Electronic Supplementary Information for

Weaving a two-dimensional fishing net of Fe_3O_4 nanocrystals-embedded titanoniobate nanosheet for highly efficient capture and isotope labeling of phosphopeptides

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Chemicals and materials. Trifluoroacetic acid (TFA), formic acid (FA), 2,5-dihydroxybenzoic acid (2,5-DHB), trypsin (from bovine pancreas, TPCK treated), 4-nitrophenyl phosphate di(tris) salt (pNPP), α-casein (from bovine milk), β-casein (from bovine milk), bovine serum albumin (BSA), formaldehyde (CH_2O, 37 wt% in H_2O), formaldehyde (CD_2O, 20 wt% in D_2O, 98 atom% D), and sodium cyanoborohydride ($NaBH_3CN$) were all purchased from Sigma (St. Louis, MO). Titanium(IV) oxide (TiO_2), niobium(V) oxide (Nb_2O_5), potassium carbonate (K_2CO_3), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), and 10 % tetrabutyl ammonium hydroxide (TBAH) were obtained from Shanghai Reagent Co. (Shanghai, China). Acetonitrile (ACN) was chromatographic grade from Merck (Darmstadt, Germany), and all other chemicals were of analytical grade.

Instrumentation and characterization. Transmission electron microscopy (TEM) images were acquired on a JEM 1011 (JEOL 2010) electron microscope at an accelerating voltage of 200 kV. Scanning electron microscopy (SEM) was done on a Hitachi S-4800 field emission electron microscope at an accelerating voltage of 5 kV. UV-vis spectra were recorded on a UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan). XRD patterns were collected on a ARL X'TRA X-ray diffractometer using a Cu-Kα radiation (λ = 0.15405 nm). X-ray photoelectron spectroscopy (XPS) analyses were carried out on a PHI5000 VersaProbe photoelectron spectrometer (ULVAC-PHI, Japan). Nitrogen adsorption-desorption isotherms were recorded on a Micromeritics ASAP 2020M automated sorption analyzer. The samples were degassed at 100 ºC for 12 h.

Superconducting quantum interference device (SQUID, Quantum Design) magnetometer was used in the magnetic measurement at 300 K. Zeta potential was tested on a Nano-Z zeta potential analyzer (Malvern Instruments, USA). Fe elemental analysis was carried out on a J-A1100 ICP-AES system.

Tryptic digestion of standard proteins. 1 mg α-, β-casein, and BSA were respectively dissolved in 1 mL 50 mM ammonium bicarbonate buffer solution (pH=8.1), denatured in 100 °C for 5 min and incubated with trypsin at the enzyme-to-substrate ratio of 1:20 (w/w) in water bath at 37 °C for 16 h.

Adsorption of *p*-nitrophenylphosphate (pNPP) on layered materials, H^+-stacked nanosheets and Fe_3O_4-TiNbNS series. For each, 2 mg affinity material was mixed with 1 mL *p*-nitrophenylphosphate (pNPP) solution in 1 % (v/v) TFA 50 % (v/v) ACN at a concentration ranging from 0.02 mg/mL to 0.2 mg/mL. After vibration for 30 min, supernatant was collected by centrifugation at 12000g for 3 min (layered materials and H^+-stacked nanosheets) or magnetic separation (Fe_3O_4-TiNbNS series), and then subjected to UV-vis analysis. The absorbance at the wavelength of 285 nm was monitored and selected as the data source for calculating the remaining pNPP in the supernatant according to the calibration curve.

Mass spectrometry. For MALDI-TOF MS analysis, 1 μL resulting solution and 1 μL 2,5-dihydroxybenzoicacid (DHB) solution (25 mg/ml in 70 % ACN) containing 1% H_3PO_4 (v/v) were deposited in turn onto MALDI target.

MALDI-TOF MS experiments were performed on a 4800 Plus MALDI-TOF/TOF Mass Spectrometer (AB Sciex) equipped with a pulsed Nd:YAG laser operated at 355 nm in positive reflection mode. A ground-steel sample target with 384 spots was utilized. MS spectra were collected by the average of 16 sub-spectra acquired from the edge bias of DHB matrix spot with sum of 25 laser shots per sub-spectrum.

Table S1. Sequence information of phosphopeptides captured from tryptic digests of β-casein by Nb_2O_5, TiO_2, HNb_3O_8, $HTiNbO_5$, H^+-stacked NbNS, H^+-stacked TiNbNS, and Fe_3O_4-TiNbNS composites.

No.	Peptide sequence	Number of phosphoryl groups	Observed m/z
β1	FQ[pS]EEQQQTEDELQDK	1	2061.80
β2	IEKFQ[pS]EEQQQTEDELQDK	1	2431.98
β3	FQ[pS]EEQQQTEDELQDKIHPF	1	2556.03
β4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR	4	3122.16

[pS]: phosphorylated serine.

Table S2. Sequence information of phosphopeptides captured from tryptic digest of α-casein by Fe_3O_4-TiNbNS-4-6.

Peptide No.	Peptide sequence	Number of phosphoryl groups	Observed m/z
α1	TVDME[pS]TEVF	1	1237.59
α2	TVD[Mo]ME[pS]TEVF	1	1253.60
α3	TVDME[pS]TEVFTK	1	1466.75
α4	TVD[Mo]E[pS]TEVFTK	1	1482.73
α5	EQL[pS]T[pS]EENSKK	2	1539.69
α6	VPQLEIVPN[pS]AEER	1	1660.89
α7	DIGSE[pS]TEDQA[Mo]EDIK	1	1863.78
α8	DIG[pS]E[pS]TEDQAMEDIK	2	1927.75
α9	DIG[pS]E[pS]TEDQA[Mo]EDIK	2	1943.76
α10	YKVPQLEIVPN[pS]AEER	1	1952.02
α11	VNEL[pS]KDIG[pS]E[pS]TEDQAMEDIK	3	2678.91
α12	VNEL[pS]KDIG[pS]E[pS]TEDQA[Mo]EDIK	3	2694.94
α13	Q*MEAE[pS]I[pS][pS] [pS]EEIVPN[pS]VEAQK	5	2703.87
α14	QMEAE[pS]I[pS][pS][pS]EEIVPNPN[pS]VEQK	5	2720.84
α15	KEKVNEL[pS]KDIG[pS]E[pS]TEDQAMEDIKQ	3	2935.98
α16	KEKVNEL[pS]KDIG[pS]E[pS]TEDQA[Mo]EDIKQ	3	2951.94
α17	NANEEEYSIG[pS][pS][pS]EE[pS]AEVATEEVK	4	3007.97
α18	NANEEEY[pS]IG[pS][pS][pS]EE[pS]AEVATEEVK	5	3088.41

[pS]: phosphorylated serine;

[Mo]: oxidation on methionine;

*Pyroglutamylation on the N-terminal Q.

