Supporting Information for

Specific Enrichment of Phosphoproteins Using Functionalized Multivalent Nanoparticles

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I. Materials, Instrumentation and Methods

I.1 Synthesis and Characterization of Ligand Molecules and Functionalized Nanoparticles

Chemicals and solvents used for the synthesis of GAPT ligand molecules and nanoparticles

All chemicals and reagents were used as received without further purification unless otherwise noted. N-(tert-Butoxycarbonyl)-L-tyrosine methyl ester (Boc-Tyr-OMe), di-(2-picolyl)amine (DPA), paraformaldehyde, trifluoroacetic acid (TFA), glutaric anhydride, N-ethyldiisopropylamine (EDPA), Iron (III) acetylacetonate (acac) (97%), benzyl ether (98%), 1,2-hexadecanediol (90%), oleic acid (OA) (99%), oleylamine (OE) (>70%), anhydrous N,N-dimethylforamide (DMF), and anhydrous dichloromethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate **(**HBTU) were obtained from TCI America (Portland, OR, USA). (3-aminopropyl)trimethoxysilane (APTMS) and 2-methoxy (polyethyleneoxy) propyl trimethoxysilane (average molecular weight =550-600, hereafter referred as Si-PEG) were purchased from Gelest (Morrisville, PA, USA). Zinc chloride ($ZnCl_2$) was obtained from Acros Organics (Morris Plains, NJ, USA). Column chromatography was performed with Silicycle 60 silica gel and thin layer chromatography (TLC) was performed with alumina pre-coated with 60 mesh silica gel.

Characterizations of molecules and materials

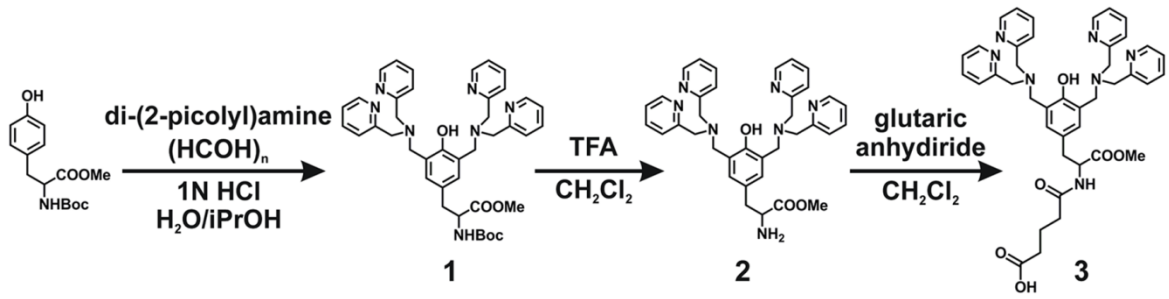
^{1}H-NMR spectra were recorded on a Hermes-Varian MercuryPlus 300 (300MHz) spectrometer at room temperature. Chemical shifts were recorded in ppm (parts per million) using residual solvent peaks as internal references [$CDCl_3$ δ: 7.26 (^{1}H)]. Electron spray ionization (ESI) mass spectra for the synthesized ligand molecules were obtained using a Waters (Micromass) LCT®

mass spectrometer. Transmission Fourier transform infrared (FT-IR) spectroscopy measurements were recorded on a Bruker Equinox 55 FT-IR spectrometer in the range of 4000 cm^{-1} to 600 cm^{-1} at about 2 cm^{-1} resolution by using potassium bromide (KBr) pellet.

Transmission electron microscopy (TEM) samples were prepared by pipetting one drop of nanoparticle (NP) solution onto a copper TEM grid with carbon film. TEM was conducted on Philips CM200 Ultra Twin instrument operated at 200 kV and images were collected using a Gatan CCD image system with digital micrograph software program. Powder X-ray diffraction (PXRD) data were collected on as-synthesized Fe_3O_4 NPs deposited on a glass substrate using a Bruker D8 Advance using Cu Kα radiation (λ= 1.5418 Å) and the background from the glass substrate was subtracted. The X-ray photoelectron spectra (XPS) were recorded on a Perkin Elmer PHI-5400 spectrometer with Mg Kα radiation (1254 eV) operated at 15 kV and 300 mA as the X-ray excitation source and a take-off angle of 45 degrees. Thermogravimetric analysis (TGA) was carried out using a TGA Q500 thermal analysis system. All TGA measurements were performed under a N_2 atmosphere at a constant heating rate of 10 °C/min from 100 ^oC to 600 ^oC. All samples were first heated to 100 °C and held at that temperature for 2 min to remove adsorbed water. Data were analyzed using the Thermal Advantage (TA) universal analysis software program. Circular dichroism (CD) spectra were recorded on an Aviv CD spectrometer (Model 420).

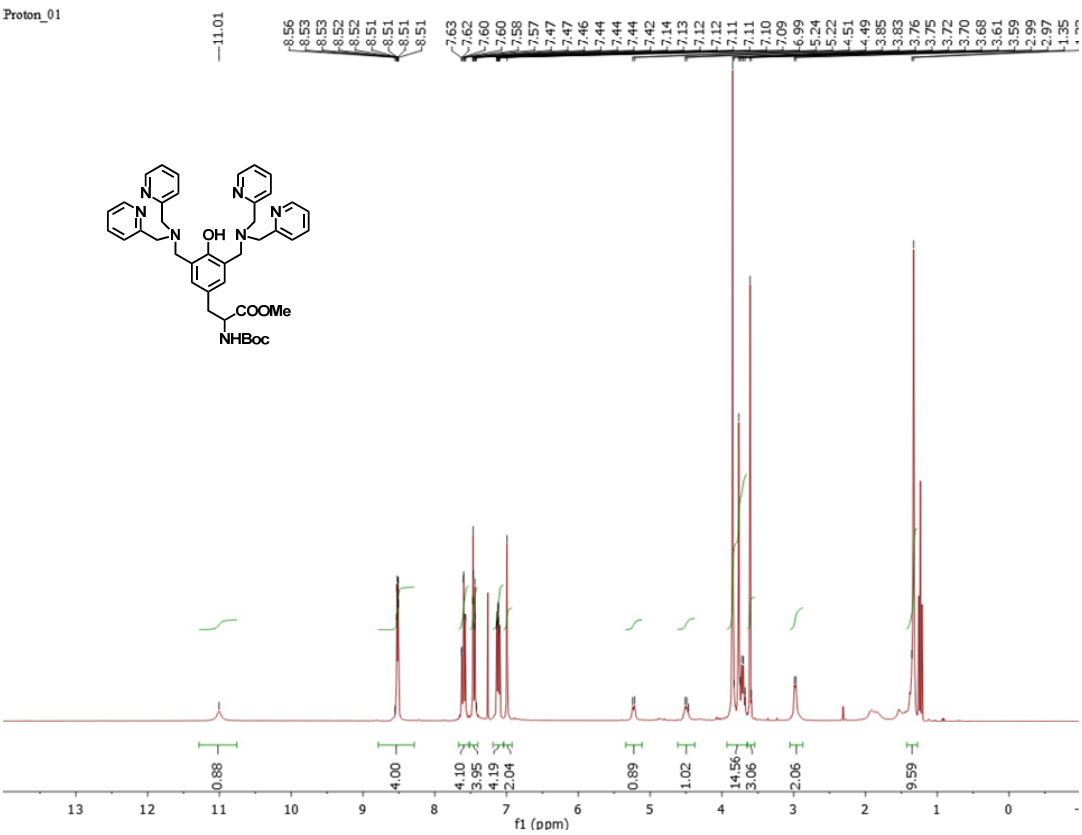
Synthesis of GAPT ligand molecules and their characterization

The GAPT ligand was synthesized using commercially available reagents following the synthesis scheme below:



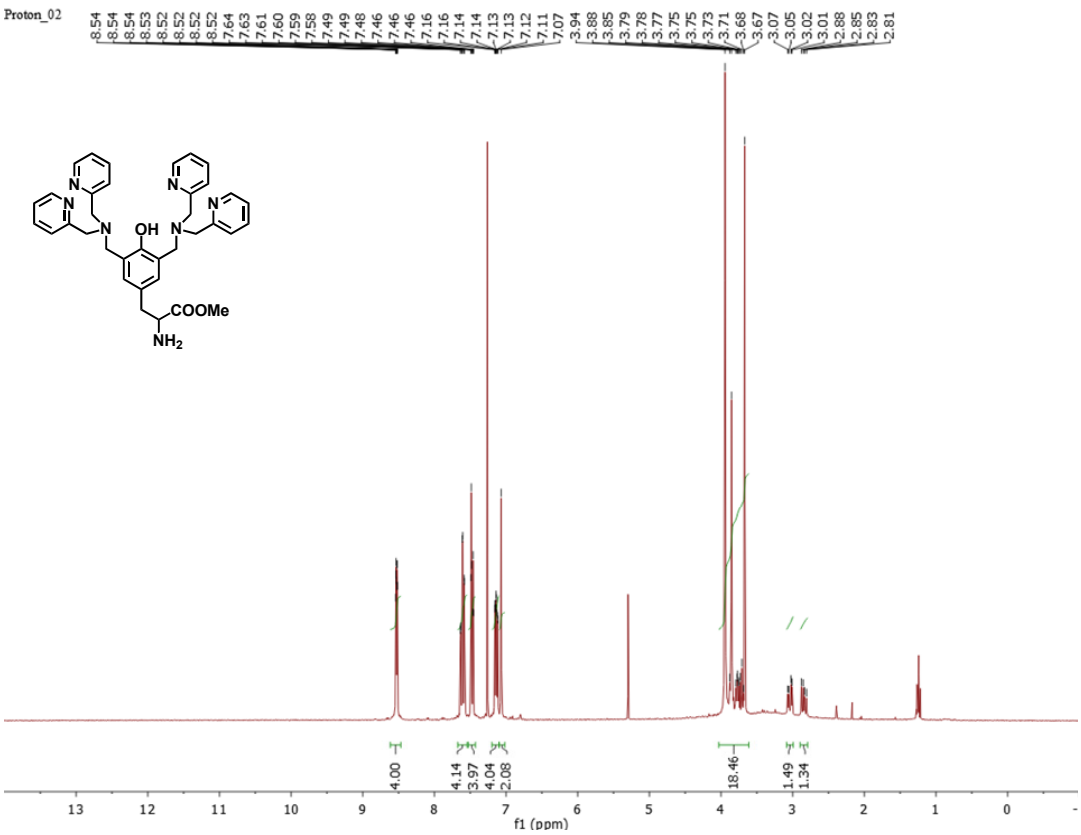
Boc-DpaTyr-OMe (1): To a solution of di-(2-picolyl)amine (3.37 g, 16.9 mmol) and paraformaldehyde (0.813 g, 27.1 mmol) in 50 mL of water/i-PrOH mixture (5:3, v/v), ~1.68 mL of 1N HCl (aq) was added. After stirring for 5 min at room temperature, Boc-Tyr-OMe (2.0 g, 6.77 mmol) was added and then stirred for 5 min at room temperature. The mixture was refluxed at ~110 °C for 48 h, then cooled to room temperature, and i-PrOH was evaporated. After cooling it in an ice-bath, the solution was decanted. The resulting viscous oil was dissolved in EtOAc. The solution was washed with saturated $NaHCO_3$ (three times) and brine (one time) followed by drying with $MgSO_4$. After the solvent was evaporated, the residue was purified by column chromatography (SiO_2, EtOH/EtOAc = 1:1 (v/v)) to produce **1** (3.44 g, 52.6 %) as a amorphous light yellow powder.

^{1}H NMR (300 MHz, $CDCl_3$), see below: δ 11.06 (1H, br s), 8.53 (4H, d, J = 6 Hz), 7.62 (4H, td, J= 7.5 Hz), 7.47 (4H, d, J= 9 Hz), 7.13 (4H, td, J= 6 Hz), 6.99 (2H, s), 5.23 (1H, d, J= 6 Hz), 4.49-4.51 (1H, m), 3.85 (8H,s), 3.76 (4H, s), 3.59-3.61(3H, s), 2.99 (2H, s), 1.35 (9H, s). ESI-MS calc'd. *m/z* for $C_{41}H_{47}N_7O_5$ $[M+H]^+$ 718.8, found: 718.1 and $[M+Na]^+$ 740.8, found: 740.1



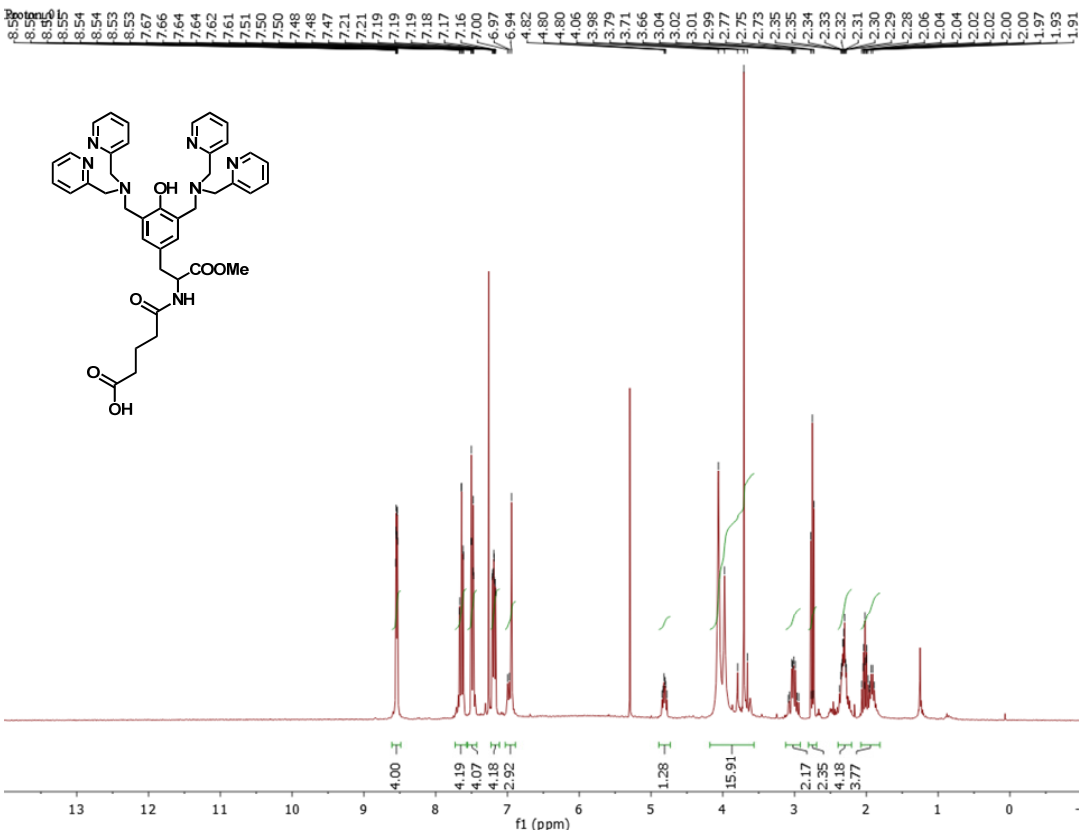
H-DPATyr-OMe (2): TFA (20.6 mL) was added dropwise to a solution of **1** (3.44 g, 4.73 mmol) in anhydrous CH_2Cl_2 (34.4 mL) in an ice bath. The solution was stirred at room temperature for 2.5 h. After removing CH_2Cl_2 by evaporation, the residue was dissolved in water and alkalized with NH_3 (aq) solution in an ice bath. The resulting mixture was extracted with CH_2Cl_2 twice. The combined organic layers were washed with brine and dried with $MgSO_4$. After evaporating the solvent, **2** was obtained quantitatively as pale yellow viscous oil, which is consistent with a previous report.[S1]

^{1}H NMR (300 MHz, $CDCl_3$), see below: δ 8.53 (4H, d, J = 6 Hz), 7.61 (4H, td, J= 6 Hz), 7.46 (4H, d, J= 9 Hz), 7.13 (4H, td, J= 6 Hz), 7.03 (2H, s), 3.86 (8H, s), 3.79 (4H, s), 3.70-3.65 (4H, m), 3.01-2.96 (1H, m), 2.80-2.75 (1H, m). ESI-MS calc'd. *m/z* for $C_{36}H_{39}N_7O_3$ $[M+H]^+$ 618.7, found: 618.1 and $[M+Na]^+$ 640.7, found: 640.1.



GA-DPATyr-OMe (3): Glutaric anhydride (0.803 g, 7.04 mmol) was added to a solution of **2** (2.89 g, 4.69 mmol) in anhydrous CH_2Cl_2 (113 mL). The mixture was stirred and refluxed at ~50 °C overnight. After removing the solvent by evaporation, **3** was obtained quantitatively as a light yellow viscous oil.

^{1}H NMR (300 MHz, $CDCl_3$), see below: δ 8.53 (4H, d, J = 6 Hz), 7.61(4H, td, J= 7.5 Hz), 7.46 (4H, d, J= 9 Hz), 7.13 (4H, td, J= 6 Hz), 7.03 (2H, s), 3.86 (8H,s), 3.79 (4H, s), 3.70-3.65 (4H, m), 3.01-2.96 (1H, m), 2.80-2.75 (1H, m). ESI-MS calc'd. *m/z* for $C_{41}H_{45}N_7O_6$ $[M+H]^+$ 732.8, found: 732.3 and $[M+Na]^+$ 754.8, found: 754.3.



