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New Materials and Techniques for Separation and Analysis of Clinically Important Proteins

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Abstract

This doctoral thesis deals with analysis of peptides and proteins using several analytical methods, mainly chromatography and mass spectrometry with following bioinformatic data processing. For the purpose of analysis of these important biomolecules, various techniques have been developed mainly for their targeted isolation from complex samples. These techniques also utilize newly introduced materials that bring a broad range of benefits such as high selectivity. Immobilization of various enzymes on selected micro- and nanomaterials is dealt with in another part of this thesis. Such created carriers with immobilized enzymes are subsequently utilized either for protein phosphorylation/dephosphorylation or for digestion of proteins.

Abstrakt

Tato disertační práce se zabývá analýzou peptidů a proteinů využívající několik analytických metod, především chromatografii a hmotnostní spektrometrii s následných bioinformatickým vyhodnocením. Za účelem analýzy těchto významných biomolekul byly vyvinuty různé techniky, a to především pro jejich cílenou izolaci z komplexních vzorků. Tyto techniky také využívají nově zavedené materiály, které přináší širokou škálu výhod, jako je například vysoká selektivita. Další součástí práce je také imobilizace různých enzymů na vybrané mikro- a nanomateriály. Takto vytvořené nosiče s imobilizovanými enzymy jsou následně využity buď pro fosforylaci/defosforylaci proteinů nebo pro jejich štěpení.

Keywords

Protein analysis, affinity chromatography, mass spectrometry, micro- and nanomaterials, TiO_2 nanotubes, recombinant proteins, protein modifications

Klíčová slova

Analýza proteinů, afinitní chromatografie, hmotnostní spektrometrie, mikro- a nanomateriály, TiO_2 nanotrubice, rekombinantní proteiny, modifikace protein

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1. Introduction

1.1 Proteins

In living organisms, combination of 20 different amino acids, which are held together via peptide bonds, creates proteins. Different sequences of these amino acids give proteins of different sizes, structures and functions. The main features of proteins in organisms are highly variable and are represented by e.g. expression, regulation, activation, transfer, interaction, modification and many other functions. The analyses of proteins and their features have shifted to more comprehensive approaches mainly after completed genome sequences became available, which resulted in a more complete picture of cells and the whole organism. The term *proteome*, representing the whole set of cellular proteins, was first devised in 1996 in a work analyzing *E. coli* proteins [1] and the term *proteomics* appeared briefly after, in 1997 [2]. Among other techniques, mass spectrometry (MS) has become more popular in this field because of its ability to analyze the variability associated with the proteome.

1.1.1 Posttranslational modifications of proteins

The organisms have only limited coding capacity for proteins and they have two major mechanisms of expanding it. The first of them, at the transcriptional level, is alternative splicing of messenger ribonucleic acid [3] and the second one, occurring after translation of ribonucleic acid into proteins, is called *covalent posttranslational modification* (PTM). PTMs refer to the (often proteolytic) cleavage, to the addition of small chemical moieties (e.g. a phosphate group or mannose) to certain amino acid residues or their connection via disulfide bonds [4]. PTMs can also be associated with different changes in living organisms such as physiological state or disease [5]. Analysis of these changes can be challenging due to high variability of PTM types and localizations.

One example of PTM is reversible phosphorylation of proteins that regulates many aspects of cell life, while abnormal phosphorylation is a cause or consequence of serious diseases [6,7]. It is estimated that about 30% of all proteins in a cell are phosphorylated during their life cycle [8]. However, relative abundance of phosphorylated proteins in organism at a designated moment is very low. Phosphoproteins are formed via a catalytic enzyme and adenosine triphosphate, serving as a donor of the phosphate group [9]. Dephosphorylation is done by phosphatases that hydrolyze the covalent bond connecting the phosphate to the protein.

Proteins are also subjected to oxidative post-translational modifications. These modifications that are reversible are involved in physiological processes and non-reversible ones may contribute to pathological situations [10]. Cysteine and methionine are the two amino acids present in proteins that contain sulphur and that both of these amino acids are subject to reversible oxidation and reduction. It was also shown that they are involved as key antioxidants important for the structure and stability of proteins [11]. Oxidation of methionine is a reversible covalent modification, similar to phosphorylation. Due to its similarity, the cyclic oxidation and reduction of methionine residues could be a part of regulatory processes, including cell signaling [12]. Cysteine is involved in the most common covalent linkage of proteins represented by disulfide links [13].

1.1.2 Genetic and chemical modifications of proteins

Beside natural modifications of proteins, the development of many artificial modifications takes place in protein analysis and production. In both simple and complex protein analyses, chemical modifications are mostly related to their sufficient proteolytic cleavage where they are reduced and alkylated. Chemical modifications are also important for understanding clinically important processes, where modifications are used prior to quantitation of proteins and their PTMs within diagnosis or therapy monitoring [14].

Prior to production of recombinant proteins and their purification, gene manipulation creates an important modification of the primary sequence. Recombinant proteins are important in life science applications because they are used for the development and production of efficient and selective biopharmaceuticals, including targeted bioactive therapeutics. Highly pure and non-aggregated biomolecules are required especially for *in-vivo* applications to prevent adverse side effects [15]. Prior to their further purification, they are commonly synthetized with an appropriate tag or modified after their expression.

Various affinity tags are available and used as highly efficient tools for protein purification. Tags are mostly represented by short additional amino acid sequences (e.g. polyhistidine affinity tag – His-tag) or by proteins (e.g. glutathione S-transferase). They are located either at the C- or N-terminal end or at both ends of the target protein. These sequences represent a defined tag for specific binding partners, e.g. high-affinity antibodies or a metal-complex [16] which is useful in affinity chromatography. Beside the large number of options, there is no universal affinity tag suitable for purification of any target protein and in many cases, the experimental confirmation of tag usability is required [17].

His-tag is a fusion tag usually composed of six histidine residues in a row attached at the N- or C-terminus of recombinant protein and it is used as a common binding motif for metal ions in immobilized-metal affinity chromatography (IMAC) [18]. Contrary to IMAC technology, where interaction is mediated by chelated metal ions, in metal oxide affinity chromatography (MOAC) the surface of the carrier itself is responsible for the interaction with separated proteins. The MOAC technology was also employed in His-tagged protein purification with materials composed mainly of nickel or cobalt oxides [19, 20]. Recently, two magnetic materials based on Fe_3O_4 nanoparticles have also been developed [21, 22].

1.2 Micro- and nanomaterials and their applications

In the past decades, there was an intensive interest to develop new nano/micromaterials with different composition, morphology or functions in order to simplify their use or to improve the results of their applications. Although microscale materials have a great impact in various research and technology fields, the further development of new materials was focused on nanostructures and nanomaterials, where identification of new functions, properties or applications became important [23]. The term of “nano” is defined by the fact that the size of structurally important units of these nanomaterials is about 1–100 nm along any single dimensional scale [24]. Nanomaterials often possess

unique optical, magnetic, electronic or mechanical properties and are strongly utilized in many areas of research and technology [25, 26]. Unique properties of nanomaterials could be exploited in analytical or large-scale separation of biomolecules.

Superparamagnetic materials bring a number of advantages, such as selective, sensitive, and controlled target molecule separation, fast separation of particles from the liquid phase in magnetic field, zero loss of the carrier or sample. Affinity based separation utilizing biofunctionalized magnetic particles could provide efficient separation and purification of target molecules with great time, labor, and yield efficiency [27].

Inorganic materials represented by silicone dioxide, graphite, hydroxyapatite, metal oxides, etc. have been widely adopted for research and applications in chromatography separations [28-32]. Mainly SiO_2 is the most important inorganic material and has been developed as the most commonly used HPLC (high performance liquid chromatography) packing. SiO_2 is also commonly involved in many composite nanomaterials, whether as a part of polymer-silicone dioxide materials [33] or as a shell layer in various types of magnetic nanoparticles e.g. for DNA extraction [34] or immobilization of proteolytic enzymes [35].