Table S3. The percentage of 4 methylated products of peptide β1 after different *in situ* labeling reaction times.

Reaction time	Percentage of 1-methyl-β1 (%)	Percentage of 2-methyl-β1 (%)	Percentage of 3-methyl-β1 (%)	Percentage of 4-methyl-β1 (%)
10 min	2.37	19.21	8.67	69.76
20 min	2.18	9.21	7.22	81.39
30 min	2.72	3.02	6.11	88.15
40 min	2.03	0.00	4.91	93.06
50 min	0.61	0.00	6.00	93.39
60 min	3.09	0.00	2.82	93.64
2 h	1.62	0.00	4.68	93.70
4 h	2.34	0.00	4.69	92.97
8 h	1.98	0.00	5.28	92.74
12 h	1.79	0.00	3.53	94.67
24 h	1.43	0.00	4.02	94.55

Table S4 Sequence information of endogenous phosphopeptides captured from human serum by Fe_3O_4-TiNbNS-4-6 and labeled by CH_2O and CD_2O according the *in situ* labeling strategy.

No.	Peptide sequence	Number of phosphoryl groups	Original mass (m/z)	Labeled by CH_2O (m/z)	Labeled by CD_2O(m/z)
HS1	D[pS]GEGDFLAEGGGV	1	1389.643	1417.535	1421.524
HS2	AD[pS]GEGDFLAEGGGV	1	1460.704	1488.572	1492.553
HS3	D[pS]GEGDFLAEGGGVR	1	1545.762	1573.654	1577.614
HS4	AD[pS]GEGDFLAEGGGVR	1	1616.801	1644.672	1648.655

[pS]: phosphorylated serine.

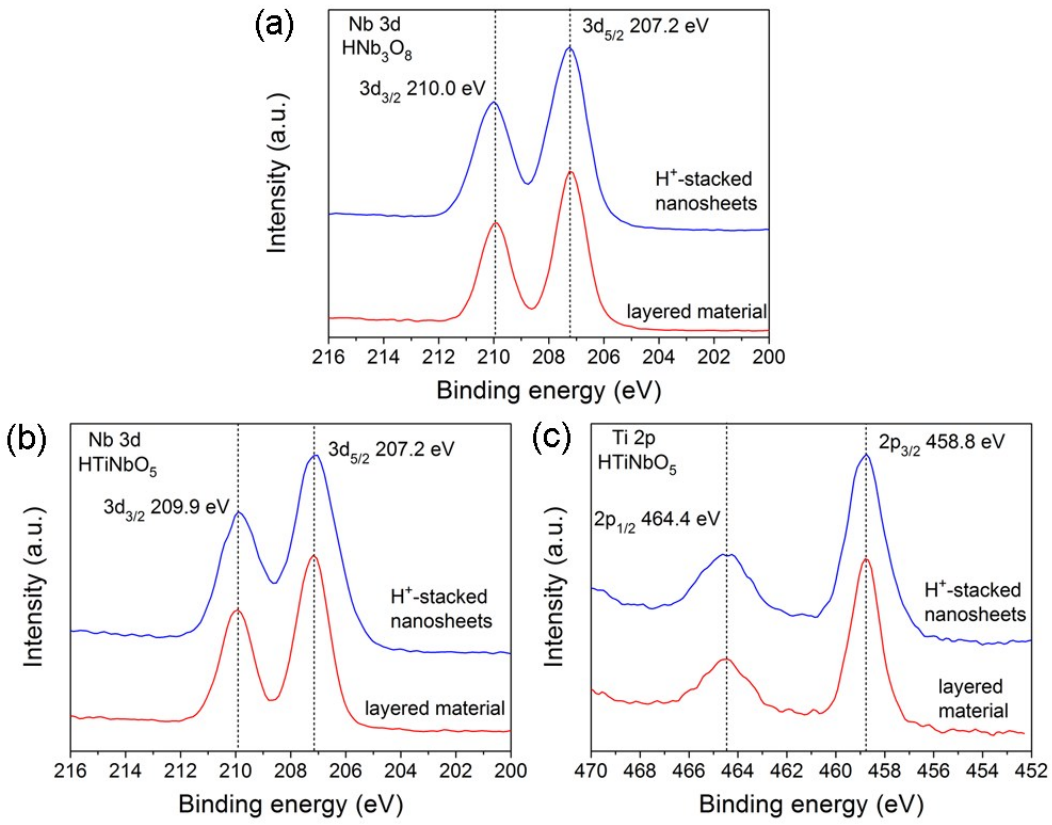

Fig. S1 XPS spectra of (a) Nb 3d in layered oxide HNb_3O_8, (b) Nb 3d and (c) Ti 2p in layered oxide $HTiNbO_5$ before and after exfoliation.

