Supporting Information

Microarray immobilization of biomolecules using a fast trans-cyclooctene-tetrazine reaction

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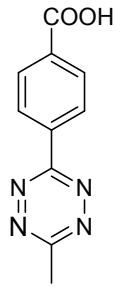
Contents

1. Material and Methods

1.1 Reagents and Instruments

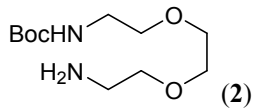
All chemicals and solvents were purchased from commercial companies and used directly without further purification, unless otherwise noted. All chemical reactions were performed under N_2 atmosphere, unless otherwise stated. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 μm thickness). TLC spots were visualized using $KMnO_4$, UV light or iodine method. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker model DPX-300 MHz NMR spectrometer. Chemical shifts are reported in parts per million with respect to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CHCl_3$ = 7.26 ppm, DMSO = 2.50 ppm). ^{1}H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet). Mass spectra were acquired on PC Sciex API 150 EX ESI-MS system. High resolution mass spectrum was carried out on ABI Qstar Elite Q-TOF.

1.2 Chemical Synthesis



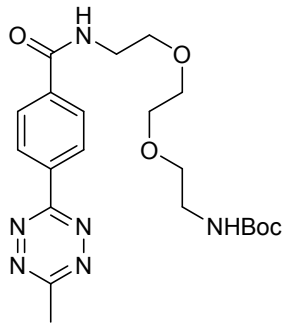
(**1**)

4-Cyanobenzoic acid (1.47 g, 10 mmol), zinc trifluoromethanesulfonate (1.82 g, 5 mmol) and methyl cyanide (5.22 ml, 100 mM) were mixed together in a round bottom flask.[1] Hydrazine monohydrate (24.3 ml, 0.5 M) was added into the solution in dropwise manner. The mixture was then allowed to stir at 60 °C under an Ar atmosphere for 24 hours. After the reaction, the mixture was cooled down, and a solution of sodium nitrite (13.8 g, 200 mM) in 50ml water was slowly added. Subsequently 1M HCl was added, and the mixture turned red and started evolving large amount of nitrogen oxide gas. The gas ceased when pH was adjusted to approximately 3.0. The red precipitate was collected by centrifugation, then washed with 1M HCl and further purified with chromatography (hexane/ethyl acetate = 15:1-10:1) to give compound **1** (1.51 g, 70%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.58 (d, J = 8.4 Hz, 2H), 8.20 (d, J = 8.4 Hz, 2H), 3.02 (s, 3H); LC-MS (Q-TOF) calcd for $[M+H]^+$:217.06, found: 217.06.



(**2**)

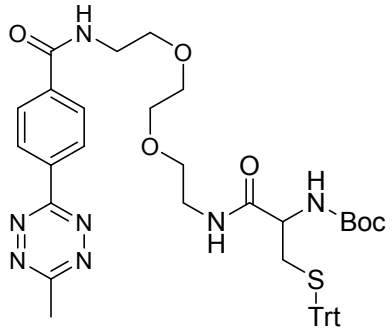
Di-tert-butyl dicarbonate (2.18 g, 10 mmol) was dissolved in 10 ml DCM in a round bottom flask and cooled down with ice-water bath. A solution of 1,2-Bis(2-aminoethoxy)ethane (3.0 g, 20 mmol) in 10 ml DCM was added into the reaction solution in dropwise manner. The mixture was stirred at 4 °C for 4h and then at room temperature for overnight. After the reaction was complete, the solvent was removed under reduced pressure. The resulting white solid was partitioned between 20% Na_2CO_3 and DCM. The DCM layer was separated and washed with water and brine respectively. Then it was dried with sodium sulfite and concentrated in vacuum to give the colorless oily product (1.78 g crude, 72%). ^{1}H NMR (500 MHz, CDCl3) δ 3.62-3.50 (m, 8H), 3.32 (t, J = 5.1 Hz, 2H), 2.88 (t, J = 5.1 Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (125 MHz, CDCl3) δ 155.9, 78.9, 73.0, 70.0, 41.4, 40.1, 28.2. LC-MS (ESI) calcd for $[M+H]^+$: 249.17, found: 249.18.



(**3**)

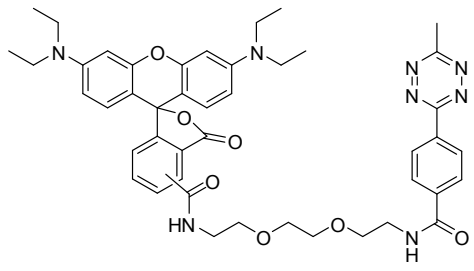
Compound **1** (480 mg, 2.22 mmol) and compound **2** (1100 mg, 4.44 mmol) were dissolved in 30 ml DMF, and stirred at room temperature overnight. Then DMF was evaporated by high vacuum pump at 50 °C. The mixture was redissolved in DCM, washed with water 3 times and dried with sodium sulfite. Then the solvent was evaporated under reduced pressure and purified with chromatography (DCM/MeOH = 97/3) to give compound 3 (849 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 (d, J = 8.4 Hz, 2H), 8.05 (d, J = 8.4

Hz, 2H), 3.72-3.60 (m, 8H), 3.59 (t, *J* = 5.0 Hz, 2H), 3.33 (t, *J* = 5.1 Hz, 2H), 3.14 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 167.45, 166.59, 163.49, 137.98, 127.85, 77.39, 76.97, 76.54, 70.17, 40.19, 28.31, 21.12; LC-MS (ESI) calcd for $[M+H]^+$:447.23, found: 447. 23.



