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In Situ Enrichment of Phosphopeptides on MALDI Plates Functionalized by Reactive Landing of Zirconium(IV)–*n*-Propoxide Ions

Grady R. Blacken[†], Michael Volný[†], Tomáš Vaisar[‡], Martin Sadílek[†], and František Tureček^{*,†}

[†]*Department of Chemistry, University of Washington, Box 351700, Seattle, Washington 98195-1700*

[‡]*Department of Medicine, University of Washington, Box 351700, Seattle, Washington 98195-1700*

Abstract

We report substantial in situ enrichment of phosphopeptides in peptide mixtures using zirconium oxide coated plates for detection by MALDI-TOF mass spectrometry. The novel feature of this approach rests on the specific preparation of zirconium oxide coatings using reactive landing on stainless steel support of gas-phase positive ions produced by electrospray of zirconium(IV)–*n*-propoxide solutions in 1-propanol. Reactive landing was found to produce durable functionalized surfaces for selective phosphopeptide capture and desorption–ionization by MALDI. Enrichment factors on the order of 20–90 were achieved for several monophosphorylated peptides relative to abundant nonphosphorylated peptides in tryptic digests. We demonstrate the ability of the zirconium oxide functionalized MALDI surfaces to facilitate detection of enriched phosphopeptides in mid-femtomole amounts of α -casein digests per MALDI spot.

Reversible protein phosphorylation is an important mechanism for cellular signaling in eukaryotic organisms.^{1,2} The misregulation of enzymes that control cellular phosphorylation activities, specifically kinases and phosphatases, has been implicated in a wide range of diseases such as Alzheimer's and many cancers.³ Thus, the ability to define and quantify specific phosphorylation events can greatly impact the ability to selectively treat several human disease states. Detection and quantitation of phosphorylated proteins is complicated as the modified forms are typically present at low, sometimes substoichiometric, levels compared to unmodified proteins. Furthermore, mass spectrometric (MS) characterization of phosphopeptides is hampered by inefficient positive ion formation from phosphopeptides due to the increased acidity of the phosphorylated residue relative to the unmodified form. Tandem MS techniques have been developed to selectively detect phosphopeptides from complex tryptic mixtures by scanning for losses of the labile phosphate. Since phosphorylated serine and threonine residues readily eliminate phosphoric acid upon collision-induced dissociation (CID) then the loss of H_3PO_4 or H_2PO_4^- can be targeted in neutral loss or parent-ion scanning experiments, respectively.^{4,5} These techniques are unable to detect phosphorylated tyrosine, as phosphoric acid is not readily eliminated from this residue in CID. Recently, selective detection methods have been proposed which both increase positive ionization efficiency of phosphopeptides and allow for the simultaneous detection of all three commonly phosphorylated amino acid residues.^{6,7} Despite these advances in phosphopeptide detection by MS, further simplification of the sample is typically required to obtain robust proteomic data.

*Corresponding author. E-mail: turecek@chem.washington.edu.

To aid in phosphoproteome characterization, many chromatographic techniques have been developed to selectively enrich the phosphoprotein or phosphopeptide content of a complex biological sample. Phosphopeptide enrichment by immobilized metal-ion affinity chromatography (IMAC) was first introduced in 1987 and remains the standard for the analysis of complex phosphopeptide mixtures.⁸⁻¹¹ However, recent studies have reported enhanced enrichment using other strategies such as selective phosphopeptide capture on titanium and zirconium oxides or capture via covalent linkage of the phosphate residue to a chemically activated, solid-phase material.¹²⁻¹⁵ The novelty of titanium and zirconium oxides, relative to IMAC, is due to their stability over a broader pH range. Thus, loading and washing buffers can contain high concentrations of TFA which minimizes nonspecific binding by more efficiently protonating the acidic carboxylates of peptide Glu and Asp residues and the C-terminus. Despite their apparent advantages for phosphopeptide enrichment, it is sometimes reported that titanium and zirconium oxides suffer from complications in eluting the captured phosphopeptides. A recent study compared the relative merits of the various enrichment techniques and found that, although IMAC was able to identify the greatest number of phosphorylation sites in the *Drosophila melanogaster* proteome, the metal oxide resin was able to identify a comparable number of phosphorylation sites that were largely unidentified by IMAC.¹⁶ Thus, the study argues, titanium and zirconium oxide can provide information that is complementary to analyses using IMAC.