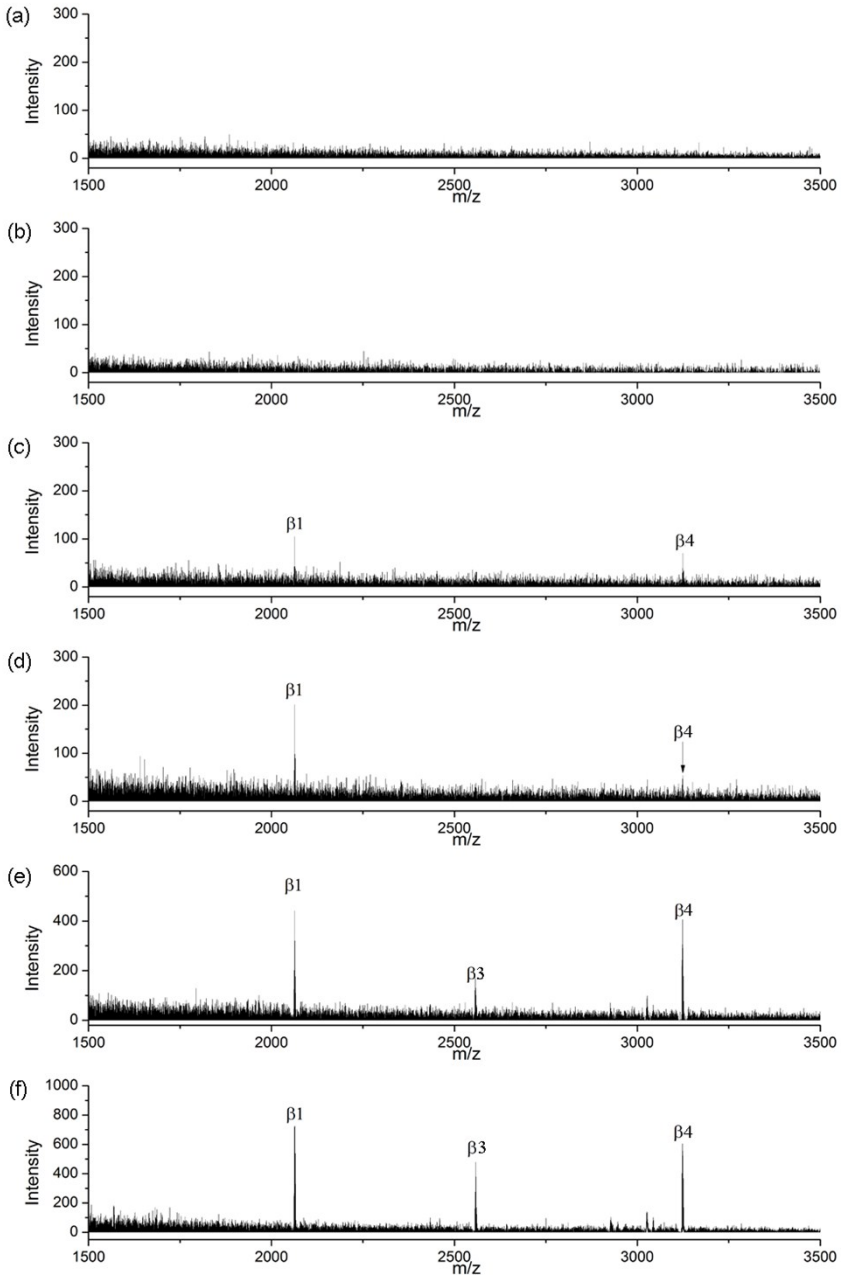

Fig. S2 MALDI-TOF MS spectra of tryptic digests of β-casein (160 fmol) enriched by (a) Nb_2O_5, (b) TiO_2, (c) HNb_3O_8, (d) $HTiNbO_5$, (e) H^+-stacked NbNS, and (f) H^+-stacked TiNbNS.

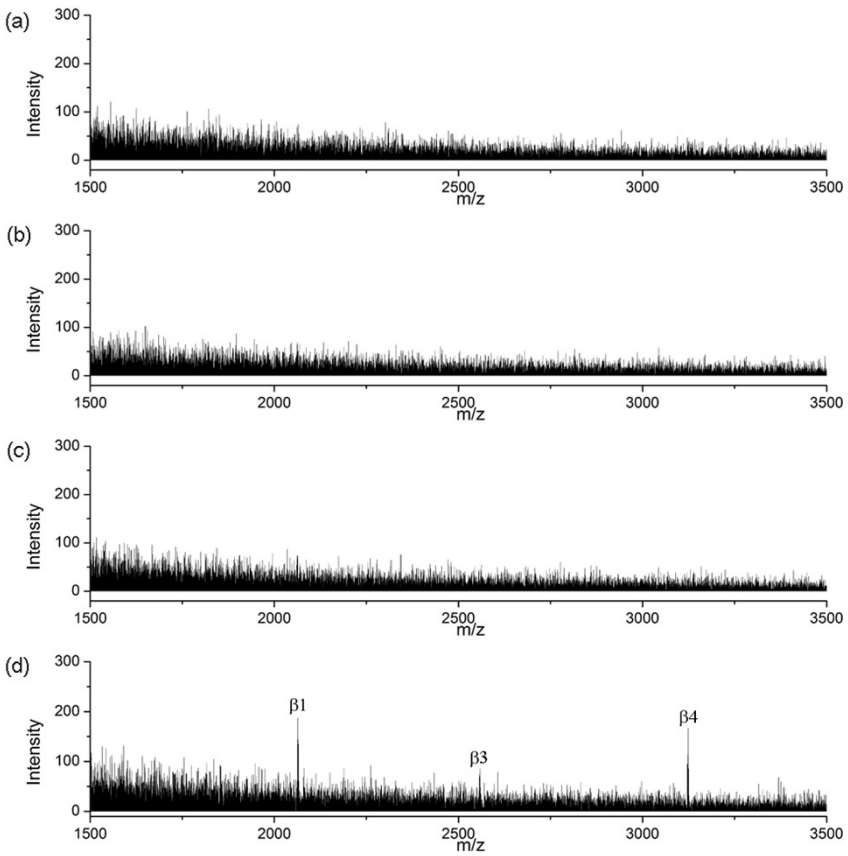

Fig. S3 MALDI-TOF MS spectra of tryptic digests of β-casein (40 fmol) enriched by (a) HNb_3O_8, (b) $HTiNbO_5$, (c) H^+-stacked NbNS, and (d) H^+-stacked TiNbNS.

