Comprehensive protocol to simultaneously study protein phosphorylation, acetylation and N-linked sialylated glycosylation

Marcella Nunes Melo-Braga[*1], María Ibáñez-Vea[*2], Katarzyna Kulej[*3,4] and Martin R. Larsen[‡5]

1. Department of Biochemistry and Immunology. Institute of Biological Science. Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.
2. Genetics, Genomics and Microbiology Research Group. Health Science Department. Public University of Navarre. Pamplona, Navarra, Spain.
3. Division of Protective Immunity and Division of Cancer Pathobiology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA.
4. Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
5. Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

**Authors contributed equally to this chapter

‡Corresponding author: Martin R. Larsen, Ph.D., Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark.

Phone: +45 60111872; Email: mrl@bmb.sdu.dk

Running head: Workflow for simultaneously analysis of various PTMs

i. Summary/Abstract

Post-translational modifications (PTMs) such as phosphorylation, acetylation and glycosylation are an essential regulatory mechanism of protein function and interaction and they are associated with a wide range of biological processes. Since most PTMs alter the molecular mass of a protein, mass spectrometry (MS) is the ideal analytical tool for studying various PTMs. However, PTMs are often present in substoichiometric levels and therefore their unmodified counterpart often suppresses their signal in MS. Consequently, PTM analysis by MS is a challenging task requiring highly specialized and sensitive PTM specific enrichment methods. Currently, several methods have been implemented for PTM enrichment and each of them has its drawbacks and advantages as they differ in selectivity and specificity toward specific protein modifications. Unfortunately, for the vast majority of the more than 400 known modifications we have no or poor tools for selective enrichment.

Here, we describe a comprehensive workflow to simultaneously study phosphorylation, acetylation and N-linked sialylated glycosylation from the same biological sample. The protocol involves an initial titanium dioxide (TiO_2) step to enrich for phosphopeptides and sialylated N-linked glycopeptides followed by glycan release and post-fractionation using sequential elution from immobilized metal affinity chromatography (SIMAC) to separate mono-phosphorylated and deglycosylated peptides from multi-phosphorylated ones. The IMAC flow-through and acidic elution is subsequently subjected to a next round of TiO_2 enrichment for further separation of mono-phosphopeptides from deglycosylated peptides. Furthermore, the lysine-acetylated peptides present in the first TiO_2 flow-through fraction are enriched by immunoprecipitation (IP) after peptide cleanup. Finally, the samples are

fractionated by High pH Reversed Phase Chromatography (HpH) or Hydrophilic Interaction Liquid Chromatography (HILIC) to reduce sample complexity and increase the coverage in the subsequent LC-MS/MS analysis. This allows the analysis of multiple types of modifications from the same highly complex biological sample without decreasing the quality of each individual PTM study.

ii. Key Words

Protein post-translational modification (PTM) enrichment, phosphorylation, acetylation, sialic acid (SA) N-linked glycosylation, immunoprecipitation (IP), TiSH, comprising of titanium dioxide (TiO_2), sequential elution from immobilized metal affinity chromatography (SIMAC) and hydrophilic interaction liquid chromatography (HILIC)/High pH reversed phase chromatography (HpH), and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

1. Introduction

Proteins are frequently modified by post-translational modifications (PTMs) such as phosphorylation, acetylation and glycosylation. PTMs regulate protein structure, function and lifetime, modulating their activity in dynamic cells. Aberrations of protein regulation by PTMs can lead to the development of various disorders and diseases. Despite the pivotal role of PTMs in a range of biological processes, PTM analysis by mass spectrometry (MS) is a challenging task since modified peptides are present in sub-stoichiometric amount in comparison to their unmodified counterpart in proteolytic digests from cells or tissues. However, recent advances in PTM enrichment methods combined

with the new generation of MS instrumentation have tremendously contributed to large-scale studies of PTMs.