TiO_2 can be produced either in different crystalline phases including rutile, anatase, brookite or in amorphous state [36]. One of the shape forms, TiO_2 nanotubes, has been employed e.g. in biomedical applications as a TiO_2 porous coatings on bone implants [37] or for a drug delivery [38]. The production of TiO_2 nanotubes using anodization process yields highly ordered and uniform structure with controllable size of pores and it can be produced in amorphous state or in crystalline form with further annealing [37]. The pore sizes of anodically grown nanotubes are ranging from 15 to 230 nm. They could be prepared with various nanotube lengths, commonly of tens to hundreds of micrometers [21, 37, 39, 40].

In general, supports for creation of stationary phase for affinity chromatography can be selected from either polymeric or inorganic materials with high variability of shapes and sizes, or monolith can be employed [19, 41, 42]. The affinity ligand immobilized to the matrix could be selected from biological agents, such as proteins, or from synthetic molecules. The materials, immobilization techniques and immobilized molecules are all together the basis for utilization in many fields of life sciences e.g. diagnostics, affinity chromatography, immobilized enzyme reactors or biosensors [43, 44], where advantages of immobilized enzymes are most obvious.

1.2.1 Enrichment of phosphopeptides/proteins using different types of materials

Difficulties during MS analysis of phosphopeptides are mainly caused by their low efficiency of ionization as compared with that of nonphosphorylated peptides [45, 46]. Various methods and various commercial materials are routinely used for enrichment of mono- and multi-phosphorylated peptides. Most common enrichment techniques are, among others, IMAC [47] and MOAC, which is mostly represented by TiO_2 forming bidentate complexes with phosphates [48]. In order to enrich of mono- and multi-phosphorylated peptides/proteins from biological materials, an IMAC separation technique with immobilized trivalent ions (e.g. Fe^{3+}, Ga^{3+}, Al^{3+}) further extended to

tetravalent ions (e.g. Ce^{4+}, Zr^{4+}, Ti^{4+}) is utilized [49]. However, in many studies, MOAC is preferably used for the specific enrichment of phosphopeptides due to its selectivity. MOAC is mainly represented by TiO_2 microspheres, which plays a key role in many large-scale phosphoproteomic studies [50, 51]. Another one, TiO_2 nanotubes, have recently been shown for selective phosphopeptides enrichment in complex samples [52].

1.3 Protein analysis

The main task of proteomics is the analysis of the proteomes of various organisms, single organs, cells or physiological fluids. Commonly, the analysis of primary structure of proteins is performed by MS with previous cleavage of proteins with proteolytic enzymes into shorter peptides and with various methods of peptide separation. The knowledge about genetic information is essential for proteomic analysis, as after translation and integration of these data sets through bioinformatics will yield a comprehensive database subsequently used for protein identification. Analytical methods for analysis of intact protein forms are still not fully developed and used as analysis on peptide level but can also offer information about isoform and PTM diversity in biological samples [53, 54]. The study of the structure, quantitative dynamics of proteins and their posttranslational modifications in current proteomics are of prime interest in this field. One of the key features of MS is high sensitivity and its ability to detect, identify and characterize individual peptides in complex mixtures especially if the MS is combined with HPLC.

Gel-based approach is mostly represented by polyacrylamide gel electrophoresis in 1D or 2D mode. Proteins after separation by polyacrylamide gel electrophoresis are then visualized using various staining techniques and the identity of proteins can be confirmed using in-gel digestion followed by MS or liquid chromatography–MS (LC-MS). 2D gel electrophoresis allows to fractionate protein mixtures and identify protein forms differing in physicochemical properties and modifications [55].

LC-MS approach exploits HPLC techniques. Peptides and proteins are usually fragmented, and MS/MS (tandem mass spectrometry) spectra are acquired. In the case of automatic database search, MS and MS/MS spectra of peptides are used in the search for protein database matches to achieve exact protein identification using well known software (e.g. Mascot, Sequest, X!Tandem) [56]. Theoretical and experimental spectra are compared looking for a statistically significant match. In some cases, e.g. where the desired protein sequence absents from the database, or where the encoding gene is spliced alternatively, the database search may provide unclear results, which can be refined by using *de novo* sequencing [57].

1.3.1 Analytical separation of proteins and peptides

Some samples of peptides/proteins can be directly analyzed by MS in the case of simple mixtures or analysis of protein from gel electrophoresis. However, most of the proteomic samples are very complex in their composition and have to be fractionated prior to introduction to the mass spectrometer [58]. Reversed-phase liquid chromatography (RPLC), ion exchange chromatography (IEX) and hydrophobic interaction liquid chromatography (HILIC), are the most common liquid

chromatography approaches applied to analysis of proteins and peptides [59]. Separation can be applied offline, independently of the mass spectrometer, but the main part of analyses is performed on-line with direct injection into the MS. To get a higher performance of analysis, gradient nanoflow RPLC separation of peptides is usually utilized or the separation is extended by additional preceding dimension [60, 61].

1.3.2 The role of mass spectrometry in protein modifications study

When the analytes are separated, the two main methods used for ionization and transferring the peptides into mass spectrometer in bottom-up proteomics are nano-electrospray ionization (nano-ESI) [62] or, to a lesser extent, matrix-assisted laser desorption/ionization (MALDI) [63]. The complexity of posttranslational modifications in samples therefore requires modern methodologies including LC separation of peptides or their selective isolation. MS-based analysis has become the most utilized method in large scale phosphoproteomics studies as well as in studies of other PTMs.

The main part of analyses of proteins in this thesis was performed using the Orbitrap-based mass spectrometers (LTQ Orbitrap XL and Q Exactive). The Orbitrap analyzer was described as a suitable mass analyzer for proteins and peptides [64] and is part of many hybrid instruments. On of them, LTQ Orbitrap XL uses a high resolution Orbitrap cell and a linear ion trap (LIT) for accumulation, selection, fragmentation or analysis of ions. However, this instrument is also equipped with a dedicated collision cell for higher-energy collision dissociation (HCD) fragmentation. The LTQ Orbitrap XL is able to analyze peptides or other analytes with high resolution (100,000 at m/z 400) in the Orbitrap cell as well as peptide fragments. Beside ESI, MALDI source can also be interfaced to LTQ Orbitrap XL instrument, which was also shown for proteomic analysis [65]. The Q Exactive is a high-resolution mass spectrometer with an Orbitrap cell able to detect both precursor and fragment ions [66]. This instrument is equipped with stacked-ring ion guide (S-lens) in the source region, a quadrupole mass filter, a C-trap, Orbitrap mass analyzer and also HCD cell enabling fragmentation. As compared to LTQ Orbitrap XL instrument, Q Exactive is benchtop, has a shorter ion path and has improved electronics enabling faster analysis with the same resolution as LTQ Orbitrap XL [66].

One of the key components in proteomics protocols is the generation of MS/MS spectra mainly by peptide fragmentation using collision-induced dissociation (CID). CID is the most-widely used technique of gas phase fragmentation of peptides in proteomics [67]. Alternatively, but in increasing extent, peptide fragmentation methods such as HCD [68] and electron capture/transfer dissociation (ECD/ETD) provide significant improvements in the identification of certain groups of peptides, proteins, and post-translational modifications. ETD technique [69] is based on electron transfer to a multicharged peptide from negative ions (electron transfer dissociation). Peptides containing any labile posttranslational modification (e.g. phosphorylation) or peptides that are longer and contain more internal basic residues are poorly fragmented by CID [70]. HCD or ETD improve the analysis of long peptides with a higher number of charges and that of peptides containing many or highly labile PTMs [71].