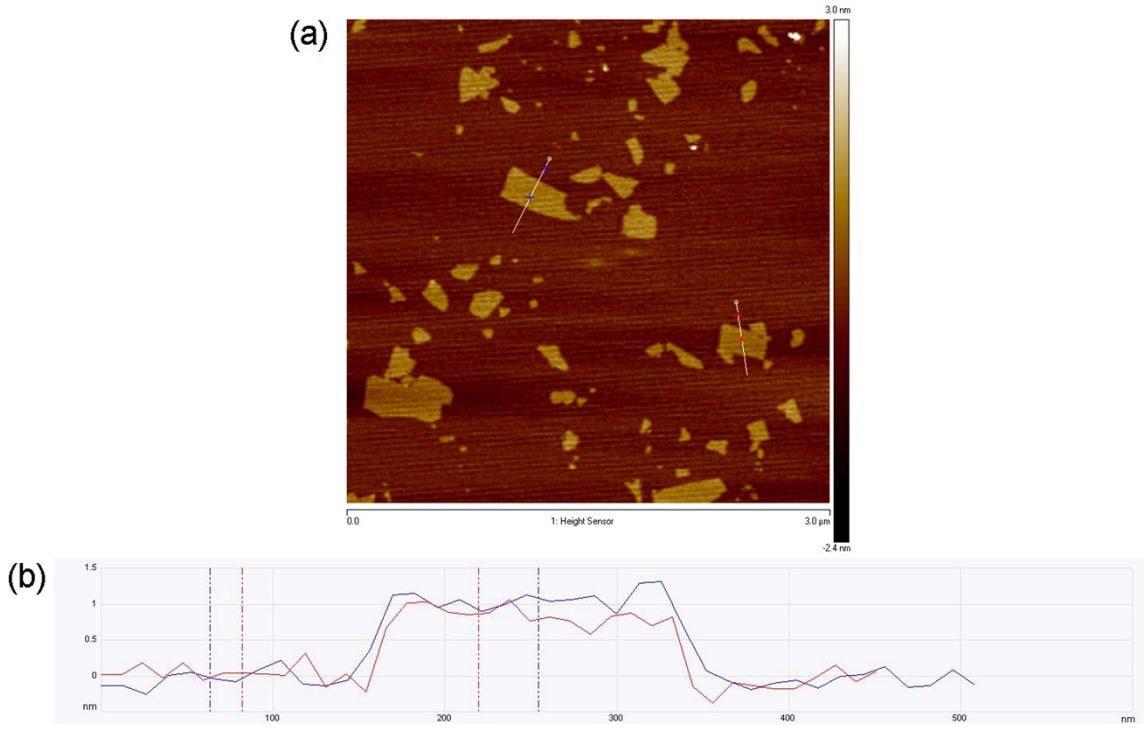

Fig. S4 (a) AFM image of TiNbNS and (b) cross-section analysis along the chosen lines.

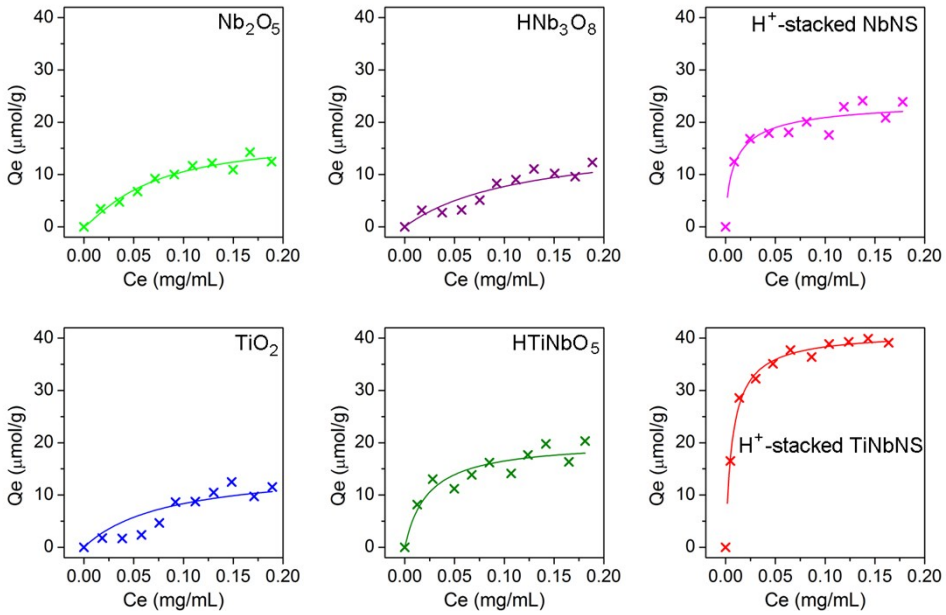

Fig. S5 Saturated adsorption isotherm for pNPP adsorbed on Ti/Nb based bulk oxides, layered oxides and H^+-stacked nanosheets. Ce: equilibrium concentration; Qe: amount adsorbed at equilibrium.

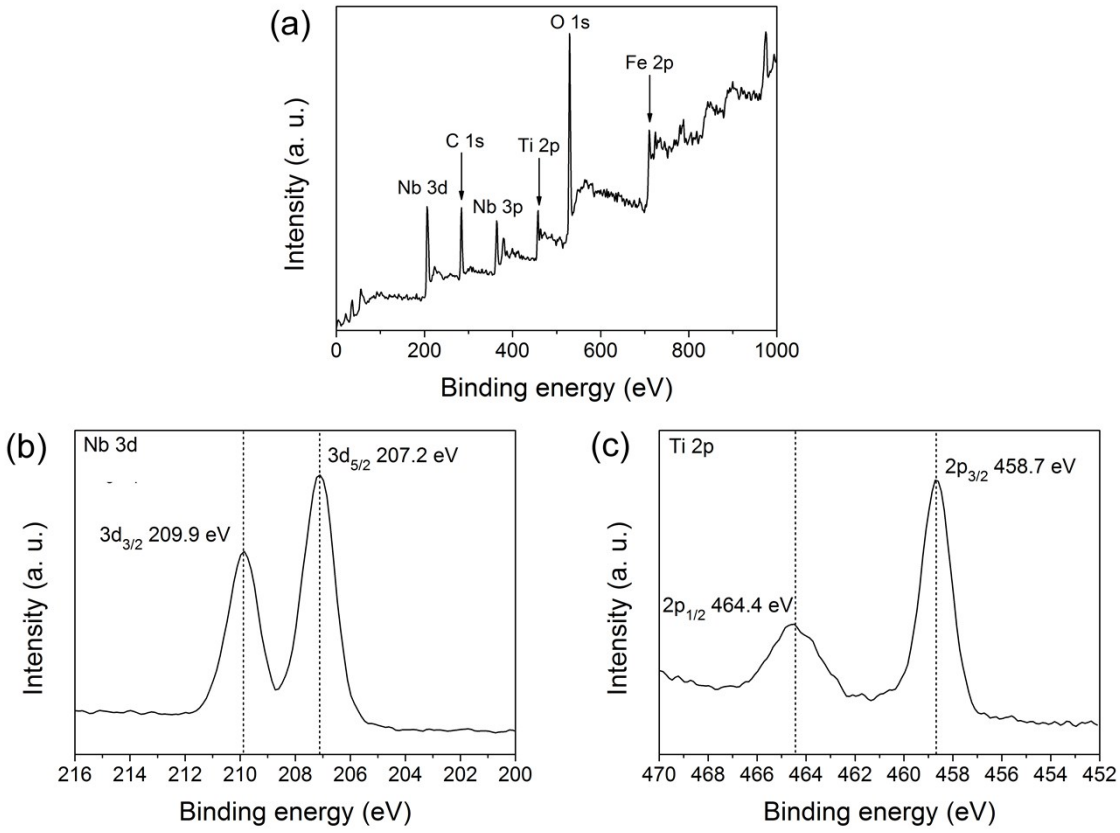

Fig. S6 (a) XPS survey spectrum of Fe_3O_4-TiNbNS-4-6. High resolution XPS spectra of (b) Ti 2p and (c) Nb 3d.