Nowadays, several techniques exist for phosphopeptide enrichment prior to MS analysis. The most extensively used methods involve metal ions for the binding of negatively charged phosphopeptides, *i.e.* immobilized metal affinity chromatography (IMAC) (1, 2), and metal oxide affinity chromatography such as titanium dioxide (TiO_2) (3-5). However, studies comparing various phosphopeptide enrichment methods showed that each method allows the isolation of distinct subset of phosphopeptides whereas none of the methods are able to provide a whole phosphoproteome, even though they partially overlap (6). On the other hand, this is highly dependent on the individuals performing the analysis and on the numerous protocols that exist for the phosphopeptide enrichment. The possibility to combine the strength of different enrichment methods by serial performance has substantially improved the phosphoproteomic field. For instance, the combination of IMAC and TiO_2 chromatography, known as “Sequential elution from IMAC” (SIMAC), has been employed to separate mono-phosphorylated peptides from multiply phosphorylated peptides in large-scale studies (7). IMAC has a stronger selectivity for multi-phosphopeptides than for mono-phosphopeptides, leading to a better characterization of the first ones. Following these enrichment methods, a new multi-dimensional large scale phosphopeptide enrichment strategy, known as the TiSH protocol (TiO_2-SIMAC-HILIC), was proposed to study phosphorylation from low amount of starting material (300 µg). The TiSH strategy includes an initial TiO_2 enrichment followed by SIMAC and HILIC fractionation to decrease the complexity of mono-phosphopeptide fraction [8]. The TiSH approach lead to several advances: (I) the TiO_2-based pre-enrichment step improves the

enrichment specificity compared to a setup utilizing only SIMAC; (II) TiO_2 is more robust than IMAC being compatible with several chemical reagents (8) and (III) HILIC fractionation of the mono-phosphorylated peptides increase the phosphopeptide coverage from complex mixtures. This increases throughput and allows for performing large-scale phosphoproteomics from low amounts of cells or biological tissues. The HILIC fractionation step of the monophosphorylated peptides can be replaced with High pH reversed phase (HpH) fractionation (9) of phosphopeptides to achieve a modified TiSH protocol, although the binding of phosphopeptides to RP material at higher pH can be significantly reduced, resulting in phosphopeptides in the HpH flow-through fraction (Larsen MR, unpublished observation).

Despite the high selectivity of TiO_2 towards phosphopeptides, it was later shown that TiO_2 also has a high selectivity toward sialylated N-linked glycopeptides, presumably due to the high negative charge of these molecules (10). Although there are several methods to enrich glycopeptides such as lectins (11) and hydrazine affinity purification (12), only TiO_2 has the capability to selectively isolate sialylated glycopeptides (10, 13). Due to these additional properties, TiO_2 has been successfully applied to simultaneously enrich phosphopeptides and sialylated N-linked glycopeptides (14-16).

Conversely to protein phosphorylation and glycosylation there is only one enrichment strategy available for Lysine acetylation, that it is antibody-based immune-precipitation (IP) (17). This strategy has allowed the identification of hundreds to thousands of lysine acetylation sites in different organisms such as bacteria (18), plant (19), parasite (20) and human (21). In addition, the IP of lysine acetylation has been combined with strong cation exchange (SCX) to simultaneously study acetylation and phosphorylation (22), as well as

with TiO_2 to simultaneously study phosphorylation, sialylated N-glycosylation and lysine acetylation in mouse brains (15, 23). However, the relative low amount of lysine acetylation sites from biological material compared to phosphorylation sites, despite the generous amount of starting material, suggests that lysine-acetylation is a rather low abundant PTM compared to phosphorylation. This means that the enrichment of peptides carrying lysine acetylation is highly dependent on the amount of starting material. The more starting material the higher the amount of Lysine acetylated peptides identified.

The simultaneous study of different PTMs allows the discovery of potential interplays between them, also termed PTM cross-talking (24), which are known to co-regulate a range of biological processes (25). Therefore, a number of strategies for comprehensive enrichment strategies have been developed in order to study different PTMs from the same sample (e.g., (14, 19, 25, 26)).

Here, a modified protocol of the TiSH approach, to simultaneously study phosphopeptides, sialylated glycopeptides, and lysine acetylated peptides, is presented. Using this strategy, a comprehensive overview of proteomes and their selected PTMomes can be achieved from relatively low amount of biological material.

2. Materials

All the solutions and buffers should be prepared with Milli-Q water (UHQ), analytical grade reagents and highest purity chemicals. Organic solutions should be prepared fresh or stored for no more than 2 weeks before performing the protocol to avoid changes in buffers composition.

2.1. Sample model - HeLa cells

2.2. Lysis, reduction, alkylation and digestion of model sample

1. Lysis buffer: 6 M urea, 2 M thiourea, 10 mM dithiotreitol (DTT), phosphatase inhibitor (PhosSTOP, Roche, Mannheim, Germany), 0.1 mM sodium pervanadate and 0.04 AU of Lys-C (lysyl endopeptidase, WAKO Pure Chemical Industries, Ltd., Osaka, Japan). Protease inhibitors can be added if needed.
2. Triethylammonium bicarbonate (TEAB).
3. Iodoacetamide.
4. Trypsin (Sigma porcine trypsin, St. Louis, MO, US) (*see* **Note 1**).
5. Formic acid.

