Experimental Details

Materials and chemicals

Bovine β-casein, bovine serum albumin, ovalbumin, trypsin (from bovine pancreas, TPCK treated), ammonium bicarbonate (NH_4HCO_3), DTT, iodoacetamide and 2, 5-dihydroxybenzoic acid (DHB) were purchased from Sigma Chemical (St. Louis, MO). ACN and TFA were purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA). All other chemicals and reagents were of analytical grade and were purchased from Shanghai Chemical Reagent.

Synthesis of alumina hollow spheres

First, carboxylate-rich carbonaceous microspheres were synthesized according to a reported work. Glucose is used as carbon source and its concentration in water is 10 w/v %, and add acrylic acid to make the concentration of acrylic acid is 10 wt %. The reaction mixtures were then transferred into a Teflon-lined stainless-steel autoclave with a capacity of 200 mL. The autoclave was sealed and heated at 190℃ for 16h and naturally cooled to room temperature. Then the obtained materials were washed several times by water and dried under vacuum at 50℃ for 12h for further use. Carboxylate-rich carbonaceous microspheres (0.1g) was ultrasonicated in 40 mL 0.2 M Al $(NO_3)_3$ solution for 70 min, followed by washing with deioned water three times. The product was then dried under vacuum at 50 ℃ and calcined in nitrogen at 500℃ for 1h. After the furnace was left to cool to room temperature, the final products were

transferred to tubes and preserved under room temperature.

Characterization of alumina hollow spheres

The morphologies of the as-synthesized carboxylate-rich carbonaceous microspheres and alumina hollow spheres were studied using their powers before modified onto the target plate under transmission electron microscopy (JEM-2100F) and scanning electron microscopy (XL30) respectively. Fourier-transform infrared (FT-IR) spectra were collected on a Nicolet Fourier spectrophotometer, using KBr pellets (USA). Powder X-ray diffraction (XRD) patterns were recorded on a Bruker D4 X-ray diffractometer with Ni-filtered Cu K_R radiation (40 kV, 40 mA). Nitrogen adsorption and desorption isotherms were measured using Micromeritics ASAP 2020. The samples were degassed in a vacuum at 200^{o}C for 8 h prior to measurement. The Brunauer-Emmett-Teller (BET) method was utilized to calculate the specific surface areas (S_{BET}) using adsorption data in a relative pressure range from 0.18 to 0.35. By using the Barrett-Joyner-Halenda (BJH) model, the pore volumes and pore size distributions were derived from the desorption branches of the isotherms, and the total pore volumes (V_t) were estimated from the adsorbed amount at a relative pressure P/P0 of 0.992.

Preparation of alumina hollow spheres modified target plate

The stainless-steel plate was washed with water and ethanol, and then dried at room temperature. Alumina hollow spheres were suspended in an aqueous solution of

ethanol (20%v/v, alumina hollow spheres concentration was 20 mg/mL), followed by sonication for 10min. The suspension of alumina hollow spheres was dropped as an array of spots (~2μL for each spot) on the clean target plate. The resulting modified plate was subsequently heated in an oven at 200℃ overnight and naturally cooled-down.

Preparation of standard protein digests

Bovine β-casein, ovalbumin and Bovine serum albumin (BSA) were dissolved in 25mM NH_4HCO_3 buffer at pH 8.3 and treated with trypsin (2%, w/w) for 16 h at 37 °C respectively. Before the digestion, ovalbumin and BSA were reduced with DTT and carboxamidomethylated with iodoacetamide respectively. Bovine β-casein was digested directly.

On-plate enrichment of phosphopeptides

The digested products of β-casein and BSA were diluted in 50% ACN/0.1% TFA to certain peptide concentrations. Then 1.0μL digests were deposited onto the alumina hollow spheres modified target plate surface and incubated for 15 min at room temperature and washed with a micropipette by drawing in and expelling the 50% ACN/0.1% TFA solution onto the deposited sample. Finally, 0.5μL of DHB solution (DHB at 12.5 mg/mL in ACN/water/H_3PO_4,50/49.5/0.1% v/v) was added at the spots to desorb the bounded phosphopeptides as well as serve as MALDI matrix. The phosphopeptides captured on the target plate were then analyzed by MALDI-TOF MS. For regeneration of the functionalized MALDI target plate, the plate was immersed in

ACN/water/TFA (20/75/5% v/v) solution and gently agitated for 30 min. And then the target plate was washed with water and dried at room temperature.

