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ORIGINAL PAPER

Iron oxide/tantalum oxide core–shell magnetic nanoparticle-based microwave-assisted extraction for phosphopeptide enrichment from complex samples for MALDI MS analysis

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Abstract A new type of metal-oxide-coated magnetic nanoparticles (NPs)—tantalum-oxide-coated magnetic iron oxide (Fe_3O_4@Ta_2O_5) NPs—which are used as affinity probes for selectively trapping phosphopeptides from complex samples, is demonstrated in this study. In this approach, phosphopeptide enrichment was achieved by incubating the NPs with sample solutions under microwave heating within 1 min. The NP–target species conjugates were readily isolated from samples by magnetic separation followed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometric analysis. When using human serum as the sample, phosphorylated fibrinopeptide-A-derived ions are the only ions observed in the MALDI mass spectra after enrichment by the Fe_3O_4@Ta_2O_5 NPs. Furthermore, only phosphopeptides appear in the MALDI mass spectra after using the affinity probes to selectively trap target species from the tryptic digest of a cell lysate and milk sample. The results demonstrated that the Fe_3O_4@Ta_2O_5 NPs have the capability of selectively trapping phosphorylated peptides from complex samples. The detection limit of this approach for a phosphopeptide (FQpSEEQQQTEDELQDK) was ~10 fmol.

Keywords Tantalum oxide · Magnetic nanoparticles · Phosphopeptides · MALDI MS

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Introduction

Protein phosphorylation, which is one of the most common posttranslational modifications, plays an important role in regulating cellular processes. Thus, exploring efficient methods for rapidly enriching and characterizing phosphoproteins from complex samples has been one of the main targets in phosphoproteomics analysis. Many transition metal oxides are capable of interacting strongly with small phosph(on)ates [1–6]. Thus, metal oxide affinity chromatography has been employed to enrich phosphopeptides selectively from complex samples with high specificities [7–26]. Spenser and coworkers [4] demonstrated that alkyl phosphates self-assemble onto the surfaces of several metal oxides, including titanium oxide, zirconium oxide, aluminum oxide, niobium oxide, and tantalum oxide. Titanium oxide [7–12, 19–21], zirconium oxide [13, 14, 20], aluminum oxide [15–17], and niobium oxide [18, 26] have been successfully employed to enrich phosphopeptides. These findings suggested to us that tantalum oxide might also be suitable for use as affinity probes for phosphopeptides.

Employing affinity probes having magnetic properties for extracting target species is convenient. After extraction, it is easy to isolate the target species–NP conjugates from sample solutions through magnetic separation. Thus, tantalum-oxide-coated magnetic nanoparticles (Fe_3O_4@Ta_2O_5 NPs) as affinity probes for selective enrichment of phosphopeptides were generated after demonstrating the capability of tantalum oxide powder for interacting with phosphopeptides in this study. Additionally, it has been demonstrated that the extraction of target species by using functional magnetic iron oxide NPs under microwave heating is very efficient [27] because iron oxide NPs are good microwave absorbers and its presence in the solution can accelerate

microwave heating, leading to effective extraction. Thus, we expected that Fe_3O_4@Ta_2O_5 NPs play two roles: as trapping agents for phosphopeptides and microwave absorbers for accelerating microwave-assisted extraction in this approach. Using this NP-based microwave-assisted extraction for phosphopeptide enrichment is rapid and quite convenient because multiple samples can be used simultaneously in a microwave oven for a very short period of time (1 min) and magnetic isolation is easily manipulated. Tryptic digests of standard phosphorylated proteins such as α- and β-caseins were first used as the samples for examination of the feasibility of using Fe_3O_4@Ta_2O_5 NPs for phosphopeptide enrichment. To demonstrate the approach in a real-world application, cell lysates and biological fluids such as serum and milk containing abundant phosphorylated peptides/proteins were also used as the model samples for examination.

Experimental

Reagents and materials

Ammonium hydroxide solution (25%) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Tetraethoxysilane (TEOS) was obtained from Fluka (Seelze, Germany). Iron(III) chloride hexahydrate, ammonium bicarbonate, ammonium sulfate, trifluoroacetic acid (TFA), urea, and phosphoric acid (85%) were purchased from Riedel de Haën (Seelze, Germany). α- and β-Caseins (from bovine milk), bradykinin, insulin, dithiothreitol (DTT), iodoacetic acid (IAA), 2,5-dihydroxybenzoic acid (DHB), potassium phosphate monobasic anhydrate, ethylenediaminetetraacetic acid, trypsin (from bovine pancreas, tosylphenylalanyl-chloromethyl-ketone-treated) were purchased from Sigma (St. Louis, MO, USA). Tantalum butoxide was purchased from Aldrich (Milwaukee, WI, USA), while ethanol and acetic acid (99.8%) were obtained from Showa (Tokyo, Japan). Nonfat milk was purchased from a local grocery store, while human serum samples were donated by healthy individuals. Dulbecco's modified Eagle's medium was obtained from Biowest (Miami, FL, USA). Carcinomic alveolar basal epithelial A549 cells were a gift from Prof. Jui-I Chao (NCTU).

