Magnetic metal phenolic networks：Expanding application of a promising nanoprobe to phosphoproteomics research

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Experimental section

Materials. Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and sodium acetate (NaAc) were purchased from Sinopharm Chemical Reagent Co., Ltd. Ethanol and methanol were purchased from GENERAL-REAGENT. Dihydroxy bis (Ammonium Lactato) Titanium (IV) (Ti-DBAL, 50 wt. % aqueous solution) and phenylmethanesulfonyl fluoride (PMSF) were bought from Alfa. Ethylene glycol was bought from Adamas. Tannic acid (TA), horseradish peroxidase (HRP), Immunoglobulin G (IgG), bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (DHB), trypsin from bovine pancreas, dithiothreitol (DTT), indoacetamide (IAA), trifluoroacetic acid (TFA), urea, thiourea and ammonium bicarbonate (NH_4HCO_3) were bought from Sigma- Aldrich. Acetonitrile (ACN) was purchased from Merck. Human serum was provided by Shanghai Zhongshan Hospital from a healthy volunteer. Ultrapure water used in all aqueous solutions was purified by using a Milli-Q system (Millipore, Bedford, MA). All other reagents were analytical grade.

Synthesis method of Fe_3O_4@TiTA nanoparticles. Fe_3O_4 nanoparticles were prepared via a solvothermal synthesis method according to our previous report.[1] In brief, $FeCl_3 \cdot 6H_2O$ (1.35 g) was dissolved with ethylene glycol (75 mL). Then, 3.6 g of NaAc was added. The obtained mixture was heated at 200 °C for 16 h. The obtained magnetic nanoparticles were washed by deionized water and ethanol several times and dried in a vacuum oven at 50 °C overnight. Fe_3O_4 (30mg) were dispersed in 100 mL H_2O followed by 10 min of ultrasound. Next, the suspension was vigorously stirred after the individual additions of TA solution with 10 min stirring for each addition. Then a certain concentration of Ti-DBAL was added with 5 min stirring. The obtained Fe_3O_4@TiTA nanoparticles were washed by deionized water four times and dried in a vacuum oven at 50 ℃ overnight.

Synthesis method of Fe_3O_4@FeTA nanoparticles. Fe_3O_4 nanoparticles were prepared same as above. Fe_3O_4 (30mg) were dispersed in 100 mL H_2O followed by 10 min of ultrasound. Next, the suspension was vigorously stirred after the individual additions of $FeCl_3$ with 10 min stirring for each addition. Then a certain concentration of TA solution was added with 5 min stirring. The obtained Fe_3O_4@FeTA nanoparticles

were washed by deionized water four times and dried in a vacuum oven at 50 ℃ overnight.

Synthesis method of Fe_3O_4@ZrTA nanoparticles. Fe_3O_4 nanoparticles were prepared same as above. Fe_3O_4 (30mg) were dispersed in 100 mL H_2O followed by 10 min of ultrasound. Next, the suspension was vigorously stirred after the individual additions of $ZrCl_4$ with 10 min stirring for each addition. Then a certain concentration of the TA solution was added with 5 min stirring. The obtained Fe_3O_4@ZrTA nanoparticles were washed by deionized water four times and dried in a vacuum oven at 50 ℃ overnight.

Sample preparation of standard proteins and human serum. 2 mg of β-casein was dissolved in 500 μL 50 mM NH_4HCO_3 and denatured in boiling water for 10 min. After cooling down to room temperature, 500 μL Milli-Q was added and then trypsin was added with the ratio of trypsin: protein at 1: 40 (w/w). The solution was incubated at 37 ℃ for 16 h. The 2 mg/mL β-casein was diluted to 0.5 mg/mL and stored at −20 ℃ for further use.

1 mL serum centrifuged for 10 min at the speed of 1738g. The supernatant was collected and stored at -20 ℃ for further utilization.

Cell culture and lysis. HeLa cells were cultured in DMEM with addition of 10% fetal bovine serum and 1% penicillin and streptomycin at 37 ℃ in 5% CO_2. Cell lysates were collected in triple biological replicates. After cells were overgrown on culture dishes with a radius of 10 cm, the culture mediums were removed. Cells were washed with PBS (1x) twice and added by trypsin for two minutes. Whereafter, cells were blown down with the usage of PBS and transferred to a clear tube. The cells were washed using PBS (1x) twice at 170g for 2 min, followed by the addition of 500 μL lysate (7M urea, 2M thiourea, 1mM PMSF). The mixture was shaken vigorously for 5 min and then underwent sonicate with an ultrasonic disintegrator for 15min. The supernatant was taken after centrifugation at 10000g for 15 min. The above solution was diluted with 50 mM ammonium bicarbonate until the concentration of urea was

lower than 0.1 M. The solution was transferred to a new tube and boiled for 10 minutes. The protein concentrations were determined via BCA protein assays. The concentration of protein was diluted to 10 μg/μL.

500 μg protein was reduced with DTT at 10 mM final concentration at 50 ℃ for 1 h. Then concentrated IAA was mixed and reacted in the dark for 30 min at 55mM final concentration. Afterwards, DTT was used to consume excess IAA so that the total molar ratio of DTT to IAA was 1: 1, followed by the supplement of 10 μg trypsin to make sure the mass ratio of protein to trypsin was 50:1. Whereafter, a certain amount of 50 mM NH_4HCO_3 was added to make the total volume of the system 100 μL and the reaction was carried out at 37 ℃ for 16 h. Finally, 1 μl of 10% TFA was added to stop the reaction to prevent excessive enzymatic hydrolysis. The protein digestion was stored at -80 ℃ for further use.

Live subject statement. All the experiments in this work were carried out in compliance with the ethical standards, and conducted according to the Declaration of Helsinki and approved by the Ethics Committee of Fudan University.

