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Enrichment and desalting of tryptic protein digests and the protein depletion using boron nitride

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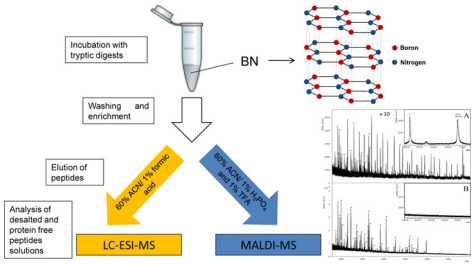
HIGHLIGHTS

- Protein tryptic digests were desalted and enriched utilizing hexagonal boron nitride.
- Phosphopeptides were desalted with high recovery rates.
- Boron nitride exhibits high wettability allowing fast sample preparation.
- Boron nitride shows protein depletion capability applied for peptide purification.

GRAPHICAL ABSTRACT



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ABSTRACT

Sample preparation still remains a great challenge in modern bioanalysis and the interest in new efficient solid phase extraction (SPE) materials still remains high. In this work, hexagonal boron nitride (h-BN) is introduced as a new SPE material for the isolation and enrichment of peptides. The h-BN is isoelectronic and structurally similar to graphite. It has remarkable properties including good thermal conductivity, excellent thermal and chemical stability and a better oxidation resistance than graphite. BN attracts increasing interest because of its wide range of applicability. In the present work, the great potential of h-BN, as a new SPE-material, on the enrichment, preconcentration and desalting of tryptic digest of model proteins is demonstrated. A special attention was dedicated to the efficient enrichment of hydrophilic phosphopeptides. Two elution protocols were developed for the enrichment of peptides compatible for subsequent MALDI-MS and ESI-MS analysis. In addition, the recoveries of 5 peptides and 3 phosphopeptides with wide range of pI values utilizing h-BN materials with different surface areas were investigated. 84–106% recovery rate could be achieved using h-BN materials. The results were compared with those obtained using graphite and silica C18 under the same elution conditions, and lower recoveries were obtained. In addition, h-BN was found to have a capability of protein depletion, which is requisite for the peptide profiling.

1. Introduction

Proteomics attempts to study all proteins present in a cell or an organism at a defined time and conditions using biochemical methods [1]. The proteome is highly dynamic and it can be modified by various influences such as environmental factors, diseases, their therapies, etc. For these reasons, plenty of effort is invested to explore the changes of the proteome during or before a disease to find new biomarkers. For efficient protein identification, peptide fingerprint plays a crucial role, after cutting into several smaller peptides using proteolytic enzymes [2]. The accurate mass of these newly formed peptides can be analyzed with mass spectrometer such as MALDI-MS or ESI-MS. The digestion protocol requires the addition of non-volatile salts and denaturation agents.

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However, mass spectrometers are very sensitive to these additives and impurities and therefore a suitable desalting procedure is necessary [3]. Several methods have been reported for sample purification, enrichment and desalting prior to MS analysis [4]. The techniques vary from ultrafiltration [5], dialysis [6], solid-phase extraction [7], etc. Nowadays the most common method is solid phase extraction with reversed-phase (RP) materials for example C 18 silica [8]. These materials are effective at retaining most tryptic peptides but are reported to fail at retention of many phosphopeptides, which tend to be more hydrophilic than other peptides [9]. Accordingly, graphite powder packed in GELoader tip microcolumns [10] and porous graphitic carbon columns [11] were used as alternatives or complementary to RP-microcolumns to avoid the loss of small and hydrophilic peptides.

The aim of this work was to investigate the suitability of hexagonal boron nitride (h-BN) as a SPE material for the enrichment and desalting of hydrophilic and hydrophobic peptides. BN exists in three crystalline forms: graphite analog α-BN (hexagonal BN), diamond analog β-BN (cubic BN or also zincblende BN) and γ-BN (wurzite-BN) [12]. h-BN is also known as white graphite and consists of a honeycomb structure of covalently bound boron and nitrogen atoms. In comparison to graphite, the layered BN hexagons are arranged vertically and each nitrogen is surrounded by 2 boron atoms of the adjacent layers. It combines unique electronic features with exceptional thermal and mechanical properties. It is chemically inert, nontoxic and has good environmental compatibility [13]. h-BN has numerous applications, ranging from low and high temperature lubricants up to various cosmetic products. BN also has enhanced physisorption properties due to the dipolar character [14,15]. Considering these excellent properties, h-BN was applied in this work as a SPE material for the enrichment and desalting of peptides solution prior to MS analysis. Tryptic digest of model proteins (α-casein, human and bovine serum albumin (HSA and BSA) were desalted under different conditions to explore the wide field of applications of h-BN as a SPE material and the recovery rates of selected peptides and synthetic phosphopeptides were evaluated.

In addition to salts, buffers or detergents also high abundant and high molecular weight proteins may interfere with the analysis of peptides [16]. As stated in literature, the fingerprint of this low molecular area of serum, urine, etc. might reflect the pathological state of organs and help in early cancer diagnosis [17,18]. For the screening of the peptidome, LC–ESI-MS and MALDI-MS are the methods of choice [19]. For an efficient identification of peptides in biological sample, the SPE materials must fulfill several criteria. The high salt concentrations of the fluids must be removed quantitatively and furthermore it is desirable to abscise bilirubin and other degradation products of the heme catabolism, which are responsible for the color of urine and serum. Biological fluids generally contain highly water soluble peptides, therefore the SPE material should have a high affinity to hydrophilic peptides. The desalting efficiency of h-BN was tested using urine spiked with BSA tryptic digest.

