Electronic Supplementary Information for

Mesoporous Zirconium Oxide Nanomaterials Effectively Enrich Phosphopeptides for Mass Spectrometry-based Phosphoproteomics

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I. Experimental Details.

Materials.

Chemicals for mesoporous material synthesis. Block copolymer $HO(CH_2CH_2O)_{106}(CH_2CH(CH_3)O)_{70}(CH_2CH_2O)_{106}H$ (designated as EO106-PO70-EO106, or Pluronic F127) was provided as a gift from BASF (Florham Park, NJ). Anhydrous precursors zirconium ethoxide ($Zr(OEt)_4$), zirconium chloride ($ZrCl_4$) and ethanol (200 proof) were purchased from Sigma Aldrich (St. Louis, MO).

Materials for enrichment. α-Casein from bovine milk, bovine serum albumin (BSA), porcine troponin from skeletal muscle, bovine ubiquitin, bovine ribonuclease B, and bovine β-lactoglobulin were purchased from Sigma (St. Louis, MO). Trypsin was a gift from Promega (Madison, WI). All proteins were used as received without further purification. Ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), acetic acid, acetonitrile (ACN), ammonium hydroxide (NH_4OH) and isopropanol were purchased from Fisher Scientific (Fair Lawn, NJ), phthalic acid from Acros Organics (Morris Plains, NJ) and used without further purification.

Preparation of mesoporous metal oxides. Mesoporous ZrO_2 was synthesized by adding Pluronic® F127 (0.5g), $ZrCl_4$ (1.6 mmol) and $Zr(OEt)_4$ (4.3 mmol) in that order to ethanol (10 g 200 proof). The resulting solution was stirred for 2 hrs and then was transferred to petri-dishes and aged 4 days in a 40 °C incubator with humidity controlled by a saturated KCl solution. Then the as-made ZrO_2 was calcined at 370 °C for 2 hrs (6 hr ramp).

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Characterization of mesoporous materials. The nanostructures of the synthesized mesoporous materials were confirmed with a combination of small angle x-ray scattering (SAXS) on a Rigaku SAXS instrument (Rigaku, Texas, USA) and transmission electron microscopy (TEM) which was taken with a Philips CM200UT (Philips Electron Optics, Eindhoven, The Netherlands) with an accelerating voltage of 200 kV. SAXS samples were prepared by grinding the samples and placing them in a u-capillary for analysis. For TEM, samples were ground and suspended in ethanol and then dispersed onto lacy carbon TEM grids for analysis. Brunauer-Emmett-Teller (BET) measurements were performed on an Autosorb-1 gas sorption system (Quantachrome Instruments, Boynton Beach, Florida) using nitrogen as the adsorbate. Samples were degassed for one hour at 213 °C prior to measurement. The nitrogen sorption curve was taken as 20 pts adsorption/20 pts desorption, with the BET surface area calculated using a 7 point BET analysis. The scanning electron microscopy (SEM) images were taken with a LEO 1530 FESEM on samples dispersed on conducting carbon tapes (Fig. S1).

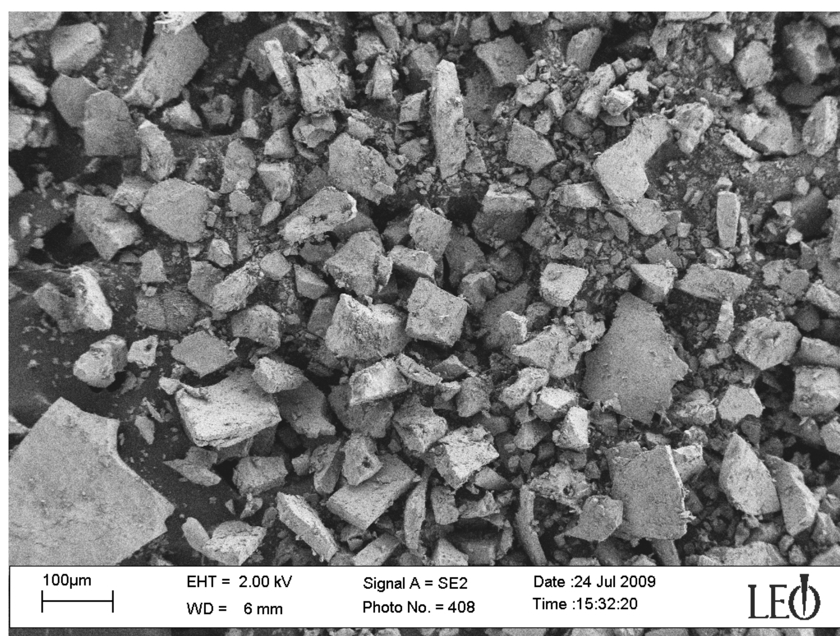

Fig. S1 Representative SEM image of the as-synthesized mesoporous ZrO_2 nanomaterials. The mesoporous structure evident in TEM is too small to be resolved by SEM.

