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Titanium Dioxide Coated MALDI Plate for On Target Analysis of Phosphopeptides

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INTRODUCTION :

Titanium oxide is a widely investigated material for application in several fields such as: pigment, photovoltaics¹, photocatalysis and self cleaning surfaces², gas sensing³, photo-electronics devices⁴, biology and medicine.

Among what are commonly addressed as bio-applications it is possible to identify more specific areas. Nanostructures with high surface area have been studied for protein immobilization and biosensing applications⁵⁻⁶. Recently it has been shown that titanium dioxide has a peculiar affinity for the phosphate group and this property has been exploited in phosphorylated proteins enrichment⁷.

Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in all organisms. Early estimates are that one third of cellular proteins are phosphorylated⁸ and it is a reasonable conclusion that all cellular functions are directly or indirectly affected by protein phosphorylation. Great interest in acquiring a complete knowledge of phosphorylation mechanisms can be due also to the fact that more than a hundred different protein kinases have been implicated in human cancer⁹. As a branch of proteomics, phospho-proteomics involves the analysis of protein phosphorylation and makes use of mass spectrometry (MS) and specific methods to purify phosphorylated proteins and peptides. The main goals of a phospho-proteomic experiment are the enrichment of phosphate-containing proteins or peptides and their characterization by MS. Thus, the

major challenge for the application of phospho-proteomics lays in successfully subtracting phospho-proteins from the whole cell lysate (phosphorylation is usually substoichiometric) with a focus on identification of low-abundant phospho-proteins. To date, several strategies have been developed to enrich samples for phosphopeptides and to remove non-phosphorylated acidic peptides. One strategy is based on the specific affinity between antibodies and phosphorylated amino acids. Another commonly used strategy is immobilized metal ion affinity chromatography (IMAC)¹⁰, a standard method for specifically enriching phosphorylated peptides prior to MS, where Fe^{3+} or Ga^{3+} ions are commonly used. Alternatively, metal oxides such as ZrO_2 , TiO_2 , and Al_2O_3 have been used to selectively concentrate phosphopeptides, where the phosphate functional groups can bind to the surface of metal oxide particles. Because of their stability over a wide pH range, acidic buffers can be employed to avoid non-specific binding¹¹.

Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry¹² is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. MALDI is based on the bombardment of sample molecules with a laser light to induce sample ionisation. The sample is pre-mixed with a highly absorbing matrix compound and deposited onto a stainless steel plate. The time-of-flight (TOF) analyser separates ions according to their mass(m)-to-charge(z) (m/z) ratios by measuring the time it takes for ions to travel through a field free region known as the flight tube. Common for MS strategies is that the proteins are enzymatically digested into peptides, which are then analyzed by MS. The analysis of the molecular mass of a phosphopeptide will indicate the presence or absence of a phosphate group. The verification of a phosphate group and location of the exact phosphorylation site on a phosphopeptide can be performed using MS/MS, where the peptide is activated in the gas phase and fragmented to produce informative fragment ions. As stated before, nanostructured titania could be an ideal candidate for phosphorylated protein enrichment before MS analysis.

Pulsed Laser Deposition (PLD) is a versatile technique for the deposition of nanostructured films even with complex stoichiometry and with a fine control on the material properties. In PLD an intense pulsed laser beam is focused on a solid target placed in a vacuum chamber. Energy absorption from the laser leads to target ablation and to formation of plasma that expands inside the chamber and then recondenses on a substrate where film growth takes place¹³. The control on plasma expansion dynamics allows to obtain films with tailored morphology¹⁴, pores size¹⁵ and structure¹⁶.

Our objective goes in the direction of simplifying phosphopeptides analysis procedures providing a unique tool, a Titanium dioxide coated MALDI plate, designed to permit enrichment and detection with the same platform, eliminating tedious washing and centrifugation steps, speeding up analysis and giving at the same time a high sensitivity due to the benefits given by the use of a nanoscaled technology.

EXPERIMENTAL :

Preparation of Standard Protein Digests. α - and β -casein, RNase, BSA, and myoglobin were reduced, alkylated and digested using trypsin. **MALDI-TOF Mass Spectrometry.** Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired on a Voyager DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). **MALDI-TOF/TOF Mass Spectrometry.** A 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) was used to perform MS/MS experiments were in reflectron mode with CID gas on. **MALDI T-Plate Fabrication.** Standard stainless steel MALDI TOF and MALDI TOF/TOF plates were coated with titanium oxide thin films (200 nm thickness) by pulsed laser deposition. The stainless steel deposition chamber was equipped with a turbomolecular pump connected to a primary pump and with a gas inlet system with pressure gauges for fine pressure control from high vacuum (10⁻⁵ Pa) to the atmosphere. Here a Ti target was ablated in 20 Pa O₂ pressure exploiting UV laser pulses (duration \approx 7 ns) from a quadrupled Nd:YAG laser (λ) 266 nm, 10 Hz repetition rate). Then 2600 laser pulses were focused on the target with an energy density (fluence) of about 3 J/cm². Titanium oxide was deposited at room temperature and subsequently annealed in air at 400°C for 1 h. A copper grid was applied to the MALDI plates for titanium dioxide deposition in specific wells.