(**4**)

Compound **3** (500 mg, 1.11 mmol) was dissolved in 10 ml DCM with 5% TFA and stirred at room temperature for 5 hours. Then TFA and DCM were removed under reduced pressure. The resulting compound was redissolved in DMF, following by addition of Boc-Cys(trt)-OH (772 mg, 1.67 mmol), HOBT (226 mg, 1.67 mmol), EDC (323 mg, 1.67 mmol) and Et_3N (234 ul, 1.67 mmol). The resulting mixture was allowed to stir at room temperature overnight. After the reaction was complete, DMF was evaporated under high vacuum at 50 °C and purified with chromatography (DCM/MeOH = 97/3) to give compound 5 (526 mg, 60%). ^{1}H NMR (400 MHz, MeOD) δ 8.54 (d, J = 8.4 Hz, 2H), 8.04 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 7.6 Hz, 6H), 7.23 (t, J = 7.2 Hz, 6H), 7.16 (t, J = 7.2 Hz, 3H), 4.02 (t, J = 6.4Hz, 1H), 3.70-3.58 (m, 8H), 3.51 (t, J = 5.2Hz, 2H), 3.36-3.31 (m, 2H), 3.01 (s, 3H), 2.58-2.48 (m, 2H), 1.41 (s, 9H); ^{13}C NMR (400 MHz, MeOD) δ 171.60, 167.66, 167.61, 163.32, 155.82, 144.55, 137.59, 134.73, 129.34, 127.94, 127.72, 127.51, 126.61, 117.25. 110.38, 79.52, 70.02, 69.30, 69.23, 66.58, 63.40, 53.85 39.74, 39.09, 34.18, 27.49, 24.04, 20.05; LC-MS (IT-TOF) calcd for $[M+H]^+$: 792.35, found: 792.35.



(5)

Compound **3** (446 mg, 1 mmol) was dissolved in 10 ml DCM with 5% TFA and stirred at room temperature for 5 hours. Subsequently TFA and DCM were removed under reduced pressure. TER (487 mg, 1 mmol) was dissolved in 10 ml DCM and activated with HOBT (203 mg, 1.5 mmol), EDC·HCl (288 mg, 1.5 mmol) and Et_3N (418 µl, 3 mmol) for 15 mins. Then deprotected compound 3 was added into the reaction solution and stirred at r.t. overnight. DCM was evaporated under reduced pressure and purified with chromatography (DCM/MeOH = 97/3) to give compound **5** (456 mg, 56%). ^{1}H NMR (400 MHz, MeOH) δ 8.60(s, 1H), 8.53(d, J = 2.4 Hz, 2H), 8.51 (d, J = 2.4 Hz, 2H), 8.17 (d, J = 8.0 Hz, 1H), 8.11 (d, J = 8.0 Hz, 2H), 8.01 (d, J = 8.8 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.81 (d, J = 1.6 Hz, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.11 (d, J = 8.6 Hz, 4H), 6.97-6.92 (m, 4H), 6.89 (d, J = 2.0 Hz, 2H), 6.84 (d, J = 2.4 Hz, 2H), 3.76-3.70 (m, 8H), 3.69-3.59 (m, 32H), 3.05 (s, 3H), 3.04 (s, 3H), 1.29 (t, J = 7.2 Hz, 24H); LC-MS (IT-TOF) calcd for [M+H]+: 815.38, found: 814.9.

1.3 Protein Expression, Purification and Labeling

Bacteria cultures were grown at 37 ºC overnight in LB medium supplemented with the corresponding antibiotics (50 µg/ml kanamycin and 34 µg/ml chloramphenicol for bromodomains; 100 µg/ml ampicillin for BRCT BRCA1, CHK2 FHA and 14-3-3 Sigma protein, 50 µg/ml kanamycin for SRC SH2 protein). The overnight culture was diluted in 1: 100 with fresh LB medium with antibiotic. The diluted culture was shaken at 37 ºC until the optical density (OD) reached 0.6~0.8 (OD600). Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added into culture to induce protein expression at 18 ºC for 16 h.

1.3.1 For His-tagged protein

Cells were harvested through centrifugation and lysed in lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole) by sonication (10 x pulses of 10 s each at half maximum power on ice). The cellular lysate was clarified by centrifugation at 13,000 rpm (30 min, 4 ºC) before incubation with Nickel-NTA agarose beads (Qiagen). Beads were washed with wash buffer (50 mM NaH_2PO4, 300 mM NaCl, 25 mM imidazole) followed by PBS. Then Cy3 N-hydroxysuccinimide ester (Amersham, G.E. Healthcare, USA) was added and incubated with the beads in PBS for 1 h on ice. Unreacted Cy3-NHS was quenched with excess of hydroxylamine for another 30 min. Following that, the labeled proteins were washed with PBS and eluted with elution buffer (50 mM NaH_2PO4, 300 mM NaCl, 300 mM imidazole). Removal of excess dye and imidazole was carried out by buffer exchange using 3 kDa MWCO centrifugal devices (Millipore) at 4 ºC according to manufacturer's protocols. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Protein purity was determined by separation on a 15 % SDS-PAGE gel, followed by in-gel fluorescence scanning.

1.3.2 For GST-tagged protein

For GST-tagged BRCT BRCA1 and 14-3-3 Sigma protein, the preparation process was similar to His-tagged protein except the following steps. GSH beads was used for purification, the cells were harvested and lysed in PBS buffer (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, *p*H = 7.4), PBS buffer with 50mM Glutathione was used as elution buffer. [2]

1.3.3 For intein-EGFP

pTwin1-intein-EGFP plasmid was transformed into an expression host ER2566. The transformed bacterial cells were grown in LB medium supplemented with ampicillin (100 µg/mL) at 37 ºC in an orbital shaker. A solution of IPTG (0.5 mM) was added to the cell culture when the cell density reaches 0.6. Cells were then left shaking over night at room temperature. Cells were harvested by centrifugation at 4,000 rpm for 30 min. The cell pellets collected were resuspended in lysis buffer (20 mM Tris buffer, pH = 8.5, 500 mM NaCl, 1mM EDTA) and lysed using sonication method on ice. Subsequently the cell debris was pelleted down by centrifugation at 13,000 rpm for 30 min at 4 ºC.

The column was added with chitin beads and washed with column buffer (20 mM Tris buffer, pH =8.5, 500 mM NaCl, 1mM EDTA). The cellular lysates were added onto the column and incubated for 30 min at 4 ºC. The beads were then washed with column buffer extensively. Subsequently 1 ml cleavage buffer (20 mM Tris buffer, pH = 7.0, 500 mM NaCl, 1mM EDTA) was added to the beads and incubated for 20 h at room temperature with slight agitation. The proteins were finally eluted out and dialyzed into PBS buffer (20 mM, pH = 7.4).