Proteolytic sample preparation. α-Casein was dissolved in 200 mM NH_4HCO_3 to a final concentration of 1 μg/μL to be used as a stock solution. Trypsin digestion was performed with an enzyme-to-protein ratio of 1:100 and incubated at 37 ºC for 2 hrs. The 6-protein mixture was

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prepared using BSA, α-casein, troponin, ubiquitin, ribonuclease B, and β-lactoglobulin. These proteins were combined just before digestion, desalted, reduced with dithiothreitol (85 mM) for 3 hrs and then alkylated with iodoacetic acid (90 mM) for 1 hr and finally digested by trypsin (1:50) overnight. For both pure α-casein and the 6-protein mixture, the resulting digest solution was quenched with 6 µL of acetic acid, aliquoted, and stored at −20 ºC. The peptide solutions were diluted 10 times with 20 mg/mL phthalic acid solution in 0.1% TFA in 50/50 water/ACN (pH 2.0) just before enrichment. This brought the final concentration of α–casein before enrichment to 4 pmol/µL for the pure α-casein. The final protein concentrations and quantities of the proteins present in the 6-protein mixture before enrichment are shown in Table S1.

Table S1. Final protein concentrations and the quantity (moles) present in 100 µL of the 6-protein mixture used for enrichment.

	BSA	β-Lactoglobulin	Ubi	RNase B	Troponin C[1]	**α-casein**
Average Molecular Weight (kDa)	69.2	19.9	8.6	15	18	**25.2**
Concentration (µg/µL)	0.2	0.02	0.02	0.02	0.02	**0.02**
Concentration (pmol/µL)	2.9	1	2	1	1	**0.8**
Protein quantity (mole)	3E-10	1E-10	2E-10	1E-10	1E-10	**8E-11**

Procedures for enrichment of phosphopeptides using mesoporous metal oxides. In a 1.5 mL centrifuge tube, 1 − 4 mg of the calcined mesoporous material was weighed out and pretreated with 200 µL of a binding solution. The binding solution consisted of a 20 mg/mL phthalic acid solution in 0.1% TFA in 50/50 water/ACN (pH 2.0). The tubes were vortexed for 1 min, centrifuged for 1 min, and then the equilibrating solution was pipetted out and discarded. 100 µL of peptide solutions digested from α–casein (4 pmol/µL) or the 6-protein mixture (Table S1) in the binding solution were added to the mesoporous materials. The samples were mixed thoroughly for 5 mins and then centrifuged for 1 min before the supernatant was pipetted off. Then the metal oxide was rinsed twice with 1 mL of a 50 mM solution of NH_4HCO_3 in 50/50 water/ACN mixture (pH 8.5) following the same procedure of vortexing, spinning down, and discarding the supernatants. Finally, the phosphopeptides were eluted from the mesoporous oxide powder with an aqueous solution of ammonium hydroxide at pH 11.5 and the supernatant

[1] Troponin C is the major component (95%) of porcine troponin purchased from Sigma suggested by ESI/FTMS and SDS-PAGE analysis of the intact proteins.

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was collected. The eluted peptides were either directly used for negative ion mode MS analysis or dried down and reconstituted in a solution of 0.1 − 5% formic acid or acetic acid in 50:50 ACN/H_2O for positive ion mode MS analysis.

Mass spectrometry analysis. Mass spectra were acquired on a stand alone LTQ linear ion trap mass spectrometer and a 7 T linear trap/Fourier transform ion cyclotron resonance (FTICR) hybrid mass spectrometer (LTQ FT Ultra, Thermo Scientific Inc., Bremen, Germany).