Synthesis of functionalized Fe_3O_4 nanoparticles (NPs)

OA/OE capped Fe_3O_4 NPs. The magnetite NPs were prepared following the procedure reported by Sun *et.al*.[S2] Briefly, $Fe(acac)_3$ (703.4 mg, 2 mmol), 1,2-hexadecanediol (2.584 g, 10 mmol), OA (1.659 g, 6 mmol), and OE (1.61 g, 6 mmol) were mixed in benzyl ether (20 mL). The mixture was heated to 200 °C for 2 h with stirring under N_2 (g), and then heated to reflux at ~300 °C for 1 h. After the mixture was cooled to room temperature and EtOH (40 mL) was added to the mixture. A black precipitate was obtained by centrifugation, which was subsequently redissolved in hexane in the presence of OA (100 μL) and OE (100 μL). Undissolved residue was removed by centrifugation (5000 rpm, 15 min). The supernatant was precipitated with EtOH, centrifuged (5000 rpm, 20-30 min) to remove the solvent, and then redispersed in hexane. The step was repeated three times to wash the NPs and remove unreacted OA and OE.

Fe_3O_4-NH_3/PEG NPs. The NP functionalization by trialkoxysilane molecules was performed based on previously published methods[S3,S4] with minor modifications. Briefly, under ambient conditions, 0.5% (v/v) trialkoxy silane solution in hexane containing APTMS and Si-PEG in a ~2:1 molar ratio was added to the Fe_3O_4 NPs dispersed in hexane (~11-12 mg in 300 mL). The mixture was stirred and refluxed at 70-72 °C for 48 h, during which a black-brown precipitate was formed. The precipitate was washed three times with hexane and three times with EtOH to remove excess silane molecules in solution. Finally, the NPs were dried for the next reaction.

Fe_3O_4-GAPT NPs. GAPT (560 mg, 0.765 mmol) was dissolved in anhydrous DMF (6.9 mL), and then HBTU (435.8 mg, 1.15 mmol) and EDIPA (741.5 mg, 5.74 mmol) were added to the solution. During the time the GAPT solution was stirred, the Fe_3O_4-NH_3/PEG NPs were redispersed in DMF at a concentration of ~10 mg/mL. EDIPA (77.5 mg, 0.6 mmol) was added to the NP solution, it was sonicated for 1 min, and then the mixture containing GAPT, HBTU, and EDIPA was added and the whole mixture was stirred at room temperature for 24 h. The resulting black-brown precipitate was washed two times with DMF, one time with DMF/MeOH (v/v 3:1), one time with DMF/MeOH (v/v 1:1), and one time with MeOH to remove unreacted GAPT, HBTU, and EDIPA. The resulting Fe_3O_4-GAPT NPs were redispersed in EtOH at a concentration of ~50 mg/mL.

Fe_3O_4-GAPT-Zn NPs. A 150-180 µL aliquot of Fe_3O_4-GAPT NP stock solution (at ~50 mg/mL) was transferred to a 2 mL vial. The NPs (~7.5-9 mg) were washed with de-ionized water three times to remove EtOH. After 1.5 mL of 10 mM $ZnCl_2$ (aq) was added to the Fe_3O_4-GAPT NPs, the mixture was agitated for 3.5 h at room temperature to chelate Zn^{2+} ions with the pyridine groups on the GAPT ligand molecules. The NPs were precipitated by a magnet

(centrifugation was also used to collect the NPs if needed) and then sonicated in de-ionized water to wash. The final product, Fe_3O_4-GAPT-Zn NPs, were used for enriching phosphoproteins.

I.2 Phosphoprotein Enrichment Procedures using Fe_3O_4-GAPT-Zn NPs and Evaluation of the Enrichment Performance

Chemicals and materials for phosphoprotein enrichment experiments

Bovine serum albumin (BSA), β-casein, and pepsin were purchased from Sigma-Aldrich. All proteins were used without further purification or modification. 50 mM HEPES (4-(2-hydroxyethyl)-piperazineethanesulfonic acid) buffer was made by directly diluting 0.5 M HEPES buffer (pH=7.7; home-made with HEPES obtained from Fluka) with 18 MΩ nanopure de-ionized water from Milli-Q water (Millipore, Corp., Billerica, MA, USA). 100 mM sodium phosphate buffer was prepared by diluting 0.5 M sodium phosphate (pH 7.3; home-made with sodium phosphate dibasic obtained from Sigma-Aldrich). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA). Trypsin was obtained from Promega (Fitchburg, WI, USA). Ammonium bicarbonate, water (HPLC grade), and formic acid were purchased from Fischer Scientific (Rockford, IL, USA) and used as received. 12.5% mini-gel (10 comb well, 10.0 cm × 10.0 cm) for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was home-made. 12.5% Criterion precast gel (18 comb well, 13.3 cm × 8.7 cm (W×L)) was purchased from Bio-Rad (Hercules, CA, USA). Amicon ultra-0.5 mL centrifugal filter with ultracel-10, or -30 membrane was purchased from Millipore. Sypro Ruby and Pro-Q Diamond staining solutions were from Invitrogen (Carlsbad, CA, USA). A commercial Pierce® phosphoprotein enrichment kit was purchased from Thermo Fischer Scientific (Rockford, IL, USA)

Procedures for protein enrichments and SDS-PAGE analysis

Standard protein sample preparation. Each of the standard proteins BSA, β-casein, and pepsin was dissolved in 50 mM HEPES buffer (pH 7.7, with 150 mM NaCl) at a concentration of about ~15-20 mg/mL to be used as stock solutions. For the test of enrichment performance of Fe_3O_4-GAPT-Zn NPs, a simple mixture containing 350 μg of BSA and 350 μg of β-casein dissolved in 500 μL of 50 mM HEPES buffer (pH 7.7 ± 0.1, 150 mM NaCl) was prepared.

Preparation of cell lysates. Human Embryonic Kidney (HEK) 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum in 150 mm plates (Gibco). HEK 293 cells were detached from the plates using trypsin (Invitrogen), and centrifuged to pellet the cells. The cell pellets were subsequently washed twice by resuspending them in cold PBS buffer followed by centrifugation. Cells were lysed by incubation with 1 mL per 1×10^7 cells of a lysis buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA) containing a cocktail of protease and phosphatase inhibitors for 45 min on ice. The vials were vortexed and pipetted up and down every 10 min. The resulting cell lysates were centrifuged at 10,000 ×g for 20 min and then the supernatant, which contains soluble proteins, was collected and used for subsequent experiments (the pellet was discarded). The concentration of the cell lysate was determined by Bradford protein assay.

Swine heart tissue extraction. Swine hearts were excised from healthy Yorkshire domestic pigs, snap frozen in liquid N_2, and stored under -80 ^oC before use. First, the frozen pig tissue samples (~1 g) were cut into small pieces and immediately washed twice in cold 25 mM HEPES buffer (pH 7.4). The tissue samples were homogenized 5-6 times using a Polytron electric homogenizer (Model PRO200, PRO Scientific Inc., Oxford, CT, USA) for 5-7 secs on ice in buffer containing 25 mM HEPES (pH 7.4), 50 mM NaF, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 2.5

mM EDTA, and protease inhibitor cocktail (Roche, Switzerland). After centrifugation, the remaining pellet was briefly homogenized two times in the same buffer followed by 30 min of centrifugation at 16,000 ×g at 4 ^{o}C. The supernatants were collected, the concentration of the tissue lysate was determined by Bradford protein assay. The samples were stored in -80 ^{o}C freezer for later study.

General procedure for enrichment experiments. The complex protein mixtures (HEK 293 cell lysate and swine heart tissue extract) were diluted in loading buffer consisting of 50 mM HEPES buffer (pH 7.7) with 150 mM NaCl to achieve a final protein concentration of ~4 mg/mL (based on protein assay). The loading mixture (total volume = 1 mL) was mixed with Fe_3O_4-GAPT-Zn NPs and then agitated on an end-to-end shaker at 4 °C for 2 h (if needed, it was agitated for 6 h). Then the NPs were collected using a magnet (centrifugation was also used to isolate the NPs if needed) and the supernatant was removed and saved as the flow-through (FT). The isolated NPs were rinsed with the same HEPES buffer three times to remove non-phosphoproteins. To elute the phosphoproteins that were bound to the NPs, 150 µL of 100 mM sodium phosphate buffer (pH 7.3, with 200 mM NaCl) was added, the reaction was agitated for 10 min, and the supernatant was collected as the elution. The elution step was repeated three more times. All elution fractions were combined, concentrated to ~50 µL and desalted for further analysis by MS. Phosphoprotein enrichement using the Pierce® phosphoprotein enrichment kit was carried out according the protocol provided with the kit.