2. Experimental part

2.1 Aims and objectives of Ph.D. study

All topics dealt with in this Ph.D. study have a common aim, to use selected nano- and micromaterials for advanced separation, modification and analysis of target proteins. The aims of the work included, among others, development of new methodological approaches for enrichment and purification of modified or hydrophobic biomolecules from complex matrices using appropriate materials and/or procedures.

The modification of the available materials was another aim, showing strategies of how to improve enzyme catalyzed reactions. To get appropriate information about a protein's identity, chemistry or behavior, a spectrum of analytical methods including MS and gel electrophoresis can be successfully applied.

The doctoral thesis contains a comprehensive set of methodological approaches utilizing different materials, either modified commercial ones or newly developed ones. Current analysis of protein sequence or its modifications often consists of well designed and precisely performed preanalytical and analytical phases starting at the protein level and continuing to peptide or amino acid level. Within this process, proteins or peptides could be specifically modified and the resulting modified molecules can be analyzed with the same procedure. This thesis consists of 5 published papers, 1 submitted manuscript and 1 patent covering several parts of analytical procedure of proteins and peptides. The scheme in **Figure 1** shows a selected part of the general sample processing in proteomic analysis with emphasized sections covered by the research work carried out in the presented doctoral thesis.

For evaluation of all methods and materials mentioned in this study several analytical techniques were applied. This panel consists of both separation and detection methods, respectively. MS as the prevailing analytical technique was adapted for various types of analytes, differentiated according to the complexity of sample, sample type and expected molar mass range, time management or simplicity of analysis. MS with MALDI ionization was the preferred technique in the experiments described here and two types of mass spectrometers with this source were used. First, hybrid mass spectrometer MALDI-LIT-Orbitrap was preferred for vast majority of analyses of peptides and their modifications in samples of low complexity and for monitoring of enzymatic reactions. As a second MS instrument, MALDI-TOF/TOF mass spectrometer (TOF – time-of-flight) operating in the linear mode served as the device for analysis of intact proteins. Another important analytical technique suitable for intact proteins was gel electrophoresis. Using one of its forms Glycine-sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [72] or Tricine-SDS-PAGE [73] – it was possible to cover theoretically the analysis of proteins in the mass range 1–500 kDa. Mainly Tricine–SDS-PAGE was the optimal strategy for our experimental purpose due to our focus on lower mass range proteins.

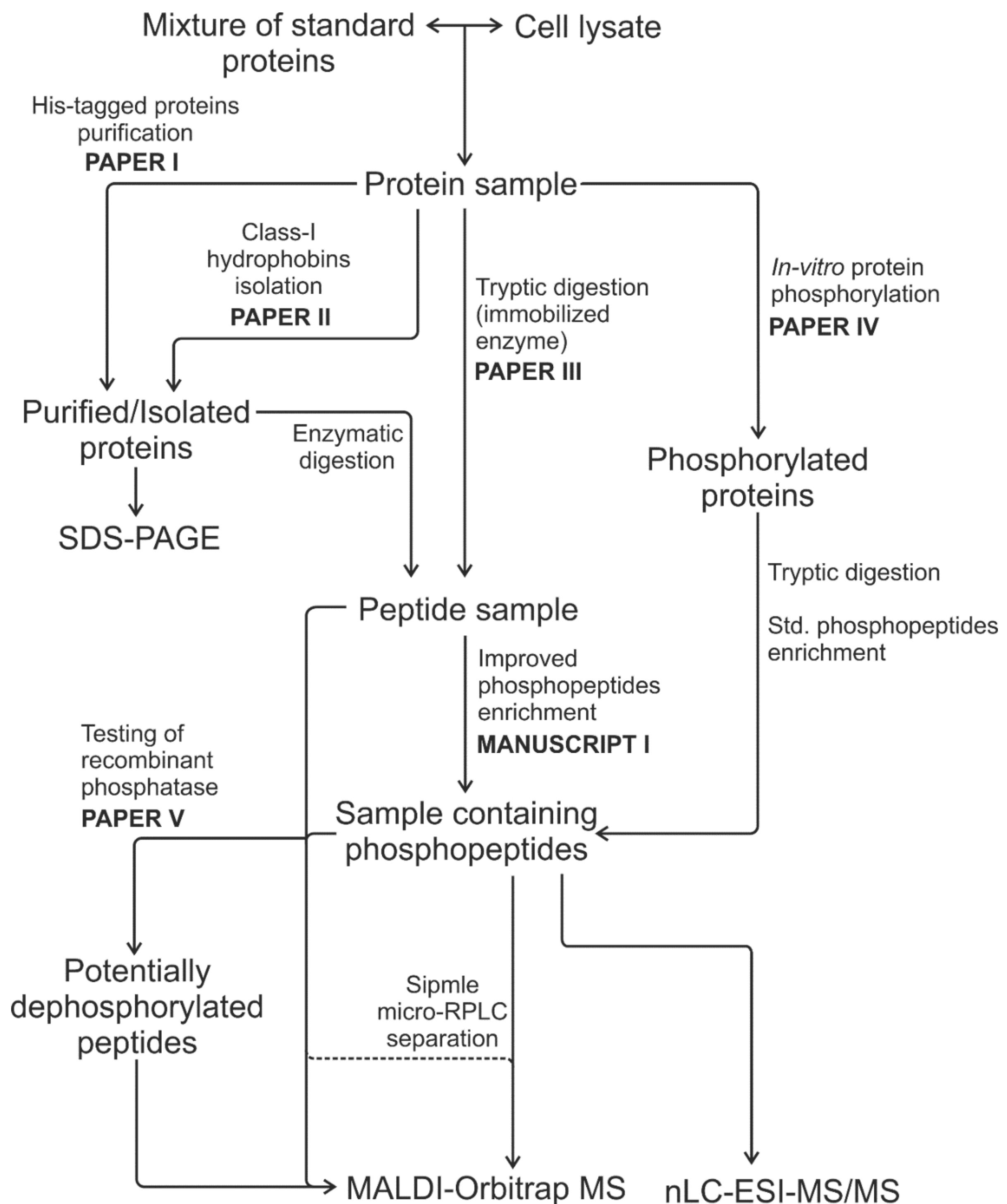

Figure 1.: An overview of selected part of proteomic sample preparation including the experimental design, methodology and materials used; papers addressing particular aspects of the overall analysis workflow are included.

The separation of intact proteins using gel electrophoresis serves for their effective detection and identification. On the other hand, liquid chromatography is more suitable for peptide separation followed by the MS/MS-based identification. In the case of simple peptide mixtures, simple microcolumn RPLC approach was applied to increase sequence coverage of proteins or to detect low abundant peptides with MALDI-MS. This

technique consists of self-made microcolumn packed with reversed stationary phase. Fractions of the original sample were obtained by application of nonlinear gradient elution formed in a microsyringe by aspirating several mobile phases with gradually decreasing acetonitrile (ACN) content. Peptides eluted from RP microcolumn were directly applied onto the MALDI plate with subsequent mixing with MALDI matrix solution [74-76].

Phosphopeptide fractions generated by their selective isolation from complex peptide mixtures were then analyzed using nanoLC-ESI-MS/MS with Orbitrap mass analyzer in the Q Exactive instrument. The system is capable of analysis of many complex samples in a short span of time. This allowed a comprehensive analysis of prepared samples resulting in identification of thousands of phosphopeptides. The capability of the analytical system to cover a sufficient part of the phosphopeptide sample was essential for obtaining informative results enabling comparison of enrichment procedures using different types of materials.

The following part of doctoral thesis includes the obtained results published in peer-reviewed journals with impact factor (IF) or in manuscript submitted for publication as well as in one granted patent. Author’s contribution on presented results is included.