Samples were introduced to LTQ with an Eksigent nano 2D HPLC system (Eksigent Technologies, Dublin, CA). The phosphopeptides were detected in a neutral loss MS^3 acquisition mode in which the mass spectrometer was set as a full scan MS followed by data dependent MS/MS. Subsequently MS^3 spectrum was automatically triggered when the neutral loss of 98 Da for detection of phosphoric acid, H_3PO_4, (Δm/z of 98, 49, and 32.7 for 1+, 2+, 3+ charge states, respectively) and 80 Da for metaphosphoric acid (HPO_3) (Δm/z of 80, 40, and 26.7 for 1+, 2+, 3+ charge states, respectively). The data dependent MS/MS and MS^3 spectra were searched against the SwissProt non-redundant bovine and porcine protein database in Bioworks using SEQUEST algorithm considering variable phosphorylations of *Ser, Thr, and Tyr* residues.

The samples were introduced to the LTQ FT mass spectrometer using an automated chip-based nanoESI source, the Triversa NanoMate (Advion BioSciences, Ithaca, NY) with a spray voltage of 1.2-1.6 kV versus the inlet of the mass spectrometer, resulting in a flow of 50 − 200 nL/min. Ion transmission into the linear trap and further to the FTICR cell was automatically optimized for maximum ion signal. The target values (the approximate number of accumulated ions) for a full MS scan linear trap (LT) scan, FTICR cell (FT) scan, MS^n linear trap scan and MS^n FTICR scan were 3×10^4, 10^6, 10^4, and 5×10^5, respectively. The resolving power of the FTICR mass analyzer was set at 100,000 $m/\Delta m_{50\%}$ at m/z 400, resulting in an acquisition rate of one scan/s. Individual charge states of the protein molecular ions were first isolated and then dissociated by electron capture dissociation (ECD) using 5-6% “electron energy” and a 150-250 ms duration time with no delay. Up to 1000 transients were averaged per spectrum to ensure high quality ECD spectra from low abundant precursor ions. For collisionally activated dissociation (CAD) precursor ions were activated using 10 − 35% normalized collision energy at the default activation q of 0.25 and dissociated in the linear ion trap followed by detection in FTICR cell. All FTICR spectra were processed with Xtract Software (FT programs

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2.0.1.0.6.1.4, Xcallibur 2.0.5, Thermo Scientific Inc., Bremen, Germany) using a signal-to-noise threshold of 1.5 and fit factor of 60% and validated manually. The resulting monoisotopic mass lists were further searched using in-house "ion-assignment" software.

II. Identification of phosphopeptides by tandem mass spectrometry

Both ECD and CAD spectra were taken in positive ion mode to sequence and characterize phosphopeptides. CAD cleaves CO-NH bonds to produce ***b*** and ***y*** fragment ions.[1] Phosphopeptides are identified in the CAD spectra by the presence of $[M+nH-H_3PO_4]^{n+}$ and $[M+nH-HPO_3]^{n+}$ fragment ions formed by metastable decomposition from phosphorylated Ser/Thr/Tyr residues. For example, CAD of the phosphopeptide DIGpSEpSTEDQAMEDIK was taken with 10% “collision energy” and was confirmed to be phosphorylated because of a 98 Da neutral loss from the precursor and fragment ions (Fig. S2, top). ECD was employed to fully characterize phosphopeptides and localize the phosphorylation sites. ECD cleaves NH-CHR bonds to produce mainly ***c*** and ***z·*** ions,[2] complementary to CAD. ECD is nonergodic,[2,3] known to preserve labile phosphorylation in peptides or intact proteins.[4,5] As shown in Fig. S2 (bottom), ECD was collected from a doubly charged precursor ion at *m/z* 706.2585 with 6% “electron energy” and an irradiation time of 250 msec. This yielded complete sequence coverage of the peptide EQLpSTpSEENSK and the peptide was confirmed to be diphosphotylated at the two serine residues close to the N-teminus. Peaks in both CAD and ECD spectra were manually assigned with very high mass accuracy (<5 ppm) (Table S2 and S3). The list of phosphopeptide identified in Fig. 2, 3 and S3, negative ion mode FT-ICR mass spectra is shown in Table S4. All the assignments of phosphopeptides were confirmed by CAD and ECD collected in FTICR manually. A previously reported peptide at 2702.8559 was found to match a different sequence

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that corresponds to a loss of ammonia from the N-terminal glutamine residue condensing to form pyroglutamate.

