Supporting Information

Complementary multiple hydrogen-bond-based magnetic composite microspheres for high coverage and efficient phosphopeptide enrichment in bio-samples

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Materials

4-aminobenzoic acid, 2-isocyanatoethylmethacrylate, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), trisodium citrate dehydrate (Na_3CT), ammonium acetate (NH_4Ac), tetraethoxysilane (TEOS), aminopropyl-trimethoxysilane, bromoisobutyryl bromide (BIBB), triethylamine, N,N-dimethylamino-2-ethyl methacrylate (DMAEMA), copper bromide (CuBr), N, N, N′,N′,N″-pentamethyl-diethylenetriamine (PMDETA), tetrabutylammonium hydroxide, β-casein (from bovine milk), α-casein (from bovine milk), bovine serum albumin (BSA), trypsin (TPCK treated), dithiothreitol (DTT), iodoacetamide (IAA), and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Commercial TiO_2 microspheres was purchased from Aladdin (Shanghai, China). PuriMag Si-TiO_2 MagBead was obtained from PuriMag Biotech company (Fuzhou, China). Acetic acid (HAC), dimethylsulfoxide (DMSO), ammonium hydroxide ($NH_3 \cdot H_2O$, 28 wt%), ethylene glycol (EG),

acetonitrile (ACN), formic acid, trifluoroacetic acid (TFA), urea, ammonium bicarbonate (NH_4HCO_3), anhydrous ethanol, dichloromethane (CH_2Cl_2), and dimethylformamide (DMF) were purchased from Forest Science and Technology Development Co. Ltd (Chengdu, China). Nonfat milk was obtained from a local supermarket.

Instruments

Hydrogen and carbon (^1H and ^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 600-MHz spectrometer. The size distribution of samples were obtained by dynamic light scattering (DLS, Zetasizer NanoZS90, Malvern Instruments Ltd, UK). Scanning electron microscopy (SEM) spectra were recorded on a Hitachi S-4800 SEM (Hitachi Corp., Toyko, Japan). Transmission electron microscopy were recorded on a JEM-100CX TEM (JEOL, JEM-100CX, Japan). Infrared spectra (FT-IR) were recorded on a PE spectrometer with wavenumber in the range of 500-4000 cm^{-1}. X-ray photoemission spectroscopy (XPS) was conducted using a Kratos XSAM 800 instrument equipped with a monochromatic Al anode X-ray gun (12 kV, 15 mA, 10^{-5} Pa). The magnetization of the sample was measured by vibrating sample magnetometer (VSM, model BHV-525, Riken Japanese Electronics Company) with field from 0 Oe to 18000 Oe at 300 K. The mass loss of sample was analyzed at temperature ranging from 35 to 900 °C with the heating rate of 10 K/min by simultaneous thermal analysis (STA449 C Jupiter, NETZSCH). Mass spectra were obtained with a MALDI-TOF/TOF mass spectrometer (Bruker Daltonics).

Synthesis and Characterization of MUBA Monomer

15 g of 4-aminobenzoic acid was dissolved in 200 mL of dichloromethane, and then 13.25 g of 2-isocyanatoethylmethacrylate in 50 mL dichloromethane was dropwise added at room temperature and the reaction was conducted overnight. The sampling point of the silica gel plate showed that the reaction was complete. The reaction liquid was filtered and then leached with 50 mL of dichloromethane to obtain 23.8 g of white solid. ^1H NMR (400 MHz, DMSO) δ 12.50 (s, 1H), 8.98 (s, 1H), 7.82 (d, J = 8.8 Hz, 2H), 7.50 (d, J = 8.8 Hz, 2H), 6.45 (t, J = 5.6 Hz, 1H), 6.07 (t, J = 5.2 Hz, 1H), 5.71 (dd, J = 14.0, 12.4 Hz, 1H), 4.28 – 4.04 (m, 2H), 3.45 – 3.36 (m, 2H), 1.90 (s,

3H). ^{13}C NMR (101 MHz, DMSO) δ 167.08, 166.50, 154.79, 144.63, 135.77, 130.46, 125.93, 122.91, 116.63, 63.75, 38.14, 17.97.

Synthesis of SPMMs

1) Synthesis of the Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

SPIONs were synthesized by hydrothermal reaction. Briefly, 1.157 g $FeCl_3 \cdot 6H_2O$, 3.303g NH_4Ac, and 0.400 g Na_3CT were dissolved in EG (60 mL) under magnetic stirring. After stirring for one hour, the mixture was added in the Teflon-line stainless-steel autoclave and maintained at 200 °C for 16 h. Then, the sediment were collected under an external magnetic field. After that, the sediment was thoroughly washed with ethyl alcohol and water for several times. Finally, SPIONs were re-dispersed in deionized water (15 mL) for subsequent use.

2) Synthesis of the SPION@SiO_2 Nanospheres

Firstly, 5 mL the above SPION suspension was added to 20 mL HCl (0.1 M) and sonicated for 30 min. Then, the suspension were added into the mixed solvent containing deionized water (8 mL) and ethanol (32 mL). Followed by a sequential addition of ammonia (28%, 0.5 mL) and TEOS (0.5 mL), the resulting mixture was sonicated for another 10 min and stirred dramatically for 4 h at room temperature. The obtained SPION@SiO_2 nanospheres were rinsed with ethanol, deionized water, and then re-dispersed in 1 mL ethanol for subsequent use.