2.2 Results

2.2.1 New interface for purification of proteins: 1D TiO_2 nanotubes decorated by Fe_3O_4 nanoparticles (Paper I)

To address limitations of IMAC technique, a high surface area interface based on anodic 1D TiO_2 nanotubes homogeneously decorated with Fe_3O_4 nanoparticles (TiO_2NTs@Fe_3O_4NPs) was developed and tested for purification of His-tagged recombinant proteins. The whole concept arose from observed co-isolation of His-tag containing peptides in experiments focused on phosphopeptide enrichment with TiO_2NTs@Fe_3O_4NPs. Compared to the isolation of phosphopeptides, it was necessary to address several crucial changes in the protocol because recombinant proteins cannot be purified under such harsh conditions as used for phosphopeptides enrichment.

First of all, binding conditions were optimized using different buffers with pH varying from 3.2–7.5. In contrast to conditions of phosphopeptides enrichment, binding solution did not contain any organic solvent or carboxylic acid, which is usually used for reduction of peptide non-specific binding. The pH 6.5 was identified as the most suitable value regarding the specificity of the isolation. The second part of protocol customization consisted in choosing of proper elution conditions, because imidazole only (commonly used elution solvent in IMAC based purification protocol (Fig. 2. – lanes b3, b4)) was ineffective for this purpose (Fig. 2. – lane a3). It was found that key component of elution solution was Na_2HPO_4, optimally in the presence of imidazole (Fig 2. – lanes a4, a5). Ammonia solution was avoided as a component of elution solution because it could affect protein stability. The lower effectivity of imidazole to elute His-tagged proteins from TiO_2NTs@Fe_3O_4NPs allowed to use higher imidazole concentrations during washing steps to reduce non-specific protein binding.

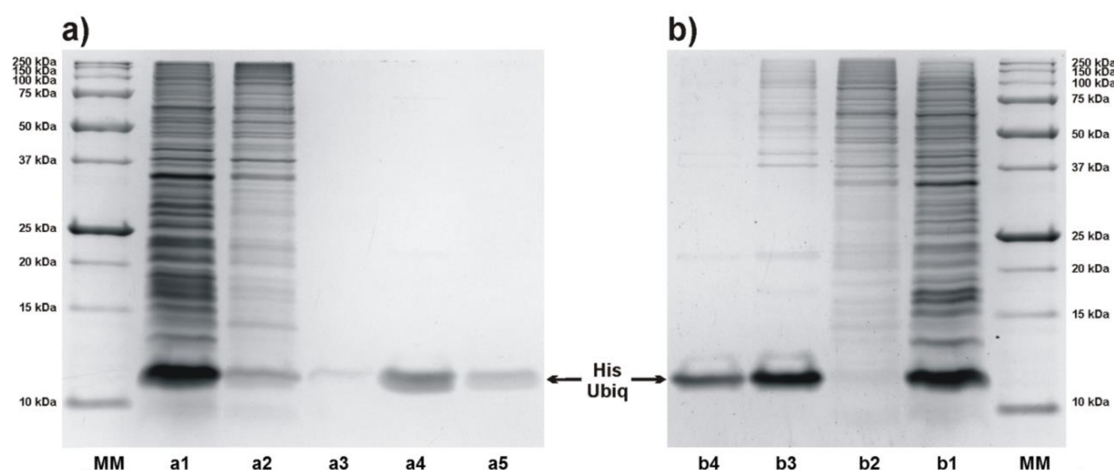

Figure 2.: Tris-Tricine-SDS-PAGE analysis of the His-tagged protein purification experiment with Jurkat cells lysate spiked with His-tagged ubiquitin (His Ubiq) where (a) represents purification on the TiO_2NTs@Fe_3O_4NPs, followed by silver staining. Lanes: MM, marker of molecular weights; a1, initial cell lysate; a2, flow-through; a3, first elution (300 mM imidazole in 50 mM Bis-Tris-HCl buffer pH 6,5); a4, second elution (100 mM imidazole, 200 mM Na_2HPO_4 in 10 mM Bis-Tris-HCl buffer pH 6,5); and a5, third elution (same as second elution). (b) represents purification on IMAC resin (HisPur Ni-NTA resin), followed by silver staining. Lanes: MM, marker of molecular weights; b1, initial cell lysate; b2, flow-through; b3, first elution (300 mM imidazole in 50 mM Bis-Tris-HCl buffer pH 6,5); and b4, second elution (same as first elution).

Taken together, excellent purification results of His-tagged protein were achieved both for the model protein mixture used for initial testing, as well as for the whole cell lysate due to the application of $TiO_2NTs@Fe_3O_4NPs$ combined with tailored purification protocol. This new material for specific isolation of His-tagged proteins significantly enhanced the purity of target protein as compared with a conventional IMAC system (Fig. 2.). $TiO_2NTs@Fe_3O_4NPs$ can also offer other interesting features and benefits. $TiO_2NTs@Fe_3O_4NPs$ consists of two basic metal oxides possessing specific isolation properties, magnetic features, biocompatibility, non-toxicity, recyclability and easy decontamination by UV-light. These features make it a suitable candidate for future directions in purification of recombinant proteins applied for *in-vivo* clinical applications or for rapidly expanding biological therapy.

Author's contribution: The feasibility of purification of His-tagged protein on $TiO_2NTs@Fe_3O_4NPs$. Testing different pH conditions, binding buffer composition, washing conditions of unwanted non-specifically bound proteins as well as development of new composition of elution buffer. Determination of binding capacity and recyclability of the system. Partially on selection of diameter of TiO_2 nanotubes, description of supposed binding mechanism and manuscript design and writing.

2.2.2 Selective isolation of hydrophobin SC3 by solid-phase extraction with polytetrafluoroethylene microparticles and subsequent mass spectrometric analysis (Paper II)

In this work, a simple, effective and inexpensive method for isolation of highly hydrophobic protein – hydrophobin SC3 of *Schizophyllum commune* as the most common representative of class I hydrophobins. A solid phase extraction method utilizing commercially available polytetrafluoroethylene (PTFE) microparticles has been developed. More effective and selective separation method for class I hydrophobins may be crucial for their analysis and production. The selectivity of this method is based on the extremely strong interaction between hydrophobin SC3 and PTFE microparticles tolerant to SDS solution washing [77], which enabled the removal of all other contaminating proteins. Due to class I hydrophobin's potential to self-assemble on hydrophilic/hydrophobic interfaces, all procedures and storage of the protein were performed in glass vials to prevent adverse losses caused by adsorption on the adhesive plastic material. To dissolve the SC3 hydrophobin in water-based solution, the sample was treated with pure trifluoroacetic acid, dried and redissolved in water or Tris-HCl buffer. For the isolation of SC3 hydrophobin from the standard protein mixture, polytetrafluoroethylene (PTFE) microparticles were packed in the GELoader Tip as dried particles as it was difficult to resuspend them in common solvents. The isolation protocol consisted of a binding and of washing steps with gradually increased elution strength of washing solutions to remove remains of weakly bound proteins. Finally, SC3 hydrophobin was released from PTFE microparticles using pure formic acid to get the target protein with high purity (Fig. 3.). Analysis of hydrophobin SC3 was performed using SDS-PAGE and MALDI-MS at the protein level. The protein was also studied at peptide level after the application of the advanced digestion protocol comprising reduction of disulfide bonds that was done with a combination of reducing

agents dithiothreitol and tris(2-carboxyethyl)phosphine in the presence of a denaturing agent at high reaction temperature. Chymotrypsin, pepsin and thermolysin were chosen as potentially suitable enzymes for hydrophobin SC3 digestion due to the presence of potential cleavage sites and chymotrypsin was finally identified as the most suitable enzyme for hydrophobin digestion.

