Supporting Information for

A combination strategy of two novel cerium-based nanocomposites affinity probes for selective enrichment of mono- and multi-phosphopeptides in mass spectrometric analysis

Xing-yu Long,[ab] Zi-jin Zhang,[a] Jia-yuan Li,[a] Dong Sheng[a] and Hong-zhen Lian[a*]

a. State Key Laboratory of Analytical Chemistry for Life Science, Collaborative Innovation Center of Chemistry for Life Sciences, School of Chemistry & Chemical Engineering and Center of Materials Analysis, Nanjing University, 163 Xianlin Avenue, Nanjing 210023, P. R. China.

b. Editorial Department of Journal, Guizhou Normal University, 180 Baoshan North Road, Guiyang 550001, P. R. China.

* To whom correspondence should be addressed. E-mail: hzlian@nju.edu.cn (H. Z. Lian).

Part 1. Experimental section

1. Materials

Bovine α-/β-casein (MW = 25 kDa), bovine serum albumin (BSA, MW = 67 kDa), 2,5-dihydroxybenzoic acid (DHB) (puriss. p.a.), acetonitrile (ACN) and trifluoroacetic acid (TFA) of HPLC grade, and phosphoric acid (85%) were bought from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Ferric chloride ($FeCl_3 \cdot 6H_2O$), iron dichloride ($FeCl_2 \cdot 4H_2O$), sodium acetate (NaAc), ethylene glycol (EG), anhydrous ethanol and ammonium hydroxide ($NH_3 \cdot H_2O$) were obtained from Nanjing Chemical Reagent (Nanjing, China). Polyethylene glycol 6000 (PEG 6000) was bought from Xilong Chemical Co., Ltd. (Shanghai, China). Cerium (IV) sulfate hydrate ($Ce(SO_4)_2 \cdot H_2O$) was purchased from Alfa Aesar (China) Chemicals Co., Ltd. (Shanghai, China). All these reagents were used as received without further purification. Non-fat milk was purchased from Jiangsu Province Educational Supermarket; Human serum sample from healthy volunteer was provided by Nanjing University and obtained according to the standard clinical procedures. The samples were stored at -80 ^{o}C before analysis. Ultrapure water (18 MΩ·cm) was prepared with a Milli-Q water purification system (Millipore, Billerica, MA, USA). All of other chemicals were of analytical grade unless otherwise noted.

2. Synthesis of two Ce-based composites

2.1 Preparation of PEG-modified magnetic nano-cerium and ceria particles (PEG-Ce/CeO_2-Fe_3O_4, P-CCF). P-CCF was prepared by a co-precipitation method in low temperature. $FeCl_2 \cdot 4H_2O$ (0.6338 g, 0.05 mol) and PEG 6000 (2.0 g) were completely dissolved in ethanol aqueous solution (60 mL, *v*/*v*=1:2). 40 mL of 1 mol/L $NH_3 \cdot H_2O$ was added drop by drop into the above jade-green solution to adjust the pH of the mixture to 12-13, and 20 mL of $Ce(SO_4)_2$ solution (0.5 mol/L, prepared by 1 mol/L H_2SO_4) was added dropwise into the above mixture. Subsequently, the mix-

solution was heated at 90 °C using a water bath for 12 h under mechanical stirring and then air-cooled to room temperature. The resultant black products were collected with the help of a magnet, and sequentially washed with ultrapure water and ethanol three times each and finally dried at 60 °C for stay over.

2.2 Preparation of Ce/CeO_2 doped SO_4^{2-}/Fe_2O_3 nanosolid superacid (Ce/CeO_2-SO_4^{2-}/Fe_2O_3, CSF) affinity probe. CSF was synthesized through a solvothermal route at 200 °C for 8 h. Briefly, NaAc (3.6 g) was first completely dissolved in EG (40 mL) *via* ultrasonication for 10 min to form clear solution, followed by the addition of $Ce(SO_4)_2 \cdot H_2O$ (0.8306 g, 0.0025 mol) and $FeCl_3 \cdot 6H_2O$ (1.35 g, 0.005 mol). The resultant yellowish mixture was transferred into a Teflon-lined stainless-steel autoclave and sealed to heat at 200 °C for 8 h. After cooling to room temperature naturally, the offwhite particles were primordially obtained from autoclave through filtration but part of them changed into light-peachblow instantaneously for the air-oxidation. After fully rinsing with ultrapure water and ethanol, the products entirely formed steady light-peachblow. Thereafter the resultant products were dried at 60 °C for stay over.

3. Sample preparation

Bovine α-/β-casein or BSA (1.0 mg) was dissolved in 1 mL of 50 mmol/L NH_4HCO_3 aqueous solution (pH 8.2) to form a substrate solution, and digested at 37 °C for 18 h with trypsin at the ratio of enzyme-to-substrate of 1:40 (*wt*/*wt*). 30 μL non-fat milk was diluted with 1 mL 50 mM of NH_4HCO_3 aqueous solution (pH 8.2) and centrifugated at a speed of 16000 rpm for 15 min. Then, the albuminous supernatant was degenerated at 100 °C for 15 min. Subsequently, 20 μg/mL of trypsin was added for proteolysis at 37 °C for 16 h. All of above aliquots of proteolytic digests were frozen at -80 °C for standby application. The frozen digests were thawed and diluted to the target concentration with an aqueous solution of 50% ACN and 5.0% TFA (*v*/*v*, loading buffer) before use.

4. Selective enrichment of phosphopeptides from tryptic digestion of standard

proteins, non-fat milk and human serum

A schematic representation of the workflow on the two diverse Ce-based composites affinity probes (P-CCF and CSF) for serially extracting and enriching phosphopeptides from different samples is shown in Scheme 1. For enrichment of the phosphopeptides from protein samples, the first step: P-CCF/CSF (5.0 μL, 20 mg/mL) were added into a peptide mixture (100 μL), which was originating from tryptic digests of the mixture of α-/β-casein as a model (2.5 μL, *v*:*v* = 1:1) or non-fat milk (2.5 μL), or human serum (5.0 μL) diluted to 1 mL with 50% ACN and 5.0% TFA solution. The mixture was vortexed for 20 min at room temperature, and then the phosphopeptide-loaded P-CCF were collected by magnetic separation with a magnet, while CSF by centrifugal separation at 14000 r/min for 5 min (Step I); the second step: flowthrough fractions of P-CCF and CSF had been inversely enriched by each other, *i.e.*, after enrichment by P-CCF/CSF (Step I), the supernatant was transferred to another tube for further enrichment by CSF/P-CCF, respectively (Step II). The operation was the same as the above-mentioned enrichment loading process. Next, after the first and/or second step, the particles were respectively washed three times with 100 μL of 50% ACN and 0.1% TFA (*v*/*v*, washing buffer) to remove unspecific adsorbates and other unbound impurities and took the way of corresponding separation. Afterwards, the captured phosphopeptides were eluted with 5.0 μL of 10% $NH_3 \cdot H_2O$ (for P-CCF) or 10 mM $(NH_4)_2HPO_4$ (for CSF) by sonication for 5 min. Finally, the supernatants were collected with the relevant magnetic/centrifugal separation for further MALDI-TOF MS analysis.

