Supporting Information

Hydrazide functionalized monodispersed silica microsphere: a novel probe with tunable selectivity for versatile enrichment of phosphopeptides with different numbers of phosphorylation sites

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Experimental Section

Materials

Trypsin, α-casein, β-casein, acetonitrile (ACN), and methanol were purchased from Sigma-Aldrich. 2,5-dihydroxybenzoic acid (DHB) and 3-aminopropyl triethoxysilane (APTES) were purchased from J&K Scientific Ltd. Ammonium bicarbonate (NH_4HCO_3) was purchased from Fluka. Ethanol and phosphoric acid (H_3PO_4) were from Beijing Chemical Works. Formic acid (FA) and hydrazine hydrate were from Sinopharm Chemical Reagent Co., Ltd. Tetraethyl orthosilicate (TEOS) was purchased from Xilong Chemical Co., Ltd. Methyl acrylate was from Beijing Yili Fine Chemicals Co., Ltd. Toluene and ammonia solution ($NH_3 \cdot H_2O$) were from Beijing Tong Guang Fine Chemicals Company. Non-fat milk was purchased from a local supermarket. Water used for digestion and enrichment analysis was from Wahaha Group Co., Ltd. All chemicals were of analytical grade except ACN and methanol, which were of HPLC grade.

Preparation of hydrazide functionalized monodispersed silica microsphere (HFMSM)

Synthesis of monodispersed silica microsphere (MSM)

100 mL ethanol, 8 mL $NH_3 \cdot H_2O$, and 4 mL TEOS were mixed in a 250 mL flask and stirred for 12 h at room temperature. After washing by ethanol for 5 times, the product was

dried at 40 °C overnight.

Synthesis of amino functionalized monodispersed silica microsphere (AFMSM)

0.4 g MSM was dispersed in 20 mL toluene. The mixture was heated to 65 °C, and 0.44 g APTES was added. Then the mixture was heated and refluxed at 120 °C for 2 h. After cooling to room temperature, the product was washed by ethanol and dried at 40 °C overnight.

Synthesis of HFMSM

0.3 g AFMSM was added to 7 mL methanol in a 25 mL flask. The mixture was sonicated for a while for better dispersion, and cooled in an ice-water bath. Then 210 μL methyl acrylate in 5.25 mL methanol was added dropwise. The mixture was stirred in ice-water bath for 15 min, and then stirred at room temperature for another 24 h.

After washing and drying, 0.25 g of the obtained product was mixed with 7.5 mL methanol by sonication. Another solution containing 600 μL hydrazine hydrate and 7.5 mL methanol was added dropwise under stirring at room temperature, and then the mixture was refluxed at 120 °C for 3 h. Finally the product was collected by centrifugation, washed by ethanol and then dried at 40 °C overnight.

Enrichment of phosphopeptides

In typical experiments, the peptides in tryptic digest were diluted to a certain concentration by loading buffers (ACN/H_2O 3:2, *v*/*v*, with varied concentration of FA). 50 μL HFMSM suspension (typically 8 mg mL^{-1}) were added into 200 μL diluted peptides solution, and the mixture was gently vibrated for 30 min. The adsorbent could be easily centrifugated at 10000 rpm for 2 min (Eppendorf centrifuge 5417R). After removal of the supernatant and being washed with loading buffers for 3 time, HFMSM with phosphopeptides adsorbed was dispersed in 4 μL matrix solution (20 mg/mL DHB, 5% H_3PO_4, ACN/H_2O 1:1, v/v) or buffers containing higher concentration of FA for phosphopeptide release. After centrifugation the supernatant was subjected to MALDI-ToF-MS analysis.

Instrumentation

For characterization of synthesized materials, transmission electron microscopy (TEM) images were acquired by FEI Tecnai G2 T20 TEM operated at 120 kV, scanning electron

microscopy (SEM) images were acquired by Hitachi S-4800 FESEM operated at 2 kV, infrared spectra (IR) were measured with KBr pellet by Bruker Tensor 27 FT-IR, and elemental composition analyses were performed using Vario Micro Cube Elemental Analyzer.

MALDI-Tof-MS spectra were obtained by a Bruker Daltonics ultraflex ToF mass spectrometer in reflection mode with following parameters: ion source 1, 25.00 kV; ion source 2, 22.15 kV; lens, 10.55 kV; reflector, 26.30 kV; reflector 2, 14.10 kV. The laser frequency was set on 20 Hz. For phosphopeptides eluted by matrix buffers, 1 μL elute was directly deposited and dried on a target plate, while for phosphopeptides eluted by FA solution, 1 μL elute was mixed with 0.5 μL matrix solution on plate and dried before MALDI-ToF-MS analysis.