Protocol of enrichment process. Fe_3O_4@TiTA nanoparticles (100 μg) were dispersed in 100 μL loading buffer (ACN/H_2O/TFA= 50/49/1, v/v/v) containing 100 fmol β-casein digests followed by incubation at 37 ℃ with 30 min. After that, the nanoparticles were separated from the mixture by using a magnet and washed with 200 μL loading buffer for three times to remove non-phosphopeptides. Then the captured phosphopeptides were eluted by 10 μL 0.4 M ammonium hydroxide for 30 min at 37 ℃. Eventually, the eluent was analyzed directly by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with the help of 2,5-dihydroxybenzoic acid (DHB) (ACN/H_2O/H_3PO_4, 50/49/1, V/V/V).

For phosphopeptide enrichment from real bio-samples, 10 μL of human serum supernatant was added in 100 μL loading buffer (ACN/H_2O/TFA = 50/49/1, v/v/v), and then 200 μg Fe_3O_4@TiTA nanoparticles were added to the solution. The mixture

was incubated for 30 min at 37 ℃. Then the deposition was washed with 200 μL loading buffer by magnetic separation three times. After that, phosphopeptides were eluted with 10 μL of eluting buffer (0.4 M ammonium hydroxide). Subsequently, the eluent was analyzed directly by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with the help of DHB matrix.

20 μL of HeLa digestion was added in 200 μL loading buffer (ACN/H_2O/TFA = 50/49/1, v/v/v), and then 400 μg Fe_3O_4@TiTA nanoparticles were added to the solution. The mixture was incubated for 40 min at 37 ℃. Then the deposition was washed with 200 μL loading buffer three times. After that, phosphopeptides were eluted with 30 μL of eluting buffer (0.4 M ammonium hydroxide) at 37 ℃ (2x30min). The samples were desalted, lyophilized and redissolved for Nano-LC-MS/MS analysis.

Nano-LC-ESI-MS/MS.

First of all, solvent A (water containing 0.1% formic acid) and solvent B (ACN containing 0.1% formic acid) were prepared. The lyophilized eluent was dissolved with 10 μL solvent A. The captured peptides were separated by Nano-LC, and on-line electrospray tandem mass spectrometry was used to analyze them. The experiments were performed on an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an online nano-electrospray ion source. A 5 μL peptide sample was loaded on the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm ×2 cm) and separated on the analytical column (Acclaim PepMap C18, 75 μm × 25 cm) with a linear gradient, from 2% B to 40% B in 110 min. The column was re-equilibrated at initial conditions for 15 min with the column flow rate at 300 nL min^{-1} and column temperature at 40 °C. A data-dependent mode was adopted in the Orbitrap Fusion mass spectrometer to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350–1500) were gained in the Orbitrap with a mass resolution of 120 000 at m/z 200. The AGC target was set to 1000 000 with maximum injection time at 50 ms. MS/MS acquisition was performed in the Orbitrap with a cycle time of 3 s, the resolution was 15000 at m/z 200. The threshold value of

intensity was 50 000 and maximum injection time was 80 ms. Ions with charge states from 2+ to 5+ were sequentially fragmented by higher energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 30%. The AGC target was set to 100 000 with the isolation window at 1.8 m/z. In all cases, one microscan was recorded using dynamic exclusion of 21 seconds. MS/MS fixed first mass was set at 110.

Database search.

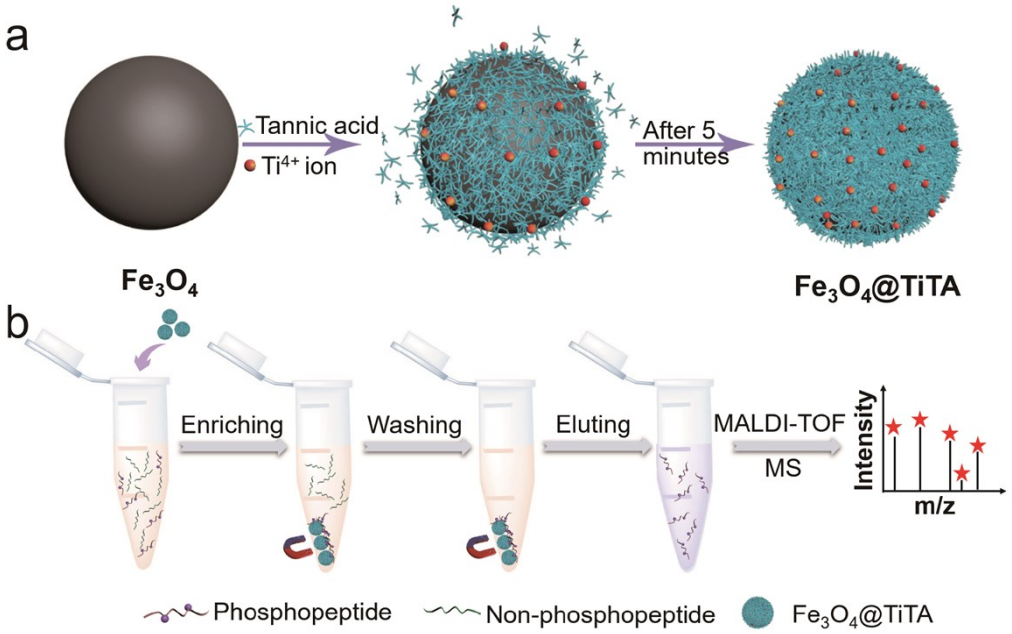
The raw mass spectrometry data files were extracted by the Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.0.288) with the MASCOT searching engine version 2.3.2. Human UniProtKB/Swiss-Prot database (Release 2015_12, with 20 199 sequences) was chosen as the database.

The Orbitrap Fusion instrument was applied to generate raw S3 files. Search parameters were precursor and fragment mass tolerance (10 ppm and 0.05 Da, respectively). The retained peptides contained at least seven amino acids. Carbamidomethyl on cysteine was set as a fixed modification. Variable modifications include oxidation (M) and phosphorylation (STY). The probability of phosphorylation site was calculated by the phosphoRS 3.0 algorithm. The target-decoy based strategy was used to control peptide level FDRs < 1%, which confirmed the reliability of the obtained results in this research.

