Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis

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We have developed a new offline chromatographic approach for the selective enrichment of phosphorylated peptides that is directly compatible with subsequent analysis by online nano electrospray ionization tandem mass spectrometry. In this technique, a titanium dioxide (TiO_2)-packed pipette tip is used as a phosphopeptide trap that acts as an offline first-dimension separation step in a two-dimensional chromatography system. This is followed by online nano reversed-phase high-performance liquid chromatography. Here, we present suitable methods for enrichment, optimized separately for each step: sample loading, washing and elution from the TiO_2-filled tips. To increase the trapping selectivity of the TiO_2 column, we used the sodium salt of 1-octanesulfonic acid combined with 2,5-dihydroxybenzoic acid as ion-pairing agents and displacers for acidic peptides. These agents also improve the binding of phosphorylated peptides and block the binding of non-phosphorylated ones. This enrichment procedure takes 30 min, followed by a 100-min HPLC program, including washing and an elution gradient.

INTRODUCTION

The majority of biochemical processes are induced and controlled by post-translational modifications on certain proteins. One of the most frequent modifications is phosphorylation of the amino acids—serine, threonine and tyrosine. It is this reversible phosphorylation that regulates the major (or even the majority of) cellular processes, and it has been estimated that almost 30% of all proteins in mammalian cells are phosphorylated at some point during their expression[1,2]. This alone would be reason enough to invest time and resources in the analysis of phosphopeptides.

Despite the fact that there are so many phosphoproteins in the cell, phosphorylated residues remain at very low concentrations physiologically and are present in substoichiometric quantities. The presence of high-abundance peptides in samples of biological origin makes it necessary to develop an efficient separation and enrichment method for phosphopeptides.

Earlier established methods for phosphorylation site detection relied on Edman degradation of ^{32}P-labeled peptides. Edman degradation is a robust and well-established method, but it shows some limitations when analyzing complex samples and cannot fulfill the requirements for sensitive detection of low concentrations of phosphopeptides[1–3]. Besides the additional needed labeling of the phosphate residues, it would take more than a hundred fold in time to chemically treat and analyze a complex sample related to other techniques for sequencing and phosphorylation site mapping.

Identifying phosphorylation sites in different proteins through mass spectrometry (MS) requires a proper pre-separation and enrichment of the phosphopeptides performed online by the HPLC, because of the often highly complex sample composition.

Some phosphopeptide-selective techniques have been developed, such as immobilized metal affinity chromatography (IMAC)[4–6] or two-dimensional HPLC systems using strong cation-exchange chromatography (SCX) and reversed-phase (RP) chromatography[7–10].

IMAC separation uses the affinity of the negatively charged phosphate group in phosphopeptides for positively charged Fe^{3+} or Ga^{3+} ions, which are bound on the stationary phase of the separation column. However, not only phosphopeptides but also acidic groups in peptides will form complexes. The result is nonselective binding of many acidic peptides due to a higher complex building constant with metal ions and displacement of phosphorylated peptides from the IMAC column, resulting in the loss of phosphorylated peptides. *O*-methyl esterification will block acidic groups (carboxylic groups of the peptide) and represents a possible solution for this problem. Ficcaro *et al.*[5] described the methylation of phosphopeptides and the resulting decrease in nonspecific binding of acidic peptides to the IMAC column. Esterification neutralizes the majority of carboxylic groups in a peptide backbone, but can also lead to other reactions such as deamidation and methylation of Asn and Gln residues.

One of the methods for enriching phosphopeptides and reducing the complexity of the biological sample is to perform ion-exchange chromatography before HPLC nano electrospray ionization tandem mass spectrometry analysis. SCX proved useful for separating major phosphopeptides within a complex sample[7–10]. During SCX separation, the phosphorylated peptides elute in early fractions at the mobile phase (pH 2.7) owing to the lack of positive charge or a very low positive charge (+1). Most of the remaining tryptic peptides would have a charge of +2 or +3, and would therefore bind more strongly to the column. However, despite the enrichment achieved, a significant number of non-phosphorylated peptides will also elute in early fractions and present a possible obstacle in later phosphopeptide analysis. Another possibility is to use anion-exchange chromatography and to separate the phosphorylated peptides according to the (negative) charge they bear. Depending on the pH value of the mobile phase, sample

TABLE 1 | Amino-acid sequence of synthetic phosphopeptides used throughout the experiment.

Name	M_r	Sequence	[M-H]+	$[M-2H]^{++}$	$[M-3H]^{+++}$
PP1	2,093.8613	TASDTDSSYpAIPTAGMSPSR	2094.86	1047.93	698.95
.PP1ox.	*2,109.8562*		*2110.86*	*1055.93*	*704.29*
PP2	1,758.7938	SVENLPEAGITpHEQR	1759.79	880.4	587.26
PP3	1,137.5292	NSpVEQGRRL	1138.53	569.76	380.18
PP4	1,343.5871	APPDNLPSPGGSpR	1344.59	672.79	448.86
PP5	1,302.5493	LIEDNEYpTAR	1303.55	652.27	435.18
NP6	1,263.6208	APPDNLPSPGGSR	1264.62	632.81	422.21
PP8	1,931.8296	ENIMRSpENSESQLTSK	1932.83	966.91	644.94
.PP8ox.	*1,947.8245*		*1948.82*	*974.91*	*650.27*
PP9	2,229.9807	QLGEPEKSpQDSSPVLSpELK	2230.98	1115.99	744.33
PP10	2,309.9471	QLGEPEKSpQDSpSPVLSpELK	2310.95	1155.97	770.98
PP11	2,329.1736	KFLSpLASNPELLNLPSSpVIK	2330.17	1165.59	777.39
PP13	2,026.0176	THILLFLPKSpVSDYEGK	2027.02	1014.01	676.34
GluFib	1,569.6696	EGVNDNEEGFFSAR	1570.67	785.83	524.22

Synthetic phosphopeptides were used to set up the method and test the quality of separation system. All peptides were synthesized in-house (**Box 1**).

complexity and the number of phosphorylated sites, the next step is the specific enrichment of phosphopeptides. Depending on the next separation step, samples containing phosphopeptides must be either desalted or otherwise prepared for the following analysis steps. Failure to perform proper sample treatment can lead to a loss of analytes and false results.