MALDI-TOF MS analysis

MALDI-TOF MS experiments were performed in positive ion mode on a 4700 Proteomics Analyzer (Applied Biosystems, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200Hz and an acceleration voltage of 20 kV. All spectra were taken from signal-averaging of 800 laser shots with the laser intensity kept at a proper constant.

Detailed information of enrichment of phosphopeptides using ovalbumin

We used another protein sample ovalbumin to further confirming the usefulness of this method and successfully enriched one phosphopeptide. A direct analysis of 40 fmol ovalbumin by MALDI-TOF MS indicates that nonphosphopeptides dominate the spectrum and the signal of the detected phosphopeptide (marked with an asterisk) was quite weak. (Figure S5a). However, after enrichment by alumina hollow spheres, the signal for the ovalbumin phosphopeptide significantly increases and dominates the spectrum and nearly no nonphosphorylated peptides were observed, indicating high enrichment efficiency of the alumina hollow spheres. (Figure S5b). The signals marked with a circumflex could be assigned to a dephosphorylated fragment of phosphopeptide through loss of H_3PO_4. The detailed information of the observed phosphopeptides was listed in the Table S2.

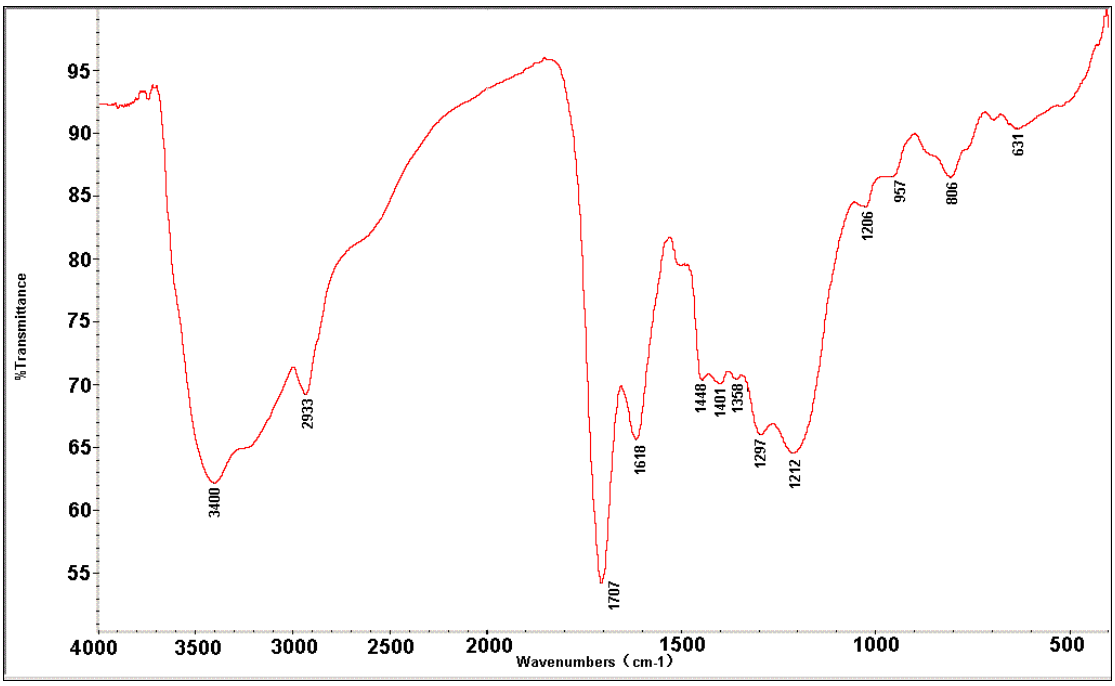

Figure S1. FT-IR of the carboxylate-rich carbonaceous microspheres

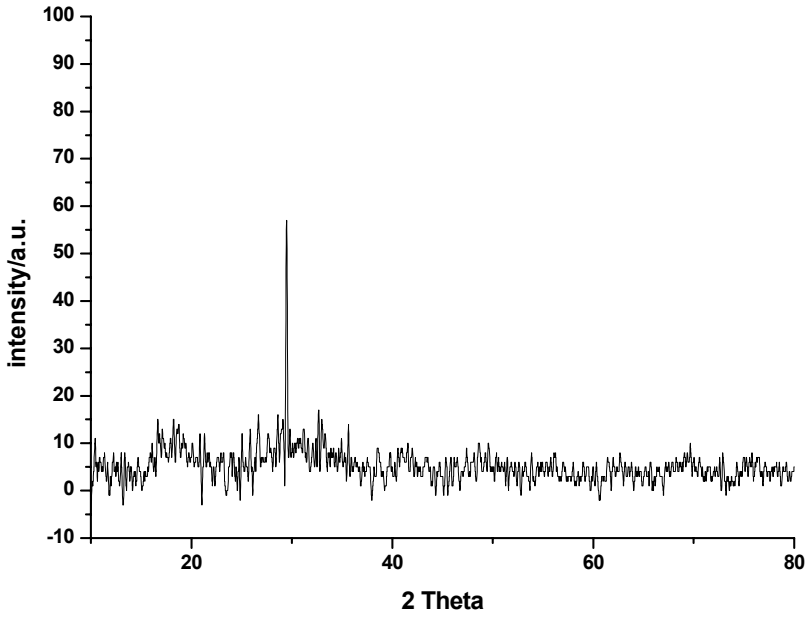

Figure S2. The wide-angle X-ray diffraction (WAXRD) pattern of the alumina hollow spheres.

