Supporting Information:

Nanodiamond-Based Two-Step Sampling of Multiply and Singly Phosphorylated Peptides for MALDI-TOF Mass Spectrometry Analysis.

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Preparation of PA-coated and TiO_2-coated NDs. Abrasive diamond powders with sizes in the range of 100 nm were purified in concentrated H_2SO_4-HNO_3 mixture (3:1, v/v) at 100 °C in a microwave reactor (Discover BenchMate, CEM) for 3 h, after which the diamond nanoparticles were rinsed extensively with deionized water and recovered by centrifugation. PA-coated NDs were prepared by a carbodiimide-based cross-linking method for covalent conjugation of poly-L-arginine (PA) with the carboxyl groups on the oxidized ND surfaces, following previous reported procedures.[31] This was made by mixing an EDC solution (22 mg/mL, 63.7 μL), a PA solution (4 mg/mL, 155 μL), and the acid-treated NDs (1 mg) together in 5 mM H_3BO_3/NaOH buffer (pH 8.5). The mixture was shaken gently at room temperature for 2 h. The resulting PA-coated NDs were recovered by centrifugation,

washed thoroughly with deionized water, and prepared as a suspension at a concentration of 1 mg/mL.

TiO_2-coated NDs were prepared by a two-step sol-gel reaction route, as proposed by Chen *et al.* [38] Briefly, 26 mL of 1.0 M BH_3-THF were added dropwise with stirring to a suspension of 500 mg acid-treated NDs in 30 mL of dry THF. The mixture was refluxed for 24 h, hydrolysed with 1 N HCl later, cooled to r.t. until the hydrogen development ended. The hydroxylated NDs were thoroughly washed with water until pH 7 and isolated by centrifugation. Reduction NDs (300mg) were suspended in ethanol (10 mL) under sonication. Ammonia (30%, 14 mL), tetraethyl orthosilicate (0.2 mL), and water (11 mL) were added sequentially to the particle suspension. The mixture was stirred vigorously for 2 h in a bath sonicator at 40°C, followed by sonication for 1 h. The SiO_2-coated NDs were thoroughly washed with ethanol, recovered by centrifugation, and resuspended in ethanol (6 mL). The suspension was heated under reflux at 60°C for 12 h to strengthen the adhesion of SiO_2 to the surfaces of the particles. The SiO_2-coated NDs were collected by centrifugation and were resuspended in ethanol (6 mL), followed by sonication for 1 h. Ethanol (50 mL) and water (0.15 mL) were added into this solution, which was then subjected to vertex-mixing for 15 min. Subsequently, the suspension was mixed with a solution of ethanol (13 mL) and titanium butoxide (0.15 mL) and then stirred vigorously at 45°C for 4 h, followed by sonication for 1 h. When the reaction was complete, and TiO_2-coated NDs were collect by

centrifugation, cleaned with ethanol (3 x 3.0 mL) and with H_2O (3 x 3.0 mL), and were resuspended in H_2O (6 mL). The suspension was heated under reflux at 60°C for 12 h to strengthen the adhesion of TiO_2 to the surfaces of the particles. The TiO_2-coated NDs were collected by centrifugation, and resuspended in H_2O at a concentration of 1.0 mg/mL before use.

Enrichment of Phosphopeptides using commerical TiO_2 beads and Fe^{3+}-IMAC beads

Fe^{3+}-IMAC and TiO_2 purification of phosphorylated peptides were performed according to manufacturer's instructions. For Fe^{3+}-IMAC purification, the sample solution (1 μL) was mixed with 20 μL of binding solution and with 10 μL of commercial Fe^{3+}-IMAC magnetic bead suspension provided by manufacturer. After incubation for 1 h, the Fe^{3+}-IMAC magnetic beads were collected by magnetic separation, cleaned twice with 100 μL of washing solution provided by the manufacturer to remove unbound peptides. The phosphopeptides extracted by Fe^{3+}-IMAC magnetic beads were then eluted from the particles with 3 μL of elution solution provided by the manufacturer. The elution solution was transferred into a fresh tube and vacuum dried. The eluted sample was then mixed with 1 μL of DHB (30 mg/mL) containing 5% phosphoric acid and 66% ACN and deposited on a MALDI plate for MS analysis. For TiO_2 purification, the sample solution (1 μL) was 10-fold diluted with binding buffer provided by the manufacturer and mixed with 10 μL of commercial TiO_2

