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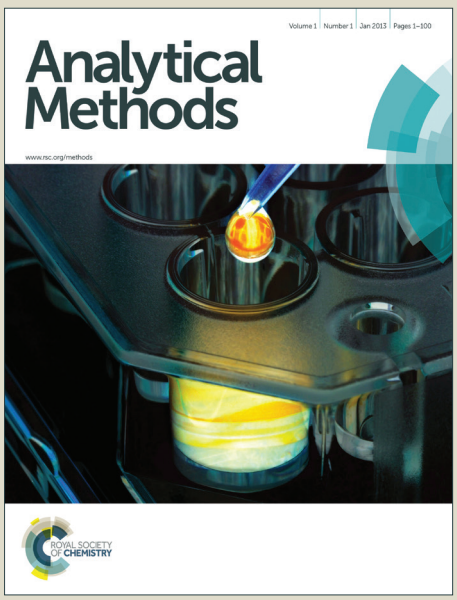

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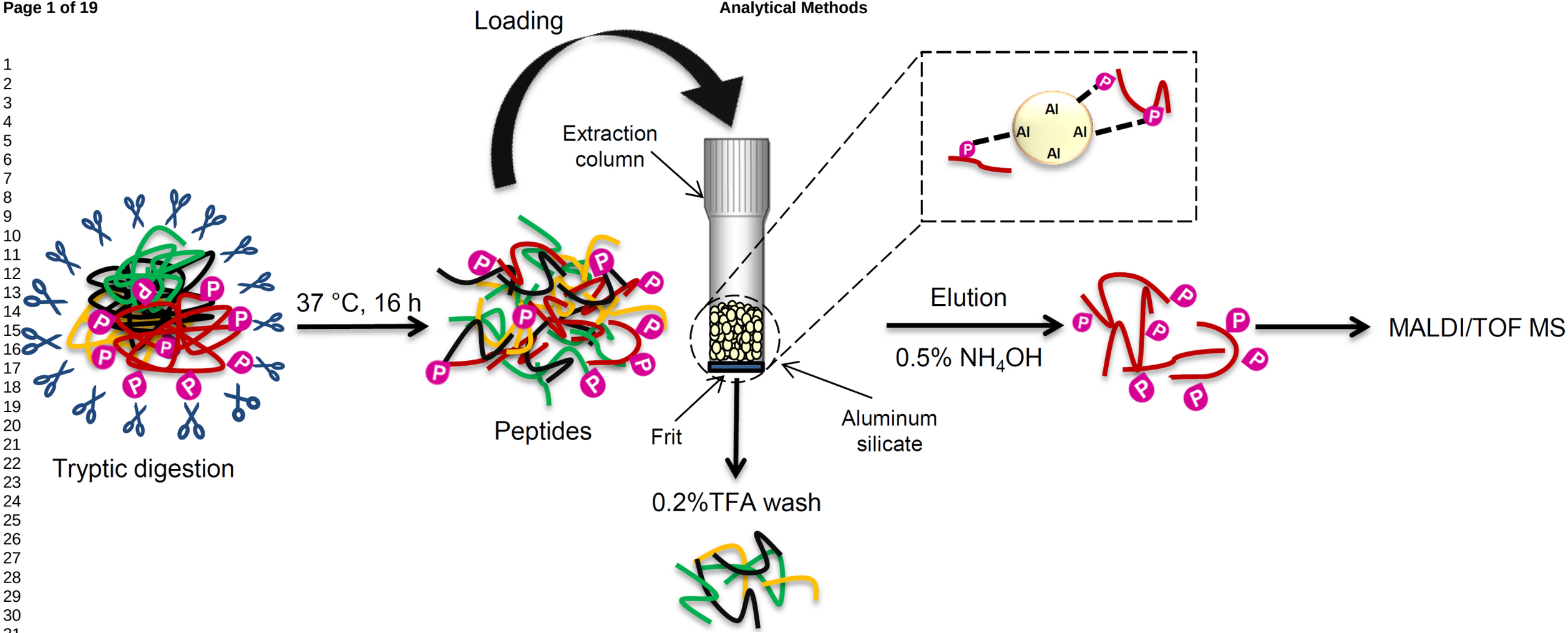