Pinkse *et al.*[12] described the use of TiO_2 columns for online coupling with the RP separation column. Interaction and binding of non-phosphorylated peptides was observed and a few acidic peptides could also be enriched by this approach.

The previously published method by Larsen *et al.*[11] uses 2,5-dihydroxybenzoic acid (DHB) to improve trapping of phosphopeptides by using it at high concentrations ($\geq$300 mg ml^{-1}). However, in our hands, DHB alone caused high background in MS and ionization suppression at these concentrations, and at lower concentrations it was not able to enhance the trapping of phosphopeptides. Hence, we introduced the sodium salt of 1-octanesulfonic acid (OSA) as an alternative to DHB. OSA proved to be a very useful ion-pairing agent for peptide and protein analysis in RP-chromatography[16,17]. Moreover, the addition of OSA increases the hydrophobic character of the peptides and enables better trapping on the RP trap column in the following chromatographic separation step.

In an offline approach, Larsen *et al.*[11] enriched phosphopeptides by using self-packed TiO_2 tips, but we decided to use commercially available pre-packed "TopTips" from Glygen, which are pipette tips filled with bare TiO_2, spherically formed with a diameter of 5 μm, because of their easy availability and reproducible packed bed. Additionally, packing columns, even tips, require some experience and very often this step is responsible for poor, inconsistent or irreproducible results.

In comparison to the methods described earlier, no chemical treatment of the sample is necessary and no additional separation or desalting steps prior to HPLC are needed. This lowers the possible sample loss due to chemical reactions, adsorption to the surface of the stationary phase or poor sample handling. In fact, the sample processing described in this paper is very rapid, uncomplicated and straightforward.

However, a slight unselectivity of the TiO_2 trap column still remains, even though it is significantly lowered by the use of additives such as DHB[11] or, in our case, OSA. When used at high concentrations, DHB produces a higher background in UV chromatograms and MS spectra (data not shown) and needs a high organic wash step ($\sim$80% acetonitrile) to be removed from the TiO_2 column.

On reviewing the results obtained, a decision was made to combine both agents for loading the sample. This led to improved recovery of phosphopeptides and to the almost complete removal of non-phosphorylated peptides during the loading step.

The elution of trapped phosphopeptides was performed using an ammonium bicarbonate (ABC) elution buffer to which 50 mM ammonium phosphate was added.

To mimic a complex sample and introduce acidic peptides onto the TiO_2 column, BSA-derived tryptic peptides were added to a test mix of synthetic peptides. Trypsinized BSA contains several acidic peptides that show a high affinity for TiO_2 and thus compete with phosphopeptides for binding to the column. The test mix consists of several phosphopeptides (see **Table 1**) and also contains two unphosphorylated peptides (NP6 and Glufib) for negative control. Two different methods were used for analysis of test samples and the real biological sample. For test samples containing synthetic phosphorylated peptides and BSA tryptic peptides, short chromatographic gradients were used. For the complex biological sample, a longer chromatographic wash step upon sample loading onto the trap column was programmed before sample separation. We wanted to circumvent possible problems with phosphate buffer used for sample elution and properly desalt the sample before HPLC separation and MS detection. Additionally, two different nano HPLC systems were used in this study: an UltiMate Plus nano HPLC system coupled to a Deca XP mass spectrometer was used to perform the method development and tests, and an UltiMate 3000 nano HPLC system coupled to an LTQ Fourier transform mass spectrometer was used to perform analysis of biological samples. To achieve the best possible MS results, and enhance the fragmentation, a multistage activation (MSA) procedure was used. The typical fragmentation pattern of phosphopeptides is neutral loss (NL) due to the dissociation of phosphoric acid, and only minor fragmentation along the peptide backbone will occur. As the NL of *m/z* 49 (doubly charged ion) and *m/z* 32 (triply charged

ion) is not sufficient for successful identification of phosphorylated peptides, further fragmentation of the peptide backbone is induced. This happens through further fragmentation of the expected NL masses after the initial CAD event on the precursor ion. This technique is named MSA and is described by Schroeder and Zumwalt[20,21]. Using the MSA method on the Fourier transform ion cyclotron resonance mass spectrometer enables both the measurement of precursor masses with accuracy in the low ppm range and the production of phosphopeptide-derived MS^2 spectra containing fragments not normally produced until the MS^3 stage by conventional methods.

The anaphase-promoting complex (APC), purified from HeLa cells, is used to demonstrate the utility of this method for analyzing a complex biological sample. However, this method can be used for trapping and separation of phosphorylated peptides of any origin.

