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# A novel titanium dioxide-polydimethylsiloxane plate for phosphopeptide enrichment and mass spectrometry analysis

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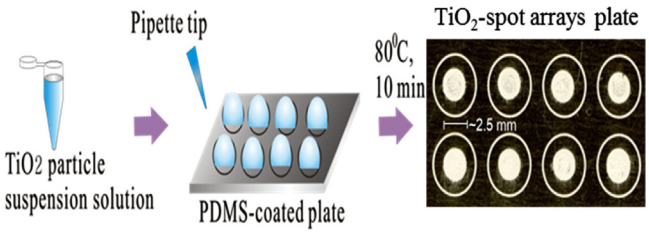
## HIGHLIGHTS

- TP plate is a novel approach to purify phoshphopeptides.
- The sensitivity of TP plate is superior to that of packed-tips.
- TP plate has high sample capacity of ~10 μg β-casein on 2.5 mm diameter spots.
- ~82% of identified proteins are phosphorylated in the analysis of SCC4 cell lysate.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The phosphorylation of proteins is a major post-translational modification that is required for the regulation of many cellular processes and activities. Mass spectrometry signals of low-abundance phosphorylated peptides are commonly suppressed by the presence of abundant non-phosphorylated peptides. Therefore, one of the major challenges in the detection of low-abundance phosphopeptides is their enrichment from complex peptide mixtures. Titanium dioxide ($TiO_2$) has been proven to be a highly efficient approach for phosphopeptide enrichment and is widely applied. In this study, a novel $TiO_2$ plate was developed by coating $TiO_2$ particles onto polydimethylsiloxane (PDMS)-coated MALDI plates, glass, or plastic substrates. The $TiO_2$-PDMS plate (TP plate) could be used for on-target MALDI-TOF analysis, or as a purification plate on which phosphopeptides were eluted out and subjected to MALDI-TOF or nanoLC-MS/MS analysis. The detection limit of the TP plate was ~10-folds lower than that of a $TiO_2$-packed tip approach. The capacity of the ~2.5 mm diameter $TiO_2$ spots was estimated to be ~10 μg of β-casein. Following $TiO_2$ plate enrichment of SCC4 cell lysate digests and nanoLC-MS/MS analysis, ~82% of the detected proteins were phosphorylated, illustrating the sensitivity and effectiveness of the TP plate for phosphoproteomic study.



*Abbreviations:* PDMS, polydimethylsiloxane; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; DHB, 2,5-dihydroxybenzoic acid; PA, phosphoric acid; TFA, trifluoroacetic acid; FA, formic acid.

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## 1. Introduction

The reversible phosphorylation of proteins is a major post-translational modification that is required for the regulation of a wide range of biological process. Analyses of the dynamics of protein phosphorylation cascades can help reveal significant signaling events that are correlated with the regulation of specific cellular functions. MS-based approaches have proven to be powerful tools for phosphoprotein characterization and phosphorylation site mapping [1,2]. However, the MS signals of phosphorylated proteins in low abundance are commonly suppressed by the presence of abundant non-phosphorylated peptides. Furthermore, because phosphopeptides have a higher degree of acidity than unmodified peptides, the positive-ion formation of phosphopeptides is less efficient, resulting in the greater difficulty in identifying phosphopeptides by MS. Therefore, the purification of phosphopeptides from unmodified peptides prior to MS analysis is crucial. To improve the detection sensitivity and enrichment specificity for phosphopeptides, novel approaches have been continuously reported [3–5]. One commonly used approach is immobilized metal ion affinity chromatography (IMAC), which uses metal ions such as $Fe^{3+}$ and $Ga^{3+}$ to chelate with the phosphate group [6,7]. However, because the IMAC approach is based on chelation, the selectivity is usually limited by the nonspecific binding of acidic non-phosphorylated peptides [8]. Because metal oxides are stable in a wide pH range, allowing the use of acidic buffers to prevent nonspecific binding, metal oxide affinity chromatography (MOAC) shows better specificity for enriching phosphopeptides. Owing to its high stability and specific affinity toward phosphopeptides, titanium dioxide ($TiO_2$) is gaining more use in MOAC approaches and it is fabricated as columns, pipette tips, or coating layers on magnetic nanoparticles for purification purposes. In the $TiO_2$-LC column system, a large quantity of sample is often used, with the requirement of a high-pressure pumping device. A pressurized system or repeated aspirate-eject cycles of pipetting is operated for packed micro-tips (e.g., StageTips) [9]. Complicated synthesis process of magnetic $TiO_2$ particles could increase the experimental cost and difficulty. However, these off-probe methods are often time-consuming and laborious, and suffer sample loss during multistep sample handling [10]. In addition, the purification reproducibility may be affected by the beads-packing quality, elution flow rate, and manual operation. In contrast, the direct enrichment and detection of phosphopeptides on a MALDI target is a high-throughput process, and could be more sensitive owing to the fewer steps in sample handling with minimal sample loss [11]. One simple approach to immobilize $TiO_2$ on MALDI plates is to directly sinter the $TiO_2$ particles onto it [12]. However, a drawback of this approach was presented as the lack of control and full characterization of the structure of the $TiO_2$ spots. $TiO_2$ films with a more controllable thickness can by fabricated by the pulsed laser deposition method [13]. However, because $TiO_2$ particles or layers are not feasible for reuse and are not easily removed from the plate, these on-probe approaches consume expensive commercial MALDI targets. Disposable $TiO_2$ plates have been fabricated by using aluminum foil [14] and glass slides [15,16] instead of commercial plates. Printing, spraying, or deposition/calcination [10] processes were used to fabricate the $TiO_2$ film on the substrates. However, these disposable $TiO_2$ plates have to be equipped with modified MALDI targets or homemade sample stages.