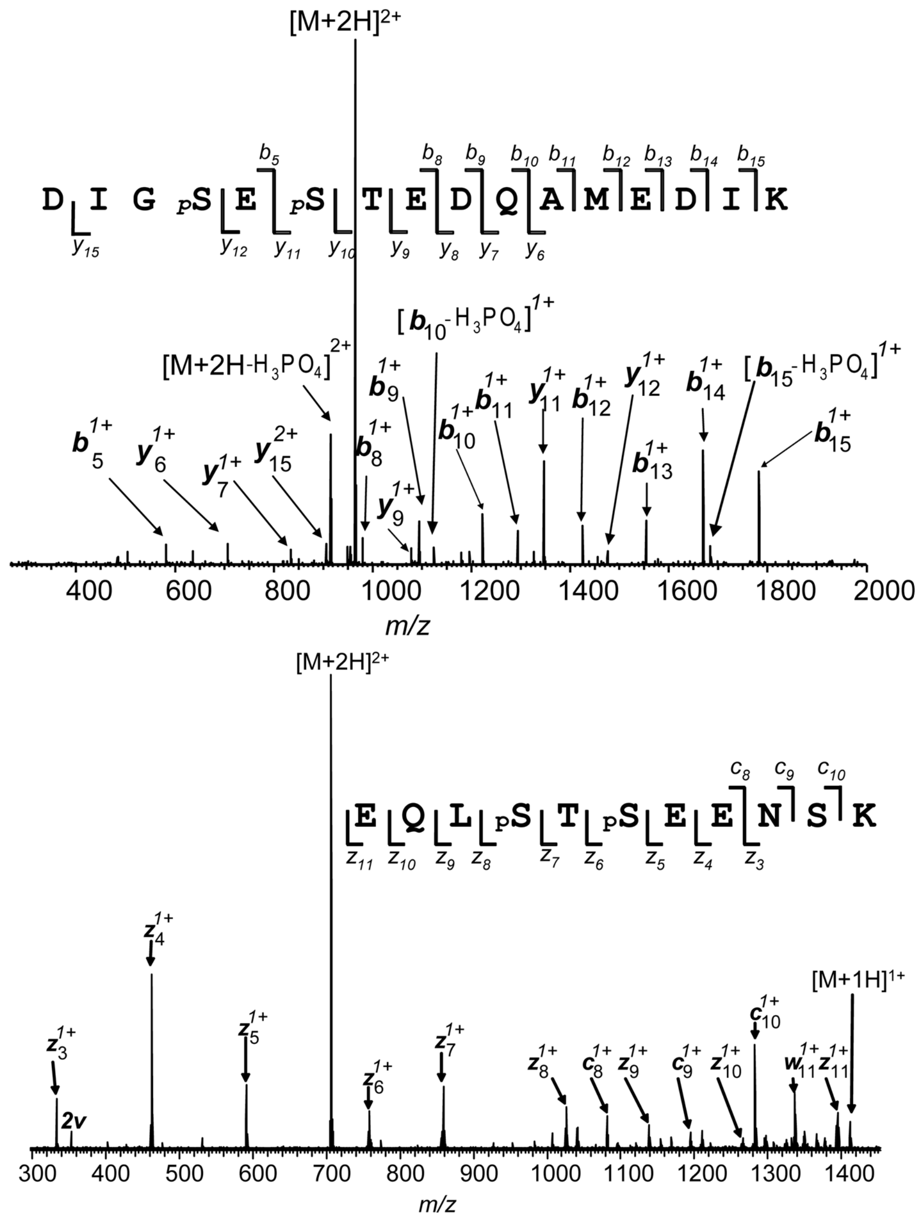

Fig. S2 Representative positive ion mode CAD and ECD spectra of phosphopeptides from an α-casein digest after mesoporous ZrO_2 enrichment. *2v* corresponds to the second harmonic of the parent peak and W_{11}^{1+} is resulted from side chain loss from the glutamic acid residue on the N terminus.