Recently, methods have been introduced which simplify the analysis of phosphopeptides with the use of a MALDI plate that can be used for both enrichment and detection. For example, a recent report described the use of gold-coated silicon wafers derivatized with a nitrilotriacetic acid moiety for phosphopeptide enrichment by Fe(III)-IMAC.¹⁷ These IMAC-MALDI wafers allowed for the enrichment and detection of low-picomole quantities of ovalbumin and β -casein tryptic phosphopeptides. Additionally, a separate study illustrated the enrichment and direct detection of casein tryptic phosphopeptides on a porous silicon MALDI target derivatized with zirconium phosphonate.¹⁸ Such techniques eliminate the complications that may arise with an elution step and allow for a more facile assay strategy with detection by MALDI-MS.

Here we demonstrate the use of a MALDI surface coated with zirconium oxides for the enrichment of synthetic phosphopeptides as well as tryptic phosphopeptides from α -casein. Zirconium dioxide is known to be one of the most stable materials both chemically and photochemically, and it is a very suitable material for different applications ranging from catalysis to ceramics. Zirconium dioxide has a number of superior properties, such as high refractive index, high hardness, durability, alkali and heat resistance, as well as resistance against oxidation.¹⁹ That is why there has been intensive investigation of zirconium dioxide coatings on different materials, mostly by suitable variations of the sol-gel process.²⁰⁻²² The novelty of our technology is that the surface is generated by exposing plasma-oxidized stainless steel surfaces to collisions with gas-phase cations generated by electrospray ionization of zirconium(IV)-*n*-propoxide.

The principles of ion soft and reactive landing to achieve surface coating and analyte immobilization were reported previously.^{23,24} Briefly, ions are generated from solution by electrospray and guided to a vacuum chamber by an ion optics system prior to colliding with a surface that has been pretreated inside an in situ coupled plasma reactor. The instrument was described in detail in the previous reports.²³⁻²⁵ It was demonstrated that reactive landing represents a unique method for modification of metal/metal oxide surfaces including stainless steel.²⁶ Whereas it is impossible to electrospray zirconium dioxide directly because it is insoluble, it is known that reactive organozirconium compounds can readily undergo decomposition that leads to stable zirconium dioxide as a product. For instance, zirconium(IV)-*n*-propoxide in glass pores can be converted into zirconium dioxide by drying at room temperature and then heating. Thus, zirconium(IV)-*n*-propoxide is commonly used as a

precursor for the preparation of zirconium dioxide,²⁷⁻²⁹ an approach that was also adopted here.

Zirconium(IV)-*n*-propoxide is commonly used for zirconium oxide thin film preparation by atmospheric-pressure electrospray, which is combined with thermal decomposition on a hot substrate surface,³⁰⁻³³ as reviewed.³⁴ Reactive landing differs from the previous atmospheric-pressure techniques in that decomposition on the cold surface is achieved by collisions of ion projectiles at hyperthermal kinetic energies in vacuum.

EXPERIMENTAL SECTION

Materials

Zirconium(IV)-*n*-propoxide was obtained from Sigma-Aldrich as 70% solution in 1-propanol. Solvents were purchased from Fisher, U.S.A. The peptides used to characterize phosphopeptide enrichment on the zirconium oxide functional surfaces include the synthetic peptides NQLLPPLR, MpSGIFR, MSGIFR, and pYWQAFR that were purchased from Genscript Corporation, Piscataway, NJ, as well as peptides from the tryptic digestion of α -casein. Sequence grade trypsin and α -casein were purchased from Sigma-Aldrich. The tryptic digestion of α -casein was performed at a 1:50 enzyme to substrate weight ratio in 50 mM NH_4HCO_3 and incubated overnight at 37 °C. Organic acids used as MALDI matrixes (α -cyano-4-hydroxycinnamic acid, CHCA, and 2,5-dihydroxybenzoic acid, DHB) were purchased from Sigma-Aldrich.

MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired on an Applied Biosystems 4700 proteomics analyzer system operated in positive-ion mode. Optimum precursor ion signals in MALDI/time-of-flight (TOF) analyses, operated in reflectron mode, were acquired with matrix solutions consisting of either 1 mg/mL CHCA in 70% acetonitrile with 0.1% trifluoroacetic acid (TFA) or 1 mg/mL DHB in 50% acetonitrile with 0.1% TFA. The laser power was set to 3500 instrument units for analyses using CHCA and 5000 units for analyses with the DHB matrix.