Fabrication of tantalum oxide powder and Fe_3O_4@Ta_2O_5 NPs

The tantalum oxide sol was prepared by stirring ethanol (2 mL) with tantalum butoxide (0.5 mL) for 0.5 h, followed by the addition of acetic acid (9.8%, 200 μL) and ethanol (2 mL) under and stirring for another 1 h [28]. Tantalum oxide gel particles were generated by calcining the mixture in an oven at 300 °C for 2 h.

Magnetic iron oxide NPs were generated via coprecipitation. That is, $FeCl_3$ (2 g) and $FeCl_2$ (5.4 g) were dissolved in aqueous hydrochloric acid (2 M, 25 mL) at room temperature under sonication. After the mixture was degassed using a vacuum pump, the flask was filled with nitrogen gas and aqueous ammonia (25%, 40 mL) was slowly injected into the mixture under nitrogen while stirring at room temperature. The generated NPs were rinsed with deionized water three times and resuspended in ethanol (40 mL). The magnetic Fe_3O_4 NP solution (40 mg/mL, 23 mL) obtained above was mixed with ethanol (35 mL) and deionized water (6 mL) under sonication for 10 min. The mixture was added with TEOS (1.5 mL) and sonicated for 10 min followed by slowly adding with aqueous ammonia (10%, 1.4 mL) over 5 min and stirred in a water bath (40 °C) for 12 h. The magnetic Fe_3O_4@SiO_2 NPs in the mixture were rinsed with ethanol, and resuspended in ethanol (40 mL). The concentration of the Fe_3O_4@SiO_2 NPs in the suspension was 14 mg/mL; it was stored at 4 °C in a refrigerator prior to use.

Tantalum oxide sol was prepared by stirring tantalum butoxide (0.5 mL) with ethanol (2.0 mL) for 0.5 h and then adding acetic acid (99.8%, 200 μL) and ethanol (2 mL) and stirring for another 1 h [28]. The Fe_3O_4@SiO_2 (14 mg/mL, 1 mL) suspension obtained above was sonicated with ethanol (15 mL) for 30 min and then the tantalum oxide sol (2 mL) was added. The mixture was stirred in a water bath at 60 °C for 6 h. The generated Fe_3O_4@Ta_2O_5 NPs were rinsed several times with ethanol and deionized water and resuspended in deionized water. The concentration of the Fe_3O_4@Ta_2O_5 NPs in the suspension was 0.36 mg/mL; it was stored at 4 °C in a refrigerator prior to use.

Tryptic digests of proteins

α- (or β-)Caseins (2 mg/mL, 500 μL) were reacted with trypsin (2 mg/mL, 10 μL; i.e., weight ratio=50:1) in aqueous ammonium bicarbonate (50 mM, pH 8) and incubated at 37 °C for 24 h. Prior to extraction, a portion of the digestion product (5 μL) was diluted in 0.15% TFA solution (44 μL). The proteins in the nonfat milk were denatured prior to tryptic digestion by mixing the protein mixture (0.25 mL) with aqueous ammonium bicarbonate solution (0.25 mL, 50 mM) containing urea (8 M) and then incubating at 38 °C for 30 min. DTT solution (50 μL, 90 mM) in aqueous ammonium bicarbonate (50 mM) was added and the mixture was maintained at 55 °C for 1 h. After cooling to room temperature, the sample solution was mixed with IAA

solution (50 μL, 200 mM) prepared in aqueous ammonium bicarbonate (50 mM) and cooled in an ice bath. The sample vial was wrapped with aluminum foil and subjected to vortex mixing for 2.5 h at room temperature. The sample solution (0.6 mL) was incubated with trypsin (1 mg/mL, 10 μL) in aqueous ammonium bicarbonate (50 mM) at 38 °C for 18 h.

Microwave-assisted phosphopeptide enrichment

The tryptic digest sample (or human serum; 5 μL) that had been diluted in 0.15% TFA (45 μL) was mixed with Fe_3O_4@Ta_2O_5 NPs (12 μg). The enrichment was performed under microwave heating (power 900 W) for 1 min by placing the mixture in a domestic microwave oven. The target species–Fe_3O_4@Ta_2O_5 NP conjugates were then isolated by magnetic separation followed by rinse with 0.15% TFA solution (3×160 μL) in acetonitrile/deionized water (1:1, *v*/*v*). Magnetic separation was achieved by placing a magnet (~4,000 G) on the edge of the sample vial and the magnetic NPs were then readily aggregated on the wall of the vial. The rinse step was performed by mixing the NPs with the rinse solvent vigorously each time with a pipette, to wash out any unbound impurities. The target species–NP conjugates were then isolated by magnetic separation. 2,5-DHB solution (30 mg/mL, 0.8 μL) prepared in acetonitrile/deionized water (2:1, *v*/*v*) containing 0.5% phosphoric acid was added to the isolated conjugates. The mixture was deposited on a matrix-assisted laser desorption/ionization (MALDI) sample plate. The sample was ready for MALDI mass spectrometry (MS) analysis after evaporation of the solvent.