3) Synthesis of the SPION@SiO_2@BiBB Nanospheres

The surface of above SPION@SiO_2 nanospheres were cleaned by soaking in 5.0% (v/v) Decon-90 solution under gentle rocking. After 30 min, the SPION@SiO_2 nanospheres were sonicated for 5 min and rinsed with deionized water. Then, the SPION@SiO_2 nanospheres were added in the mixed solvents of 18 mL of ethanol and 2 mL of water. APTES (0.4 mL) was dissolved into the mixed solution with stirring for 6 h. Then, 0.4 mL of TEA was added and stirred for another 24 h. The SPION@SiO_2@NH_2 nanospheres was separated and washed with water and ethanol for three times. To immobilize the ATRP initiator, SPION@SiO_2@BiBB nanospheres were synthesized by reacting SPION@SiO_2@NH_2 nanospheres with bromoisobutyryl bromide. The above SPION@SiO_2@NH_2 nanospheres were dissolved in 20 mL of

dry CH_2Cl_2, and then 1.8 mL of TEA were added under N_2 protection and magnetic stirring. After cooling down to 0℃, a pre-mixed solution of 1.8 mL BiBB in 8 mL dry CH_2Cl_2 was added dropwise. The solution was stirred at 0℃ for 1 h and then at room temperature for 24 h. The obtained SPION@SiO_2@BiBB nanospheres were rinsed with CH_2Cl_2, ethanol, deionized water, and then re-dispersed in 1 mL DMF for subsequent use.

4) Synthesis of SPMMs

The P(DMAEMA-co-MUBA) polymer was grafted onto SPION@SiO_2@BiBB nanospheres through a surface initiated ATRP. Briefly, DMAEMA (3.46g), MUBA (1.07g) (molar ratio: MUBA: DMAEMA=1:6) were dissolved in 15 mL dry DMF, then the above SPION@SiO_2@BiBB nanospheres were added. The above mixture was injected into the flask under N_2 protection and magnetic stirring, and then PMDETA (176 μL) and copper bromide (0.11 g) in 5 mL dry DMF were added. The solution was continued to stir for 8 h at 60 °C. The obtained SPMMs were rinsed with DMF, ethanol, deionized water, and then re-dispersed in 1 mL deionized water for subsequent use.

Phosphopeptide Enrichment Experiments

1) Preparation of Tryptic Digests of Standard Proteins

One milligram α-casein or β-casein was dissolved in 1 mL 50 mM NH_4HCO_3 solution (pH 8.2) and digested with trypsin (40:1, w/w) at 37 °C for 16 h. BSA (10 mg) was dissolved in 1 mL 50 mM NH_4HCO_3 solution containing urea (8 mol L^{-1}). After the addition of 100 μL of DTT (1 mol L^{-1}), the mixture was incubated at 60 °C for 1 h to reduce the disulfide bonds of proteins. Subsequently, 37 mg of IAA was added and the mixture was incubated at room temperature in the dark for 45 min. Finally, the mixture was diluted with NH_4HCO_3 (50 mmol L^{-1}, pH = 8.2) and incubated at 37°C for 16 h with trypsin at the mass ratio of enzyme to protein of 1:40 (w/w).

2) Preparation of Tryptic Digests of Proteins Extracted From Nonfat Milk

Nonfat milk (30 μL) was dissolved in 1 mL 25 mM NH_4HCO_3, and this solution was centrifuged at 14000 rpm for 20 min. The supernate was collected and proceeded at

100 °C for 10 min to make the protein degeneration. The supernate was digested with trypsin (40 μg) at 37 °C for 16 h. This tryptic digest of nonfat milk was diluted by loading buffer for further use.

3) Preparation of Tryptic Digests of Proteins Extracted From Oral Carcinoma Patients' Saliva, Oral Epithelial Tissue and Rat Brain

The study was permitted by the Ethics Committee of Sichuan University. Participants had signed the informed consent to all studies and their data were linked to the registry. Fresh saliva was taken from oral carcinoma patient volunteers. Briefly, 2 mL saliva was diluted with 2 mL 0.2% TFA and centrifuged for 5 min at the speed of 8000 rpm. The supernatant was collected and freezed for further use. Then, the saliva samples were taken out from the -80 °C refrigerator, then thawed at 4 °C and centrifuged at 13000 × g for 10 min. The supernatant was transferred to a 10 kD ultrafiltration tube and centrifuged at 13,000 g for 10 min. All samples were ultrafiltered to a final volume of 50 μL. The sample was collected and added 50 μL of SDT lysis buffer. The lysate was vortexed and boiled at 100 °C for 10 min. Next, the sample was cooled to room temperature for BCA quantification. The oral epithelial tissues of oral carcinoma patients were obtained from West China Hospital of Stomatology with the protocol approved by the Ethics Committee of West China Fourth Hospital, Sichuan University. The oral epithelial tissues were cut into bits, and washed with saline. A Sprague Dawley male rat was sacrificed, and its brain was promptly taken out, then cut into bits, and washed with saline. Then, the oral epithelial tissues and rat brain tissues were minced with scissors and homogenized for 30 min. The animal experiments were performed at the National Engineering Research Centre for Biomaterials (Sichuan University, China) in compliance with the guidelines of the Animal Experiment Center of Sichuan University and approved by the West China Hospital of Sichuan University Biomedical Research Ethics Committee.