1.4 Slide Preparation

Glass slides (Sigma-Aldrich) were immersed into a piranha solution (sulfuric acid/hydrogen peroxide, 7:3) before slide derivatization. An amine handle was first introduced onto glass slides by reacting with (3-aminopropyl) triethoxysilane in ethanol. The amine slide was then used to react with TCO-$(PEG)_4$-NHS to obtain TCO-functionalized slide. These slides were then washed with ethanol, dried and ready for printing.

1.5 Microarray Preparation

For the small molecule immobilization experiment, different concentrations of small molecules in the spotting buffer (PBS: DMSO = 1:1) were prepared in 384 well before the spotting process. Various concentrations of proteins were prepared in PBS buffer prior to the spotting process. The spotting process was performed using an ESI SMA arrayer (Ontario, Canada) followed by incubation in a humidity chamber with different time period. After the incubation process, the slides were washed with PBST buffer, rinsed with deionized water and dried with nitrogen. The slides were finally scanned with a microarray scanner (Tecan Trading AG).

1.6 Peptide Synthesis

Peptides were synthesized using standard Fmoc chemistry with a microwave peptide synthesizer (Liberty 1). Rink amide resin was used as solid support. Amino acid residues and tetrazine building blocks were coupled to the resin through HOBT/HBTU/DIEA activation method. The Fmoc group was deprotected with 20% piperidine. At the end of the peptide coupling, the peptides were cleaved from the resin using a TFA cleavage cocktail (86% TFA, 5% EDT, 2% H_2O, 4% thioanisole and 3% phenol). The peptides were then purified by HPLC before slide coating process. The HPLC running condition is as follows: 2% B to 100% B in 0-7 min, then 100% B in 7-9 min, followed by 100% B to 2% B in 9-15 min (Phase A: Water with 0.1% TFA, Phase B: ACN with 0.1% TFA).

2. Supplementary Figures

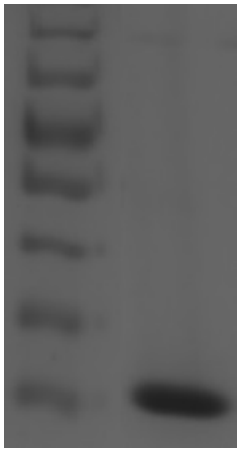


Fig. S1. SDS page analysis of pTwin1-EGFP.

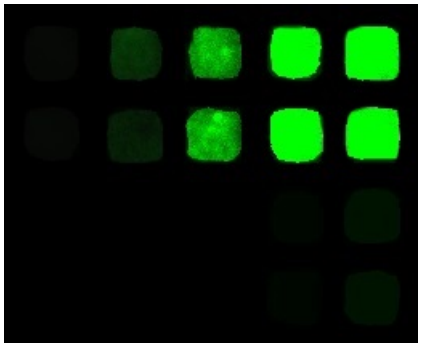


Fig. S2. Time course experiment of tetrazine-EGFP (from left to right: 1, 2, 5, 15, 30 min).

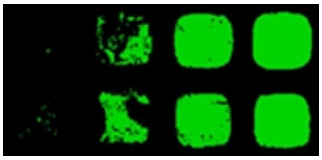


Fig. S3. Different concentrations of tetrazine- EGFP were immobilized onto TCO-functionalized slides (From left to right: 0.2 , 2, 20, 50 μM).

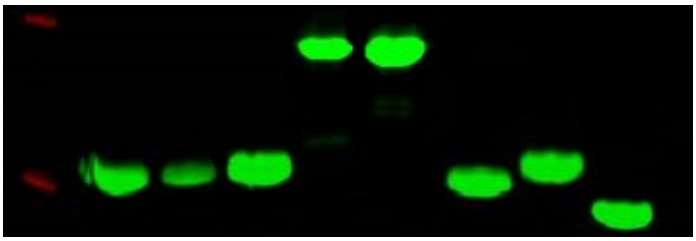


Fig. S4. SDS page analysis of eight PTM readers.