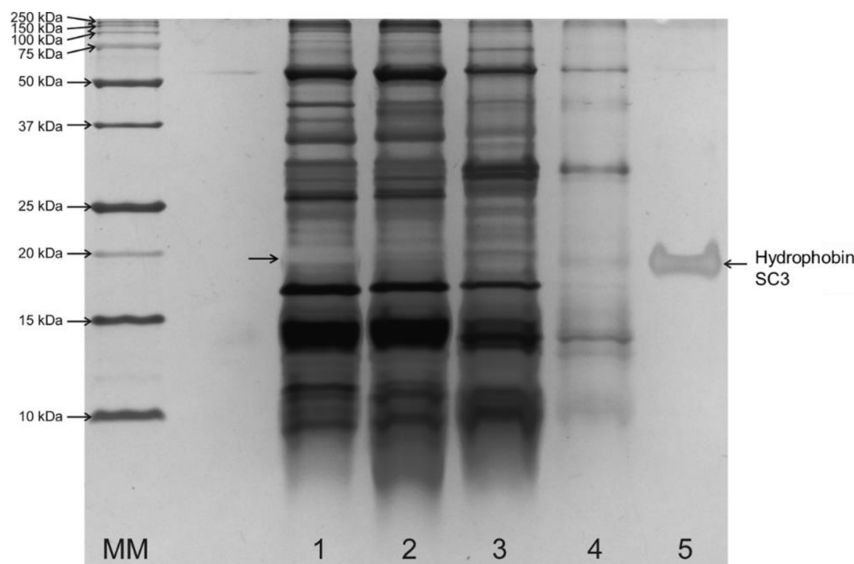

Figure 3.: Tris-tricine-SDS-PAGE analysis of fractions collected during the hydrophobin SC3 protein purification experiment from the model mixture of 10 proteins with PTFE microparticles loaded in microcolumn followed by silver staining. Lanes: MM – marker of molar masses, 1 – model protein mixture, 2 – flow-through, 3 – first washing with 80% ACN/0.1% TFA, 4 – second washing with 1% SDS in water, 5 – elution with formic acid. The protein band in the lane 5 corresponds to the selectively isolated hydrophobin SC3.

The proposed system exhibits excellent parameters for hydrophobin isolation. This work represents an application of a well-established material to a different purpose based on basic knowledge protein' structure and behavior in liquid phase or on the liquid/solid interface. Despite some difficulties concerning digestion of SC3 hydrophobin due to its unfavorable properties (such as low solubility, high hydrophobicity, very stable disulfide bonds and absence of basic residues), the effective reduction and alkylation of disulfide bonds with subsequent enzymatic cleavage with chymotrypsin was proven to be applicable to further bottom-up proteomic analysis.

<u>Author's contribution:</u> The design of isolation and digestion protocols, MS analysis and data evaluation. Manuscript writing.

2.2.3 Application of trypsin $Fe_3O_4@SiO_2$ core/shell nanoparticles for protein digestion (Paper III)

The presented work relates to the development of new magnetic nanomaterial suitable for covalent immobilization of proteolytic enzymes. The commonly used proteolytic enzyme trypsin was covalently immobilized by attaching it to porous $Fe_3O_4@SiO_2$ core/shell nanoparticles decorated with free amino groups by reaction with 3-aminopropyltriethoxysilane.

Firstly, the specific enzyme activity of trypsin immobilized on $Fe_3O_4@SiO_2$-NH_2 was estimated by low-molecular substrate. Michaelis–Menten constant and v_{max}, storage stability and reusability of the porous magnetic $Fe_3O_4@SiO_2$ nanoparticles with immobilized trypsin were determined. Relatively lower value of the apparent Michaelis–Menten constant K_M (0.399–0.658 mM) indicates improvement in enzyme–substrate affinity after its immobilization compared with its soluble form. Furthermore, in comparison with commercially available SiMAG-Amine microparticles used for immobilization, the newly developed nanomaterial with immobilized enzyme showed more than three times higher specific enzyme activity per mg of particles. The activity and specificity of trypsin–$Fe_3O_4@SiO_2$-NH_2 nanoparticles were also evaluated using α-casein proteolytic digestion experiments followed by high-resolution MS analysis using MALDI-LIT-Orbitrap MS (Fig. 4).

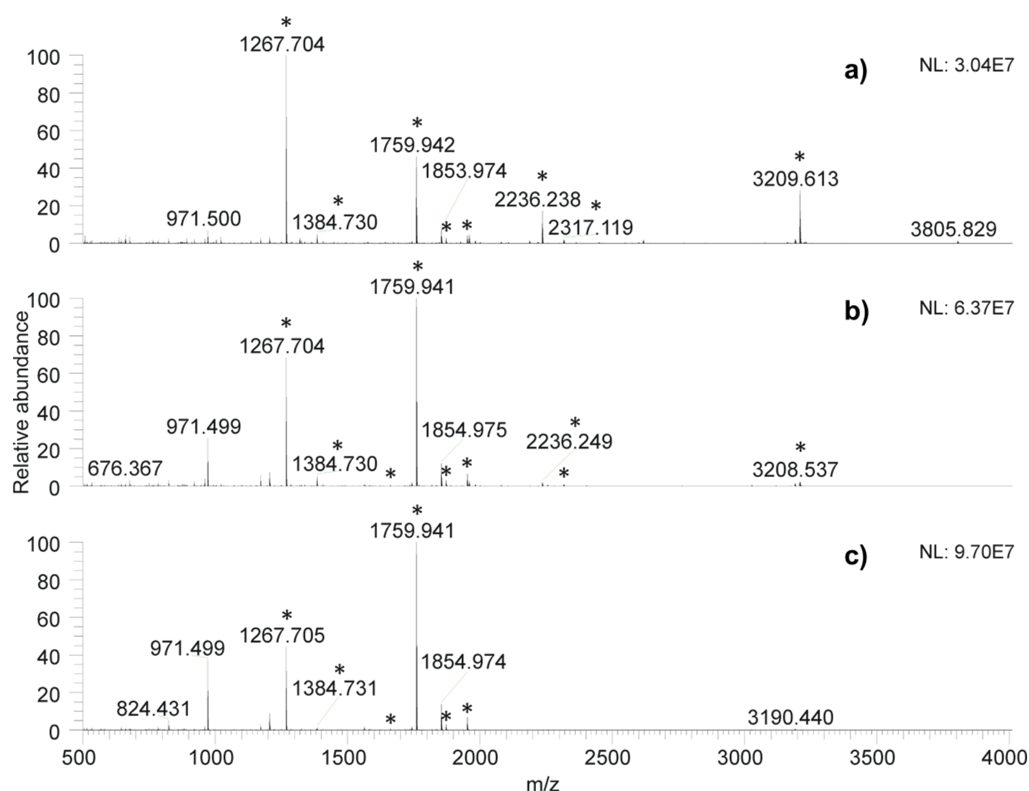

Figure 4.: MALDI-Orbitrap mass spectra (m/z 500–4000) of peptides obtained by digestion of α-casein with trypsin $Fe_3O_4@SiO_2$-NH_2 nanoparticles in different times. Digestion times: (a) 15 min, (b) 1 h, and (c) 3 h. Asterisks show specifically cleaved peptides (0 or 1 missed cleavage).

Regarding the set of identified peptides, the mass spectra of the α-casein digested with soluble sequencing grade trypsin and with the trypsin–$Fe_3O_4@SiO_2$-NH_2 nanoparticles were comparable. SiMAG-Amine microparticles with the immobilized trypsin showed higher intensity of peptides having missed cleavage sites in their primary sequence. High proteolytic activity and specificity of trypsin–$Fe_3O_4@SiO_2$-NH_2 nanoparticles was confirmed by the increased abundance of tryptic peptides. Furthermore, the enzyme immobilized in this way remained active at least 2 weeks after the immobilization when stored at 4°C without any significant decrease of its proteolytic activity.

