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Removal of Polyethylene Glycols from Protein Samples using Titanium Dioxide

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Introduction

Polyethylene glycols (PEGs) are widely used in biological and pharmaceutical analysis to stabilize proteins for separation, purification, storage, and delivery¹ and they also are used as the precipitants in crystallization process for X-ray crystallography of proteins.² However, the analysis protein and peptide samples contaminated with PEG using mass spectrometry either using electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is very challenging due to ion signal suppression and interference from the PEGs. Many off-line and on-line sample cleanup methods including LC column, capillary electrophoresis, membrane filtration, solid-phase extraction, dialysis and ion exchange cartridge, *etc.* have been implemented to remove salts, detergents, or other small molecules from complex biological samples before ESI and MALDI mass analysis.^{3–7}

Unfortunately, samples with high concentration PEGs are still problematic. They can easily carry over to the sample fraction and frequently contaminate LC columns. Furthermore, sample purification procedures are time consuming and low throughput.

Very recently, a titanium dioxide (TiO₂) microcolumn has been developed as an efficient method to enrich phosphorylated peptides.^{8–12} In this study, this same microcolumn is shown to assist rapid removal of high concentration PEG contaminants from protein and peptides samples.

Methods

Materials

Protein samples (cytochrome *c* and α -casein¹³) and all the solvents used were obtained from Sigma (St. Louis, MO). Trypsin was obtained from Promega (Madison, WI). The three wall-coated titanium dioxide micropipette tips (Trap'nTipsTM, TiO₂) were obtained from New Objective (Woburn, MA).

Sample preparation

For the whole protein example, 2 μ M α -casein or cytochrome *c* was mixed with 20 μ M PEG 4000 before TiO₂ cleanup. For the peptides example, 1 μ g α -casein was digested with trypsin at 1:25 (w/w) in 100 mM ammonium bicarbonate (pH 8.0) overnight at 37°C and then 20 μ M PEG 4000 was added into this digestion mixture before TiO₂ cleanup.

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TiO₂ cleanup procedure

For the phosphorylated protein and peptides (α -casein and α -casein digest), the sample was dissolved in a 10 μ l solution of 20 mg/ml 2, 5-dihydroxy-benzoic acid (DHB) with 80% acetonitrile, 0.1% TFA, and then was loaded onto the TiO₂ tip which had been previously wetted by the solution of 80% acetonitrile, 0.1% TFA. After loading the sample, the TiO₂ tip was washed by both 20 mg/ml DHB with 80% acetonitrile, 0.1% TFA (3 times) and 80% acetonitrile, 0.1% TFA solutions (7 times) and then the sample was eluted by 75 mM ammonium hydroxide (NH₄OH, PH = 10.5) in water. For unphosphorylated proteins (cytochrome *c*), the sample was dissolved in 10 μ l solution of 20 mg/ml DHB with 10% acetonitrile, 0.1% TFA, and then was loaded onto the TiO₂ tip which had been previously wetted by the solution of 10% acetonitrile, 0.1% TFA. After loading the sample, the TiO₂ tip was washed by both 20 mg/ml DHB with 10% acetonitrile, 0.1% TFA (3 times) and 10% acetonitrile, 0.1% TFA solutions (7 times) and then the sample was eluted by 80% acetonitrile, 0.1% TFA. All the protein and peptide samples after cleanup were dried under the speedvacuum and reconstituted in 50% acetonitrile, 1% formic acid.

FTMS instrumentation

A custom built qQq-FTMS with a nanospray source and 7T actively shielded magnet (Cryomagnetics Inc., Oak Ridge, TN) was utilized in this study.^{14, 15} A ~2–3 μ l sample was loaded into a glass capillary tip pulled with a micropipette puller (Model P-97, Sutter Instruments Co., Novato, CA) to ~1 μ m orifice diameter and a stainless steel wire was inserted into the distal end of the tip to form the electrical connection. For all the samples, around 1100 V spray voltage was used to form the spray.