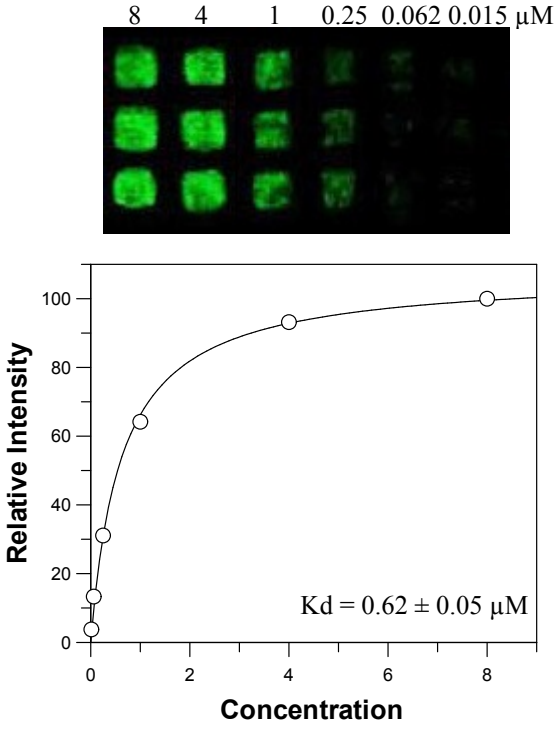
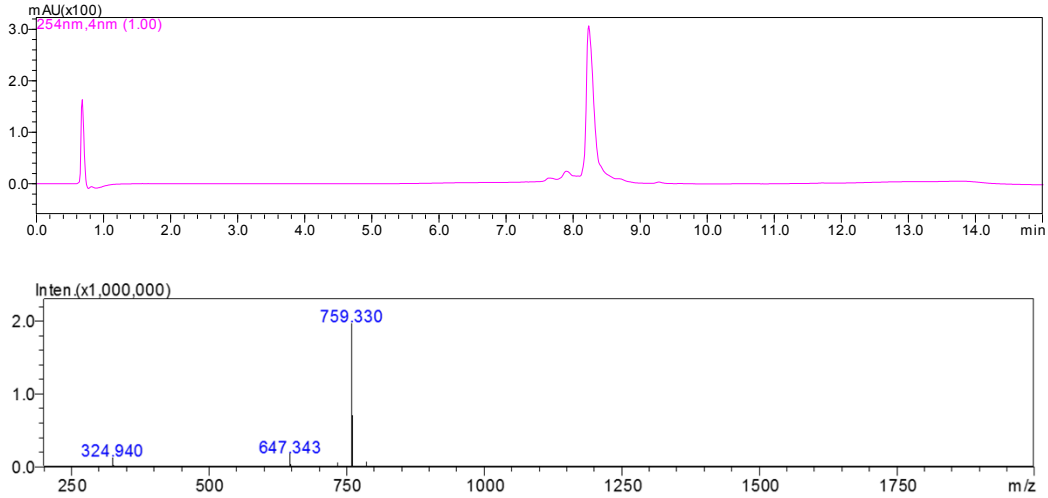
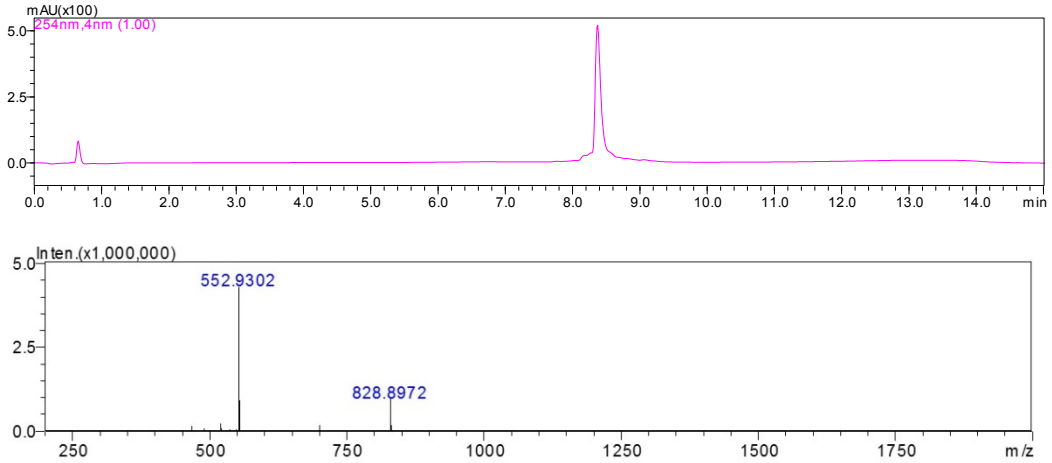


Fig. S5. Studies of binding between BRCT BRCA1 protein and its peptide ligand.

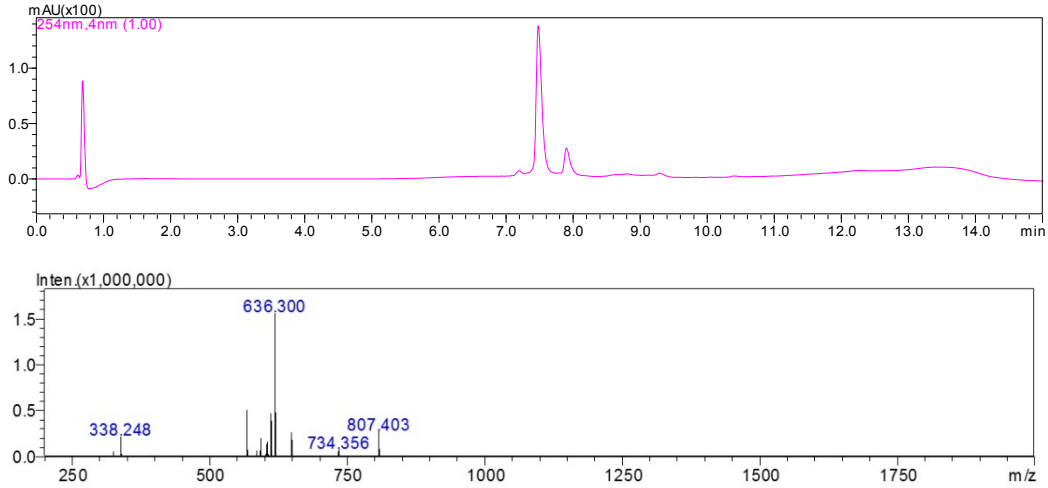
Peptide GGSRST(pS)PTFNK, expected Mw: 1516.3, observed Mw (M^{2+}): 759.3.



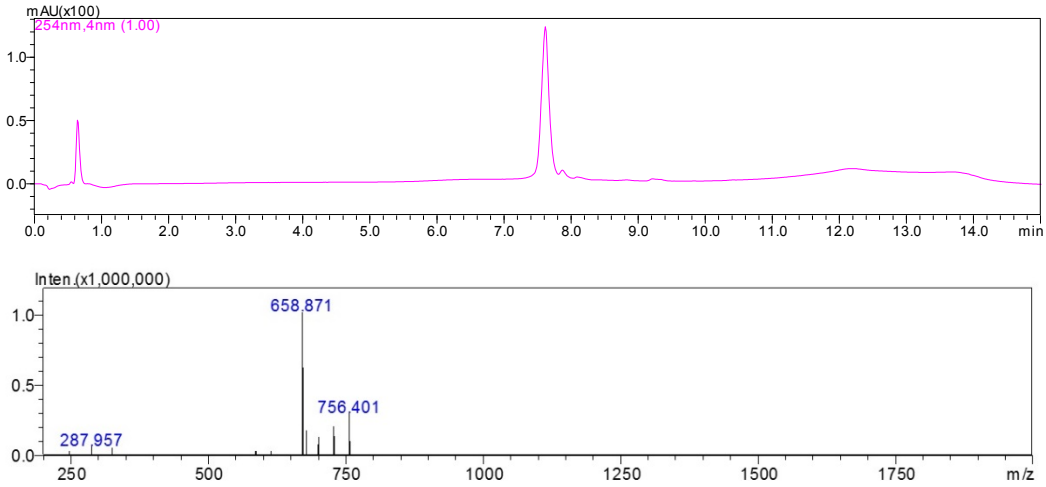
Peptide RHFD(pT)YLIRR, expected Molecule Weight: 1654.7, observed Mw (M^{2+}): 828.9.