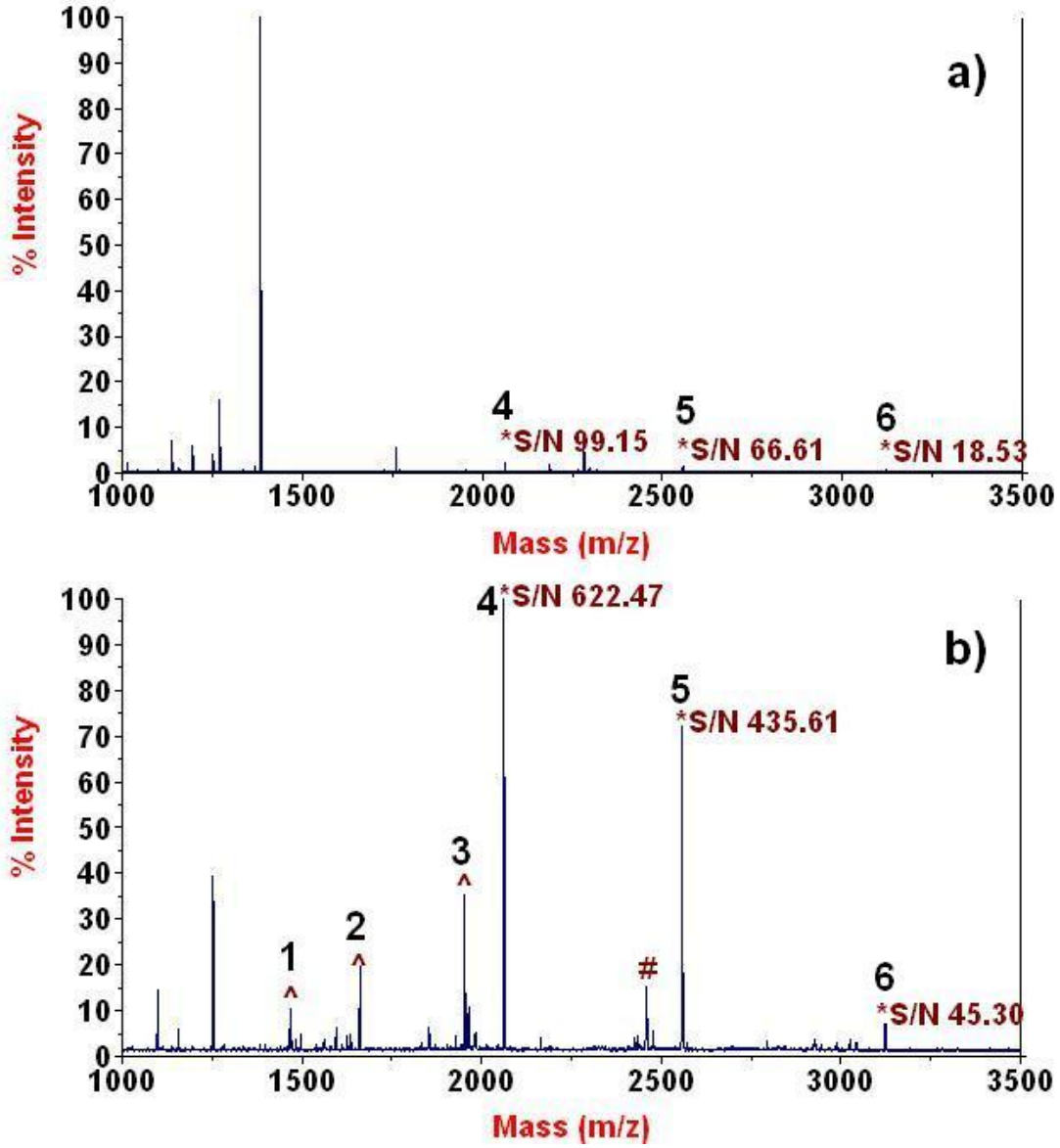

Figure S3. MALDI mass spectrum of peptides derived from β-casein a) without enrichment b) enriched by mesoporous TiO_2 microspheres. *phosphopeptides of β-casein, ^ phosphopeptides