magnetic bead suspension. After incubation for 1 h, the TiO_2 magnetic beads were collected by magnetic separation, cleaned four times with 200 μL of binding buffer provided by the manufacturer to remove unbound peptides. The phosphopeptides extracted by TiO_2 magnetic beads were then eluted from the particles with 10 μL of elution buffer provided by the manufacturer. The elution solution was transferred into a fresh tube and vacuum dried. The eluted sample was then mixed with 1 μL of DHB (30 mg/mL) containing 5% phosphoric acid and 66% ACN and deposited on a MALDI plate for MS analysis.

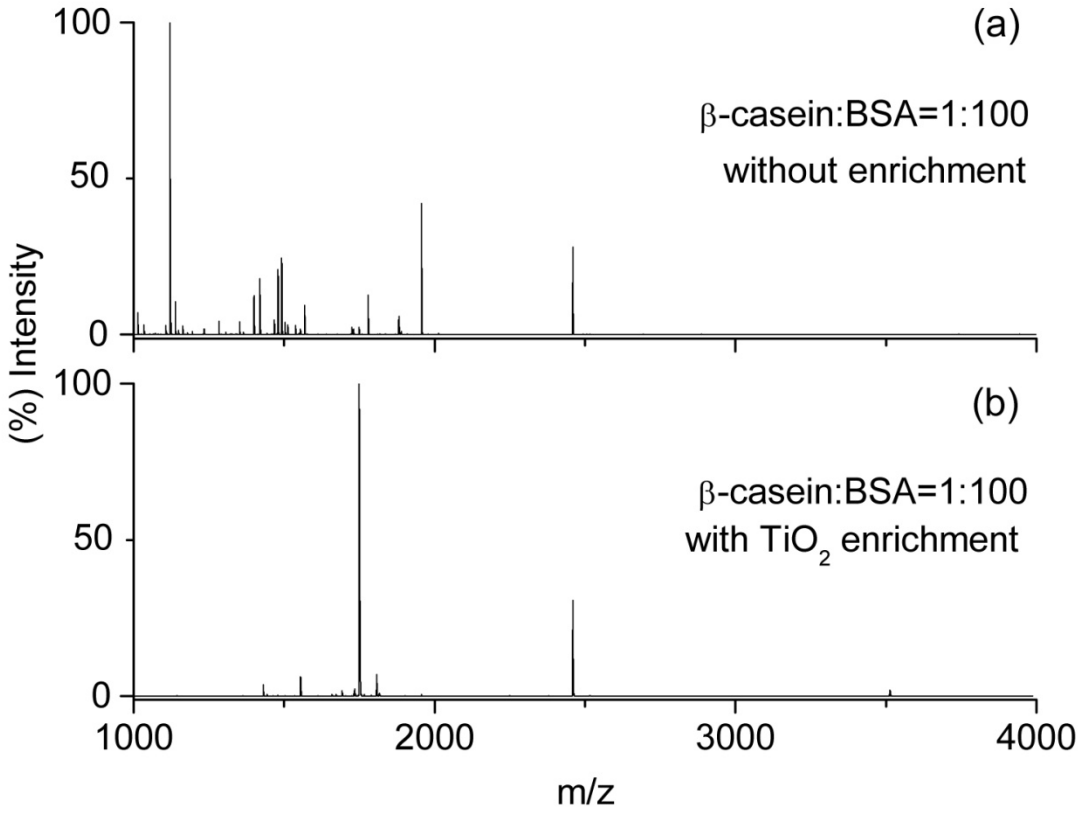

Figure S1. MALDI mass spectra of a tryptic digest of a mixture of β-casein (1 pmol) and BSA (100 pmol). The mass spectra were obtained by (a) direct analysis; (b) analysis using commercial TiO_2 beads;