2.3. TiO_2 enrichment

1. TiO_2 beads (5 µm, GL Science Inc., Japan).
2. Low-binding Eppendorf tubes 1.5 mL (Sorenson BioScience, Inc., Utah, US).
3. Acetonitrile (ACN) HPLC grade.
4. Formic acid.
5. TiO_2 loading buffer: 80% ACN, 5% trifluoroacetic acid (TFA) and 1 M glycolic acid.
6. TiO_2 washing buffer 1: 80% ACN, 1% TFA.
7. TiO_2 washing buffer 2: 10% ACN, 0.1% TFA.
8. TiO_2 elution buffer: 1.5% ammonium hydroxide (pH $\geq$ 11.3), always prepare fresh solution.

2.4. Deglycosylation of N-linked glycopeptides

1. Glyko®Sialidase A (ProZyme®, Inc., San Leandro, CA, US - 1 unit).
2. PNGase F – glycerol free (New England Biolabs Inc., Ipswich, MA, US – 500,000 U/mL).

3. TEAB.

2.5. SIMAC enrichment

1. Iron-coated PHOS-select metal chelate beads (Sigma, Missouri, US).

2. GELoader tips 200 µL (Alpha Laboratories, Hampshire, UK).

3. Loading buffer: 50% ACN, 0.1% TFA.

4. Elution buffer 1: 20% ACN, 1% TFA.

5. Elution buffer 2: 1.5% ammonium hydroxide ($pH \geq 11.3$), always prepare fresh solution.

6. Formic acid.

7. TFA.

2.6. Second TiO_2 enrichment

1. TiO_2 loading buffer: 70% ACN, 2% TFA.

2. TiO_2 washing buffer: 50% ACN, 0.1% TFA.

3. TiO_2 elution buffer: 1.5% ammonium hydroxide ($pH \geq 11.3$), always prepare fresh solution.

4. Formic acid.

5. TFA.

2.7. Desalting peptide mixtures on reversed-phase (RP) columns

1. POROS Oligo R3 reversed phase material (Applied Biosystems, Foster City, CA, US) or Oasis HLB cartridges (Waters, Milford, MA, US) (*see* **Note 2**).

2. p200 pipette tips.

3. 3M Empore C8 disk (3M Bioanalytical Technologies, St. Paul, MN, US).

4. Syringe for HPLC loading (P/N 038250, N25/500-LC PKT 5, SGE, Ringwood, Victoria, Australia) to create a small plug of C_{18} membrane material.

5. Plastic syringe of 1 mL and 5 mL (4606051V, B. Braun Medical Inc., US) to create the pressure of the micro-columns.

6. Loading buffer: 0.1% TFA.

7. Elution buffer: 60% ACN, 0.1% TFA.

2.8. Acetyl lysine IP

1. Acetyl Lysine Antibody, Agarose (ImmuneChem Pharmaceuticals Inc., Burnaby, British Columbia, Canada) against epsilon amino group of lysine residues, K-Ac.

2. Loading buffer: 50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM sodium chloride (pH 8.0).

3. Elution buffer: 0.15% TFA.

2.9. HILIC fractionation

1. MicroHPLC - Agilent 1200 Series HPLC (Agilent, Santa Clara, CA, US).

2. Buffer B: 90% ACN, 0.1% TFA.

3. Buffer A: 0.1% TFA.

4. In-house packed TSKGel Amide 80 (3 µm; Tosoh Bioscience, Stuttgart, Germany) (15 cm x 0.3 mm) column.

3.0 Methods

The complete workflow for the multiple PTM enrichment strategy described in this chapter is illustrated in the **Figure 1**. The workflow consists of an initial simultaneous enrichment of phosphopeptides and N-linked sialylated glycopeptides using the first TiO_2 step. The

flow-through (FT) from this step is used to enrich acetylated peptides by IP after an RP cleanup. The K-Ac antibodies capture acetylated peptides, whereas the non-modified peptides are present in the IP-FT. After enzymatically release of N-linked glycan structures from the sialylated N-linked glycopeptides in the TiO_2 eluate, the multi-phosphopeptides are separated from the mono-phosphopeptides and deglycosylated peptides by SIMAC. Three fractions are obtained from IMAC: (I) IMAC-FT, (II) an acidic elution fraction (pH $\leq$ 2.3) and (III) a basic elution fraction (pH $\geq$ 11.3). The FT and acidic elution that contains deglycosylated peptides and mono-phosphopeptides are combined and subjected to a second milder TiO_2 chromatographic step to separate both types of modified peptides. Finally, in order to decrease the complexity of mono-phosphopeptides, deglycosylated peptides and non-modified peptides, HILIC fractionation is performed prior LC-MS/MS analysis (this could have been done with any other fractionation method such as HpH fractionation).

