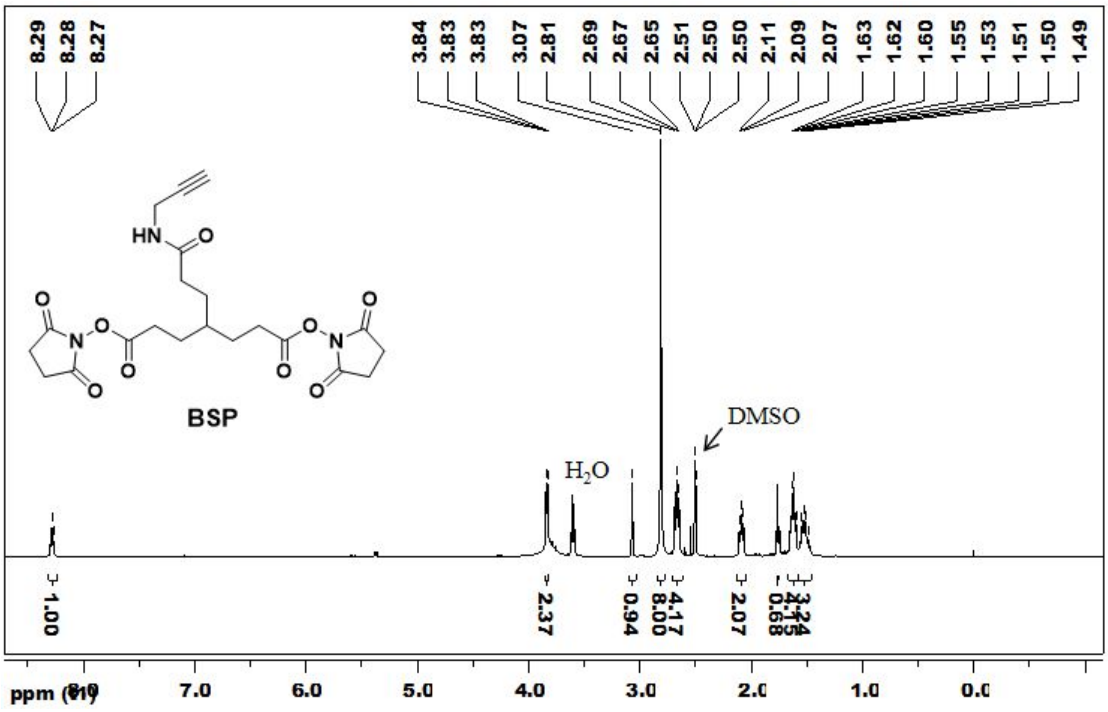


Figure S9. [1]H-NMR of BSP

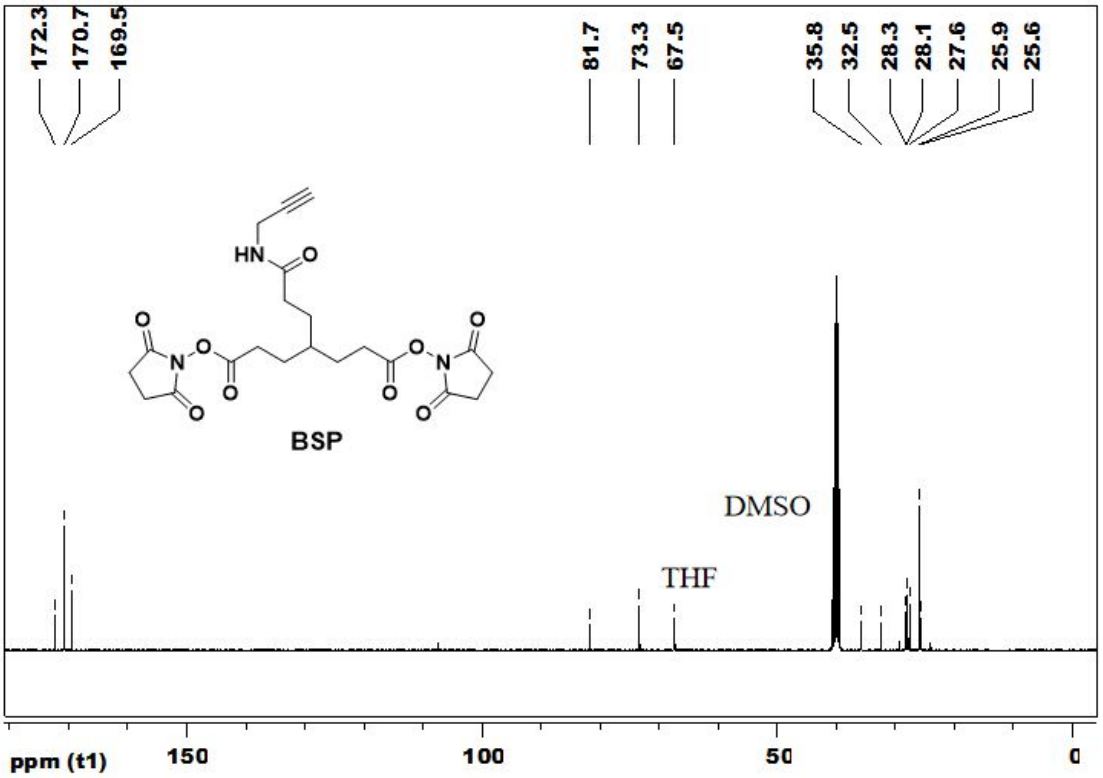


Figure S10. [13]C-NMR of BSP

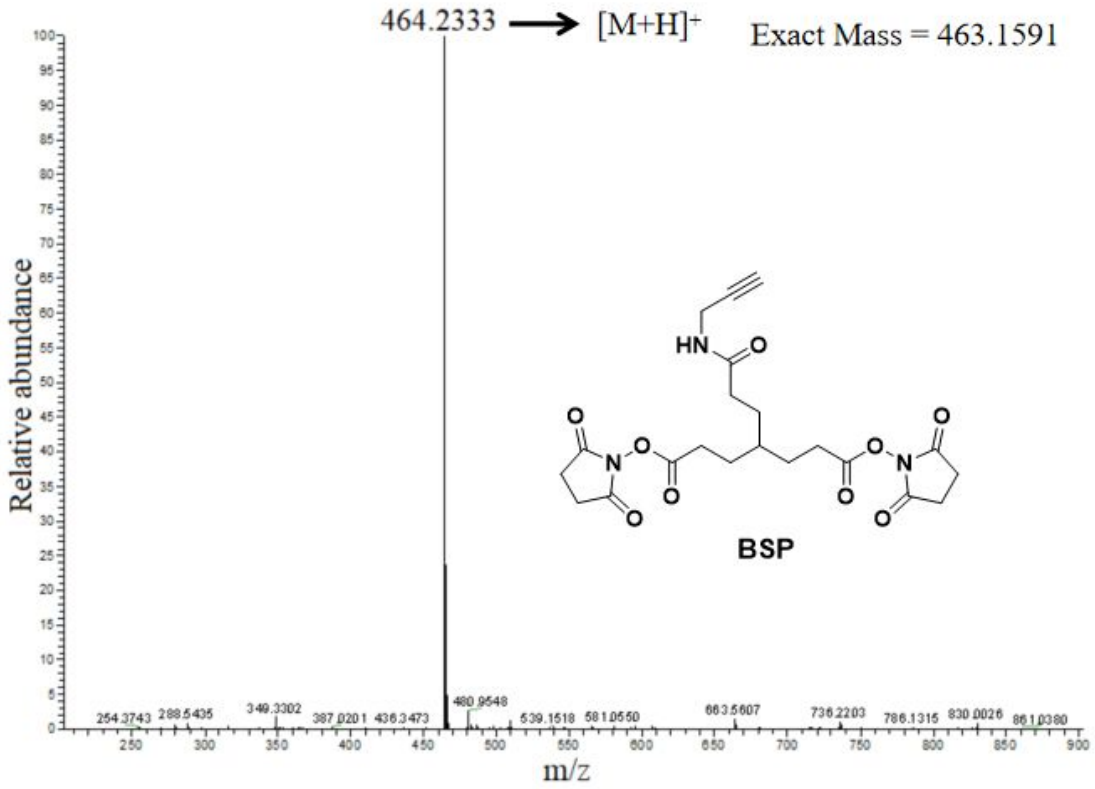


Figure S11. LTQ-mass spectra of BSP

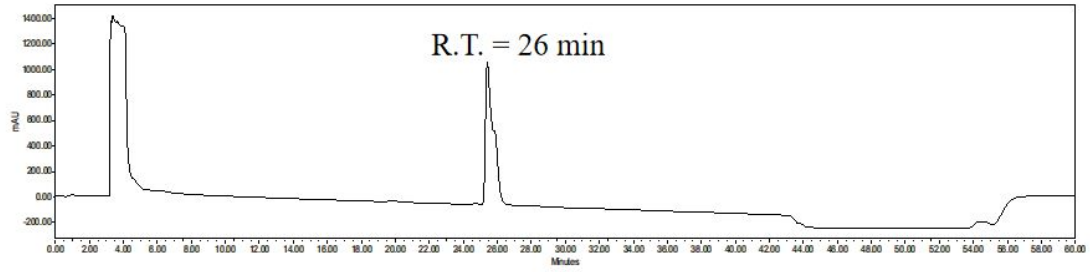


Figure S12. HPLC analysis of BSP

Supplemental methods

Reagents and materials

Standard peptide Ac-SAKAYEHR was custom-synthesized by Chinapeptides (Shanghai, China). Bovine serum albumin (BSA), azo biotin-azide (ABA), (+)-sodium L-ascorbate (Vc), copper (II) sulfate ($CuSO_4$), tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA), dimethyl sulfoxide (DMSO), ammonium bicarbonate (ABC), ammonium hydroxide, tris(2-carboxyethyl)phosphine (TCEP), 1,4-dithio-D-threitol (DTT), iodoacetamide (IAA), formic acid (FA), urea, thiourea, sodium hydrosulfite were purchased from Sigma-Aldrich (St. Louis, MO USA). Trypsin, sequencing grade, was obtained from Promega (Madison, WI USA). Dulbecco's phosphate buffered saline (PBS, pH 7.4), streptavidin agarose resin and C18 tips were ordered from Thermo Fisher Scientific (Rockford, USA). Cell counting kit-8 (CCK8), BCA protein assay kit, 12% SDS-PAGE resolving gel master mix, coomassie blue staining solution were purchased from Beyotime (Shanghai, China). Acetonitrile (ACN, HPLC grade) was ordered from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Milford, MA). Mix-mode cation exchange (MCX) columns were purchased from Waters (Oasis, Ireland). All other chemicals were obtained as analytical grade and used without further purification.

Crosslinking of standard peptide

Synthetic peptide Ac-SAKAYEHR 1 mg was dissolved in 100 μl anhydrous dimethyl sulfoxide (DMSO) containing 1% triethylamine. BSP was reconstituted in anhydrous DMSO and mixed with peptide solution in a molar ratio of 1:2. After a quick vortex, the crosslinking reaction was performed at room temperature for 5 min. Adding 20 volumes of 0.1% (v/v) FA/H_2O to quench the reaction. DMSO was removed by C18 tips to afford the crosslinked peptide.

The biotinylated peptide was obtained by click chemistry as described in the section of '**Click chemistry at the peptide level.**' and the reductive released peptide after enrichment was obtained as described in the section of '**Enrichment of crosslinked peptides.**'

The resulting crosslinked peptides were diluted to 1 μg/μl in 0.1% FA/H_2O prior to MALDI-TOF MS and LC-MS/MS analysis.