All above sample were diluted in 4% SDS, 100 mM Tris-HCl (pH 8.0), and 100 mM DTT and heated at 100 ºC for 5 min. The sample was then cooled to room temperature and loaded onto an ultrafiltration filter followed by centrifugation at 14000×g for 30 min. One hundred microliters of 50 mM iodoacetamide was

subsequently added to the filter, and the samples were then incubated for 30 min at room temperature. Next, 100 μL of 25 mM ABC (Applied Biosystems, Foster City, CA, USA) was added to each filter, followed by centrifugation at 14000 × g for 30 min. The protein suspensions were then digested with 40 μL of trypsin buffer at 37 ºC for 18 h. Finally, the filter unit was transferred to a new tube, added 40 μL 25 mM ABC and centrifuged at 14000 × g for 30 min. The resulting peptides were collected, and lyophilized for further use.

4) Selective Enrichment of Phosphopeptides with SPMMs

SPMMs were washed with ethanol and then suspended in deionized water at 10 mg/mL. Tryptic digests of α-casein, β-casein, BSA, or nonfat milk were dissolved in 200 μL of loading buffer (75% ACN containing 0.1% TFA); then 10 μL of SPMMs suspension was added, and the mixture was incubated at room temperature for 20 min, respectively. After that, SPMMs with captured phosphopeptides were separated from the mixed solutions by applying an external magnet. After washing with 200 μL of loading buffer to remove the nonspecifically adsorbed peptides, the trapped phosphopeptides were eluted with 10 μL of loading buffer (50% ACN containing 2% TFA) for further MS analysis.

For the phosphopeptide enrichment from tryptic digests of proteins extracted from oral carcinoma patients' saliva or tissue and rat brain, the amount of SPMMs was adjusted to 1 mg. The peptide sample (0.5 mg) was diluted with 500 mL loading buffer, and the enrichment processing was same as that in the peptide mixture enrichment. The eluted solution was then lyophilized to dryness, desalted with Zip-Tip C18, and used for the Elite-LC-MS/MS analysis (Thermo Orbitrap-Elite). The data were searched against the Uniprot rat reference proteome database.

5) MS Analysis

For MALDI-TOF MS analysis: The MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflector positive mode was used to analyze the eluent samples. Matrix 2, 5-dihydroxybenzoic acid (DHB) was dissolved in 70% ACN-H_2O containing 1% H_3PO_4 (25 mg mL^{-1}). A 0.5 μL aliquot of the eluent and 0.5 μL of DHB matrix were sequentially dropped onto the MALDI plate for MS analysis.

For Elite-LC-MS/MS analysis: The eluent were analyzed using Easy-nLC nanoflow HPLC system connected to Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The original mass spectrometry data were extracted and analyzed using the Proteome Discoverer software and Mascot software. Peptide level false discovery rates (FDRs) were controlled lower than 1% by the percolator algorithm.

Molecular Simulation Study

The 100 ns molecular dynamics simulation of the composite system was carried out by YASARA. NPT (constant temperature and pressure) was used in the process of dynamics simulation. The force field was Amber14 and the boundary was set as periodic boundary. Pressure was controlled as 1 ATM via parrinello-rahman barostat and temperature was controlled as 298K via nose-hoover thermostat. The cut-off value of van der waals force was set at 1 nm, and the electrostatic interaction was treated by PME method. The YASARA program and VMD1.9.1 were used for analysis, and the interaction visualization was achieved by PyMol 1.7.0. The Copyright information of YASARA software was as follows: Hu Wei (huwei@moldesigner.com) Chengdu FenDi Technology Co., Ltd. Room 515, Building B, Gaofa Mansion, No.6, Jiuxing Avenue, Hi-tech Zone, Chengdu, 610000, Sichuan, China.

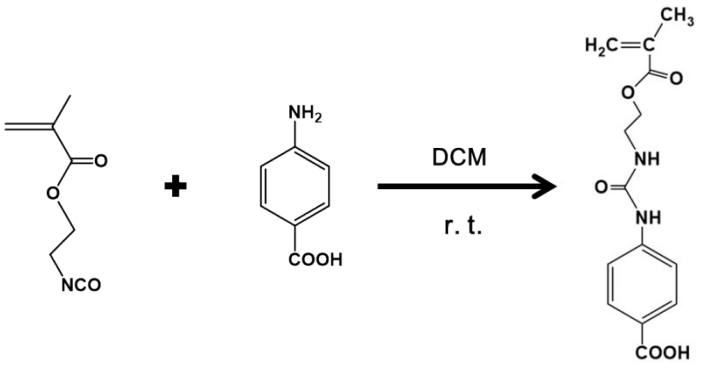

Fig. S1 Synthesis of 4-(3-(2-(methacryloyloxy)ethyl)ureido)-benzoic acid (denoted as MUBA).