Characterization

The transmission electron microscopy (TEM) images of Fe_3O_4@TiTA nanoparticles were conducted on a JEOL 2011 transmission electron microscopy. Scanning electron microscopy (SEM) images were investigated with the usage of a Philips XL30 electron microscope and the element analysis was measured by energy dispersive X-ray (EDX) spectroscopy with a Philips XL30 electron microscope at 20 kV. Fourier transform infrared (FT-IR) spectrum was acquired on a Nicolet Fourier spectro photometer (Thermo Fisher). The zeta-potential and dynamic light scattering (DLS) were measured with the utilization of a Malvern Nano Z Zetasizer. Magnetization measurement was conducted on an S-(SQUID) VSM (Quantum Design, USA). All MALDI-TOF MS experiments were performed on AB Sciex 5800 MALDI TOF/TOFTM mass spectrometer (AB Sciex, USA) in a reflector positive mode with a 355 nm Nd-YAG

157 laser, 200 Hz frequency, and acceleration voltage of 20 kV. Thermogravimetric
158 analysis (TGA) was operated by SDT Q600 thermogravimetric analyzer with nitrogen
159 atmosphere, temperature range: 25-800 °C, heating rate: 10 °C·min^{-1}.



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161 **Scheme S1** (a) The dynamic synthesis procedure of Fe_3O_4@TiTA and (b) workflow of

162 phosphopeptide enrichment with Fe_3O_4@TiTA nanoparticles.

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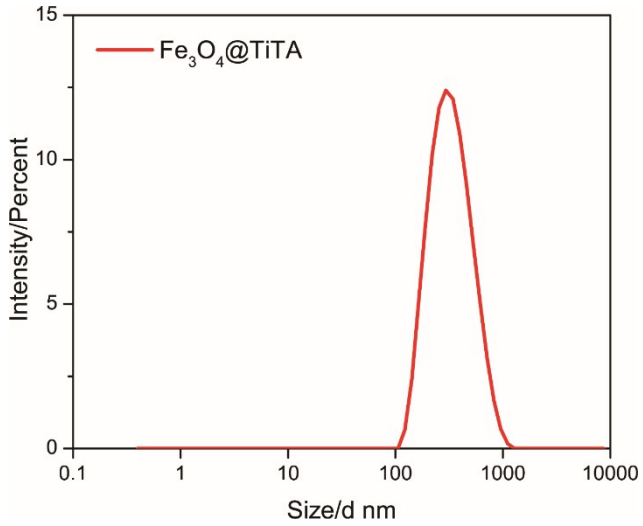

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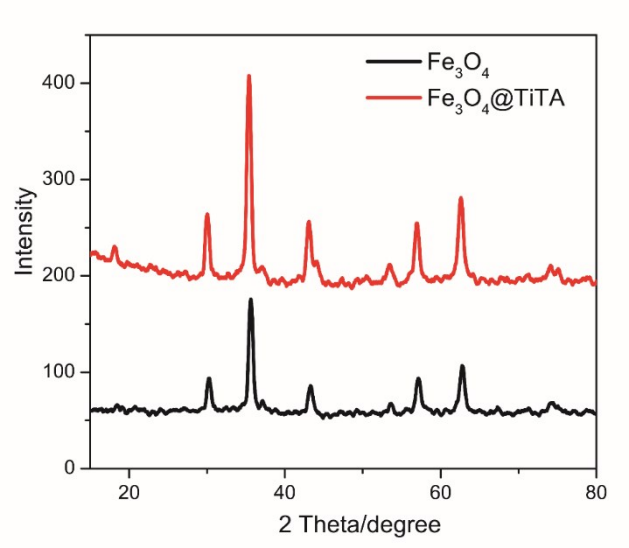
165 **Fig. S1** Dynamic Light Scattering of Fe_3O_4@TiTA nanoparticles.



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167 **Fig. S2** The X-ray diffraction of Fe_3O_4 and Fe_3O_4@TiTA nanoparticles.

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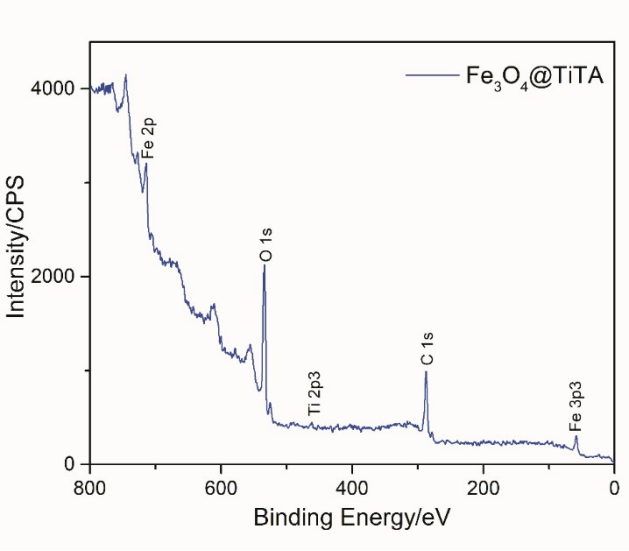

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170 **Fig. S3** XPS patterns of Fe_3O_4@TiTA nanoparticles.