Figure and Table

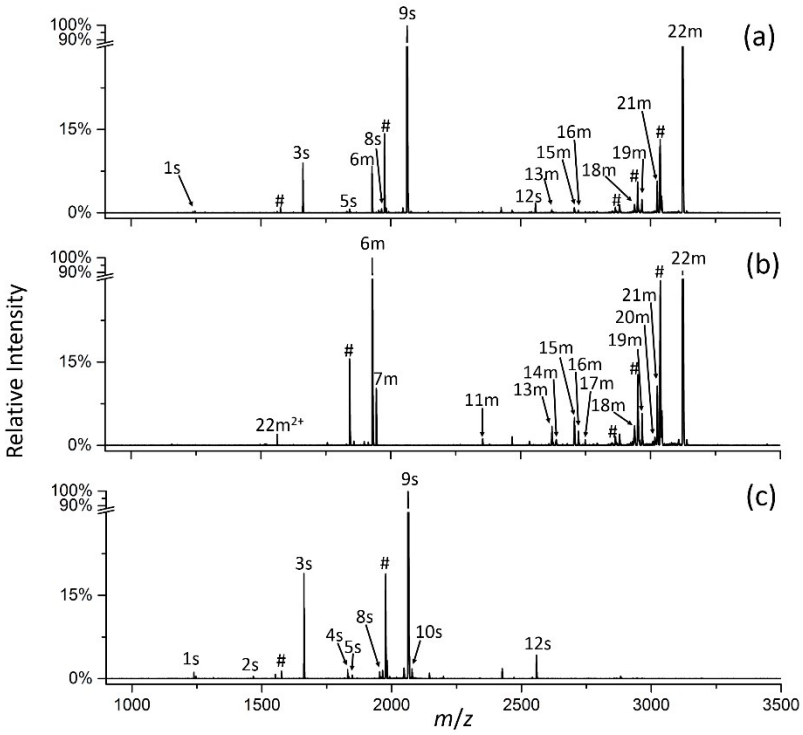

Figure S1MALDI-ToF-MS spectra of digest of nonfat milk. (a) Global analysis of both mono- and multi- phosphopeptides; (b) Selective analysis of multiphosphopeptides; (c) Selective analysis of monophosphopeptides. Monophosphopeptides were marked with "s"; multiphosphopeptides were marked with "m"; "#" denoted dephosphorylated fragments.

Table S1 Phosphopeptides identified in digest of standard phosphoproteins

No.	*m/z*	Numbers of phosphorylation sites	Sequence
β1	2061.8	1	FQpSEEQQQTEDELQDK
β2	2431.0	1	IEKFQpSEEQQQTEDELQDK
β3	2556.4	1	FQpSEEQQQTEDELQDKIHPF
β4	2966.2	4	ELEELNVPGEIVEpSLpSpSpSEESITR
β5	3122.3	4	RELEELNVPGEIVEpSLpSpSpSEESITR
α1	1237.3	1	TVDMEpSTEVF
α2	1253.3	1	TVDoMEpSTEVF
α3	1466.6	1	TVDMEpSTEVFTK
α4	1482.6	1	TVDoMEpSTEVFTK
α5	1660.8	1	VPQLEIVPNpSAEER

α6	1833.1	1	YLGEYLIVPNpSAEER
α7	1847.7	1	DIGpSESTEDQAMEDIK
α8	1927.7	2	DIGpSEpSTEDQAMEDIK
α9	1943.7	2	DIGpSEpSTEDQAoMEDIK
α10	1952.0	1	YKVPQLEIVPNpSAEER
α11	2618.9	4	NTMEHVpSpSpSEEpSIISQETYK
α12	2634.9	4	NToMEHVpSpSpSEEpSIISQETYK
α13	2678.0	3	VNELpSKDIGpSEpSTEDQAMEDIK
α14	2704.9	5	pyro-QMEAEpSIpSpSpSEEIVPNpSVEQK
α15	2720.9	5	QMEAEpSIpSpSpSEEIVPNpSVEQK
α16	2736.9	5	QoMEAEpSIpSpSpSEEIVPNpSVEQK
α17	2747.0	4	KNTMEHVpSpSpSEEpSIISQETYK
α18	2935.2	3	EKVNELpSKDIGpSEpSTEDQAMEDIK
α19	3008.0	4	NANEEEYSIGpSpSpSEEpSAEVATEEVK
α20	3088.0	5	NANEEEYpSIGpSpSpSEEpSAEVATEEVK

Table S2 Phosphopeptides identified in digest of standard phosphoproteins

No.	*m*/*z*	Numbers of phosphorylation sites	Sequence	Proteins
1s	1237.3	1	TVDMEpSTEVF	α-S2-casein
2s	1466.6	1	TVDMEpSTEVFTK	α-S2-casein
3s	1660.8	1	VPQLEIVPNpSAEER	α-S1-casein
4s	1833.1	1	YLGEYLIVPNpSAEER	α-S1-casein
5s	1847.7	1	DIGpSE**S**TEDQAMEDIK	α-S1-casein
6m	1927.7	2	DIGpSEpSTEDQAMEDIK	α-S1-casein
7m	1943.7	2	DIGpSEpSTEDQAoMEDIK	α-S1-casein
8s	1952.0	1	YKVPQLEIVPNpSAEER	α-S1-casein
9s	2061.8	1	FQpSEEQQQTEDELQDK	β-casein
10s	2080.1	1	KYKVPQLEIVPNpSAEER	α-S1-casein
11m	2352.6	4	NVPGEIVEpSLpSpSpSEESITR	β-casein
12s	2556.4	1	FQpSEEQQQTEDELQDKIHPF	β-casein
13m	2618.9	4	NTMEHVpSpSpSEEpSII**S**QETYK	α-S2-casein
14m	2634.9	4	NToMEHVpSpSpSEEpSII**S**QETYK	α-S2-casein
15m	2704.9	5	pyro-QMEAEpSIpSpSpSEEIVPNpSVEQK	α-S1-casein
16m	2720.9	5	QMEAEpSIpSpSpSEEIVPNpSVEQK	α-S1-casein
17m	2747.0	4	KNTMEHVpSpSpSEEpSII**S**QETYK	α-S2-casein

18m	2935.2	3	EKVNELpSKDIGpSEpSTEDQAMEDIK	α-S1-casein
19m	2966.2	4	ELEELNVPGEIVEpSLpSpSpSEESITR	β-casein
20m	3008.0	4	NANEEEY**S**IGpSpSpSEEpSAEVATEEVK	α-S2-casein
21m	3025.5	2	FPQpYLQpYLYQGPIVLNPWDQ VKR	α-S2-casein
22m	3122.3	4	RELEELNVPGEIVEpSLpSpSpSEESITR	β-casein