Crosslinking of BSA for SDS-PAGE analysis and LC-MS/MS acquisition

For optimization of crosslinking time, the BSA (0.1 mM final concentration) and BSP crosslinker (5 mM final concentration) were freshly dissolved in 1% (v/v) DMSO/PBS and the crosslinking reaction were carried out at room temperature for different time (Figure. S16A). For optimization of the amount of BSP, the crosslinking reaction were carried out at room temperature for 5 min at different BSA to BSP molar ratios from 1:5 to 1:50 (Figure. S16B) and compared that with DSS. The control of BSA was carried out without addition of crosslinker.

50 mM ABC was added to stop the reaction. After addition of 5-fold pre-cold acetone, the proteins were precipitated at -20 ℃ overnight to remove the non-reacted crosslinkers. The samples were centrifuged at 4000 g for 5 min and washed once with cold acetone. The resulting proteins were resuspended in 1 × loading buffer and boiled at 95 ℃ for 5 min. A separation gel of 12% loaded with 10 μg protein for each aisle was run at 120 V for 90 min. After fixed in 50% MeOH and 10% AcOH for 30 min, the gel was stained with coomassie brilliant blue for 1 h and destained in deionized water overnight. The pictures were taken by Molecular Imager (Bio-Rad, USA) and handled with Image Lab software, as shown in Figure. S16.

For LC-MS/MS acquisition, the optimized crosslinking was conducted for 5 min at a BSA to BSP molar ratio of 1:50. After acetone precipitation, the sample was divided into two pieces and

subjected to click chemistry at the protein level and peptide level, respectively. The detailed click chemistry and sample preparation methods were performed as described in the section of '**Click chemistry at the protein level and peptide level.**'

Procedures for using C18 tips and MCX columns

The C18 tips and the MCX were used according to manufacturer's instructions. For C18 tips, one column volume of solvent B (20 % H_2O, 80% ACN, 0.1% TFA, v/v) was added to activate the C18 silicon particles by gravity flow and repeated it once. After activation, the C18 tips were equilibrated by one column volume of solvent A (0.1% TFA/ H_2O, v/v) for three times. The samples were loaded onto the C18 tips and washing with solvent A for three times (desalting step). Finally, the resulting peptides were eluted by two column volume of solvent B. The elution procedure was repeated once more for high peptide recovery.

