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New materials and techniques for separation and analysis of clinically important proteins

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In Pardubice on 17th May 2019 .....

Mgr. Rudolf Kupčík

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ANNOTATION

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.

KEYWORDS

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

ANOTACE

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.

KLÍČOVÁ SLOVA

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

ABSTRACT

The main aim of my work was to develop research techniques utilizing materials in both nano and micro sizes for their use in protein isolation and modification. Currently available materials applied in protein analysis still have limits and those which are highly valuable for e.g. protein purification are the subject of continuous research. However, rapid advances in material engineering over the past decades has created many unique materials like carbon nanorods or TiO_2 nanotubes.

In this thesis, I have included five papers, one manuscript and one patent from research related to my Ph.D. study. The thesis is divided into several parts according to material type and the group of protein for which the material is used. Special attention is paid to sample preparation, mainly to the development of new methods for protein/peptide isolation and modification.

The materials of various shapes and sizes are studied and utilized in novel methods of highly selective isolation of proteins/peptides. The material with improved architecture that is composed of 1D (one-dimensional) TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles significantly improves purification of recombinant proteins from cell lysates. The TiO_2 nanotubes-based materials serve also for a substantial reduction of the amount of co-enriched non-phosphorylated peptides in phosphoproteomic analysis. Another approach utilizing polytetrafluoroethylene microparticles suitable for selective isolation of highly hydrophobic proteins like hydrophobin SC3 is presented.

Immobilization of enzymes brings many benefits that are shown to be particularly useful in proteomics. The research involved in this thesis deals with enzymatic phosphorylation and dephosphorylation of suitable substrates. First, systems with magnetic microparticles with immobilized kinases suitable for phosphorylation of recombinant proteins or peptides *in vitro* have been created and successfully applied. Second, polymeric resins with immobilized recombinant phosphatase have been tested for dephosphorylation of various substrates. In order to facilitate preanalytical stage of protein analyses, newly developed nanoparticles have been successfully applied to speed up and improve the enzymatic digestion of proteins.

All these issues were addressed in this work and the applicability of various materials for sample preparation in proteomics was demonstrated together with various analytical techniques suitable for analysis and modification of proteins and peptides.

ABSTRACT IN CZECH

Hlavním cílem mé práce bylo vyvinout výzkumné techniky využívající materiály jak v nano, tak v mikro měřítcích za účelem jejich použití při izolaci a modifikaci proteinů. Materiály, které jsou v současné době dostupné a zároveň jsou využívány při analýze proteinů, mají stále určité limity. Materiály, které jsou vysoce hodnotné např. pro čištění proteinů, jsou stále předmětem výzkumu. Nicméně, rychlý pokrok v materiálovém inženýrství v posledních desetiletích umožnil vytvořit mnoho jedinečných materiálů, jako jsou uhlíkové nanotyčky nebo TiO_2 nanotrubičky.

Do této práce jsem zahrnul pět článků, jeden rukopis a jeden patent, které byly součástí výzkumu při mém Ph.D. studiu. Práce je rozdělena do několika částí podle druhu materiálu a skupiny proteinů, pro které jsou tyto materiály použity. Zvláštní pozornost je věnována přípravě vzorků, zejména vývoji nových metod izolace a modifikace proteinů/peptidů.

Pro nové způsoby vysoce selektivní izolace proteinů/peptidů jsou studovány a využívány materiály různých tvarů a velikostí. Materiál s vylepšenou architekturou, který se skládá z 1D TiO_2 nanotrubiček pokrytých nanočásticemi Fe_3O_4, významně zlepšuje čištění rekombinantních proteinů z buněčných lyzátů. Materiály na bázi nanotrubiček TiO_2 také slouží pro výrazné snížení množství spoluobohacených nefosforylovaných peptidů při fosfoproteomové analýze. Pro selektivní izolaci vysoce hydrofobních proteinů, jako je hydrofobin SC3, je zde prezentován další přístup využívající polytetrafluorethylenové mikročástice.

Imobilizace enzymů přináší mnoho výhod, které jsou zvláště užitečné v proteomice. Výzkum zahrnutý do této práce se zabývá enzymatickou fosforylací a defosforylací vhodných substrátů. První z nich umožnil vytvoření a úspěšnou aplikaci systému s magnetickými mikročásticemi, na které byly imobilizovany kinázy vhodné pro fosforylaci rekombinantních proteinů nebo peptidů *in vitro*. Druhý přístup využíval polymerního nosiče s imobilizovanou rekombinantní fosfatázou pro testování defosforylace různých substrátů. Dále byly pro usnadnění preanalytické fáze proteinových analýz úspěšně použity nově vyvinuté nanočástice, což vedlo k urychlení a zlepšení enzymatického štěpení proteinů.

V rámci práce byla řešena veškerá tato problematika a také využitelnost různých materiálů pro přípravu vzorků v proteomice. Ta byla předvedena společně s různými analytickými technikami vhodnými pro analýzu a modifikaci proteinů a peptidů.

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LIST OF ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
2DE	two-dimensional gel electrophoresis
ADP	adenosine triphosphate
ATP	adenosine triphosphate
CID	collision-induced dissociation
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ERK2	mitogen-activated protein kinase 1
ERLIC	electrostatic repulsion-hydrophilic interaction chromatography
ESI	electrospray ionization
ETD	electron-transfer dissociation
ETHcD	electron-transfer/higher-energy collision dissociation
FT-ICR	Fourier-transform ion cyclotron resonance
GSK-3β	glycogen synthase kinase 3β
GST	glutathione *S*-transferase
HCD	higher energy collisional dissociation
HILIC	hydrophobic interaction liquid chromatography
HPLC	high performance liquid chromatography
IEX	ion exchange chromatography
IMAC	immobilized-metal affinity chromatography
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography
LIT	linear ion trap

MALDI	matrix assisted laser desorption ionization
MBP	maltose-binding protein
MetO	oxidized methionine
MOAC	metal oxide affinity chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSA	multi-stage activation
MsrA	methionine sulfoxide reductase A
MsrB	methionine sulfoxide reductase B
MudPIT	multidimensional protein identification technology
m/z	mass-to-charge ratio
NPs	nanoparticles
NusA	N-utilization substance protein A
pI	isoelectric point
pS	phosphoserine
pT	phosphothreonine
PTFE	polytetrafluoroethylene
PTM	post-translational modification
pY	phosphotyrosine
ROS	reactive oxygen species
Q	quadrupole
QqQ	triple-quadrupole
QqTOF	quadrupole-quadrupole-time-of-flight
RP	reversed phase
RPLC	reversed-phase liquid chromatography
SALDI-MS	mass spectrometry ionization laser attended surface

SCX	strong cation exchange chromatography
SDS	sodium dodecyl-sulfate
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SILAC	stable isotope labeling by amino acids in cell culture
SIMAC	sequential elution from IMAC
SUMO	small ubiquitin-like modifier
TCEP	tris(2-carboxyethyl)phosphine
TiO_2NTs	plain TiO_2 nanotubes
TiO_2NTs@Fe_3O_4NPs	TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles
TMT	tandem mass tags
TOF	time of flight mass analyzer
Trx	thioredoxin
UHPLC	ultrahigh pressure liquid chromatography
WAX	weak anion exchange chromatography

1. INTRODUCTION

1.1 Proteins and their modifications

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.

On top of primary sequence variability of the proteins of an organism, extension by splice isoforms and post-translational modifications (PTMs) is essential. PTMs of the target protein may play an important role, e.g. in signaling pathways, and thus they may be responsible for changing protein activities in the cell. Study of such modifications may reveal changes with biological and clinical importance. The technologies developed and used in proteomics cover the latest improvements in both MS instrumentation and separation techniques, as well as appropriate bioinformatic approaches. At present, research teams are able to routinely identify thousands of proteins/peptides in a single experiment within large scale studies utilizing multilevel analytical approaches. The crucial importance of enrichment of phosphopeptides is obvious from experiments obtained in the past and also in current studies [3, 4]. Beside undisputable advantages and performance of currently utilized materials for selective sample preparation in proteomics, there are still many issues to be solved by technological development in materials used in proteomic sample preparation.

1.1.1 Posttranslational modifications of proteins

Proteins have diverse biological functions ranging from deoxyribonucleic acid (DNA) replication, forming cells and tissues, body protection, synthesis of molecules, coordination of biological processes to transferring of signals. The list of functional properties of proteins is

almost unlimited and new protein functions are continually being discovered as proteomic and genomic research studies are trying to find new subjects. Beside this functional variability, 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 [5, 6] and the second one, occurring after translation of ribonucleic acid into proteins, is called *covalent posttranslational modification* (Fig. 1). It takes place at one or more sites of the protein [7, 8]. Thus, PTMs represent one of the basic mechanisms of increasing the chemical and biological diversity of the genome. PTMs refer to the (often proteolytic) cleavage (e.g. activation of insulin) or to the addition of small chemical moieties (e.g. a phosphate group or mannose) to certain amino acid residues. In general, protein modification results in changes of the protein properties. Among others, PTMs include hydroxylation, acetylation, methylation, phosphorylation, S-S bond formation, ubiquitination, and glycosylation etc., and expand the proteome structurally and functionally [8]. PTMs can also be associated with different changes in living organisms such as physiological state or disease [9]. One example is reversible phosphorylation of proteins that regulates many aspects of cell life, while abnormal phosphorylation is a cause or consequence of serious diseases [10-12]. Phosphorylation often involves changes of enzyme activity and protein signaling processes. Therefore, changes in PTMs both at qualitative and quantitative level can provide us with a prediction or verification of many diseases. It is very important to characterize PTMs successfully and to understand the function of modified protein, which can be challenging due to high variability of PTM types and localizations.