Alteration of Standard MALDI Plates

A standard stainless steel MALDI plate was modified by milling off several layers of material from the area of approximately 20 mm $\times$ 20 mm and 2 mm depth to accommodate the zirconium oxide coated substrate plates. The substrate plate was mounted inside the depression with scotch tape and thus leveled with the rest of the MALDI plate surface. In principle, reactive landing could be done on the MALDI plate directly without having to mount externally prepared surfaces. The reason for our current approach is the higher price of commercial MALDI plates as well as the fact that smaller chips of cut stainless steel are easier to handle inside the soft-landing instrument, which has relatively narrow chambers.²³

X-ray Photoelectron Spectroscopy

XPS spectra were taken on a Surface Science Instruments (SSI) S-Probe ESCA instrument. This instrument uses a monochromatized Al K α X-ray source for photoemission stimulation and a low-energy electron flood gun for charge neutralization. The Service Physics ESCAVB Graphics Viewer program was used to determine peak areas.

Surfaces

Metal targets used as substrates for deposited zirconium dioxide were made of approximately 15 mm $\times$ 15 mm 316L stainless steel plates. The plates were mechanically polished with a

diamond paste, rinsed successively with hexane, methanol, and water, sonicated in 1:1 chloroform/methanol for 15 min, rinsed with methanol, and exposed to oxygen plasma immediately after cleaning.

In Situ Plasma Treatment

The custom-made plasma reactor is similar to that described by Ratner³⁵ and was operated at 13.56 MHz. It is an integral part of the soft and reactive landing instrument as described previously.²³ The surface treatment was carried out at 60 W rf power for 10 min in 250 mTorr of flowing oxygen gas.

Preparation of Surfaces by Reactive Landing

For reactive landing experiments, 70% zirconium(IV)-*n*-propoxide in 1-propanol was diluted in the same solvent to the final concentration of 10^{-5} M. The solution was electrosprayed in positive mode, and the ions were landed on a freshly plasma-treated stainless steel surface. The landing experiment lasted for 3 h, and the landing substrate was biased at -50 V corresponding to a landing energy of 50 eV for singly charged ions. The gas-phase ions consisted of a mixture of alkoxyated Zr clusters similar to those reported for negative-ion electrospray of Zr(IV) alkoxides.³⁶ The cations were not mass-separated before reactive landing. The sample surface was then removed from the instrument and further examined. The areas on the landing substrate to be tested for phosphopeptide enrichment and MALDI analysis are sketched in Figure 1a. The landed material formed a compact spot that was visible by the naked eye (Figure 1b). The spot was successively rinsed with water, methanol, propanol, and hexane and then soaked in water/propanol mixture for 4 h. All samples that were prepared and used in the presented study did not visibly change upon multiple washings. The zirconium oxide coating remained intact even after it was rubbed with wet soft material such as filtration paper or cotton wool.

In a control experiment, zirconium(IV)-*n*-propoxide was dried on a stainless steel surface that was previously treated by plasma oxidation. No zirconium oxide coating was achieved as no Zr was detected by XPS after the sample was rinsed. In another experiment zirconium(IV)-*n*-propoxide was electrosprayed and deposited at atmospheric pressure on a stainless steel surface that was previously treated by plasma oxidation. The electrospray needle was kept at 4 kV, and the target surface was grounded and kept at room temperature. Under these conditions, the ions produced by electrospray acquire thermal kinetic energies, as reported by Morozov and Morozova.³⁷ However, electrospray at atmospheric pressure and room temperature produced no permanent zirconium oxide coating. Thus, we conclude that to form an adherent zirconium oxide coating on the target surface the zirconium-carrying ions must strike the surface at hyperthermal kinetic energies, e.g., 50 eV in our reactive landing method. The structure and more detailed composition of surfaces with reactively landed zirconium(IV) oxides, as well as studies focused on comparison with other, thermally assisted, zirconium oxide deposition techniques,³⁰⁻³⁴ are currently under investigation in this laboratory.