Phosphopeptide enrichment from the tryptic digest of cell lysate by Fe_3O_4@Ta_2O_5 NPs

Carcinomic alveolar basal epithelial A549 cells (approximately 2×10^7 cells per milliliter, 0.5 mL) were placed into a tank containing liquid nitrogen for 5 min and then incubated in a water bath maintained at 70 °C for another 5 min. This step was repeatedly operated three times. The cell sample was then centrifuged at 4,000 rpm (4 °C) for 10 min. The supernatant was collected as the cell lysate sample. The cell lysate (100 μL) was acidified by mixing with 0.3% TFA (200 μL). C18-coated magnetic iron oxide nanoparticles (140 μg) were added to the mixture to concentrate proteins under microwave heating (900 W) for 1 min. After rinsing with 0.15% TFA, the NP–target species conjugates were isolated by magnetic separation and reacted with trypsin (0.1 mg/mL, 20 μL) prepared in aqueous ammonium bicarbonate (50 mM, pH 8) under microwave irradiation for 1 min. The supernatant (20 μL) was pooled with the digest products eluted from the NPs by acetonitrile/deionized water (1/1, *v*/*v*) containing 0.1% TFA (20 μL×2), followed by mixing with 0.15% TFA (140 μL). Fe_3O_4@Ta_2O_5 NPs (9 μg) were added to the solution and phosphopeptide enrichment was performed under microwave irradiation (900 W) for 1 min. After rinsing with 0.15% TFA, the NP–target species were magnetically isolated and directly mixed with 2,5-DHB (30 mg/mL, 0.5 μL) to avoid sample loss. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

Database searches

Fragment peaks (S/N>3) resulting from precursor ions (S/N > 3) were submitted via Biotools (v. 3.0) to MASCOT (www.matrixscience.com) using the following search parameters: the database searched was SwissProt 56.2; taxonomy was limited to *Homo sapiens* (human); the enzyme was trypsin; MS and MS/MS tolerances were set at ±0.5 and ±0.8 Da, respectively; the number of missed cleavages was set to three; the significance threshold was set at $p<0.05$ because the score was over 41. Only one MS/MS spectrum resulting from a precursor ion was searched in the protein database search each time.

Instrumentation

Mass spectra were obtained by a Bruker Daltonics Biflex III (Germany) time-of-flight mass spectrometer (TOF MS) equipped with a 337-nm nitrogen laser. The following voltage parameters were employed: IS1, 19.0 kV; IS2, 15.85 kV; ref1, 20.0 kV; lens, 8.8 kV. The laser power was adjusted for obtaining the optimized data with a shot frequency of 20 Hz. A Bruker Daltonics Autoflex III (Germany) TOF/TOF MS equipped with a 355-nm Nd:YAG laser was also employed for obtaining MS spectra. The following voltage parameters were employed: ion source 1, 19.06 kV; ion source 2, 16.61 kV; lens, 8.78 kV; reflector 1, 21.08 kV; reflector 2, 9.73 kV. The laser power was set to 50% with a frequency of 10 Hz, while each mass spectrum was collected from 500 laser shots. When MS/MS analyses were performed, the following voltage settings were used: ion source 1, 6.01 kV; ion source 2, 5.30 kV; lens, 3.01 kV; reflector 1, 27.10 kV; reflector 2, 11.74 kV; lift 1, 19.16 kV; lift 2, 4.42 kV. The laser power was set to 50% with a shot frequency of 10 Hz. Parent ions were obtained by collecting the mass spectra from 500 laser shots (50% power intensity) at a frequency of 10 Hz; daughter ions were obtained by collecting the mass spectra from 1,000 laser shots (65% power intensity) at a frequency of 10 Hz.

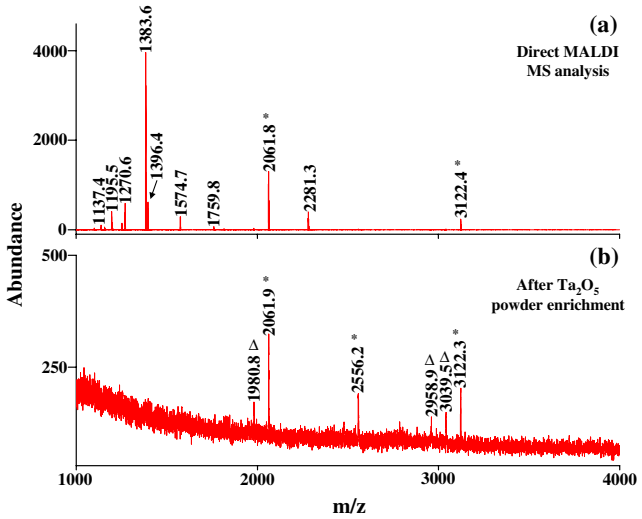

Fig. 1 **a** Direct MALDI mass spectrum of the tryptic digest product of β-casein (4×10^{-7} M) prior to enrichment. **b** MALDI mass spectra of samples obtained when using tantalum oxide powder to selectively enrich target species from the tryptic digest of β-casein (4×10^{-7} M, 50 μL). Phosphopeptide ions are marked with *asterisks*, while the fragments derived from the phosphopeptides by loss of HPO_3^- are marked with *empty triangles*