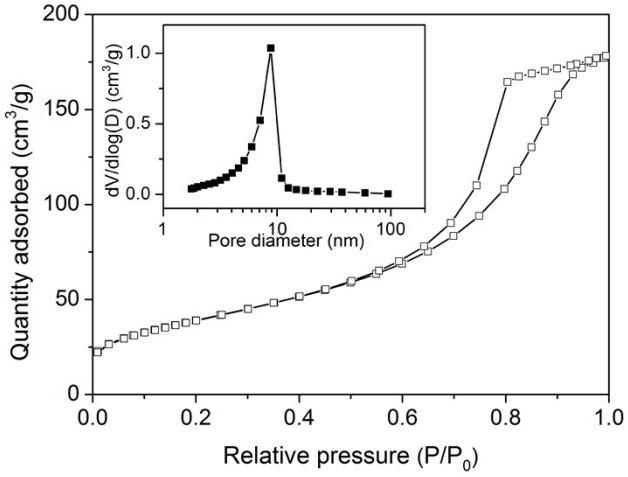

Fig. S7 Nitrogen adsorption-desorption isotherms and pore size distribution curves (inset) calculated from the desorption branches of control Fe_3O_4 nanocrystals.

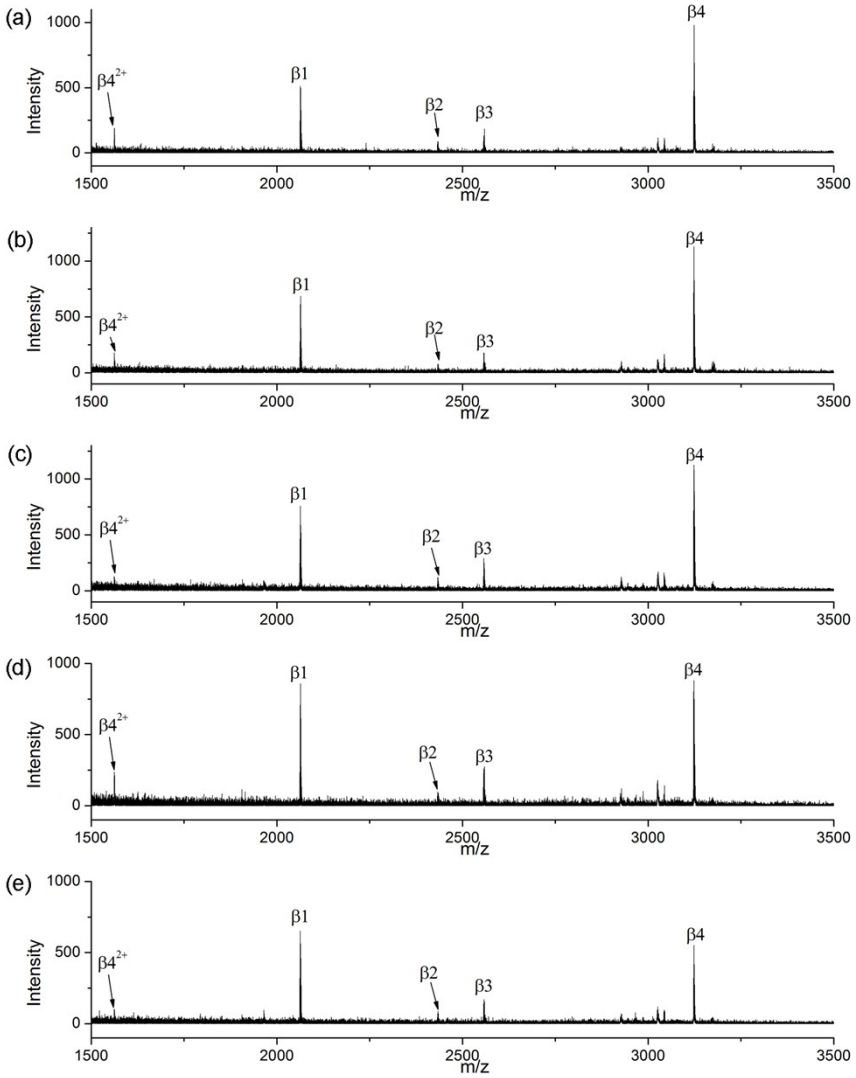

Fig. S8 MALDI-TOF MS spectra of tryptic digests of β-casein (160 fmol) enriched by Fe_3O_4-TiNbNS-*x*-*y* (a-e are successively assigned to 1:6, 2:6, 4:6, 4:3, and 6:3).