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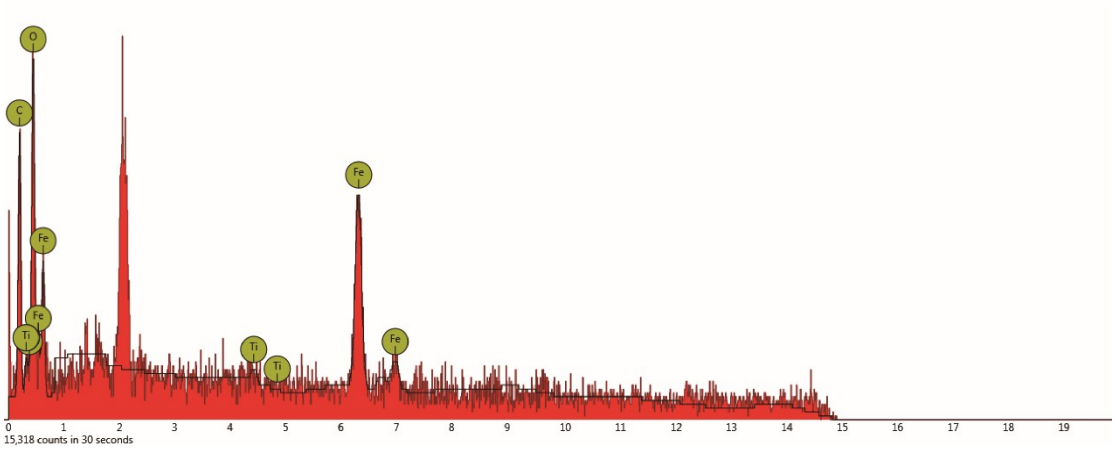


Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
26	Fe	Iron	12.33	36.44
6	C	Carbon	53.54	34.02
8	O	Oxygen	33.75	28.57
22	Ti	Titanium	0.38	0.96

Fig. S4 Energy dispersive X-ray (EDX) spectrum data of Fe_3O_4@TiTA.

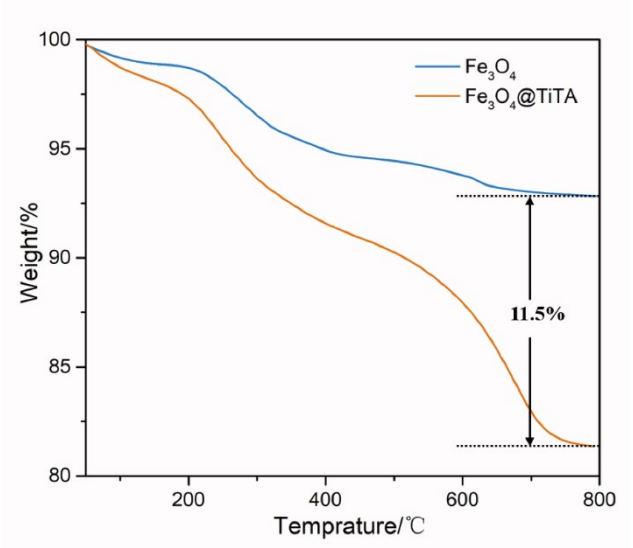


Fig. S5 The thermogravimetric analysis of Fe_3O_4 and Fe_3O_4@TiTA nanoparticles.

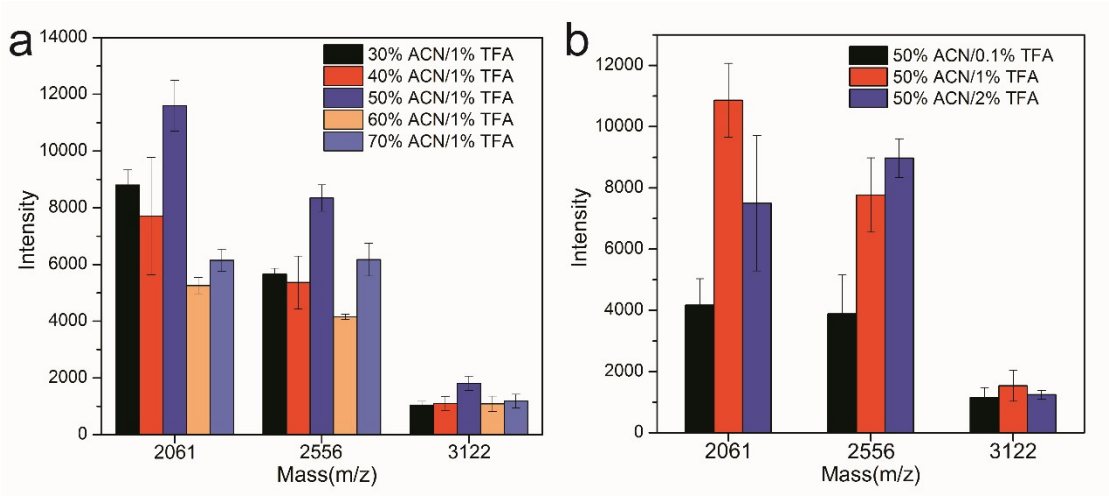


Fig. S6 Peak intensities of three selected phosphopeptides enriched by Fe_3O_4-CA nanoparticles in 100 fmol/μL β-casein digestion: a) in different concentration of ACN in loading buffer; b) in different concentration of TFA in loading buffer. N=5.