MATERIALS

REAGENTS

- Acetic acid (AA) 100% Suprapur, Merck
- Acetonitrile (ACN) (HPLC grade), Merck
- Ammonia 25% p.A., Merck
- ABC Ultra, Fluka
- Formic acid (FA) 98–100% Suprapur, Merck
- OSA, Sigma, p.A., Aldrich
- DHB Part-No. # 201346, Bruker, Bremen, Germany
- *o*-Phosphoric acid 85% puriss, Riedel-de-Haën
- Trifluoroacetic acid (TFA), Pierce
- Trypsin Gold was purchased from Promega
- DL-Dithiothreitol (DTT) and iodoacetic acid (IAA) were purchased from Sigma
- Cdc27 peptide antibodies crosslinked to Affi-Prep protein A beads (Bio-Rad, Hercules)
- Phosphorylated amino acids used for the synthesis of phosphopeptides were purchased from Novabiochem
- Non-phosphorylated amino acids for the synthesis of phosphopeptides were purchased from MultiSynTech GmbH
- Bovine serum albumin (minimum 99%) was purchased from Sigma
- Synthetic phosphorylated and non-phosphorylated peptides (**Table 1**, **Box 1**) IP buffer: 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.01% Tween, 20 mM β-glycerophosphate, 5% glycerol, 5 mM EDTA, 1 mM NaF, 0.5 mM DTT, 0.2 mM Na_3VO_4, 1 μm okadaic acid
- IP buffer without detergent is the same as above but lacks 0.01% Tween330 nM nocodazole (Sigma-Aldrich) in DMSO (Fluka)
- PBS (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4)
- TBS-T 0.01 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Tween)
- TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl)
- 100 mM glycine-HCl, pH 2.2
- 1.5 M Tris-HCl, pH 9.2
- Load solution: AA 20%; octanesulfonic acid sodium salt 300 mM, DHB 20 mg ml^{-1}
- Wash solution: 70% water; 30% ACN

EQUIPMENT

- 145-mm tissue culture dishes (Greiner bio-one, Frickenhausen)
- Biofuge (Heraeus)
- Potter-Elvejhem glass-Teflon homogenizer (Wheaton Scientific Products)
- Beckman Optima MAX Ultracentrifuge using a Beckman TLA 45 rotor (Beckman Coulter)
- TiO_2-filled tips: TopTips, produced by Glygen, purchased from SunChrom (purchase order number 668-TT2TIO.96)

REAGENT SETUP

Extraction buffer 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 1 μM okadaic acid, 1 mM DTT, 0.2% NP-40, 10% glycerol, 10 μg ml^{-1} each of chymostatin, leupeptin and pepstatin A (Sigma-Aldrich)

Elution buffer for TiO_2 TopTips ABC buffer (pH 10.5; 125 mM) with ammonium phosphate 50 mM. Dissolve 0.5 g ABC in ~35 ml water and add ammonia solution to reach a basic pH. Then, add 140 μl phosphoric acid and adjust pH with ammonia solution and water to

BOX 1 | PREPARATION OF SYNTHETIC PEPTIDES.

All synthetic phosphorylated and non-phosphorylated peptides were synthesized in-house using standard Fmoc solid-phase chemistry on an ABI 433A (Applied Biosystems) peptide synthesizer and purified on a BioCAD (Applied Biosystems) preparative C18 RP-HPLC system. The quality of the synthesis was tested by MALDI mass spectroscopy analysis. The steps of this procedure are listed below.

Inject protected amino acids (MultiSynTech GmbH) in reverse order at the concentration of 1 mM each, as synthesis takes place from the C-terminal to the N-terminal end. The first amino acid is already bound to the TCP-resin (Pepchem, Goldammer & Clausen).

250 mg resin of substitution grade (0.5 mmol g^{-1}) is used for the entire synthesis. The peptide synthesis is monitored by conductivity measurement. All coupling steps are performed automatically by using the programmed routine "FastMoc 0.10 ΩMonPrevPk" supplied with the instrument.

Release and deprotect the completed peptide by treating the resin with 11 ml of 90% TFA (v/v), 5% triethylsilane (v/v), 5% H_2O for 2–3 h. Isolate the peptide by rinsing and filtering the resin (MultySynTech GmbH) with TFA.

Precipitate the peptide by adding it dropwise to a 35 ml solution of cold 60% *t*-butyl-methylether (v/v)and 40% heptane (v/v). Store for 60 min at −80 °C.

Collect the precipitate by centrifugation and wash three times with 40 ml *t*-butyl-methylether. Lyophilize the peptide under vacuum.

Dissolve the peptide in 5 ml of 0.1% (v/v) TFA in water. Inject into an HPLC machine (Vision, Applied Biosystems) and purify by reverse-phase chromatography on a C18 column (Phenomenex, Luna 5C18 or Phenomenex Jupiter 5C18) using a 45 min, 2–45% gradient of water + 0.1% TFA to acetonitrile + 0.1% TFA. The positive fractions are identified by mass spectroscopy in linear and reflector mode (Bruker Reflex III MALDITOF).

Freeze the peptide at −80 °C and lyophilize for 2 days.

During the synthesis of phosphopeptides, some oxidations at Met can occur and these oxidized peptides sometimes display a higher abundance than the non-oxidized starting peptides. The introduction of oxidized peptides was not anticipated during the synthesis; however, it occurs to some extent for two peptides described, PP1 and PP8. These peptides show a different chromatographic behavior and are clearly separated from their non-oxidized counterparts.

TABLE 2 | Settings used for operating the FT-ICR mass spectrometer.

1 Survey scan: in ICR cell (resolution 100,000, AGC target = 5×10^5, 1 μScan)

2 MS^2 scans (five repeats): in linear ion trap (AGC target 10,000), isolation width of precursor 4 Da, excitation: 35 normalized collision energy for 30 ms, with multistage activation on neutral loss peaks of precursor mass −32.659 and −48.98840 Da

3 Single-charged ions were excluded for MS^2 experiments

4 Masses used for MS^2 experiments were excluded (in a range of 5 ppm) for further fragmentation for 60 s

5 Ionizing spray voltage: 1.5 kV

The settings for the mass spectrometer were evaluated through a number of experiments (data not shown) to enable proper detection and fragmentation of the phosphorylated peptides.

a final volume of 50 ml and a pH 10.5. The buffer should not be older than 2 days.

Trypsin digestion buffer Dissolve 100 μg trypsin ("Gold" from Promega) in 1 ml (50 mM) AA to a final concentration of 100 ng μl^{-1}. ▲ CRITICAL Trypsin aliquots should be stored at −80 °C.

Chymotrypsin digestion buffer Dissolve 25 μg chymotrypsin (sequencing grade from Roche) in 250 μl of 1 mM HCl to a final concentration of 100 ng μl^{-1}. ▲ CRITICAL Chymotrypsin aliquots should be stored at −80 °C.