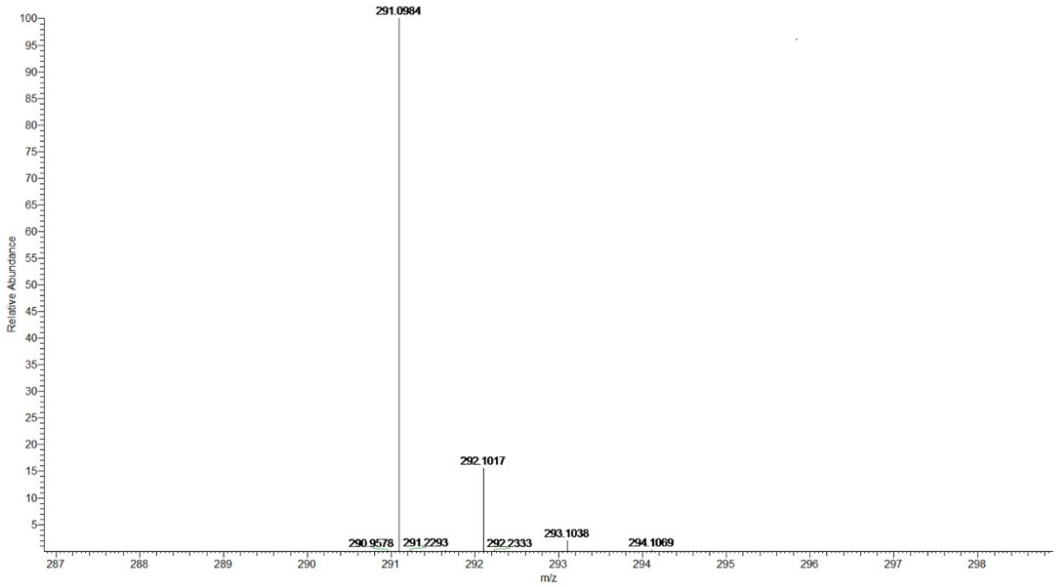

Fig. S2 MS spectra of MUBA monomer.

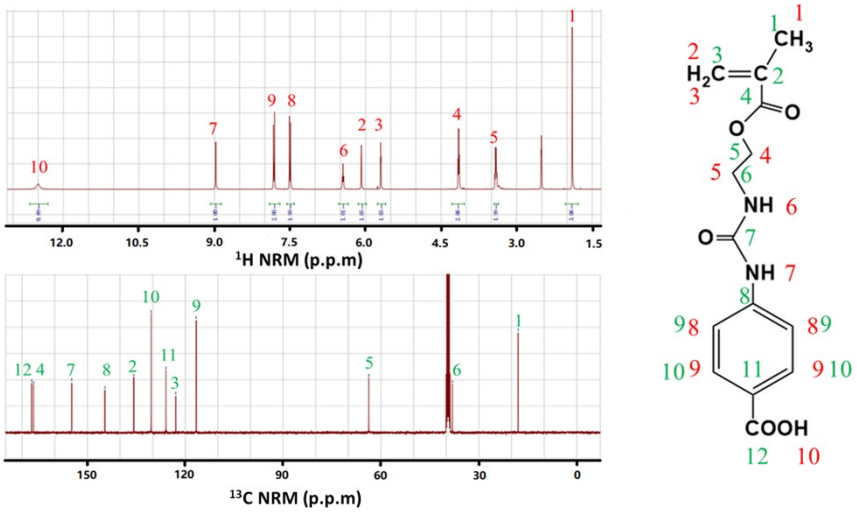

Fig. S3 Chemical structure of 4-(3-(2-(methacryloyloxy) ethyl) ureido)-benzoic acid, denoted as MUBA. Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra and ^{13}C NMR spectra of MUBA.

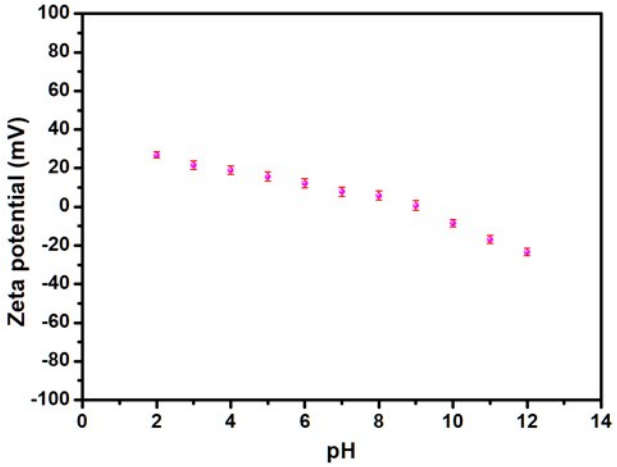

Fig. S4 Zeta potential change of SPMMs with pH values.