SDS-PAGE analysis and staining. Standard protein mixtures (before and after enrichment) were separated by SDS-PAGE (12.5% gel). 15 µg of protein mixtures before and after enrichment from HEK 293 cell lysate and 10 µg of protein mixtures before and after enrichment from swine heart tissue extract were separated on a Criterion Tris-HCl (10.5-14 %) precast gel

(Bio-Rad) and stained with Pro-Q Diamond (Invitrogen) followed by Sypro Ruby (Invitrogen) according to the manufacturers' protocols. To visualize phosphoproteins, the gel was stained with Pro-Q Diamond first and imaged using a Typhoon 9200 imager (GE Healthcare, Bio-Science, Piscataway, NJ, USA) with excitation at 532 nm and emission filter of 580 nm. The same gel was subsequently stained with Sypro Ruby fluorescent dye and visualized on the Typhoon 9200 scanner under the same conditions.

Top-down MS analysis. Reverse-phase liquid chromatography (RPC) was conducted on a Thermo EASY nano-LC 1000 system (Thermo Fisher Scientific) equipped with a PicoFrit™ PLRP-S column (100 mm × 100 μm i.d., 5 μm, 1000 Å) (New Objective, Inc., Woburn, MA). The buffers utilized for RPC were as follows: Buffer A, water with 0.25% formic acid; Buffer B, acetonitrile with 0.25% formic acid. The nano-LC was operated at a flow rate of 500 nL/min and 5 μL of sample was injected with an autosampler after equilibration of the capillary column. For the separation of both the loading mixture (before enrichment) and elution (after enrichment) of the heart tissue lysate, a 420-min optimized RPC gradient was used, which includes the following concentrations of buffer B: 5% for 15 min, 25% at 30 min, 60% at 330 min, 95% at 360 min, and then back to 5% at 410 min. Samples were electrosprayed[S5] with a "nanoflex" ionization source into a Q Exactive™ benchtop Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany)[S6,S7] for online nano-LC-MS/MS experiments, as described previously.[S8] Heated metal capillary temperature and s-lens voltage were experimentally optimized to 300 ºC and 50 V, respectively. Both LC-MS and LC-MS/MS data were collected with 8 and 6 microscans at high mass resolving power of 140,000 and 70,000, respectively (theoretical maximum resolving power setting on QE: m/Δm50% = 140,000 at m/z 200, in which Δm50% denotes mass spectral peak full width at half-maximum peak height). In top 2 data-

dependent MS/MS scans, the intact protein ions are injected into the collision cell for higher-energy collision dissociation (HCD)[S9,S10] at a previously optimized setting of 27 V and all ions moved back into the C-trap and then into the Orbitrap for analysis. Here, intact protein ions with more than 8 positive charges were quadrupole-isolated for top-2 data dependent HCD and other ions with fewer charges were discounted. Data were collected using the Xcalibur™ 2.2 software (Thermo Fisher Scientific, Bremen, Germany) and the total RPC-MS data acquisition period was 420 min per sample. The deconvoluted masses for all observed ions were searched against the swine database generated from NCBI (Sscrofa10.2, containing 24,476 protein sequences) using the MS-Align+ search engine (release version: 0.7.1). Protein identification results with statistically significant lower P and E values (<0.00005) and a high fragment number were manually validated.

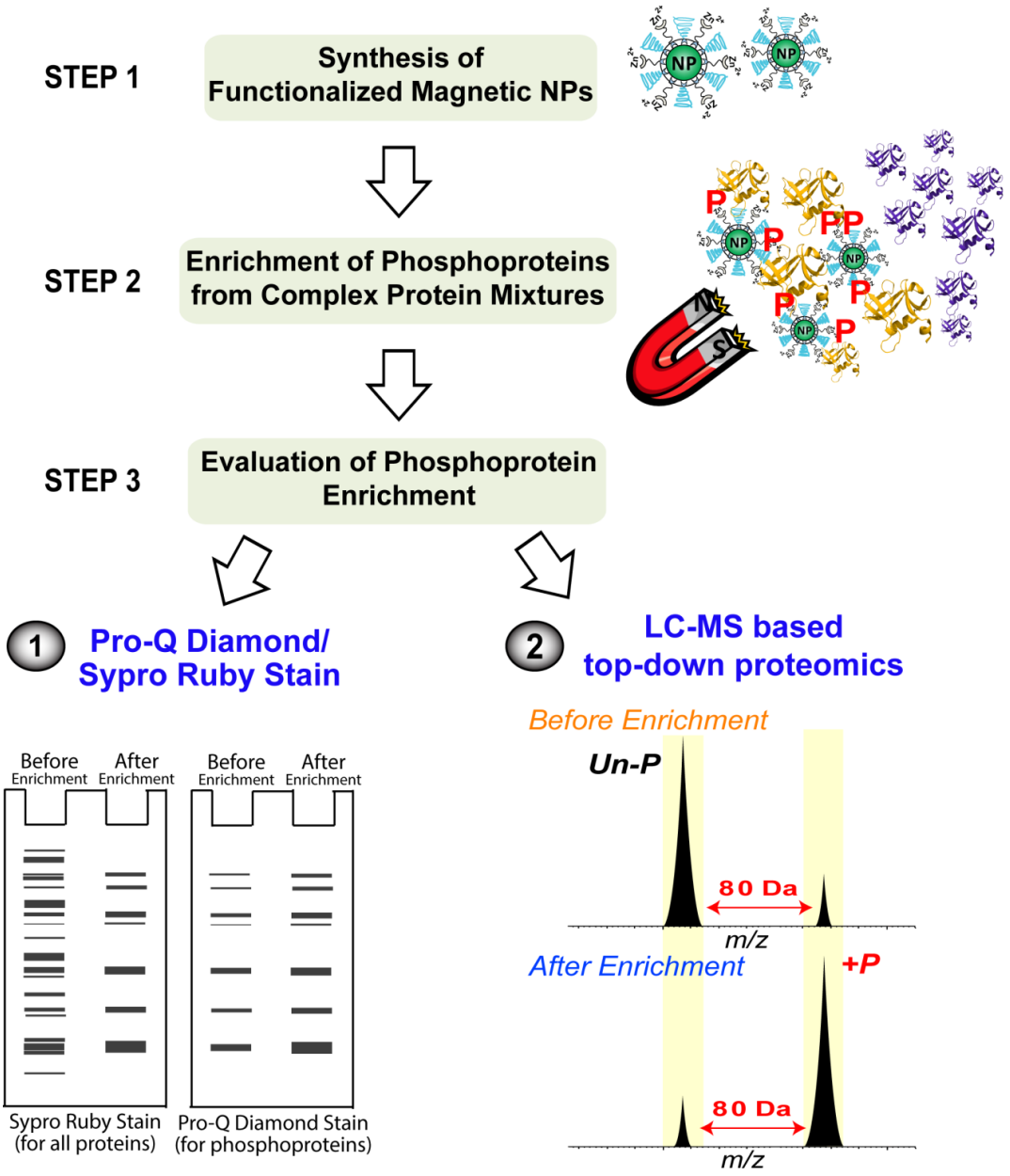


Figure S1. Schematics of the work-flow including synthesis of functionalized multivalent NPs, phosphoprotein enrichment, and evaluation of the enrichment performance by SDS-PAGE and top-down MS. **Step 1**: synthesize magnetic NPs that were functionalized by multivalent ligand molecules that can capture and release phosphoproteins; **Step 2**: enrich phosphoproteins from standard protein mixtures and complex protein mixtures (i.e. cell lysate and tissue extract); and **Step 3**: evaluate the enrichment performance by 1) SDS-PAGE analysis with Pro-Q Diamond (for phosphoproteins) and Sypro Ruby (for total proteins) staining and 2) top-down MS analysis, to detect the level change of phosphorylation before and after enrichment.

II. Characterization of the Functionalized Nanoparticles (NPs)

Estimate the total surface area of each NP. We have estimated the surface area of the NPs reported herein using the size distribution of these functionalized NPs (5.4 ± 0.62 nm, Figure 1C) based on TEM image analysis. By assuming spherical NPs, the volume and surface area of a single NP are:

v (volume of a single particle) = $4/3\pi \times$ (r, radius)^3 = m (mass) /{ρ (density of Fe_3O_4 = 5.18 g/ cm^3) × N (# of total particles)}

sa (surface area of a single particle) = $4\pi r^2$

Therefore the surface to volume ratio is:
sa/ v = $4\pi r^2 / 4/3\pi \times r^3 = 3/r$
which is inversely proportional to the radium (or diameter) of the NPs.
We can also calculate the surface area to mass ratio:
sa/ m = *sa*/ v ρ = 3/r ρ

Table S1. Estimation of the total volume and surface area for 1 gram of NPs with a diameter of 5 nm (r = 2.5 nm) and for microparticles with a diameter of 500 nm (r =250 nm)

V (total volume)	m/ ρ = 1g/ 5.18g * cm^3 = 0.193 cm^3
N (# of total particles)	V/v = 3m /4πr^{3}ρ = 0.193cm^3× 3/ 4πr^3
SA (total surface area)	N× 4πr^2 = 0.193cm^3× 3/ r
SA (total surface area) of a particle with a diameter 5 nm (r= 2.5 nm)	= 0.193cm^3 × 3/ (2.5 * 10^{-9} m) = **232 m^2/g**
SA (total surface area) of a particle with a diameter 500 nm (r=250 nm)	= 0.193cm^3 × 3/ (2.5 * 10^{-7} m) = **2.32 m^2/g**

Estimate the nmber of GAPT (with PEG) ligand molecules on the surface of each NP. The size distribution of the NP-GAPT-Zn based on TEM image analysis (Figure 1B) and TGA (Figure 1F) were used to roughly estimate the coverage of ligand molecules on the surface of each nanoparticle. Table S2 summarized the result.