Polydimethylsiloxane (PDMS) is chemically inert, hydrophobic, transparent, and widely applied in microfluidic devices for separation [17] and detection [18]. The desorption of proteins adsorbed onto surfaces of unmodified, oxidized and fluorinated PDMS has been studied with MALDI-TOF [19]. Wang et al. [20] have used thin PDMS stamp with punched holes in fabricating of microspots of phosphopeptide-binding polymer brushes for phosphopeptide enrichment. Recently, we have developed a PDMS-coated target for increased sample sensitivity and homogeneity in MALDI-TOF analysis [21]. Herein, a simple approach was developed to fabricate removable $TiO_2$ spot arrays on substrates for phosphopeptide purification or on-target MALDI-TOF analysis. By first coating a polydimethylsiloxane (PDMS) film on the MALDI target or other substrates, $TiO_2$ particles can then be adhered onto the PDMS-coated plates, followed by on-probe MALDI-TOF detection or phosphopeptide purification. The sensitivity, selectivity, reproducibility, and sample capacity toward phosphopeptides of this novel design and its application in phosphoproteomics were demonstrated.

## 2. Materials and methods

PDMS (polydimethylsiloxane) prepolymer was purchased from Dow Corning (Sylgard® 184, Midland, MI, USA). Acetonitrile (ACN), formic acid (FA), and $NH_4OH$ were purchased from J.T. Baker (Phillipsburg, NJ, USA). Dithiothreitol (DTT), iodoacetamide (IAA), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), phosphoric acid (PA), TFA, α-casein, β-casein, ovalbumin, myoglobin, cytochrome C, angiotensin II, adrenocorticotropic hormone (ACTH) fragment 18–39 (RPVKVYPNGAEDESAEAFPLEF, 2465.2 m/z), and $TiO_2$ nanoparticles (<150 nm, part no. 700347) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tryptic BSA and 2,5-dihydroxybenzoic acid (DHB) were purchased from Bruker Daltonics (Germany). Trypsin (modified, sequencing grade) was from Promega (Madison, WI, USA). A mixture of 4 phosphopeptides (part no. 186003285) was purchased from Waters (Milford, MA, USA). $TiO_2$ particles (5 μm) were purchased from GL Sciences (Tokyo, Japan). BSA, ammonium bicarbonate (ABC), Tris-HCl, and SDS were purchased from Bio Basic Inc. (Toronto, Canada). Deionized water (Milli-Q, Millipore, USA) was used to prepare the sample and matrix solutions.

### 2.1. Protein digestion

Ovalbumin, α-casein, β-casein, BSA, myoglobin, or cytochrome C protein was individually dissolved in 50 mM ABC and heated to 90 °C for 20 min. The denatured proteins were reduced with 10 mM DTT for 20 min at 56 °C, followed by the addition of 55 mM IAA for 30 min in the dark at 25 °C. Trypsin was added to the protein solution at an enzyme-to-substrate ratio of 1:50 (w/w) for 12 h at 37 °C. The peptide solution was dried in a centrifugal concentrator (miVac Duo Concentrator; Genevac, Stone Ridge, NY, USA).

### 2.2. Phosphopeptide enrichment by $TiO_2$-PDMS plate (TP plate)

The flow chart for TP plate fabrication and phosphopeptide enrichment is shown in Fig. 1. In step 1, a PDMS-coated plate was first fabricated. Prepolymer (reagent A) was mixed in an Eppendorf tube with its curing agent (reagent B) in a 10:1 volume ratio and spun down. An aliquot (3–5 μL) of the mixture was smeared on a glass/plastic plate, or a stainless steel MALDI plate (ground target; Bruker Daltonics). A roller (part no. 165-1279; Bio-Rad) was used to flatten the PDMS prepolymer to make a thin layer on the plate, which was then incubated in an oven for polymerization at 80 °C for 1 h. Once the PDMS film had been formed, the plate was washed with a 0.1% FA solution to effectively remove incompletely polymerized monomers, which may be detected by MALDI-TOF [21]. In step 2, aliquots (5 μL) of a $TiO_2$ particle solution (1 mg in 500 μL, 70% ACN) were deposited on a PDMS-coated plate to make the spot arrays. The plate was then incubated in an oven at 80 °C for 10 min,