Subtilisin digestion buffer Prepare a solution of 1 mg ml^{-1} subtilisin (Fluka) in 1 mM HCl and dilute it with 6 M urea and 1 M Tris by a factor of 1:50 to a final concentration of 20 ng ml^{-1}.

DTT Dissolve 1 mg of DTT in 2 ml of 50 mM ABC buffer (pH 8.3). ▲ CRITICAL DTT must be prepared fresh before every digestion.

IAA Dissolve 5 mg IAA in 2 ml of 50 mM ABC buffer (pH 8.3). ▲ CRITICAL IAA must be prepared fresh before every digestion.

HeLa culture medium High glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories GmbH), 0.3 μg ml^{-1} L-glutamine (Sigma-Aldrich), 100 U ml^{-1} penicillin (Sigma-Aldrich) and 100 μg ml^{-1} streptomycin (Sigma-Aldrich)

EQUIPMENT SETUP

HPLC system Chromatographic system: UltiMate Plus Nano-LC system (used for method development).

UltiMate 3000 Dual-gradient Nano-LC system (used for biological sample measurement). Both systems are from LC Packings—A Dionex Co.

Injection system: FAMOS μ-autosampler equipped with a 250 μl sample loop, a user-defined injection method was used, LC Packings—A Dionex Co.

HPLC detection system: UltiMate Plus UV Detector, 3 nl flow cell, 214-nm detection wavelength.

MS: Thermo Finnigan LCQ DECA XP mass spectrometer (used for method development). Thermo Finnigan LTQ-Fourier Transform Ion Cyclotron Resonance mass spectrometer (used for measurement of biological sample).

Separation column: PepMap C18, 75 μm ID × 150 mm length, 3 μm particle size, 100 Å pore size, LC Packings—A Dionex Co.

Trap (guard) columns: PepMap C18, 300 μm ID × 5 mm length, 3 μm particle size, 100 Å pore size, LC Packings—A Dionex Co.

Mobile phase A for RP column: 95% water; 5% ACN; 0.1% FA.

Mobile phase B for RP column: 20% water; 80% ACN; 0.1% FA.

Loading solvent for HPLC: water; 0.1% TFA

RP-column flow rate: 0.300 μl min^{-1}.

RP gradient for method testing This is shown in **Table 3**. The sample is loaded for 45 min onto an RP trap column (which is not online with the separation column) at a flow rate of 20 μl min^{-1} and washed free of ion-pairing agents, basic elution agent and other impurities. The gradient described starts at 45 min (the trap column is switched online with the separation column) and continues for 30 min. After applying a high organic wash step (95% mobile phase B), the trap column is switched back to offline mode and equilibrated with the loading mobile phase[15]. The MS data are recorded only for the time when both columns are online.

RP gradient for separation of the complex biological sample This is shown in **Table 4**. The sample is loaded for 40 min onto an RP trap column (which is not online with the separation column) at a flow rate of 20 μl min^{-1} and is washed free of ion-pairing agents, basic elution agent, IAA and DTT used in the digestion step and other impurities. Owing to a higher complexity of the sample and the presence of many highly abundant peptides, which often mask the low-abundant peptides, the gradient is significantly different in comparison to the gradient used for method tests and separation of less complex samples.

The gradient described starts at 38 min (the trap column is switched online with the separation column) and continues for 120 min with a very shallow increase to 25% B. The majority of peptides will elute on an RP column (under given conditions) at 20–35% B (16.5–31.5% ACN). By slowly increasing the amount of mobile phase B in the gradient, a better separation of co-eluting peptides is possible. Finally, this approach results in a more sensitive detection in MS. After applying a high organic wash step (95% mobile phase B), the trap column is switched back to offline mode and equilibrated with the loading mobile phase[15]. The MS data are recorded only for the time when both columns are online.

The method used for analysis of the simple test mixture was intentionally kept shorter for reasons of time saving and faster separation. However, the analysis of complex biological samples requires shallower gradients and longer wash times after the sample loading.

All chromatographic data have been acquired using Chromeleon HPLC software from Dionex.

MS equipment and settings for analyzing standard samples and method test

Thermo Finnigan—LCQ DECA XP operated in positive nano-ESI mode.

Ionizing spray voltage: 1.5 kV

Enhanced MS full-scan range: 220–2,000 amu

MS equipment and settings for analyzing the biological sample:

Thermo Finnigan—LTQ Fourier Transform mass spectrometer operated in positive nano-ESI mode using the settings described in **Table 2**.

Full scan was conducted in the ICR cell, yielding a survey scan with a resolution of 100,000 and a typical mass accuracy <2 ppm (rms); detection of the CAD fragment spectra (MS^2) was carried out in the linear ion trap. Using this method, the advantages of a highly accurate precursor mass and the high scan speed and low duty cycle time, respectively, for MS^2 spectra were combined.

Mascot search parameters

Type of search	MS/MS Ion Search
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M)
Phospho_no_P-NL (STY)	
Mass values	Monoisotopic
Protein mass	Unrestricted
Peptide mass tolerance	± 5 ppm
Fragment mass tolerance	± 0.6 Da
Max. missed cleavages	3

PROCEDURE

Preparation of HeLa cell lysates

1| Grow adherent HeLa cells in 145-mm tissue culture dishes at 37 °C in the presence of 5% CO_2 in HeLa culture medium.

TABLE 3 | Gradient used for method tests and separation of less complex sample of BSA and synthetic phosphopeptides.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0–45	100	0
45–74	100–50	0–50
74–75	50–5	50–95
75–80	5	95
80–83	5–100	95–0
83–100	100	0

TABLE 4 | Gradient used for separation of a complex biological sample.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0–50	100	0
50–178	100–75	0–25
178–238	75–50	25–50
239–267	5	95
268	100	0

2| To arrest logarithmically proliferating cells in mitosis, plate at 6×10^6 for 4–6 h for attachment. Then, treat them at 60–80% confluency for 16 h with 330 nM nocodazole.