Here, a total of 500 µg of whole HeLa cell lysates was used for a qualitative analysis to illustrate the average outcome of the protocol; although, the protocol can be optimized to different amounts (lower or higher) and applied also for quantitative studies such as TMT or iTRAQ.

3.1. Model sample preparation: lysis, reduction, alkylation and digestion

1. Add 50 µL lysis buffer to the HeLa pellet (lysis buffer volume depends on the pellet size; here we used 1 x 10^7 cells). Vortex well and incubate for 2 hours at room temperature (RT) (*see* **Note 3 and 4**).

2. After incubation, dilute the sample 10 times with 20 mM TEAB, pH 7.5 and sonicate on ice. In order to alkylate the sample, 20 mM iodoacetamide is added and the sample is incubated for 20 min in the dark at RT.

2. Digest the sample using trypsin (enzyme to substrate ratio 1:50) overnight (12 - 16 h) at room temperature (RT).

3. After incubation, quench the reaction with formic acid to a final concentration of 5% and leave for 5 min at RT. Then, centrifuge for 15 min at 14000 x g in order to pellet lipids.

4. Transfer the supernatant to another low-binding Eppendorf tube.

3.2. HLB cartridge purification to desalt and concentrate the sample (*see* Note 5 and 6)

1. Add 0.1% TFA to the tryptic peptide sample to achieve approximately 1 mL of final volume and adjust the pH to below 2.0.

3. Activate the HLB cartridge with 1 mL of methanol followed by 1 mL of ACN.

4. Equilibrate the cartridge twice with 2 mL of 0.1% TFA.

5. Load the sample onto the HLB cartridge slowly and collect the FT.

6. Pass again the FT through the same HLB cartridge.

6. Wash the cartridge twice with 2 mL of 0.1% TFA.

7. Elute the peptides in a new low-binding Eppendorf tube with 1 mL of 60% ACN/0.1% TFA and lyophilize the sample.

8. Reconstitute the sample in 0.1% formic acid.

9. Take an aliquot for amino acid analysis (AAA) to determine peptide concentration (*see* **Note 7**).

3.3. TiO_2 batch mode enrichment of phosphopeptides and sialylated N-linked glycopeptides

1. To reduce nonspecific binding to the TiO_2 resin, it is important to adjust the amount of TiO_2 beads to the amount of sample. The optimal quantity was previously found to be 0.6 mg TiO_2 beads per 100 µg peptide solution derived from HeLa cells (27). This can be optimized for a given sample.
2. Transfer 3 mg of TiO_2 beads to a new low-binding Eppendorf tube (for 500 µg of starting material).
3. Dilute the peptide sample at least 10 times in TiO_2 loading buffer (v/v) and add the TiO_2 beads (*see* **Note 8**). Alternatively, adjust the sample volume to achieve the proper loading buffer concentration such as for 100 µL sample add 50 µl water, 50 µL of 100% TFA, 800 µL of 100% ACN and 76 mg of glycolic acid.
4. Incubate the sample on the shaker for 10 min at RT. Then, centrifuge to pellet the beads using a table centrifuge for 15 s (*see* **Note 9**).
5. Transfer the supernatant to another low-binding Eppendorf tube containing half of the amount of the TiO_2 beads as used in the first round. Repeat the incubation as described above to increase the yield of peptides modified by phosphorylation and sialylated N-linked glycosylation (*see* **Note 10**).
6. Save the supernatant (TiO_2 – FT) for further enrichment of acetylated and non-modified peptides (see section 3.8).

7. Pool the TiO_2 beads from the two incubations using 100 µL of TiO_2 loading buffer and transfer to a new low-binding Eppendorf tube. This step is performed to avoid contamination with non-modified peptides that may bind to the Eppendorf tube surface. Vortex the sample for 10 sec and then centrifuge to pellet the beads. The supernatant is removed and pooled together with the TiO_2 - FT.

