Chapter 9

The Use of Titanium Dioxide for Selective Enrichment of Phosphorylated Peptides

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Abstract

Titanium dioxide (TiO_2) has very high affinity for phosphopeptides and in recent years it has become one of the most popular methods for phosphopeptide enrichment from complex biological samples. Peptide loading onto TiO_2 resin in a highly acidic environment in the presence of 2,5-dihydroxybenzoic acid (DHB), phthalic acid, lactic acid, or glycolic acid has been shown to improve selectivity significantly by reducing unspecific binding of non-phosphorylated peptides. The phosphopeptides bound to the TiO_2 are subsequently eluted from the chromatographic material using an alkaline buffer. TiO_2 chromatography is extremely tolerant towards most buffers used in biological experiments, highly robust and as such it has become the method of choice in large-scale phosphoproteomics. Here we describe a batch mode protocol for phosphopeptide enrichment using TiO_2 chromatographic material followed by desalting and concentration of the sample by reversed phase micro-columns prior to downstream MS and LC-MS/MS analysis.

Key words Protein phosphorylation, Phosphopeptide enrichment, Titanium dioxide chromatography, Mass spectrometry

1 Introduction

For some years the adsorption of proteins to titanium dioxide (TiO_2) films has been studied with the aim of determining a method for pursuing bioelectrochemical studies of protein functions [1]. More interestingly for phosphoproteomic studies, TiO_2 has been shown to have affinity for phosphate ions from aqueous solutions [2, 3] and recently, TiO_2 chromatography has been adapted as an efficient alternative to already existing methods for phosphopeptide enrichment from complex samples. In 2004 several groups introduced the enrichment of phosphopeptides using TiO_2 material in combination with mass spectrometric (MS) analysis [4–6]. For example, Pinkse and coworkers described the ability of TiO_2 to selectively bind phosphorylated peptides using an online two-dimensional liquid chromatography (LC) MS setup

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with spherical particles of TiO_2 (Titansphere) as the first dimension and reversed phase (RP) material as the second dimension [5]. The sample was loaded onto a TiO_2 column in acidic conditions (pH 2.9) to promote the binding of phosphopeptides to the TiO_2 particles. The unbound non-phosphorylated peptides were trapped on the RP column. After elution from the RP column the non-phosphorylated peptides were analyzed using nanoLC-ESI-MS/MS. The phosphopeptides were subsequently eluted from the TiO_2 column using an alkaline buffer (pH 9.0), concentrated on the RP pre-column and analyzed using nanoLC-ESI-MS/MS [5]. However, the buffers used for these initial TiO_2 enrichments all resulted in significant binding of non-phosphopeptides. In 2005, we introduced an offline setup for TiO_2 chromatography in which much stronger buffer conditions were applied and included the use of 2,5-dihydroxybenzoic acid (DHB) and high concentration of trifluoroacetic acid (TFA) in the loading buffer, which significantly reduced unspecific binding from non-phosphorylated peptides [7]. In addition, ammonia solution at even higher pH (pH 11.3) was shown to elute phosphorylated peptides from the TiO_2 column more efficiently than pH 9 and thereby improve phosphopeptide recovery [7]. Due to problems associated with the use of high amount of DHB, such as polymerization and contamination of the LC system we investigated other multifunctional acids such as phthalic acid [8] and glycolic acid [9] and found the latter to be ideal for large-scale phosphoproteomic studies.

The high selectivity of TiO_2 towards phosphorylated peptides makes it a powerful tool for phosphoproteomic studies, also when used in combination with other phosphopeptide enrichment methods such as Immobilized Metal Affinity Chromatography (IMAC) (*see* Chapter 8) termed Sequential elution from IMAC (SIMAC) [10] (*see* Chapter 10) or TiSH [11] (*see* Chapter 11) methods. In addition, TiO_2 chromatography of phosphorylated peptides is extremely tolerant towards most buffers and salts used in biochemistry and cell biology laboratories [9]. The offline setup is simple and fast, and does not require expensive equipment.