3| Harvest the cells by scraping with a rubber policeman and by subsequent centrifugation at ~ 260 g for 5 min at 4 °C in a Biofuge. The cells need no treatment before removal. Wash the cell pellets twice with ice-cold PBS.

4| Resuspend the pellet obtained from 20 dishes (approximately 1 ml containing 2×10^8 cells) (there is no minimum cell number for making extracts, but this is the amount required to perform this kind of MS analysis) in one volume (~1 ml) of ice-cold extraction buffer and homogenize in a pre-chilled Potter–Elvejhem glass-Teflon homogenizer.

5| To remove the bulk of cell debris and chromatin, centrifuge the lysates at 100,000*g* and 4 °C for 20 min in a Beckman Optima MAX ultracentrifuge using a Beckman TLA 45 rotor (42,000 rpm).

Protein purification

6| Immunopurify the APC from extracts of nocodazole-arrested HeLa cells (approximately 25 mg ml^{-1}) using Cdc27 peptide antibodies crosslinked to Affi-Prep protein A beads as described previously[13,14,22].

7| For MS analysis, wash the APC immunoprecipitates five times with 20 volumes of TBS-T0.01 buffer on a rotary shaker for 5 min at 4 °C and subsequently three times with 20 volumes of TBS.

8| Elute the APC-bound antibody beads by resuspending in 1.5 bead volumes of 100 mM glycine-HCl, pH 2.2.

9| Rebuffer the sample by adding 1.5 M Tris-HCl, pH 9.2, to suspension until pH is approximately 8. Measurement with pH paper is sufficient. The expected concentration of immunoprecipitated APC is approximately 40 ng μl^{-1}, which can be estimated by comparison of a BSA dilution series to the APC dilution series on silver-stained SDS–PAGE.

Sample digestion

10| Reduce 100 µl of sample (in our case, immunopurified APC isolated from about 10 mg of total HeLa protein) by incubation with 1 µg of DTT for 1 h at 37 °C.

11| After reduction, alkylate the sample by incubation with 5 µg of iodoacetamide for 30 min at room temperature (25 °C) in the dark.

12| Digest the proteins in solution with one of the following proteases: 200 ng (2 µl of the prepared digestion buffer) of trypsin at 37 °C overnight, 200 ng (2 µl of the prepared digestion buffer) of chymotrypsin (Fluka) for 5 h at 25 °C or 800 ng (40 µl of the prepared digestion buffer) of subtilisin for 1 h at 37 °C.

13| Stop every digest with 1 µl of concentrated TFA.

14| Lyophilize the digested sample over a few hours almost to dryness. A volume of approximately 10 µl should remain. If it accidentally becomes dry, treat it as usual but be aware of sample loss.

Phosphopeptide enrichment on titanium oxide trap column

15| Prepare the load and wash solutions. See details in the REAGENTS section.

16| Dissolve the sample (in our case of method development: 500 fmol BSA tryptic digest and 50 fmol of synthetic phosphopeptides, or any other complex sample) in loading solvent to a total volume of 50 μl. Wash the TopTip by pipetting 20 μl of wash solution onto the material and applying pressure using a pipette or a syringe. Discard the flow-through. Repeat this step twice.

17| Equilibrate the material by applying 3 × 20 μl of loading solvent. Discard the flow-through.

18| Load the column by applying 2 × 25 μl of the sample. After each load, wait for 2 min to allow the sample to bind to TiO_2. Collect the whole flow-through in a 0.2 ml vial (labeled "Flow-through") for further analysis.
▲ **CRITICAL STEP** Use very gentle pressure to load the sample (= 1 drop per 30 s).

19| Wash the column by applying 2 × 25 μl of the wash solution. Collect the whole flow-through in a 0.2 ml vial (labeled "Wash") for further analysis. Keep the column wet throughout the complete procedure.

20| Elute the sample from the column by applying 3 × 13.3 μl of elution buffer onto the column. Wait for at least 5 min after each elution step to allow proper absorption of solvents.
▲ **CRITICAL STEP** Use very gentle pressure to elute the sample = 1 drop per 30 s.

21| Collect the whole eluate in a 0.2 ml vial (labeled "Eluate") for further analysis.

22| Cool the eluate on ice or in a fridge for acidification afterwards. Do not freeze it and do not wait for more than 1 h to acidify the sample.

23| While cooling the sample, apply 3 × 20 μl of wash solution to the TopTip for proper storage. The tips should be stored in a sealed bag and kept free of dust.

24| Acidify the eluate by adding 10 μl of concentrated TFA to neutralize the ABC buffer and enable direct injection onto the nano HPLC system for further analysis (HPLC-MS/MS).
! CAUTION Pay attention when handling concentrated TFA. Heat and gas development can occur.
■ **PAUSE POINT** The former sample is now separated into three 50 μl fractions, "Flow-through", "Wash" and "Eluate", ready to perform further experiments or to be stored at −80 °C.

HPLC-MS/MS analysis of the prepared sample

25| Inject the acidified sample onto the RP trap column and wash with 0.1% TFA for 50 min. This will remove the phosphate buffer originating from elution and other impurities. The sample itself will bind to the RP trap column[15]. The sample is injected by using the "user-defined injection protocol—UDP": (i) Aspirate 16 μl of transport liquid into the injection needle (needle volume = 15 μl). The injection valve of the autosampler is in the "Inject" position. (ii) The injection valve switches to the "Load" position and 50 μl of sample is aspirated. (c) Aspirate an additional 16 μl of transport liquid to transport the complete sample from the needle into the sample loop. (d) The injection valve switches to "Inject" position and the sample is transported to the RP trap column.

26| Elute the sample from the trap column onto the separation nano column and detect with a mass spectrometer. Use the gradient described in **Table 4** for complex samples. See the EQUIPMENT section for MS settings.

