## Experimental Section

**Chemicals and Materials.** Bovine α-casein, β-casein, trypsin (TPCK treated) and ethanol were purchased from Sigma-Aldrich. Acetonitrile was purchased from Mreda Technology Inc. Dithiothreitol (DTT) was purchased from INALCO SPA MILANO ITALY. Iodoacetamide (IAA) was purchased from Beijing Biodee Biotechnology Co., Ltd. Phosphoprotein extraction kit was purchased from BestBio. Bovine serum albumin (BSA) was purchased from Beijing Jingkehongda Biotechnology. 304 L stainless steel wires (0.3 mm in diameter) were purchased from XinHuaShi Yunlian Stainless Steel Products Co., Ltd. Sulfuric, ammonia, PEG 400 and acetylacetone were purchased from Beijing Chemical Works. Tetra-n-butyl titanate and ammonium bicarbonate were purchased Sinopharm Chemical Reagent Co., Ltd. Skim milk was purchased from a local supermarket in Beijing.

Borosilicate glass capillary with an outer diameter of 1.0 mm and an inner diameter of 0.59 mm was purchased from Vitalsense Scientific Instruments Co., Ltd. The capillary was pulled using a P-2000 laser puller (Sutter Instrument Co.) preprogrammed to fabricate nanopipettes with the inner diameter of ~ 1 μm. Parameters used were: Heat = 300, Fil = 5, Vel = 30, Del = 128, and Pul = 70.

Mass measurements were accomplished on Orbitrap MS (Q-Exactive, Thermo Scientific, San Jose, CA). Capillary temperature: 320 °C, tube lens voltage: 50 V, mass resolution: 70000, maximun inject time: 50 ms, and microscans: 1. The commercial ionization source of ESI was removed ahead of our experiments.

**Preparation of Stainless Steel Needles Coated with Titania ($TiO_2$).** Titania sol was prepared from tetra-n-butyl titanate (TBT) according to the following process: 1 mL TBT was added dropwise to 47 mL ethanol followed by addition of 1 mL acetylacetone and 0.1 mL PEG 400 at room temperature. The solution was continuously stirred for 10 min and it was subsequently aged for 24 h.

One end of the 304 L stainless steel needles (8 cm in length) were dipped in newly prepared 50% sulfuric acid for 5 min. Afterwards, the stainless steel needles were thoroughly rinsed with distilled water and ethanol.

Nano-sized $TiO_2$ was coated on the stainless steel needles via dipping one end of the stainless steel needles in the sol solution 30 s and pulling it up. After drying in the air for 10 min, the samples were heated in the resistance furnace at 400 °C for 10 min. The stainless steel needles coated with four layers were obtained by repeating such an operation four times.

**Preparation of Tryptic Digestion of Sample.** β-casein (1 mg) was dissolved in 50 mM ammonium bicarbonate (1 mL) and trypsin was added into the solutions at a weight ratio of 1:40 (w/w) with the proteins. The mixture was digested by incubating 16 h at 37 °C. Bovine serum albumin (BSA) and skim milk were denatured by 50 mM ammonium bicarbonate (1 mL) containing 8 M urea at 37 °C for 30 min. The DTT solution (200 mM, 0.1 mL) was added into the solution and the solution was incubated for 1 h at 37 °C in order to reduce the disulfide bonds. Then, the reduced cysteine residues were alkylated with IAA (400 mM, 0.1 mL) in the dark at room

temperature for 1 h. Finally, the obtained mixture was incubated with trypsin (2.5% w/w) at 37 °C for 16 h.

All the peptide mixtures were diluted with 50 % acetonitrile and 1 % TFA aqueous solution (v/v).

**Extraction of Phosphopeptides.** The stainless steel needles coated with titania were immersed into digested peptides mixtures (1% TFA, water/acetonitrile, 1/1, v/v) at room temperature for 5 min. After that, the stainless steel needles were washed with 50% acetonitrile (v/v) aqueous solution containing 1% TFA for three times to wash out any unbound impurities. Then the peptide-loaded stainless steel needles were put into a new nanopipette preloading 2 μL of 2% ammonia and 50% acetonitrile aqueous solution (v/v) to elute the bound peptides at room temperature for 40 min.

**Test of Extraction Time and Elution Time.** To carry out the extraction time and elution time test, 10 ppm synthetic phosphopeptide (MW: 2089.13 Sequence: EVVG[pS]AEAGVDAASVSEEFR) was added as internal standard. The stainless steel needles were dipped into digested peptides (β-casein, 10ppm) for different time 5 s, 10 s, 30 s, 45 s, 60 s, 120 s and 1200 s. Afterwards, the eluent was detected by nanoESI after 40 min elution time. In the similar way, the eluent with different elution time (2 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min and 50 min) was detected after enrichment for 120 s.