2 Materials

2.1 Model Proteins

1. Transferrin (human) was a gift from ACE Biosciences A/S. Serum albumin (bovine), α-lactoglobulin (bovine), carbonic anhydrase (bovine), α-casein (bovine), β-casein (bovine), ovalbumin (chicken), ribonuclease B (bovine), alcohol dehydrogenase (Baker's yeast), myoglobin (whale skeletal muscle), lysozyme (chicken), α-amylase (*Bacillus* species) were from Sigma (St. Louis. MO, USA). Of these 12 proteins 3 are phosphorylated: α-casein, β-casein, and ovalbumin, yielding about 15 phosphorylated peptides depending on the purity of the batches.

2.2 Reduction, Alkylation, and Digestion of Model Proteins

1. Triethylammonium bicarbonate.
2. Dithiothreitol (DTT).
3. Iodoacetamide.
4. Modified trypsin.
5. Acetone.

2.3 Cell Lysis and Titanium Dioxide (TiO_2) Chromatography

1. Titanium dioxide (TiO_2) beads (Titansphere, 5 μm, GL sciences Inc.).
2. Low-binding microcentrifuge tubes 1.7 mL.
3. Acetonitrile, HPLC Grade.
4. TiO_2 Loading Buffer: 1 M glycolic acid in 5 % trifluoroacetic acid (TFA), 80 % acetonitrile.
5. TiO_2 Washing Buffer 1: 1 % TFA, 80 % acetonitrile.
6. TiO_2 Washing Buffer 2: 0.1 % TFA, 10 % acetonitrile.
7. TiO_2 Elution Buffer: 1 % ammonia water (40 μL ammonia Solution (25 %) in 960 μL UHQ water).
8. Formic acid.
9. Milli-Q water (UHQ water) (*see* **Notes 1** and **2**).
10. PhosStop.

2.4 Reversed Phase (RP) Micro-columns

1. POROS Oligo R3 reversed phase material (PerSeptive Biosystems, Framingham, MA, USA).
2. GELoader tips (Eppendorf, Hamburg, Germany) or p200 pipette tips depending on the size of the column needed.
3. 3 M Empore C18 disk (3 M, Bioanalytical Technologies, St. Paul, MN, USA).
4. 1–5 mL disposable syringes fitted to GeLoader tip or p200 tips by using a pipette tip cut in both ends.
5. RP Washing Buffer: 0.1 % TFA.
6. RP Elution Buffer (for LC-ESI MS/MS analysis): 70 % acetonitrile, 0.1 % TFA.
7. 2,5-dihydroxybenzoic acid (DHB) Elution Buffer (for MALDI MS analysis): 20 mg/mL DHB in 50 % acetonitrile, 1 % ortho-phosphoric acid.

2.5 Other Materials

1. Tabletop centrifuge.
2. pH meter.
3. Thermomixer.
4. Shaker.
5. Vacuum centrifuge.

2.6 Analysis by Mass Spectrometry

1. Mass spectrometer capable of performing MS/MS—preferentially a high-resolution/high mass accuracy instrument (Q-TOFs (Waters, ABSciex, Bruker, and Agilent) or Orbitrap based mass spectrometer (Thermo Fisher Scientific)) interfaced to a nanoHPLC (e.g., Dionex 3000 ultimate LC system (Thermo Fisher Scientific)) with a 50–100 μm i.d. RP capillary column setup for highly sensitive online peptide separation can be used. For simpler samples a MALDI MS instrument can be used (e.g., Bruker Ultraflex (Bruker Daltonics, Bremen, Germany)).
2. Software for processing of raw mass spectrometry data files and generation of peak lists for searching against a protein database (e.g., Uniprot) Analysis software such as Mascot/Mascot Distiller (Matrix Science, London, UK) (data from most vendors and instruments), Proteome Discoverer (Thermo Scientific, Bremen, Germany) (data from Thermo instruments), MaxQuant [15] (high resolution data from Thermo Orbitrap instruments and certain Bruker and ABSciex Q-TOFs) and the TransProteomicPipeline [16] (vendor independent).