For the use of MCX to remove the excess ABA reagent, the column was activated by two column volume of solvent A (1% FA in methol, v/v) by gravity flow. The biotinylated peptides dissolved in 50% ACN/H_2O containing 1% FA were loaded onto the MCX column and washing with solvent A until the effluent was colorless (The yellow ABA was thoroughly washed away). Finally, the resulting peptides were eluted by two column volume of solvent B (5% $NH_3 \cdot H_2O$ in methol, v/v). Repeat the elution until the effluent was colorless to ensure the high recovery.

CCK8 assay for cell viability during BSP *in vivo* crosslinking

Bel-7402 cells were grown in 96-well plates and cultured for 24 h to achieve complete attachment (approximately 10000 cells per well). The culture medium was removed and the cells were washed 3 times with PBS, followed by crosslinking with 5 mM BSP at room temperature for 1 min, 3 min, 5 min, 10 min, 30 min and 60 min, respectively. After removing the reaction mixture and washing 2 times with PBS, the cells were incubated with RPMI 1640 medium containing 10% (v/v) CCK8 for 2 h. The two blank controls were carried out in PBS or 1% DMSO/PBS which were the only difference from the experimental groups. Each group was repeated 5 times to reduce the experimental error. The absorbance value of cell viability was recorded on a spectrophotometer (Thermo Fisher Scientific) with measure wavelength of 450 nm. The histograms of cell viability affected by crosslinking time are depicted in Figure 1D.

Label-free quantitative proteomics of protein expression differences during BSP *in vivo* crosslinking in 5 min

After *in vivo* crosslinking of 5 mM BSP in 5 min, Bel-7402 cells were subjected to sample preparation by *i*-FASP workflow[2] for shot-gun proteomics analysis. Briefly, the crosslinked Bel-7402 cells were lysed in 10% (v/v) C12Im-Cl and 6 M guanidine hydrochloride and the extracted proteins were reduced with 100 mM DTT at 95 ℃ for 5 min. After cooled to room temperature, the samples were transferred to 10 kDa filter devices and washed with 50 mM ABC, followed by alkylation with 150 mM IAA for 30 min at room temperature in the dark. The samples were then washed with 10 mM ABC and digested with trypsin at an enzyme-to-protein ratio of 1:50 (w/w) at 37 ℃ for 12 h. The digested sample was collected by centrifugation and subjected to LC-MS/MS acquisition. The control was performed in 1% DMSO/PBS for 5 min and each experiment was carried out 3 times in parallel.

Fluorescent confocal microscopy of BSP cell membrane permeability

For fluorescent confocal microscopy, Bel-7402 cells were grown in 35-mm Petri dishes with number 1.5 cover glass bottom for 2 days until they reached 70-80% confluence. The adherent cells

were washed 3 times with PBS and reacted with 5 mM BSP at room temperature for 0.5 min, 1 min, 3min and 5 min, respectively. Then the cells were again washed 3 times and fixed by 1 ml 10% HCHO/PBS for 15 min at room temperature with constant shaking. After fixation, the cells were incubated with 0.1％ (v/v) triton X-100 in 1ml PBS for 60 min for cell membrane perforation. The click chemistry reaction was processed with 1μg/ml FITC-N_3 in the mixture of 4 mM Vc, 4 mM THPTA ligand and 4 mM $CuSO_4$ for 3 h at room temperature in the dark. After extensive washing, the cells were incubated with 1 μg/ml DAPI for 15 min for cell recognition and localization. The confocal microscopy samples were finally prepared after washing the cells with PBS for 3 times. The control was carried out in 1% DMSO/PBS for 5 min. Confocal fluorescent imaging was performed separately in 405 nm and 488 nm channels using a 100 × objective by andor live cell confocal imaging platform (Nikon Instruments Inc., USA). Images were all included in Fig. 1E.

Mass Spectrometry Acquisition

MALDI-TOF analysis of the standard crosslinked peptides

The resulting crosslinked samples from the standard peptide (crosslinked peptide, biotinylated peptide after click chemistry and reductive released peptide after enrichment) were diluted in 0.1% FA/H_2O. The samples were spotted onto the MALDI plate and mixed with 2,5-Dihydroxybenzoic acid (DHB). Mass spectra were manually acquired in a positive linear mode by using a UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) and the FlexControl software (version 3.3). The instrument parameters were set as follows: positive linear mode, 200 Hz for laser frequency; 800-3000 Da for molecular weight range; 4.0 × detector gain). Each mass spectrum was generated from 200-shot from different positions of the sample to generate the spectra with an intensity ≥ 10^4. The MALDI mass spectra were summarized in Figure S14.

Orbitrap Fusion Lumos acquisition of crosslinked peptides

The crosslinked standard peptides and BSA digestion were analyzed using an Easy-nano LC 1200 system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The crosslinked standard peptides were separated using a 30 min gradient as follows: 0-20 min (7-23% B), 20-25 min (23-35% B), 25-26 min (35-80% B), 26-30 min (80% B). The BSA digestion was separated using a 85 min gradient as follows: 0-15 min (10-20% B), 15-50 min (20-35% B), 50-75 min (35-50% B), 75-76 min (50-95 % B), 76-85 min (95% B). Other parameters were set as described in the section of '**LC-MS/MS acquisition.**'

Orbitrap Exploris 480 acquisition for label-free quantitative proteomics

The samples obtained from *i*-FASP workflow were automatically loaded on a C18 trap column as described above and separated using 85 min gradient as follows: 0-15 min (10-20% B), 15-55 min (20-35% B), 55-80 min (35-50% B), 80-81 min (50-100 % B), 81-85 min (100% B). The peptides were analyzed using an Orbitrap Exploris 480 mass spectrometer with FAIMS device (Thermo Fisher Scientific). FAIMS separations were set as the following settings: 100 ℃ for inner and outer electrode temperature, 4 L/min for FAIMS gas flow, -45/-65 V for compensation voltage. The MS was operated in data-dependent acquisition mode with a full MS scan 350-1500 at R = 60, 000 (m/z = 200), RF Lens (%) = 50, followed by MS/MS scans at R = 15, 000 (m/z = 200), with an isolation width of 1.6 m/z. The maximum injection time for MS^1 and MS^2 were 20 ms and 30 ms. The precursors with charge states 2 to 7 with an intensity higher than 25, 000 were selected for HCD fragmentation, and the dynamic exclusion was set to 30 s. The normalized HCD collision energy was set as 30%.