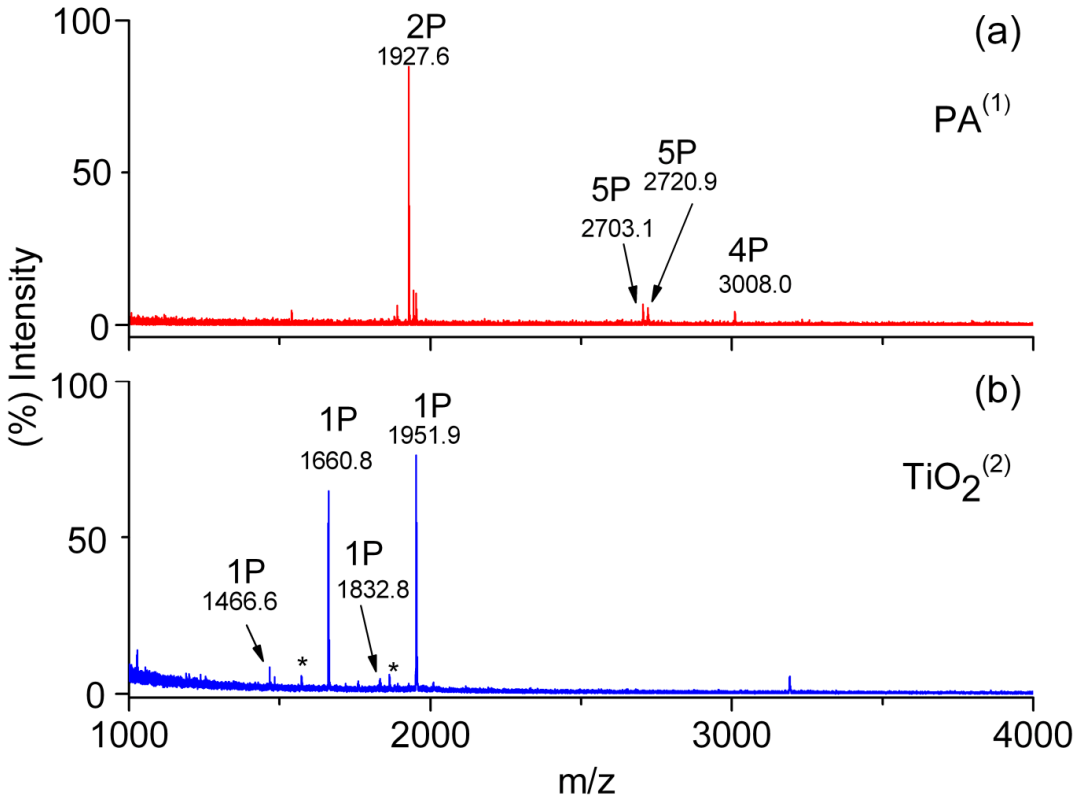

Figure S2. MALDI mass spectra of a tryptic digest of β-casein (1 pmol). (a) Analysis after isolation using PA-coated NDs; (b) analysis after successive isolation using TiO_2–coated NDs. An asterisk indicates the loss of phosphoric acid, and "#P" denotes the number of phosphate group per peptide. The number in the parentheses denotes the number of the purification steps.