● **TIMING**

Preparation of HeLa cell lysates: depending on the amount to be harvested, this step needs up to 2 days

Protein purification:

Immunopurification, 90 min
Wash, 60 min
Elution, 30 min

Sample digestion:

Step 10, 60 min
Step 11, 30 min
Step 12, Between 1 and 5 h (depends on protease used for digestion)

Phosphopeptide enrichment on titanium oxide trap column:

Steps 14–17, 20 min
Steps 18–21, 30 min
Steps 22–24, 10 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Tables 5** and **6**.

TABLE 5 | Troubleshooting table.

Problem	Possible reason	Solution
Air bubbles running through the titanium oxide trap column bed	Solvent was not applied directly onto the bed	Experiments showed that one need not worry about air bubbles running through the bed, but they can be avoided: do not elute all of the prior-applied solvent but keep a small volume of supernatant before applying the next solvent volume. This will keep the column free of air bubbles
Titanium oxide trap column bed is interspersed with air bubbles	Most of the times this problem occurs if the samples or solvents react, accompanied by gas formation, for example, use of bicarbonates with an acidic reagent	Avoid the use of such solvents, or prevent them coming into contact by washing the column with neutral solvents between the two reagents
Contamination owing to electrostatic attraction	Owing to electrostatic attraction, the column and vials are often littered with dust and other small particles	Work in a hood, or if not available, protect the vial opening with Parafilm and puncture it with the column. This is also helpful for fixing the column inside the vial

TABLE 6 | Recovery of synthetic phosphorylated peptides obtained by using different ion-pairing agents for peptide trapping.

	20% AcOH, 300 mM OSA (%)	80% ACN, 0.1% TFA, 20 mg ml^{-1} DHB (%)	20% AcOH, 300 mM OSA, 20 mg ml^{-1} DHB (%)
PP7	100	100	72
PP3	57	100	87
PP8_ox	97	74	100
PP5	78	88	56
PP1	100	100	100
PP4	100	100	80
PP2	87	100	75
PP9	74	58	62
PP10	42	33	47
NP6	4	100	0
GluFib	30	94	9

ACN, acetonitrile; DHB, 2,5-dihydroxybenzoic acid; OSA, 1-octanesulfonic acid; TFA, trifluoroacetic acid. By combining OSA and DHB, we were able to reduce the loss of phosphopeptides during the loading process.

ANTICIPATED RESULTS

To evaluate the utility of our TiO_2-chemo-affinity chromatography technique for phosphopeptide enrichment and to optimize procedural conditions, the method was performed on peptide and phosphopeptide samples of different complexities and origins, and the resulting product peptides were analyzed by LC-MS/MS.

First, to test the selectivity of the TiO_2 column for phosphopeptides, a mix of synthetic phosphorylated and non-phosphorylated peptides was subjected to the method. Non-phosphorylated peptides failed to bind the TiO_2 column, being detected in the flow-through or wash fractions (**Fig. 1**), whereas all the phosphopeptides bound the column and were

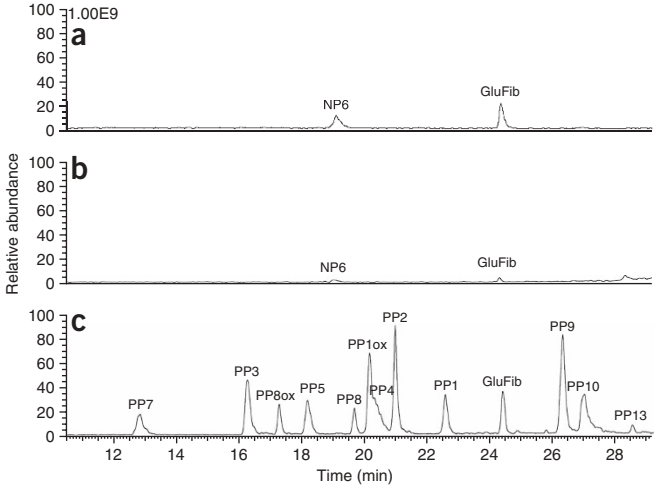


Figure 1 | Purification of phosphopeptides by TiO_2 chemo-affinity chromatography. Shown are typical reversed-phase high-performance liquid chromatography base-peak chromatograms after treating the above-described standard peptide mix (500 fmol injected) with TiO_2-packed pipette tips are shown. (**a**) Flow-through, showing the two non-phosphorylated peptides that were not retained; (**b**) wash fraction, showing no phosphopeptides eluting during the wash procedure; (**c**) phosphopeptides eluted from the TiO_2-packed tip column using 125 mM ABC and 50 mM ammonium phosphate at pH 10.5.

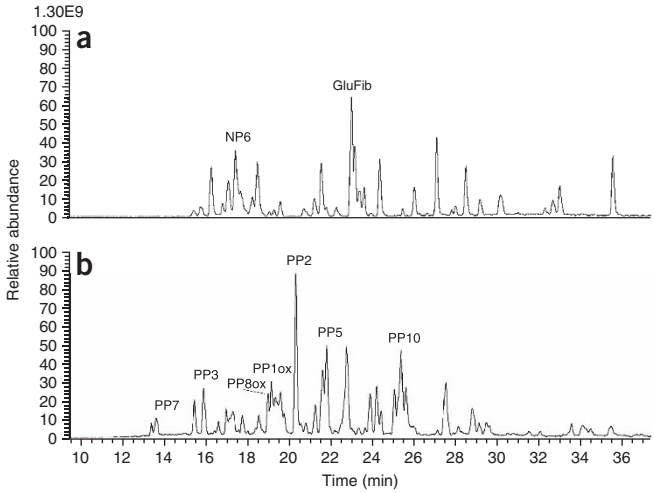


Figure 2 | Selectivity of TiO_2 towards phosphopeptides. A sample containing 500 fmol BSA spiked with 500 fmol phosphopeptide mix was subjected to TiO_2 chemo-affinity chromatography as described. Reversed-phase high-performance liquid chromatography base-peak chromatograms are shown. (**a**) The flow-through fraction contained the two non-phosphorylated synthetic peptides accompanied by some BSA peptides; (**b**) phosphopeptides eluted in a fraction after treating the TiO_2 column with 125 mM ABC and 50 mM ammonium phosphate at pH 10.5.