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Table S2. Fragment ion assignments for CAD spectrum of a phosphopeptide from an α-casein digest after mesoporous ZrO_2 enrichment as shown in Fig. S2, top.

fragment type	Expt'l mass	Calc'd mass	Mass error (Da)	Mass error (ppm)
b_5	581.1739	581.1734	-0.00052	0.89
b_8	978.2620	978.2620	-2E-05	0.020
b_9	1093.2891	1093.2889	-0.00018	0.16
b_{10}	1221.3497	1221.3475	-0.0022	1.8
b_{11}	1292.3863	1292.3846	-0.0017	1.3
b_{12}	1423.4265	1423.4251	-0.0014	0.98
b_{13}	1552.4702	1552.4677	-0.0025	1.6
b_{14}	1667.4963	1667.4946	-0.0017	1.0
b_{15}	1780.5809	1780.5787	-0.0022	1.2
y_7	833.3957	833.3953	-0.00041	0.49
y_8	948.4233	948.4222	-0.0011	1.1
y_{11}	1345.5124	1345.5108	-0.0016	1.2
y_{12}	1474.5548	1474.5534	-0.0014	0.94
y_{16}	1926.6853	1926.6842	-0.0011	0.56
b_{10}-H_3PO_4	1682.603	1682.6041	0.0011	-0.65
b_{15}-H_3PO_4	1828.709	1828.7071	-0.0019	-0.65

Table S3. Fragment assignments for ECD spectrum of a phosphopeptide from an α-casein digest after mesoporous ZrO_2 enrichment as shown in Fig. S2, bottom.

fragment type	Expt'l mass	Calc'd mass	Mass error (Da)	Mass error (ppm)
z'_3	332.1684	332.1690	-0.00064	-1.9
z'_4	461.2108	461.2116	-0.00083	-1.8
z'_5	590.2528	590.2542	-0.0014	-2.4
z'_6	757.2508	757.2526	-0.0018	-2.3
z'_7	858.3016	858.3002	0.0014	1.6
z'_8	1025.2965	1025.2986	-0.0021	-2.0
$z^\cdot_5$	589.245	589.2469	-0.0019	-3.3
$z^\cdot_9$	1137.3745	1137.3753	-0.00084	-0.74
$z^\cdot_{10}$	1265.4342	1265.4339	0.00028	0.22
$z^\cdot_{11}$	1394.475	1394.4765	-0.0015	-1.1
c_8	1080.3379	1080.3413	-0.0034	-3.1
c_9	1194.3786	1194.3842	-0.0056	-4.7
c_{10}	1281.4133	1281.4162	-0.003	-2.3
w_{11}	1335.4604	1335.4637	-0.0033	-2.5

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III. Enrichment of phosphopeptides from α-casein digest using mesoporous ZrO_2 nanomaterials.

The enrichments using mesoporous ZrO_2 are extremely effective as shown by the high resolution Fourier transform (FT) mass spectra of the α-casein digest before and after the enrichment (Fig. S3). Only 8 MS peaks corresponding to 6 phosphopeptides were detected before enrichment (Fig. S3a); all of which are low abundance peaks owing to ion suppression from abundant non phosphopeptides. In contrast, after enrichment with mesoporous ZrO_2 (Fig. S3b), 30 multiply charged MS peaks corresponding to 20 phosphopeptides were detected in a single mass spectrum with much higher signal-to-noise ratios. When enriched with ZrO_2 nearly all of the non phosphopeptides were removed leaving only phosphorylated peaks, which substantially enhanced the signal of phosphopeptides. The insets in Fig. S3 highlight a quintuply phosphorylated peptide, p14, which was completely suppressed by non phosphopeptides without enrichment (Fig. S3a) and was observed only after enrichment (Fig. S3b) underscoring the effectiveness of this enrichment procedure.