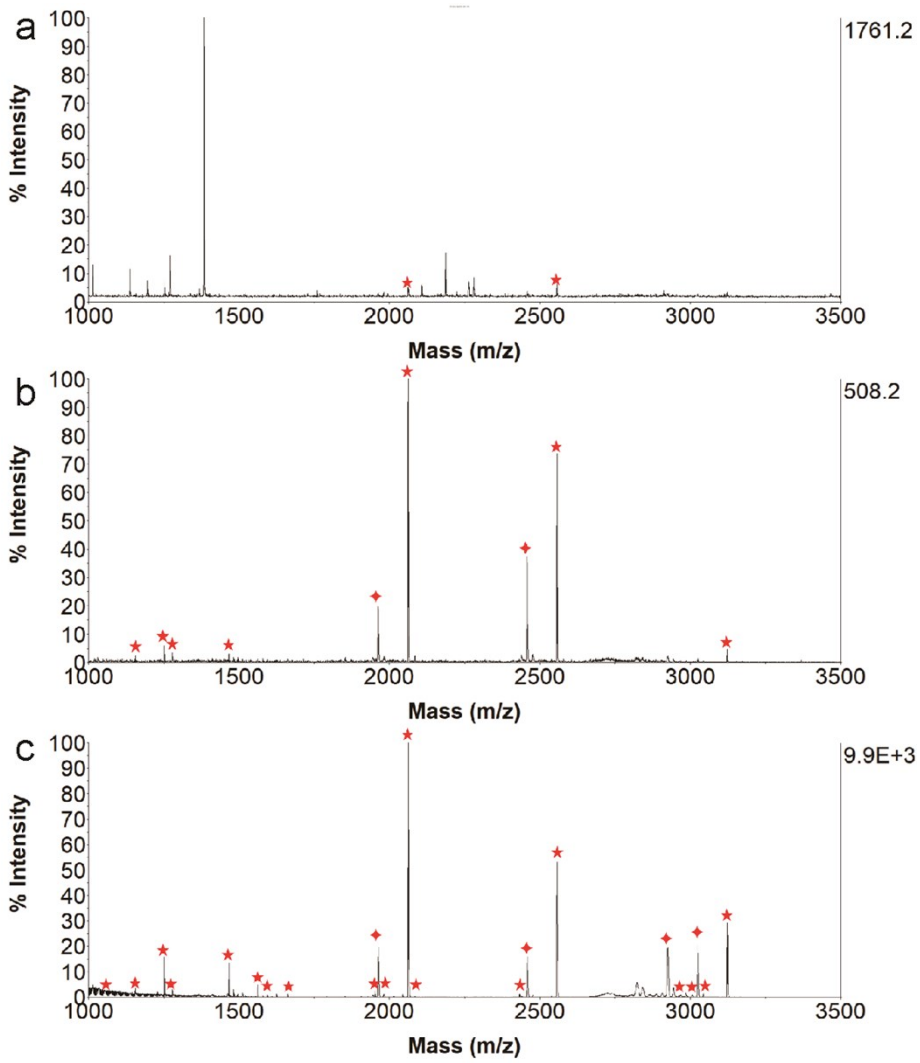
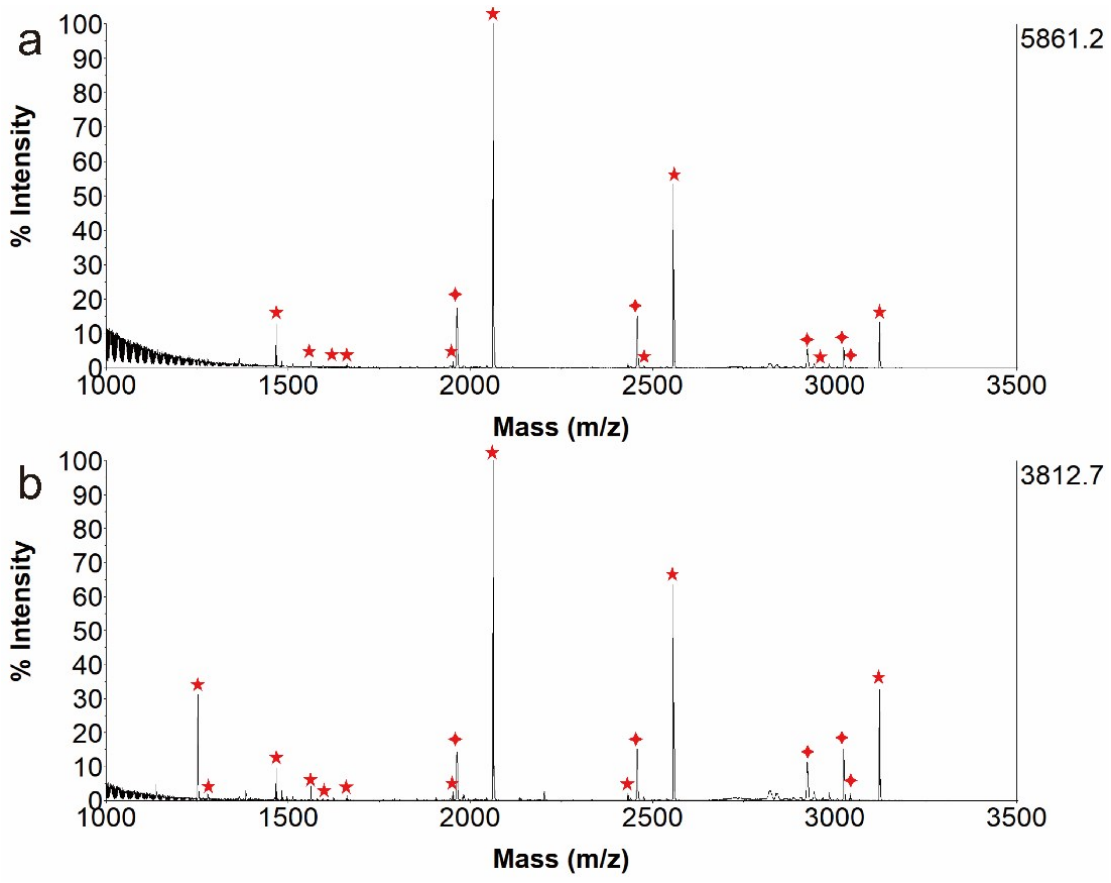


Fig. S7 The MALDI-TOF mass spectra of 100 fmol/μL β-casein digestion: before treatment (a), after treatment with Fe_3O_4 nanoparticles (b) and Fe_3O_4@TiTA nanoparticles (c). The identified phosphopeptides and dephosphorylated peptides are marked with ★and ✦, respectively.



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198 **Fig. S8** The MALDI-TOF mass spectra of 100 fmol/μL β-casein digestion after

199 treatment with Fe_3O_4@FeTA nanoparticles (b) and Fe_3O_4@ZrTA nanoparticles. The

200 identified phosphopeptides and dephosphorylated peptides are marked with ★

201 and ✦ , respectively.