Highly Selective Enrichment of Phosphopeptides using Aluminum Silicate

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Abstract

This study presents a novel strategy for highly selective enrichment of phosphopeptides using aluminium silicate (mullite) powder. Mullite is a nontoxic and inexpensive material and offers excellent performance for the purification of phosphopeptides from complex samples. The selectivity of the method was investigated by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The method was validated with tryptic peptides from model proteins, bovine milk and human saliva samples. For sample preparation, the digested samples were loaded on self-assembled extraction columns containing mullite as sorbent. Non-phosphorylated compounds could be easily removed by several washing steps, while phosphorylated peptides were successfully immobilized on the mullite substrate. In a final step, phosphopeptides were eluted from the extraction column at alkaline conditions. To further assess the enrichment efficiency of the presented method, HeLa cell lysates were spiked with two synthetic phosphopeptides at different ratios. The method showed high selectivity and allowed the detection of phosphopeptides at ratios of 1:1000. In a further study, the performance of the presented approach was compared with conventional phosphopeptide enrichment by TiO_2 and revealed superior results for aluminium silicate.

Introduction

Protein phosphorylation is one of the most important post-translational modifications and is involved in complex biological processes including signalling, transductions, growth and cell differentiation. Approximately, one-third of all proteins are phosphorylated in mammalian cells at some point during their expression [1, 2]. Phosphorylation takes mainly place on serine, threonine and tyrosine amino acid residues. A study on HeLa cell phosphoproteins demonstrated that the distribution of phosphoserine, phosphothreonine and phosphothyrosine sites was 86.4%, 11.8% and 1.8%, respectively [3]. During the last years, mass spectrometry has emerged as the most powerful analytical tool for characterizing protein phosphorylation [4-6]. Very often mass spectrometric analysis is complicated by the low abundance and significant ion suppression of phosphoproteins. Thus, the application of selective and sensitive tools for enrichment of phosphorylated peptides and proteins is highly demanded [7, 8]. In literature there have been reported several strategies for the isolation of phosphorylated peptides including immunoprecipitation using specific antibodies [9, 10], chemical modification [11, 12], selective co-precipitation by metal cation [13-15] and chromatographic methods such as immobilized metal-ion affinity chromatography (IMAC) [16-21] or metal-oxide affinity chromatography (MOAC) [22]. Numerous metal oxides including TiO_2 [23, 24], ZrO_2 [25, 26], or mixed TiO_2-ZrO_2 [27] have been described as excellent sorbents for many phosphoproteomic studies. In MOAC, phosphorylated molecules are specifically isolated through co-ordination between metal atoms and phosphate groups by Lewis acid-base interactions [28]. A major challenge of MOAC represents the undesired binding of non-phosphorylated acidic peptides [6]. To prevent unspecific binding, protein digests are usually acidified in order to neutralize most of acidic residues (glutamic or aspartic acid), while keeping the phosphate groups negatively charged [29, 30]. Very often so-called displacers are employed which prohibit any unspecific binding by replacing acidic peptides [23, 31]. Most

frequently used displacers include phthalic acid, glycolic acid or 2,5-dihydroxybenzoic acid (DHB).

During the last years, aluminium based sorbents such as aluminum hydroxide [32] , aluminum oxide [33] and some composites of aluminium [34-38] have found great application in phosphoproteomics. Aluminium containing materials are described to exhibit a high preference for phosphate groups and have been successfully applied in several research studies for the enrichment of phosphopeptides [39-41].

In this study, we present an efficient and selective solid-phase extraction (SPE) method for the isolation of phosphopeptides using mullite as a sorbent. Mullite is an aluminium silicate with the chemical formula $Al_{4+2x}O_{2-2x}{\cdot}SiO_{10-x}$, where x ranges between 0.2 and 0.9 (about 55 to 90% Al_2O_3). Owing to its high thermal and chemical stability, mullite is an outstanding ceramic material with high resistance, strength and corrosion stability [42]. Tryptically digested protein mixtures containing model phosphoproteins, bovine milk, human saliva and spiked HeLa cell lysate samples were selected for the immobilization of phosphopeptides. According to the author's knowledge, mullite has been used for the very first time as sorbent in phosphoproteomics.