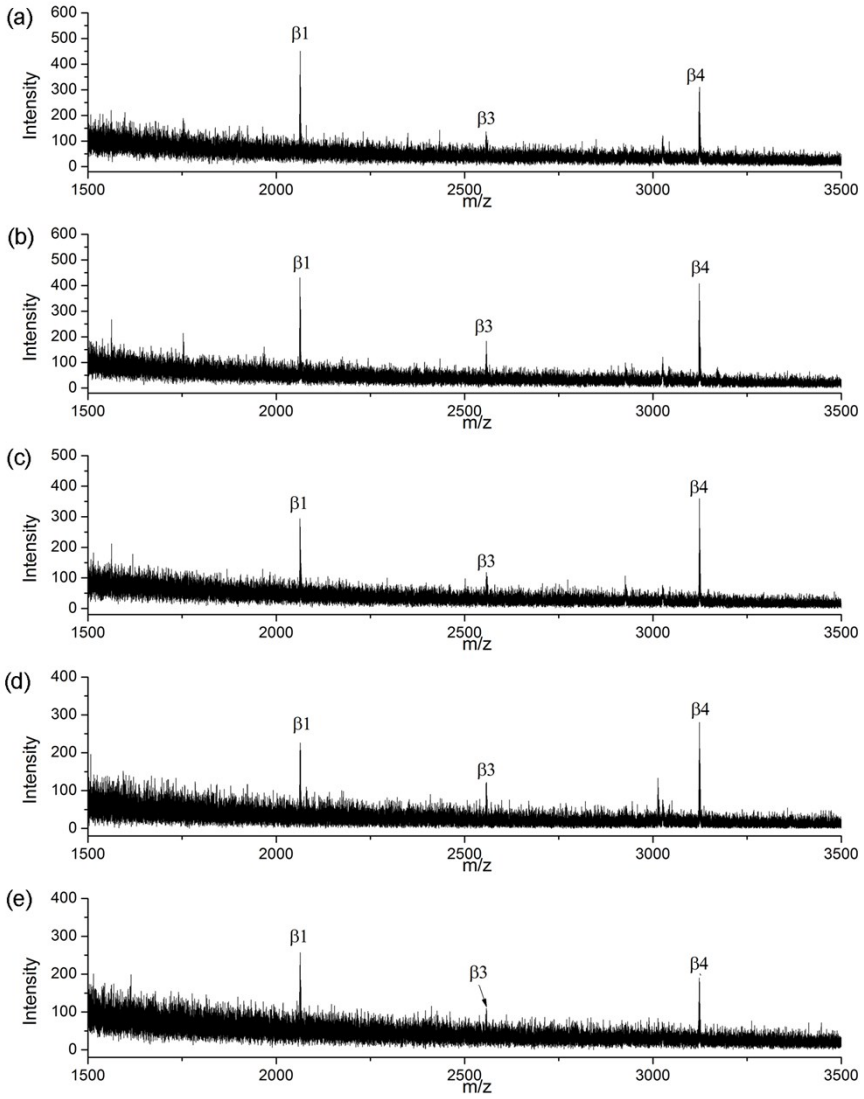

Fig. S9 MALDI-TOF MS spectra of tryptic digests of β-casein (16 fmol) enriched by Fe_3O_4-TiNbNS-*x*-*y* (a-e are successively assigned to 1:6, 2:6, 4:6, 4:3, and 6:3).

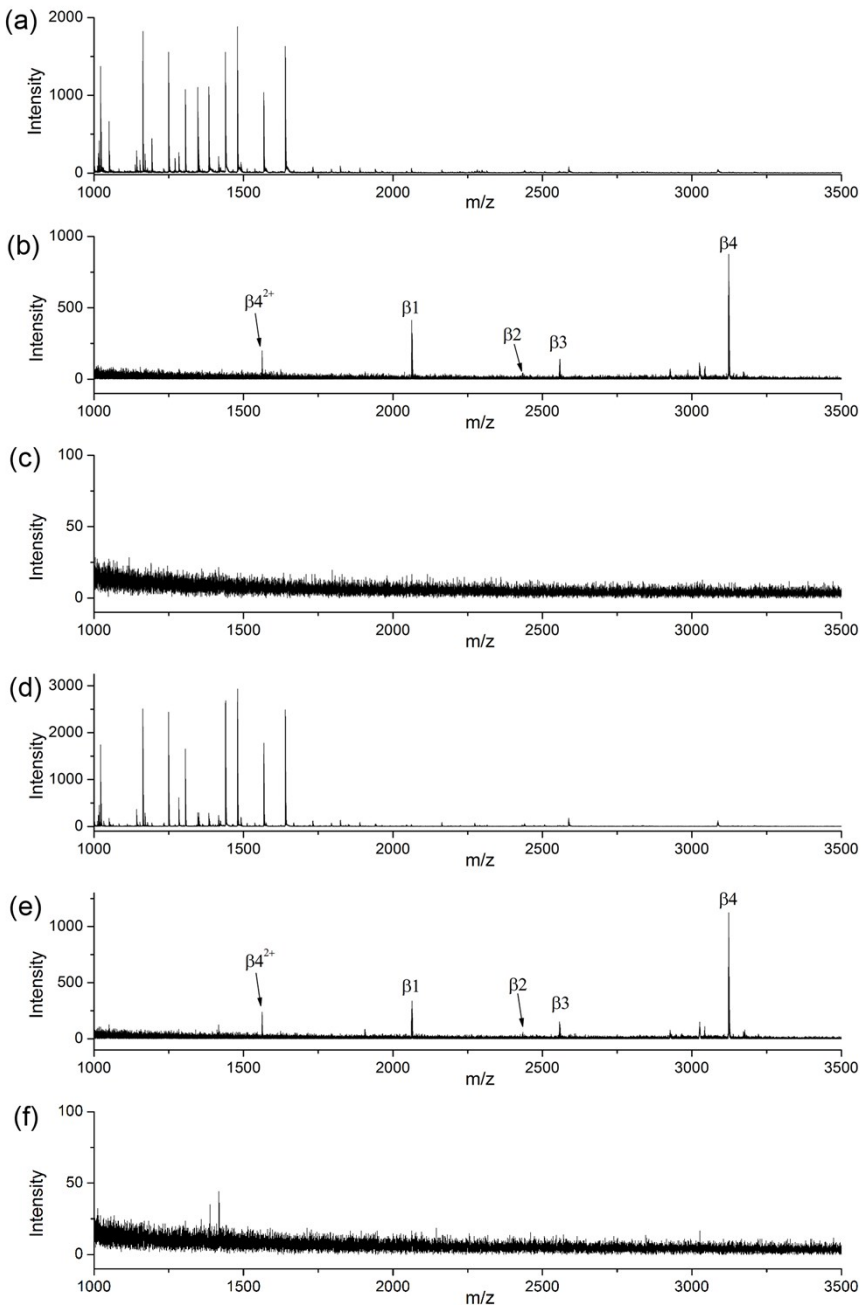

Fig. S10. MALDI-TOF MS spectra of tryptic digests of β-casein (160 fmol) mixed with BSA at mass ratio of (a, b and c) 1:10 and (d, e and f) 1:100. (a) and (d), direct analysis; (b) and (e), after enrichment by Fe_3O_4-TiNbNS-4-6; (c) and (f), after enrichment by layered oxide $HTiNbO_5$.