5. MALDI-TOF MS analysis

0.5 μL of the eluate was deposited on a MALDI plate, and then 0.5 μL of 20 mg/mL DHB aqueous solution contained 50% ACN and 1% H_3PO_4 was introduced as a matrix to perform the MALDI-TOF MS analysis in a positive ion mode on a 4800 Proteomics Analyzer (Applied Biosystems, Framingham, CT, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. Mass spectra were obtained by accumulation of 2000-3000 consecutive laser

shots.

6. Characterizations

The transmission electron microscopic (TEM) images were obtained on a JEM-2100 TEM (JEOL, Tokyo, Japan). Scanning electron microscopic (SEM) images were gained with a Hitachi S-3000 N SEM (JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV. Fourier-transform infrared (FT-IR) spectra of P-CCF and CSF were collected on NEXUS 870 FTIR spectrometer (Nicolet, Madison, WI, USA) using KBr pellets. The compositions of two Ce-based composites were characterized on energy dispersive X-ray spectroscopy (EDX) (Hitachi, Tokyo, Japan). XRD patterns of the two materials were collected on an ARL X'TRA X-ray power diffractometer (Karlsruhe, German) using Cu Kα radiation (λ = 0.154 05 nm) at a scanning rate of 10°/min and a detection range from 10° to 80°. Magnetization measurement was carried out by a magnetometer at 300 K using a superconducting quantum interference device (SQUID) (Quantum Design, USA).

Fig. S1A shows the FT-IR absorbance spectra of P-CCF and CSF. The strongest characteristic peaks of metal oxides under 800 cm^{-1} were assigned to the metal–oxygen bonds (Fe-O/Ce-O) in these two materials. Moreover, the broad adsorption bands centered at around 3375.0 and 1614.7 cm^{-1} corresponded to the surface hydroxyl (-OH) and the adsorbed water molecule. The characteristic peaks from P-CCF at about 1045.5 and 881.0 cm^{-1} were ascribed to the -CH_2 bending vibration of PEG molecules,[S1] while that from CSF at 1190.9 and 1097.6 cm^{-1} were attributed to the O-S-O vibration of SO_4^{2-}.[S2]

As for P-CCF, from comprehensive analysis of its TEM and SEM images, it is obvious that single large particles (200 nm) consisted of the independent small Fe_3O_4 particles (10-20 nm) and numerous tiny metal oxides particles (Fe_3O_4, Fe_2O_3, CeO_2 and Ce), which were linked closely by PEG. According to selected area electron diffraction (SAED) pattern (Fig. S2A, inset) and high-resolution TEM (HRTEM) images (Fig. S2B and C), the *d* spacings of the polycrystalline Fe_3O_4 and CeO_2 nanoparticales (*i.e.* 0.239 and 0.300 nm, and 0.294 and 0.308 nm) can be indexed to

the (311) and (220), and (200) and (111) crystal planes of Fe_3O_4 and CeO_2, respectively. While as for CSF, the discussion is similar to above-mentioned. The polycrystalline structure of CSF also is the composition of numerous mischcrystals (Fe_2O_3, CeO_2 and Ce nanocrystallites) from their spacing of lattice fringe in HRTEM images (Figs. S2F and G). The short rod-like or globelike CSF particles were kinked each other (Fig. S2H), which was dispersed by the sonication for enough time (Fig. S2E). Moreover, the displayed dispersion ring of SAED pattern (Fig. S2E, inset) resulted from the tiny-crystalline and/or the poor degree of crystallinity. XRD patterns of P-CCF and CSF are showed in Fig. S3. The main diffraction peaks of P-CCF and CSF can be perfectly indexed to magnetite with hexagonal structure Fe_3O_4 (JCPDS 03-0863, marked with "▲", Fig. S3A) and hematite with corundum-type structure Fe_2O_3 (JCPDS 89-0597, marked with "*", Fig. S3B), respectively. Some diffraction peaks, which were indexed to cerianite (CeO_2) (marked with "#") (JCPDS 04-0593) and monatomic Ce (marked with "●") (JCPDS 04-0707) entering Fe_3O_4 or Fe_2O_3 crystal lattice, also can be observed in Fig. S3. In addition, two peaks (2θ = 10.9° and 21.1°) marked with "○" in Fig. S3B maybe resulted from unknown impurities.

As illustrated in the hysteresis loop (Fig. S4), the probe possessed a strong magnetism with negligible coercivity and remanence, as well as a saturation magnetization value of 43.1 emu/g. Thus, the P-CCF can be rapidly isolated from the solution within 20 s with the help of a magnet, and can be easily dispersed by shaking in the absence of the magnetic field (inset in Fig. S4). The excellent magnetic response and redispersibility of this P-CCF probe contribute to rapid and convenient magnetic separation.

7. Limits of detection (LOD)

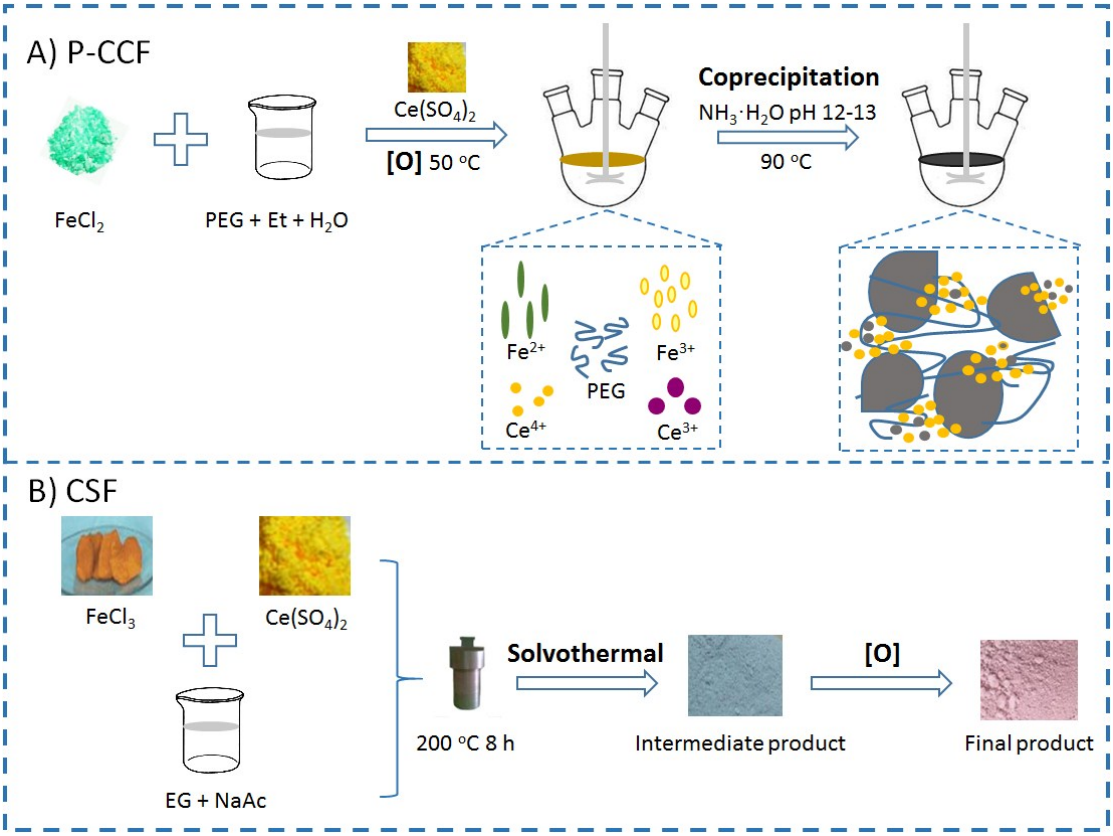
The LOD for phosphopeptide enrichment by CSF and P-CCF were evaluated, respectively. Using the mixture of tryptic digests of α-/β-casein (*v*/*v* = 1:1) as analytes, the concentration of phosphopeptides are expressed in terms of β-casein tryptic digest concentration. As shown in Fig. S4, the multiply and single phosphorylated peptides were separately extracted by CSF and P-CCF treatment in different concentrations. At

a concentration of 50 fmol, the multi-phosphopeptide (*m*/*z* 1927.6, 2P) with signal-to-noise ratio (*S/N*) of 3.43 was identified for CSF treatment, while the mono-phosphopeptide (*m*/*z* 2061.7, 1P) with *S/N* of 4.24 was detected with P-CCF treatment.