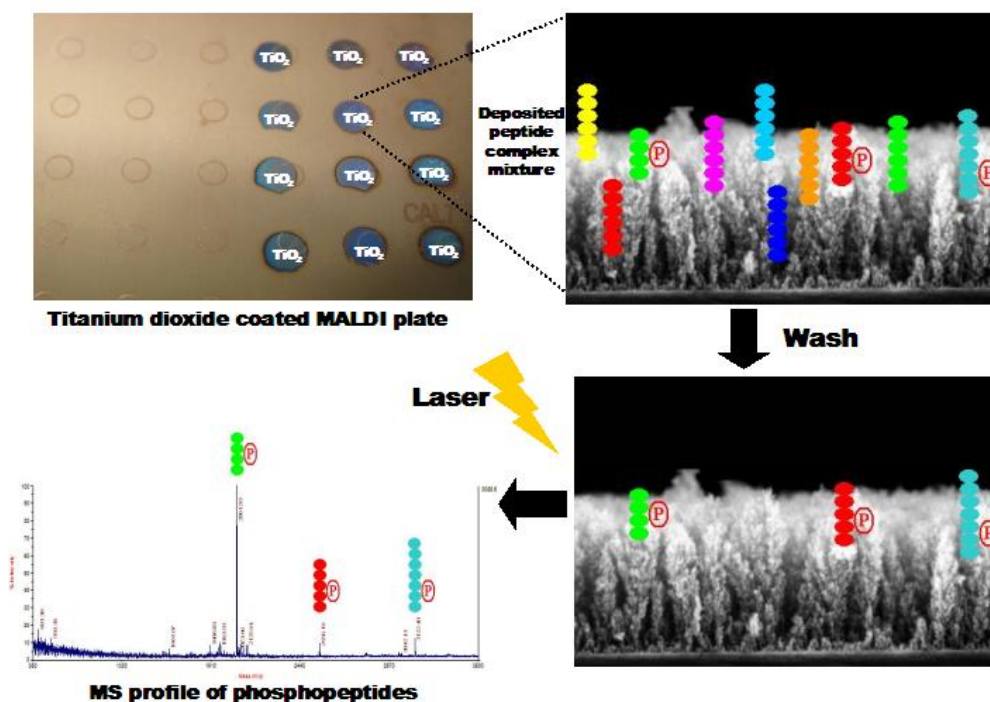
RESULTS AND DISCUSSION :

The tool we developed consists in a new MALDI plate which is selectively coated by means of PLD with a titanium dioxide film with a thickness of about one hundred of nanometers. This support (T-plate) works both as a device for the purification and for the analysis of phosphorylated and non-phosphorylated peptides. Performances of this coated MALDI plate in the enrichment of tryptic phosphopeptides were tested by using α - and β -casein (two standard phosphorylated proteins) in complex mixtures together with non-phosphorylated proteins. The experimental strategy consisted in depositing a small amount of this mixture containing both phosphorylated and non-phosphorylated peptides onto the T-plate. As only peptides containing phosphogroups interact with the Titanium dioxide coating, after washing away from the surface the non-specific bound peptides, only the species of interest are detected by MS. Part of the work consisted in the

optimization of the enrichment and analysis conditions, to reach high selectivity and good sensitivity towards phosphopeptides.

Samples containing protein digests were mixed with a loading solution (that improve the specific binding) and deposited onto the T-plate. After rinsing the deposited sample with the loading solution most of the non-phosphorylated peptides were washed away and, following matrix deposition, the most abundant signals detected by MALDI-TOF were originated from phosphopeptides. The matrix used for the detection has been also optimized and a 4:1 mixture of dihydroxybenzoic acid and α -cyano-hydroxycinnamic acid was found to give the best selectivity towards phosphopeptides. The new plate showed a good sensitivity as it was possible to detect the presence of phosphopeptides even with 25 fmol starting material.

Adaptability of the T-plate to different platforms was demonstrated as it has been used both with MALDI-TOF and TOF/TOF instruments; in this last case the aminoacidic sequence and the identification of the phosphorylated sites were obtained by MS/MS measurements. Moreover it was possible to elute the phosphorylated peptides from the T-plate, after deposition and enrichment, by applying 0.5% NH_4OH and analyze the recovered phosphorylated peptides by liquid chromatography (LC)-MS/MS. Thus the T-plate platform also offers the possibility of being re-used after cleaning the active surface.



CONCLUSIONS :

A new MALDI target, called T-plate, was produced by exploiting pulsed laser deposition of a nanostructured titanium dioxide thin film onto a stainless steel plate. The advantages of using the T-plate involve practical use, fast experimental steps and the possibility of using the same active surface many times. The compatibility with a MALDI-TOF/TOF instrument could then open the perspective of using it for the identification of phosphosites in complex biological samples, exploiting the high mass accuracy in the MS/MS mode and the possibility of coupling an LC device for the separation and automated deposition of sample fractions.

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