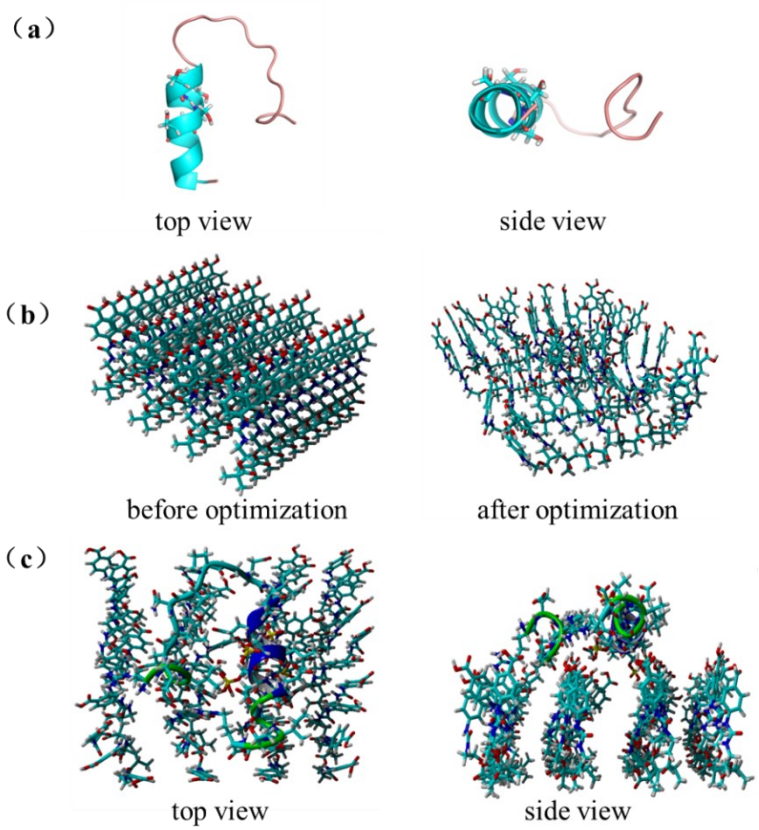

Fig. S5 a) Three-dimensional structure of the phosphopeptide; b) Three-dimensional diagram of layered polymer structure; c) The dominant conformations of the complex obtained by the docking of phosphopeptide with the layered polymer structure.

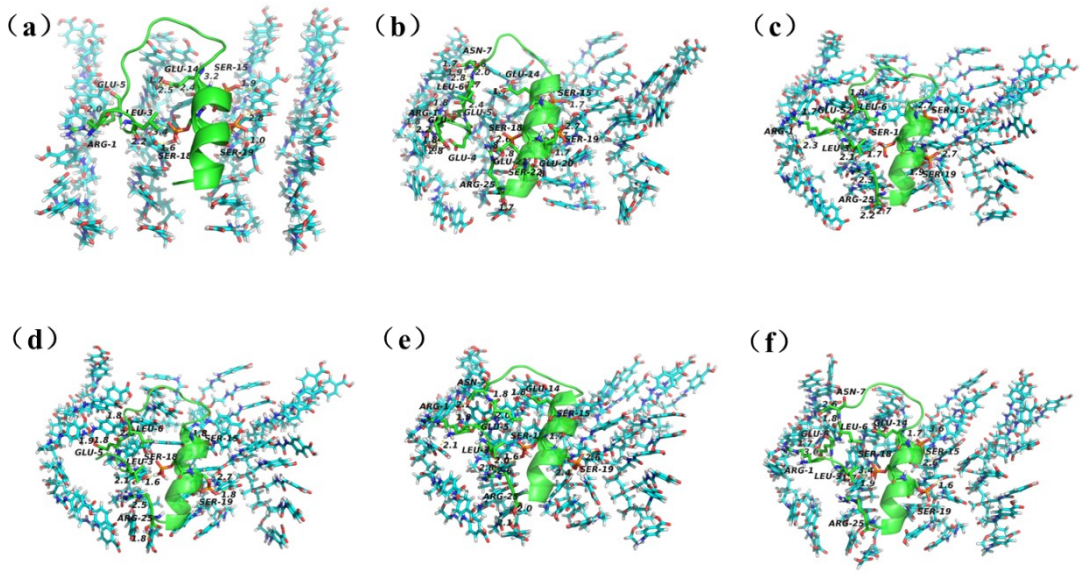

Fig. S6 The conformational of the complex and the hydrogen bond interaction during the dynamic simulation : a) initiating structure, b) 20 ns, c) 40 ns, d) 60 ns, e) 80 ns, f) 100 ns.