3 Methods

The principle of the TiO_2 method described in this chapter is illustrated by the purification of phosphopeptides from a peptide mixture originating from tryptic digestions of 12 standard proteins (Model proteins) and from 150 μg of tryptic peptides from a HeLa cell lysate (*see* **Notes 3** and **4**).

The TiO_2 purification method is simple and straightforward. It is fast and efficient for enrichment of phosphopeptides even from highly complex samples (e.g., [11, 12]) (*see* **Note 5**). The experimental setup of the method is illustrated in Fig. 1. For illustrating the anticipated results, first a peptide mixture originating from tryptic digestions of 12 standard proteins was subjected to TiO_2. Secondly, the TiO_2 procedure was applied to 150 μg of tryptic peptides derived from a HeLa cell lysate.

3.1 Digestion of Model Proteins and the HeLa Cell Lysate

1. Dissolve each protein in 50 mM triethylammonium bicarbonate (TEAB), pH 7.8, 10 mM DTT and incubate at 37 °C for 1 h. After reduction, add 20 mM iodoacetamide and incubate the samples at room temperature for 1 h in the dark.
2. Digest each protein using trypsin (1–2 % w/w) at 37 °C for 12 h.
3. Lyse HeLa cells in 6 M urea, 2 M thiourea containing phosphatase inhibitors. Precipitate the proteins using 10 volume excess of ice-cold acetone and incubate overnight at −20 °C. Centrifuge the sample at 14,000 × *g* and wash the pellet twice with ice-cold acetone. Redissolve the pellet in 50 μL 6 M urea,

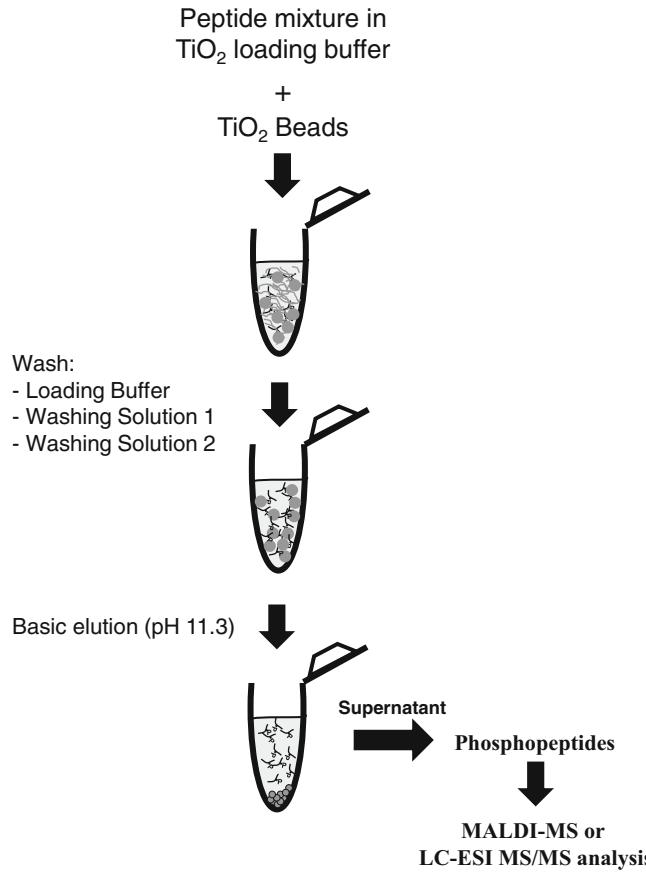

Fig. 1 The strategy used for the enrichment of phosphopeptides using TiO_2 material. The peptide sample is diluted with the TiO_2 Buffer and TiO_2 beads are added. The sample is incubated for 5–10 min. The TiO_2 beads are washed with washing solutions and finally the phosphopeptides are eluted from the TiO_2 material using basic elution conditions

2 M thiourea, 10 mM DTT containing 1 μg endoproteinase Lys-C and incubate at room temperature for 2 h. After incubation dilute the sample 10× with 50 mM TEAB, pH 7.8 containing 20 mM iodoacetamide and incubate for 1 h in the dark at room temperature. After incubation, add trypsin (1–2 % w/w) and incubate the sample overnight at room temperature.