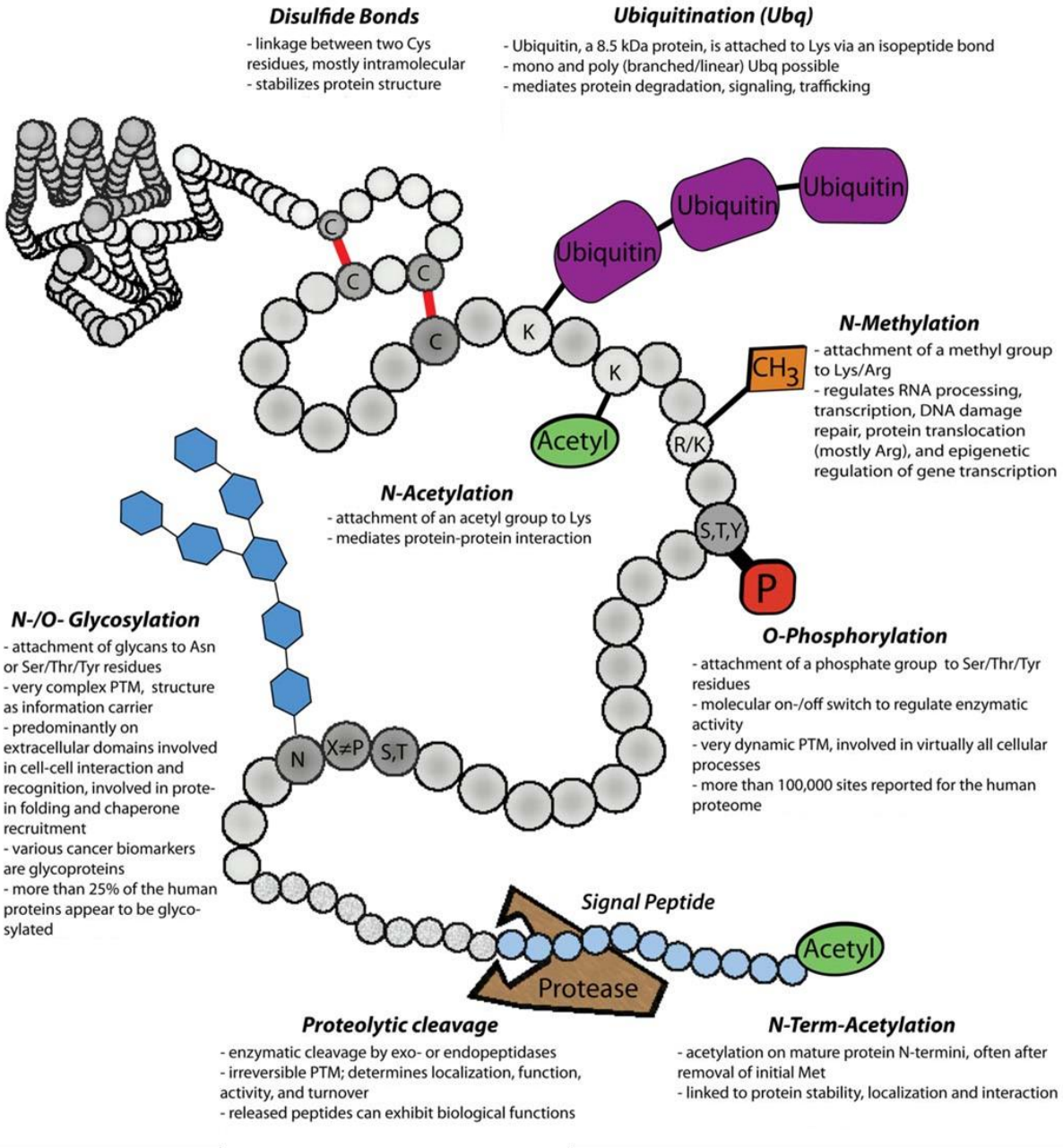

Figure 1.: Frequently reported post-translational protein modifications. Adapted from [13].

1.1.1.1 Protein phosphorylation

The functions of phosphorylation of proteins and their dynamic changes under physiological conditions have already been described in more detail in the literature and it still attracts the research interest [4, 14, 15]. Protein phosphorylation is one of the most prevalent intracellular protein modifications while having a crucial importance in various cellular processes including cell differentiation, proliferation, and migration. Reversible protein phosphorylation works as a molecular switch and is also part of metabolism regulation and signal transduction in cells

[16]. As has been found over past two decades, changes in protein phosphorylation are related to certain pathological states in the human body and that is why they are diagnostically and prognostically very important. It is estimated that about 30% of all proteins in a cell are phosphorylated during their life cycle [17]. However, relative abundance of phosphorylated proteins in organism at a designated moment is very low. An enzymatic and biologically meaningful phosphorylation in mammalian cells usually takes place at the hydroxyl side chain of serine (pS), threonine (pT) and tyrosine (pY) in the relative frequency of pS:pT:pY = 90:10:0.5 [18]. These three phosphorylated amino acids undergo stable O-type phosphorylation and they are usually characterized with LC-MS analysis [19]. Other phosphorylation types, including histidine and other types of N-, S- and acyl-phosphorylation are still poorly investigated due to their chemical instability; however, a considerable progress has been achieved in the past years regarding the development of new synthetic and/or MS-based proteomic tools to study these PTMs [20].

Phosphoproteins are formed via a catalytic enzyme and adenosine triphosphate (ATP), serving as a donor of the phosphate group [21]. Dephosphorylation is done by phosphatases that hydrolyze the covalent bond connecting the phosphate to the protein (Fig. 2). In cells, there is a constant balance between phosphorylation and dephosphorylation events mediated by kinases and phosphatases, respectively [22, 23]. Intracellular functions of phosphorylation affect protein interactions or modulate enzymatic activity through changes in protein conformation. Not only are many changes in protein interactions and conformations mediated by phosphorylation but also regulatory function is dependent on phosphorylation events. Phosphorylation can also regulate the subcellular localization, the turnover of its targets, and it also impacts signaling by other PTMs, which is called *crosstalk* [15]. An example of such crosstalk was observed between methionine oxidation and phosphorylation as a part of cellular defense against oxidative stress. It was evidenced that the oxidation of methionine localized within phosphorylation motifs was a highly selective process [24]. The protein phosphorylation and dephosphorylation by kinases and phosphatases serve as a rapid molecular switch, capable to change or modify the feature of its targets either directly or vicariously [15].

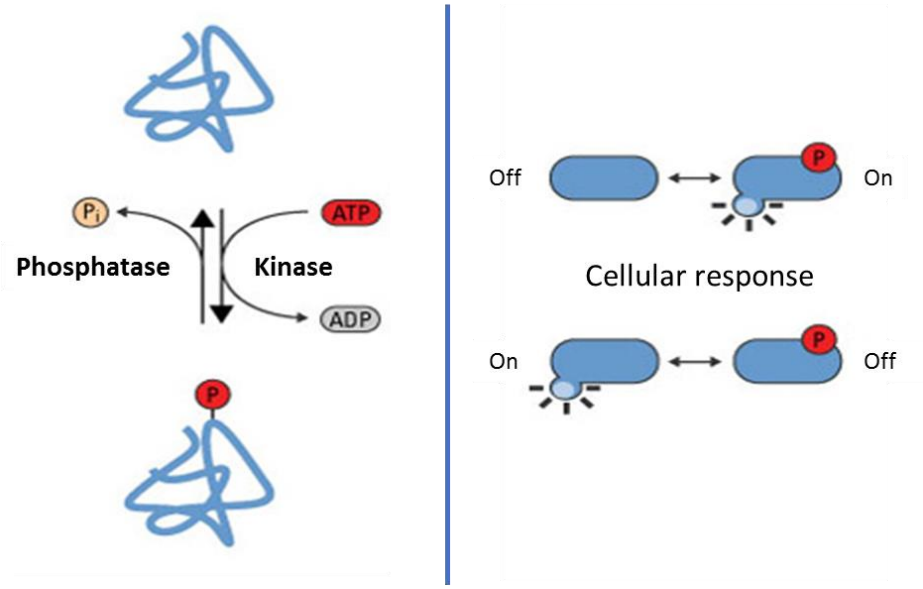

Figure 2.: General scheme of protein phosphorylation and dephosphorylation. *Abbreviations: ADP – adenosine triphosphate*. Adapted from [25].

The importance of protein phosphorylation is commonly associated with the pathogenesis and development of a wide variety of serious human diseases [4]. Aberrant phosphorylation has been commonly confirmed in cancer [26], diabetes [27], heart disease [28], and neurodegenerative diseases [11]. Alzheimer's disease is one of phosphorylation-related pathologies where tau hyperphosphorylation and also aberrant phosphorylation of proteins of signaling cascade, apparently caused by the imbalance between kinase and/or phosphatase functions, was observed [11, 12]. Several proteomic studies have been carried out to profile alteration of protein phosphorylation in the brain, suggesting that not only central pathways regulated by kinases/phosphatases were involved, but also pro-survival or cell death pathways play a central role in disease pathology [11]. Many studies in recent years have revealed the role of deranged phosphorylation of proteins in neurodegeneration process and, what is also very important, in manifestation of cancer [29-32].

1.1.1.2 Protein oxidation and S-S bond formation

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 [33]. Cysteine and methionine are the two amino acids present in proteins that contain sulphur. Most important common characteristic is 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 [34].

Oxidation of methionine (MetO) 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 [35, 36]. Methionine oxidation is achieved by forming methionine sulfoxide by the addition of an oxygen atom to its sulfur atom. Oxidized methionine is naturally reduced back by methionine sulfoxide reductases (Fig. 3) [37, 38]. In contrast to cysteine, methionine cannot create disulfide bridges and it might be difficult to list other functions of methionine residues, other than its well-known role in protein initiation. However, in a recent study it has been shown that oxidation of methionine to MetO may provide the basis for regulating the specificity of protein kinase-substrate interactions. The described crosstalk between sulfoxidation and phosphorylation is more prevalent among stress-related proteins [24].

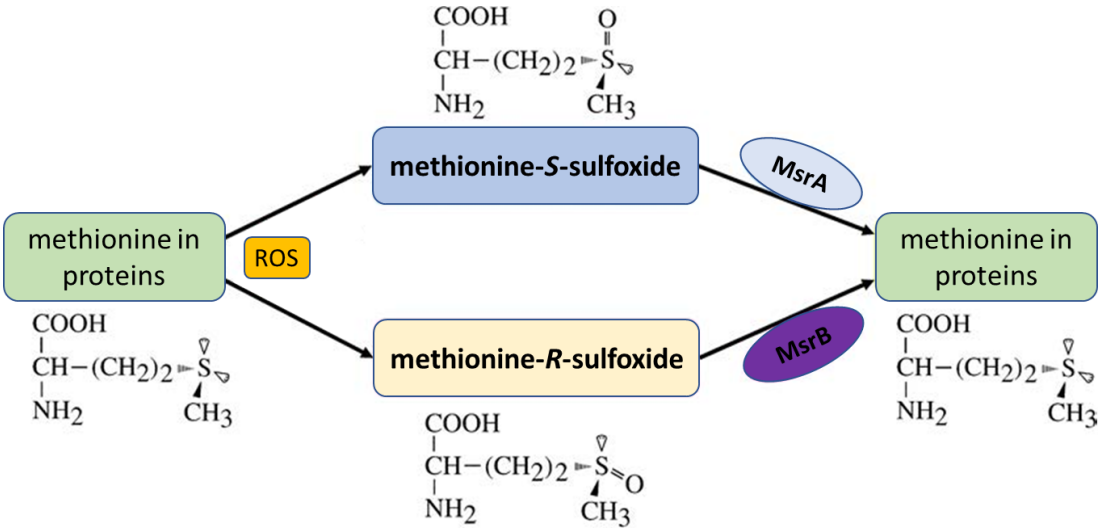

Figure 3.: A pathway of methionine sulfoxide oxidation and reduction. *Abbreviations: ROS – reactive oxygen species; MsrA and MsrB – methionine sulfoxide reductase A and B.* Adapted from [39].