8. Selectivity

To further test the selectivity of these two affinity probes, they were used separately to capture phosphopeptides from a more complex peptides mixture consisting of α-/β-casein and BSA (in different mass ratios). The results are shown in Figs. S6 and S7, respectively. The phosphopeptides in the mixtures before enrichment cannot be trapped and recognized because of the suppression of the abundant non-phosphopeptides. However, after being enriched by the CSF affinity probe, tens of multi-phosphopeptides were clearly recognized with no other interference peaks under the existence of 200-fold BSA. When the interference degree is up to 2000-fold, several multiply phosphorylated peptides were still obtained and dominated the mass spectra. Furthermore, 5 phosphopeptides including 4 multi- and 1 mono-phosphopeptide were extracted from 5000-fold of interfering substance (BSA). While for the P-CCF probe, several phosphopeptides were faintly visible in mass spectra and the dominant peaks were other peptides from 50- to 200-fold BSA interferences. Therefore, it is further concluded that highly efficient capture and extraction of multi-phosphopeptides from high-fold interference complex mixture samples were realized with CSF probe treatment. However, the PEG-modified P-CCF probe exhibits partial non-special adsorption due to the hydrophobicity of carbon chains.

Part 2. Supplementary figures and tables



Scheme S1. Synthesis of P-CCF (A) and CSF (B).

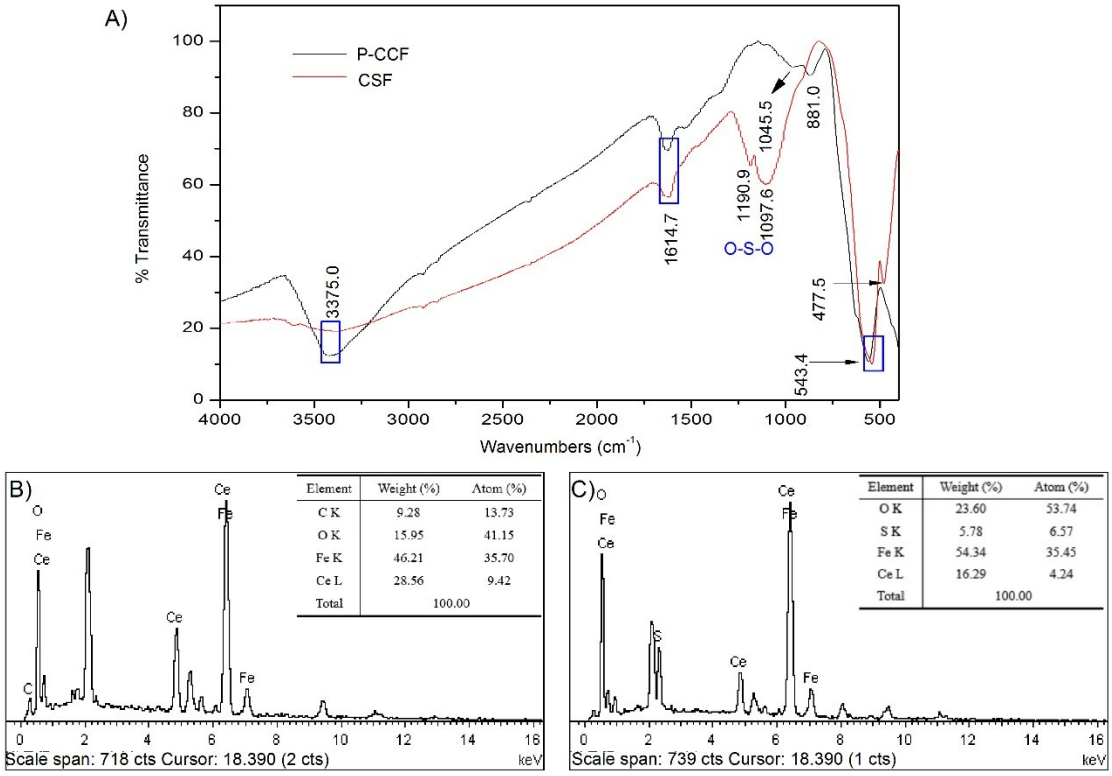


Fig. S1. FT-IR spectra (A) of P-CCF (black line) and CSF (red line), and EDX spectra of P-CCF (B) and CSF (C).