8. Add 70 - 100 µL of TiO_2 washing buffer 1, vortex for 15 sec and centrifuge to pellet the beads. This step is performed to remove the contaminating hydrophobic non-modified peptides.

Repeat this step using 50 -100 µL of TiO_2 washing buffer 2 in order to remove the hydrophilic non-modified peptides and potential neutral glycopeptides (*see* **Note 11**).

9. Dry the beads for 10 min in the vacuum centrifuge or on the table (*see* **Note 12**).

10. Elute the phosphopeptides and sialylated N-linked glycopeptides by adding 100-150 µL of TiO_2 elution buffer. Vortex and incubate the solution in the shaker for 10 min to allow an efficient elution.

11. Centrifuge the solution for 1 min and pass the supernatant over a filter (C_8 stage tip) into a new low-binding Eppendorf tube to avoid the presence of TiO_2 beads in the solution. Wash the TiO_2 beads with 30 µL of elution buffer for 5 min, pass over the C_8 filter and pool with the other TiO_2 eluate. To recover any peptide that bound to the C_8 filter, add 5 µL of 30% ACN to the C_8 stage tip and pass this solution through and collect with the TiO_2 eluates.

12. Lyophilize the eluted peptides. Any remnant of ammonia can interfere with the subsequent steps.

13. Lyophilize also the TiO_2 - FT to perform the lysine acetylation enrichment (see section 3.8 and 3.9).

3.4. Deglycosylation: cleavage of N-linked glycans from glycopeptides

1. Redissolve the lyophilized peptides from the TiO_2 enrichment in 40 µL of 20 mM TEAB pH 7.5 and add 1 µL of PNGase F and 0.5 µL of Sialidase A.
2. Incubate at 37°C overnight.
3. To quench the reaction, add 1 µL of 10% TFA.

3.5. SIMAC enrichment

1. Slowly, dilute the acidified deglycosylated peptide solution with 200 µL of SIMAC loading buffer (*see* **Note 13**). Adjust the pH to 1.6 - 1.8 using 10% TFA solution.
2. As in the TiO_2 enrichment, the amount of IMAC beads per sample amount is a crucial factor to avoid non-specific binding. The optimal quantity is 60 - 80 µL beads per 300 µg of starting material (27).
3. Transfer 130 µL of IMAC beads (for 500 µg of starting material) to a low-binding Eppendorf tube and wash twice with 200 µL of IMAC loading buffer by mixing and table centrifugation (*see* **Note 14**).
4. Add the peptide solution to the IMAC beads and incubate for 30 min at RT under slow rotation shaking.
5. Centrifuge the sample for 15 sec at 14000 x g and transfer half of the supernatant to a new low-binding Eppendorf tube called SIMAC-FT.
6. Squeeze the tip of a 200 µL GELoader tip to retain the IMAC beads.

7. Transfer the remaining solution with IMAC beads to the constricted 200 µL GELoader tip. Use a syringe to press the liquid through into the SIMAC-FT tube and pack the IMAC micro-column.

8. Wash the IMAC column with 50 µL of loading buffer and collect it in the SIMAC-FT tube.

9. Elute the mono-phosphorylated peptides from the IMAC column with 70 µL of SIMAC elution buffer 1. The eluate is collected together with the SIMAC - FT from the above steps 5, 7 and 8. This step should be performed slowly (around 1 drop/s) to allow mono-phosphorylated peptides to fall off the IMAC beads whereas multi-phosphorylated peptides still are retained on the IMAC resin.

10. Elute the multi-phosphorylated peptides with 80 µL of SIMAC elution buffer 2 directly down into a pre-equilibrated p200 stage tip blocked with a C_8 filter plug and packed with R3 material (1 - 2 cm of R3) (see section 3.9). It is important to acidify the eluate with 8 µL of 100% formic acid and 2 µL of 10% TFA prior the contact with R3 material (*see* **Note 15**).

11. Elute the multi-phosphorylated peptides after washing the column (see section 3.9.) and lyophilize the sample.

3.6. Second TiO_2 enrichment

1. Dilute the SIMAC FT at least 10 times with second TiO_2 loading buffer or adjusts the concentration by adding 100 % ACN and 100 % TFA to achieve a final concentration of 70% ACN/1% TFA. Add the loading buffer or the ACN slowly to avoid peptide precipitation.