Material

Chemicals and Reagents

Acetonitrile (for HPLC, ≥99.9%), n-octyl β-D-glucopyranoside (nOGP, 98%), iodoacetamide (IAA, ≥98.0%), α-casein from bovine milk (≥70.0%), β-casein from bovine milk (bio ultra ≥90.0%), myoglobin from horse heart (≥90.0%), cytochrome c from bovine heart (≥95.0%), lysozyme from chicken egg white, albumin from bovine serum (BSA, ≥96.0%, electrophoresis), Aluminum silicate powder ($Al_6Si_2O_{13}$) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA, for protein sequence analysis), dithiothreitol (DTT, ≥99.0%), and ammonium bicarbonate (ultra, ≥99.5%) were obtained from Fluka (Buchs, Switzerland). Deionized water (for chromatography) was purchased from Merck KGaA (Darmstadt, Germany). Columns (11 µm frit size) were provided by PhyNexus, Inc. (San Jose, CA, USA). Trypsin (sequencing grade modified) was obtained from Promega Biosciences (San Luis Obispo, CA, USA). The protein and peptide standards were bought from Bruker Daltonics Care (Bremen, Germany). Bovine milk was obtained from a local supermarket. For phosphopeptide enrichment by TiO_2, TopTip columns (Glygen, Columbia, MD) were employed. The two synthetic phosphopeptides (VYGKTpSHLR $[M+H]^+$ = 1140.20, and KIGEGTpYGVVYK $[M+H]^+$ = 1392.67) were a gift from Karl Mechtler (Institute of Molecular Pathology, Dr. Bohr-Gasse 7, 1030 Vienna, Austria). Human saliva was obtained from one of the authors. All experiments were performed in compliance with the relevant laws and institutional guidelines

Methods

Preparation and digestion of protein mixture and milk samples

For protein standards, 1 mg of each protein (α-casein, β-casein, myoglobin, cytochrome c, lysozyme, and bovine serum albumin) was dissolved in 1 mL of 0.1% TFA solution. Bovine milk was diluted 1:10 using deionized water. For denaturation, 1 mL of protein standard and milk sample was treated with 100 µL of 40 mM nOGP and 100 µL of 45 mM DTT. The samples were sonicated for 1 min in an ultrasonic bath and additionally centrifuged for 10 min at 14,000 rpm at room temperature (Eppendorf Centrifuge 5415 D, Hamburg, Germany). Afterwards, the supernatants were denatured at 37 °C on a thermomixer (Eppendorf Thermomixer Comfort, Hamburg, Germany) for 30 min at 800 rpm. In a next step, 160 µL of 1 M NH_4HCO_3 were added to adjust the pH at 8. Alkylation was performed after adding 25 µL of 100 mM iodoacetamide for 30 min under the exclusion of light. Then, proteolysis was carried out by adding 2 µg of trypsin (20 µL of 0.1 µg/mL) for 16 h at 37 °C on a thermomixer (600 rpm). Finally, proteolysis was stopped by adding 120 µL of 1% TFA (pH~3). The protein digest were stored at -20 °C before performing enrichment.

Proteolysis of human saliva and HeLa cell extract

Human saliva was collected from a healthy non-smoking volunteer in the morning from 8 - 10 a.m. The volunteer was informed not to eat, drink, or brush the teeth but to rinse the mouth five times with water during the two hours sample collection period. In case of HeLa cell extracts, dephosphorylation was carried out as described in a previously published protocol [21]. The dephosphorylated HeLa lysates were divided into 500 µL fractions (total protein concentration 1 mg/mL), snap frozen in liquid nitrogen and stored at -80 °C.

For denaturation, 1 mL of dephosphorylated HeLa cell extract or collected saliva was treated with 200 µL of 40 mM nOGP and 150 µL of 45 mM DTT. After 1 min ultra-sonication, the diluted samples were centrifuged for 10 min at 14,000 rpm. Additionally, 100 µL of the supernatants were placed on a thermomixer (800 rpm) and denaturation was carried out for 20 min at 70 °C and 10 min at 90 °C. Afterwards, alkylation was performed by adding 25 µL of 100 mM iodoacetamide, followed by 30 min incubation in dark at room temperature. In a next step, 70 µL of H_2O and 8 µL of 1 M NH_4HCO_3 were added to the samples in order to achieve basic pH (pH~8). Finally, 20 µL of 0.1 µg/mL concentrated trypsin were added and proteolysis was carried out for 16 h at 37 °C on a thermomixer.

Before enrichment, the digested HeLa cell lysate was spiked with two synthetic phosphopeptides (0.5 µg/ml) at a ratio of 1:1000.