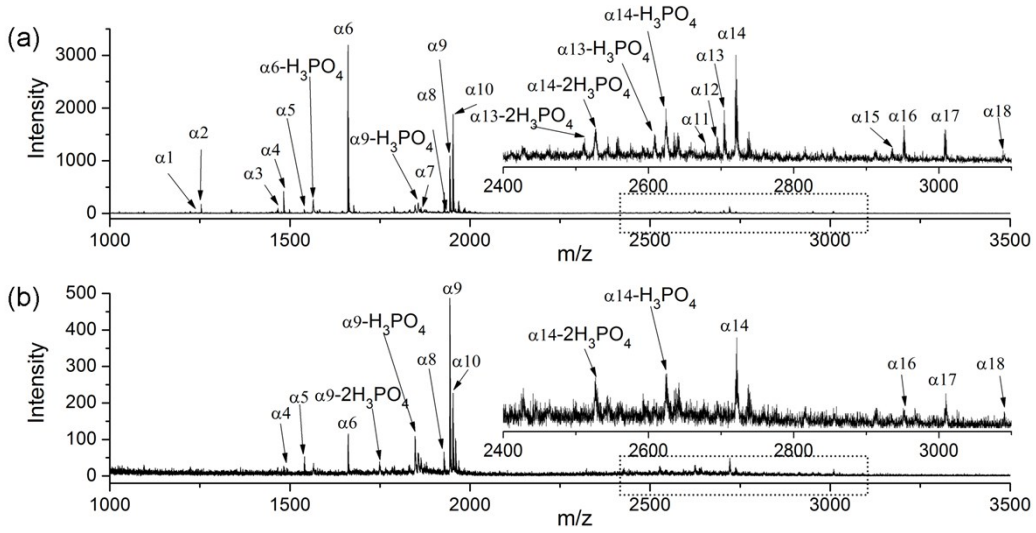

Fig. S11 MALDI-TOF MS spectra of tryptic digests of α-casein (1.6 pmol) enriched by (a) Fe_3O_4-TiNbNS-4-6 and (b) layered oxide $HTiNbO_5$.

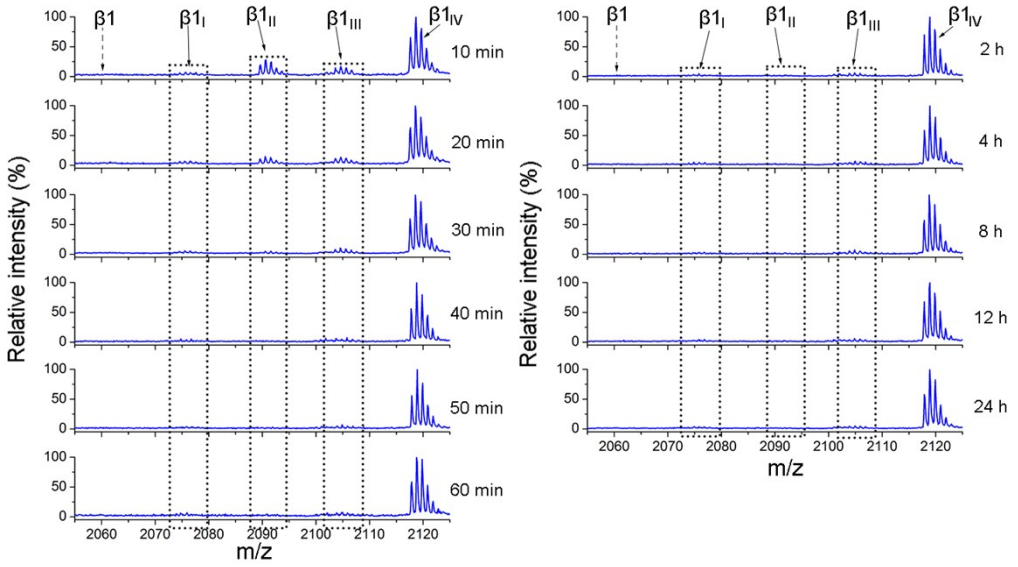

Fig. S12 MALDI-TOF MS characterization of 4 products $\beta1_{I}$, $\beta1_{II}$, $\beta1_{III}$, and $\beta1_{IV}$ after different *in situ* labeling reaction times.