Table S2. The estimated number of GAPT (with PEG) ligands on the surface of each NPs.

Nanoparticle	Fe_3O_4
Average radius (based on TEM image)	2.7 nm

The weight loss of GAPT (with PEG) ligand molecules	29% × 5.356 mg (Sample size for TGA) = 1.553 mg
Total # of the ligand molecules = $m(MM)N_A$ MM: a molar mass of ligands, N_A: Abogadro #	~7.07×10^{17}
The weight of Fe_3O_4 (ρ = 5.18 g/cm^3)	3.803 mg
Total # of the particles = $3m/4\pi r^3\rho$	~8.9×10^{15}
# of ligand molecules per particle	~ 79

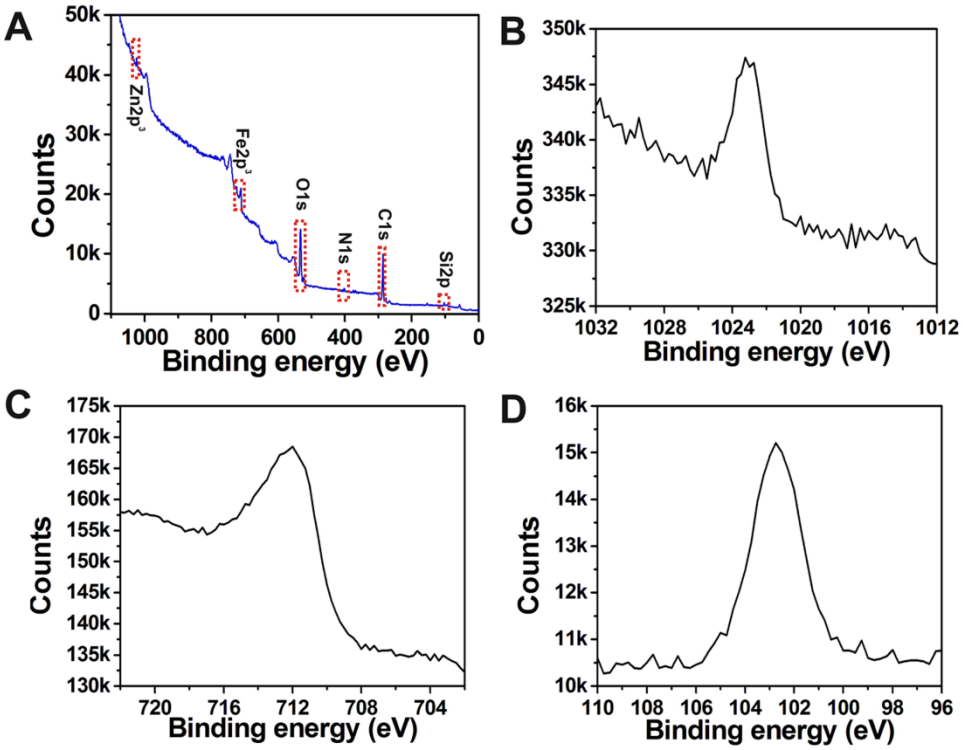


Figure S2. X-ray photoelectron spectroscopy (XPS) of the Fe_3O_4 NPs functionalized with all ligands and Zn ions, i.e. the Fe_3O_4-GAPT-Zn NPs. (A) Survey spectrum for scanning all major elements including Zn, Fe, O, N, and Si; the detailed scan of (B) Zn 2p3/2 at 1023 eV, (C) Fe 2p3/2 at 712 eV, and (D) Si 2p3/2 at 102-103 eV.

III. Additional Evaluation of the Enrichment Performance using SDS-PAGE Analysis

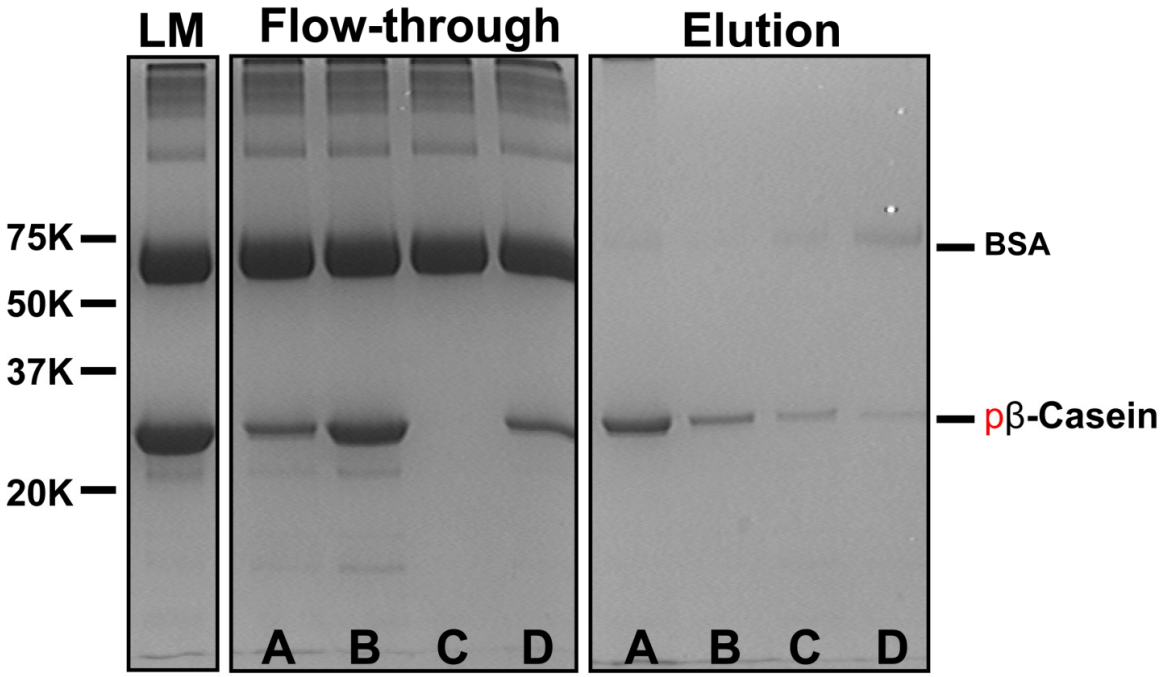
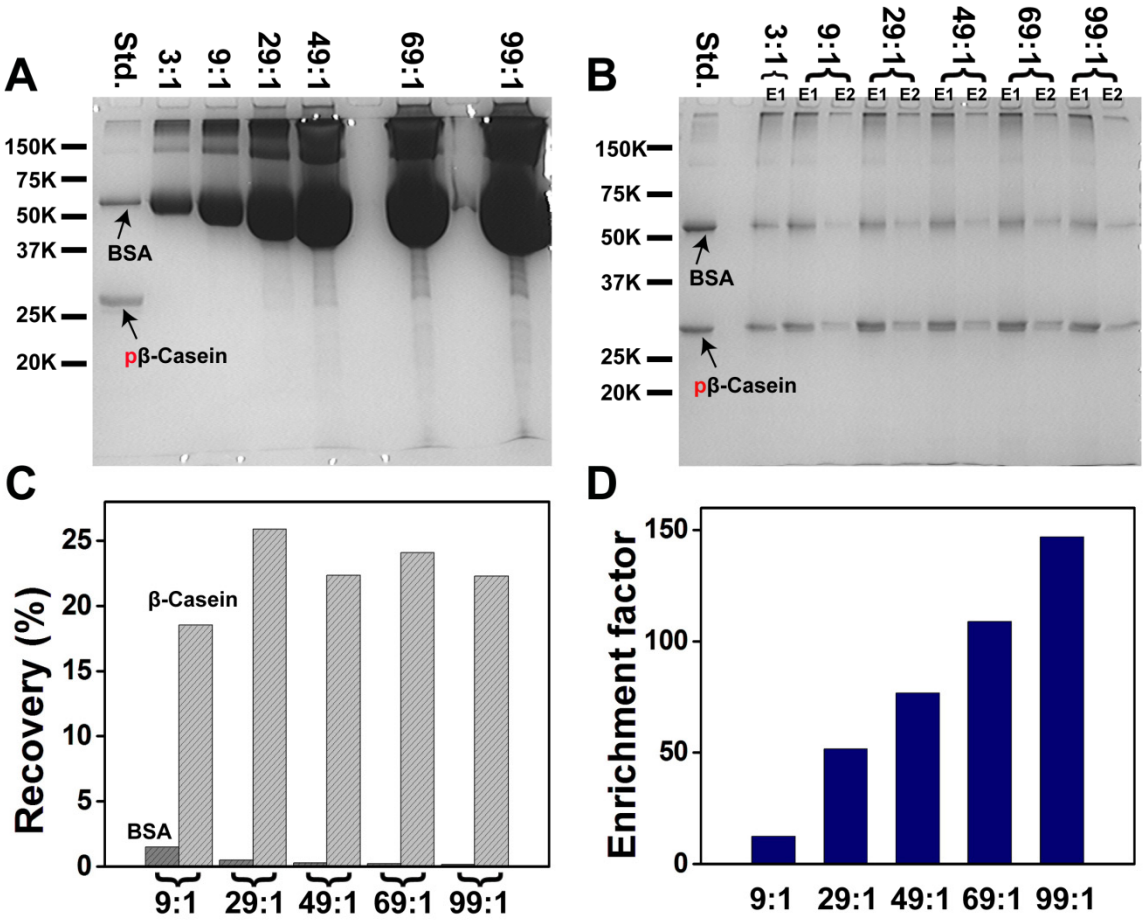