Data Analysis

The raw data of the crosslinked standard peptides and BSA samples were searched by pLink 2.0 software[3] (version 2.3.9) to identify the crosslinking information. For the crosslinked standard peptides, acetyl on protein *N*-term was set as the fix modification. The database was the peptide sequence of SAKAYEHR. The MS/MS spectra were annotated by pLabel 2.0 software[4] (version 2.4.1). For BSA samples, BSA sequence downloaded from UniProtKB (P02769) was used as the database with a separate FDR ≤ 0.01 at peptide-spectrum match (PSM) level and searching results were manually filtered by *E*-value ≤ 0.0001, PSM ≥ 3 for the identified residue pairs. Other parameters were set in accordance with the cell samples searching method.

For label-free quantitative proteomics analysis, the raw data were analyzed using Maxquant (Version 1.6.5.0) against the Human UniprotKB database from April 2019 containing 42432 proteins. The search parameters were set as follows: 20 ppm for precursor mass tolerance, 20 ppm for fragment mass tolerance, 10 ppm for precursor filter tolerance, up to 2 missed cleavages for trypsin digestion, carbamidomethyl on [C] for fixed modification, oxidation on [M] and acetyl on Protein *N*-term for variable modification. LFQ algorithm was applied for protein quantification. The function of 'match between runs' was selected. The false discovery rate (FDR) estimated by a target-decoy approach was set to 1% at both the peptide and the protein level.

Structural refinement of HNRNPA1

The structure of RNA chain in original PDB file (4YOE) of HNRNPA1[5] was removed before the calculation. The structural refinement was performed using Xplor-NIH[6] software package with the overlength restraints. The maximum distance between two crosslinked Cβ atoms of 20 Å was adopted to refine the structure[7]. In addition, bond, angle, and improper energy term were also used during the calculation. The van der walls repulsion energy function was also used to prevent atom clash. As most of the amino acids involved in crosslinks were in the loop region of the protein, the non-loop region (that is, the amino acids formed secondary structure) was fixed during the calculation. The loop region moved freely under the guidance of CXMS restraints until all CXMS restraints were satisfied.

Supplemental Figures

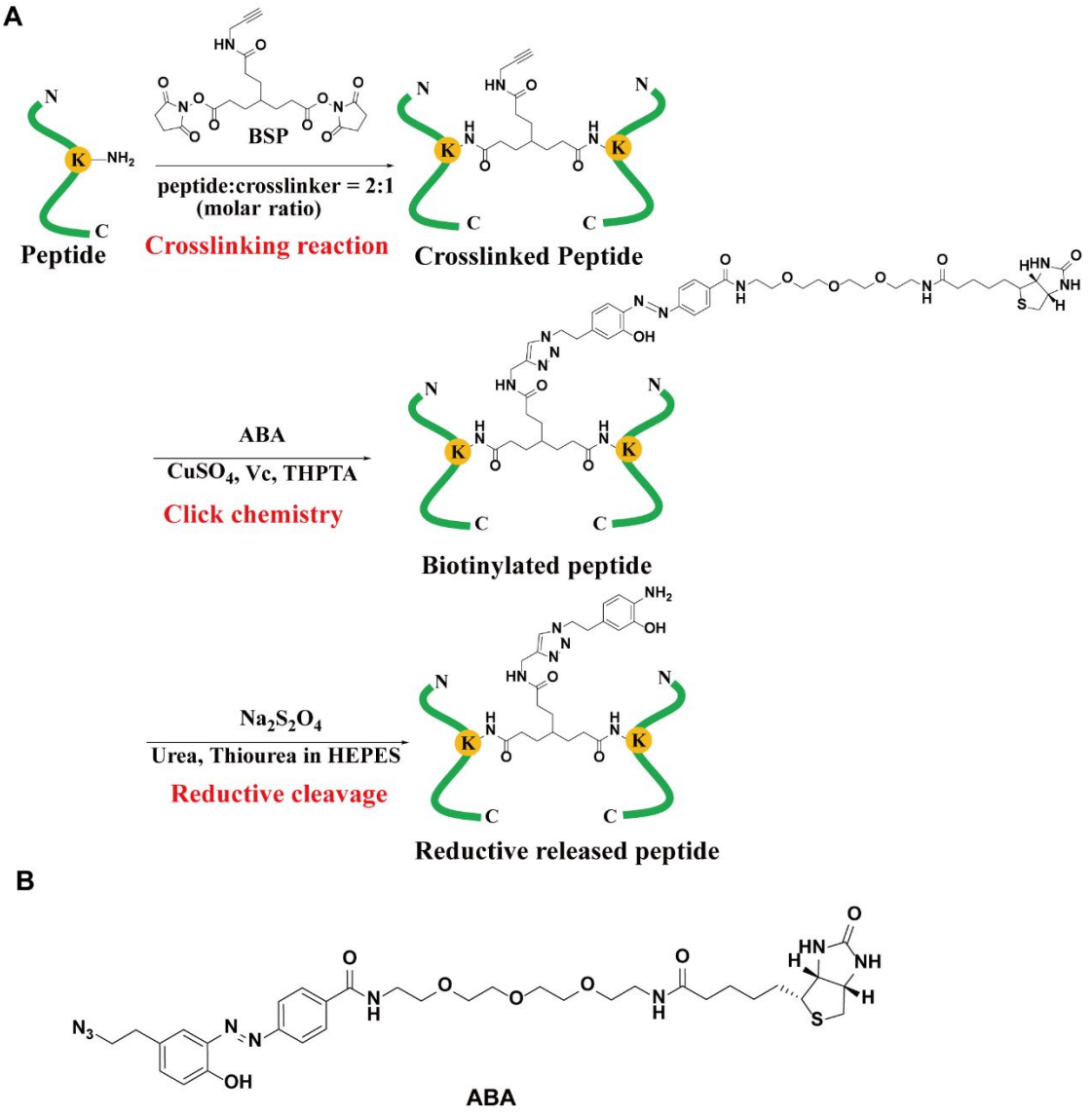


Figure S13. (A) Details of the chemical reactions, including chemical crosslinking, click chemistry and reduction cleavage. (B) The chemical structure of commercially available azo biotin−azide (ABA) probe.