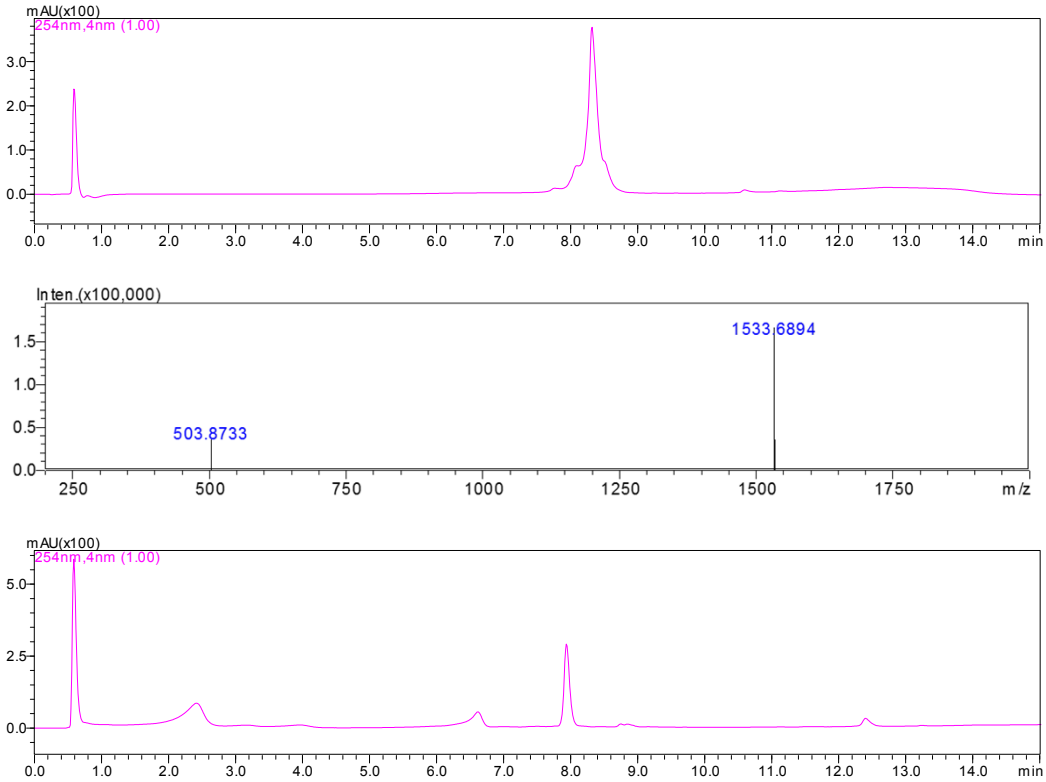
Peptide GGPI(pY)EEID, expected Molecule Weight: 1270.5, observed Mw (M^{2+}): 636.3.



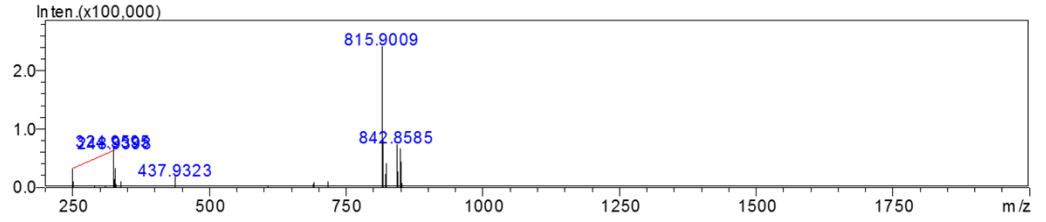
Peptide GGRFR(pS)YPP, expected Molecule Weight: 1314.5, observed Mw (M^{2+}): 658.9.



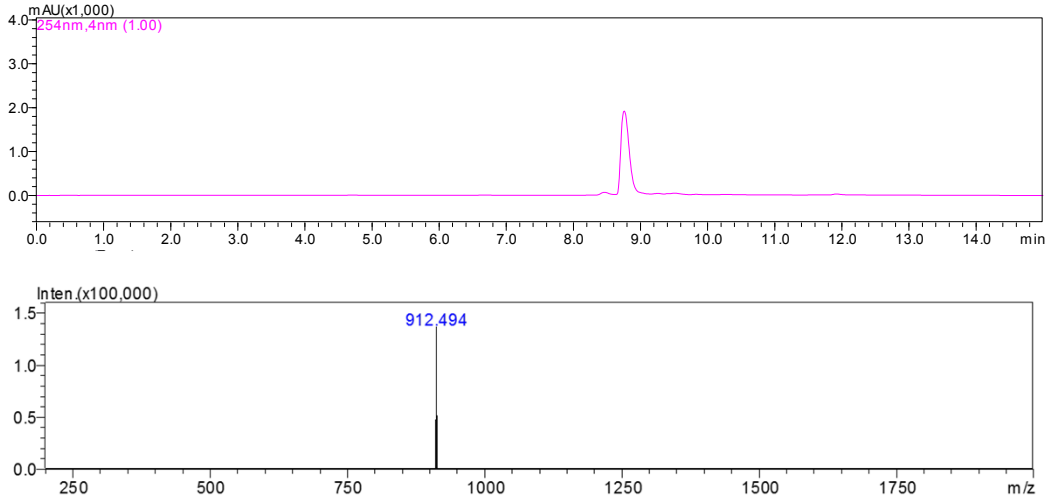
Peptide GGSGRG***Kac***GG***Kac***GLGY, expected Molecule Weight: 1532.7, observed Mw (M^{+}) 1533.7.



Peptide GGG***Kac***GGA***Kac***RHR***Kac***V, expected Molecule Weight: 1631.8, observed Mw (M^{2+}) 815.9.