successfully eluted. To test whether acidic peptides could also bind the TiO_2 column and affect its selectivity for phosphopeptides, the peptide mix was supplemented with BSA-derived tryptic peptides. Here, the BSA peptides were found in both the flow-through and the eluate (**Fig. 2**), indicating that some non-phosphorylated peptides can also bind the TiO_2 column, but they failed to prevent selective enrichment of the phosphopeptides. Although some BSA peptides were present at higher concentrations compared to synthetic phosphopeptides, a reduction in complexity of the sample is clearly demonstrated.

Peak area comparison of peptides before and after TiO_2 enrichment (**Fig. 3**) showed a high percentage recovery of phosphopeptides at around 80–100%, whereas the recovery for non-phosphorylated peptides was significantly lower. Upon elution with basic ABC solution, the two non-phosphorylated peptides ("NP6" and "GluFib") appeared with a recovery below 2 and 10%, respectively. This example shows that the binding of acidic non-phosphorylated peptides can be successfully inhibited. Regarding the recovery of PP1, its oxidized form has to be taken into account, and PP8 is present in an oxidized form only under current separation conditions.

Combining OSA and DHB resulted in a more effective removal of non-phosphorylated peptides. For example, the non-phosphorylated peptide NP6 was always present in the eluate when DHB was used. A significantly lower amount of NP6 was observed with OSA alone, and none was found when both agents were combined. For GluFib, the amount detected after basic elution was $<10\%$, with 300 mM OSA $\sim 30\%$ and with 20 mg ml^{-1} DHB $>90\%$. The results demonstrate that the combination of both agents led to high sequence coverage and reduced the binding of non-phosphorylated peptides. The non-phosphorylated peptides "NP6" and "Glufib" were successfully used as a negative control.

It was mentioned earlier that BSA peptides bound to the TiO_2 column in all three cases: when using OSA, DHB or a combination of both agents. The combination of these ion-pairing agents lowered the amount of BSA peptides (calculated as areas of peptides found) in comparison to amounts when each agent was used alone. **Table 7** shows data obtained by analyzing the mass spectra acquired with different loading solvents. We were able to reduce the number of peptides bound to the TiO_2 column. Intriguingly, the Mascot score (calculated with $-10\log_{10}(P)$, where P is the absolute probability that the observed match is a random event) and the sequence coverage (a percent value how much of a given sequence was obtained by sample analysis) did not decrease in the same manner. We assume that the combination of both agents neutralizes the acidic groups on non-phosphorylated peptides more effectively in comparison to the use of either agent alone.

The suitability of our method for the enrichment of phosphopeptides from complex samples was confirmed by processing and analyzing a mix of peptides of biological origin, in this case a proteolyzed sample of human APC, for which we identified several phosphorylation sites previously[13,14]. TiO_2 enrichment of APC-derived peptides enabled the identification of nine additional phosphorylation sites in this complex (**Table 8**).

An example of an MS/MS spectrum for one novel phosphorylation site is shown in **Figure 4**, which shows the spectrum and corresponding fragment ion series of the additionally found phosphopeptide on the CDC26 subunit of the APC. Both *b*- and *y*-ion series are almost complete and the NL of the b(9) ion is visible in the spectrum. Similar results were obtained for other phosphopeptides identified.

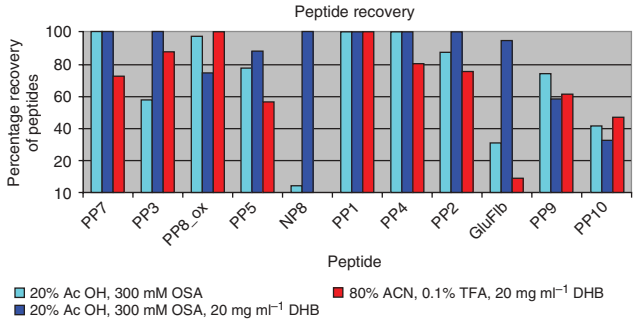


Figure 3 | Recovery of phosphopeptides separated from a simulated complex mixture. Synthetic peptide mix (50 fmol) together with a tenfold excess of BSA (500 fmol) was purified using TiO_2-packed tips as described, and the percentage recovery was determined by peak area comparison in selected ion monitoring.

TABLE 7 | Sequence coverage, Mascot score and peptide count for the non-phosphorylated BSA peptides when trapped under different loading conditions.

	Average		
	Sequence coverage (%)	Score	Peptide found
20% AcOH, 300 mM OSA	50	1,430	165
80% ACN, 0.1% TFA, 20 mg ml^{-1} DHB	46	1,123	92
20% AcOH, 300 mM OSA, 20 mg ml^{-1} DHB	42	1,105	70

ACN, acetonitrile; DHB, 2,5-dihydroxybenzoic acid; OSA, 1-octanesulfonic acid.

TABLE 8 | Additional phosphorylation sites found in APC.