Phosphopeptide enrichment by aluminium silicate in extraction columns

Ten mg of mullite were placed into empty PhyTip^{TM} columns (11 µm frit size, PhyNexus Inc., San Jose, CA, USA). Activation of the sorbent was performed twice with 100 µL of 0.2% TFA solution. Before enrichment, the protein digests (protein standard, bovine milk, human saliva and spiked cell lysate) were diluted 1:1 with 0.2% TFA. Afterwards, 50 µL of each sample was loaded onto the activated mullite sorbent. To remove non-phosphorylated compounds, three washing steps with 200 µL of 0.2% TFA were carried out. Finally, the enriched phosphopeptides were eluted using 50 µL of 0.5% NH_4OH solution. All eluted fractions were analyzed by MALDI-TOF MS and MS/MS using 1 µL of DHB matrix (20 mg/L in 50% ACN/0.1% TFA/1% H_3PO_4 solution).

MALDI-TOF MS and MS/MS analysis

One µL of protein digest was spotted on a stainless steel target (Bruker Daltonics, Bremen, Germany), followed by the addition of 1 µL of DHB as MALDI matrix. All measurements were recorded on an Ultraflex I (Bruker Daltonics, Germany) MALDI-TOF/TOF MS in the positive reflectron mode. An external calibration was performed by spotting 0.5 µL of peptide calibration standard (Bruker Daltonics, Bremen, Germany) and 0.5 µL of DHB matrix. All mass spectra were recorded by summing 500 laser shots. Laser power was adjusted between 30 and 50% of its maximal intensity, using a 337 nm laser at 50 Hz. The Flex Analysis version 2.4 and BioTools 3.0 software packages provided by the manufacturer were used for data processing. Database searching analysis was performed with Mascot software (http://matrixscience.com) and SwissProt as database. For PMF database searching analysis, the parameters were set as following: C-carbamidomethyl (fixed modification), oxidation (M), phosphorylation (ST), phosphorylation (Y, variable modification), mass value (monoisotopic), peptide mass tolerance (120 ppm), mass tolerance (0.6 Da), missed cleavage (1 to 3) and taxonomy ("other mammalian").

For MS/MS analysis of phosphopeptides from human saliva samples the Mascot search parameters were fixed as following: SwissProt as database; taxonomy limited to Homo sapiens (human); enzyme, semiTrypsin; fixed modifications, carboxymethyl; variable modifications, phosphorylation (ST), phosphorylation (Y), oxidation (M), Gln - pyro-Gln (N-term Q), Gln - pyro-Gln (N-term E); missed cleavage, 2; and MS and MS/MS tolerances were 0.5 and 0.1 Da, respectively.

Results and Discussion

In this study, mullite was used for the first time as a selective sorbent for enriching phosphopeptides from tryptically digested protein samples. Mullite is an excellent ceramic material, revealing high thermal and chemical stability, low thermal expansion, good conductivity, high creep resistance and corrosion stability together with suitable strength and fracture toughness [42]. During the last years, aluminium based materials have been successfully applied for the immobilization of phosphorylated peptides. Aluminium was shown to act as a Lewis acid which interacts with different types of Lewis bases such as phosphate groups [28, 43]. In the mullite's structure, the electropositive aluminium accounts for the increased interaction for phosphate containing compounds such as phosphorylated peptides.

Fig. 1 shows the sample preparation workflow of the presented enrichment strategy. Mullite was placed in empty extraction tips (PhyTip™, PhyNexus Inc., CA) for further enrichment on column. Tryptic digested proteins were diluted with 0.2% TFA solution to achieve acidic loading conditions. At pH~2 the carboxyl groups of acidic amino acid residues (aspartic and glutamic acid) are mostly protonated, while phosphate groups remain deprotonated [44]. The enrichment ability of the presented method was first tested with a protein mixture containing lysozyme, cytochrome c, myoglobin, bovine serum albumin and the two model phosphoproteins α- and β-casein. After sample loading, mullite was washed with 0.2% TFA solution to remove non-phosphorylated peptides. Finally, all enriched phosphopeptides were eluted with 0.5% NH_4OH. Fig. 2 shows the mass spectra of the protein-mixture digest before and after enrichment using mullite. The high selectivity of the method can be demonstrated by the high number of recovered phosphopeptides. Almost all detected mass signals derive from caseins. Peaks with the mass signals at *m/z* 1660.7, 1832.8, 1847.7, 1927.7, 1951.9, 2678.0, 2703.5, 2720.9, 2856.5 and