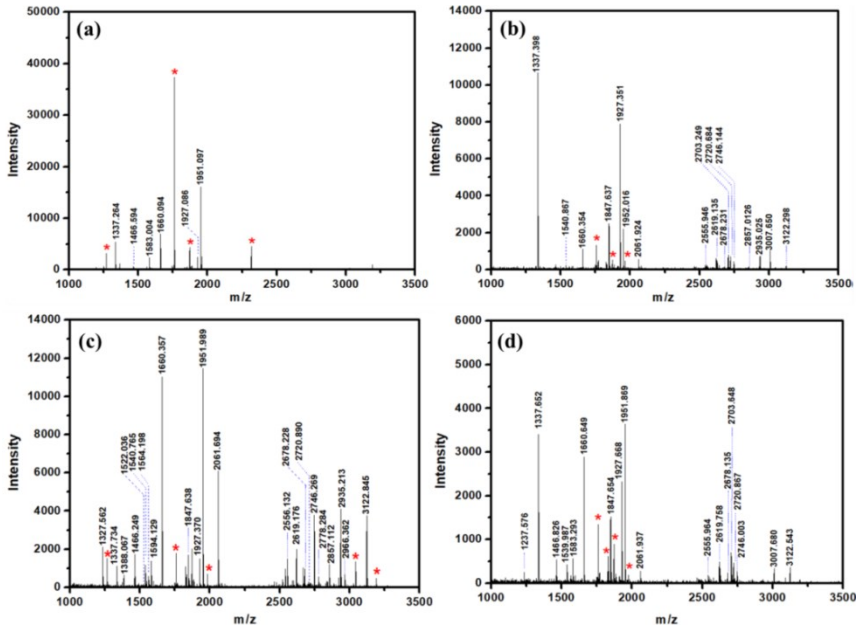

Fig. S7 MALDI-TOF mass spectra of the tryptic digests of α-casein (10^{-6} M) analysis after enrichment using SPMMs with different buffer solution (a) ACN/H_2O/FA 95:4.9:0.1; (b) ACN/H_2O/FA 85:14.9:0.1; (c) ACN/H_2O/FA 75:24.9:0.1, and (d) ACN/H_2O/FA 65:34.9:0.1. The phosphopeptides are labeled with their m/z values and non-phosphopeptide peaks are marked with symbol *.

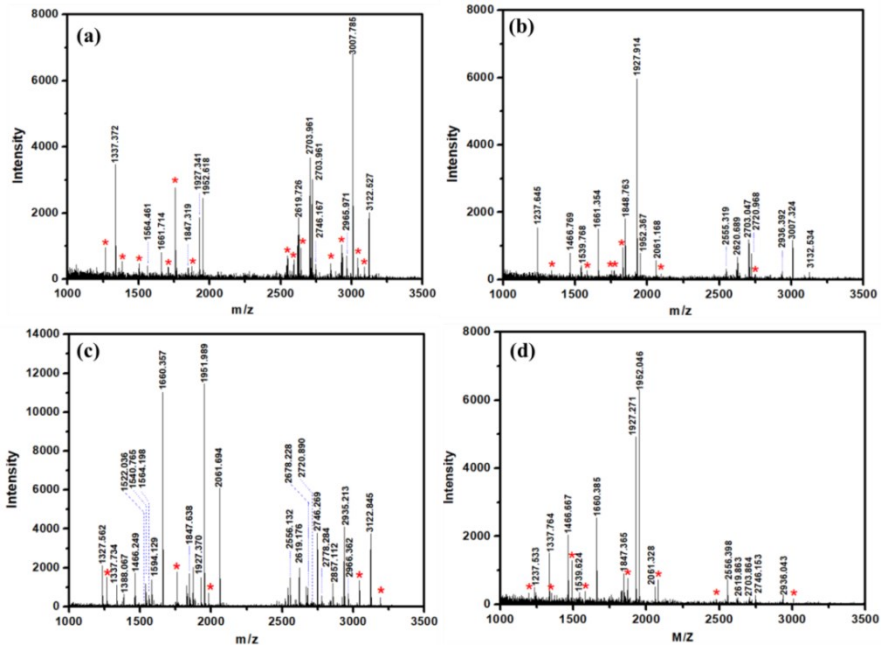

Fig. S8 MALDI-TOF mass spectra of the tryptic digests of α-casein (10^{-6} M) analysis after enrichment using SPMMs with molar ratio of MUBA monomer to DMAEMA monomer: A) 1:2, B) 1:4, C) 1:6, C) 1:10. The phosphopeptides are labeled with their m/z values and non-phosphopeptide peaks are marked with symbol *.

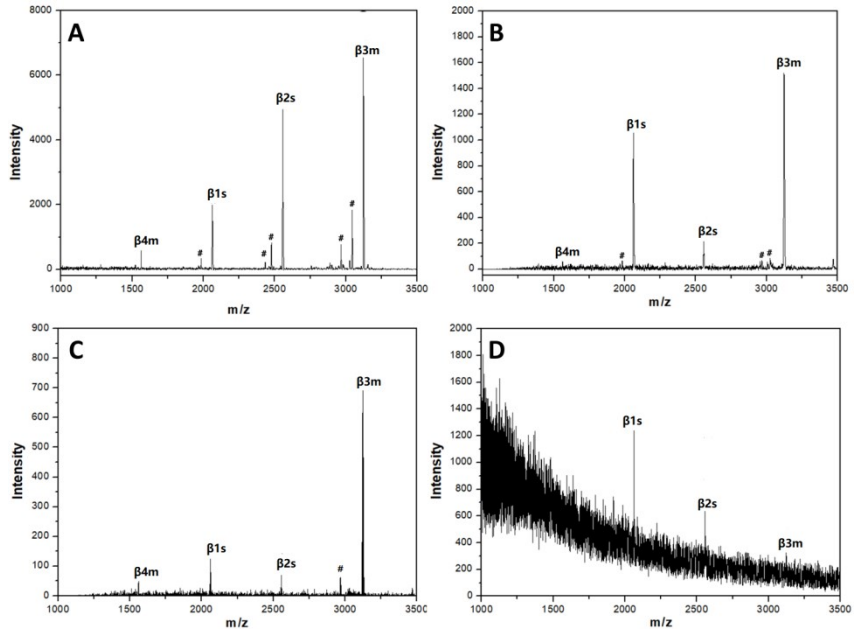

Fig. S9 MALDI-TOF mass spectra of the tryptic digest mixture of β-casein with different concentrations after enrichment with the SPMMs: A) 10^{-8} M, B) 10^{-9}M, C) 10^{-10} M, and D) 10^{-11} M. (s, monophosphopeptide; m, multiphosphopeptide; #, dephosphorylated peptide).