2. Add the same amount of TiO_2 used previously in section 3.3 (the same TiO_2 beads can be used if they are washed extensively with pH 11). Vortex and incubate for 10 min on a shaker.

3. Centrifuge to pellet the beads and transfer the supernatant to a new low-binding Eppendorf tube containing half of the amount of TiO_2 beads. Then, incubate for 10 min as described in section 3.3.

4. Centrifuge to pellet the beads and save the supernatant that contains the deglycosylated peptides.

5. Pool the TiO_2 pellets using 100 µL of second TiO_2 washing buffer, vortex and centrifuge. Remove the supernatant and combine it with the deglycosylated peptides.

6. Dry the beads for 10 minutes in the vacuum centrifuge or on the table (*see* **Note 12**).

7. Add 100 µL of TiO_2 elution buffer, vortex and incubate for 10 min on a shaker to elute the mono-phosphopeptides.

8. Centrifuge the solution for 1 min and pass the supernatant over a C_8 stage filter repeating the item 10 from section 3.5 but using two R3 micro-columns of 1.5 cm each (the number of R3 micro-columns depends on the amount of starting material) (*see* **Note 16**). This step is performed sequentially being the solution passed through the first R3 and collected directly on top of the second R3 micro-column. Wash and elute the mono-phosphopeptides from both columns as described in the section 3.9. Pool the eluates in the same tube. Lyophilize the mono-phosphopeptides. Step 8 can be omitted for HpH fractionation.

9. Lyophilize the second TiO_2 - FT that contains the deglycosylated peptides. Subsequently, reconstitute the sample in 80 µL of 0.1% TFA prior to R3 micro-purification as described in section 3.9.

3.7. Purification of the FT from the first TiO_2

1. The FT from the first TiO_2 is used to enrich acetylated peptides by immunoprecipitation (see section 3.8).
2. Resuspend the sample in 1 mL of 0.1% TFA.
3. Purify the sample by an HLB RP cartridge as described in the section 3.2 in order to remove glycolic acid present in the TiO_2 loading buffer.

3.8. Acetyl lysine IP in batch mode to enrich for lysine-acetylated peptides

1. Reconstitute the above dried peptide sample in 200 µL of IP loading buffer and adjust the pH to 8.0.
2. For 100-200 µg of sample use 20 µL of slurry of anti-Lys acetylated antibody immobilized on agarose beads.
3. Transfer 40 µL of Anti-K-Ac antibody beads (for 500 µg of starting material) to a new low-binding Eppendorf tube and wash twice with 200 µL of IP loading buffer by mixing and table centrifugation (*see* **Note 17**).
4. Incubate the reconstituted sample with the antibody for 12 h under rotation at 4°C.
5. Pellet the antibody beads using a table centrifuge and transfer the supernatant to a new low-binding Eppendorf tube. The supernatant (K-Ac – FT) contains the “non-modified” peptides.

4. Wash the anti-K-Ac-antibody beads 4 times with 200 µL of IP loading buffer as mentioned above. Pool all the supernatants together into the K-Ac-FT tube.

5. Wash twice the antibody beads with 200 µL of water as described above.

6. Elute the K-Ac peptides with 200 µL of elution buffer and subsequently purify the eluate as described in section 3.9. Lyophilize the purified K-Ac peptides.

7. Lyophilize the K-Ac – FT that contains the non-modified peptides to be used for subsequent whole proteome analysis.

3.9. Poros Oligo R3 Reversed Phase (RP) micro-purification to desalt and concentrate the sample

1. Prepare a micro-column using p200 tip by plugging the constricted end with C_8 material filter. Pack 2 cm column with R3 material slurry in 100% ACN by applying air pressure using a syringe.

2. Equilibrate the column with 50 - 100 µL of 0.1% TFA.

3. Load the acidified sample (pH < 2.0) into the column and make a gently air pressure. It is important to perform this step slowly. In case the sample is dried, add 80 µL of 0.1% TFA to reconstitute the sample prior to load. Collect the FT.