of α-casein, # the metastable losses of phosphoric acid.

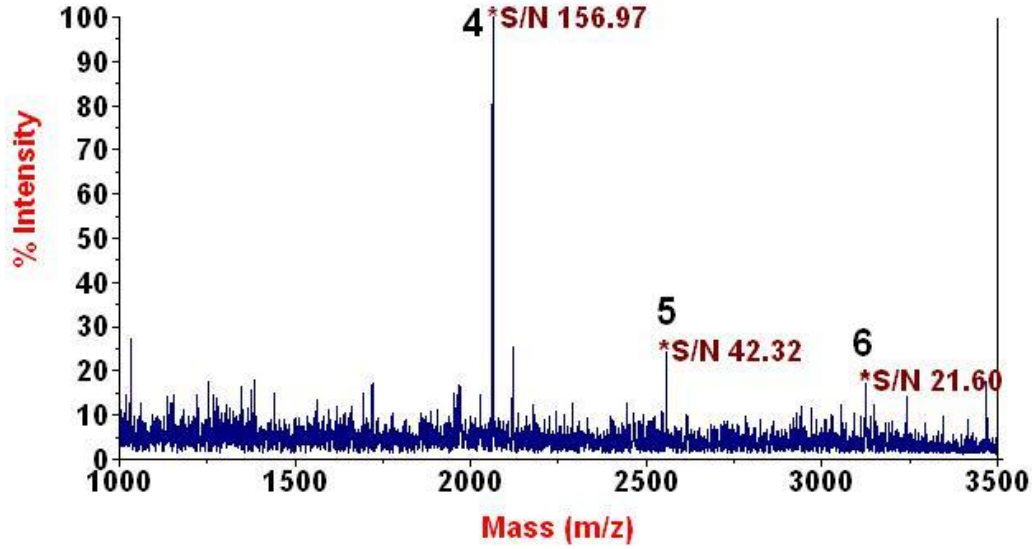

Fig. S4 MALDI mass spectrum of 5 fmol β-casein digest enriched by alumina hollow spheres. *phosphopeptides of β-casein.