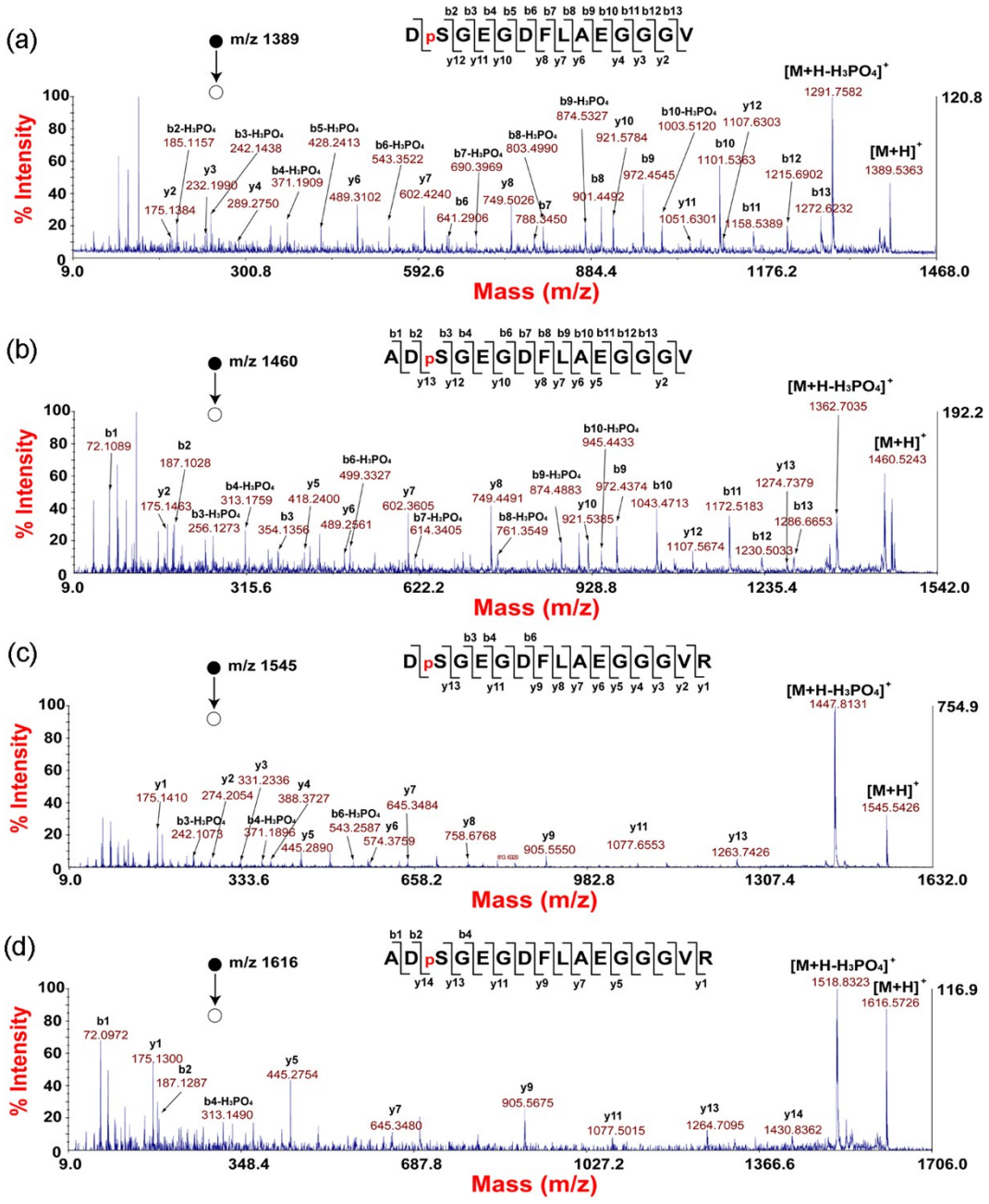

Fig. S13 CID MS/MS spectra of the 4 phosphopeptides captured from human serum (a) m/z 1389, (b) m/z 1460, (c) m/z 1545 and (d) m/z 1616.

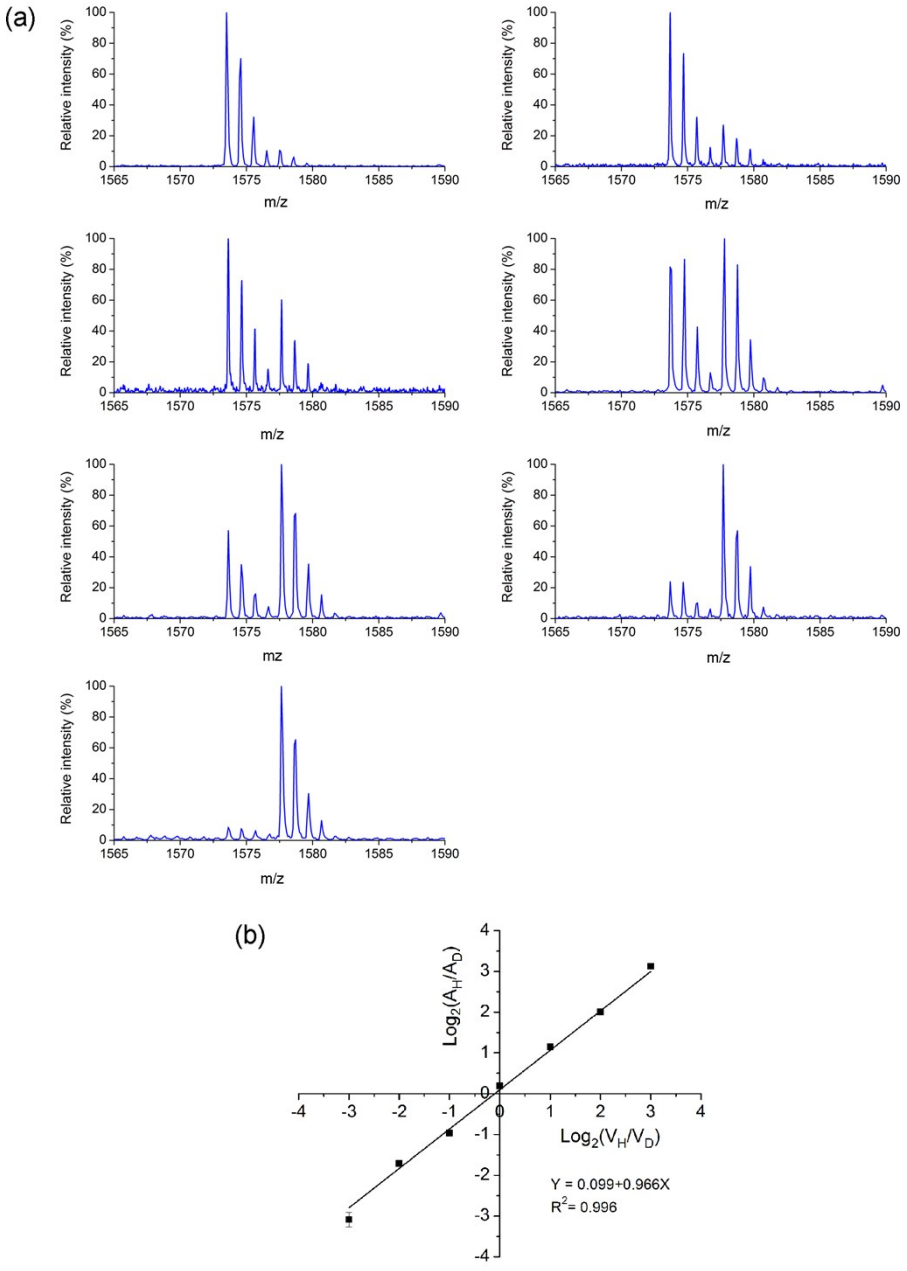

Fig. S14 (a) Isotope cluster area ratios (A_H/A_D) of light-labeled to heavy-labeled phosphopeptide HS3. The loading volume ratio (V_H/V_D) for enrichment and isotope dimethyl labeling was set as 8:1, 4:1, 2:1, 1:1, 0.5:1, 0.25:1, and 0.125:1, respectively. (b) Linear relationship between the logarithms of A_H/A_D and V_H/V_D.