Peptide GGKASG***Kac***G***KacKacKac***RGSN, expected Molecule Weight: 1825.8, observed Mw (M^{2+}): 912.5



Peptide GGART***Kac***QTARKSTGGKAPRKQLATKAA, expected Molecule Weight: 2980.6, observed Mw (M^{3+}): 993.9

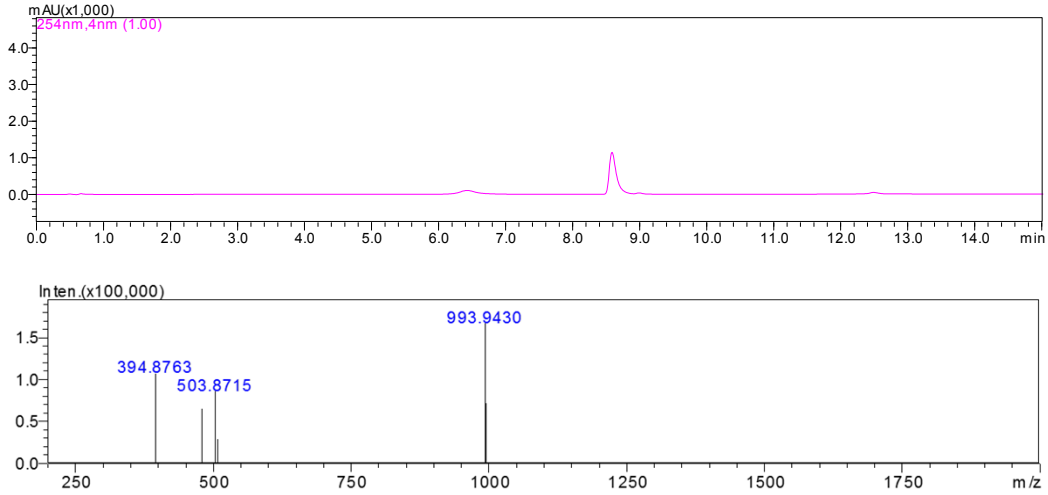


Fig. S6. LC-MS analysis of the peptides in this study.

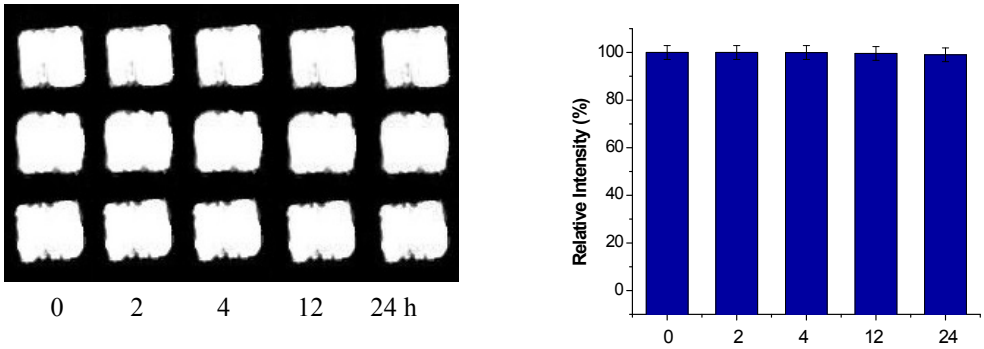
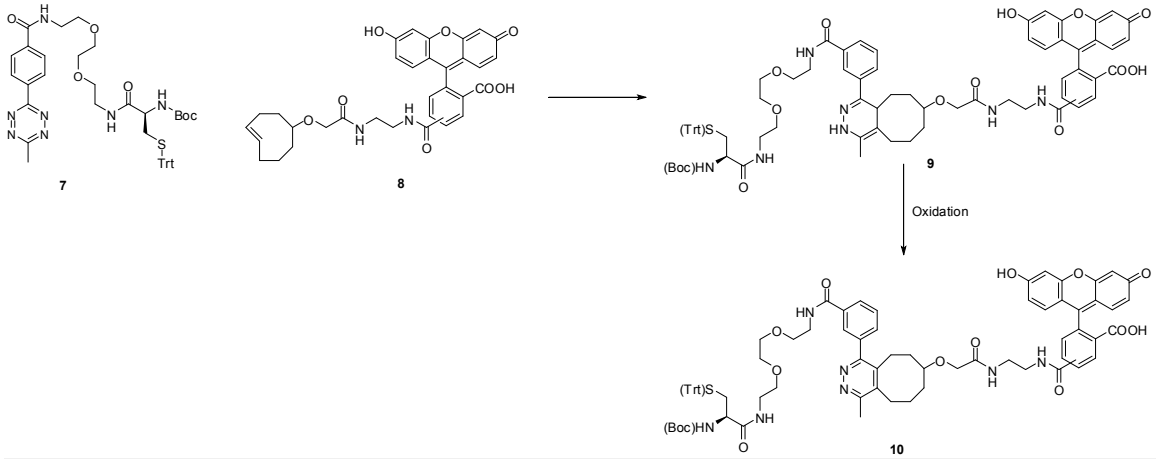
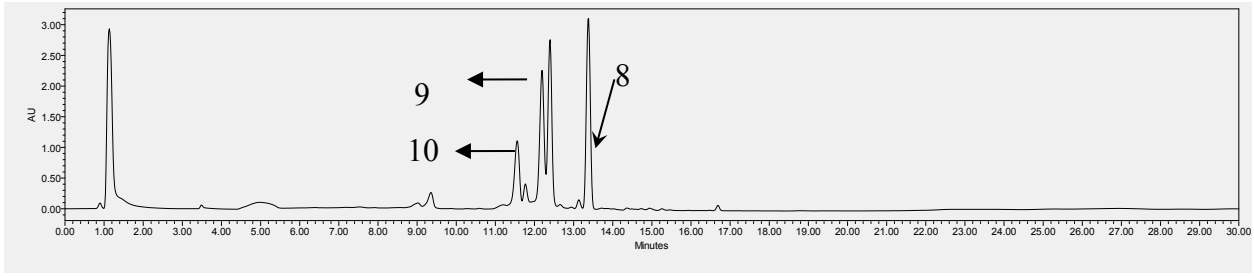
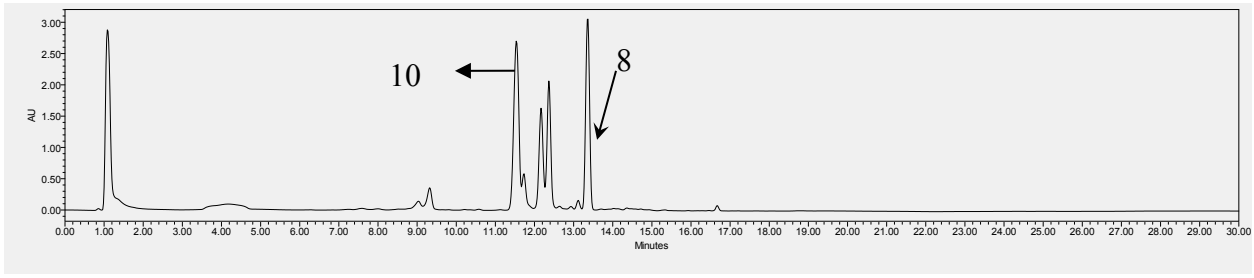


Figure. S7. Fluorescent intensity of the immobilized EGFP on slide after storing the slides at 4 ºC for different time intervals (2-24h).