2935.2 belong to αS1-casein, while *m/z* values at 1466.6, 2747.1, 3008.0, 3087.9 and 3132.2 correspond to αS2-casein including one missed cleavage. Mass signals at *m/z* 2061.8, 2432.0, 2966.1, 3042.2 and 3122.2 can be attributed to β-casein including one missed cleavage. Signals at *m/z* 2080.0, and 2716.2 are phosphopeptides with two missed cleavages and can be assigned to αS1-casein. Methionine oxidations (+16 Da) were observed for *m/z* 1943.6 (1927.6), which derives from αS1-casein, and for *m/z* 1610.7 (1594.7), a phosphopeptide from αS2-casein [45]. The mass signals at *m/z* 1927.6, 2061.8, 2080.0, 2678.0, and 2935.1 could be assigned to neutral losses of HPO_3 (-80 Da) [46] from the phosphopeptides *m/z* 1847.6, 1981.8, 2598.0, and 2856.5, respectively. Moreover, the peak at *m/z* 3024.2 derives from the phosphopeptide at *m/z* 3122.2 after a neutral loss of H_3PO_4 (-98 Da) [47]. The signal of a doubly charged peptide from the presented tetra-phosphorylated peptide (*m/z* 3122.2) was located at *m/z* 1561.1 and labelled with double asterisk within the spectra. Table 1 provides an overview about all enriched phosphopeptides and includes a comparative study with conventional enrichment by TiO_2. All in all, 14 phosphopeptides could be enriched from the digested protein standard using TiO_2. However, enrichment by aluminium silicate resin resulted into the recovery of 12 more phosphopeptides.

Enrichment of phosphopeptides from milk and human saliva

The analytical method was further applied to bovine milk, which contains the phosphoproteins αS1-casein (11.9 g/L), αS2-casein (3.1 g/L), β-casein (9.8 g/L), κ-casein (3.5 g/L), γ-casein (1.2 g/L) the and non-phosphorylated proteins α-lactalbumin (3.2 g/L), β-lactoglobulin (1.2 g/L), serum albumin (0.4 g/L), immunoglobulins (0.8 g/L) and proteose-peptones (1.0 g/L) [48]. The mass spectra of the enriched phosphopeptides from digested bovine milk caseins are displayed in Fig. 3. Almost all signals could be assigned to phosphorylated peptides and were labelled

within the spectra. Table 1 presents an overview about all phosphopeptides which could be immobilized from milk using mullite as sorbent. Moreover, a comparison with commercial TiO_2 enrichment was performed. Enrichment by TiO_2 resulted in a total of 16 phosphopeptides. In case of mullite based enrichment, 14 additional phosphopeptides could be recovered. This underlines the high capacity and performance of the presented sample preparation approach. During the last years, the interest in human saliva as a diagnostic fluid has increased due to its convenient availability and minimally invasive collection [49]. Saliva contains a large number of proteins and peptides that maintain homeostasis in the oral cavity [50]. The identification of salivary proteins contributes to the understanding of oral pathophysiology by providing a foundation for the recognition of potential biomarkers of human disease. Protein biomarker discovery demands rapid, sensitive and highly specific analytical methods in order to analyse potential candidates. Thus, the enrichment of phosphopeptides by mullite was investigated for human saliva sample. Fig. 4 depicts the MALDI-TOF MS spectra of selectively enriched phosphopeptides from tryptically digested human saliva before and after enrichment using mullite as resin. Mass signals of phosphopeptides and neutral losses of H_3PO_4 (-98 Da) or HPO_3 (-80 Da) are tagged within the spectra. The peaks at *m/z* 1155.6, 1270.5, and 1426.5 derive from statherin isoform-a, a salivary phosphoprotein. Furthermore, the signals at *m/z* 1404.6, 1461.8, 1576.5, and 3521.7 belong to salivary acidic proline-rich phosphoprotein 1/2. Phosphopeptides from basic salivary proline-rich protein-4 were observed at *m/z* 2454.0, 2535.0, 2670.0, and 2830.5 Da. Another signal at *m/z* 1663.6 could be assigned to proline-rich phosphoprotein, which was also described by Chen *et. al* in 2010 [51]. Neutral losses of H_3PO_4 (-98 Da) could be observed for *m/z* 1328.5 (1426.5), 1565.6 (1663.6) and 2437.0 (2535.0), respectively. One more signal at *m/z* 3441.7 derives from the phosphopeptide at *m/z* 3521.7 after a neutral loss of HPO_3 (-80 Da). The peak at *m/z* 1753.2 could be assigned to a double-phosphorylated peptide but could not be identified by database search analysis. All

phosphopeptides were identified by additional tandem MALDI MS measurements (“Electronic Supplementary Material”). Table 2 provides an overview about the identified salivary phosphoproteins.