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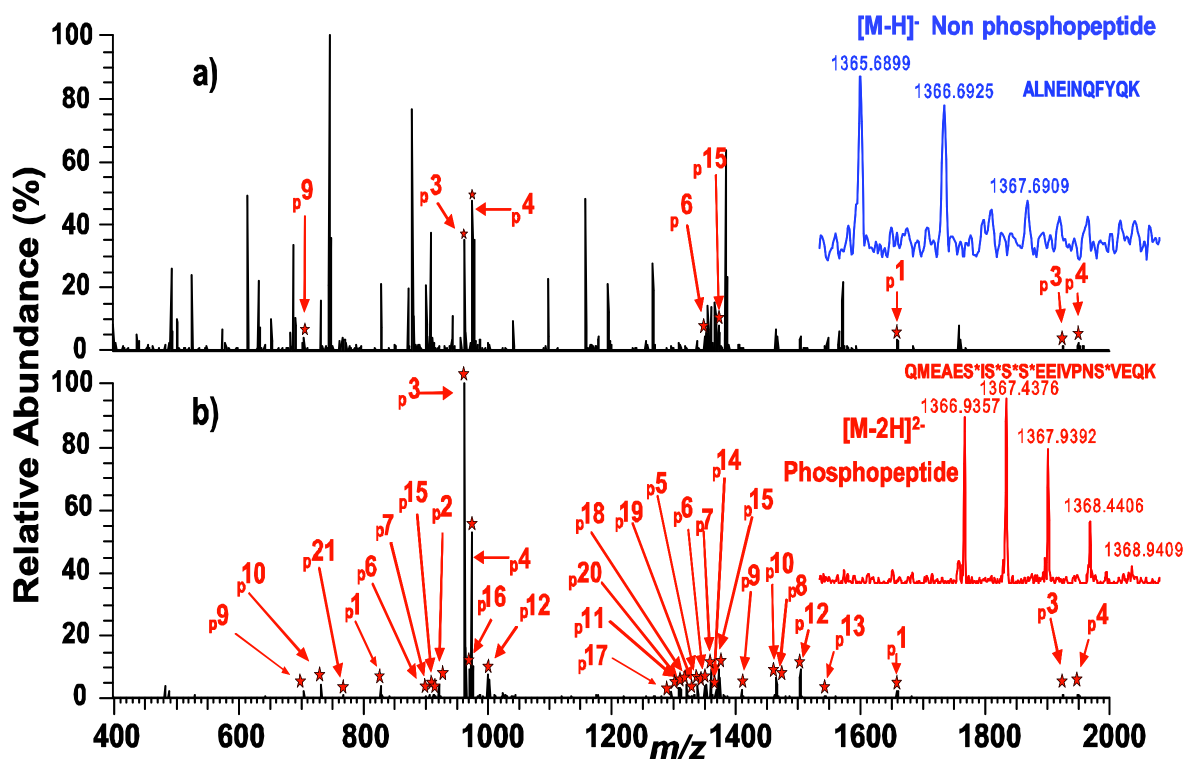

Fig. S3 ESI/FTMS spectra of peptide mixtures digested from α-casein with trypsin (a) before and (b) after enrichment using mesoporous ZrO_2. Phosphopeptides are labeled with numbers that are listed in Table S42. Insets show a singly charged non phosphopeptide in (a) and a doubly charged quintuply phosphorylated peptide, p14, in (b).

II. List of the identified phosphopeptides in Fig. 2 and 3 and S3.

Table S4. List of phosphopeptide identified in the negative ion mode FTMS spectra (Fig. 2, 3 and S3) of peptide mixtures digested from α-casein with trypsin.

Peptide #	Expt'l (m/z)	Charge State	Expt'l Mass	Calc'd Mass	Error (ppm)	Sequence Identified	Phosphory-lation state	Assignment
p1	828.8824	-2	1659.7759	1659.7869	-6.6	VPQLEIVPNS*AEER	1	α-S1[121-134][b]
	1658.7689	-1	1659.7779	1659.7869	-5.4	VPQLEIVPNS*AEER	1	α-S1[121-134][a,b]
p2	922.3468	-2	1846.7159	1846.7179	-1.1	DIGS*ESTEDQAMEDIK	1	α-S1[58-73][b]
p3	962.3299	-2	1926.6759	1926.6842	-4.3	DIGS*ES*TEDQAMEDIK	2	α-S1[58-73][a,b]
	1925.6644	-1	1926.6679	1926.6842	8.5	DIGS*ES*TEDQAMEDIK	2	α-S1[58-73][a,b]
p4	974.462	-2	1950.9359	1950.9451	-4.7	YKVPQLEIVPNS*AEER	1	α-S1[119-134][a,b]
	1949.9218	-1	1950.9279	1950.9451	-8.8	YKVPQLEIVPNS*AEER	1	α-S1[119-134][a,b]
p5	1337.4917	-2	2676.9959	2677.0155	-7.3	VNELS*KDIGS*ES*TEDQAMEDIK	3	α-S1[52-73][b]