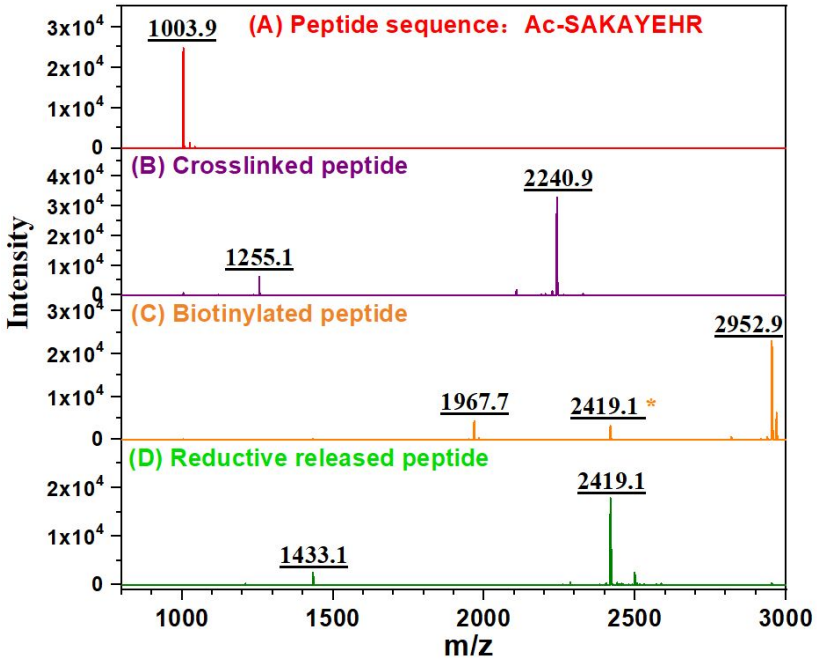


Figure S14. MALDI-TOF analysis of BSP crosslinked standard peptides. (A) MALDI mass spectra of the synthetic peptide Ac-SAKAYEHR (exact mass: 1002.4883). (B) MALDI mass spectra of the crosslinked peptide before click chemistry. The strong peak of 2240.9 was assigned to the crosslinked peptide (exact mass: 2240.0974, covalently binding two peptides with a linker mass of 235.1208). The weak peak of 1255.1 was belonged to the monolinked peptide (exact mass: 1254.6119, one peptide with a hydrolysis modification of 252.1236). (C) MALDI mass spectra of the biotinylated peptide after click chemistry. Similarly, the strong peak of 2952.9 (exact mass: 2951.4137, covalently binding two peptides with a linker mass of 946.4371) and the weak peak of 1967.7 (exact mass: 1965.9281, one peptide with a linker mass of 963.4398) were identified as the crosslinked peptide and hydrolyzed peptide, respectively. To be note that the MALDI laser can partially break the cleavable azo group, resulting in a weak peak of 2419.1 (marked with asterisk). (D) MALDI mass spectra of reductive released crosslinked peptide after enrichment. Similarly, the strong peak of 2419.1 (exact mass: 2951.4137, covalently binding two peptides with a linker mass of 413.2063) and the weak peak of 1433.1 (exact mass: 1965.9281, one peptide with a linker mass of 430.2090) were identified as the crosslinked peptide and hydrolyzed peptide, respectively. These results demonstrated better crosslinking and enrichment efficiency of BSP in the standard peptide level.

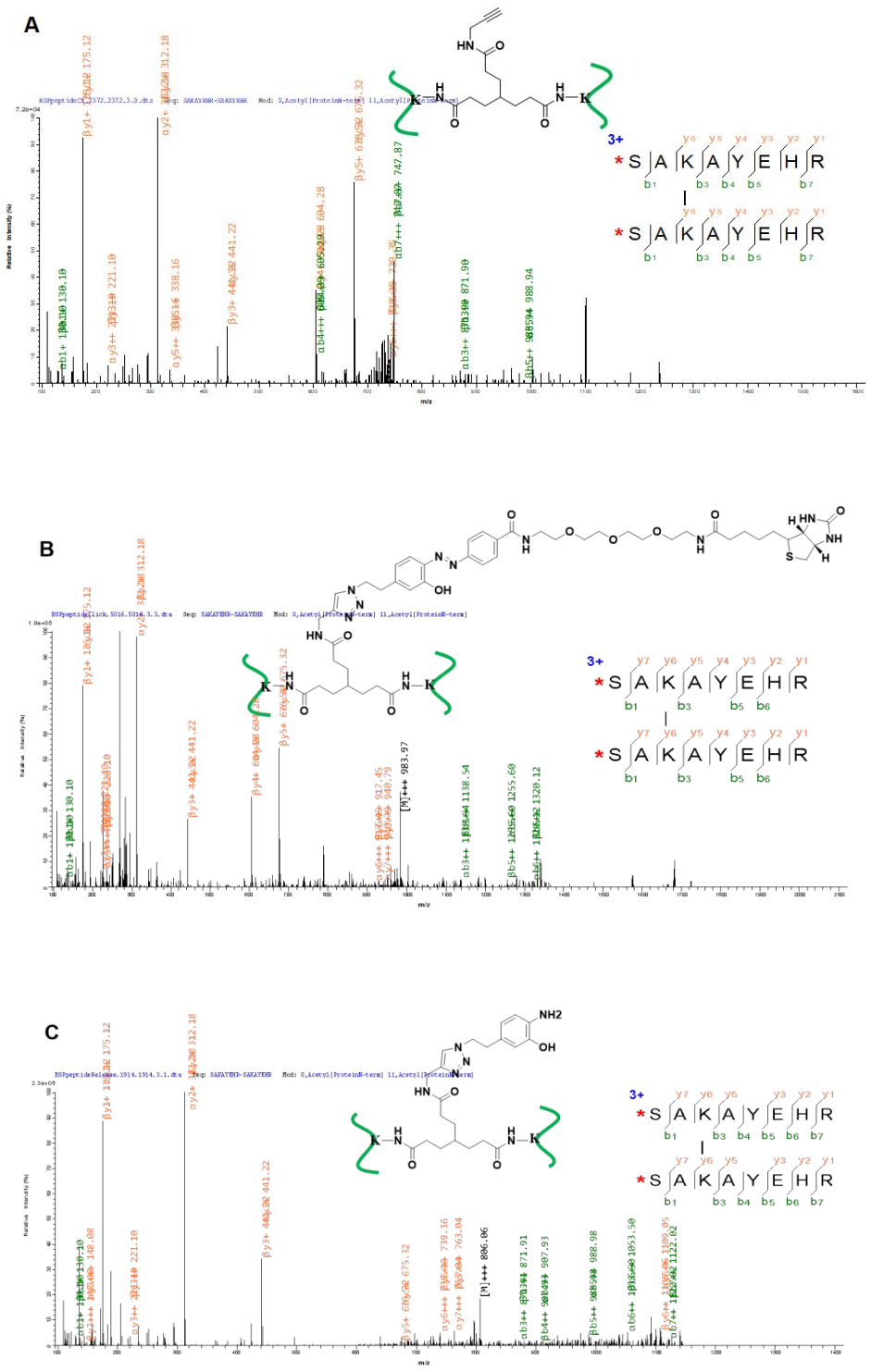


Figure S15. LC-MS/MS analysis of the crosslinked peptides. (A) MS^2 spectra of the crosslinked peptide before click chemistry. (B) MS^2 spectra of the biotinylated peptide after click chemistry. (C) MS^2 spectra of the reductive released crosslinked peptide after enrichment. The MS^2 peaks of 3^+ precursor ion corresponds to fragmentation of the amido bond of the crosslinked peptides, producing abundant b^+ ions and y^+ ions. Peak assignments are shown in orange if they correspond to y^+ ions, and green if they correspond to b^+ ions. These results demonstrated high quality of

fragment ion matches during LC-MS/MS analysis.