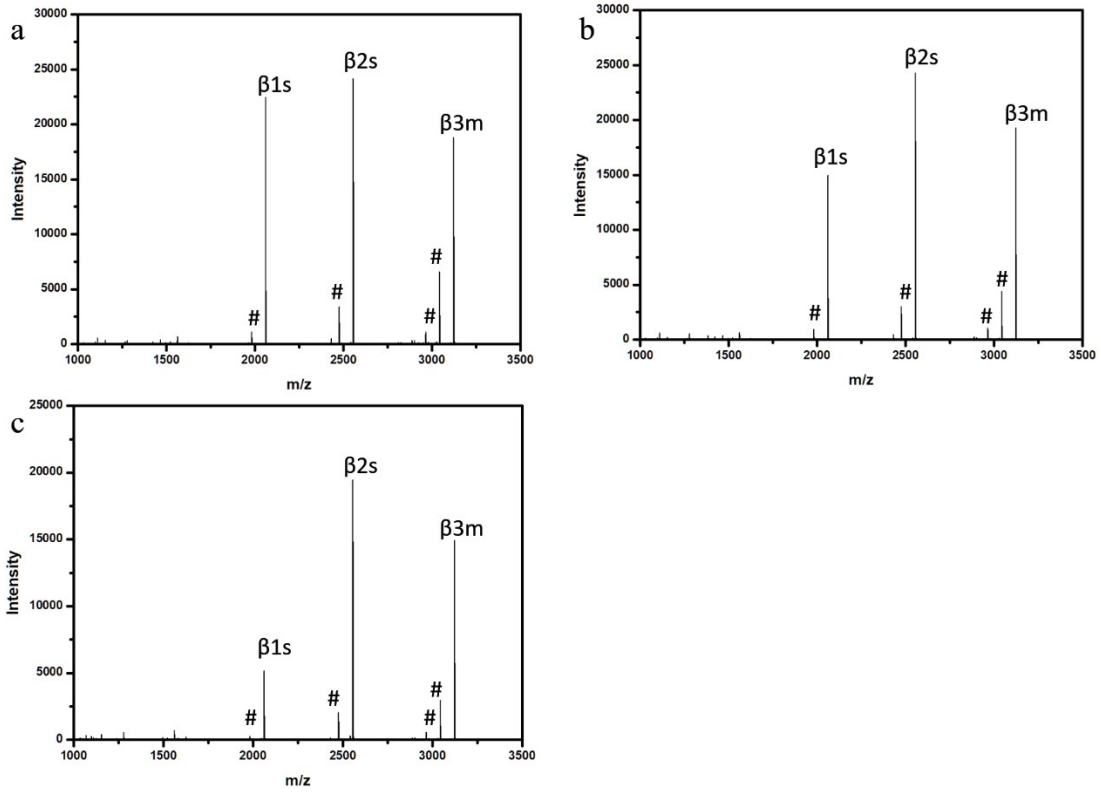

Fig. S10 MALDI mass spectra of β-casein digest (10^{-6} M) after enrichment with the SPMM nanospheres. Cycling 1st (a), cycling 3rd (b), and cycling 5th (c). (s, monophosphopeptide; m, multiphosphopeptide; #, dephosphorylated peptide).

Table S1. The comparison of the SPMM nanospheres with the other reported affinity materials.

No.	Type of materials	Selectivity	Sensitivity	Journal
1	SPIOs@SiO_2@MOF	β-casein:BSA 1:400	0.1fmol/ul (β-casein)	ACS Sustainable Chem. Eng., 2019, 7, 6043-6052
2	Fe_3O_4/PDA/PAMA-Arg	β-casein:BSA 1:500	0.001fmol/ul (β-casein)	J. Mater. Chem. B, 2018, 6, 3969-3978
3	SPMA nanospheres	β-casein:BSA 1:1000	0.1fmol/ul (β-casein)	Nanoscale, 2018, 10, 8391-8396
4	2-D Ti-based MOF nanosheets	β-casein:BSA 1:10000	0.1fmol/ul (β-casein)	Anal. Chem. 2018, 90, 22, 13796-13805
5	2-D Hf-BTB nanosheets	---	0.4fmol/ul (β-casein)	Anal. Chem. 2019, 91, 14, 9093-9101
6	MCNC@COF@Zr^{4+} composites	β-casein:BSA 1:200	0.1fmol/ul (β-casein)	ACS Appl. Mater. Interfaces 2019, 11, 14, 13735-13741
7	SPMM nanospheres	β-casein:BSA 1:5000	0.01fmol/ul (β-casein)	This work

Table S2. Detailed information for the observed phosphopeptides obtained from tryptic digests of α-casein after enrichment by SPMMs in MALDI-TOF mass analysis. S*: phosphorylated site.