3.2 Batch Mode TiO_2 Phosphopeptide Purification

1. Add acetonitrile, TFA, and glycolic acid to your peptide mixture to obtain TiO_2 Loading Buffer conditions (80 % acetonitrile, 5 % TFA, and 1 M glycolic acid) (*see* **Note 6**) or dilute the sample at least 10× with the TiO_2 Loading Buffer.
2. Add 0.6 mg TiO_2 beads per 100 μg peptide solution (*see* **Note 7**).

3. Place the tubes on a shaker (highest shaking) at room temperature for 5–10 min.
4. After incubation centrifuge to pellet the beads (table centrifuge <15 s).
5. Transfer the supernatant to another low-binding tube and incubate it for a second round of incubation with 0.3 mg TiO_2 beads. This step may be repeated more times in order to recover as many phosphopeptides as possible.
6. Pool the TiO_2 beads from the 2–3 incubations using 100 µL Loading Buffer and transfer them to a new low-binding microcentrifuge tube (*see* **Note 8**).
7. Vortex the solution for 10 s and then centrifuge to pellet the beads. Remove the supernatant.
8. Wash the beads with 70–100 µL (*see* **Note 9**) Washing Buffer 1—mix for 10 s and then centrifuge to pellet the beads.
9. Wash the beads with 70–100 µL Washing Buffer 2—mix for 10 s and then centrifuge to pellet the beads. This step is important to remove peptides that bind in a HILIC mode to TiO_2 (*see* **Note 10**).
10. Dry the beads for 5–10 min in the vacuum centrifuge or on the table for 30 min.
11. Elute the phosphopeptides with 100–200 µL Elution Buffer—mix well and leave the solution on a shaker for 10 min to allow an efficient elution.
12. Centrifuge the solution for 1 min and pass the supernatant (TiO_2-eluate) over a small stage tip filter [13] (C8 stage tip) into a new low-binding tube to recover the liquid without TiO_2 beads.
13. Wash the beads with 30 µL Elution Buffer and pool the wash with the TiO_2-eluate from the previous step.
14. Elute potential bound peptides from the C8 filter with 5 µL 30 % acetonitrile and pool with the TiO_2-eluate from **steps 12** to **13**.
15. Lyophilize the eluted peptides (*see* **Note 11**) or acidify the eluate with 1 µL Formic Acid per 10 µL eluate for direct cleanup of the phosphopeptides using RP material prior to downstream analyses (e.g., HILIC fractionation [11]).

3.3 Poros Oligo R3 Reversed Phase (RP) Micro-column Desalting/ Concentration of the Sample

Use RP GELoader tip micro-columns of ~6–10 mm or p200 pipette tips micro-columns (1–2 cm) depending on the amount of material to be purified. Here, it is illustrated for the p200 pipette tip.

1. Suspend Poros Oligo R3 reversed phase (RP) material in 200 µL 100 % acetonitrile.

2. Prepare a p200 pipette tip micro-column by stamping out a small plug of C_{18} material from a 3 M Empore™ C_{18} extraction disk and place it in the constricted end of the tip.
3. Pack Poros Oligo R3 RP beads on top of the p200 stage tip until the size of the column is 1–2 cm.
4. Load the acidified phosphopeptide sample slowly onto the RP micro-column (~1 drop/s).
5. Wash the RP micro-column using 60 µL RP Washing Buffer.
6. Elute the phosphopeptides from the RP micro-column using 40–60 µL RP Elution Buffer, followed by lyophilization of the phosphopeptides (N.B. For MALDI MS analysis the peptides can be eluted off the GeLoader tip RP micro-column directly onto the MALDI target using 1 µL DHB solution. After crystallization the sample is ready for MALDI MS analysis).
7. Redissolve the lyophilized phosphopeptides in 0.5 µL 100 % formic acid and dilute immediately to 10 µL with UHQ water. The sample is then ready for LC-ESI-MS^n analysis.