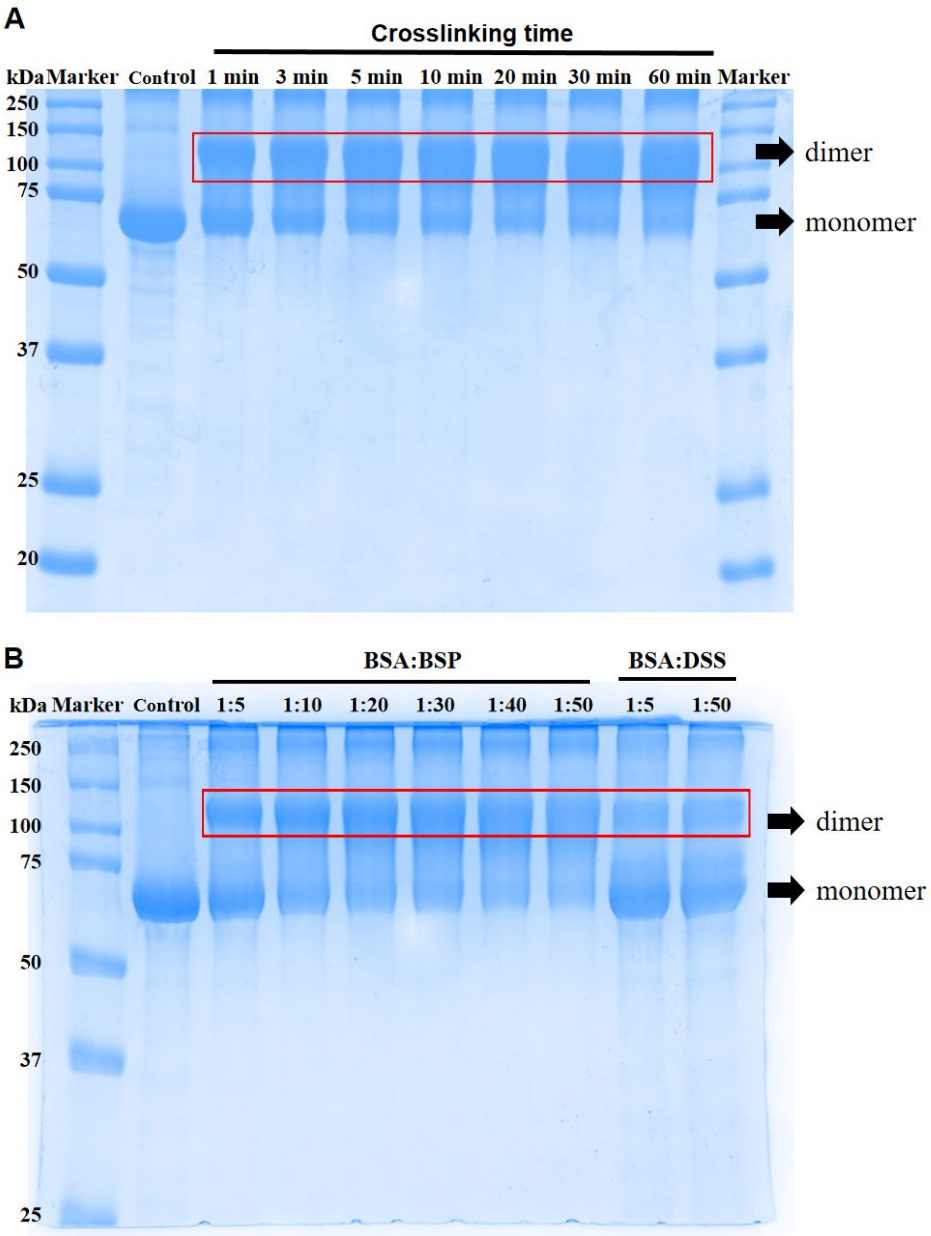


Figure S16. SDS-PAGE for BSP crosslinking of BSA. (A) Optimization of crosslinking time. Expected dimer bands were clearly detected on SDS-PAGE compared to the control in 5 min, and the proportion of dimer bands were not significantly increased with the increase of crosslinking reaction time. (B) optimization of the amount of BSP, and compared that with DSS at the same time. The single BSA bands gradually weakened with the increase amount of BSP. Besides, the crosslinking performance of BSP was obviously superior to that of DSS by comparing the dimer band. These results demonstrated better crosslinking efficiency (in minutes) of BSP in the protein level.