Selectivity and sensitivity study

The identification and characterization of phosphopeptides is usually complicated due to their low abundance in biological samples. Therefore, the applied enrichment methods must offer high sensitivity by providing adequate selectivity. In this regard, the enrichment performance of the demonstrated strategy was carried out for a complex sample background containing two synthetic phosphopeptides at low-concentration levels. Thus, a dephosphorylated HeLa cell extract (1 mg/mL) was tryptically digested and subsequently spiked 1:1000 with two synthetic phosphopeptides (0.5 µg/mL). The final concentration of the spiked synthetic phosphopeptides was around 200 and 150 fmol/µL. Fig. 5a shows the MALDI-TOF MS spectrum of the digested HeLa cell lysate containing the two synthetic phosphopeptides before enrichment. The identification of both phosphopeptides was found to be impossible without a prior purification step. After a clean-up step using aluminium silicate both synthetic phosphopeptides could be easily isolated from the complex HeLa cell lysate background (Fig. 5b).

Conclusions

The study of protein phosphorylation is of utmost importance as it controls several cellular processes including growth, proliferation, apoptosis, differentiation and transcription. This requires highly sensitive and selective sample preparation methods before mass spectrometry. In this study, mullite proved to be a selective sorbent for the enrichment of phosphopeptides from bovine milk and human saliva. Mullite revealed strong affinity towards phosphopeptides due to the co-ordination between aluminium and phosphate groups by Lewis acid-base interactions. The enrichment of phosphopeptides by mullite is easily applicable and was found to be highly reproducible. In comparison with conventional TiO_2, mullite also allows the enrichment

of multiple phosphorylated peptides and shows better performance in terms of number of retained phophopeptides. The overall sensitivity was found to be in the femto molar range and was demonstrated for two synthetic phosphopeptides, which were spiked into a dephosphorylated and digested HeLa cell lysate. All in all the method enables a convenient and rapid immobilization of phosphopeptides from biological samples without using complex enrichment protocols.

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Figures

Figure1 Sample preparation workflow for the enrichment of phosphorylated peptides using mullite as sorbent

Figure 2 MALDI-TOF MS spectra of a tryptically digested protein mixture before (A) and after enrichment using mullite (B). Phosphopeptides are labelled with asterisks. **doubly charged phosphopeptide $[M+2H]^{2+}$=1561.1 Da

Figure 3 MALDI-TOF MS spectrum of enriched phosphopeptides from tryptically digested bovine milk using mullite. α-S1 and α-S2 are the first and second subunits of α-casein, respectively. β represents phosphopeptides from β-casein. **doubly charged phosphopeptide $[M+2H]^{2+}$=1561.1 Da

Figure 4 MALDI-TOF MS spectra of tryptically digested human saliva before (A) and after enrichment using mullite (B). Phosphopeptides are labelled with asterisks.

Figure 5 Enrichment of synthetic phosphopeptides (0.5 µg/mL) from a digested HeLa cell lysate (1 mg/mL) at a ratio 1:1000. MALDI-TOF MS spectrum of dephosphorylated and tryptically digested HeLa cell lysate before (A) and after selective enrichment using mullite. Synthetic phosphopeptides: VYGKTpSHLR, $[M+H]^+$ = 1140.20; KIGEGTpYGVVYK, $[M+H]^+$ = 1392.67.

Tables

Table 1 Overview of enriched phosphopeptides from a protein mixture (lysozyme, cytochrome c, myoglobin, bovine serum albumin, α- and β-casein) and bovine milk after enrichment by mullite. The table includes a comparative study using conventional TiO_2 enrichment. α-S1 and α-S2 represent the first and second subunits of α-casein, respectively. β-C represents peptides from β-casein.

"s" represents a phosphorylated serine in the peptide sequences

"M*" oxidized methionine

Table 2 Overview of enriched phosphopeptides from tryptically digested human saliva.

"s" represents a phosphorylated serine in the peptide sequences

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