Cysteine is involved in the most common covalent linkage of proteins represented by disulfide links from the oxidation of cysteinyl residue by thiolate side chains [40, 41]. Cysteinyl residues are well protected against oxidation due to the reducing microenvironment in cytoplasmic and nuclear compartments in eukaryotic cells [42]. When proteins reach the cell's outer surfaces or are excreted into the extracellular spaces, the stabilization of protein architecture becomes desirable and disulfide links predominate. The mechanism of disulfide bridge creation consists in oxidation of protein thiols on the side chains of Cys residues.

Selected oxidants are able to perform the one-electron oxidation of thiols to thiyl radicals that could dimerize to the disulfides. Alternatively, the two-electron oxidation of thiols yields sulfenic acids (-SOH) on side chains followed by capture of the sulfenate by a neighboring Cys-S, which together generates disulfides. Furthermore, cysteine can exist in thiol form, or sulfinic and sulfonic acids are generated by irreversible addition of 2 or 3 oxygen atoms, respectively [43]. This type of reaction can be utilized for irreversible oxidation of disulfide bonds stabilized proteins e.g. hydrophobins in which commonly used protocols for reduction of disulfide bonds are ineffective [44]. Addition of oxygen atoms to the amino acid side chain also reduces hydrophobicity of proteins (hydrophobins, hordeins etc.) and can simplify the proteomic analysis [45].

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 these fields, chemical modification or expression of additional sequence of amino acids with desired properties in protein synthesis are required. 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 suitable for understanding clinically important processes, where modifications are used prior to quantitation of proteins and their PTMs within diagnosis or therapy monitoring [46]. Beside the label-free quantitation [47] and/or targeted multiple reaction monitoring MS methodologies [48], the chemical or metabolic labeling of proteins/peptides with stable isotopes [49, 50] are the key approach to protein quantification.

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 [51]. Prior to their further purification, they are commonly synthetized with an appropriate tag or modified after their expression. Tagging proteins of interest has become a routine practice with the advances of cloning techniques.

1.1.2.1 Affinity tags

Affinity chromatography has become an increasingly important method in pharmaceutical science, biochemistry, biotechnology and environmental sciences as a specific and effective technique for protein purification [52, 53]. 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. 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 [54]. The affinity tags are widespread in preparation of several biotechnologically created proteins, from *de novo* synthetized uncharacterized proteins to *in vitro* production of clinically important proteins. Whereas many tagged proteins retain their structural integrity and biological activity, other proteins may clearly behave differently [55, 56]. In specific examples, of the use of an affinity tag could also positively influence the properties of synthesized proteins. Affinity tags could be responsible for increased solubility of proteins, correct protein folding, enabling protein immobilization or preventing of the protein cleavage [57-59]. On the other hand, in most of the cases, affinity tags are not desirable and have to be removed, e.g. in many clinical applications. For that purpose, several tag-removal techniques have been developed. They employ e.g. highly specific enzymes such as tobacco etch virus protease or thrombin, which need a specific amino acid motif for a successful cleavage and these sequence motifs are usually added to recombinant proteins prior to their expression [57].

Within the gene expression procedure, it is also necessary to design a purification strategy. At current state of the art, many types of affinity tags are available and can be chosen for affinity purification of target protein (as summarized in several reviews [60-62]). According to the tag size, these tags may be divided into two groups. First group of small peptide tags is represented by the poly-His-, poly-Arg-, FLAG-, Strep-, S-tags etc. [62]. Small tags are typically chosen for their lower impact on folding, biological activity and immunogenicity of the produced protein [51]. The second group of tags with higher molecular weight may bring an extra advantage and they may also work as solubility enhancers [63]. From this group, most popular tags are represented by the glutathione *S*-transferase (GST), maltose-binding protein (MBP), ubiquitin, cellulose-binding tag, N-utilization substance protein A (NusA), thioredoxin (Trx) or small ubiquitin-like modifier (SUMO) [62, 64, 65]. However, 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 [66]. In the following

paragraph, the polyhistidine affinity tag (His-tag) will be described in more detail due to research focus of this work.

1.1.2.1.1 Polyhistidine affinity tag

Polyhistidine affinity tag (His-tag) is a fusion tag composed of six to eight 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 peptide chemistry [7]. His-tag is utilized for recombinant protein purification exclusively and in the vast majority of cases, immobilized-metal affinity chromatography (IMAC) is employed as an affinity carrier [67, 68]. Histidine amino acid exhibits a strong interaction with immobilized metal ions. Histidine possesses electron donor groups on the imidazole ring, which forms coordination bonds with the immobilized transition metal and thus proteins containing sequences of 6-8 consecutive histidine residues are efficiently bound on the IMAC resin. Commonly used nitrilotriacetic acid modified resin forms a quadridentate chelate and is suitable for metal ions with coordination numbers of 6 (nickel, cobalt), since two valences are available for the reversible binding of histidine-containing protein [62]. However, the conventional IMAC approach with polymer or agarose beads functionalized with nitrilotriacetic, iminodiacetic acid, carboxymethylated aspartic acid or 8-hydroxy-quinoline as chelating agents for metal ions [6] reports several drawbacks. Among others, the insufficient purity of separated proteins [68], limited surface area of microspheres suitable for binding [69] and the toxicity of released metal ions [70] could be included. The purification of His-tagged protein using IMAC materials could be hindered by impurities including bacterial proteins with cysteine and histidine-rich regions or metal centers [8].

Recently, due to the technical development in materials engineering, various materials have been developed for the specific separation of His-tagged proteins. By using a core made of a more rigid material, like SiO_2, these materials have the potential to remove some disadvantages of standard IMAC system, e.g. chemical stability [71]. Other designed materials have added a new feature by employing magnetic core of nanoparticles, e.g. Ni^{2+}-functionalized Fe_3O_4@polydopamine magnetic nanoparticles for the purification and immobilization of His-tagged protein [72] or utilizing magnetic nanoparticles dispersed homogeneously in silica matrix, further functionalized with Ni^{2+} or Co^{2+} [74]. 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 (Fig. 4) with materials composed mainly of nickel or cobalt oxides [73, 74-77]. For example, Lee and co-workers (2006) designed and prepared Ni/NiO core/shell nanoparticles for the selective binding and magnetic separation of His-tagged proteins [77]. The systems used, however, suffer from nickel or cobalt toxicity as they adopted IMAC technology. Recently, the new advanced material prepared from biocompatible and non-toxic material based on 1D TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles (TiO_2NTs@Fe_3O_4NPs) have been developed. This material combines advantages from other materials, like mechanic stability of the support, magnetic activity, chemical resistance and high selectivity for isolation of His-tagged proteins [78]. The mechanism of interaction of His-tagged proteins with Fe_3O_4 described in this work was subsequently adopted for large scale green fluorescent protein purification using bare Fe_3O_4 nanoparticles [79]. Other materials suitable for analysis of proteins and peptides will be described in next paragraphs.

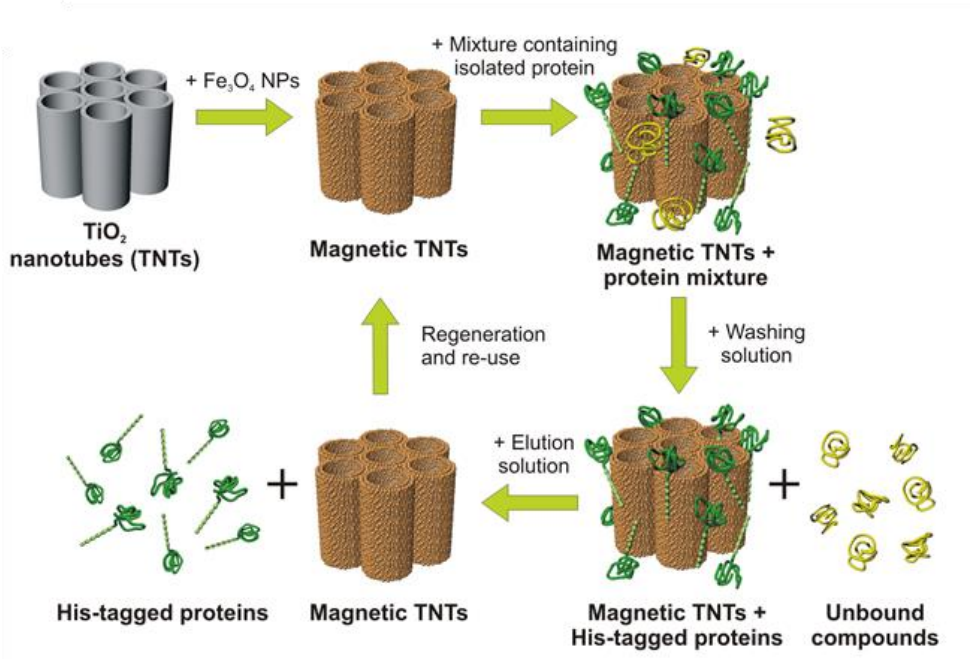

Figure 4.: Sequence of the purification of His-tagged proteins using the TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles. *Abbreviations: NPs – nanoparticles.* Adapted from [78].

1.2 Nano- and micromaterials 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. In microscale range, mainly microparticles have emerged as advanced functional materials for a wide range of applications, such as drug delivery, diagnostics, environmental application, separation of (bio)molecules, tissue engineering, or biosensing [80-85]. Properties of microparticles correlate with their size, structure, composition and configuration. 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 [86]. Nanomaterials are relatively novel material forms that started arising in the 1980s and have been produced and studied intensively in recent years. 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 [87]. Nanomaterials often possess unique optical, magnetic, electronic or mechanical properties and are strongly utilized in many areas of research and technology [88, 89].

Unique properties of nanomaterials could be exploited in analytical or large-scale separation of biomolecules. However, it is usually difficult to handle nanomaterials during separation due to their physical properties (size, weight, electrostatic repulsion etc.). There are two ways how of solving this problem: i) using magnetic core or preparing magnetic composite material that allows to separate materials from liquid phase, ii) immobilizing the nanoparticles on a solid support, e.g. iron oxide nanoparticles coated, organic polymer-based monolithic columns [90]. Nanomaterials used in enrichment protocols offer several advantages such as higher specific surface and, in the case of magnetic nanomaterials, very fast magnetic response. Nowadays, preparation of new materials for protein or peptide enrichment is a dynamically developing field in material science.

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 [91]. Separations have commonly been performed in batch (off-column) configuration or separation occurring as an integral part of systems such as microfluidic devices and in chromatography as so-called *magnetically stabilized fluidized bed* [80, 91]. Affinity based separation utilizing biofunctionalized magnetic

particles could provide efficient separation and purification of target molecules with great time, labor, and yield efficiency [91].