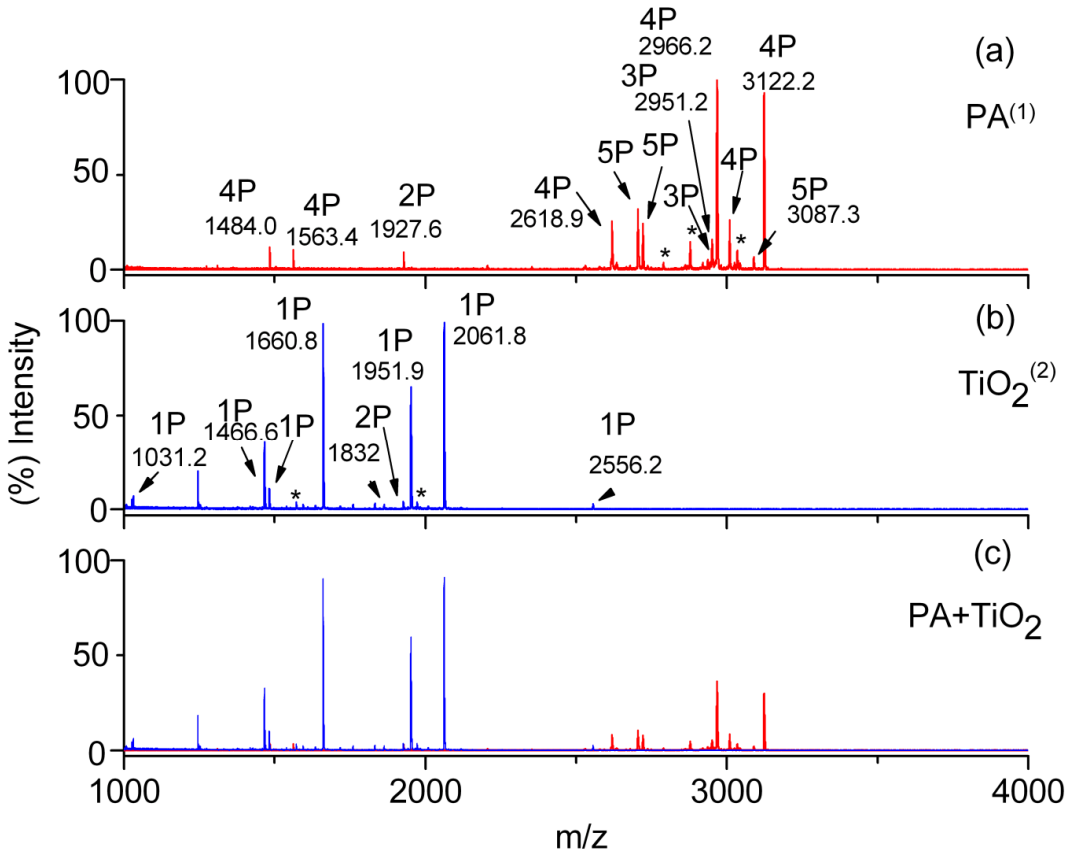

Figure S3. MALDI mass spectra of a tryptic digest of a tryptic digest of nonfat milk with an estimated casein concentration of 2.33 x 10^{-8} M (100 µL). (a) Analysis after enrichment using PA-coated NDs; (b) Analysis after successive enrichment using TiO_2–coated NDs; (c) An overlay of two mass spectra. An asterisk indicates the loss of phosphoric acid, and P denotes the number of phosphate group per peptide. The number in the parentheses denotes the number of purification steps.

Table S1. List of phosphorylated peptides identified from the tryptic digest of nonfat milk using the two-step isolation strategy. The phosphorylation sites are indicated in bold and underlined.