Results and discussion

We first examined the feasibility of using tantalum oxide powder as the affinity probe for selectively trapping target species from the tryptic digest of β-casein, followed by MALDI MS characterization. Figure 1a displays the direct MALDI mass spectrum of the tryptic digest of β-casein prior to enrichment. The peaks at *m/z* 2,061.8 (FQsEEQQQTEDELQDK/#48-63) and 3,122.4 (RELEELNVPGEIVEsLsssEESITR/#1-25) marked with asterisks are phosphopeptides. The rest of the peaks are nonphosphopeptides. Figure 1b presents the MALDI mass spectrum obtained after using tantalum oxide powders as affinity probes to selectively enrich their target species from the tryptic digest of β-casein. The phosphopeptides are marked with asterisks. When using tantalum oxide powder as the affinity probe to selectively enrich phosphopeptides from the same sample, the peaks at *m/z* 2,061.9 (FQsEEQQQTEDELQDK/#48-63), 2,556.2 (FQsEEQQQTEDELQDKIHPF/#48-67), and 3,122.3 (RELEELNVPGEIVEsLsssEESITR/#1-25) are all protonated pseudomolecular ions of phosphopeptides, those at *m/z* 3,039.5 and 2,958.9 are fragments of the phosphopeptide at *m/z* 3,122.3 (having lost one and two phosphorylated groups, respectively), and the peak at *m/z* 1,980.8 resulted from the loss of a phosphorylated group from the peak at *m/z* 2,061.9 (Fig. 1b). The results indicate that Ta_2O_5 powder has the capability of selectively binding with phosphopeptides from complex samples.

For ease of operation, we generated tantalum-oxide-coated magnetic iron oxide (Fe_3O_4@Ta_2O_5) NPs as affinity probes for phosphopeptides. The NPs were generated by immobilizing a thin film of tantalum on the surface of iron oxide NPs, which had been coated with thin film of silicate. Figure 2a–c presents the transmission electron microscopy (TEM) image of Fe_3O_4 NPs, Fe_3O_4@SiO_2 NPs, and Fe_3O_4@Ta_2O_5, respectively. The particle size of the Fe_3O_4 NPs is ~10 nm, while aggregated NPs are also observed. After coating a thin film of silicate on the surface of the NPs, the particle size of the NPs was further increased. The particle size of the Fe_3O_4@Ta_2O_5 NPs was increased to ~25 nm.

Figure 3a presents the direct MALDI mass spectrum of the tryptic digest of α-casein prior to enrichment. Only three phosphopeptides peaks (marked with asterisks) at *m/z* 1,660.7, 1,927.7, and 1,952.0 appear in this mass spectrum; the rest of the peaks are nonphosphopeptides (see Table 1). Figure 3b presents the MALDI mass spectrum obtained after using the Fe_3O_4@Ta_2O_5 magnetic NPs as affinity probes to selectively trap target species from the tryptic digest of α-casein under microwave heating (power 900 W) for 1 min.

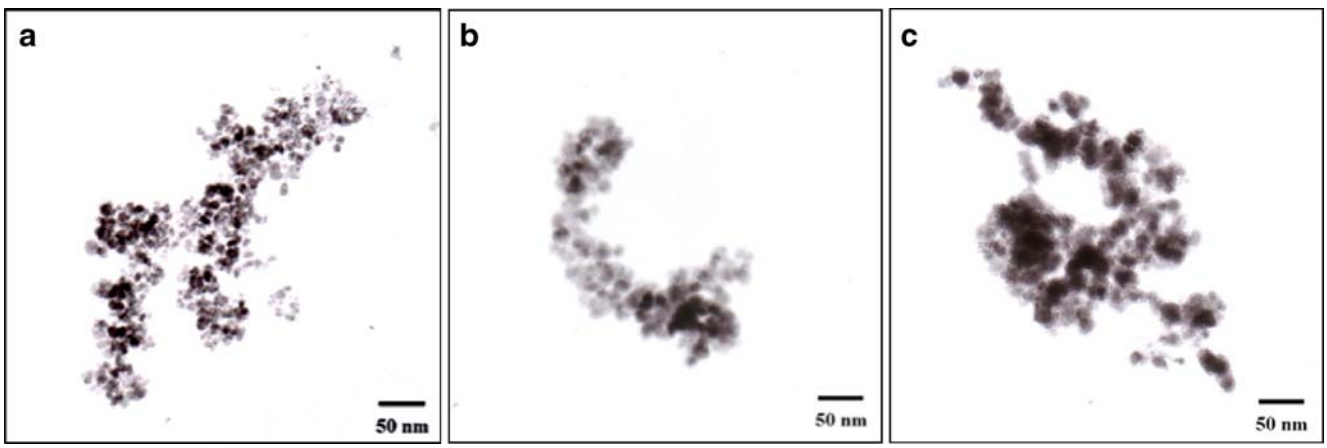

Fig. 2 TEM images of **a** Fe_3O_4 NPs, **b** Fe_3O_4@SiO_2 NPs, and **c** Fe_3O_4@Ta_2O_5 NPs