In summary, it was shown that porous $Fe_3O_4@SiO_2$-NH_2 nanoparticles are a suitable carrier for proteolytic enzymes immobilization and moreover they possess several advantages, e.g. enhanced stability, higher activity and specificity as compared to soluble form of trypsin.

Author's contribution: MS analysis of α-casein digests and the evaluation of acquired data. Partially the setting of digestion protocol.

2.2.4 Kinase-loaded magnetic beads for sequential *in vitro* phosphorylation of peptides and proteins (Paper IV)

Protein phosphorylation impacts the physiological function of proteins, especially those that are natively unfolded and implicated in many neurodegenerative diseases. In studies related to structure analysis of proteins participating in the pathogenesis of these diseases, the standards with defined phosphorylation are needed. Some of these protein forms are very rare and it may be difficult or impossible to isolate them from natural tissues, e.g. human brain. Recombinant proteins which are further modified to approximate natural post-translational modifications may be beneficial.

The main goal of this paper was to phosphorylate recombinant proteins or peptides *in vitro* in a controlled manner with kinases of proper specificity. To get phosphorylated proteins with minimal enzyme contamination, kinases immobilized on various magnetic microparticles were tested. The advantages of immobilized enzymes consist in their recoverability, stability and possibility of sequential use of different enzymes which can inhibit each other.

In detail, this work deals with non-oriented covalent immobilization of proline-directed kinases glycogen synthase kinase 3β (GSK-3β) and mitogen-activated protein kinase 1 (ERK2), both of which are Ser/Thr kinases expressed in human central nervous system. As GSK-3β kinase strongly prefers pre-phosphorylated substrates with motif SXXXpS (S – serine, X – any residue, pS – phosphoserine), tau protein had to be prephosphorylated with ERK2 kinase to take full advantage of phosphorylation with GSK-3β kinase. For optimization and for reusability demonstration, low-moleculare peptide substrates were phosphorylated such as tyrosine hydroxylase 24–33 (TH 24-33) with amino acids sequence KQAEAVTSPR specific for ERK2 kinase testing (Fig. 5.). In Figure 5, non-phosphorylated TH 24-33 peptide with m/z 1086.59 and its phosphorylated form with m/z 1166.56 are visible. Immobilized systems were also tested against recombinant tau, a clinically relevant model protein, to obtain sequentially

phosphorylated products of high purity, without contamination of final product with soluble kinases. Another benefit is reusability and storage stability of immobilized system, which for GSK-3β kinase is at least 10 phosphorylation cycles and 42 days of storage at 4 °C. Thus, such modified magnetic beads may prove as a capable tool for *in vitro* modification of various recombinant proteins.

In comparison with soluble kinases, the kinase-beads should be easier to handle, reusable and they possess the ability to be removed from reactions, thus contamination of phosphorylated protein with kinases is minimalized.

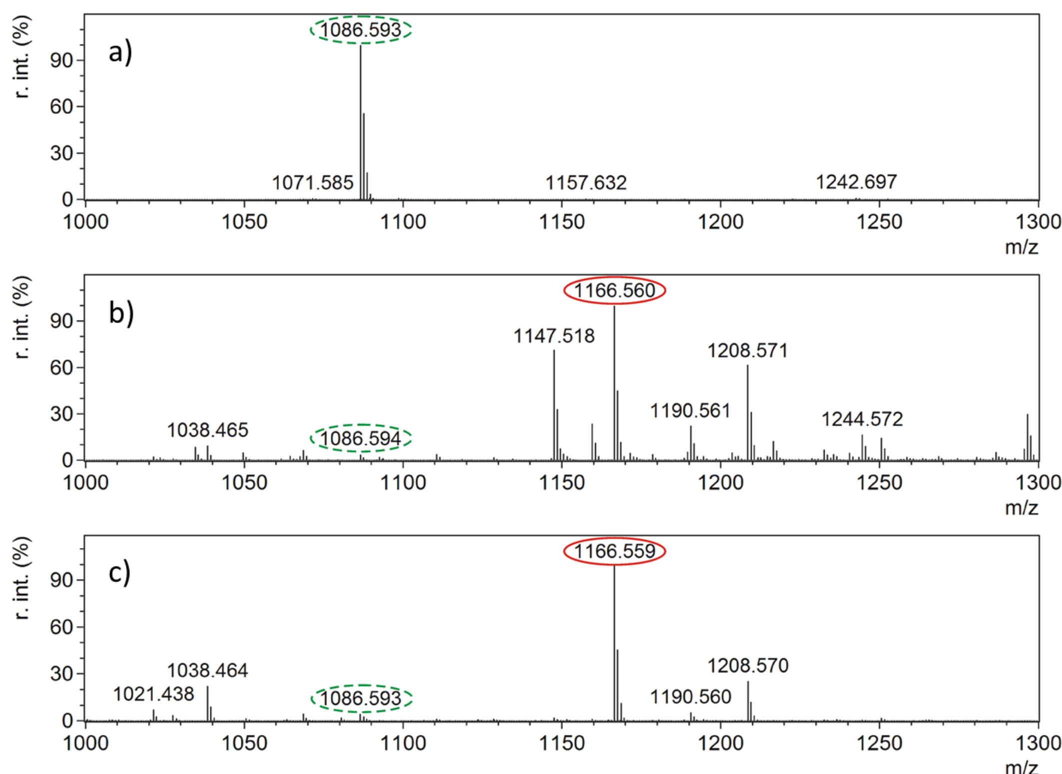

Figure 5.: Representative MALDI-Orbitrap mass spectra of (a) non-phosphorylated TH 24-33 low-molecular weight substrate, and TH 24-33 phosphorylated, for 6 h at 30 °C with (b) soluble ERK2 kinase as reference, and (c) 0.2 mg ERK2-loaded SeraMag beads. Non-phosphorylated peptides are marked with a green dashed circle, and phosphorylated forms are circled in red circle.

Author's contribution: Part of MS analysis of substrates, the evaluation of acquired data. Analysis of phosphorylated tau protein. Writing of appropriate parts of manuscript.

2.2.5 *PHO15* genes of *Candida albicans* and *Candida parapsilosis* encode HAD-type phosphatases dephosphorylating 2-phosphoglycolate (Paper V)

In bioinformatic databases, there is a huge number of predicted proteins from DNA sequence that have never been synthesized or isolated. Most of the phosphatases of human fungal pathogens *Candida albicans* (CaPho15p) and *Candida parapsilosis* (CpPho15p) belong to these predicted-only proteins. The group of *PHO15* genes

encodes proteins that have been predicted as alkaline phosphatases catalyzing dephosphorylation of 4-nitrophenylphoshate. It was based only on homology to *PHO13* gene from *Saccharomyces cerevisiae* and *PHO15* phosphatases have never been synthesized, isolated or even more, tested.