1.2.1 Polymeric materials

Polymeric materials have an emerging role in analytical chemistry, biotechnology and environmental applications as they serve as a support for chemical or biological ligands used for separation, purification or removal of various compounds [92-94]. The polymeric materials have been utilized in low-pressure liquid chromatography where the polymeric beads are usually created from polysaccharides or polyacrylamide [95]. For the same purpose, in a wide range of applications, soft-gel matrices made of these polymers have been widely adopted. Agarose and other polysaccharides, for instance cellulose, possess the advantage of biocompatibility and are ready for a surface activation prior to further biofunctionalization. Materials based on biopolymers are commonly employed in IMAC as a conventional support to which a chelating ligand is connected by a linker [62]. Synthetic polymers, e.g. bis-imidazolium functionalized porous polymer beads, have recently been utilized for capture and enrichment of phosphopeptides and phospholipids from digests or plasma [92]. Important application of synthetic polymers is also the creation of macroporous monolithic materials, with implication in affinity chromatography [96]. Recently, polymeric monoliths have been combined with molecularly imprinted polymers technology for specific material recognizing and separating phosphorylated peptides [97].

Polymers are also commonly utilized as a coating shell for magnetic oxides or pure metals forming magnetic particles. They protect the analyte from a direct contact with the magnetic material [98]. Pure magnetic metals possess the highest saturation magnetization extremely sensitive to oxidation [99]. Functionalization of magnetic nanoparticles with a suitable polymer material (e.g. poly(ethylene glycol)-terminated dihydrolipoic acid and -OH or modified to an $-NH_2$ group at the other end) can result in their good aqueous dispersion and $-NH_2$ could be further used for bioconjugation [100]. The biofunctionalized magnetic beads could be utilized for microfluidic immunomagnetic tumor cells capturing, e.g. in the case of macroporous crosslinked poly(glycidyl methacrylate) microspheres [101].

1.2.2 Inorganic materials

Other material types with a great prospect in biotechnology and analytical chemistry are represented by micro/nanomaterials prepared from metalloids or metal oxides. They can be prepared and utilized at a wide range of structures and may have exceptional properties, while making them useful for a large variety of applications. Some of the most utilized materials are based on either silicon oxide, hydroxyapatite or oxides of transition metals. The group of transition metal oxides can be represented by TiO_2, ZrO_2, Al_2O_3, Ta_2O_5, WO_3, SnO_2, HfO_2, Nb_2O_5, MoO_2, MoO_3, ZnO, V_2O_5, Fe_2O_3, Fe_3O_4, NiO or Co_3O_4 [102]. Inorganic materials represented by silicone dioxide, graphite, hydroxyapatite, metal oxides, etc. have been widely adopted for research and applications in chromatography separations [103-107]. TiO_2, SiO_2 and Fe_3O_4 are oxides that have been studied extensively and their application was involved in this study due to their exceptional properties for biomolecular applications.

Silicon dioxide

SiO_2 nanoparticles are used in a broad range of fields e.g. in catalysis, drug delivery, chromatography, and biomedicine [108-110]. In the field of chromatography, SiO_2 is the most important inorganic material and has been developed as the most commonly used HPLC (high performance liquid chromatography) packing. From chromatographic point of view, silicon dioxide is almost an ideal support due to its good mechanical strength, high chemical and thermal stability, controllable pore structure and surface area [111, 112]. High mechanical and chemical stability is also required for development of new materials in affinity chromatography where SiO_2 replace agarose and other polymeric supports common in affinity chromatography [113]. Relatively small particle diameters of silicon dioxide particles in high performance affinity chromatography result in short diffusional distances for solutes, which helps to perform highly efficient separations. However, as compared to larger-diameter supports, the use of silicon dioxide microparticles results in greater back pressures [114]. As separation is performed in columns, the whole process has to be maintained under HPLC conditions [53]. Main advantage of SiO_2 particles lies in high concentration of hydroxyl groups on their surface, which allows easy functionalization [115]. SiO_2 has also been widely produced in nanoscale level as nanoparticles with high surface area and enhanced surface reactivity [116]. SiO_2 is also commonly involved in many composite nanomaterials, whether as a part of polymer-silicone

dioxide materials [117] or as a shell layer in various types of magnetic nanoparticles e.g. for DNA extraction [118] or immobilization of proteolytic enzymes [119].

Titanium dioxide

TiO_2 is known for its numerous and diverse applications, ranging from common products, such as sunscreens, to advanced devices, such as batteries, sensors, electrocatalysts, self-cleaning and antibacterial surfaces [120-122]. A series of environmental and biomedical applications, such as photocatalytic degradation of pollutants, water purification, biosensing, and drug delivery were reviewed in the past [123, 124]. The diversity and significance of related applications have induced extensive interest of researchers resulting in advances in the fabrication, and characterization of TiO_2 nanomaterials in the past decades [125-127]. In proteomics, TiO_2 has been proven to be an excellent material for selective enrichment of phosphorylated peptides [128-130]. One of the undervalued benefits of TiO_2, related to biological applications, is the possibility to produce TiO_2 either in different crystalline phases including rutile, anatase, brookite or in amorphous state [131]. One of the most preferred shape forms, TiO_2 nanotubes, has been employed in biomedical applications as a TiO_2 porous coatings on bone implants [132], for a drug delivery [133] or phosphopeptide enrichment [134]. Beside assisted-template method [135], the sol-gel process [136], hydrothermal treatment [137] and electrochemical anodic oxidation of Ti foil [138] are the most-utilized methods of fabrication of TiO_2 nanotubes. 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 (Fig. 5) [132]. 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 [78, 132, 139, 140]. The morphology TiO_2 nanotube array depends on the size and shape of the template and within the removal process from the template, nanotube arrays are subsequently destructed [87]. Such created bundles of nanotubes might be useful for in-batch applications in the isolation of biomolecules from complex sample mixtures.

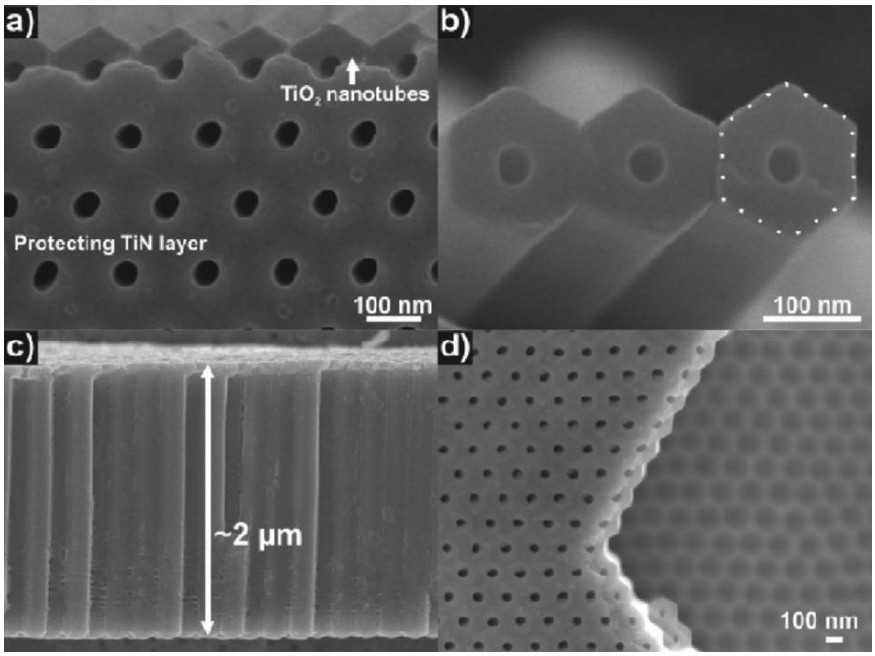

Figure 5.: Scanning electron microscopy images of TiO_2 nanotubes obtained on different patterns in an electrolyte containing 44 mM NH_4F: a) top view, S=150 nm; b) single nanotubes, S=150 nm; c) cross-section, S=180 nm; d) dimples, S=180 nm. Adapted from [141].

1.2.3 Magnetically active materials

As magnetically active particles possess advantages such as fast separation from media, they have been extensively studied and produced for nanotechnology, biological and biomedicine applications [142]. These nanoparticles or microspheres were also used as separation media in different fields of chemistry, biochemistry, biology, environment protection [143]. Nowadays, we can find many companies worldwide which are producers of magnetic particles for a biomedical, analytical or biotechnological use, such as Chemicell (Germany), GE Healthcare Life Sciences (USA), Thermo Fisher Scientific (USA) etc. Most of them allow convenient covalent coupling of target molecules. In the past years, many non-commercial magnetically active materials were developed that were synthetized from metal oxides mainly Fe_3O_4, Fe_2O_3, Fe_xO_y, $CoFe_2O_4$, NiO, FePt or $MnFe_2O_4$ [144]. They are commonly used in many research fields such as cell separation, electrochemistry, energy storage, drug delivery separation of biomolecules [145]. The most widely used structure type available on the market are core-shell particles coated or encapsulated in either polymeric or inorganic materials, respectively [145]. For the core-shell magnetic nanoparticles or microspheres or for other shapes of magnetically active materials, high magnetic responsiveness of the magnetic part is required. Superparamagnetic properties could be used for fast and gentle separation of material from the

sample and in combination with its easy manipulation and automation possibility they provide unique advantages over other techniques [146]. Superparamagnetic nanoparticles enable manipulation with composite materials by external magnetic field but show no remanence at room temperature after field removal [147]. This is an important feature that allows to quickly remove suspended superparamagnetic particles adhered to target analytes from the original matrix using an external magnetic field and without any agglomeration after removal of the field [148]. Magnetic nanoparticles are often coated with polymers or organic molecules to enhance stability in aqueous media due to the tendency of magnetic nanoparticles to aggregate [149]. The opposite way to improve physical and chemical stability of magnetic nanoparticles was to use solid support of another metal oxide, such as TiO_2. Although character and an exact stoichiometry of interaction of these oxides remain unclear, it prevents disintegration or loss of magnetism in magnetic nanoparticles [78]. In analytical chemistry, biotechnology and life sciences, magnetic separations are probably one of the most versatile separation processes. The whole cells, proteins and nucleic acids could be directly isolated from raw samples in batch-wise arrangement [150, 151].