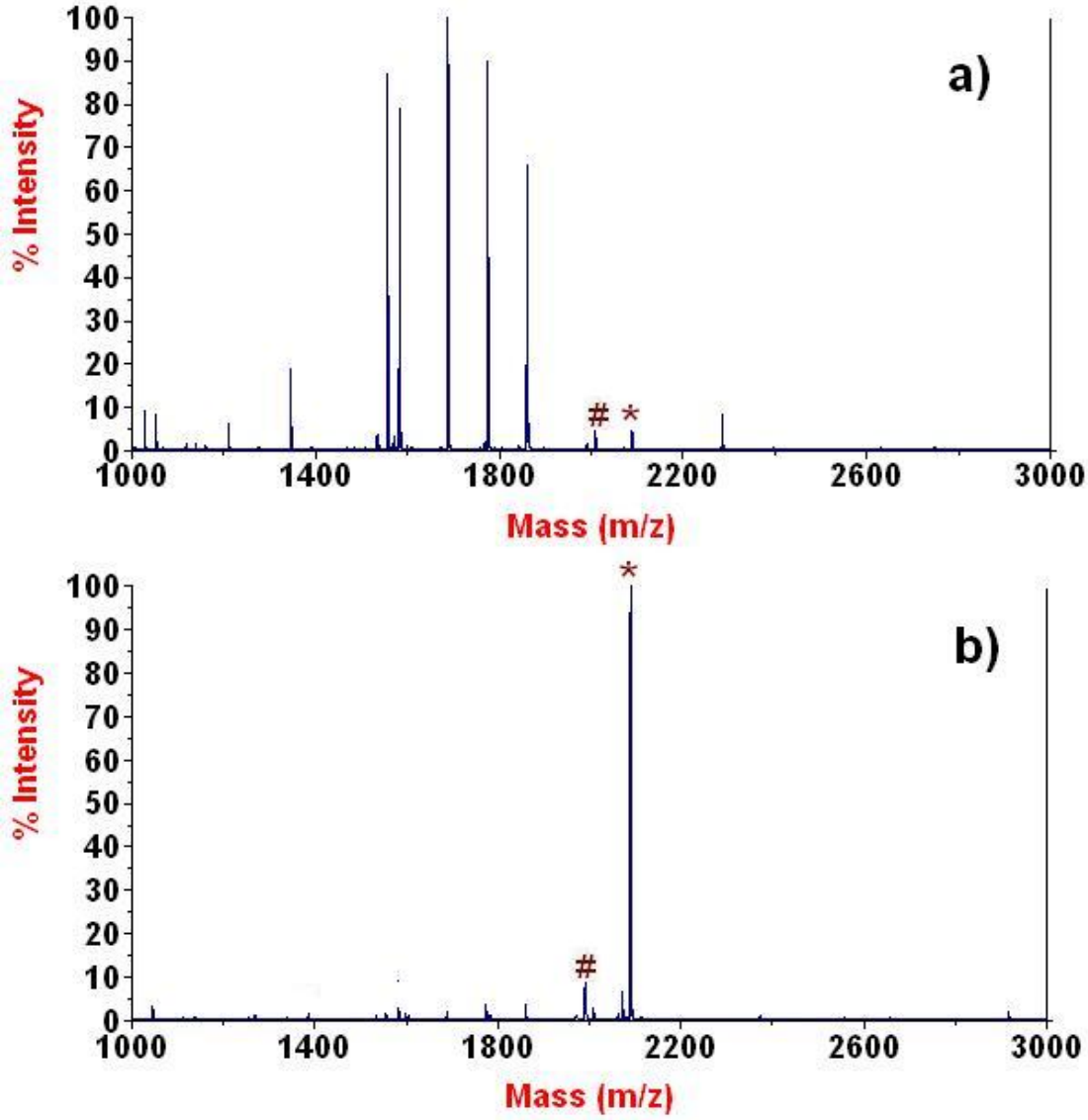

Figure S5. MALDI mass spectrum of peptides derived from ovalbumin a) without enrichment b) enriched by alumina hollow spheres. *phosphopeptides of ovalbumin, # the metastable losses of phosphoric acid.

Table S1 Detailed information of the observed phosphopeptides obtained from tryptic digestion of α-casein S1 and S2, and β-casein. [a]

Peak number	Theoretical m/z	Observed m/z	aa	Peptide sequence
1	1466.6	1466.7	α-S2/153–164	TVDMSTEVFTK
2	1660.8	1660.9	α-S1/106–119	VPQLEIVPNSAEER
3	1952.0	1952.1	α-S1/104–119	YKVPQLEIVPNSAEER
4	2061.8	2062.0	β/33–48	FQSEEQQQTEDELQDK
5	2556.1	2556.3	β/33–52	FQSEEQQQTEDELQDKIHPF
6	3122.3	3122.6	β/1–25	RELEELNVPGEIVESLSSSEESITR

[a] The phosphorylation sites are underlined.

Table S2. Detailed information of the observed phosphopeptide obtained from tryptic digestion of ovalbumin. [a]

Theoretical m/z	Observed m/z	aa	Peptide sequence
2088.9	2088.9	Ova/340-359	EVVGSAEAGVDAASVSEEFR

[a] The phosphorylation sites are underlined.