In this work, the real biological function of these potential phosphatases from *Candida* spp. was examined. Recombinant phosphatases were prepared using expression in *Escherichia coli* transformed with vector containing sequences for expression of five different chaperones. The identification of primary structure of phosphatases using combination of gel electrophoresis, in-gel digestion and MS was performed. After confirmation of the protein's origin, dephosphorylation potential was tested. The conditions were tested according to dephosphorylation reactions of similar phosphatases from *Saccharomyces cerevisiae* that hydrolyze 4-nitrophenyl phosphate at pH 8–8.3 (Fig. 6.) in presence of Mg^{2+}, Mn^{2+} or Co^{2+} as cofactors. Recombinant phosphatases were isolated with IMAC purification system represented by Ni-NTA Agarose (Qiagen). In contrast with standard purification protocol, phosphatases were left immobilized on particles due to stability issues. The activity of the immobilized phosphatases remained stable for at least up to 3 months whereas released phosphatases were unstable and lost their activity. CaPho15p and CpPho15p were highly active and dephosphorylated 4-nitrophenylphoshate, and phosphopeptides were expected as substrates. For that purpose, dephosphorylation of phosphopeptides originating from α-casein was tested without any significant activity. As the α-casein contains only phosphorylated serine residues, commercial mixture consisted of peptides containing all O-phosphorylated amino acids but still without any effect. We concluded that neither of these phosphatases dephosphorylated phosphopeptides, thus testing of several other substrates was performed. It was found that strongly preferred substrate for both phosphatases was 2-phosphoglycolate. For CaPho15, a slight preference was observed also for glyceraldehyde phosphate and glycerol-2-phosphate, while CpPho15 dephosphorylated mainly 1,3-dihydroxyacetone phosphate. From the range of identified substrates, it was obvious that CaPho15 and CpPho15 are a part of metabolism rather than of protein signaling pathways.

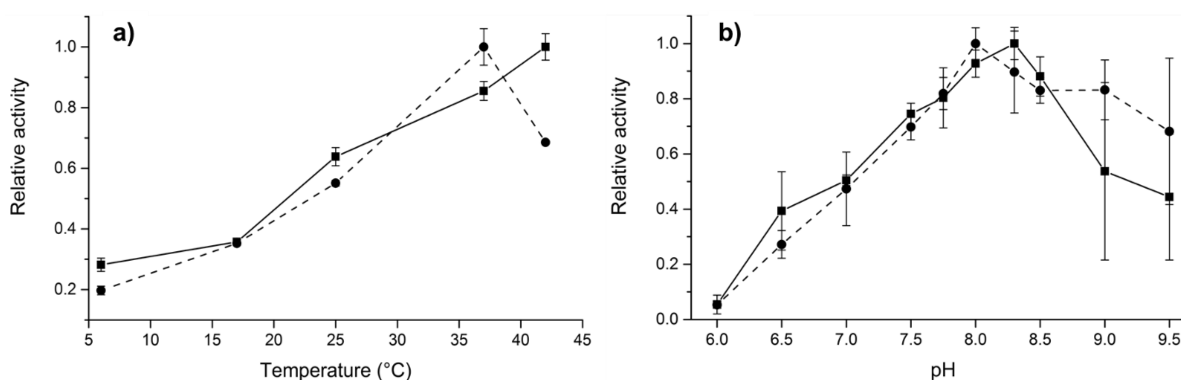

Figure 6.: Effect of temperature (a) and pH (b) on the activity of CaPho15p (solid line) and CpPho15p (dashed line). The reactions were performed in triplicate, using immobilised phosphatases and 4-nitrophenyl phosphate as a substrate.

In conclusion, synthetized phosphatases were tested for their potential for protein dephosphorylation, but it was not confirmed. However, the original substrates were identified, and it was possible due to the immobilization of phosphatases to IMAC resin, which strongly stabilized protein conformation and allowed for long term testing of several substrates.

Author's contribution: Experiments related to phosphopeptides dephosphorylation. MALDI-MS analysis of simple peptide phosphorylated and potentially dephosphorylated peptides. Confirmation of identity of phosphatases. Writing of appropriate parts of the manuscript.

2.2.6 Amorphous TiO_2 nanotubes as a platform for highly selective phosphopeptide enrichment (Manuscript I)

Protein phosphorylation is commonly analyzed after proteolytic digestion by MS at the peptide level. Analysis of phosphopeptides is complicated due to their low ionization efficiency and their suppression with more abundant non-phosphorylated peptides. This is the reason for seeking of materials for affinity chromatography that can enrich specifically phosphorylated molecules and reduce the amount of non-phosphorylated molecules. Despite the progress in material engineering, however, many new affinity materials based on metal oxides for the specific enrichment of phosphopeptides are not sufficient and there is still open space for improvement.

In this work, highly selective phosphopeptide enrichment procedure using plain TiO_2 nanotubes (TiO_2NTs, average inner diameter ≈ 230 nm) or decorated with Fe_3O_4 nanoparticles (average diameter ≈ 8 nm) – TiO_2NTs@Fe_3O_4NPs was described. These materials were subjected to extensive testing of their properties related to enrichment efficiency. After the testing of enrichment procedure on a simple peptide mixture prepared from digested BSA and α-casein, more comprehensive approach using Jurat T-cell lysate digest was performed. The testing scheme included both introduced materials and commonly used commercial TiO_2 microspheres. The course of thorough comparison of materials for phoshopeptides enrichment was described in detail and was included in the experimental part of the paper. All elution fractions from raw lysate and elution fractions after re-enrichment of supernatants were analyzed using nanoLC-ESI-MS/MS. After statistical evaluation of all data, we could conclude that both materials, TiO_2NTs and TiO_2NTs@Fe_3O_4NPs were comparable to each other. What is more important and highly beneficial for proteomic experts, these two materials withstand a critical comparison with commonly used TiO_2 microspheres in term of enriched phosphopeptides. At the same time, both introduced materials substantially outperformed commercial material in the number of non-specifically bound peptides. The amount of identified non-phosphorylated peptides in first elution fractions for introduced materials was four times lower than for TiO_2 microspheres (Fig. 7.).

The number of multiphosphorylated peptides was similar for all materials and composition of new materials did not affect the number of multiphosphorylated peptides. After re-enrichment of flow through fractions from all materials using commercial TiO_2 microspheres, the number of newly identified peptides was less than

5% for the newly introduced materials and less than 1% for the same TiO_2 microspheres. It meant that TiO_2 nanotubes in both forms had shown good binding capacity for phosphopeptides. Furthermore, $TiO_2NTs@Fe_3O_4NPs$ material was similarly useful for highly selective phosphopeptides enrichment and its superparamagnetic activity provided further added value.

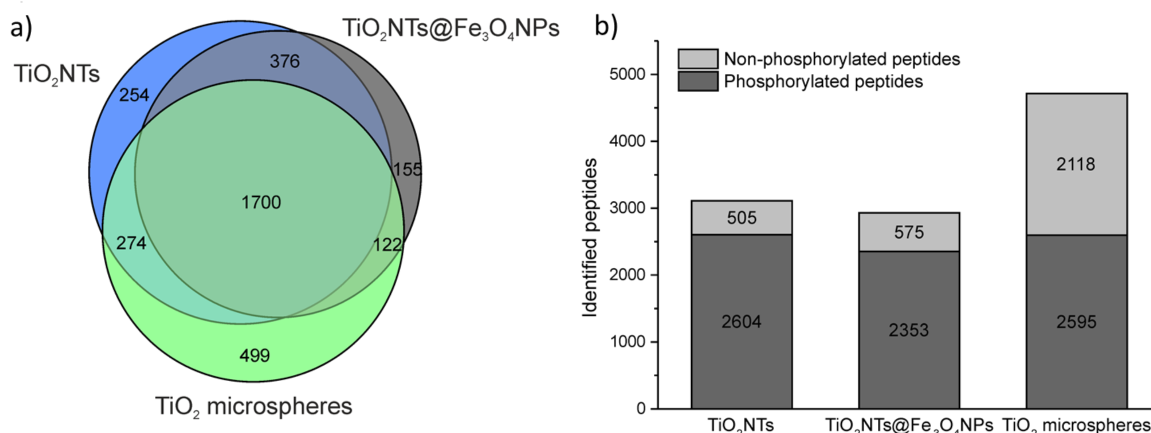

Figure 7.: A comparison of the raw lysate digest enrichment using TiO_2NTs, $TiO_2NTs@Fe_3O_4NPs$ and TiO_2 microspheres. (a) Venn diagram of identified phosphopeptides for all compared materials and (b) column diagram containing total numbers of identified phosphorylated and non-phosphorylated peptides.