3.4 µHPLC Tandem Mass Spectrometry (LC-MS/MS) Analysis

For LC-MS/MS analysis of purified phosphopeptides a standard strategy as described below can be used. A typical nanoLC setup would include a 0.075 mm × 200 mm analytical column packed with 3 µm RP resin interfaced with a high resolution/mass accuracy mass spectrometer as described in our original paper [11]. The number of phosphopeptides identified in the analysis can be increased by maximizing the resolution of the nanoLC separation via longer columns (e.g., 50 cm) and smaller chromatographic particle sizes (e.g., 1.9 µm). Alternatively, a two column system can be utilized using a 0.1 mm × 20 mm pre-column packed with RP resin (3–5 µm) combined with an analytical column as described above. A two column system is described below.

1. The phosphopeptides are redissolved in 0.1 % TFA and loaded onto a pre-column as described above using a µHPLC system (e.g., Dionex or EASY-LC) at a loading speed of 5 µL/min.
2. The phosphopeptides are eluted directly onto the analytical column (e.g., 0.075 mm × 200 mm) using a gradient (60–120 min) from 0 to 35 % B-Buffer (e.g., A-Buffer: 0.1 % formic acid; B-Buffer: 90 % acetonitrile, 0.1 % TFA) at an elution speed of 2–300 nL/min.
3. The phosphopeptides are eluted directly into a tandem mass spectrometer and analyzed by Data Dependent Analysis.

LC-ESI-MS^n analysis of multi-phosphorylated peptides can be improved by redissolving the phosphopeptides by sonication in an EDTA containing buffer prior to LC-ESI-MS^n analysis [14] (*see* **Note 12**).