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p6	899.9485	-3	2702.8673	2702.8626	1.8	pyroQMEAES*IS*S*S*EEIVPNS*VEQK[2]	5	α-S1[114-135][b]
	1350.423	-2	2702.8559	2702.8626	2.5	pyroQMEAES*IS*S*S*EEIVPNS*VEQK[3]	5	α-S1[114-135][a,b]
p7	905.6242	-3	2719.8839	2719.9055	-7.9	QMEAES*IS*S*S*EEIVPNS*VEQK	5	α-S1[74-94][b]
	1358.9367	-2	2719.8959	2719.9055	-3.5	QMEAES*IS*S*S*EEIVPNS*VEQK	5	α-S1[74-94][b]
p8	1466.06	-2	2934.1359	2934.1530	-5.8	EKVNELS*KDIGS*ES*TEDQAMEDIK	3	α-S1[50-73][b]
p9	704.2372	-2	1410.4959	1410.4952	0.47	EQLS*TS*EENSK	2	α-S2[141-151][a,b]
	1409.4785	-1	1410.4879	1410.4952	-5.2	EQLS*TS*EENSK	2	α-S2[141-151][b]
p10	731.792	-2	1465.5959	1465.6047	-6.0	TVDMES*TEVFTK	1	α-S2[153-164][b]
	1464.5881	-1	1465.5979	1465.6047	-4.7	TVDMES*TEVFTK	1	α-S2[153-164][b]
p11	1307.9331	-2	2617.8801	2617.8879	-2.3	NTMEHVS*S*S*EESIIS*QETYK	4	α-S2[17-36][b]
p12	1001.3274	-3	3007.0139	3007.0221	-2.7	NANEEEYSIGS*S*S*EES*AEVATEEVK	4	α-S2[61-85][b]
	1502.4936	-2	3006.9959	3007.0221	-8.7	NANEEEYSIGS*S*S*EES*AEVATEEVK	4	α-S2[61-85][b]
p14	1366.9350	-2	2735.8847	2735.9004	-5.8	Q**M**EAES*IS*S*S*EEIVPNS*VEQK[4]	5	α-s1[74-94][b]
p15	914.32	-3	2745.9839	2745.9923	-3.1	NTMEHVS*S*S*EESIIS*QETYKQ	4	α-S2[17-37][b]
	1371.9798	-2	2745.9742	2745.9559	6.6	NTMEHVS*S*S*EESIIS*QETYKQ	4	α-S2[17-37][a,b]
p16	970.3289	-2	1942.6724	1942.6791	-3.0	DIGS*ES*TEDQSMEDIK	2	α-S2[58-73][b]
p17	1294.9093	-2	2591.8332	2591.8332	8.7	QMEAES*IS*S*S*EEIVPNS*VEQ	5	α-S1[74-93][b]
p18	1318.9558	-2	2639.9262	2639.9392	-4.9	QMEAES*IS*S*S*EEIVPNSVEQK	4	α-S1[74-94][b]
p19	1331.9985	-2	2666.0116	2665.9896	8.2	NTMEHVS*S*S*EESIISQETYKQ	3	α-S2[17-36][b]
p20	1310.4443	-2	2622.9032	2622.8833	7.6	pyroQMEAES*IS*S*S*EEIVPNSVEQK	4	α-S1[74-94][b]
p21	768.2900	-2	1538.5945	1538.5902	2.8	EQLS*TS*EENSKK	2	α-S2[141-152][b]
p22	795.8395	-2	1593.6935	1593.6997	3.9	TVDMES*TEVFTKK	1	α-S2[153-165][b]

IV. Comparison with commercial phospho-enrichment product

A side-by-side quantitative comparison of phosphor-enrichment using two leading commercially available phospho-enrichment products, one based on immobilized metal affinity chromatography (IMAC) technology (Fig. 2A) and the other of ZrO_2 packed tips (Fig. 2B), with the ZrO_2 mesoporous materials reported herein (Fig. 2C) has been performed. The morphology of the material used in ZrO_2 packed tips is shown as supplemental Fig. S4. We have used the same quantity and concentration of the same tryptic digest of α-casein for the three enrichments

[2] This sequence corresponds to a loss of ammonia from the N-terminal glutamine residue condensing to form pyroglutamate.
[3] **M** represents oxidized methionine

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(10 μL of tryptic digest from α-casein (4 pmol/μL). The enrichment experiments with the commercial products were performed according to manufacturers' instructions (recommended optimal procedures and supplied reagent kits, if available).