Peak No.	Observed m/z	Theoretical m/z	Amino acid sequence
P1	1237.6	1237.5	TVDMES*TEVF
P2	1337.8	1337.7	HIQKEDVS*ER
P3	1388.8	1389.1	RNAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTKK
P4	1466.8	1466.6	TVDMES*TEVFTK
P5	1522.0	1521.9	VVRNANEEEYSIGS*S*S*EES*AEVATEEVKITVDDKHYQKAL
P6	1540.8	1541.7	KYKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQELAY
P7	1564.2	1563.0	RELEELNVPGEIVES*LS*S*S*EESITRI
P8	1594.0	1594.7	TVDMES*TEVFTKKR
P9	1660.2	1660.7	VPQLEIVPNS*AEER
P10	1847.8	1847.6	DIGSES*TEDQAMEDIK
P11	1927.2	1927.6	DIGS*ES*TEDQAMEDIK

P12	1951.7	1951.9	YKVPQLEIVPNS*AEER
P13	2061.8	2061.7	FQS*EEQQQTEDELQDK
P14	2548.0	2548.3	KYKVPQLEIVPNS*AEERLHSMKE
P15	2556.0	2556.1	KFQS*EEQQQTEDELQDKIHPFA
P16	2619.0	2618.7	NTMEHVS*S*S*EESIIS*QETYK
P17	2678.0	2678.0	VNELS*KDIGS*ES*TEDQAMEDIK
P18	2703.7	2703.1	QMEAESIS*S*S*EEIVPNS*VEQK
P19	2720.7	2719.9	QMEAES*IS*S*S*EEIVPNPNS*VEQK
P20	2746.0	2745.9	KNTMEHVS*S*S*EESIIS*QETYK
P21	2778.8	2779.3	KIEKFQS*EEQQQTEDELQDKIHPF
P22	2857.0	2857.2	KVNELS*KDIGS*ES*TEDQAMEDIKQME
P23	2935.0	2935.2	KEKVNELS*KDIGS*ES*TEDQAMEDIKQ
P24	2966.0	2965.2	ELEELNVPGEIVES*LS*S*S*EESITR
P25	3122.7	3122.3	RELEELNVPGEIVES*LS*S*S*EESITR

Table S3. Detailed information for the observed phosphopeptides obtained from tryptic digests of non-fat milk after enrichment by SPMMs in MALDI-TOF mass analysis. S*: phosphorylated site.

Peak No.	Observed m/z	Theoretical m/z	Amino acid sequence
P1	1103.6	1103.4	KFQS*EEQQQT
P2	1161.5	1162.5	KKIEKFQS*EEQQQTEDELQDKIHPFAQTQS
P3	1229.7	1229.5	KNTMEHVS*S*S*EESIIS*QETY
P4	1282.0	1281.1	RINKKIEKFQS*EEQQQTEDELQDKIHPFAQTQS
P5	1432.6	1432.1	RNAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTKKT
P6	1483.5	1483.8	RELEELNVPGEIVES*LS*S*S*EESITR
P7	1495.7	1495.0	RFFVAPFPEVFGKEKVNELSKDIGS*ES*TEDQAMEDIKQME
P8	1522.7	1521.9	VVRNANEEEYSIGS*S*S*EES*AEVATEEVKITVDDKHYQKAL
P9	1540.6	1541.7	KYKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQELAY
P10	1562.6	1563.0	RELEELNVPGEIVES*LS*S*S*EESITRI
P11	1628.9	1629.7	RELEELNVPGEIVES*LS*S*S*EESITRINKKIEKFQS*EEQQQTEDELQDKIHPFA
P12	1661.7	1660.7	VPQLEIVPNS*AEER
P13	1737.7	1737.0	RELEELNVPGEIVES*LS*S*S*EESITRINKKIEKFQS*EEQQQTEDELQDKIHPFAQTQS
P14	1807.2	1807.7	KNTMEHVS*S*S*EESIIS*QETYKQEKNMAINPS*KENLCSTFCKEVVR

P15	1927.0	1927.6	DIGS*ES*TEDQAMEDIK
P16	1952.5	1951.9	YKVPQLEIVPNS*AEER
P17	2062.3	2061.7	FQS*EEQQQTEDELQDK
P18	2556.0	2556.1	KFQS*EEQQQTEDELQDKIHPFA
P19	2720.7	2719.9	QMEAES*IS*S*S*EEIVPNPNS*VEQK
P20	2806.0	2806.1	KVNELS*KDIGS*ES*TEDQAMEDIKQM
P21	2856.9	2857.2	KVNELS*KDIGS*ES*TEDQAMEDIKQME
P22	2965.9	2965.2	ELEELNVPGEIVES*LS*S*S*EESITR
P23	3008.2	3008.0	NANEEEYSIGS*S*S*EES*AEVATEEVK
P24	3053.8	3054.4	KKIEKFQS*EEQQQTEDELQDKIHPFA
P25	3123.2	3122.3	RELEELNVPGEIVES*LS*S*S*EESITR