Phosphopeptide Enrichment

In preparation for phosphopeptide enrichment the surfaces were washed by agitating in 10 mL aliquots of methanol (three times), 100 mM NH_4OH (two times, 10 min each), water (three times), and then equilibrated in a wash/bind solution consisting of an aqueous mixture of 20% acetonitrile and 0.1% TFA. Peptide mixtures, diluted to the desired concentration in the wash/bind solution, were then applied to the assay surface and incubated at room temperature for 20 min. Longer incubation times did not increase phosphopeptide enrichment. Approximately 10 min after application, each peptide spot was mixed with a micropipettor by drawing in and expelling the droplet back onto the original assay surface. After 20 min of incubation the spots were washed with the wash/bind solution either by applying and removing several 30 μL portions of wash/bind solution to the assayed surface or by immersing and agitating the surface

in 25 mL of the wash/bind solution for 2 min. After the final wash the surfaces were allowed to dry and matrix was spotted directly onto the assayed area.

RESULTS AND DISCUSSION

Surface Characterization

The composition of the zirconium dioxide surfaces was examined by XPS. A composition scan (Figure 1c) confirmed that the spot of the landed material contained zirconium. The detected zirconium/oxygen ratio ranged from 1:3.3 to 1:3.8 for different samples. This is comparable to the zirconium/oxygen ratios of 1:3.3, which were previously reported for the oxide formed by thermal decomposition of zirconium(IV)-*n*-propoxide on Si substrates that was studied by XPS.²⁹ The higher oxygen content compared to the stoichiometric composition of zirconium dioxide (1:2) is probably caused by surface contamination with oxygen-containing organic compounds giving an unresolved O(1s) line at an energy close to 530.7 eV. The peak at 285 eV is due to contamination by C–C bound carbon that is detected on blank surfaces, as well. No Fe, Cr, or Ni were detected by XPS on the coated areas indicating that the stainless steel substrate was completely covered by a zirconium oxide layer that was thicker than the normal XPS sampling depth.

Phosphopeptide Retention on Zirconium Oxide Surface

To determine the ability of zirconium oxide surfaces to enrich phosphopeptides we first attempted to capture and elute model peptides from zirconium oxide spots prepared by reactive landing of zirconium(IV)-*n*-propoxide. A mixture consisting of a phosphorylated peptide (MpSGIFR) at 10 μ M and its nonphosphorylated analogue (MSGIFR, 10 μ M) was prepared in the bind/wash solution described above. This mixture, 25 μ L (250 pmol), was then applied to the zirconium oxide surface and allowed to bind. After washing away the unbound portion with bind/wash solution, the surface was eluted with 10 μ L of 100 mM NH₄OH. After diluting this elute to 100 μ L in 50% methanol the solution was directly infused into a Bruker Esquire ion-trap mass spectrometer. Neither peptide was detected in this first elution by ESI-MS analysis in the negative-ion mode (Figure S1a, Supporting Information). The surface was then eluted with 500 mM NH₄OH, and only the phosphopeptide, MpSGIFR could be detected, as evidenced by the (M – H)[–] ion at *m/z* 788 (Figure S1b, Supporting Information). The surface was eluted again with 1 M NH₄OH to reveal even more eluted phosphopeptide, whereas the peak of MSGIFR (*m/z* 709) was not detected (Figure S1c, Supporting Information). Note that the relative responses of MSGIFR and MpSGIFR are ~1:2 in a negative-ion mode, as illustrated by ESI-MS analysis of a mixture that was 1 μ M in each peptide (Figure S2, Supporting Information). These results illustrated that the zirconium oxide surface had the ability to selectively capture phosphopeptides. However, phosphopeptide elution from the surface was slow and resulted in sample dilution. In complex mixtures such as tryptic digests, tightly bound peptides, especially multiply phosphorylated forms, may elute with low efficiency, thus limiting sensitivity. Since the binding mechanism of phosphopeptides to zirconium oxide surfaces is currently not well understood to allow us to optimize the release conditions, elimination of the elution step was deemed to be advantageous as it would greatly simplify the analysis. This was achieved by direct enrichment on zirconium oxide plates followed by MALDI-TOF-MS analysis, as described next.