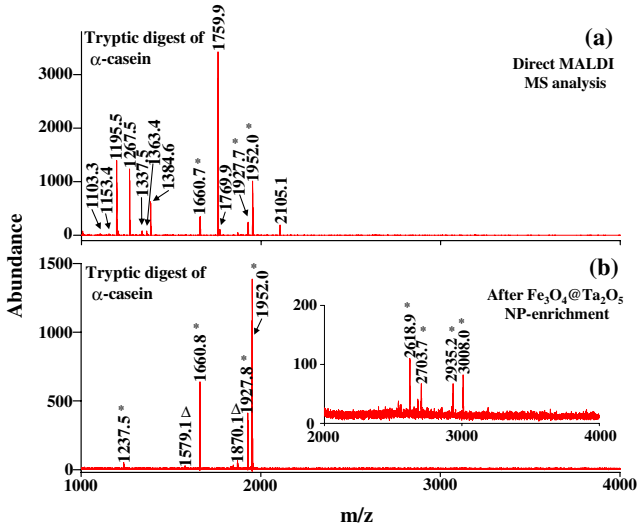

Fig. 3 **a** Direct MALDI mass spectrum of the tryptic digest of α-casein at a concentration of 4.0×10^{-7} M prior to enrichment. **b** MALDI mass spectrum of the samples obtained when using the Fe_3O_4@Ta_2O_5 NPs (12 μg) magnetic NPs to selectively enrich target species from the tryptic digest of α-casein at a concentration of 4.0×10^{-7} M (50 μL). The enrichment was performed with microwave heating for 1 min. The *inset* shows the mass spectrum at the range of *m/z* 2,000 to 4,000. Phosphopeptide ions are marked with *asterisks*, while the fragments derived from the phosphopeptides by loss of HPO_3^- are marked with *empty triangles*

The peaks at *m/z* 1,237.5, 1,660.8, 1,927.8, and 1,952.0 represent phosphopeptides derived from α-casein, while the peaks at *m/z* 1,579.1 and 1,870.1 represent the loss of a HPO_3^- from the ions at *m/z* 1,660.8 and 1,952.0, respectively. All the phosphopeptides appearing in the mass spectrum were monophosphorylated peptides, except for the peak at *m/z* 1,927.8, which represents the ion containing two phosphorylated serine moieties. We suspected that the peaks from low-abundance multiple phosphorylated peptides might have been subjected to the ion suppression effects that are observed generally in MALDI mass spectra since multiple phosphorylated peptides have lower ionization efficiency than that of monophosphorylated peptides. Thus, we used the cutoff mode to suppress all of the ions having values of *m/z* below 2,000, resulting in the mass spectrum presented in the inset to Fig. 3b, in which the phosphopeptides at *m/z* 2,618.9, 2,703.7, 2,953.2, and 3,008.0 started to appear (Table 1). These results indicate that our magnetic Fe_3O_4@Ta_2O_5 NPs had the ability to trap multiple phosphorylated peptides, although their signal intensities were weaker than those of monophosphopeptides. The absence of signals for phosphopeptides having high masses might be due to ion suppression effects. Additionally, we also examined the detection limit of this approach by using a low concentration of phosphopeptide (FQsEEQQQTEDELQDK) as the sample (10^{-9} M,

Table 1 Peptide ion peaks observed in the MALDI mass spectra of the tryptic digest of α-casein after enrichment using Fe_3O_4@Ta_2O_5 NPs

Observed *m/z*	Theoretic *m/z*	Peptide sequence[a]	Direct MALDI MS	Fe_3O_4@Ta_2O_5 Enrichment
1,103.3	1,103.5	YYVPLGTQY (α -S1/180–188)	v	
1,153.4	1,153.6	QHQKAMKPW (α -S2/200–208)	v	
1,195.5	1,195.7	NAVPITPTLNR (α -S2/130–140)	v	
1,237.5	1,237.5	TVDMEsTEVF (α -S2/138–147)		v
1,267.5	1,267.7	YLGYLEQLLR (α -S1/106–115)	v	
1,337.5	1,337.7	HIQKEDVPSER (α -S1/95–105)	v	
1,363.4	1,363.7	EPMIGVNQELAY (α -S1/148–159)	v	
1,384.6	1,384.7	FFVAPFPEVFGK (α -S1/38–49)	v	
1,660.7	1,660.8	YPQLEIVPNsAEER (α -S1/121–134)	v	v
1,759.9	1,759.7	HQGLPQEVLNENLLR (α -S1/23–37)	v	
1,769.9	1,770.0	LYQGPIVLNPWDQVK (α -S2/114–128)	v	
1,927.7	1,927.7	DIGsEsTEDQAMEDIK (α -S1/43–58)	v	v
1,952.0	1,952.0	YKVPQLEIVPNsAEER (α -S1/119–134)	v	v
2,105.1	2,105.0	TDAPSFSDIPNPIGSENSEK (α -S1/189–208)	v	
2,618.9	2,618.9	NTMEHVsssEESIIsQETYK (α -S2/2–21)		v
2,703.7	2,703.5	LRLKKYKVPQLEIVPNsAEERL (α -S1/99–120)		v
2,935.2	2,935.2	EKVNELsKDIGsEsTEDQAMEDIK (α -S1/35–58)		v
3,008.0	3,008.0	NANEEEYSIGsssEEsAEVATEEVK (α -S2/46–70)		v