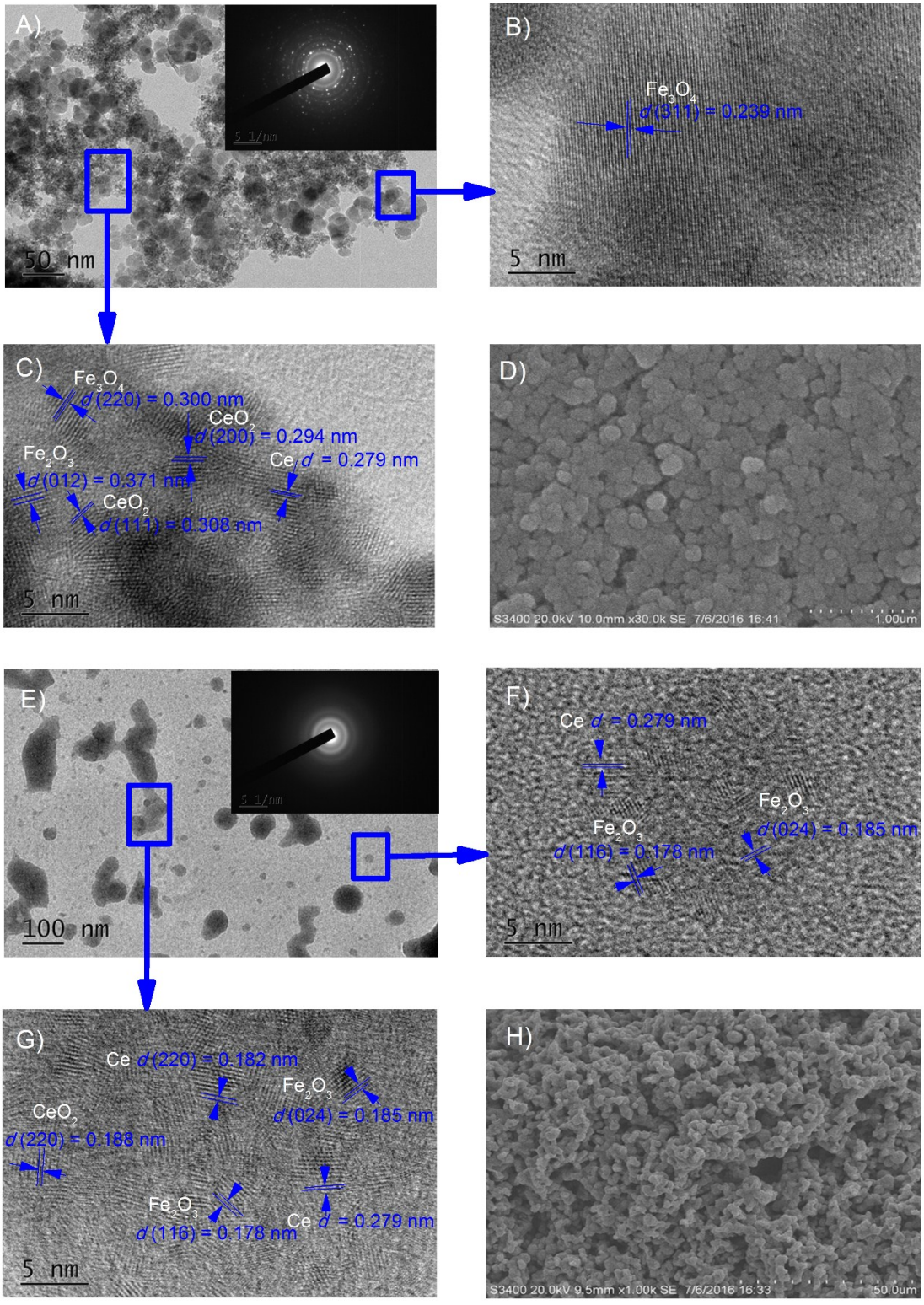


Fig. S2. TEM images (A and E), SAED patterns (A and E, inset), HRTEM images for different areas (B, C, F and G) and SEM images (D and H) of P-CCF (A-D) and CSF (E-H), respectively.

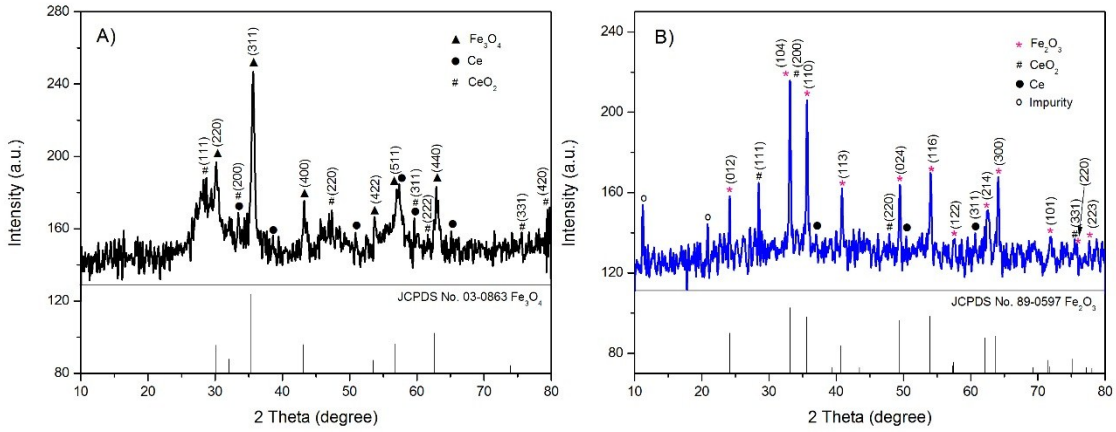


Fig. S3. XRD patterns of P-CCF (A) and CSF (B).

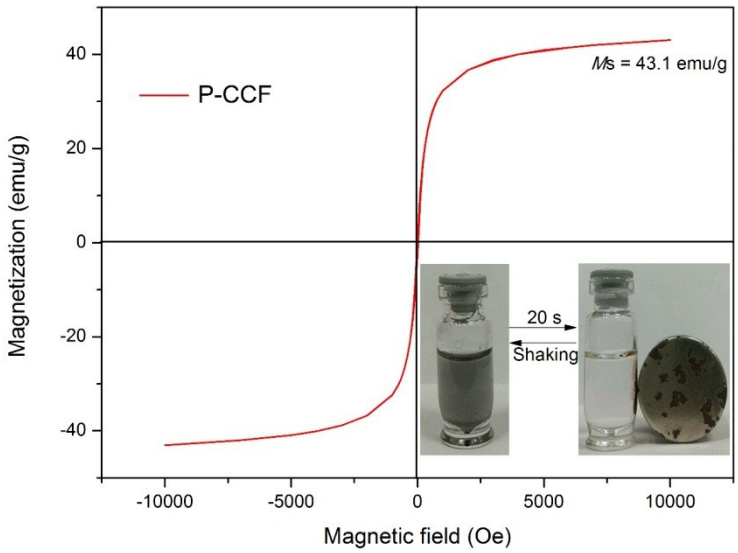


Fig. S4. Hysteresis loop of P-CCF and the dispersion and separation process of P-CCF (inset).

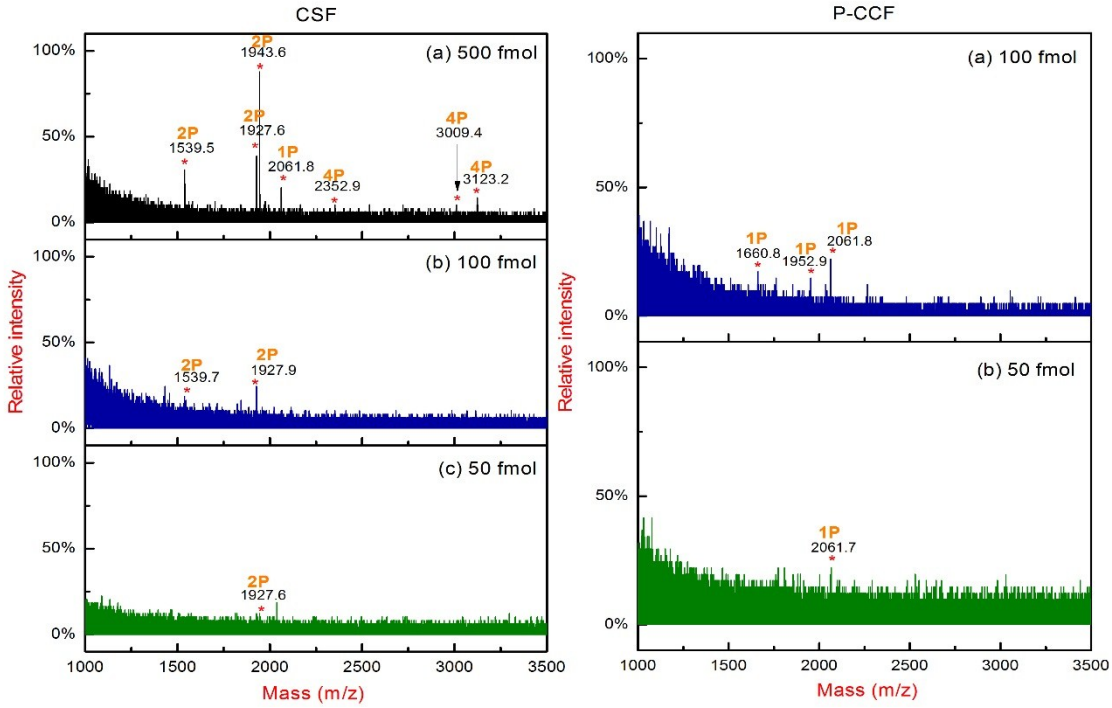


Fig. S5. MALDI-TOF MS spectra of mixture of bovine α- and β-casein digests treated with CSF (Left) and P-CCF (Right), respectively. Left: (a). 500 fmol; (b). 100 fmol; (c). 50 fmol; Right: (a) 100 fmol; (b) 50 fmol. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. "*" indicates phosphopeptides; "#" indicates the dephosphorylated fragments of phosphopeptide.