7
8
9
Oxidation
10


9
10
8
AU
Minutes

5min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8
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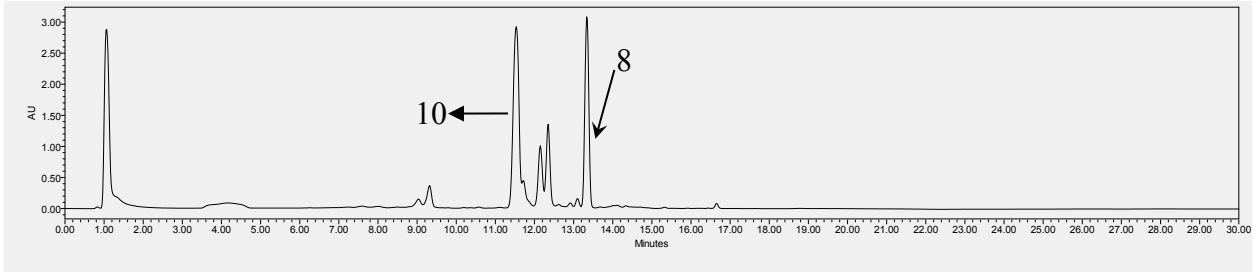
2h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10
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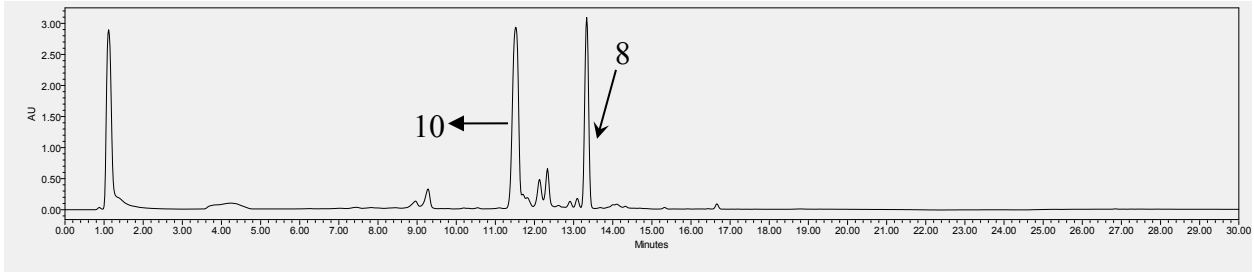
4h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10
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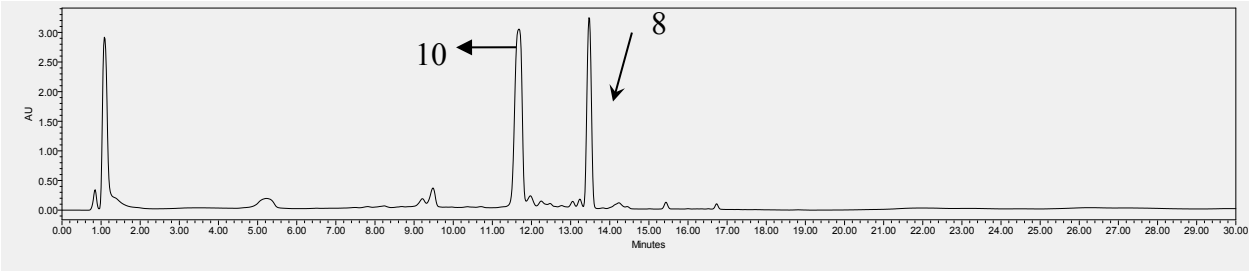
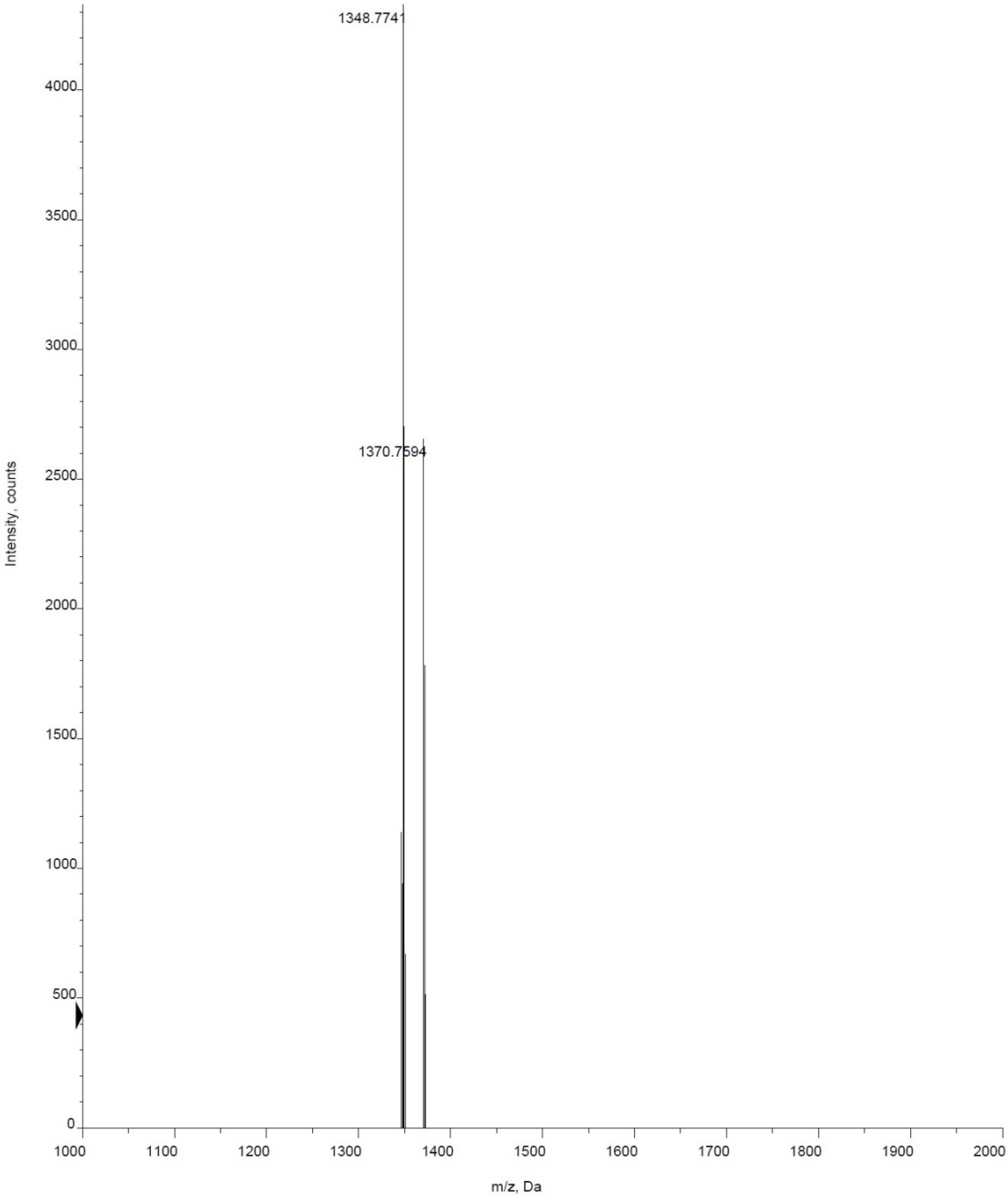
Minutes