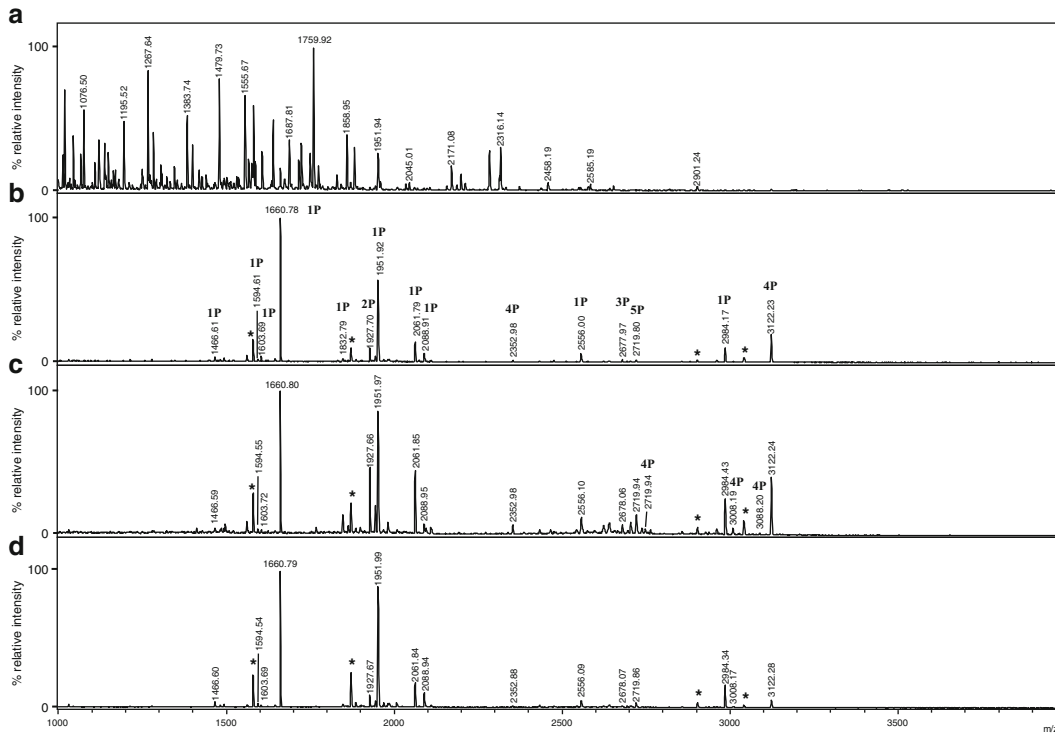

Fig. 2 Results obtained from 1 pmol peptide mixture using the TiO_2 strategy. (**a**) MALDI MS peptide mass map of the direct analysis of the tryptic peptides. (**b**) MALDI MS peptide mass map of phosphopeptides purified by TiO_2 from the standard peptides mixture dissolved in 0.1 % TFA. (**c**) MALDI MS peptide mass map of phosphopeptides purified by TiO_2 from the standard peptides mixture dissolved in 5 % SDS and 25 mM EDTA. (**d**) MALDI MS peptide mass map of phosphopeptides purified by TiO_2 from the standard peptides mixture dissolved in 1 M KCl and 25 mM EDTA. The number of phosphate groups on the individual phosphopeptides is indicated by "#P". *Asterisk* indicates the metastable loss of phosphoric acid

An example of the results obtained by TiO_2 chromatography is shown in Fig. 2. The figure shows the MALDI MS results obtained on a Bruker Ultraflex from a dried droplet sample preparation of 0.25 pmol peptide mixture without phosphopeptide enrichment (Fig. 2a). The MALDI MS peptide mass map of the TiO_2 enriched phosphopeptides from 0.25 pmol of peptide mixture is shown in (Fig. 2b). The unique tolerance towards biological buffers is illustrated by the enrichment of phosphopeptides from the peptide mixture in the presence of 50 mM EDTA which will normally strip the iron from the chromatographic material used in IMAC. The MALDI MS peptide mass map is shown in (Fig. 2c). The phosphopeptides are illustrated in (Fig. 2c) by asterisks (*see* **Note 13**).

An example of the results obtained using the present protocol for enrichment of phosphopeptides from a total of 150 μg peptides derived by tryptic digestion from a HeLa cell lysate is shown in Fig. 3. The enriched phosphopeptides were separated on a Dionex

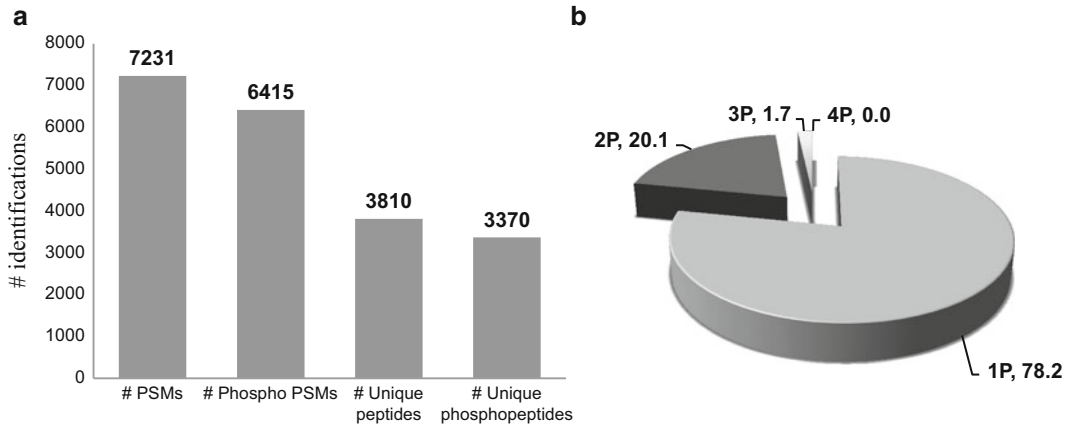

Fig. 3 Results obtained from the enrichment of phosphorylated peptides from acetone precipitated proteins from HeLa cells using TiO_2 chromatography. (**a**) Overview of the number of peptide spectral matches (PSMs) and unique phosphopeptides identified in the TiO_2 enriched fraction. (**b**) Percentage distribution of the number of phosphate groups on the phosphopeptides identified in the TiO_2 enriched fraction