Peptide sequence	Calculated	Two-step isolation	
	m/z	PA[(1)]	TiO_2[(2)]
TVDME**S**TEVFTK (α-S2. 153–164)	1466.6	*N/A* [a]	++
VPQLEIVPN**S**AEER (α-S1. 121–134)	1660.8	*N/A*	++
YLGEYLIVPN**S**AEER (α-S1. 104-119)	1832.8	*N/A*	++
DIG**S**E**S**TEDQAMEDIK (α-S1. 58–73)	1927.6	++	++
YKVPQLEIVPN**S**AEER (α-S1. 119–134)	1951.9	*N/A*	++
FQ**S**EEQQQTEDELQDK (β. 33–48)	2061.8	*N/A*	++
FQ**S**EEQQQTEDELQDKIHPF (β. 33–52)	2556.2	*N/A*	++
NTMEHV**SSS**EESII**S**QETYK (α-S2. 17-36)	2618.9	++	*N/A*
QMEAE**S**I**SSS**EEIVPN**S**VEQK (α-S1. 74–94) [b]	2703.1	++	*N/A*
QMEAE**S**I**SSS**EEIVPN**S**VEQK (α̃-S1. 74–94)	2720.9	++	*N/A*
EKVNEL**S**KDIG**S**E**S**TEDQAMEDIK (α-S1. 50–73)	2935.1	++	*N/A*
EKVNEL**S**KDIG**S**E**S**TEDQAMEDIK (α-S1. 50-73) [c]	2951.2	++	*N/A*
ELEELNVPGEIVE**S**L**SSS**EESITR (β. 17-30)	2966.1	++	*N/A*
NANEEEYSIG**SSS**EE**S**AEVATEEVK (α-S2. 61–85)	3008.0	++	*N/A*
NANEEEY**S**IG**SSS**EE**S**AEVATEEVK (α-S2. 61–85)	3087.3	++	*N/A*
RELEELNVPGEIVE**S**L**SSS**EESITR (β. 16–40)	3122.2	++	*N/A*

[a] *N/A* denotes a peak *S/N* ratio less than 3; ++ denotes a peak *S/N* ratio over 10.
[b] pyroglutamate formation from N-terminal glutamine residue
[c] Oxidation of methionine residue

The phosphorylation sites are bold and underlined. The oxidized methionine is denoted by oM. The phosphorylated peptides were derived by tyrptic digestion of α-casein S1 (α-S1) and S2 (α-S2) and β-casein (β).