In summary, it was shown that phosphopeptides could be enriched on both, TiO_2NTs or $TiO_2NTs@Fe_3O_4NPs$, respectively. These nanomaterials were able to achieve high selectivity and throughput for both singly and multiply phosphorylated peptides in one step with minimal non-specific binding. Reduction of non-specifically bound peptides might substantially reduce demands of analytical workflow or even more, may allow the elimination of one separation step with same achieved results.

<u>Author's contribution:</u> Enrichment of all samples using newly introduced and commercial materials. MALDI-MS analysis of simple peptide mixtures. Sample preparation prior to nanoLC-ESI-MS/MS. Manuscript design and writing with cooperation with other co-authors.

2.2.7 Method for separation of biopolymer molecules and a carrier for application of this method (Patent)

Nowadays, various methods and various carriers have been developed for separation of mono- and multi-phosphorylated peptides, recombinant peptides/proteins with a polyhistidine tag (His-tag), cysteine-containing peptides/proteins and nucleic acids. Nevertheless, beside indisputable utility values of these carriers and methods, they have a number of limiting characteristics and there are still open pathways to improve them.

The presented invention relates to a method for separation of biopolymers based on their affinity to the surface of patented carrier. This carrier could be composed of a

core with dimensions in nano- and/or submicro- and/or microscale that was prepared of an oxide of at least one transition metal and/or silicon oxide. Moreover, on the surface of the core at least one continuous or non-continuous layer and/or nanoparticles of magnetic metal oxide was deposited. The description perfectly fits to 1D TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles, however numerous combinations of materials are available. The main novelty of the invention relies on the fact that magnetic metal oxide or magnetic nanoparticles on the surface are responsible not only for magnetic properties of the composite, but they are directly involved in specific interaction with biomolecules. Biopolymers interacted with the solid phase under defined reaction conditions, non-specifically bound components were washed off and target molecules were eluted by elution mobile phase with changed pH and/or by using a competitive reagents. The invention describes examples of utilization of the carrier for separation of biomolecules including material types, their composition and also a protocol for application of these methods. The novel material in the form of TiO_2NTs@Fe_3O_4NPs is useful not only for enrichment of phosphopeptides and for recombinant protein purification which is constituent part this patent but the same material was also utilized for isolation of two other types of biomolecules: nucleic acids and cysteine containing peptides under specifically optimized conditions.

Author's contribution: In this patent, the author contributed to design and development of protocols for specific isolation of a selected group of molecules. Experiments for examples 1–6 to show separation ability of the materials were performed by the author. The author also participated in writing and compilation of the patent.

Conclusions

Research in the frame of this doctoral thesis is dedicated to the development of new materials and implementation of innovative approaches to purification, analysis or to modification of target proteins/peptides. Newly developed approaches attempt to improve current technologies to some extent or completely new strategies are applied. This thesis is complex and deals with several challenges in analytical chemistry, proteomics or biotechnology. New materials suitable for separation, structural analysis and controllable modification of proteins are the connecting element intersecting all experiments.

The theoretical part consists of an overview of the nanomaterials and their properties, analytical techniques including affinity chromatography and other separation techniques focused mainly on phosphoproteomics, purification of recombinant proteins and proteins that are modified either naturally or artificially. The experimental part covers all achieved results published or submitted in peer reviewed scientific journals. This part is extended by one patent with international application.

A major part of this thesis is devoted to the development or utilization of new materials that open up new pathways in the relevant field. This was represented by 1D TiO_2 nanotubes utilized in as-formed state or decorated with magnetic Fe_3O_4 nanoparticles (TiO_2NTs@Fe_3O_4NPs). Within the experiments utilizing these nanomaterials we were able to achieve high selectivity and throughput for phosphorylated peptides in one step with minimal non-specific binding and thus to outperform commonly used TiO_2 microparticles. TiO_2NTs@Fe_3O_4NPs and tailored isolation protocol were also applied to His-tag protein purification. Obtained results showed efficient and selective purification again with significantly reduced non-specific binding of contaminating proteins. On the other hand, broader use of this type of material in recombinant proteins purification for e.g. pharmaceutical use is still limited due to the small-scale production. New nanomaterials are not useful only for isolation purposes but can serve as rapid and effective digestion, as shown on the example of newly developed Fe_3O_4@SiO_2-NH_2 nanoparticles with covalently immobilized trypsin. The nanomaterial is valuable for proteolytic enzymes immobilization and this type of carrier possesses several advantages such as higher activity and specificity of immobilized enzyme and its enhanced stability.

In some cases, also commercial materials could bring innovation to well-established procedures. The main advantage of commercial particles is their easy accessibility and no necessity for the knowledge and equipment for their production. These particles can also be further modified e.g. with enzymes to get one functional unit as demonstrated on protein *in-vitro* phosphorylation of tau protein. This work described possible pathways to immobilize kinases to magnetic beads and to create an easy handling, reusable, and thus low-cost system. Furthermore, these beads are also convenient to be easily and quantitatively removed from reactions to minimize the contamination of phosphorylated products. Commercial particles served also for immobilization of biotechnologically-derived predicted phosphatases of human fungal pathogens which were successfully tested and their substrates were determined. Another commercially available material, PTFE microparticles, was recognized as a valuable material for specific isolation of highly hydrophobic protein – hydrophobin SC3. It was

an example of application of well-established material used for a different purpose based on basic knowledge of protein's behavior and features.

To conclude, new materials have an irreplaceable role in selective isolation of biomolecules. From the research presented in this study, they also bring additional value to many separation and analytical methods, mostly in bioanalysis. It was possible thanks to advances in production technology, characterization of prepared materials and vital cooperation with partners from materials engineering. Current or commercially available materials offer in many cases a useful tool, especially if they are biofunctionalized. It seems that in future, science will bring more and more new advanced micro- or nanomaterials, as many research teams are interested in their development.

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List of Students' Published Works

Publications in peer-reviewed journals with IF

Publications related to this thesis (included *in extenso*)

Kupcik, R., Rehulka, P., Bilkova, Z., Sopha, H., Macak, J. M., New Interface for Purification of Proteins: One-Dimensional TiO_2 Nanotubes Decorated by Fe_3O_4 Nanoparticles. *ACS Applied Materials & Interfaces* 2017, 9, 28233–28242. **(Paper I)**

Kupčík, R., Zelená, M., Řehulka, P., Bílková, Z., Česlová, L., Selective isolation of hydrophobin SC3 by solid-phase extraction with polytetrafluoroethylene microparticles and subsequent mass spectrometric analysis. *Journal of Separation Science* 2016, 39, 717–724. **(Paper II)**

Slováková, M., Sedlák, M., Křížková, B., Kupčík, R., Bulánek, R., Korecká, L., Drašar, Č., Bílková, Z., Application of trypsin $Fe_3O_4@SiO_2$ core/shell nanoparticles for protein digestion. *Process Biochemistry* 2015, 50, 2088–2098. **(Paper III)**

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Other work related to this thesis (included *in extenso*)

Manuscript submitted in a scientific journal peer-reviewed scientific journal

Kupcik R., Macak J. M., Rehulkova H., Fabrik I., Sopha H., Anitha V. C., Klimentova J., Murasova P., Bilkova Z., Rehulka P., Amorphous TiO_2 Nanotubes as a Platform for Highly Selective Phosphopeptide Enrichment, Manuscript revision submitted in ACS Omega, May 9, 2019, *ACS Omega*, ao-2019-00571y.R1. **(Manuscript I)**

Patent

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