"s" refers to a phosphorylated serine unit

[a] Searched from http://tw.expasy.org

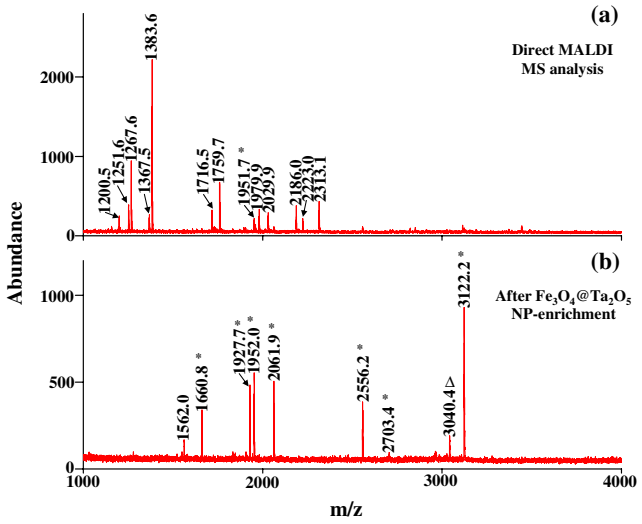

Fig. 4 **a** Direct MALDI mass spectrum of the tryptic digest of nonfat milk that had been diluted 100-fold by 0.15% TFA aqueous solution. **b** MALDI mass spectrum of the sample obtained after using the Fe_3O_4@Ta_2O_5 NPs (12 μg) magnetic NPs to selectively enrich target species from the tryptic digest of the same diluted milk sample (50 μL) as that used for obtaining **a**. The enrichment was performed with microwave heating for 1 min. Phosphopeptide ions are marked with *asterisks*, while the fragments derived from the phosphopeptides by loss of HPO_3^- are marked with *empty triangles*

10 μL); the peak at *m/z* 2,061 is visible in the MALDI mass spectrum after Fe_3O_4@Ta_2O_5 NP (9 μg) enrichment under microwave irradiation (power 900 W) for 1 min (result not shown). That is, the detection limit was estimated to be ~10 fmol.

Milk contains abundant phosphoproteins including α- and β-caseins. The tryptic digest of nonfat milk was used as the sample to examine the effectiveness of this approach. Figure 4a presents the direct MALDI mass spectrum of the tryptic digest of diluted milk. Only one phosphopeptide at *m/z* 1,951.7 with a low intensity is visible in the mass spectrum, while the rest of peaks are nonphosphopeptides. When using the Fe_3O_4@Ta_2O_5 NPs as affinity probes for phosphopeptide enrichment, a number of phosphopeptide-derived ions appear in the mass spectrum. The peak at *m/z* 3,122.2 dominates the MALDI mass spectrum and its doubly charged ion appears at *m/z* 1,562.0. The peaks at *m/z* 1,660.8, 1,927.7, 1,952.0, 2,061.9, 2,556.2, and 2,703.4 present in Fig. 4b are derived from phosphopeptides. The peak at *m/z* 3,040.4 corresponds to the fragment by loss of a HPO_3^- from the phosphopeptide at *m/z* 3,122.2. There are no nonphosphopeptides visible in the mass spectrum. The results indicate that Fe_3O_4@Ta_2O_5 NPs are capable of selectively trapping phosphopeptides from complex samples.

Additionally, we also used the tryptic digest of a cell lysate as the sample because this type of sample is commonly investigated in phosphoproteomics analysis.