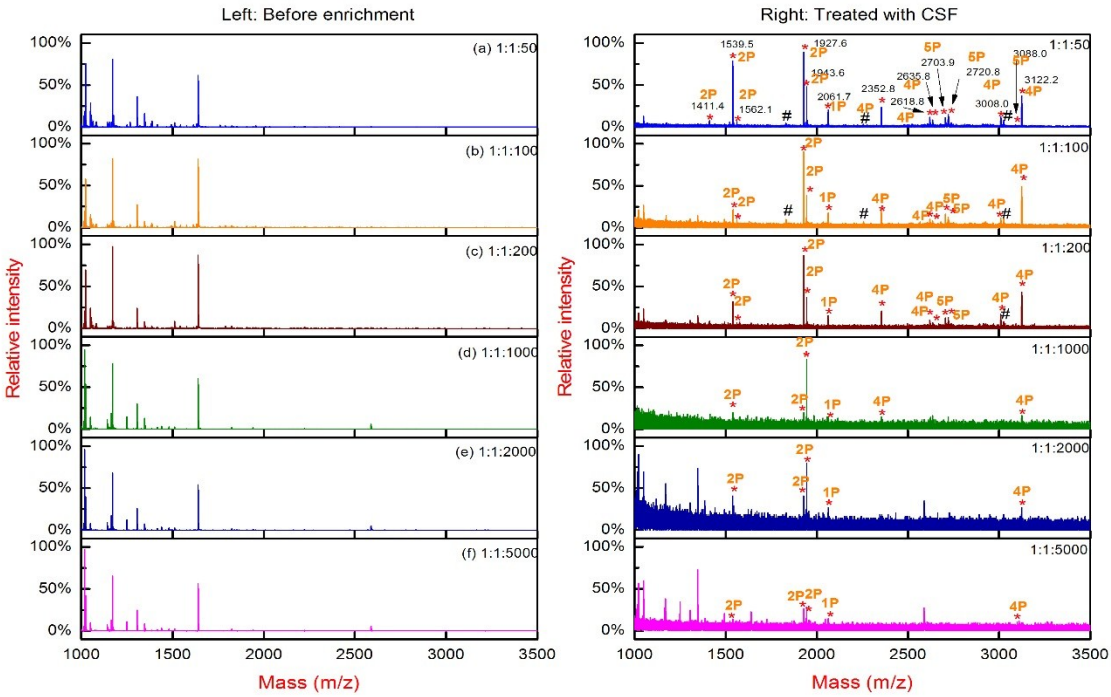


Fig. S6. MALDI-TOF MS spectra of tryptic digests of bovine α- and β-casein and BSA at mass ratios of 1:1:50, 1:1:100, 1:1:200, 1:1:1000, 1:1:2000, and 1:5000 treated without (Left: a-f) and with (Right) CSF affinity probe. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. "*" indicates phosphopeptides; "#" indicates the dephosphorylated fragments of phosphopeptide.

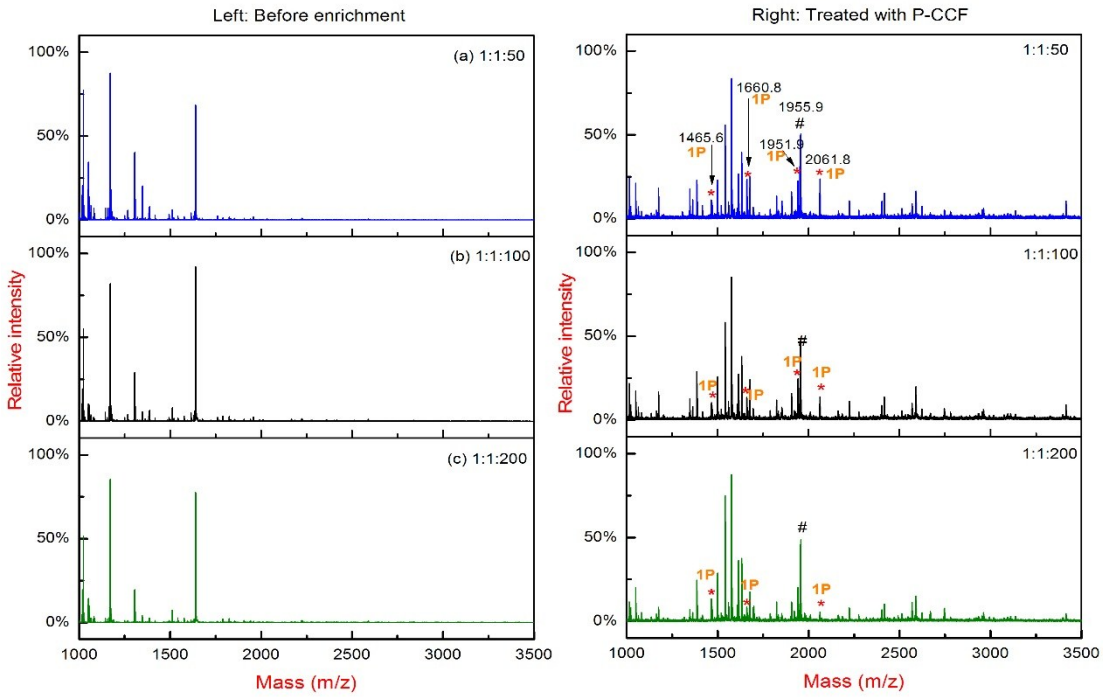


Fig. S7. MALDI-TOF MS spectra of tryptic digests of bovine α- and β-casein and BSA at mass ratios of 1:1:50, 1:1:100, and 1:1:200 treated without (Left: a-c) and with (Right) P-CCF affinity probe. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. "*" indicates phosphopeptides; "#" indicates the dephosphorylated fragments of phosphopeptide.