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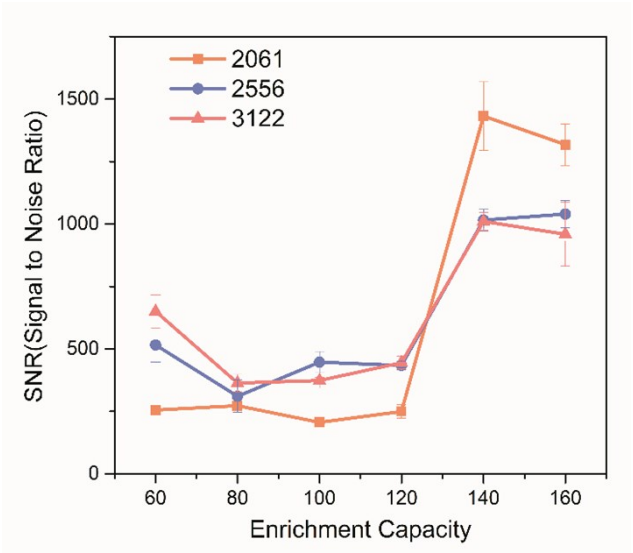

Fig. S9 The S/N ratio of phosphopeptides derived from β-casein digestion in the supernatant after enrichment by Fe_3O_4@TiTA nanoparticles. N=5.

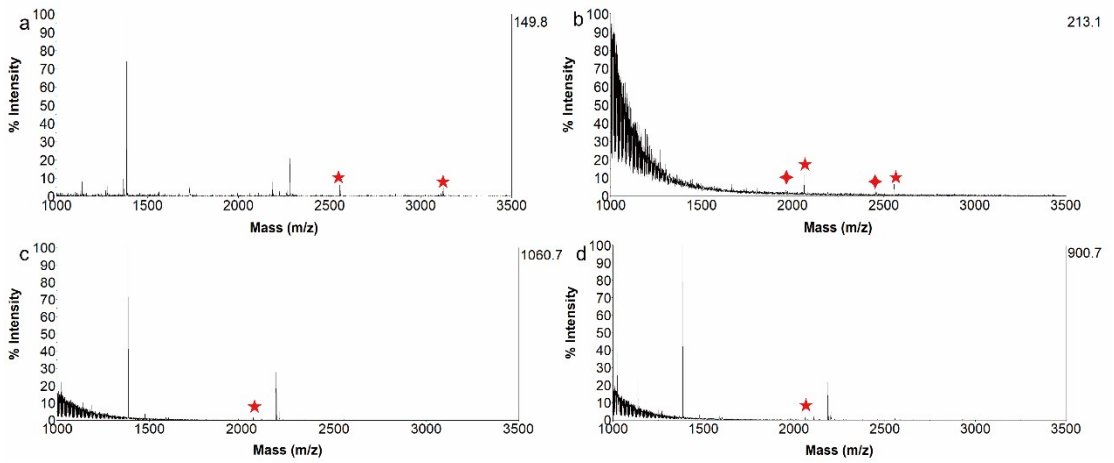

Fig. S10 The MALDI-TOF mass spectra of 0.5 fmol/μL β-casein digestion: before treatment (a), after treatment with Fe_3O_4@TiTA nanoparticles (b), Fe_3O_4@ZrTA anoparticles (c) and Fe_3O_4@FeTA nanoparticles (d). The identified phosphopeptides and dephosphorylated peptides are marked with ★ and ✦, respectively.