Direct Enrichment on Zirconium Oxide Coated MALDI Plates

For MALDI-TOF-MS experiments, phosphopeptide enrichment was investigated on four types of surfaces: (1) zirconium oxide coatings prepared by reactive landing of Zr(IV)-*n*-propoxide, (2) plasma-oxidized Zr metal, (3) plasma-oxidized stainless steel, and (4) untreated stainless-steel as a reference. Each enrichment assay was compared to blank and control assays that were performed in the arrangement illustrated in Figure 1a. The modified stainless steel shims were

affixed to the MALDI plate that had been altered to accommodate facile loading into the MALDI source chamber. The enrichment capabilities of each surface were initially tested using a mixture of synthetic phosphopeptides. To evaluate the concentration dependence and detection limits, we performed the enrichment assay on three peptide mixtures, consisting of 10 μ M MSGIFR as reference and either 10 μ M, 1 μ M, or 100 nM of each of the three synthetic phosphopeptides NQLLPPLR, MpSGIFR, and pYWQAFR. These solutions were applied to the surfaces in 10 μ L aliquots. Since the zirconium oxide spots have approximately 10-fold greater surface area than a spot on a usual MALDI plate, the surface concentration of phosphopeptides for each assay is 10 pmol, 1 pmol and 100 fmol per equivalent MALDI spot. Each enrichment on this zirconium oxide surface was not only compared to the blank and control enrichment on plasma-oxidized stainless steel but also to a standard, nonenriched sample that was spotted directly onto the MALDI plate.

The MALDI-MS spectra of the standard and enriched mixture of a 10:1 MSGIFR–phosphopeptide mixture are shown in Figure 2, top and bottom, respectively. The MALDI-MS spectra from the enrichment of the 100:1 and 1:1 MSGIFR to phosphopeptide mixtures are given as Supporting Information (Figure S3 and Figure S4, respectively). The enrichment resulted in a significant enhancement of the phosphopeptide signals, whereas the non-phosphopeptide signal was reduced to near baseline levels (Figure 2, bottom). Also notable in these spectra is the amount of oxidation observed for the Met residues and Trp residues included in this set of synthetic phosphopeptides. The Met residue is nearly completely converted to a singly oxidized sulfoxide form (m/z 806), whereas the Trp residue is less efficiently converted to both a singly (m/z 966) and doubly oxidized (m/z 982) form (Figure 2, bottom). The increased oxidation occurring on the zirconium oxide coated plates may simply be due to a longer exposure of the sample to air, or perhaps these surfaces exhibit some catalytic activity. If peptide oxidation presents an interference, it may be suppressed by adding an antioxidant such as dithiothreitol or tris-(carboxymethylphosphine) to the sample.

For quantitative purposes we defined an enrichment factor for each peptide in the enrichment assay based on a percent ratio of summed signal intensities for all ion forms of each peptide in both the standard (ΣI_0) and enriched (ΣI_{enr}) MALDI spectra. The spectral data showed that the peptide signals for MSGIFR were reduced to 0.2% of their peak area intensities in the standard sample. The peak area intensities for the phosphopeptides MpSGIFR, pYWQAFR, and NQLLPPLR, on the other hand, were only reduced to 12.1%, 11.5%, and 11.2%, respectively, of their pre-enriched peak area intensities. Thus, each phosphopeptide is enriched by a factor

$$f = \left\{ \sum I_{\text{enr}} (\text{phosphopeptide}) / \sum I_0 (\text{phosphopeptide}) \right\} / \left\{ \sum I_{\text{enr}} (\text{MSGIFR}) / \sum I_0 (\text{MSGIFR}) \right\}$$

of approximately 60 compared to the MSGIFR non-phosphopeptide.

To further test the abilities of this zirconium oxide surface to enrich phosphopeptides from complex mixtures we resorted to analyzing phosphopeptides from a tryptic digest of α -casein. Aliquots of the α -casein digest were diluted to 10 μ M, 1 μ M, and 100 nM in the bind/wash buffer described earlier. After allowing the sample to bind for 20 min with one mix at 10 min, the surface was washed by submerging and agitating for 2 min in 25 mL of bind/wash buffer. The MALDI-TOF-MS analysis of the enriched sample was compared to a standard, control, and blank sample, as described earlier. The control analysis (Figure S5a, Supporting Information) showed no phosphopeptide enrichment by the plasma-oxidized stainless steel, and the blank analysis (Figure S5b, Supporting Information) illustrated that the peptides detected after enrichment were not from carryover due to incomplete washing.