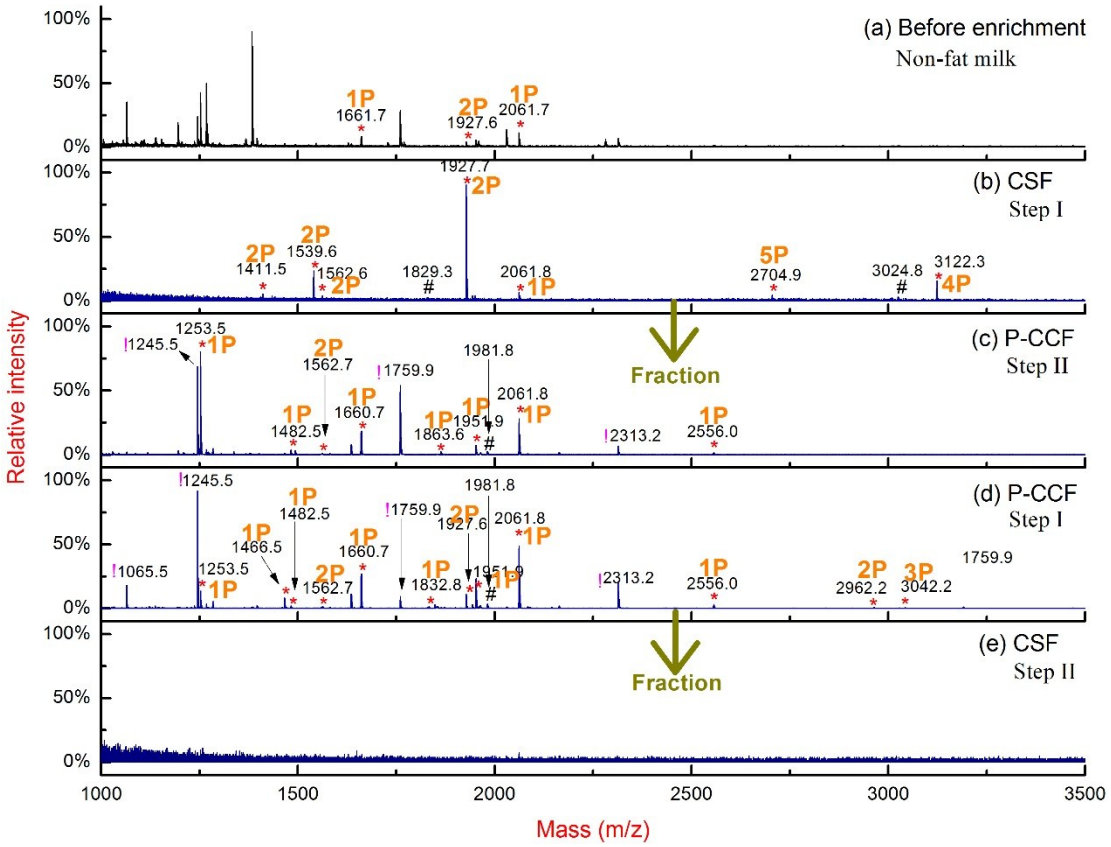


Fig. S8. MALDI-TOF mass spectra of two serial enrichment strategies (Steps I and II): Before enrichment (a); Step I: Direct enrichment from tryptic digests of non-fat milk with CSF (b) and P-CCF (d); Step II: From the flowthrough fraction of CSF or P-CCF with P-CCF (c) or CSF (e), respectively. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. Mass spectrometric peaks are marked as: phosphopeptides (*), dephosphorylated fragments (#), and non-phosphopeptides or unknown impurities (!).

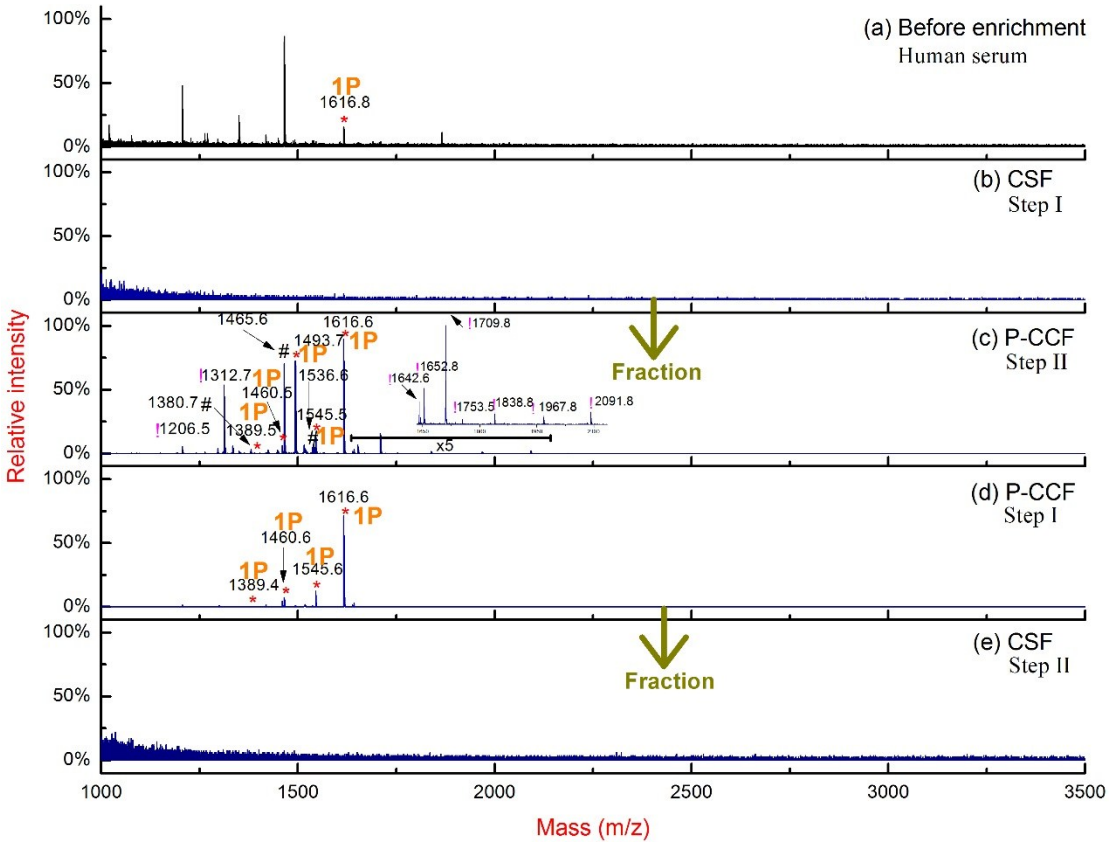


Fig. S9. MALDI-TOF mass spectra of two serial enrichment strategies (Steps I and II): Before enrichment (a); Step I: Direct enrichment from human serum with CSF (b) and P-CCF (d); Step II: From the flowthrough fraction of CSF or P-CCF with P-CCF (c) or CSF (e), respectively. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. Mass spectrometric peaks are marked as: phosphopeptides (*), dephosphorylated fragments (#), and non-phosphopeptides or unknown impurities (!).