Figure S3. Phosphoprotein enrichment using Fe_3O_4-GAPT-Zn NPs and other control NPs with a loading mixture that contains 350 µg BSA and 350 µg β-casein in 0.5 mL HEPES buffer (50 mM). SDS-PAGE analysis of flow-through and elution fractions from enrichments using: **A**. Fe_3O_4-GAPT-Zn NPs, **B**. Control NP-1 that was not activated with Zn ions; i.e. Fe_3O_4-GAPT (No Zn), **C**. Control NP-2 that was functionalized with positively charged ligands, but without metal-ion chelating ligand molecules; i.e. Fe_3O_4-NH_3^+/PEG NPs, and **D**. Control NP-3 that was functionalized only with PEG molecules; i.e. Fe_3O_4-PEG NPs. All of these control NPs **(B-D)** showed poor enrichment performance toward phosphoproteins. This comparison confirms that the specific binding and capture of phosphoproteins come from the GAPT-Zn ligand complex on the surface of the Fe_3O_4-GAPT-Zn NPs (**A**). The gels were stained with Coomassie stain solution. In the case of control NP-2 (Fe_3O_4-NH_3^+/PEG) that are charged NPs, we reasoned that the control NP-2 and have a strong electrostatic interaction with other anionic groups of β-casein (an acidic protein at pH 7.8 for our loading buffer condition), resulting in electrostatic absorption onto the surface of the control NP-2.[S11] Thus, almost no band in either FT or E can be seen because β-casein is strongly bound and cannot be eluted from the control NP-2 under the elution condition we optimized. Therefore, we have concluded that the Fe_3O_4-GAPT-Zn NPs can be

used for enrichment purpose since the proteins not only need to bind to the NPs but also have to be eluted from them under appropriate conditions.



Mass ratio	1:1 (std)	3:1	10:1	30:1	50:1	70:1	100:1
BSA (μg)/mL	200	600	1800	5800	9800	13800	19800
β-casein (μg)/mL	200	200	200	200	200	200	200

Figure S4. SDS-PAGE gel analysis of (A) flow-through solutions and (B) elution solutions for a series of protein mixtures in which the mass ratio of BSA: β-casein was systematically increased from 9: 1 to 99: 1, while the amount of β-casein is held constant at 200 μg. The β-casein was clearly enriched even in the mixture containing a highly abundant BSA (BSA: β-casein = 99: 1). (C) The recovery percentage of β-casein and BSA. The concentration of a protein in SDS-PAGE gel bands was determined using software ImageJ. The recovery percentage was calculated as

follows: % Recovery = [the amount of an obtained protein in the elution (after enrichment) / the original amount of the corresponding protein in the loading mixture (before enrichment)] × 100%. The average recovery of phosphoprotein β-casein was 23% compared to 0.53% for non-phosphoprotein BSA. (D) Enrichment factor (defined as the gain in the relative ratio of the phosphoprotein to non-phosphoprotein) was achieved over 140 folds, which could almost be considered as "purification" of phosphoproteins. The gels were stained with Coomassie stain solution.

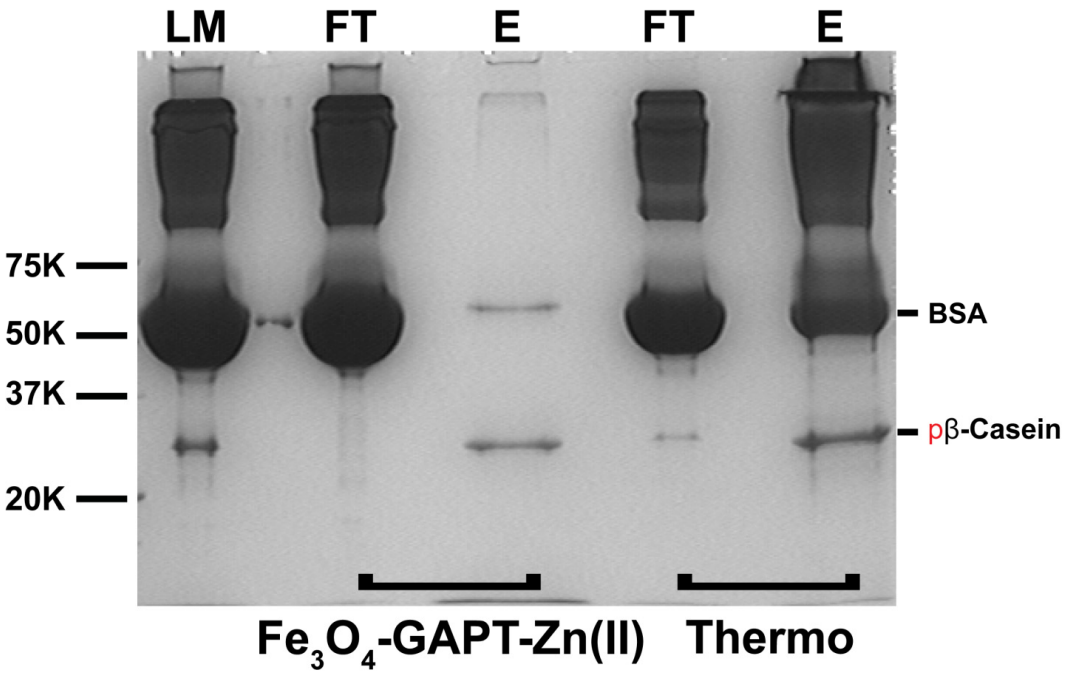


Figure S5. Comparison of the Fe_3O_4-GAPT-Zn NPs and the Pierce-Thermo enrichment kit (Thermo) for phosphoprotein enrichment from a loading mixture that contained 19.800 mg BSA and 0.2 mg β-casein (99:1 ratio of BSA and β-casein). For fair comparison, after enrichment, the elution fractions obtained using both enrichment methods were concentrated to 50 µl by using a centrifugal filter with an ultracel-10 membrane, and then an equal aliquot of the elution solutions was loaded on the gel. The gels were stained with Coomassie stain solution.