1.2.4 Materials suitable for protein immobilization and for affinity chromatography

For purification of a specific molecule or a series of molecules with same functional group from complex mixtures, several chromatographic methods have been developed. For this purpose, affinity chromatography is one of the most utilized method as it is based on highly specific biological interactions between two molecules. In this technique, reversible interactions between affinity ligand and its counterpart, e.g. enzyme-substrate, receptor-ligand, immobilized metal ion-phosphopeptide or antibody-antigen, is utilized. Purification is performed by immobilization of first part of the interaction pair onto a solid support to create a stationary phase while the second part is isolated from the mobile phase [152, 153]. Supports for creation of stationary phase can be selected from either polymeric or inorganic materials with high variability of shapes and sizes, or monolith can be employed [52, 73, 96]. The selectivity is a key feature for affinity chromatography and the carrier should specifically bind only molecular species of interest and inert to all other compounds present in the complex sample [152]. The carriers described and employed in affinity chromatography could be selected from materials have direct affinity to separated molecules or the material serves only as a matrix and affinity

interaction is mediated by immobilized ligands. In first group we can find many examples, such phosphopeptides enrichment using metal oxides [129] or molecularly imprinted polymeric spheres as affinity-based chromatography media [154]. These polyres as a HPLC stationary phases and solid-phase extraction media employed in pharmaceutical, biomedical and environmental analysis [154]. The combination of high-performance separation methods using affinity ligands with HPLC results in a technique known as high performance affinity chromatography taking advantage of both methods [53].

The affinity ligand immobilized to the matrix could be selected from biological agents, such as proteins, or from synthetic molecules. Various types of proteins have been used as affinity ligands, such as monoclonal and polyclonal antibodies, human serum albumin, protein A and G, or lectins. Common examples of synthetic molecules that have been used as affinity ligands are metal ion chelates and biomimetic dyes [6, 52, 153]. The applications of affinity chromatography include affinity purification, chiral separation, inhibitor isolation or depletion of abundant proteins form plasma samples [53, 155]. The main application, affinity chromatographic purification, is usually utilized due to its high clearance of impurities caused by high specificity. Recently, research on the utilization of advanced nanomaterials has become important also in the field of separation of biological or chemical products. Several materials have been released for this purpose, such as magnetic nanoparticles, Ni-doped magnetic mesoporous silica for His-tagged proteins purification or halloysite nanotubes for affinity enrichment of lipase inhibitors [73, 144, 156].

There are also various methods used for depletion of abundant proteins in the plasma, however only immunoaffinity-based method allows simultaneous depletion of multiple high abundant proteins [157-158] or sample pre-fractionation to improve detection of low abundance proteins in plasma [159].

Immobilization of ligands to various materials, so-called functionalization is common for many applications in protein research. 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 [156, 160], where advantages of immobilized enzymes are most obvious. Soluble enzymes commonly possess weak stability and reusability which could be generally overcome by their immobilization to selected support [161]. Another important advantage is that immobilization to solid allows to purify the products of catalyzed reaction without any contamination [162, 163]. Immobilization is also associated with other relevant advantages such as possibility of

continuous use in enzyme rector, reduction of reaction time, improved process control and stability of enzyme [164]. Immobilized proteases are common example of time reduction. When digestion of samples in bottom-up proteomics using soluble endoproteases is usually performed overnight. The time necessary for efficient digestion using immobilized enzyme reactor could be significantly reduced [119, 165].

1.2.5 Enrichment of phosphopeptides/proteins using different types of materials

One of the major goals in phosphoproteomics is to identify and describe the relationship of the phosphorylation regulation with the pathogenesis and development of serious diseases [166]. The study of phosphorylated proteins and their dynamic changes over time is complicated due to low abundance of phosphorylated proteins and phosphopeptides [167]. Difficulties during MS analysis of phosphopeptides are also caused by their low efficiency of ionization as compared with that of nonphosphorylated peptides [168, 169]. Especially, highly phosphorylated peptides can be detected only with a considerably reduced sensitivity in positive ion mode, but negative ionization is commonly avoided for reduced stability of ions [16]. All these reasons render the analysis challenging and enrichment of phosphopeptides in biological samples is one of the most crucial steps of the whole analysis.

Enrichment and analytical strategies suitable for these diagnostically important molecules, including the degree and localization of phosphorylation, are currently in great attention. Various methods and various commercial materials are routinely used for enrichment of mono- and multi-phosphorylated peptides [170]. For highly complex mixtures, the main aim is to choose a proper purification protocol and also material to achieve the desired purity of final phosphopeptide-rich sample. Most common enrichment techniques are, among others, IMAC [171] and MOAC, which is mostly represented by TiO_2 forming bidentate complexes with phosphates [129, 172]. Enrichment of pY-containing proteins can also be achieved using antibodies [173].

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 [174, 175]. IMAC is based on interaction between a negatively charged phosphate group, where the electron donor is an oxygen atom, and positively charged metal ions [174]. Metal ions can be bound with the aid of

iminodiacetic acid (IDA), nitrilotriacetic acid (NTA) for trivalent cations, or, more recently, with highly efficient chelating ligands like arsenate (-AsO_3^{2-}), phosphate (-PO_3^{2-}), ATP and dopamine for immobilization of quadrivalent metal cations [175]. However, peptides containing aspartic or glutamic acid or chains containing histidine of a higher density are after the separation of phosphopeptides by IMAC with Fe^{3+} or Ga^{3+}, contaminating substances [176]. Reduction of this non-specific binding by methyl esterification of carboxyl groups has been described [177]. However, esterification of the residue of aspartic acid worsens the interpretability of spectra and complicates data analysis. Ti^{4+} -IMAC has a high tolerance toward acidic buffers, possesses higher selectivity than Fe^{3+}-IMAC and allows for the enrichment of more basic phosphopeptides. However, it has lower affinity towards the multiply phosphorylated peptides, as compared with conventional Fe^{3+}-IMAC enrichment [178, 179]. All ligands in IMAC technology are immobilized on the support, which can be represented by polymeric resin, porous monolith in column, SiO_2 nanospheres or magnetic nanoparticles [170, 171, 181, 182] .

In many studies, MOAC is preferably used for the specific enrichment of phosphorylated peptides due to its selectivity. Among the various types of metal oxides, TiO_2 was first used as a chromatographic resin for highly selective enrichment of phosphopeptides prior to MS analysis [129]. TiO_2 plays a key role in many large-scale phosphoproteomic studies – see e.g. [183, 184]. Other oxides which were introduced and used for this purpose are ZrO_2 [185], HfO_2 [186], Al_2O_3 [187], Ga_2O_3 [188], CeO_2 [189], Ta_2O_3 [190] and La_2O_3 [191]. However, porous microspheres of TiO_2 are the most commonly utilized material, which size varies in units of micrometers [192]. TiO_2 resin is able to bind multiply phosphorylated peptides as well as monophosphorylated peptides but the elution of multiphosphorylated forms of peptides is difficult and does not occur quantitatively [193]. Sequential elution from IMAC (SIMAC) approach combines both IMAC and MOAC enrichment strategies to obtain a higher purity of sample and to prevent the loss of multiphosphorylated peptides [193]. Another, although less used alternative, is $Al(OH)_3$-based affinity chromatography [194].

Many promising types of advanced materials have been reported for effective and specific enrichment of phosphopeptides based on various types of metal oxides and exhibit various types of shapes (Fig. 6). Some of these materials were extensively reviewed in [175]. Selected examples will be presented in the following paragraphs.

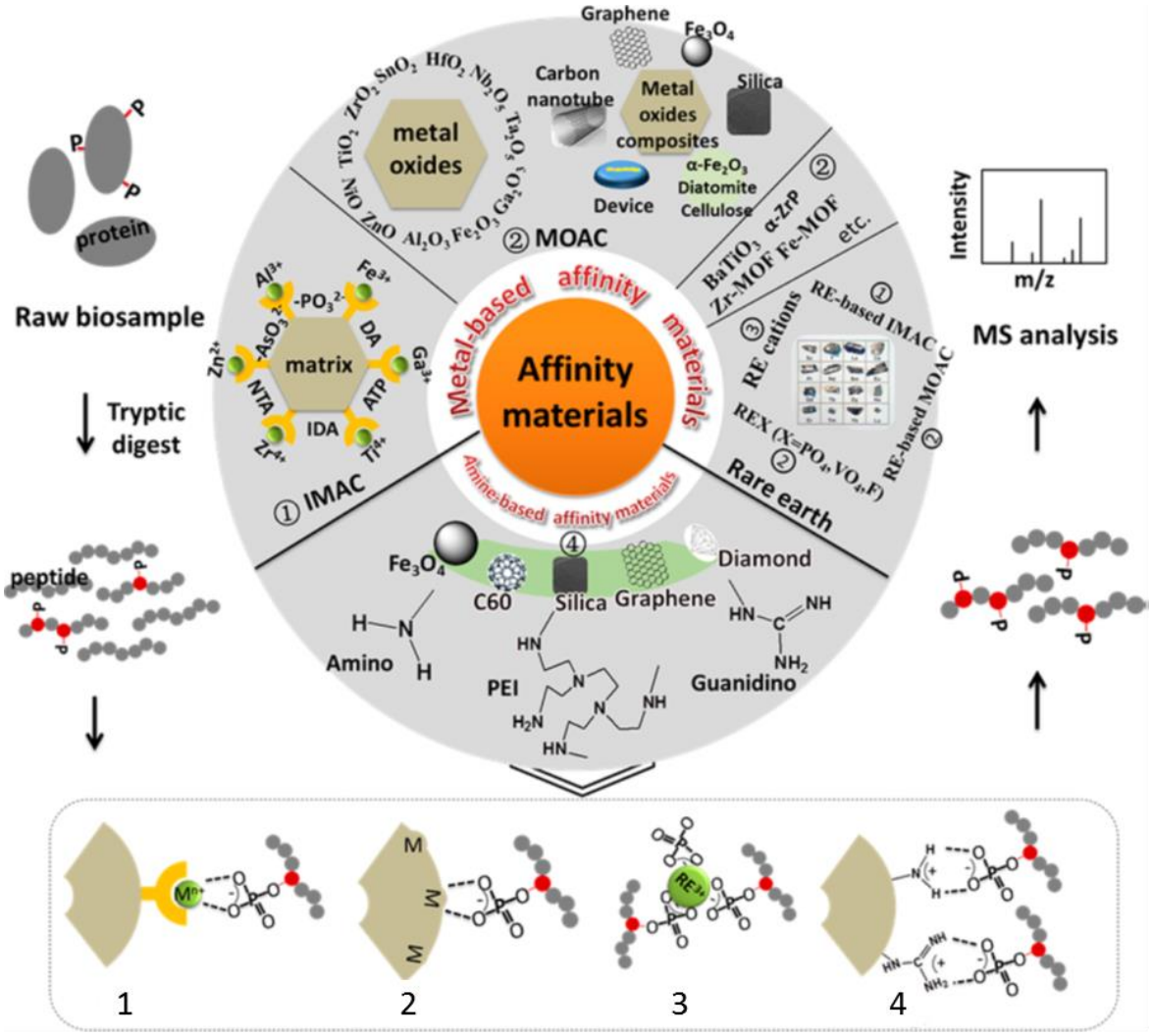

Figure 6.: Schematic illustration of phosphopeptide enrichment strategies for MS detection using diverse affinity materials. The numbers 1–4 represent the main interaction mechanisms of phosphopeptides with different affinity materials. Adapted from [175].