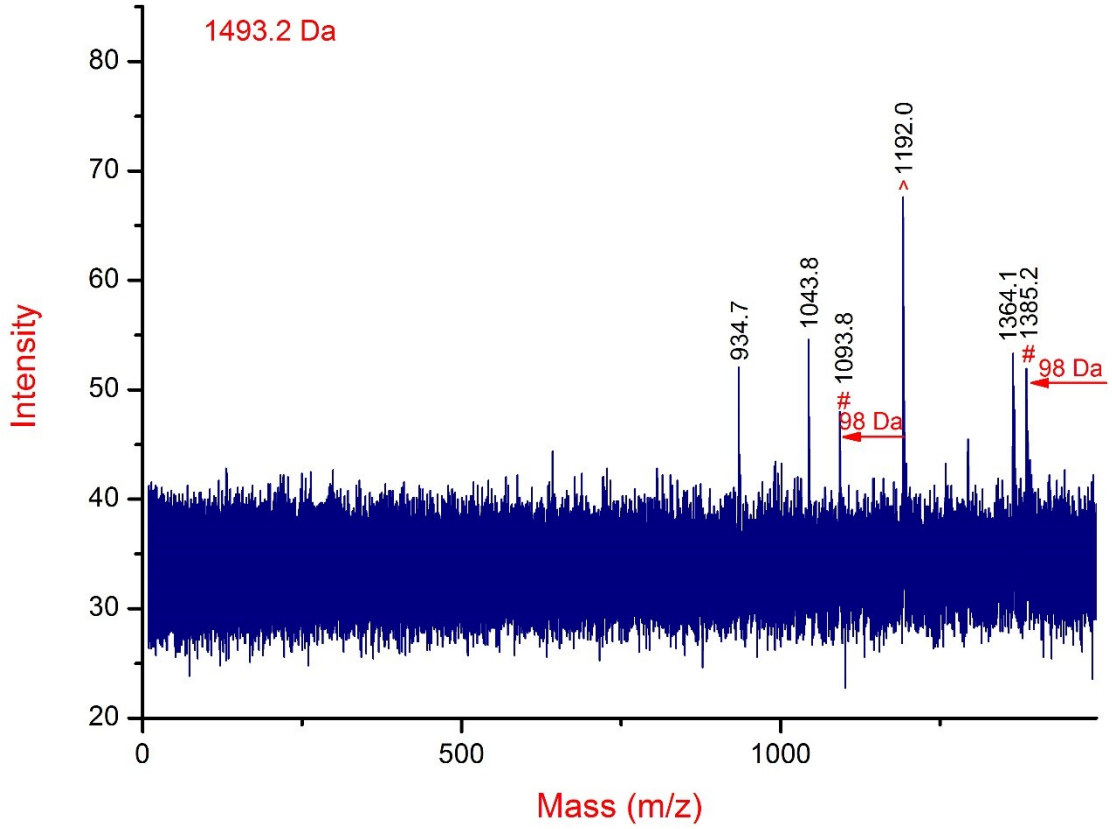


Fig. S10. MALDI-TOF mass spectra of human serum treated with P-CCF for the precursor ion at *m*/*z* of 1493.2 Da. The MS/MS experiment shows the characteristic loss of 98 Da, and also provides unambiguous identification, *e.g.* the peptide at *m*/*z* 1493.2 is phosphopeptide due to the presence of the fragment ion at *m*/*z* 1385.2 adjacent to the parent ion with a mass difference of 98 Da in MS/MS spectra as well as the pair of fragment ions at *m*/*z* 1093.8 adjacent to 1192.0 (Δ98 Da). "#" indicates fragment ion peak with a loss of 98 Da.

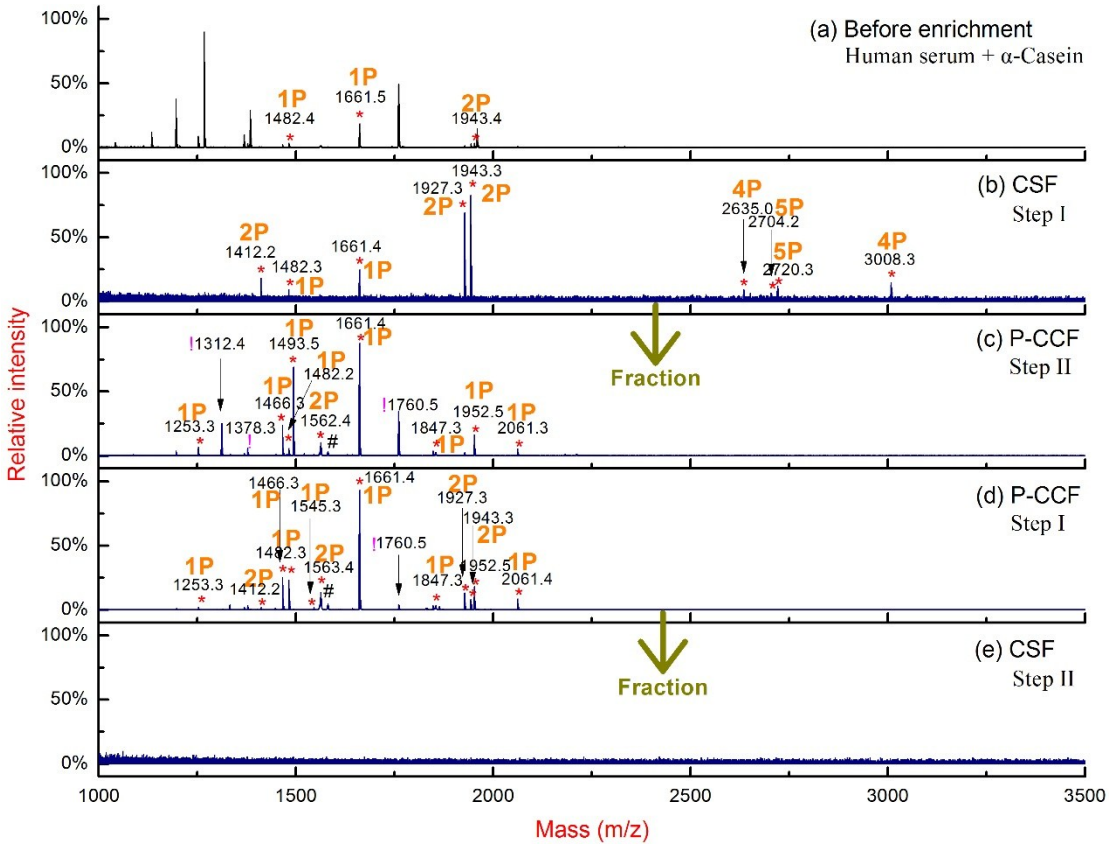


Fig. S11. MALDI-TOF mass spectra of two serial enrichment strategies (Steps I and II): Before enrichment (a); Step I: Direct enrichment from human serum strike to α-casein tryptic digest with CSF (b) and P-CCF (d); Step II: From the flowthrough fraction of CSF or P-CCF with P-CCF (c) or CSF (e), respectively. Phosphorylations were labelled as P. Arabic numeral before P represents mono-/multi-phosphopeptides. Mass spectrometric peaks are marked as: phosphopeptides (*), dephosphorylated fragments (#), and non-phosphopeptides or unknown impurities (!).

Table S1. Identified mono-/multi-phosphopeptides from the mixture of tryptic digests of *α*-/*β*-casein with P-CCF and CSF affinity probes for different strategies, respectively [S3]

Strategies		Position	Obtained (*m*/*z*)	Theoretical (*m*/*z*)	Δ(*m*/*z*)	Number of phosphoryl group	Amino acid sequence
CSF	Step I	αS2/141-152	1539.5	1539.0	0.5	2	EQL[pS]T[pS]EENSKK
		αS2/141-152	1562.1	1561.8	0.3	2	EQL[pS]T[pS]EENSKK-Na
		αS1/121-134	1660.7	1660.9	-0.2	1	VPQLEIVPN[pS]AEER
		αS1/58-73	1943.6	1943.8	-0.2	2	DIG[pS]E[pS]TEDQA[Mo]EDIK
		β-c/48-63	2061.8	2062.2	-0.4	1	FQ[pS]EEQQQTEDELQDK
		β-c/22-40	2254.8	2254.8	0.0	3	NVPGEIVESL[pS][pS][pS]EESITR
		β-c/22-40	2352.8	2352.8	0.0	4	NVPGEIVE[pS]L[pS][pS][pS]EESITR
		αS2/17-36	2619.9	2619.4	0.5	4	NTMEHV[pS][pS][pS]EESII[pS]QETYK
		αS2/17-36	2635.9	2635.3	0.6	4	NT[Mo]EHV[pS][pS][pS]EESII[pS]QETYK
		αS1/74-94	2720.9	2720.8	0.1	5	QMEAE[pS]I[pS][pS][pS]EEIVPNPN[pS]VEQK
		αS1/74-94	2736.9	2736.8	0.1	5	Q[Mo]EAE[pS]I[pS][pS][pS]EEIVPNPN[pS]VEQK
		αS2/17-37	2762.9	2762.9	0.0	4	NT[Mo]EHV[pS][pS][pS]EESII[pS]QETYKQ