The enrichment of 1 pmol of α -casein peptides per MALDI spot is shown in Figure 3, parts a and b. Comparison of the standard (Figure 3a) to the enriched sample (Figure 3b) showed that the singly phosphorylated peptides $\alpha_{S1}(121-134)(VPQLEIVNP\textbf{p}SAEER)$ at m/z 1660 and $\alpha_{S1}(119-134)(YKVPQLEIVNP\textbf{p}SAEER)$ at m/z 1951 were significantly enriched compared to the non-phosphopeptides. The enrichment factors for phosphopeptides $\alpha_{S1}(121-134)$ and $\alpha_{S1}(119-134)$ ranged between $f=20$ and $f=50$ when related to the two prevalent non-phosphopeptides at m/z 1267 and m/z 1760. For the assignment of the major peaks in the MALDI-TOF mass spectrum, see Table 1. On the basis of absolute ion intensities, the non-phosphopeptide signals at m/z 1267 and m/z 1760 were reduced to $<3.8\%$ and 1.8% , respectively, in the enriched spectrum relative to their peak areas in the standard spectrum. In contrast, the phosphopeptide signals at m/z 1951 and m/z 1660 were reduced only to 50% and 78% relative to their peak areas in the standard spectrum. Hence, the zirconium oxide surfaces showed substantial selectivity in retaining the phosphopeptides.

The MALDI-TOF mass spectrum in Figure 3b showed ions generated by postsource decay losses of phosphoric acid from the two phosphopeptides. The ion at m/z 1566 resulted from the elimination of H_3PO_4 from RS1 (121–134), whereas the ion at m/z 1856 resulted from the same loss from α_{S1} (119–134). These fragments had characteristically poor resolution and were apparent at masses higher than their actual values (m/z 1562 and 1853, respectively) as a result of an inherent limitation of the MALDI-TOF experiment. However, we note that MALDI from the zirconium oxide layer did not result in ion defocusing, as illustrated by the comparable peak widths for stable nondissociating ions in Figure 2, top and bottom, and Figure 3, parts a and b.

Although the zirconium oxide coated surface turned out to be highly efficient at enriching singly phosphorylated peptides, multiply phosphorylated peptides from the α -casein digest were not detected by MALDI-TOF. A similar result was also observed in a previous study of the enrichment of casein phosphopeptides by oxidized Zr.¹⁶ Here we tested the possibility that a DHB matrix may be more suitable for phosphopeptide detection than CHCA. It should be noted that, in our study, the optimal concentration for each matrix component is at less than saturation in their respective mixtures. Typically, saturated matrix solutions are used for MALDI analyses. However, since we are working with peptides that are bound in a thin layer on the assay surface, the use of more dilute matrix solutions prevents the sample from being buried under layers of matrix. Figure 4 shows the analysis of α -casein phosphopeptides enriched on reactively landed zirconium oxide spots by MALDI-MS using the DHB matrix solution described in the Experimental Section. Multiply phosphorylated peptides are still not detected with the DHB matrix. The inability to detect these highly phosphorylated components may simply reflect a limit of MALDI to produce cations from acidic peptides. Comparison of Figures 3 and 4 suggests that although DHB may be more selective for phosphopeptides, CHCA provides higher ion counts and thus is far more sensitive.

As another characterization of the surfaces generated by reactive ion landing, we examined the durability, ruggedness, and surface-to-surface reproducibility of the zirconium oxide coatings. Three separate spots were prepared, and each was used for phosphopeptide enrichment 20 times over the period of 4 months without generating any visible degradation or inhomogeneity in the white zirconium oxide coating. After each enrichment, the functional surfaces were regenerated by first soaking in 100 mM NH_4OH twice for 20 min with agitation, followed by soaking in excess water and methanol. After 20 enrichments each, the three spots were compared for their ability to detect phosphopeptides from 1 pmol of peptide mixture produced by tryptic digestion of α -casein. These three 4 month old spots each enriched the two singly phosphorylated peptides, $\alpha_{S1}(121-134)$ and $\alpha_{S1}(119-134)$, by a factor of 20–60-fold compared to the two abundant non-phosphopeptides. For further comparison, a new zirconium oxide surface was prepared and found to enrich the same singly phosphorylated α -casein peptides by

a factor of 90 compared to the two abundant nonphosphorylated peptides. Thus, although the newly prepared surface was more efficient at enriching phosphopeptides, the older zirconium oxide surfaces were still capable of enriching phosphopeptides even after being used repeatedly over the course of several months.