IV. Details of the Top-down Mass Spectrometry Data for Pig Heart Tissue Extracts Before and After Enrichments

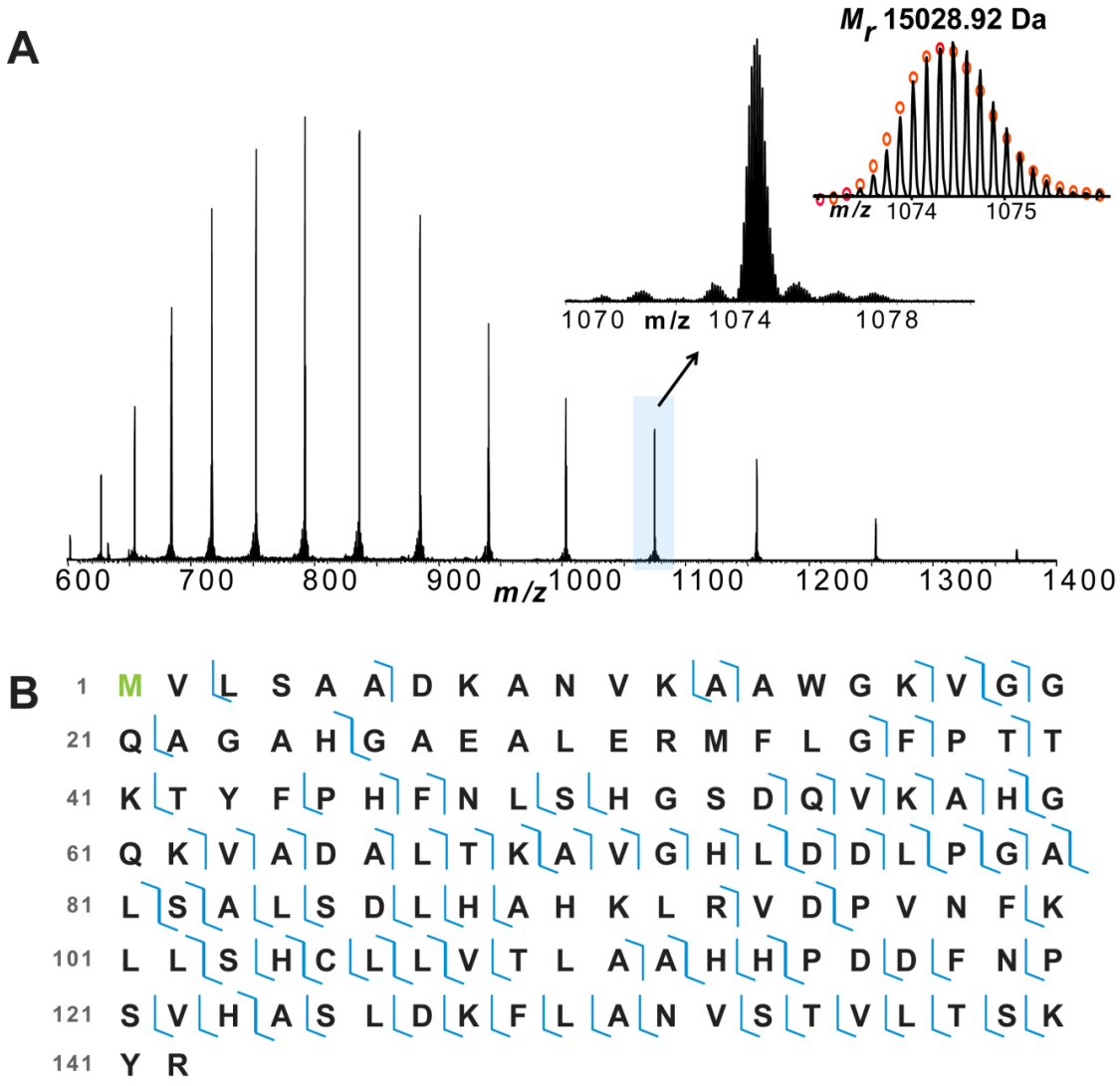


Figure S6. Comprehensive characterization of hemoglobin subunit α-like protein. (A) MS measurement with isotopic distribution of a single charge state (M^{14+}) of unmodified ions; Red circles show the theoretical isotopic abundance distribution of the isotopomer peaks correspoinding to the assigned mass. (B) fragmentation map of the hemoglobin subunit α-like protein by top-2 data-dependent HCD in the chromatographic time-scale; MS and MS/MS matched with the sequence of *Sus scrofa* Hemoglobin subunit α-like protein (UniprotKB/ Swiss-prot P01965, HBA_PIG).

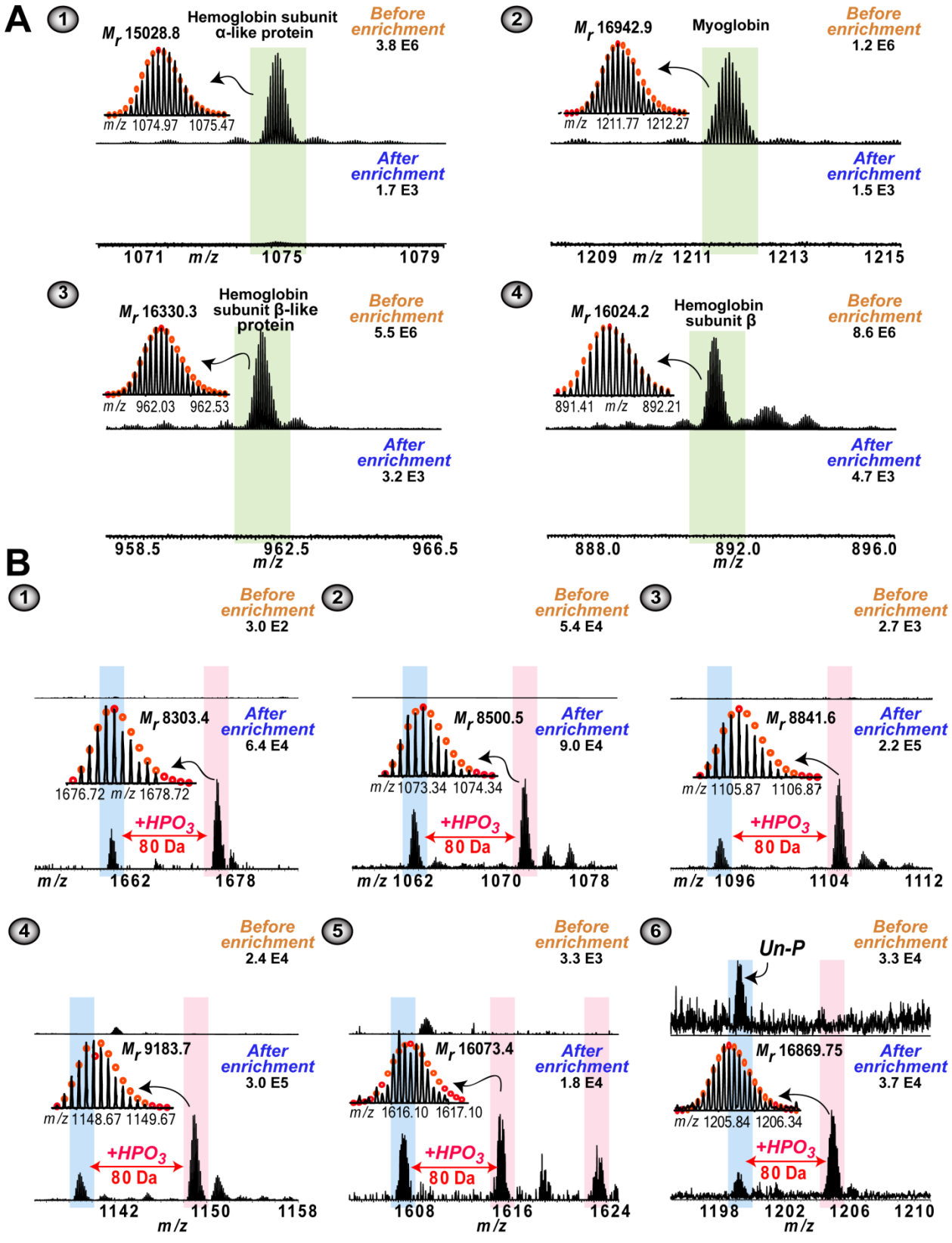

A
1
Hemoglobin subunit α-like protein
Mr 15028.8
Before enrichment
3.8 E6
m/z 1074.97 1075.47
After enrichment
1.7 E3
1071 m/z 1075 1079
2
Mr 16942.9
Myoglobin
Before enrichment
1.2 E6
m/z 1211.77 1212.27
After enrichment
1.5 E3
1209 m/z 1211 1213 1215
3
Mr 16330.3
Hemoglobin subunit β-like protein
Before enrichment
5.5 E6
m/z 962.03 962.53
After enrichment
3.2 E3
958.5 962.5 966.5
m/z
4
Mr 16024.2
Hemoglobin subunit β
Before enrichment
8.6 E6
891.41 m/z 892.21
After enrichment
4.7 E3
888.0 892.0 896.0
m/z
B
1
Before enrichment
3.0 E2
Mr 8303.4
After enrichment
6.4 E4
1676.72 m/z 1678.72
+HPO3
80 Da
m/z 1662 1678
2
Before enrichment
5.4 E4
Mr 8500.5
After enrichment
9.0 E4
m/z 1073.34 1074.34
+HPO3
80 Da
m/z 1062 1070 1078
3
Before enrichment
2.7 E3
Mr 8841.6
After enrichment
2.2 E5
m/z 1105.87 1106.87
+HPO3
80 Da
m/z 1096 1104 1112
4
Before enrichment
2.4 E4
Mr 9183.7
After enrichment
3.0 E5
m/z 1148.67 1149.67
+HPO3
80 Da
1142 1150 1158
m/z
5
Before enrichment
3.3 E3
Mr 16073.4
After enrichment
1.8 E4
m/z 1616.10 1617.10
+HPO3
80 Da
1608 1616 1624
m/z
6
Un-P
Before enrichment
3.3 E4
Mr 16869.75
After enrichment
3.7 E4
m/z 1205.84 1206.34
+HPO3
80 Da
1198 1202 1206 1210
m/z