4. Pass again the FT through the micro-column to increase the binding of peptides.

5. Wash the column with 100 µL of 0.1% TFA.

6. Elute the peptides into a new low-binding Eppendorf tube with 100 µL of 60% ACN/0.1% TFA.

7. Lyophilize the sample in a vacuum centrifuge.

3.10. HILIC fractionation for mono-phosphopeptides, deglycosylated peptides and non-modified peptides

1. Reconstitute the sample in 45 µL of HILIC solvent B by adding first 0.45 µL of 10% TFA and then 4.05 µL of milli-Q water. Vortex and finally add 40.5 µL of 100% ACN slowly to avoid precipitation. This step should be performed immediately prior to fractionation since the acetonitrile evaporates fast altering the efficiency of the method.
2. Inject 40 µL onto the microHPLC HILIC column
3. Fractionate the peptide mixture using the gradient described in the **Table 1**.
4. Collect the fractions according to the **Table 2**.
5. Pool the fractions according to the UV absorbance intensity (λ = 210 nm) and chromatographic profile (**Figure 2**). The number of analyzed fractions depends on the complexity of the sample and the accessibility to LC-MS/MS instruments.
6. Lyophilize the fractions.

3.11. LC-ESI-MS/MS analysis (*see* Note 18)

1. Reconstitute the dried samples (multi-phosphopeptides, acetylated peptides as well as the HILIC fractions from mono-phosphopeptides, deglycosylated peptides and non-modified peptides) in 0.1% formic acid.
2. Inject 5 µL of sample onto an in-house packed Reprosil-Pur C18-AQ (3 µm; Dr. Maisch GmbH, Germany) pre-column (2 cm x 100 µm) connected to an analytical column (17 cm x 75 µm) using an Easy-nLC system (Thermo Scientific, Bremen, Germany).
3. Run the samples at a flow of 250 nL/min, using 90% ACN (B) and water (A) both containing 0.1% formic acid as mobile phase.

4. Depending on the sample, the gradient was 0 – 34% solvent B in 60, 90 or 120 min, 34 – 100% solvent B in 5 min, and 8 min at 100% solvent B.

5. MS analysis was performed in an LTQ Orbitrap Velos system (Thermo Scientific, Bremen, Germany) with data-dependent acquisition for CID MS/MS analysis of the 10 most intense ions except for phosphopeptides where MSA MS/MS analysis was used.

11. Data processing and search were performed using Proteome Discoverer v1.4 (ThermoFisher Scientific). Data were searched against the Swiss-Prot human v3.53 database using an in-house MASCOT server (v2.3, Matrix Science Ltd, London, UK) and the Sequest HT. Trypsin was selected as digestion enzyme and two missed cleavages were allowed. Database searches were performed with the following parameters: precursor mass tolerance of 10 ppm, product ion mass tolerance of 0.8 Da and cysteine carbamidomethylation as fixed modification. Searches were also conducted with the following variable modifications: methionine oxidation; serine, threonine, tyrosine phosphorylation; asparagine and glutamine deamidation. Protein grouping was performed, in order to avoid presence of different proteins identified by non-unique peptides. Only peptides with up to q-value of 0.01 (Percolator), Mascot and Sequest HT rank 1, Mascot and Sequest HT search engine 1, a Sequest HT ΔCn of 0.1, a cut-off value of Mascot score $\geq$ 22 and a cut-off value of XCorr score greater than 1.5, 2, 2.5 and 3 for charge states of +1, +2, +3, and +4, respectively, were considered for further analysis.

3.12. Results

The **Figure 3** highlights the number of modified peptides identified specifically by the analysis of the dedicated enrichment step described in this chapter. By using newer and

more sensitive MS instrumentation the numbers can increase significantly. The four different steps achieved various specificity and sensitivity regarding enrichment of modified peptides. Mono- and multi-phosphorylated peptides were efficiently enriched from the elution of the IMAC and 2nd TiO_2 chromatography achieving the identification of thousands of phosphopeptides. Hundreds of sialylated N-glycopeptides were enriched from the FT of the 2nd TiO_2 chromatography. Finally, acetylated peptides were enriched from the elution of the K-Ac IP. The relative low number of identified acetylated lysines is due to the lower abundance of this PTM in comparison with phosphorylation or N-linked sialylated glycosylation in biological material. In order to increase the number of K-ac peptides the amount of starting material should be increased. Furthermore, a high number of peptides contain more than one differently modified site. Therefore, a significant part of acetylated sites might co-purify with phosphopeptides during the 1st TiO_2 enrichment step, causing the reduced number of acetylated peptides identified in K-Ac IP.

4. Notes

1. The trypsin is purified by benzamidine sepharose affinity to guarantee the same proteolytic activity for the entire batch, as well as to significantly reduce the level of autodigestion products.

2. The choice between POROS Oligo R3 or Oasis HLB cartridge depends on the amount of sample. For samples with amount higher or equal to 500 µg, the Oasis HLB cartridge is often a choice.