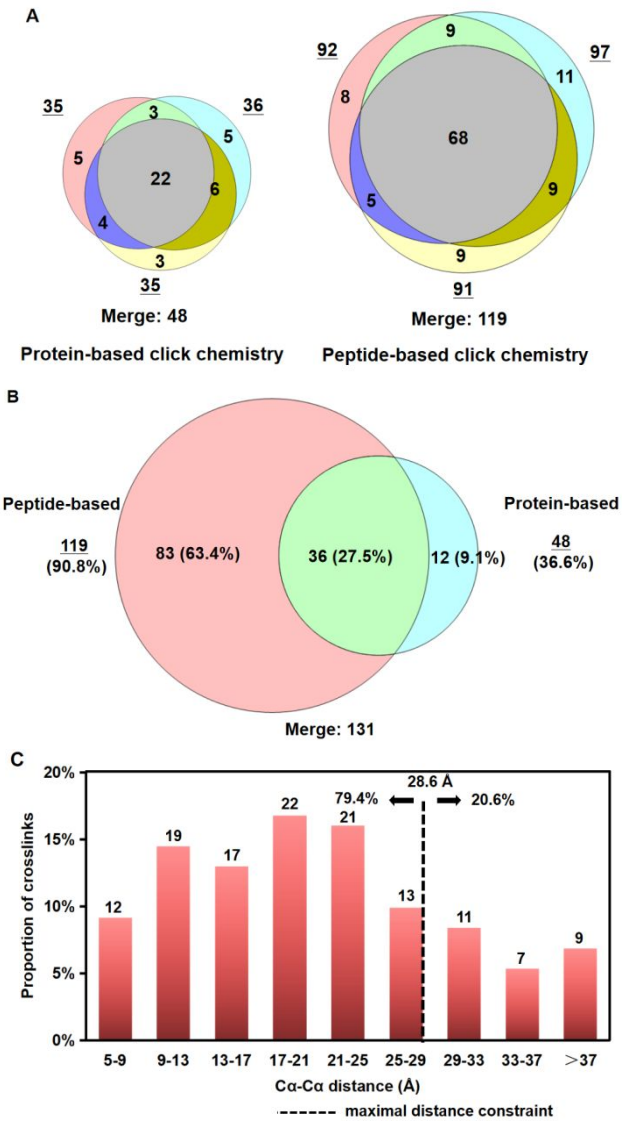


Figure S17. BSA data analysis. (A) Venn diagrams of the identified crosslinks in BSA from protein-based and peptide-based click chemistry enrichment with three replicates (1% FDR, E-value ≤ 0.0001, PSM ≥ 3). Briefly, 1.5-fold increase was obtained (119 vs 48) for crosslinked peptides identification by peptide-based click chemistry, compared to those by protein-based method, with more than 72% reproducibility for three technical replicates. (B) Comparison of the identified crosslinks in BSA from protein-based and peptide-based click chemistry enrichment. 75% (36 out of 48) of the crosslinked sites identified by protein-based method were commonly acquired by peptide-based method, while 63.4% of the total identifications were exclusively obtained with peptide-based method. (C) Histogram of the distribution of Cα-Cα solvent accessible surface distances of 131 BSP crosslinked residues, validated by comparison to the crystal structures of BSA (PDB: 3V03). The proportion of crosslinked K-K pairs within the maximum distance restraint imposed by BSP (28.6 Å) is 79.4%. These results demonstrated the better enrichment efficiency of the crosslinked peptides from BSA based on click reaction at the peptide level and good matches of BSP for structural architecture analysis.

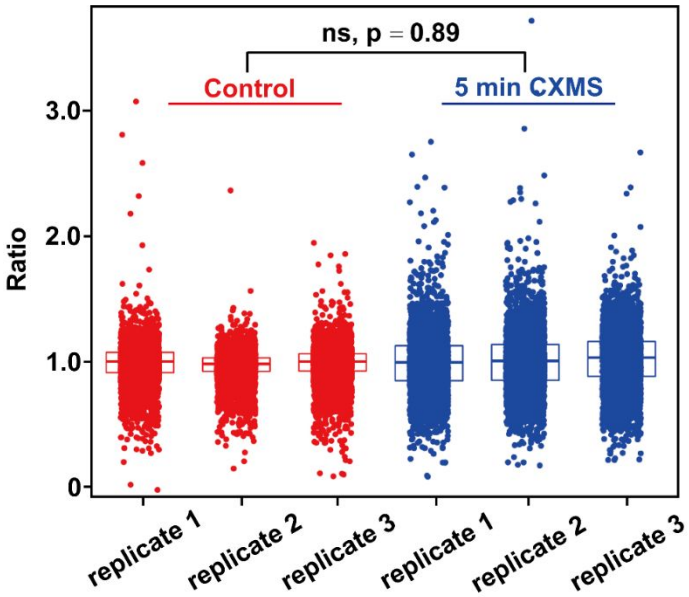


Figure S18. Label-free quantitative proteomics analysis during BSP *in vivo* crosslinking in 5 min (ns, no significance; 3 biological repeats). There were no obvious changes for protein expression during BSP *in vivo* crosslinking in 5 min compared with the control (ns: no significance, p = 0.86), which was important to acquire native assemblies of protein complexes in living cells.

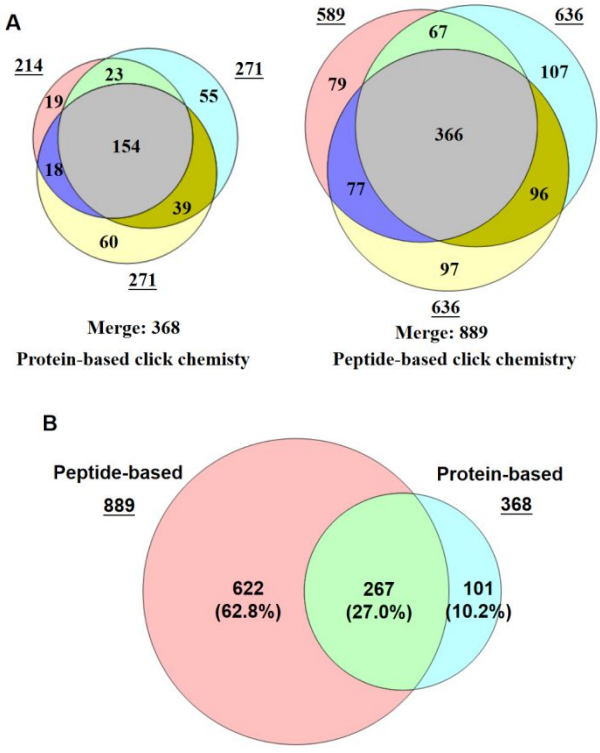


Figure S19. (A) Venn diagrams of the identified inter-crosslinking peptides in Bel-7402 cells from protein-based and peptide-based click chemistry enrichment without fractionation under 1% FDR control with three replicates. (B) Comparison of the identified crosslinks in Bel-7402 cells from

protein-based and peptide-based click reaction enrichment without peptide fractionation.

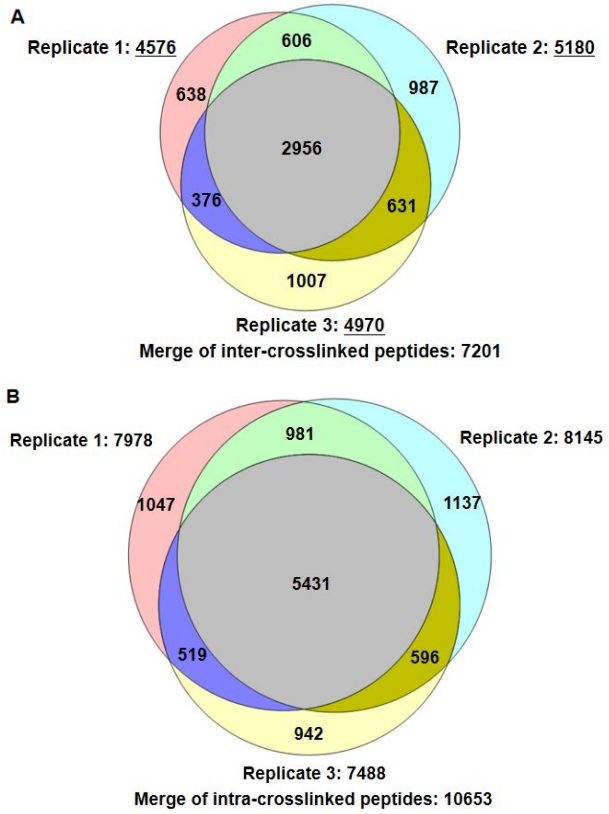


Figure S20. Venn diagrams of the identified inter-crosslinked peptides (A) and intra-crosslinked peptides (B) in Bel-7402 cells from peptide-based click chemistry enrichment with 20 fractions derived from high pH C18 fractionation under 1% FDR control with three replicates.