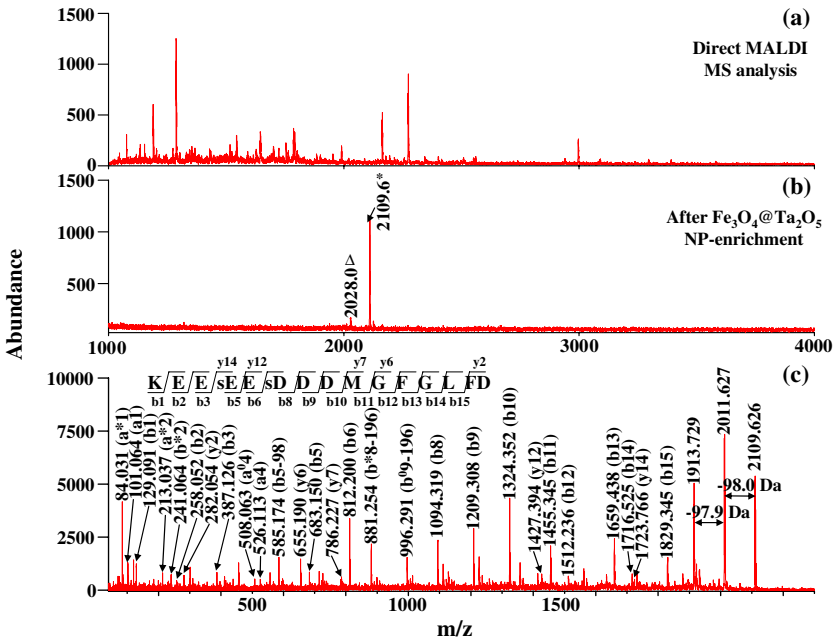

Fig. 5 **a** Direct MALDI mass spectrum of the tryptic digest of a cell lysate prepared in 0.15% TFA aqueous solution. **b** MALDI mass spectrum of the samples obtained when using the Fe_3O_4@Ta_2O_5 NPs (12 μg) to selectively enrich target species from the same sample (50 μL) as that used for obtaining **a**. The enrichment was performed with microwave heating for 1 min. Phosphopeptide ions are marked with *asterisks*, while the fragments derived from the phosphopeptides by loss of HPO_3^- are marked with *empty triangles*. **c** MALDI MS/MS spectrum obtained by selecting the peak at *m/z* 2,109.6 as the precursor ion

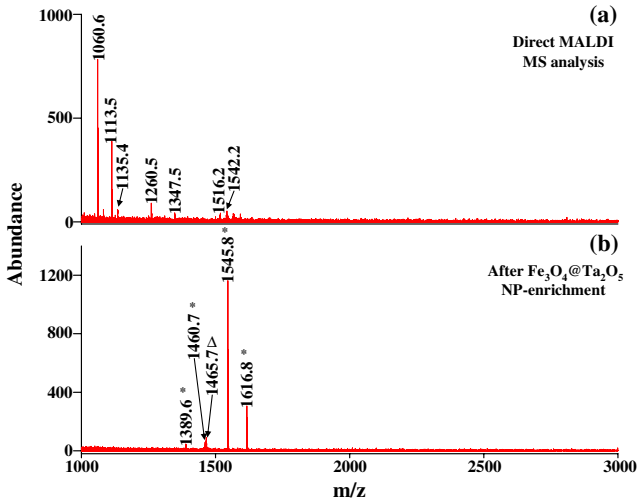

Fig. 6 **a** Direct MALDI mass spectrum of a tenfold diluted human serum sample in 0.15% TFA aqueous solution. **b** MALDI mass spectrum of the sample obtained after using the Fe_3O_4@Ta_2O_5 NPs (12 μg) to selectively enrich target species from the same serum sample (50 μL) as that used for obtaining **a**. The enrichment was performed with microwave heating for 1 min. Phosphopeptide ions are marked with *asterisks*, while the fragments derived from the phosphopeptides by loss of HPO_3^- are marked with *empty triangles*

Figure 5a presents the direct MALDI mass spectrum of the tryptic digest of an A549 cell lysate. Many ions appear in the range of *m/z* 1,000~3,000. However, after enrichment by Fe_3O_4@Ta_2O_5 NPs, only two ions at *m/z* 2,109.6 and 2,028.0 appear in the mass spectrum (Fig. 5b). The peak at *m/z* 2,028.0 (marked an empty triangle) is derived from the peak at *m/z* 2,109.6 by the loss of a HPO_3^-. We confirmed that the ion at *m/z* 2,109.6 is a phosphopeptide (gi|4506669/KEEsEEsDDDMGFGLFD(#98-114)) derived from 60 S acidic ribosomal P1 by the MALDI TOF/TOF MS result (Fig. 5c) and protein database searching with score 70. The results indicate that the affinity probes have a good affinity for phosphopeptides because they can successfully trap only phosphopeptides even in a very complex sample such as a cell lysate. A question may arise: why only two peaks derived from a phosphopeptide appear in the mass spectrum after using this approach to enrich target species from the tryptic digest of the cell lysate? Presumably, the phosphopeptide is abundant in the sample, so its derived ions dominate the mass spectrum prior to any sample treatment. If the abundant phosphopeptide is removed, we expect that traces of phosphopeptides derived from other phosphoproteins would start to appear in the mass spectrum after enrichment. That is, the same sample can be repeatedly enriched by the affinity probes. After removing abundant phosphopeptides by the affinity probes, less abundant phosphopeptides would be readily enriched. However, we herein only aim to illustrate the specificity of the tantalum oxide NPs for phosphopeptides.