3. Any protein extraction, denaturation and digestion method is compatible with the present simultaneous PTMs enrichment strategy.

4. Mix by vortexing and pipette cell pellet up and down after adding lysis buffer. Cell lysate will form a very viscous solution due to the presence of nuclear DNA.

5. POROS Oligo R3 micro-columns or Oasis HLB cartridges desalting step is required if the buffer solutions are not compatible with labeling methods such as iTRAQ, TMT, dimethyl labeling; or prior to determination of protein concentration by AAA analysis.

6. TiO_2 enrichment might be performed directly after the protein digestion step without RP cleanup since TiO_2 chromatography is compatible with most of the commonly used buffer solutions (8).

7. A labeling strategy such as iTRAQ, TMT or dimethyl labeling can be introduced in this step for quantitative studies. Remember to check the compatibility of buffer solutions with the specific labeling reagents.

8. In case the sample was lyophilized before, it is crucial to reconstitute the sample in a small amount of 0.1% TFA. Moreover, the TiO_2 loading buffer should be added slowly to avoid peptide precipitation.

9. Centrifugation time is not critic.

10. This step can be done up to 3 times, depending on the sample complexity.

11. The washing buffer 2 is used to remove the peptides that bind in the HILIC mode to TiO_2. The FT of washing buffer 2 could contain neutral glycopeptides, which can be analyzed as a separate PTM fraction.

12. It is very important to lyophilize the sample in the vacuum centrifuge. In case vacuum centrifuge is not available, it is necessary to check and adjust the pH to ≥ 11.3 in the next step.

13. Prior adding 200 µL of SIMAC loading buffer, the sample can be lyophilized and then reconstituted in a small volume of 0.1% TFA.

14. Avoid vortexing the IMAC beads in high speed.

15. The direct purification is performed to avoid loss of multi-phosphopeptides that can bind to the tube wall. However, multi-phosphopeptides can be transferred to another low-binding Eppendorf tube, following with adjustment of the pH eluate below 2.0 prior micro-purification.

16. This step uses two POROS Oligo R3 micro-columns to ensure a good recovery of phosphopeptides.

17. The anti K-Ac antibody beads stock solution and all solutions containing K-Ac antibody beads should be kept on ice constantly.

18. Different LC-MS/MS set-up can be used. However, the results will differ from those obtained using the present protocol.

Figure Legends

Figure 1 - Enrichment workflow. An initial titanium dioxide (TiO_2) step is performed to enrich phosphopeptides and sialylated N-linked glycopeptides followed by glycan release. Then, SIMAC is performed to separate mono-phosphorylated and deglycosylated peptides from multi-phosphorylated peptides. The IMAC FT together with the IMAC acidic elution is subjected to a second round of TiO_2 enrichment to separate mono-phosphopeptides from deglycosylated peptides. In addition, the first TiO_2 FT is used to enrich acetylated peptides by IP. Finally, the samples are fractionated by HILIC prior LC-MS/MS analysis.

Figure 2 - HILIC chromatogram. The figure illustrates the HILIC chromatogram of non-modified peptides in order to show how fractions were pooled in the present work. * denote individual fractions.

Figure 3 - Results obtained from the analysis of phosphopeptides, sialylated N-linked glycopeptides and acetylated peptides using the optimized workflow. The number of phosphorylated peptides (mono- and multiply phosphorylated peptides), sialylated N-glycopeptides and acetylated peptides identified from 500µg of HeLa cell Lys-C/tryptic digestion using the combination of TiO_2, SIMAC and K (Ac) IP enrichment methods.

Tables

Time (min)	**Concentration of Sol. B (90% ACN, 0.1%TFA)**	**Flow (µL/min)**
00:00	100	12
08:60	100	12
09:00	95	6
35:00	60	6
39:00	0	6
42:00	0	6
46:00	100	6
48:00	100	6

Table 1 – HILIC gradient. The gradient used for fractionation of all obtained modified and unmodified peptide samples by HILIC microHPLC, except for the multi-phosphopeptides.

Time (min)	Trigger Mode	Time slices (min)
00:00	Time-based	10
10:00	Time-based	2
14:00*	Time-based	1
36:00**	Time-based	2
48:00	off	

Table 2 – Timeline of HILIC fraction collector. The timeline used to collect all peptide samples beside the multi-phosphopeptides. In addition, alteration to *20:00 and **40:00 was done for the sample containing mono-phosphopeptides.

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