**Mass Spectrometry.** The stainless steel needle was kept into ammonia aqueous solution in the nanoESI capillary during the nanoESI analysis. A high voltage (near -

1.5 kV for negative mode) was directly applied to the stainless steel needle. The QE instrument was operated at negative ion mode. The nanoESI capillary was positioned at 1 cm in front of the inlet of a mass spectrometer. MS spectra were acquired across the mass range of 500-2000 m/z in high-resolution mode.

To test the extraction efficiency of this method, Phosphopeptide Ⅰ solutions with concentrations of 30 ppm were prepared with 10 ppm somatostatin in it as the internal standard. The SPME probe was put into 10 μL prepared solution 30 min. We analyzed the matrix solutions before and after extraction by mass spectrometry and we obtained that the extraction efficiency was 67.11±3% (n=3) through the intensity ratio between Phosphopeptide Ⅰ and somatostatin.

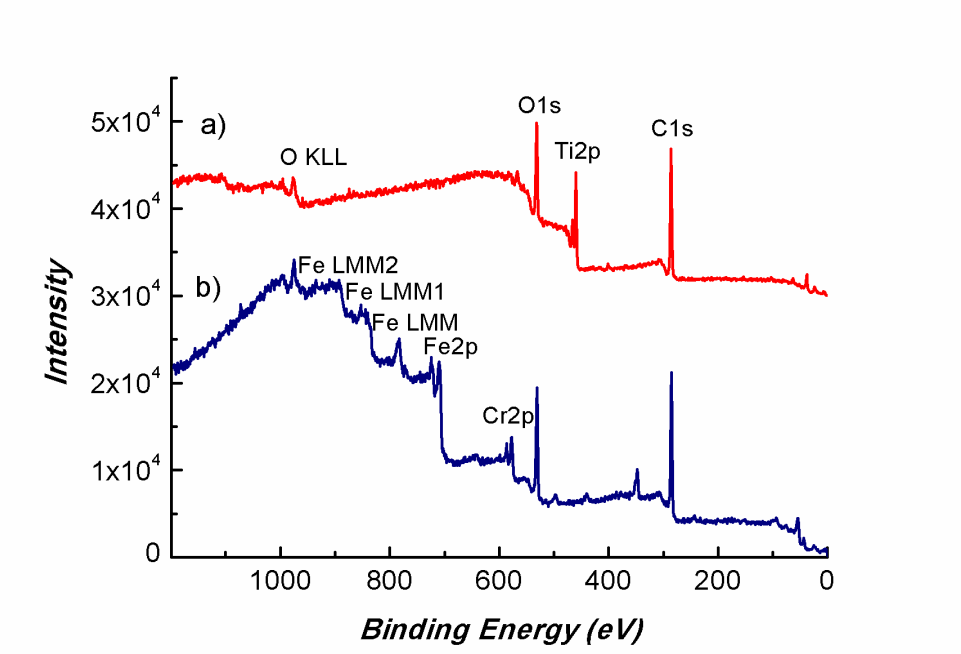


**Fig. S1** XPS characterization of stainless steel wires. a) After coated with $TiO_2$ and b) without any pretreatment.

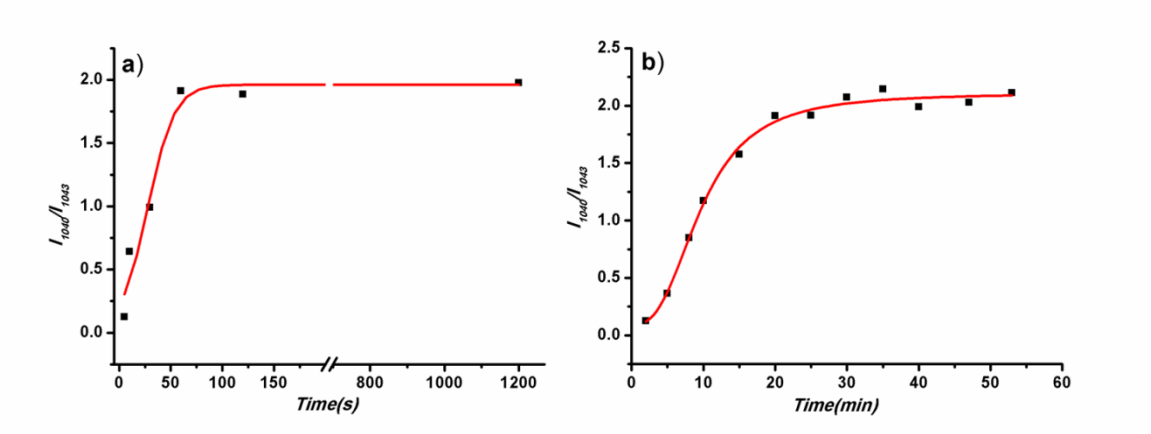


**Fig. S2** The influence of extraction time and elution time. The ratio of intensity of m/z 1040 and m/z 1043 was chosen to evaluate the degree of extraction and elution. M/z 1040 was $[\beta 3\text{-}3H]^{3-}$ and m/z 1043 was the double-charged ion of a synthetic phosphopeptide (MW: 2089.13 Sequence: EVVG[pS]AEAGVDAASVSEEFR). a) The effect of extraction time on ratio of intensity of m/z 1040 and m/z 1043. b) The effect of elution time on ratio of intensity of m/z 1040 and m/z 1043.