			αS1/50-73	2951.2	2952.4	-1.2	3	EKVNEL[pS]KDIG[pS]E[pS]TEDQA[Mo]EDIK
			αS2/61-85	3008.1	3008.6	-0.5	4	NANEEEYSIG[pS][pS][pS]EE[pS]AEVATEEVK
			β-c/16-40	3122.3	3122.9	-0.6	4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
P-CCF	Step I	Step II	αS1/39-50	1196.6	1197.3	-0.7	1	KNMAINP[pS]KENL
			αS2/153-162	1253.5	1253.1	0.4	1	TVD[Mo]E[pS]TEVF
			αS2/153-164	1482.5	1482.7	-0.2	1	TVD[Mo]E[pS]TEVFTK
			αS1/121-134	1660.7	1660.9	-0.2	1	VPQLEIVPN[pS]AEER
			αS1/58-73	1863.6	1864.4	-0.8	1	DIGSE[pS]TEDQA[Mo]EDIK
			αS1/119-134	1951.9	1952.1	-0.2	1	YKVPQLEIVPN[pS]AEER
			β-c/48-63	2061.8	2062.2	-0.4	1	FQ[pS]EEQQQTEDELQDK
			β-c/48-67	2556.1	2556.7	-0.6	1	FQ[pS]EEQQQTEDELQDKIHPF
		αS2/153-164		1466.6	1466.7	-0.1	1	TVDME[pS]TEVFTK
		αS2/141-152		1562.7	1561.8	0.9	2	EQL[pS]T[pS]EENSKK-Na
		αS1/58-73		1943.7	1943.8	-0.1	2	DIG[pS]E[pS]TEDQA[Mo]EDIK
		β-c/16-40		2962.4	2962.2	0.2	2	RELEELNVPGEIVESLS[pS][pS]EESITR
		β-c/16-40		3042.4	3042.2	0.2	3	RELEELNVPGEIVESL[pS][pS][pS]EESITR

“[pS]” shows phosphorylation on serine; “[Mo]” indicates oxidation on methionine.

Table S2. Identified mono-/multi-phosphopeptides from the mixture of tryptic digests of non-fat milk with P-CCF and CSF affinity probes for different strategies, respectively

Strategies			Position	Obtained (*m*/*z*)	Theoretical (*m*/*z*)	Δ(*m*/*z*)	Number of phosphoryl group	Amino acid sequence
CSF	Step I		αS2/141-151	1411.5	1410.8	-0.7	2	EQL[pS]T[pS]EENSK
			αS2/141-152	1539.5	1539.0	0.5	2	EQL[pS]T[pS]EENSKK
			αS2/141-152	1562.6	1561.8	0.8	2	EQL[pS]T[pS]EENSKK-Na
			αS1/58-73	1927.7	1927.9	-0.2	2	DIG[pS]E[pS]TEDQAMEDIK
			β-c/48-63	2061.8	2062.2	-0.4	1	FQ[pS]EEQQQTEDELQDK
			αS1/74-94	2704.9	2703.8	1.1	5	Q*MEAE[pS]I[pS][pS][pS]EEIVPNPN[pS]VEQK
			β-c/16-40	3122.3	3122.9	-0.6	4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR
P-CCF	Step I	Step II	αS2/153-162	1253.5	1253.1	0.4	1	TVD[Mo]E[pS]TEVF
			αS2/153-164	1482.5	1482.7	-0.2	1	TVD[Mo]E[pS]TEVFTK
			αS2/141-152	1562.6	1561.8	0.8	2	EQL[pS]T[pS]EENSKK-Na
			αS1/121-134	1660.7	1660.9	-0.2	1	VPQLEIVPN[pS]AEER
			αS1/58-73	1863.6	1864.4	-0.8	1	DIGSE[pS]TEDQA[Mo]EDIK

			αS1/119-134	1951.9	1952.1	-0.2	1	YKVPQLEIVPN[pS]AEER
			β-c/48-63	2061.8	2062.2	-0.4	1	FQ[pS]EEQQQTEDELQDK
			β-c/48-67	2556.0	2556.7	-0.7	1	FQ[pS]EEQQQTEDELQDKIHPF
		αS2/153-164		1466.5	1466.7	-0.2	1	TVDME[pS]TEVFTK
		αS1/104-119		1832.8	1833.1	-0.3	1	YLGEYLIVPN[pS]AEER
		αS1/58-73		1927.6	1927.8	-0.2	2	DIG[pS]E[pS]TEDQAMEDIK
		β-c/16-40		2962.2	2962.2	0.0	2	RELEELNVPGEIVESLS[pS][pS]EESITR
		β-c/16-40		3042.2	3042.2	0.0	3	RELEELNVPGEIVESL[pS][pS][pS]EESITR

“[pS]” shows phosphorylation on serine; “[Mo]” indicates oxidation on methionine; “Q*” represents deletion or truncation of N-terminal on glutamine.

Table S3. Identified mono-phosphopeptides from human serum with P-CCF affinity probe [S3]

No.	Obtained (m/z)	Theoretical (m/z)	$\Delta(m/z)$	Number of phosphoryl group	Amino acid sequence
HS1	1389.5	1389.3	0.2	1	D[pS]GEGDFLAEGGGV
HS2	1460.5	1460.4	0.1	1	AD[pS]GEGDFLAEGGGV
HS3	1545.5	1545.5	0.0	1	D[pS]GEGDFLAEGGGVR
HS4	1616.6	1616.6	0.0	1	AD[pS]GEGDFLAEGGGVR
HS5	1493.2	-	-	1	Identified by MS/MS

"[pS]" shows phosphorylation on serine.

References:

S1 S. Zeng, M.-K. Tsang, C.-F. Chan, K.-L. Wong and J. Hao, *Biomaterials*, 2012, **33**, 9232.

S2 (a) J. Gao, Y. Qi, W. Yang, X. Guo, S. Li and X. Li, *Mater. Chem. Phys.*, 2003, **82**, 602; (b) B. M. Reddy, P. M. Sreekanth, P. Lakshmanan and A. Khan, *J. Mol. Catal. A-Chem.*, 2006, **244**, 1.

S3 (a) F. Jabeen, M. Najam-ul-Haq, M. Rainer, Y. Güzel, C. W. Huck and G. K. Bonn, *Anal. Chem.*, 2015, **87**, 4726; (b) G Cheng, Z.-G. Wang, Y.-L. Liu, J.-L. Zhang, D.-H. Sun and J.-Z. Ni, *Chem. Commun.*, 2012, **48**, 10240.