Figure S7. Representative MS spectra before and after enrichment confirm the highly specific enrichment of phosphoproteins. (A) The highly abundant blood proteins such as hemoglobin subunit α-like protein (A1), myoglobin (A2), and hemoglobin subunit β-like protein (A3) and hemoglobin subunit β (A4), present in the original swine tissue lysate before the enrichment were nearly undetectable in the elution after the enrichment. (B) Low abundance phosphoproteins that were nearly undetectable in the original swine tissue lysate before enrichment were significantly enriched and detected in the elution after enrichment. + HPO_3 corresponding to a mass difference of + 80 Da. In the cases of B1-B5, multiple phosphorylated forms of the protein, were detected after enrichment but none of these peaks were detected before enrichment implying they are all phosphorylated protein forms that have low abundance. In the case of B6, after enrichment, the level change of phosphoprotein (M_r 16,869.75, labeled as + HPO_3, based on +80 Da mass difference from *Un-P*) was greatly increased compared to non-phosphoprotein (M_r 16,789.78, labeld as *Un-P*) before enrichment. For simplicity, only one charge state per protein is shown here. Red circles show the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. MS spectra for two other examples of enriched phosphoproteins are shown in the main text in **Figure 3**.

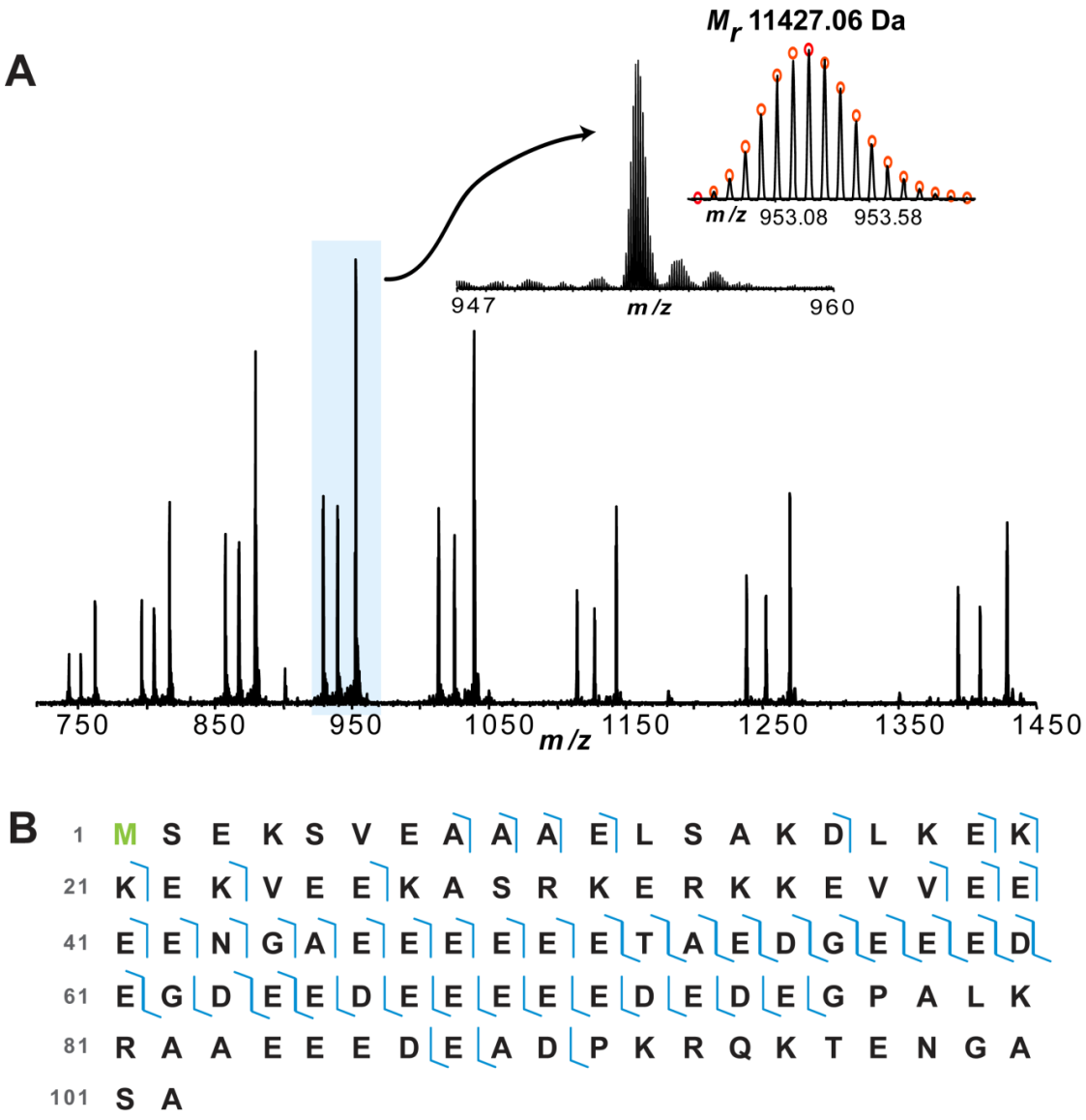


Figure S8. Comprehensive characterization of a predicted Parathymosin-like protein. (A) MS measurement with isotopic distribution of a single charge state (M^{12+}) of unmodified ions; Red circles show the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. (B) fragmentation map of the predicted Parathymosin-like protein by top-2 data-dependent HCD in the chromatographic time-scale; MS and MS/MS matched with the sequence of *Sus scrofa* predicted parathymosin-like protein UniprotKB/ Swiss-prot I3LJM0, I3LJM0_PIG, 11,351 Da (reported in Uniprot); 11,420.09 Da (observed M_r). Swine protein database is not completely annotated so the sequence is predicted based on its

human counterpart. Here we have unambiguously verified and characterized the this predicted sequence.

V. Circular Dichromism Spectra of the β-Casein Before and After Enrichment

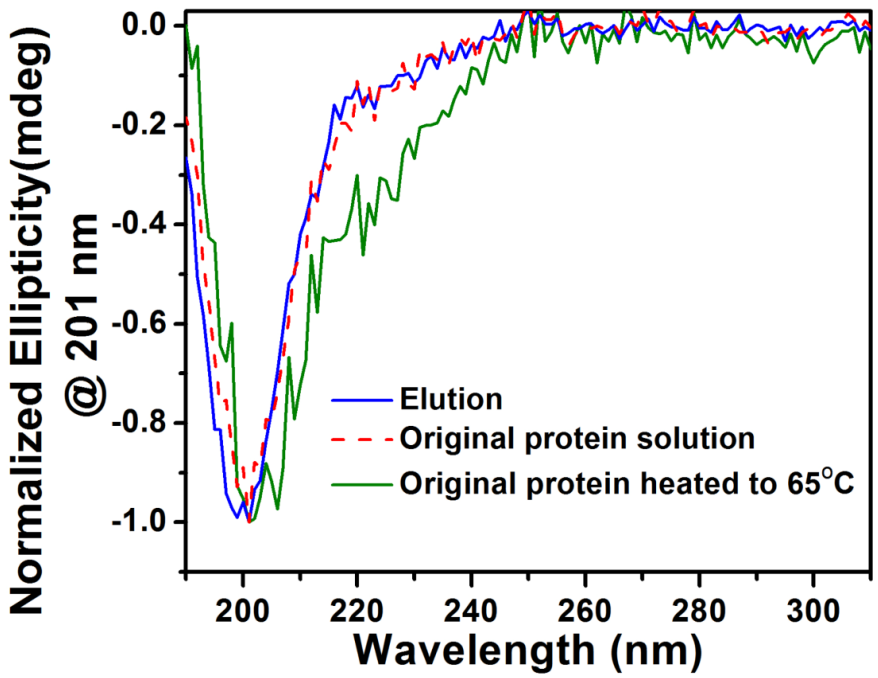


Figure S9. Circular dichroism (CD) spectra of eluted β-casein (after enrichment with Fe_3O_4-GAPT-Zn) (blue line), original β-casein protein solution (before enrichment) in 20 mM sodium phosphate buffer (red dot), and the same original protein solution which was heated to 65 °C (green line). We note that the features in the spectra of the original solution and the elution solution of β-casein were consistent with the structure study of β-casein using CD that was previously reported.[S12] We did not detect any changes in β-casein before and after enrichment but did observe changes after the protein was heated to 65 °C.

VI. References

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