Results and discussion

Recently, TiO₂ microcolumns have been widely utilized in enrichment of phosphorylated peptides, and it was discovered that the addition of 2, 5-dihydroxy-benzoic acid (DHB) helps to efficiently displace nonphosphorylated peptides from the TiO₂ while retaining the phosphorylated peptides.^{8–12} Here, this microcolumn was tested for efficient removal of high concentrations of PEG contaminants from protein and phosphorylated peptides samples. Figure 1 (left) shows the results of this study with whole phosphorylated protein. Figure 1A (left) shows the 14⁺ to 27⁺ charge states of α -casein. When 2 μ M α -casein is mixed with 20 μ M PEG 4000, the PEG charge state distributions are clear¹⁶, but the α -casein is almost unobservable (Figure 1B left). This is a classic example of the matrix effect, also known as the signal suppression effect and is common in electrospray mass spectrometry. After sample cleanup, as described above, the PEG 4000 peaks were completely eliminated (Figure 1C left) and the α -casein peaks are again observable. Furthermore, additional “chemical noise” baseline between m/z 1000–2000 is also suppressed. Based on the previous work with these columns^{8–12}, most likely, both phosphorylated protein and PEG are bound to the TiO₂ microcolumn, and then the PEG was washed off the column by 80% acetonitrile, 0.1% TFA with DHB due to the large molar excess of DHB which competes for adsorption to the surface of TiO₂. The phosphorylated protein was then eluted by NH₄OH (pH = 10.5).

TiO₂ can also separate high concentration PEG from unphosphorylated proteins. For example, Figure 1A (right) shows the 8⁺ to 18⁺ charge states of cytochrome *c*, Figure 1B (right) shows the spectrum of a mixture of 2 μ M cytochrome *c* and 20 μ M PEG 4000; the cytochrome *c* peaks are not observable. Cytochrome *c* charge state distributions are rescued after TiO₂ cleanup, albeit not to the same extent as was observed with the phosphoprotein α -casein (Figure 1C right). The conditions used to wash the TiO₂ microcolumn in this unphosphorylated protein are little different from that of phosphorylated protein. The solutions of 10% acetonitrile, 0.1%

TFA with and without DHB were utilized to get rid of PEG and the protein was eluted by 80% acetonitrile, 0.1% TFA.

Figure 2 demonstrated that, as previously reported^{8–12}, phosphopeptides are efficiently enriched by TiO₂. The tryptic digest peptides of α -casein are observable in Figure 2A. While some compete effectively for signal against PEG 4000, most of the peaks are not observed, and phosphopeptides in particular are difficult to detect (Figure 2B). However, phosphopeptides are rescued by TiO₂ microcolumn cleanup (Figure 2C).

In summary, the method described here has demonstrated that the TiO₂ microcolumn can rapidly remove PEG contaminants from protein and phosphorylated peptide samples to improve the characterization of the proteins and peptides by mass spectrometry.

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Figure 1.

(left) A. ESI spectrum of 2 μ M α -casein. B. ESI spectrum of 2 μ M α -casein with 20 μ M PEG 4000. C. ESI spectrum of 2 μ M α -casein with 20 μ M PEG 4000 after TiO₂ cleanup. (right) A. ESI spectrum of 2 μ M cytochrome *c*. B. ESI spectrum of 2 μ M cytochrome *c* with 20 μ M PEG 4000. C. ESI spectrum of 2 μ M cytochrome *c* with 20 μ M PEG 4000 after TiO₂ cleanup. Scalefactor is inverse proportion to intensity, and baseline noise is constant so that signal/noise is a valid measure of relative intensity.

Figure 2.

A. ESI spectrum of 1 μ g α -casein tryptic digest. B. ESI spectrum of 1 μ g α -casein tryptic digest with 20 μ M PEG 4000. C. ESI spectrum of 1 μ g α -casein tryptic digest with 20 μ M PEG 4000 after TiO₂ cleanup.