One major challenge of the analysis of biological samples such as urine, serum and plasma is the high dynamic range of peptide and protein concentrations of more than 10^{10} [20,21]. During LC analysis, the high molecular weight proteins can be adsorbed strongly to the RP-materials [22], resulting in carryover, peak tailing and a decreased separation efficiency [23]. Additionally in CE-analysis, the adsorption of proteins to the capillary wall can interfere dramatically [24]. Moreover, the reliable detection range of LC–MS or CE-MS is around 3–4 orders of magnitude, which is insufficient for detection of low-abundant proteins and peptides [25]. Therefore, a protein-free sample is a prerequisite for efficient separation and sensitive detection. In MALDI-MS the presence of high protein concentrations strongly suppress the ionization of peptides [26] and protein fragmentation takes place in the ion source (MALDI-in source decay (ISD)), which could interfere with peptide detection and identification [27]. In biological samples such as plasma or serum with thousands of different proteins [28], the ISD spectra become more complex and hinder the peptide identification. Several pre-fractionation strategies using chromatographic adsorbents have been employed to desalt samples and remove abundant proteins such as albumin and immunoglobulin. As an alternative to multi-stage fractionation chromatography, in this work we demonstrate the protein depletion capability of h-BN using a mixture consisting of 4 proteins and 6 peptides. Moreover, HSA protein solution spiked with its tryptic digest in a ratio of 50,000:1 was analyzed before and after protein depletion with BN.

2. Experimental

2.1. Reagents and chemicals

TRÈS BN® Cosmetic powder (h-BN-20) with 20 $m^2 g^{-1}$ specific surface area and 1 μm particle size and Combat® BN powder (h-BN-60) with 60 $m^2 g^{-1}$ surface area and 6 μm particle size were a gift from Saint-Gobain Ceramic Materials (Amherst, New York, USA). BN nano powder (h-BN NANO) with a surface area of 14 $m^2 g^{-1}$ was a gift from BORTEK Boron Technologies and Mechatronics Inc. (Eskisehir, Turkey). Graphite flakes with 15 $m^2 g^{-1}$ surface area (graphite 15) was from AMG Mining AG (Kropfmühl, Germany), graphite particle was a gift from BTR energy material co. ltd. (Shenzhen, China) and Hypersil ODS with 3 μm and 170 $m^2 g^{-1}$ surface area was from Grom (Rottenburg-Hailfingen-Germany). 2,5-dihydroxybenzoic acid (DHB) (puriss. p. a); sinapinic acid (SA) (≥99.0%); trifluoroacetic acid (TFA) (≥99.5); orthophosphoric acid (85.0%); formic acid (98–100%); acetonitrile (ACN) (G Chromasolv for HPLC); hexadimethrine bromide (≥94%); sodium hydroxide (reagent grade, ≥98%); dithiothreitol (≥99.0%); n-octylglucopyranoside (≥99.0%); iodoacetamide (≥98.0%); ammonium bicarbonate (≥99.5%); α-casein, HSA and BSA lyophilized powders (≥96%); Ubiquitin from bovine (≥98%), insulin from bovine, cytochrom C from bovine (≥95%), myoglobin from equine (≥95%), oxytocin acetate salt hydrate (≥97%); substance P acetate salt hydrate (≥95%); luteinizing hormone releasing hormone (LHRH) (≥97%); bombesin acetate salt hydrate (≥97%);bradykinin acetate salt powder (≥98%), angiotensin I human acetate (≥90%); angiotensin II human (≥93%); renin substrate tetradecapeptide porcine (≥97%) and adrenocorticotropic hormone (ACTH) fragment 18–39 (≥97%) were purchased from Sigma–Aldrich (Vienna, Austria). Disodium phosphate anhydrous (99.5%) and urea (for analysis, ≥98.0%) were from Merck (Darmstadt, Germany). Trypsin (sequencing grade modified) was purchased from Promega Biosciences (San Luis Obispo, CA). The peptide calibration standard was from Bruker (Bremen, Germany) and the synthetic phosphopeptides were a gift from Karl Mechtler and colleagues (IMP/Vienna, Austria). All chemicals were used directly, as received. The used urine was collected as midstream urine from the author. Water was purified by a Milli-Q unit (Merck Millipore, Darmstadt, Germany) and used for all experiments.

2.2. MALDI-MS analysis

One microliter of each sample was spotted on a stainless steel target (MTP-384 target ground steel TF, Bruker Daltonics, Bremen, Germany), mixed with 1 μL of the appropriate matrix and dried under ambient conditions. For peptide analysis a matrix solution consisting of 20 mg mL^{-1} DHB in ACN/H_2O 1:1 v/v containing 1% H_3PO_4 and 0.1% TFA was used and for protein samples a saturated solution of SA in ACN/H_2O 1:1 and 1% TFA was utilized. All