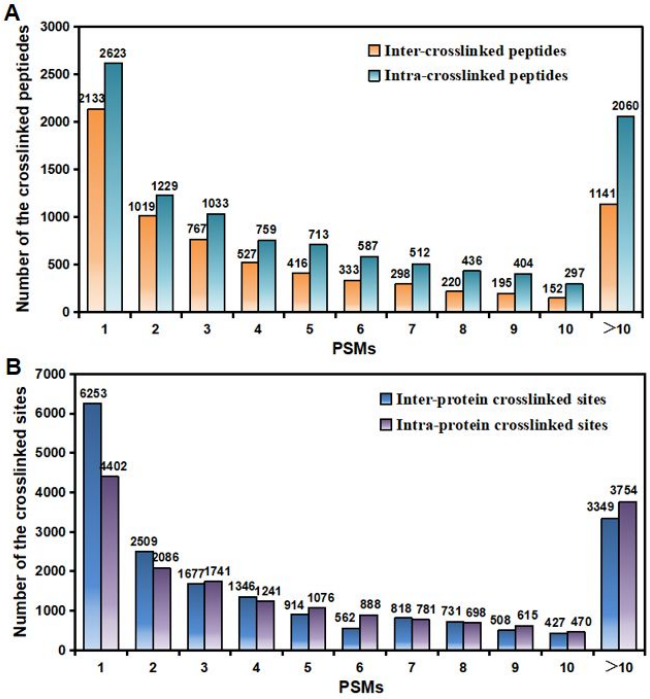


Figure S21. The PSM distribution of the crosslinked peptides (A) and the crosslinked sites (B) based on BSP *in vivo* CXMS data. In order to further improve the credibility of the identification results, we excluded the identifications with low confidence (PSM = 1), and controlled the data threshold

of PSMs ≥ 2 for identifications on the basic of the FDR setting of 1%.

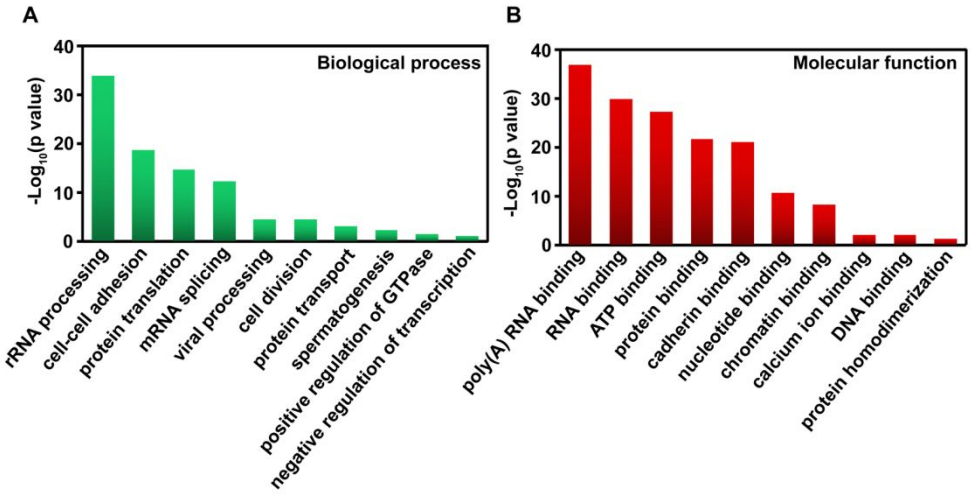


Figure S22. (A) Biological process and (B) Molecular function analysis of the crosslinked proteins according to GO annotation using DAVID program.

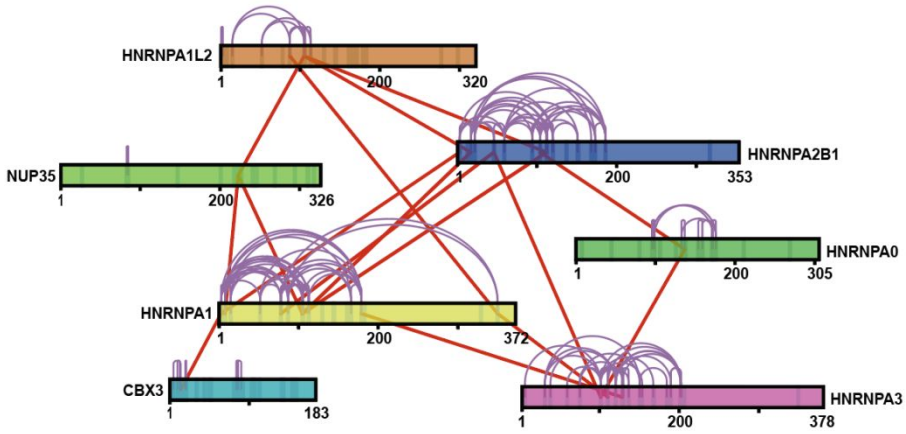


Figure S23. 2D crosslink map of identified interactions related to heterogeneous nuclear ribonucleoprotein. The inter-protein and intra-protein crosslinked pairs are labeled with red and purple lines, respectively.

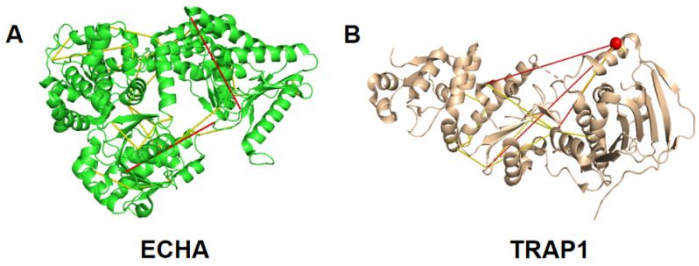


Figure S24. High confident crosslinks within crosslinking constraint (yellow dash line) and over-length crosslinks (red dash line) are mapped onto the X-ray structures of ECHA (mitochondria, 6DV2) and TRAP1 (mitochondria, 7C05). Euclidean distances were adopted for structural validity assessment.

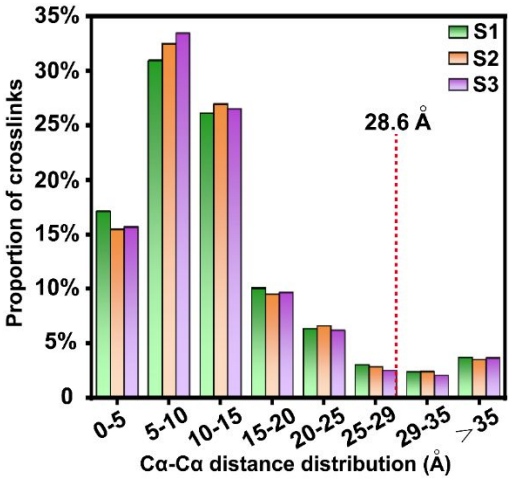


Figure S25. Histogram of the Cα-Cα distance distribution by mapping to the structures of the pre-translocation state (S1, PDB: 6Y0G), the rotated state (S2, PDB: 6Y2L) and the post-translocation state (S3, PDB: 6Y57) of human 80S ribosome[8] based on BSP *in vivo* CXMS data. Euclidean distances were adopted for structural validity assessment.

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