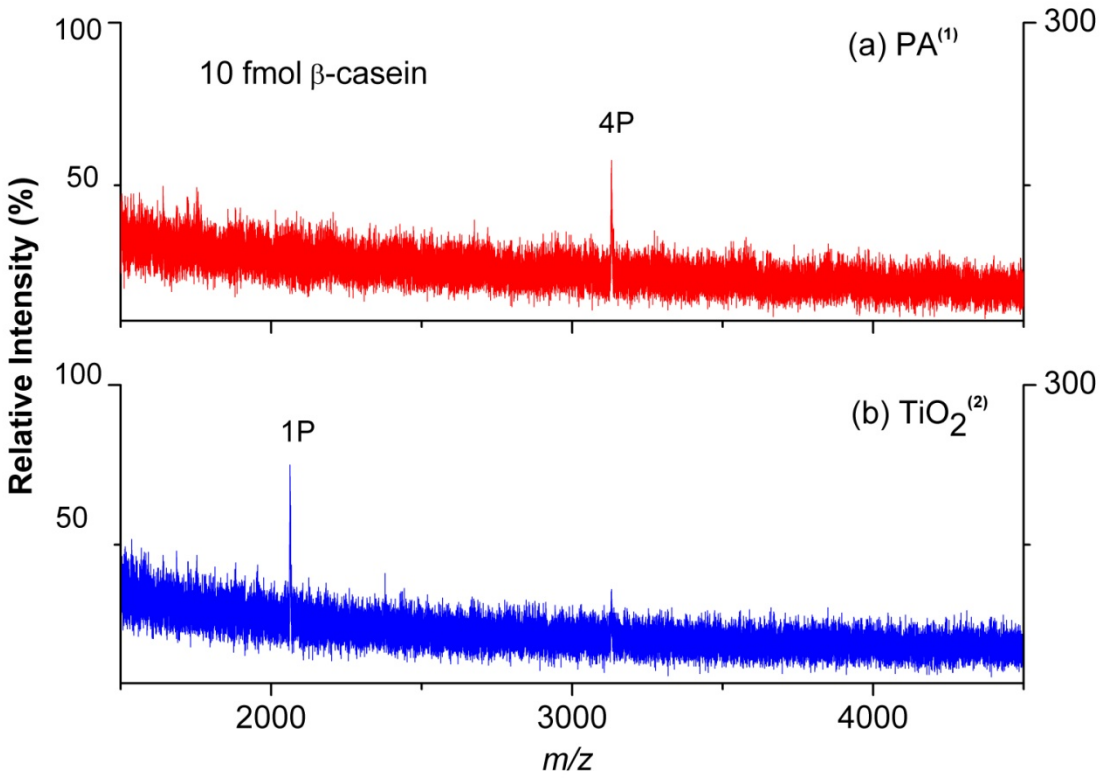

Figure S4. MALDI mass spectra of a tryptic digest of β-casein (10 fmol). (a) Analysis after isolation using PA-coated NDs; (b) analysis after successive isolation using TiO_2–coated NDs. An asterisk indicates the loss of phosphoric acid, and "#P" denotes the number of phosphate group per peptide. The number in the parentheses denotes the number of the purification steps.

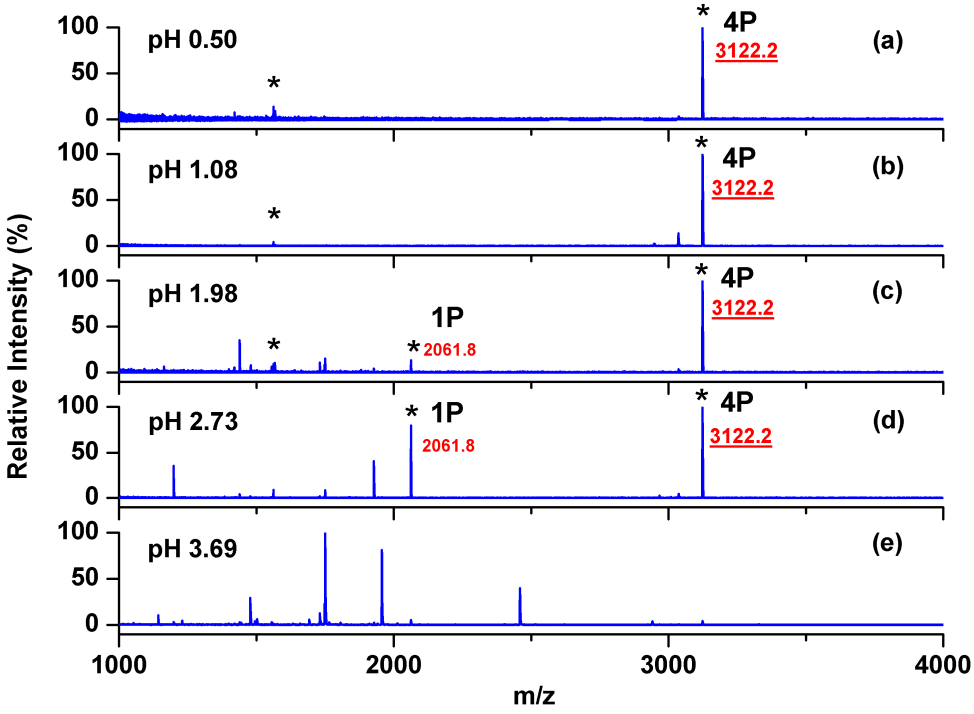

Figure S5. Effect of binding solution pH on the affinity of phosphopeptides for PA-coated NDs. MALDI-TOF mass spectra of a tryptic digest of β-casein (1.0 pmol) and BSA (100 pmol), extracted at (a) pH 0.5; 10% TFA (b) pH 1.08; 1% TFA, (c) pH 1.98; 0.1% TFA, (d) pH 2.73; 0.01% TFA, and (e) pH 3.69; 0.001% TFA with the ND treatment.