First example of TiO_2 particles with magnetically active cores composed of Fe_3O_4 for the enrichment of phosphopeptides and subsequent SALDI-MS (mass spectrometry ionization laser attended surface) was introduced in 2005 [195]. Since this time, many other magnetically active materials have been developed and utilized for phosphopeptide enrichment with various structures or compositions [175]. Interaction between the carrier based on bare Fe_3O_4 nanoparticles was described for rapid enrichment of phosphopeptides originating from β-casein [196]. Nonstoichiometric magnetic ferrites, including $NiFe_2O_4$, $ZnFe_2O_4$ and $NiZnFe_2O_4$ for enrichment of mono- and multi- phosphorylated peptides are another example of utilization of the nanomaterials in phosphoproteomics [197].

One of the most promising materials are TiO_2 nanotubes, which have recently been shown for selective phosphopeptides enrichment in [198] as non-magnetically active tubes grown on Ti wire support or TiO_2 nanotubes aligned along the bottom of the flow path in a chip suitable for enrichment of serum phosphopeptides [134]. The magnetically active variant TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles utilized in batch mode has been described in patent WO2016015690 A1 (CZ305599). Very recently, amorphous nanotubes with or without Fe_3O_4 decoration have been exploited for highly selective enrichment of phosphopeptides [199].

1.3 Protein analysis

Proteomics is one of the most rapidly developing areas of biochemistry. 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 [200, 201]. Analysis of secondary and tertiary structure can be observed by using special MS techniques, such as stable isotope exchange [202] or crosslinking with specially designed linkers [203].

MS allows for the analysis of peptides, proteins, protein complexes, and their structural and quantitative dynamics. The approach to MS analysis, type of mass spectrometer or fragmentation technique is dependent on sample type, its complexity and purpose of the analysis. 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. With recent MS instrumentation, one can achieve a sensitivity of detection at sub-femtomolar range [204], which is very important mainly in clinical proteomics.

In proteomics, key separation techniques are frequently combined with MS and they can be divided into two categories – gel-based or liquid chromatography (LC-MS) based approaches, respectively. Gel-based approach is mostly represented by polyacrylamide gel electrophoresis in 1D or 2D (two-dimensional) 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 LC-MS.

LC-MS approach exploits HPLC techniques – reversed phase, anion/cation exchange, hydrophilic interaction liquid chromatography etc. or their combination. 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) [205]. 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 (incomplete database or unknown protein), or where the encoding gene is spliced alternatively, the database search may provide unclear results, which can be refined by using *de novo* sequencing. *De novo* sequencing is a procedure for identification of peptides and proteins in which the amino acid sequence is determined only based on the MS/MS spectra [206].

1.3.1 General approaches to proteomic analysis

1.3.1.1 Bottom-up approach

The bottom-up approach is the most widely used one in proteomic analysis. This method involves the digestion of the protein of interest prior to its analysis [207]. When this approach is applied to the protein mixture it is then termed shotgun proteomics [208]. Peptide separation and their MS analysis are simpler both theoretically and experimentally compared to top-down approach. Bottom-up methods of analyzing proteins at the peptide level are also more straightforward [209]. Proteins are chemically or enzymatically digested prior to their separation or introduction to the mass spectrometer. The conversion of proteins into peptides is almost exclusively done by using trypsin as a proteolytic reagent [210]. It cleaves C-terminally to lysine and arginine residues, unless followed by a proline. The detection and typically MS/MS-based identification of the peptides is suitable for the determination of the original protein identity as well as its modifications if they occur. For example, the acquired MS/MS

data can be searched with data analysis engine against the whole or a selected part of protein database e.g. SwissProt or TrEMBL. Searching engines usually allow to set enzyme for cleavage, missed cleavages, fixed modifications (e.g. carbamidomethylation of Cys) or variable modifications (e.g. MetO or pS, pT, pY). Spectra are processed with designated precursor ion and product ion mass tolerance (in ppm or Da). Peptide spectral matches are then validated based on *q*-values at false discovery rate – usually 1 or 5% [211]. One of the main advantages of the shotgun approach is analysis of complex mixtures, which is the case when MS device is coupled with separation techniques such as HPLC. Main disadvantage lies in the fact that usually not all the peptides from the digest are analyzed, thus the information about some regions of the protein is lost [212]. In phosphoproteomics it is the most widely used technique and almost all enrichment protocols are performed on the peptide level instead of the protein one.

1.3.1.2 Top-down approach

Despite the fact that MS-based proteomics is commonly carried out in a bottom-up approach [207], several disadvantages of this approach are obvious. Mainly, a peptide or even several of them may be the same for particular proteins or protein forms. Moreover, large regions of the protein sequence may not be identified. This can cause a loss of information about PTMs or sequence variants because relation of these modifications to each other is lost when they are located in disparate peptides [212]. Top-down mass approach eliminates these limitations by measuring both, the intact protein and fragment ions during a single analysis and can provide more information about the analyzed protein. The main application of top-down approach, from the range of possible applications, is related to the identification and characterization of PTMs in proteins [213]. At the beginning, the technical difficulty of proteome analysis at the intact protein level has caused top-down proteomics to be less used in comparison with bottom-up approach. However, recent advances in separation techniques and MS instrumentation makes top-down approach more feasible [200]. The mass spectrometers primarily used for the top-down approach provide high resolution and mass accuracy, e.g. Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry, and use low energy activation methods for fragmentation, which allow for the conservation of the PTMs [212].

1.3.1.3 *De novo* sequencing

De novo sequencing approach [214] is also used in proteomics. This procedure for identification of peptides and proteins is an alternative to the database search of the primary sequence. In some cases, *de novo* sequencing is necessary because database search procedures often fail if the proteins are modified, unknown, subjected to alternative splicing, artificially created, originating from unknown species or cancerous cells [215, 216]. *De novo* sequencing is a procedure in which the amino acid sequence is determined only based on the mass spectrum without resorting to databases. It can be done manually or using specific software tools like PEAKS (Bioinformatics Solutions Inc.).

1.3.2 Analytical separation of proteins and peptides

Proteomic approaches, preanalytical and analytical approaches play a pivotal role, which is valid for both protein and peptide level, respectively. Some samples of peptides/proteins can be directly analyzed by MS in the case of simple mixtures or analysis of protein form 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 [217]. Within the past two decades many separation strategies for peptides and proteins have been developed. In this section, most common and very often used ones in proteomics will be mentioned with the focus on analysis of peptides and phosphopeptides. 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 [212]. The list of separation methods can be extended by electrostatic repulsion−hydrophilic interaction chromatography (ERLIC), which is useful for separation of phosphopeptides [218]. 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 mass spectrometer. 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 [219, 220]. Whichever method is chosen to fractionate protein/peptide in first dimension, the mixture is then injected into the mass spectrometer, usually directly from the RPLC. The milestone in separation technology further used in proteomics was the use of ultrahigh pressure liquid chromatography (UHPLC) developed by MacNair et al. [221]. In chromatographic terms, small packing materials and thus, peptide peak capacity, increased

protein identification efficiency [222]. Chromatographic separation enables effective ionization of complex samples with electrospray ionization (ESI) source, which can be negatively influenced by ionization suppression. In the case of overloading of the ESI source with analytes, it will result in preferential ionization of hydrophobic molecules [209, 223].

1.3.2.1 Gel-based separation methods

Gel-based methods commonly rely on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This allows determining molecular weight of proteins, after gel calibration with suitable standards, and also to estimate protein concentration. In combination with isoelectric focusing (separating proteins according their isoelectric point (pI)), it creates two-dimensional gel electrophoresis (2DE) useful for separation of complex protein mixtures. In combination with other methods, e.g. specific staining, and/or mass spectrometry, 2DE allows to fractionate protein mixtures and identify protein forms differing in physicochemical properties and modifications [224, 225]. Possibilities of 2DE in combination with bottom-up mass spectrometry approach for identifying protein modifications were demonstrated e.g. in a study focused on the identification of glycosylation isoforms [225]. Prior to mass spectrometric analysis, separated proteins (SDS-PAGE, 2DE etc.) must be excised, *in-gel* digested and subsequently extracted from the gel. After this procedure, either matrix assisted laser desorption ionization MS (MALDI-MS) or nanoESI-MS/MS can be employed for identification and characterization of target protein [226]. Detection of protein modifications is also possible via several specific separation or staining techniques. For detection of protein phosphorylation, the phosphoproteins can be, after their separation, stained with various phosho-specific reagents, like Pro-Q Diamond or quercetin–Al^{3+} [227]. Another option is to detect phosphoproteins after their transfer to western-blot membrane. Typically, with site-specific antibodies or with the reagent called pIMAGO, which is a stain functionalized with titanium ions and exhibiting high selectivity towards phosphorylated proteins [228]. An example of the advanced electrophoretic separation of phosphorylated proteins is the Phos-tag SDS-PAGE [229, 230]. The detection of protein phosphorylation is held by a dinuclear metal complex of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate conjugated in the gel. The complex acts as reversible phosphate binding molecule. Mobility of phosphorylated proteins is thus shifted, and they are separated from their unphosphorylated forms [229].

1.3.2.2 Reversed-phase liquid chromatography

RPLC uses a non-polar stationary phase and a polar mobile phase, that allows the peptides/proteins to stick to reverse phase of HPLC columns. These compounds are usually eluted from the columns with a gradient with increasing organic content and they are separated based on their hydrophobic character. Alkyl chains (e.g. C4, C5, C8, C18) linked to porous silica particles are commonly used as the stationary phases [212]. The length of alkyl chains depends on the application, where shorter alkyl chains are typically employed for intact proteins due to higher recovery and reduction of peak tailing [231]. RPLC is usually directly connected to mass spectrometers using electrospray ionization or can be coupled off-line in the case of MALDI ionization. In proteomics and phosphoproteomics, nanoflow RPLC with nano-electrospray (nano-ESI) source provide high sensitivity for effective analysis of peptides. In nanoflow RPLC-based separation of peptides, mainly two factors can be modified to increase peak capacity: column length and the size of stationary phase particles [232]. Typically, the gradient separation uses gradients of up to 2 h or, to achieve high performance of separation, longer gradients of up to 10 h on longer columns (25–100 cm) packed with smaller (sub-2 μm) particle sizes were demonstrated [219, 233]. Recently, it has been shown that also conventional-flow chromatography can be effective for discovery-oriented proteomics. The amount of a complex tryptic digest needed for comparable proteome coverage can be roughly only 5-fold greater after choosing the proper column dimensions and under optimized and appropriate conditions for peptide separation [234].