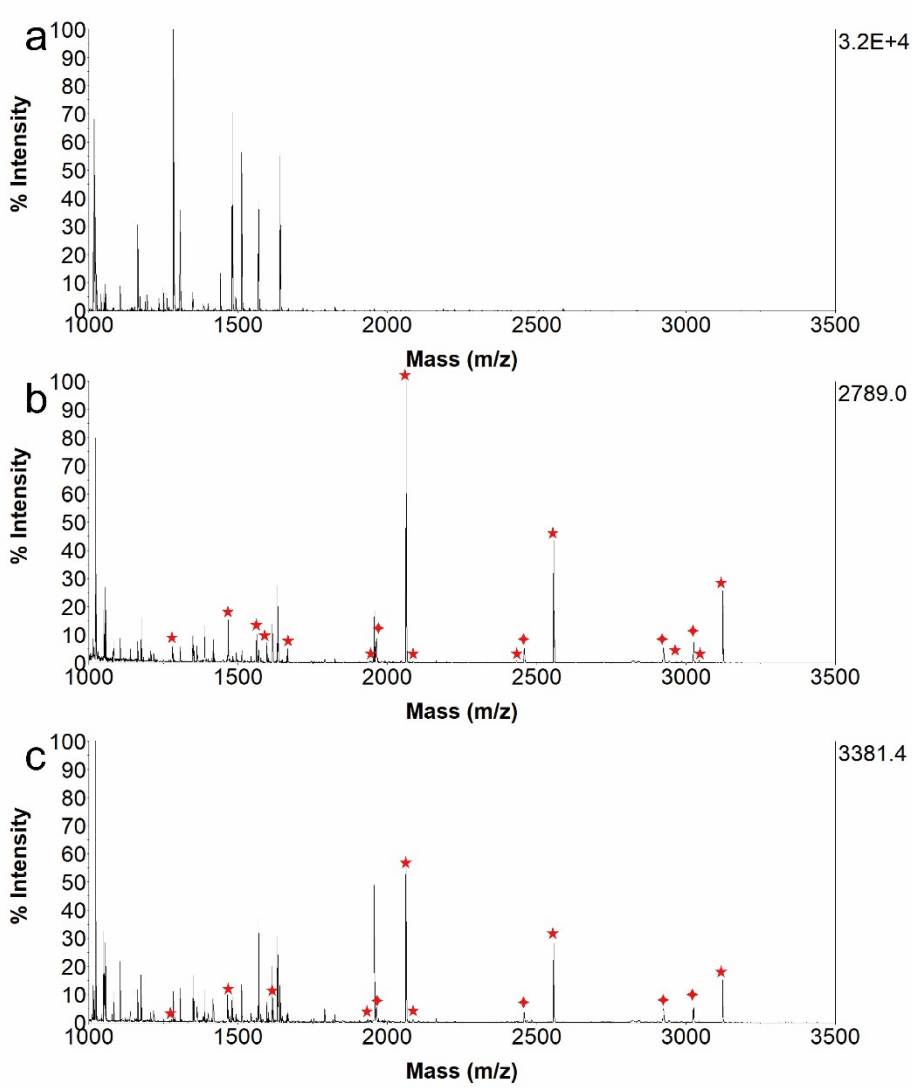

Fig. S11 The MALDI-TOF mass spectra of a mixture of BSA and tryptic digestion of β-casein: (a) before enrichment and (b) after treatment with Fe_3O_4@TiTA with the mass ratios of BSA:β-casein digestion of 40:1; (c) after treatment with Fe_3O_4@TiTA with the mass ratios of BSA:β-casein digestion of 100:1. The identified phosphopeptides and dephosphorylated peptides are marked with ★ and ✦, respectively.

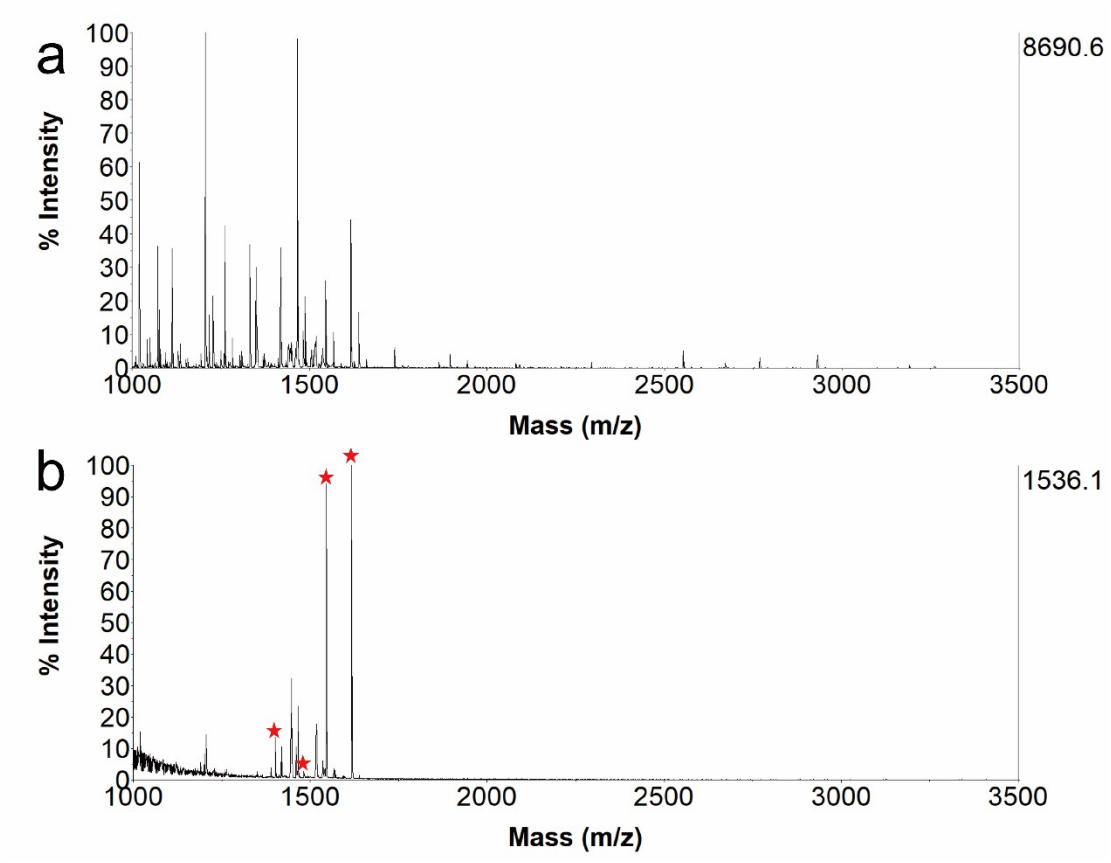

Fig. S12 The MALDI-TOF mass spectra of human serum: before treatment (a) and the eluent after enrichment with Fe_3O_4@TiTA (b).

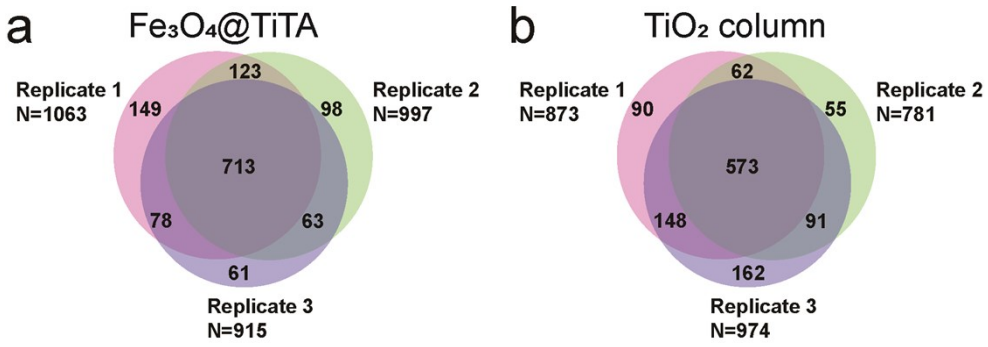

Fig. S13 Overlap of phosphoproteins captured by Fe_3O_4@TiTA (a) and TiO_2 column (b) of three biological replicates.