Table 3 Comparisons of various types of metal oxide coated magnetic NPs as affinity probes for phosphopeptides combined with MALDI MS analyses in terms of sensitivity and selectivity

Affinity probes	Sensitivity (fmol)	Selectivity
Fe_3O_4@TiO_2 [8]	50	Fair
Fe_3O_4@Al_2O_3 [15]	2.5	Good
Fe_3O_4@ZrO_2 [13]	45	Fair
Fe_3O_4@Zr(IV) [29]	50	Fair
Fe_3O_4@Nb_2O_5 [26]	10	Good
Fe_3O_4@Ta_2O_5	50	Good

Human serum contains abundant phosphorylated proteins/peptides, and it was also used as the sample for examination. Figure 6a presents the direct MALDI mass spectrum of a tenfold diluted human serum sample. There are a number of peaks appearing in the MALDI mass spectrum, but no phosphopeptides are observed. Figure 6b presents the MALDI mass spectrum obtained after using Fe_3O_4@Ta_2O_5 magnetic NPs as affinity probes to selectively trap target species from the same serum sample as that used for obtaining panel Fig. 6a. The peaks at *m/z* 1,389.6, 1,460.7, 1,545.8, and 1,616.8 marked with asterisks are derived from phosphorylated fibrinopeptide A and the peak at *m/z* 1,465.7 is the fragment by loss of a HPO_3^- from the peak at *m/z* 1,545.80. Table 2 lists the corresponding sequences of these peaks. These sequences were confirmed by MALDI TOF/TOF MS results combined with protein database searching. Phosphorylated fibrinopeptide-A-derived molecules are abundant in serum,

Table 2 Ion observed in the MALDI mass spectra after using Fe_3O_4@Ta_2O_5 probes to enrich target species from serum samples

Observed $[M+H]^+$	Theoretical $[M+H]^+$	Sequences	Identity[a]
1,389.6	1,389.5	DsGEGDFLAEGGGV	Fibrinopeptide A (2–15)
1,460.7	1,460.6	ADsGEGDFLAEGGGV	Fibrinopeptide A (1–15)
1,545.8	1,545.6	DsGEGDFLAEGGGVR	Fibrinopeptide A (2–16)
1,616.8	1,616.7	ADsGEGDFLAEGGGVR	Fibrinopeptide A (1–16)

"s" stands for phosphorylated serine

[a] The identity of these sequences was confirmed by MALDI TOF/TOF MS results with protein database searching

so it is not surprising to observe their phosphopeptide-derived ions dominate the mass spectrum after enrichment by the affinity probes. Less abundance of phosphopeptides would be revealed in the mass spectra after these abundant phosphopeptides were removed. Additionally, there are no nonphosphopeptides observed in the mass spectra. The results demonstrated again that Fe_3O_4@Ta_2O_5 NPs have high specificity for phosphopeptides.

Previously, we concluded that the specificity of the magnetic nanoparticles coated with alumina (Fe_3O_4@Al_2O_3) was superior to that of the magnetic nanoparticles coated with titania (Fe_3O_4@TiO_2) [16]. It was because that there was still one peak derived from a nonphosphopeptide resulting from albumin residues appearing in the mass spectrum after using Fe_3O_4@TiO_2 NPs to selectively enrich target species from a serum sample. However, only the peaks derived from phosphorylated fibrinopeptide A appeared in the mass spectrum after using Fe_3O_4@Al_2O_3 NPs to trap target species from the same serum sample. On the basis of the previous and current results, it seems that the Fe_3O_4@Ta_2O_5 and Fe_3O_4@Al_2O_3 have similar trapping specificity for phosphopeptides, while their trapping specificity for phosphopeptides is better than that of Fe_3O_4@TiO_2 NPs. We used the tryptic digest of β-casein as the sample to examine the selectivity and specificity of these affinity probes for phosphopeptides followed by MALDI MS characterization. The selectivity and sensitivity of various types of metal oxide magnetic NPs for phosphopeptides, which were performed previously, are summarized in Table 3. On the basis of the experimental results, the selectivity of Fe_3O_4@Ta_2O_5 magnetic NPs for phosphopeptides is good. The sensitivity of using Fe_3O_4@Ta_2O_5 NPs as affinity probes is slightly worse than that of Fe_3O_4@Al_2O_3 NPs. Nevertheless, the sensitivity of using Fe_3O_4@Ta_2O_5 magnetic NPs as affinity probes for phosphopeptides combined with MALDI MS analysis is better than that of either Fe_3O_4@ZrO_2 or Fe_3O_4@Zr(V).

Conclusions

We have demonstrated that tantalum oxide materials can be used as alternative affinity probes for phosphopeptide with desirable specificity. There are no nonphosphopeptides visible in the MALDI mass spectra after using the Fe_3O_4@Ta_2O_5 NPs to enrich target species from complex samples such as serum and cell lysates. The sensitivity and selectivity of the Fe_3O_4@Ta_2O_5 NPs for phosphopeptides are better than those of Fe_3O_4@TiO_2 and Fe_3O_4@ZrO_2 NPs. This report provides the first example of the use of tantalum oxide materials as affinity probes for phosphopeptides. The generation of the particles is straightforward. Furthermore, the extraction step only takes 1 min when combining the use of the NPs with microwave-assisted extraction. The advantages of this approach include short analysis time, simplicity, and ease of use. These merits make this approach potentially feasible for being used in phosphoproteomics analysis.

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