1.3.2.3 Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) employs polar stationary phases such as bare silica, cyano- or amino-modified silica, but the mobile phase used is similar to those employed in the RPLC [235]. In contrast with RPLC, HILIC utilizes high organic content mobile phase for sample loading and a gradient of increasing water content. It results in elution of more hydrophobic compounds at the beginning of the gradient and higher selectivity towards polar compounds [236, 237]. The mechanism of HILIC separation is based on partitioning between the mobile phase and water-rich layer partially immobilized on the stationary phase. This is the main difference from normal phase chromatography where analytes are adsorbed directly to the stationary phase surface [238, 239]. HILIC separation technique utilizes either packed [240] or monolithic [241] columns and both can be used e.g. in shotgun proteomics. As the peptides are separated based on their hydrophilic interactions with an ionic resin, it is also

suitable for analysis of PTMs [242]. In the case of a mixture of phosphorylated and non-phosphorylated peptides, the non-phosphorylated peptides are eluted at the beginning of the gradient with subsequent elution of singly and then multiply phosphorylated peptides due to the increase of peptide's hydrophilicity and retention time by phosphate group [243].

1.3.2.4 Ion exchange chromatography

For compounds with one or more charged functional groups, IEX is an ideal separation technique. It can be used for separation of solutes with net surface charge, from small inorganic ions to proteins, peptides and other biological macromolecules [238]. First variant, cation exchange chromatography, uses a negatively charged ion exchange resin with an affinity for molecules having net positive surface charges. The second variant, anion exchange chromatography, uses a positively charged ion exchange resin with an affinity for molecules having net negative surface charges (Fig. 7). Elution is, in both cases, performed by increasing the salt concentration, or by changing of pH of the mobile phase.

IEX is one the most common separation techniques in downstream processes of recombinant proteins used for contaminants removal [244]. However, it also finds its application in phosphoproteomics where its variant, strong cation exchange chromatography (SCX), is commonly used for fractionation phosphopeptides before or after their specific enrichment using MOAC [208, 245].

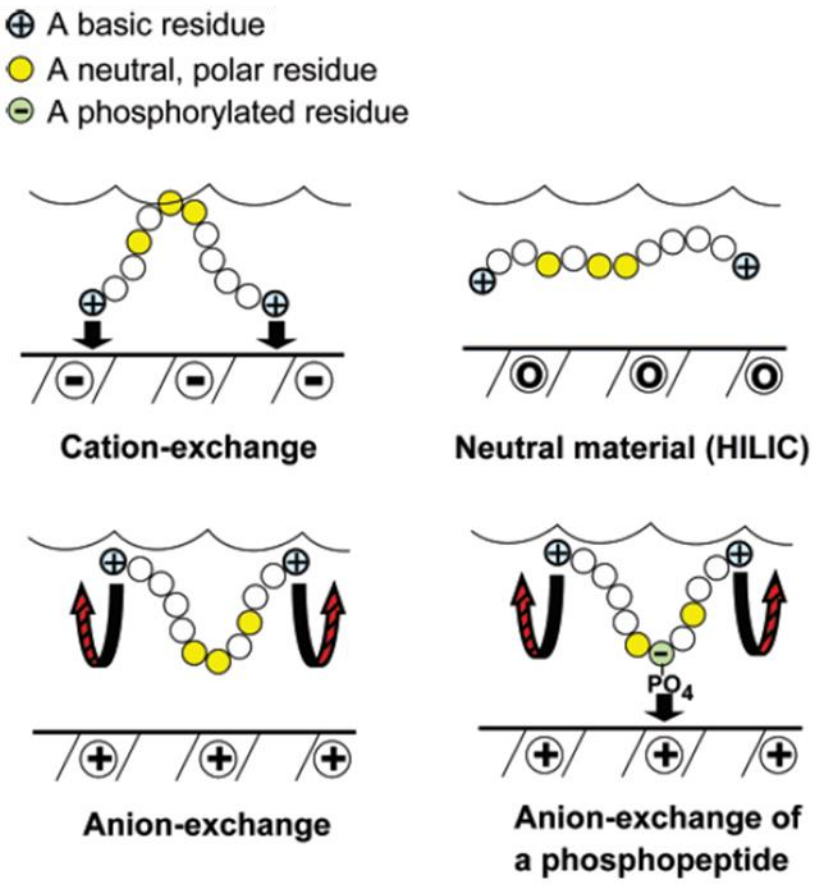

Figure 7.: Theoretical orientation of tryptic peptides with or without phosphate on various stationary phases. The basic termini are attracted in cation exchange and repelled in anion exchange. Adapted from [246].

1.3.2.5 Electrostatic repulsion−hydrophilic interaction chromatography

ERLIC is a specific form of HILIC, using a weak anion exchange (WAX) resin. For tryptic peptides, organic solvent in the mobile phase generates hydrophilic interaction sufficient for reasonable retention. Under these conditions, all peptides in a mixture would be retained through hydrophilic interaction but, at the same time, will be repelled by the stationary phase to some extent (except for neutral peptides) [246]. During separation, peptides are retained under two separation modes. At the beginning of the gradient with high concentration of organic solvent hydrophilic interactions dominate, but with increasing aqueous content of the elution buffer, basic peptides are electrostatically repelled from the WAX resin. Acidic peptides are retained until their hydrophilic interaction with the WAX resin and they are eluted at the end of the gradient. This combined mechanism distributes peptides over the gradient better than RPLC, and ERLIC separations can achieve a higher peptide identification rate and are more effective for acidic residues [209, 247]. Due to the acidic properties of phosphopeptides, ERLIC

is obviously suitable for their separation, where positively charged stationary phase of ERLIC favors retention and separation of negatively charged multiphosphorylated peptides.

1.3.2.6 2D chromatographic approaches suitable for phosphoproteomics

Due to highly complex mixtures and low abundance of phosphoproteins, an effective separation is crucial for successful analysis of these biomolecules. Beside and in cooperation with highly specific enrichment techniques, a multidimensional chromatographic separation of (phospho)peptides, generated by endopeptidase digestion of (phospho)proteins in solution, seems to be very effective. Highly orthogonal chromatographic dimensions should be chosen for 2D separation and they can be coupled either on-line or off-line [248]. Online peptide separation was shown within the multidimensional protein identification technology (MudPIT) approach, which is based on peptide mixtures separations, initially by SCX and then followed by nanoflow RPLC [220]. IEX setup usually operates with salt gradients, which interfere with RPLC-MS analysis; that is why RP capillaries are usually equipped with pre-columns for preconcentration and desalting of the peptide mixtures. More robust, and more common in use, is the off-line separation of peptide mixtures by SCX chromatography followed by the application of single fractions to the nanoflow RPLC-MS system, which can also be performed in combination with specific enrichment of phosphopeptides [249-251].

One of the orthogonal separations that works without the salt problem is the concept of RP-RPLC separations [252]. First, peptide mixtures are separated by RP chromatography at neutral or basic pH, then fractionated and the fractions analyzed by conventional nanoflow RPLC–MS under acidic conditions. This approach can also be extended with specific enrichment of phosphopeptides after separation in the first dimension [253]. The same approach was shown for combination ERLIC-TiO_2 followed by nanoflow RPLC–MS [184]. HILIC has been suggested as an alternative fractionation method to SCX and ERLIC, which also provides orthogonal separation in multidimensional chromatography [254]. Direct comparison of HILIC-TiO_2, SCX-TiO_2 and ERLIC-TiO_2 approach followed by nanoflow RPLC–MS have shown that SCX-TiO_2 identifies a higher total number of nonredundant phosphopeptides, whereas the ERLIC-TiO_2 approach identifies a higher number of multiphosphorylated peptides. HILIC-TiO_2 provided the worst performance compared to the two other approaches [184].

1.3.3 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 nanoESI [255] or, to a lesser extent, MALDI [256]. Nanoflow ESI has generally been coupled with RPLC separations, and MALDI, with gel-based separations [209]. The complexity of post-translational modifications in samples therefore requires modern methodologies including LC separation of peptides (see section 1.3.2) or their selective isolation. This step is critical for high-throughput and sensitive detection of target peptides from complex proteomic mixtures. MS-based analysis has become the most utilized method in large scale phosphoproteomics studies as well as in studies of other PTMs.

1.3.3.1 The Orbitrap based mass spectrometers

The main part of analyses of proteins in this thesis was performed using the Orbitrap-based mass spectrometers. After invention of the Orbitrap analyzer [257] it was described also as a suitable mass analyzer for proteins and peptides [258] and followed by development of several different hybrid high accuracy mass spectrometers for proteomics. The hybrid instruments that use the Orbitrap are combined with an additional mass analyzer, linear ion trap (LIT) or quadrupole (Q). The mass spectrometers that were developed and introduced to the market (Thermo Fisher Scientific, USA) with the combination of LIT and Orbitrap are called LTQ Orbitrap XL and LTQ Orbitrap Velos. The second group, form the same company, is represented by mass spectrometers combining quadrupole with Orbitrap and are represented by e.g. Q Exactive, Q Exactive plus, Orbitrap Fusion or Orbitrap Elite. Both LIT and Q combined with Orbitrap analyzer are alltogether placed in Orbitrap Fusion Tribrid [259]. The core of these instruments, Orbitrap, can be installed in 3 variants as standard, high field or ultra-high field. Comercially available ultra-high field Orbitrap instruments are characterized by either faster analysis of samples at the same resolution or by very high resolution, up to 500,000 [260]. Data mentioned in this thesis were in vast of majority generated by the LTQ Orbitrap XL and Q Exactive and they are therefore further described in detail.

LTQ Orbitrap XL is a hybrid instrument that uses a high resolution Orbitrap cell and a 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 with sequential isolation and collision induced dissociation (CID) fragmentation of the top N most intense peptides in the LIT. Mass of parental peptides is measured in parallel in the LIT or sent to Orbitrap analyzer for high resolution measurements. LTQ Orbitrap XL is usually coupled to ESI ion source, taking benefits of online LC separation. MALDI ion source is typically coupled to time of flight mass analyzers, utilizing benefits of the combination of short pulses (ns) of ultraviolet lasers and the small spatial distribution of desorbed ions, which is the ideal combination for time of flight (TOF) analyzers. However, after modification, MALDI source can also be interfaced to LTQ Orbitrap XL instrument, which was also shown for proteomic analysis [260, 261].

The Q Exactive is a high-resolution mass spectrometer with an Orbitrap cell able to detect both precursor and fragment ions [262]. 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 [262]. As compared to LTQ Orbitrap XL instrument, Q Exactive is benchtop, has a shorter ion path and has improved electronics enabling faster analysis. Another difference is the ability to fill in mass spectrometer parallely, during analysis on Orbitrap and Fourier transformation. Acquisition speed ranges from 12 Hz for resolving power 17,500 at *m*/*z* 200 (corresponding to 12,500 at *m*/*z* 400) to 1.5 Hz for resolving power 140,000 at *m*/*z* 200 (corresponding to 100,000 at *m*/*z* 400) [260, 262]. These improvements make the Q Exactive a more sensitive and faster instrument than LTQ Orbitrap XL.