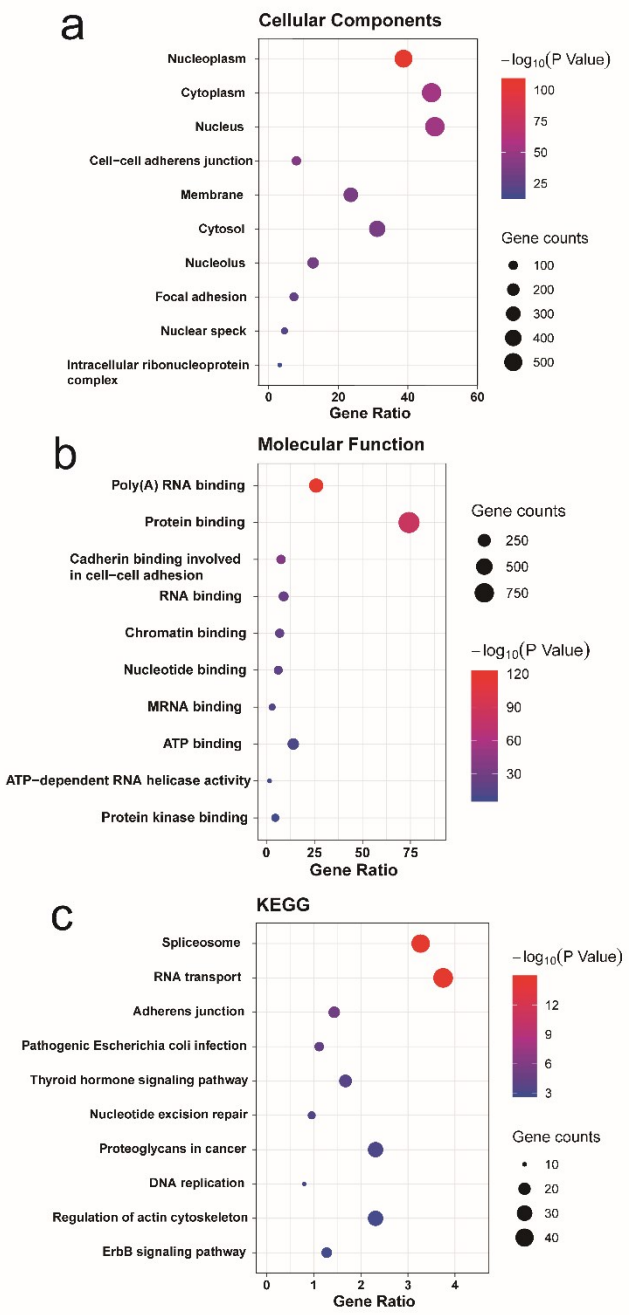

Fig. S14 The gene onthology (GO) term enrichment analysis of the cellular components (a), molecular function (b) and KEGG pathway (c).

Table S1. Detailed information of the observed phosphopeptides in β-casein digests by using Fe_3O_4@TiTA, Fe_3O_4@ZrTA and Fe_3O_4@FeTA. (S: phosphorylation site)

No.	Observed m/z	Amino acid sequence	Fe_3O_4@ TiTA	Fe_3O_4@ FeTA	Fe_3O_4@ ZrTA	Fe_3O_4
1	1031.0	FQSEEQQQTEDELQDK	√			
2	1154.6	SSEEKFLR	√			√
3	1251.6	TVD[Mo]ESTEVF	√		√	√
4	1279.0	FQSEEQQQTEDELQDKIHPF	√		√	√
5	1466.6	TVDMESTEVFTK	√	√	√	√
6	1562.1	EQLSTSEENSKK	√	√	√	
7	1594.7	TVDMESTEVFTKK	√	√	√	
8	1660.7	VPQLEIVPNSAEER	√	√	√	
9	1951.9	YKVPQLEIVPNSAEER	√	√	√	
10	1979.9	NMAINPSKENLCSTFCK	√			
11	2061.8	FQSEEQQQTEDELQDK	√	√	√	√
12	2083.8	KYKVPQLEIVPNSAEER	√			
13	2432.0	IEKFQSEEQQQTEDELQDK	√	√	√	
14	2556.1	FQSEEQQQTEDELQDKIHPF	√	√	√	√
15	2966.3	ELEELNVPGEIVESLSSSEESITR	√	√		
16	3008.2	NANEEEYSIGSSSEESAEVATEEVK	√			
17	3122.3	RELEELNVPGEIVESLSSSEESITR	√	√	√	√

Table S2. Detailed information of the observed phosphopeptides derived from 10 μL human serum. (S: phosphorylation site)

No.	Observed m/z	Phosphorylation site	Amino acid sequence
S1	1389.4	1	DSGEGDFLAEGGGV
S2	1460.4	1	ADSGEGDFLAEGGGV
S3	1545.5	1	DSGEGDFLAEGGGVR
S4	1616.5	1	ADSGEGDFLAEGGGVR

Table S3. The comparison table of Fe_3O_4@TiTA and other functional materials in identifying phosphopeptides in HeLa cell lysates.

Material	Protein amount	Mass spectrometry	Run	Identified phosphopeptides/proteins
Fe_3O_4@TiTA	100μg	Orbitrap Fusion	Three	3456/1285
PNI-*co*-$ATBA_{0.2}$@SiO_2[2]	50μg	LTQ-Orbitrap Velos	-*	631/721
MagG@PEI@PA-Ti^{4+} [3]	200μg	Q-Exactive plus	Two	574/341
Ti^{4+}-IMAC[4]	250μg	LTQ-Orbitrap Velos	Two	~4700/-
DMSNs@PDA-Ti^{4+} [5]	-*	Q-Exactive	Three	2422/-
TiO_2/Bi/Fe/Zr[6]	-*	LTQ-Orbitrap Velos	-*	434/-*
Fe_3O_4@H-TiO_2@f-NiO[7]	-*	Q-Exactive	-*	972/

-* means not mentioned

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