8h



24h

Figure S8. HPLC analysis of the tetrazine ligation reaction in PBS:CH_3CN (1:1). TCO-FITC (**8**) is in excess. Four stereoisomers were formed at the beginning (5 min). As time increased, the ligated product underwent oxidation and was converted to compound **10** in the end. Nevertheless, the ligated product was stable. No hydrolyzed product was observed.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m/z, Da
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2000

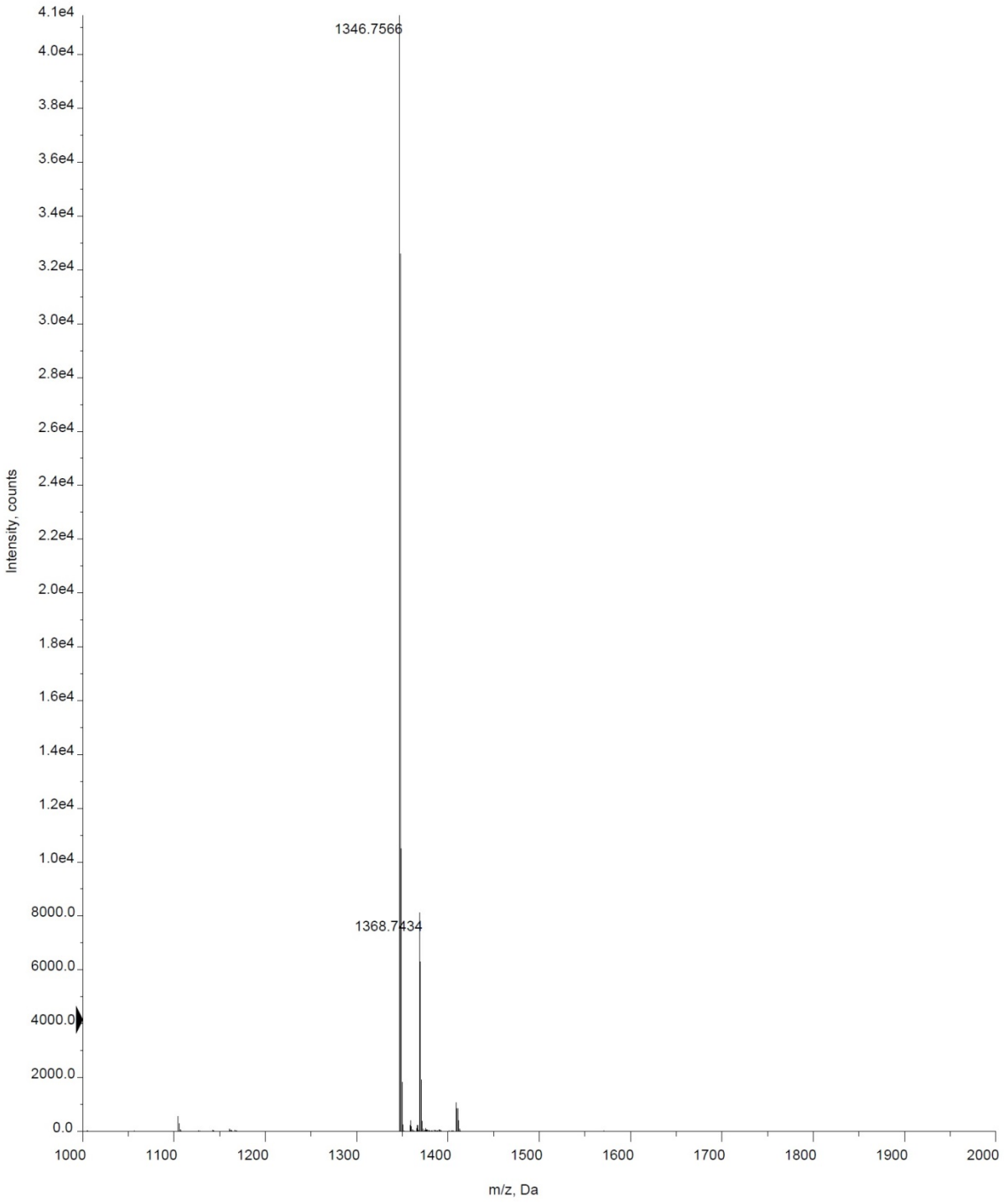

Figure S9. HRMS of the ligated product 9 and its oxidized product 10.

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