	Sequence	Protein	Mascot score	Mascot expect	Sequest Xc	Sequest Sf	Charge state	HPLC RT
1	SSQFGSpLEF	CDC26	38	0.0024	3.47	0.93	2+	108.63
2	EDVEVVGGSpDGEGAIGLSSDPK	CDC26	87	1e-07	6.79	0.99	2+	75.02
3	VGSpLQEVTIH	APC1	63	0.0017	4.60	0.97	2+	54.73
4	VGSpLQEVTpIHEK	APC1	23	22	3.84	0.85	2+	46.55
5	RVSpPLNLSSVTP	APC8 (CDC23)	28	0.058	2.68	0.59	2+	80.73
6	RVSpPLNLSSVTpP	APC8 (CDC23)	31	0.03	3.35	0.86	2+	87.20
7	QTAEETGLTPLETpSpR	APC6 (CDC16)	37	0.01	3.74	0.72	2+	54.17

APC, anaphase-promoting complex. By employing the method described, it was possible to identify additional phosphorylation sites. The complete list of detected phosphopeptides will be published elsewhere. For the phospho-sites colored blue, their exact location cannot be distinguished.

The choice of correct "displacer" or ion-pairing agent for sample loading is one of the crucial steps for this technique. To effectively keep acidic and non-phosphorylated peptides away from binding to the TiO_2 column, large amounts of ion-pairing agent must be added. We compared the previously described displacer, DHB, with an alternative, OSA, and the combination of both agents. When using DHB alone, disruption in phosphopeptide enrichment and massive interference in the MS signal occurred (data not shown), whereas this was not seen when using OSA alone or when OSA and DHB were combined for trapping of phosphopeptides.

Additionally, the method of generating of MS/MS spectra is very important for the quality of MS/MS spectra and the number of identified peptides. Therefore, we optimized not only the HPLC but also the MS methods used for detection. For proper analysis of phosphorylation sites, special fragmentation settings called MSA were used[20,21]. Compared to conventional ion dissociation, the MSA procedure increases both the number of fragments and their intensity. However, many of these ions with increased intensity or the newly produced ones had already experienced an NL of phosphoric acid. The phosphoric acid NL alone does not contribute to peptide sequence identification. The software recognizes the ions that experienced NL of phosphoric acid and activates them for further fragmentation. By this approach, the phosphorylated peptides can be distinguished from their non-phosphorylated counterparts and additionally fragmented. The additional fragmentation improves the quality of MS/MS spectra and leads to higher confidence scores for phosphorylated peptides.

Data analysis and peptide spectrum interpretation

MS/MS spectra (dta file format) were extracted from the raw data using the BioWorks 3.2 software suite.

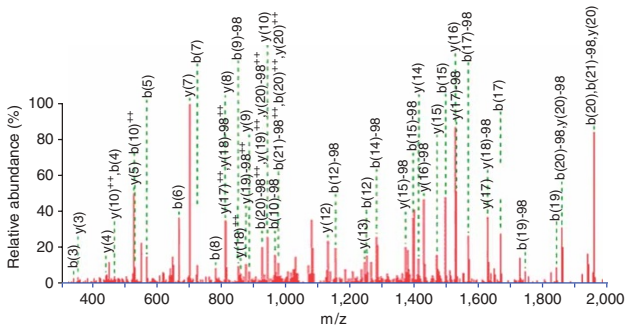
For Mascot database searches, dta files were merged using the perl script program *merge.pl* (Matrix science) into a single file (mgf file format).

Data were searched against the IPI human database v. 3.17 (ref. 18) with an MS accuracy of 7 ppm and an MS/MS accuracy of 0.6 Da.

For Sequest database searches, MS/MS spectra were analyzed by TurboSEQUEST, part of BioWorks 3.2 software, against the IPI human database with an MS accuracy of 7 ppm and an MS/MS accuracy of 1.0 Da.

To confirm the results obtained with previous searches, a search against a randomized database combined with a normal IPI human database was performed[19]. The randomized database was generated with a program perl script *decoy.pl*, which is freely available from Matrix Science. Afterwards, the real and decoy databases were concatenated. A false-positive hit is given only when the match from the decoy sequence is better than the match from the correct sequence. Searching a reversed or randomized database is an excellent validation method for MS/MS searches of large data sets or for post-translational modifications.

a



b

No.	b	b++	Seq.	y	y++	No.	No.	b	b++	Seq.	y	y++	No.
1	130.04987	65.52857	E			22	12	1155.47999	578.24364	E	1073.54730	537.27729	11
2	245.07681	123.04204	D	1969.93484	985.47106	21	13	1212.50145	606.75437	G	944.50471	472.75599	10
3	344.14522	172.57625	V	1854.90790	927.95759	20	14	1283.53856	642.27292	A	887.48325	444.24526	9
4	473.18781	237.09754	E	1755.83949	878.42338	19	15	1396.62262	698.81495	I	816.44614	408.72671	8
5	572.25622	286.63175	V	1629.79690	813.90209	18	16	1453.64408	727.32568	G	703.36208	352.18468	7
6	671.32463	336.16595	V	1527.72849	764.36788	17	17	1566.72814	783.86771	L	646.34062	323.67395	6
7	728.34609	364.67668	G	1428.66008	714.83368	16	18	1653.76017	827.38372	S	533.25656	267.13192	5
8	785.36755	393.18741	G	1371.63862	686.32295	15	19	1740.79220	870.89974	S	446.22453	223.61590	4
9	854.38900	427.69814	S	1314.61716	657.81222	14	20	1855.81914	928.41321	D	359.19250	180.09989	3
10	969.41594	485.21161	D	1245.59570	623.30149	13	21	1952.87190	976.93959	P	244.16556	122.58642	2
11	1026.43740	513.72234	G	1130.56876	565.78802	12	22			K	147.11280	74.06004	1

Figure 4 | Identification of a novel APC-derived phosphorylation site. This shows the spectra (**a**) and corresponding product ion series (**b**) of the newly identified phosphopeptide in the digested APC sample. Ser42 was found on the CDC26 peptide EDVEVVGGSpDGEGAIGLSSDPK.

We anticipate that this method could be widely used to enrich phosphopeptides from protein samples from diverse biological origins, facilitating the identification of additional sites of phosphorylation in proteins of interest. This technology could contribute to further improving our understanding of cell-signaling pathways and could have application in large-scale phospho-proteomic studies.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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Erratum: Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis

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