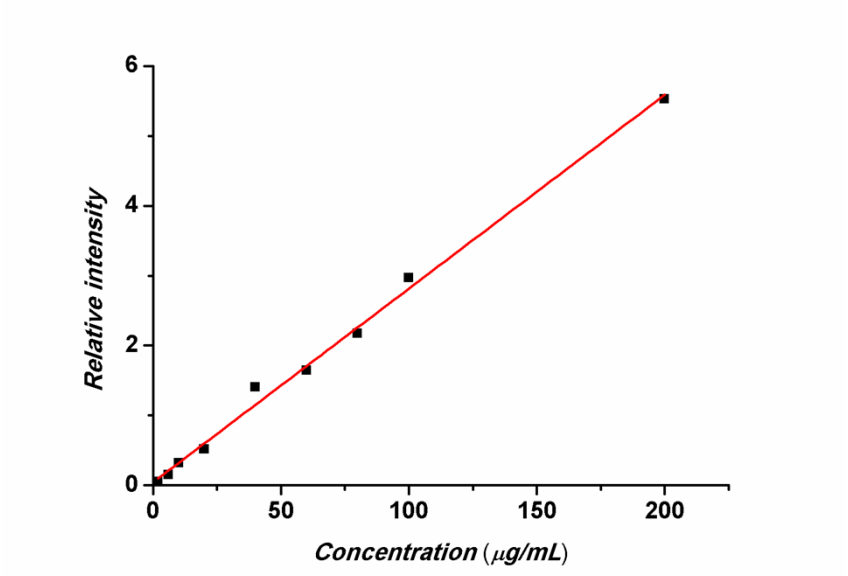


**Fig. S3** The standard curve for the quantification of phosphopeptide Ⅰ.

**Table S1** Detailed information of phosphopeptides isolated from the tryptic digest of β-casein

| No. | Observed m/z | | | | | Theoretical m/z | Residues | Peptide sequences |
|---|---|---|---|---|---|---|---|---|
| | $[M-2H]^{2-}$ | $[M-3H]^{3-}$ | $[M-4H]^{4-}$ | $[M-5H]^{5-}$ | $[M-6H]^{6-}$ | | | |
| $\beta_1$ | 1029.79 | 686.45 | 514.45 | | | 2060.83 | β/33-48 | FQ[pS]EEQQQTEDELQDK |
| $\beta_2$ | 1277.18 | 851.09 | | | | 2555.09 | β/33-52 | FQ[pS]EEQQQTEDELQDK IHPF |
| $\beta_3$ | 1560.18 | 1039.91 | 779.73 | 623.64 | 519.55 | 3121.27 | β/1-25 | RELEELNVPGEIVE[pS]L [pS][pS][pS]EESITR |