In view of the good performance of the zirconium oxide surfaces made by ion reactive landing, we also examined the possibility of phosphopeptide enrichment on plain plasma-oxidized sheets of zirconium metal. Those were prepared by standard plasma oxidation treatment, and no soft or reactive ion landing was involved in their preparation. Plasma oxidation is a well-established and widely available technique and could be used for the rapid production of MALDI surfaces for phosphopeptide enrichment. The assay described above for enrichment on soft-landed Zr(IV)-*n*-propoxide was repeated on the plasma-oxidized Zr metal. Again, phosphopeptides from a mixture of synthetic phosphopeptides could be enriched down to 100 fmol per MALDI spot in the presence of 100 pmol of the non-phosphopeptides (Figure S6, Supporting Information). However, when handling smaller amounts of phosphopeptides, the zirconium oxide coating produced by reactive ion landing showed far superior enrichment capabilities than the plasma-oxidized Zr metal. In fact, the plasma-oxidized Zr surface was inadequate to achieve enrichment from more complex mixtures such as the tryptic digest of α -casein (Figure S7, Supporting Information).

CONCLUSIONS

Zirconium oxide surfaces provide a useful tool for the in situ enrichment and detection of phosphopeptides by MALDI-TOF mass spectrometry. In particular, zirconium oxide coated spots that were prepared by reactive landing of Zr(IV)-*n*-propoxide ions on stainless steel substrates proved to be efficient in enriching singly phosphorylated peptides in mid-femtomole amounts for both synthetic peptide mixtures and a tryptic digest of α -casein. Enrichment factors on the order of 20–90-fold were achieved when compared to the abundant non-phosphopeptides. The zirconium oxide coated surfaces showed good reproducibility even after having been reused up to 20 times without significant loss in enrichment efficiency. An advantage of zirconium oxide coatings is that they can be produced on stainless steel supports in a variety of sizes by focusing or defocusing the ion beam used for reactive landing.²⁶ Further characterization of the reactively landed zirconium oxides and their ability to enrich a wide variety of phosphopeptides is necessary for more complete characterization of these novel materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

(a) Layout of a typical enrichment assay on modified surfaces. (b) Digital picture of a zirconium oxide spot from reactively landed Zr(IV)-*n*-propoxide. (c) XPS composition scan taken from a zirconium oxide spot from reactively landed Zr(IV)-*n*-propoxide.

Figure 2.

Top: MALDI-MS analysis of 10 μM MSGIFR (MH^+ m/z 710.3), 1 μM MpSGIFR (MH^+ m/z 790.3), 1 μM pYWQAFR (MH^+ m/z 950.3), and 1 μM NQLLP TPLR (MH^+ m/z 1033.5).

Bottom: Enrichment of the same peptide mixture on zirconium oxide prepared by the reactive landing of Zr(IV)-*n*-propoxide. Peptides with an oxidized Met or Trp are denoted with (ox) per oxygen atom.

Figure 3.

(a) Standard: Positive-ion MALDI-MS analysis of nonenriched α -casein tryptic peptides (1 pmol). A single asterisk denotes a phosphopeptide, whereas double asterisks indicate peptides derived from PSD loss of phosphoric acid during the MALDI-MS experiment. (b) Enrichment: MALDI-MS analysis of α -casein tryptic peptides (1 pmol) enriched for phosphopeptides on zirconium oxide modified MALDI surface. Peak annotation as in (a). Known peak assignments are given in Table 1.

Figure 4. Positive-ion MALDI-MS analysis using a DHB matrix of α -casein tryptic peptides (1 pmol) enriched for phosphopeptides on zirconium oxide modified MALDI surface. A single asterisk denotes a phosphopeptide, whereas double asterisks indicate peptides derived from PSD loss of phosphoric acid during the MALDI-MS experiment.

Table 1List of Abundant Peptide Peaks in the MALDI-MS Spectrum of α -Casein Tryptic Digest

<i>m/z</i>	α -casein variant	residues	sequence
1195	S2	130–140	(R)/NAVPTPTLNR/(E)
1267	S1	106–115	(K)/YLGYLEQLLR/(L)
1337	S1	95–105	(K)/HIQKEDVPSEK/(Y)
1385	S1	38–49	(H)/FFVAPFPEVFGK
1660	S1	121–134	(K)/VPQLEIVPNpSAEER/(L)
1760	S1	23–37	(K)/HQGLPQEVLENENLLR/(F)
1951	S1	119–134	(K)/YKVPQLEIVPNpSAEER/(L)