Briefly, for the enrichment with the IMAC-based product, the spin column was washed with 50 μL supplied Bind/Wash Solution (250 mM acetic acid in 30% acetonitrile). The sample was added and incubated at room temperature for 15 minutes, then washed 3 times with 50 μL Bind/Wash Solution and once with 50 μL water to remove residual Bind/Wash solution. The phosphopeptides was eluted by centrifugation with Elution Solution (0.4 M ammonium hydroxide) and dried down in a speedvac to remove excessive ammonium hydroxide and reconstituted in 0.5% ammonium hydroxide. The enrichment with the commercial ZrO_2 packed tips was performed with Loading Buffer of 0.3% formic acid, Wash Buffer as Loading Buffer or water and Elution Buffer of ammonium hydroxide (pH 9.5-11). Tips were conditioned by aspirating the Loading Buffer 5 times. Then the tips were aspirated in air to remove excess Loading Buffer. Samples were aspirated/expelled 50 times to allow the peptides to adsorb to the ZrO_2 material, washed 10 times with 20μL of Wash Buffer and eluted with the Elution Buffer (0.5% ammonium hydroxide). The enrichment with the mesoporous ZrO_2 nanomaterials uses a binding solution of 20 mg/mL in 0.1% TFA 50/50 H_2O/ACN, a wash buffer of 50 mM NH_4HCO_3 in 50/50 ACN/H_2O and an elution buffer of 0.5% NH_4OH (the optimal conditions discussed in the manuscript).

As shown in Fig. 2, the mesoporous ZrO_2 materials showed significantly higher efficiency and specificity for phosphopeptide enrichment over these two leading commercial products. After enrichment with the IMAC-based enrichment product (Fig. 2a), 7 multiply charged MS peaks corresponding to 7 phosphopeptides were identified in one MS spectrum.

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Nevertheless, it suffers from severe non-specific binding of potentially acidic peptides since many highly abundant non-phosphopeptides still dominate the spectrum. Enrichment with the ZrO_2 packed tips (Fig. 2b) revealed 6 multiply charged MS peaks corresponding to 6 phosphopeptides in one MS spectrum. In contrast, an enrichment with the mesoporous ZrO_2 nanomaterials detected 27 multiply-charged MS peaks corresponding to 19 phosphopeptides (Fig. 2c), which demonstrated significantly higher efficiency and unparalleled specificity for phosphopeptides as nearly all the non-specific bindings were suppressed.

IV. The Nanoparticle morphology of the ZrO_2-based Commercial phosphoenrichment Product

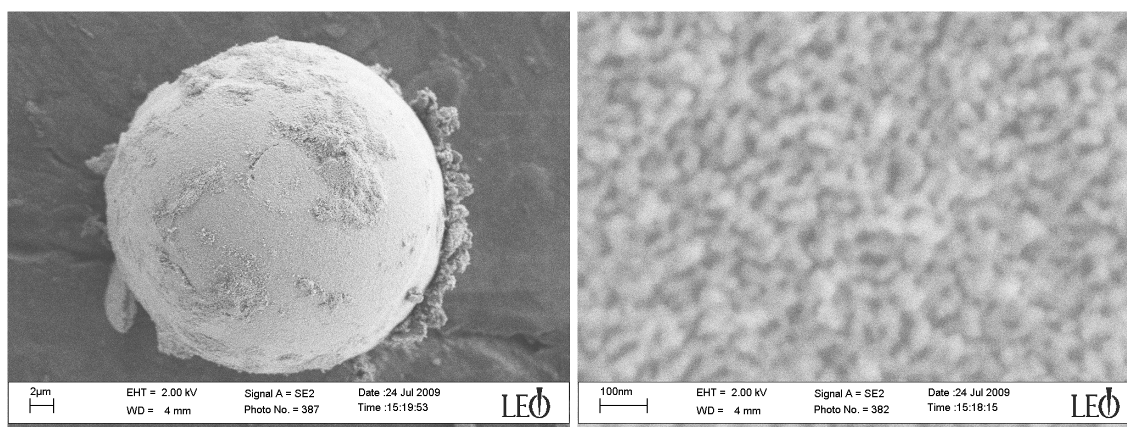

Fig. S4 Representative SEM images of the commercial phosphoenrichment materials based on ZrO_2, which consist of microspheres of aggregates of ZrO_2 nanoparticles of about 20 nm diameter. This was the materials used for enrichment experiment shown in Fig. 2B.

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