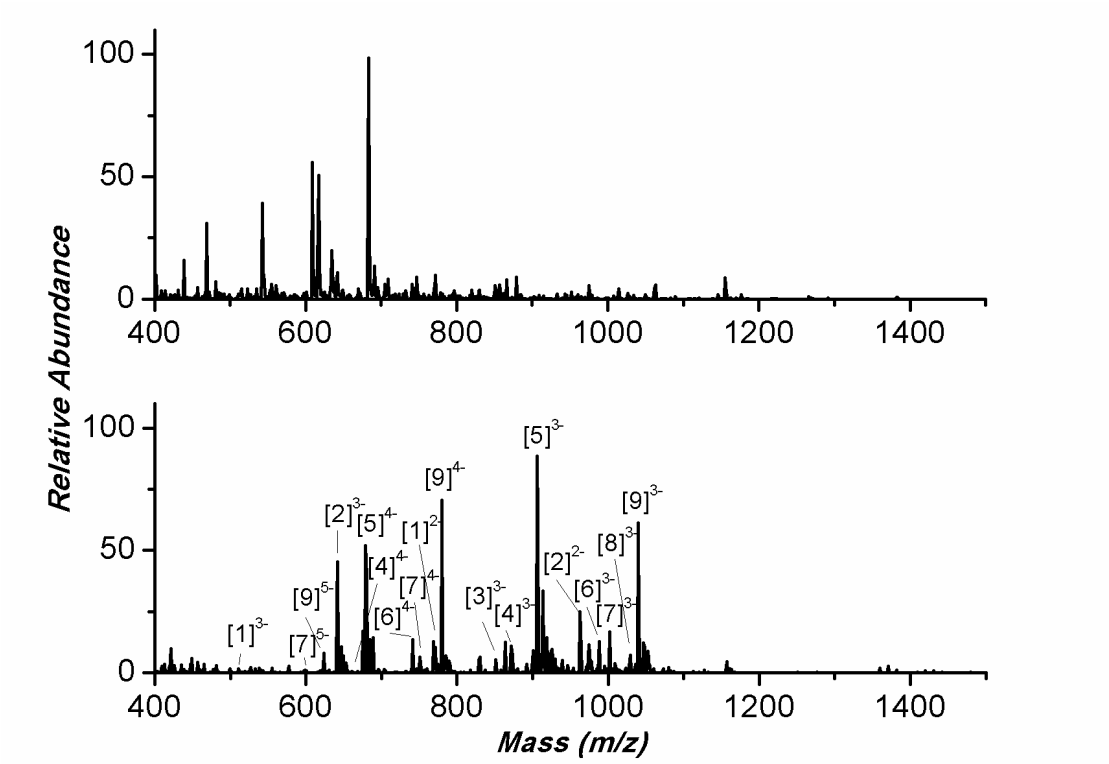


**Fig. S4** Negative ion nanoESI mass spectrum obtained from the tryptic digest of skim milk. a) Direct analysis of the tryptic digest of skim milk and b) after enrichment using the probe coated with $TiO_2$. Numbers indicate the phosphopeptides obtained from the tryptic digest of the non-fat milk listed in Table S2.

**Table S2** List of phosphopeptides obtained from a tryptic digest of skim milk

| No. | M (m/z) | No. of phosphorylations | Protein | Sequence |
|---|---|---|---|---|
| 1 | 1538.96 | 2 | αS2-casein | EQL[pS]T[pS]EENSKK |
| 2 | 1926.66 | 2 | αS1-casein | DIG[pS]E[pS]TEDQAMEDIK |
| 3 | 2555.04 | 1 | β-Casein | FQ[pS]EEQQQTEDELQDKIHPF |
| 4 | 2617.86 | 4 | αS2-casein | NTMEHV[pS][pS][pS]EE[pS]IISQETYK |
| 5 | 2719.86 | 5 | αS1-casein | QMEAE[pS]I[pS][pS][pS]EEIVPN[pS]VEQK |
| 6 | 2965.11 | 4 | β-Casein | ELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR |
| 7 | 3006.99 | 4 | αS2-casein | NANEEEYSIG[pS][pS][pS]EE[pS]AEVATEEVK |
| 8 | 3086.96 | 5 | αS2-casein | NANEEEY[pS]IG[pS][pS][pS]EE[pS]AEVATEEVK |
| 9 | 3122.22 | 4 | β-Casein | RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR |

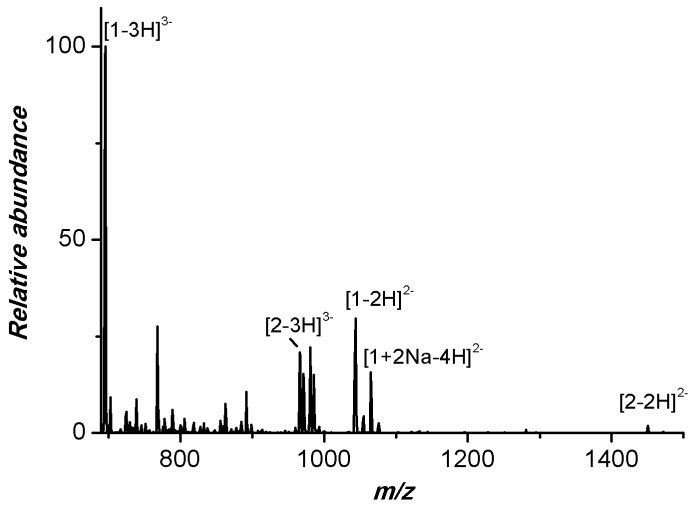


**Fig. S5** Negative ion nanoESI mass spectrum obtained after enrichment using the probe coated with TiO2. Numbers indicate the phosphopeptides obtained from the tryptic digest of egg white were listed in Table S3.

**Table S3** List of phosphopeptides obtained from tryptic digested egg white

| No. | M (m/z) | No. of phosphorylations | Sequence |
|---|---|---|---|
| 1 | 2087.88 | 1 | EVVG[pS]AEAGVDAASVSEEFR |
| 2 | 2901.30 | 1 | FDKLPGFGD[pS]IEAQCGTSVNVHSSLR |