3000 ultimate LC system using homemade RP capillary columns (20–25 cm) directly into a Q-Exactive Plus MS instrument from Thermo Fisher Scientific. The peptides were separated using a 90 min gradient from 0 to 25 % B Buffer (90 % acetonitrile in 0.1 % formic acid). The MS instrument was set to isolate and fragment 12 parent ions per MS cycle (MS and MS/MS resolution was set to 70,000 and 35,000 at 200 *m/z*, respectively; MS and MS/MS AGC target was 1E6 and 5E4, respectively; normalized collision energy was 30; isolation window was 1.5 Da). Here a total of 3370 unique phosphopeptides were identified from the 150 μg of starting material, using the Proteome Discoverer 1.4.1.14 (SwissProt_2014_04 (20,340 entries)) with an enrichment percentage of about 88 % phosphopeptides (*see* Fig. 3a). Of these, 2634 were singly phosphorylated and 735 had two or more phosphate groups (*see* Fig. 3b).

4 Notes

1. It is important to obtain the highest purity of all chemicals used.
2. All solutions should be prepared in UHQ water.
3. Always start by testing the method using a model peptide mixture. It is important to freshly prepare the peptide mixture as peptides bind to the surface of the plastic tubes in which they are stored. In addition, avoid transferring the peptide sample between different tubes to minimize adsorptive losses of the sample to the plastic surfaces.

4. The peptide mixtures used for the experiments illustrated in this chapter contained peptides originating from tryptic digestions of 1 pmol of each of 12 standard proteins and of 150 μg of a HeLa cell lysate. Experiments have shown that the presented method is sensitive down to the low femtomole level [8].
5. Frequently, the TiO_2 enrichment method described here is combined with either peptide pre- or post-fractionation methods such as strong cation exchange (SCX) (e.g., [15, 16]) or Hydrophilic Interaction Liquid Chromatography (HILIC) (e.g., [11, 12, 17]) in order to increase the phosphopeptide coverage in large-scale phosphoproteomic studies.
6. If you have 100 μL peptide sample, you can add 50 μL water, 50 μL 100 % TFA, 800 μL acetonitrile, and 76 mg glycolic acid to obtain the proper TiO_2 Loading Buffer conditions.
7. The optimal amount of TiO_2 beads to use in order to reduce nonspecific binding and optimize phosphopeptide yield is 0.6 mg TiO_2 per 100 μg of peptide starting material (*see* [11] for further information). This will of course change depending on the source of biological material used as TiO_2 selectively enriches other biomolecules (reviewed in [18]) such as sialylated glycopeptides [19] and acidic lipids [20] commonly found in membrane fractions.
8. The transfer to a new tube is performed due to the fact that peptides stick to plastic and can be eluted from the plastic surface in the last elution step resulting in contamination with non-modified peptides.
9. For large-scale analysis, where more TiO_2 beads are used, larger volumes of the buffers should be used.
10. TiO_2 is an efficient HILIC material and hydrophilic peptides can bind to the material when loaded in high organic solvent. The inclusion of 5 % TFA and 1 M glycolic acid should prevent most hydrophilic non-modified peptides from binding; however, some can still be found in the elution from TiO_2. Therefore in order to eliminate any binding from non-phosphorylated hydrophilic peptides this last Washing Buffer is important. For membrane preparations the last washing supernatant will contain neutral glycopeptides which can then be analyzed further.
11. If ammonia is left in the solution, it will interfere with deglycosylation procedures (membrane fractions) or a subsequent SIMAC purification [11].
12. Titanium dioxide was originally thought to have a preference for mono-phosphorylated peptides likely due to the fact that it is difficult to elute multi-phosphorylated peptides from the

TiO_2 micro-column once they have bound [9], as performed in the original DHB TiO_2 procedure [7]. However, since the procedure is now performed in batch mode the elution takes longer time and plenty of multi-phosphorylated peptides can be eluted with the TiO_2 Elution Buffer described here [11]. The combination of sequential elution from Immobilized Metal Affinity Chromatography (IMAC) and TiO_2 has made it possible to enrich for mono- as well as multi-phosphorylated peptides as described in the SIMAC protocol in Chapter 10.

13. The results obtained using this protocol will differ according to the mass spectrometer used for the analysis of the phosphopeptides, not only between MALDI MS and ESI MS but also within different MALDI MS instruments, depending on laser optics, laser frequency, instrumental configuration, sensitivity, etc.

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