1.3.3.2 Fragmentation techniques used in proteomics and phosphoproteomics

The fragmentation methods of peptides using diverse procedures aimed at identification of the peptides and subsequent identification of proteins have been comprehensively studied and developed. One of the key components in proteomics protocols is the generation of tandem mass (MS/MS) spectra mainly by peptide fragmentation using CID. This approach is currently used in the vast majority of experiments to routinely identify up to thousands of proteins from a single MS run. CID is the most-widely used technique of gas phase fragmentation of peptides in proteomics [263]. CID fragmentation with trypsin digestion, which generates short peptides mainly doubly charged, is shown as a suitable combination for peptide identification.

Alternatively, but in increasing extent, peptide fragmentation methods such as HCD [264] 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 [265] is based on electron transfer to a multicharged peptide from negative ions (electron transfer dissociation). During this process, same as in the ECD, the N-Cα bond is mainly cleaved and the c- and z-ions (Fig. 8, Tab. 1) are formed [265]. 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 [266]. 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 [267].

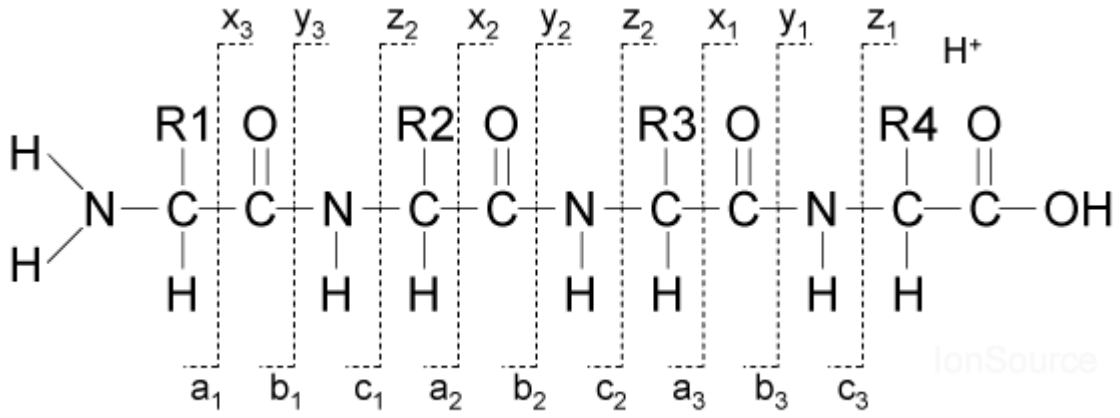

Figure 8.: Roepstorff and Fohlman nomenclature system for protonated peptide fragmentation [268].

Within CID, dissociation can be achieved by vibrational activation of a peptide ion by collisions with neutral gas molecules [269] and results in cleavage of the C–N (peptide) bond, which results in the emergence of amino acid sequence-informative b- and y-ions (Table 1). CID can be performed either in ion traps (Trapping CID – very slow activation), in quadrupoles or other multipoles (‘‘Low-energy’’ CID – slow activation) and in TOF/TOF analyzers (‘‘High-energy’’ CID – fast activation), the differences in energy deposition result in differences in the appearance of mass spectra [270]. Beam-type CID commonly used in quadrupole-time-of-flight instruments or triple-quadrupole (~100 eV; QqTOF or QqQ), has also been introduced on the Orbitrap hybrid instruments as HCD (“higher-energy CID”) [264]. HCD employs higher energy dissociations than those used in ion trap CID, enabling a wider range of fragmentation pathways resulting in higher quality MS/MS spectra [271]. Within HCD fragmentation in Orbitrap based instruments, ions are fragmented in a collision cell and then transferred back through the C-trap for analysis at high resolution in the Orbitrap. As compared to CID, the labile phosphate groups

on the peptide backbone are better preserved during HCD fragmentation and allow for a more confident phosphosite assignment. That is why the combination of trypsin digestion with phosphopeptide enrichment and HCD is such a widely used phosphoproteomics platform, even though tryptic peptides produce y-ion rich spectra [210]. For the LTQ Orbitrap devices equipped with HCD cell, no low-mass cut-off of HCD fragmentation allows to use low mass region in the MS/MS spectra as compared with CID [272]. This makes the combination of HCD with quantitation isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tag (TMT) reagents producing small m/z reporter ions possible [273, 274]. Hybrid Orbitrap instruments including linear ion trap are able to perform HCD fragmentation independently, [275] or in combination with low-energy CID as shown in the glycoproteomic study, and allow to get information about glycan and peptide sequence in a single experiment [276]. Other advantages of HCD with the Orbitrap analyzer detection compared to CID with ion trap detection include assignment of neutral loss ions, and the presence of reporter ions such as the 216.041 ion for pY [277].

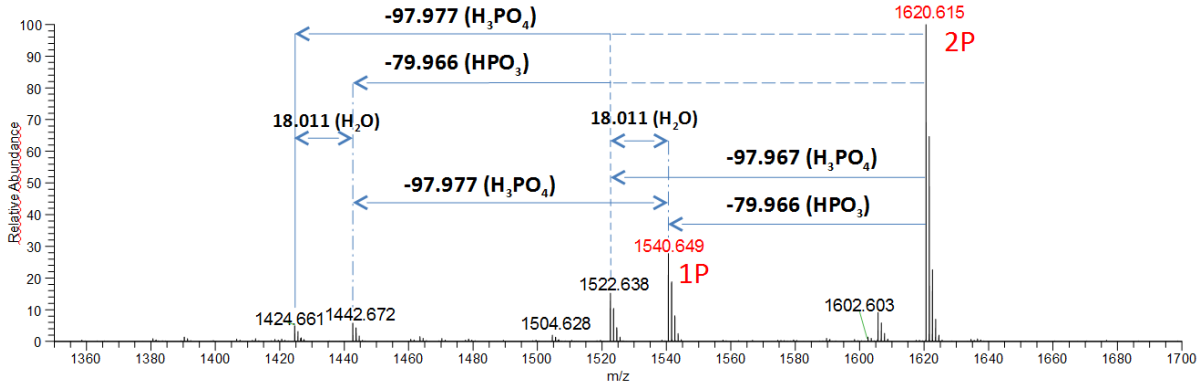

Figure 9.: Phosphopeptides with different level of phosphorylation (m/z 1620.615 doubly phosphorylated; m/z 1540.649 singly phosphorylated) and demonstration of in-source phosphorylation loss. Tryptically digested phosvitin (Sigma-Aldrich) analyzed by MALDI LTQ Orbitrap XL mass spectrometer.

MS-based phosphoproteomics has yielded information about thousands of phosphorylation sites per experiment through global high-throughput analyses. However, in these approaches, information on the defined succession of phosphorylation events and the co-occupation of phosphorylation sites in one protein is largely lost through the digestion of the protein into peptides [278]. Sequence analysis of phosphopeptides and exact localization of phosphorylation is challenging due to the lability of the phosphate group (Fig. 9) and, mainly during CID, many pS and pT exhibit a significant neutral loss of 98/*z* (H_3PO_4, *z* being the charge

state of the selected precursor ion) as the dominant fragmentation pathway. In some cases, the neutral loss peak is dominant, and no other sequence informative ions can be observed. In contrast, pY modification remains stable in the peptide sequence. Neutral loss of H_3PO_4 is assumed to be a β-elimination reaction [279] or a formation of the neutral loss product ion via a charge-directed S_N2 mechanism involving the participation of a neighboring group [280].

Table 1.: *Calculation of masses of fragmentation ions: where [N] is the mass of the N-terminus [C] is the mass of the C-terminus. [M] is the sum of the amino acid residue masses that are contained within the fragment ion. CO is the combined mass of oxygen plus carbon atoms (27.9949 Da).*

Ion type	Masses calculation of 1x positively charged fragment ions
a-ion	[N] + [M] - CO - [e]
b-ion	[N] + [M] - [e]
c-ion	[N] + [M] + NH_3 - [e]
x-ion	[C] + [M] + CO - [e]
y-ion	[C] + [M] + H_2 - [e]
z-ion	[C] + [M] - NH - [e]
d-ion	[*a*-ion] - [part of side chain]
v-ion	[*y*-ion] - [whole side chain]
w-ion	[*z*-ion] - [part of side chain]
immonium ion	[M] + H - CO - [e]

To obtain more sequence information from phosphopeptides with CID technique, the use of a second step of activation is necessary (MS^3). MS^3 is implemented in many tandem mass spectrometers containing LIT, including LTQ Orbitrap XL devices. The second approach which has been introduced in ion trap-based systems is multi-stage activation (MSA)[281]. Within this technique, instead of two separate isolation and fragmentation steps, the precursor and neutral loss events are simultaneously activated and stored generating a single mass spectrum comprising product ion species derived from multiple ion activation events. Therefore, spectra obtained in MSA mode contain MS^2 and MS^3 information, while only one level of fragmentation data needs to be analyzed within automatic data analysis [281, 282]. Behavior of HCD within fragmentation of phosphopeptides was described above.

The ETD fragmentation technique offers widespread possibilities for large-scale phosphoproteomic analysis. In contrast to CID, ETD preserves labile PTMs during fragmentation, which is crucial for confident PTM site localization assignment [283]. Contribution of ETD to accurate localization of phosphorylation on a peptide chain is very

important because determination of the exact site of phosphorylation can be even more demanding than identifying the phosphopeptide itself. However, ETD strongly depends on charge density, and ETD tandem mass spectra are often dominated by unreacted and charge reduced precursors [284, 285]. Direct comparison of CID, HCD and ETD on an LTQ Orbitrap Velos shows that in all analyses the success rate for HCD was significantly higher than for other fragmentation techniques [286]. It was shown that HCD is most suitable for doubly charged peptides, singly phosphorylated peptides, and N-acetylated peptides. ETD with Orbitrap detection outperforms the other techniques for peptides with charged states >2. It was confirmed that ETD also has some limitations, such as lower scan speeds (due to longer reaction times), lower fragmentation efficiencies and substantial dependence on m/z and charge state.

One of the most advanced alternatives to increase fragmentation efficiency is sequential combination of ETD and HCD in a single event called EThcD [287]. In EThcD, the initial ETD event is followed by HCD fragmentation, yielding c/z and b/y fragment ions in a single MS/MS spectrum. The generated dual fragmentation ion series provides richer fragmentation spectra compared with HCD and ETD alone, which leads to increased sequence coverage. EThcD is commercially available on Orbitrap Fusion platform.

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 10 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.

MS-based analysis has become a standard approach for investigating protein phosphorylation and several phosphopeptide enrichment procedures were developed recently. However, there are still uncovered issues of the phosphopeptide enrichment and the further development should focus e.g. on selectivity of these procedures or the ability to enrich multiphosphorylated peptides, which may be important for clinical analyses. One aim of our work thus was to introduce a new material meeting these requirements.

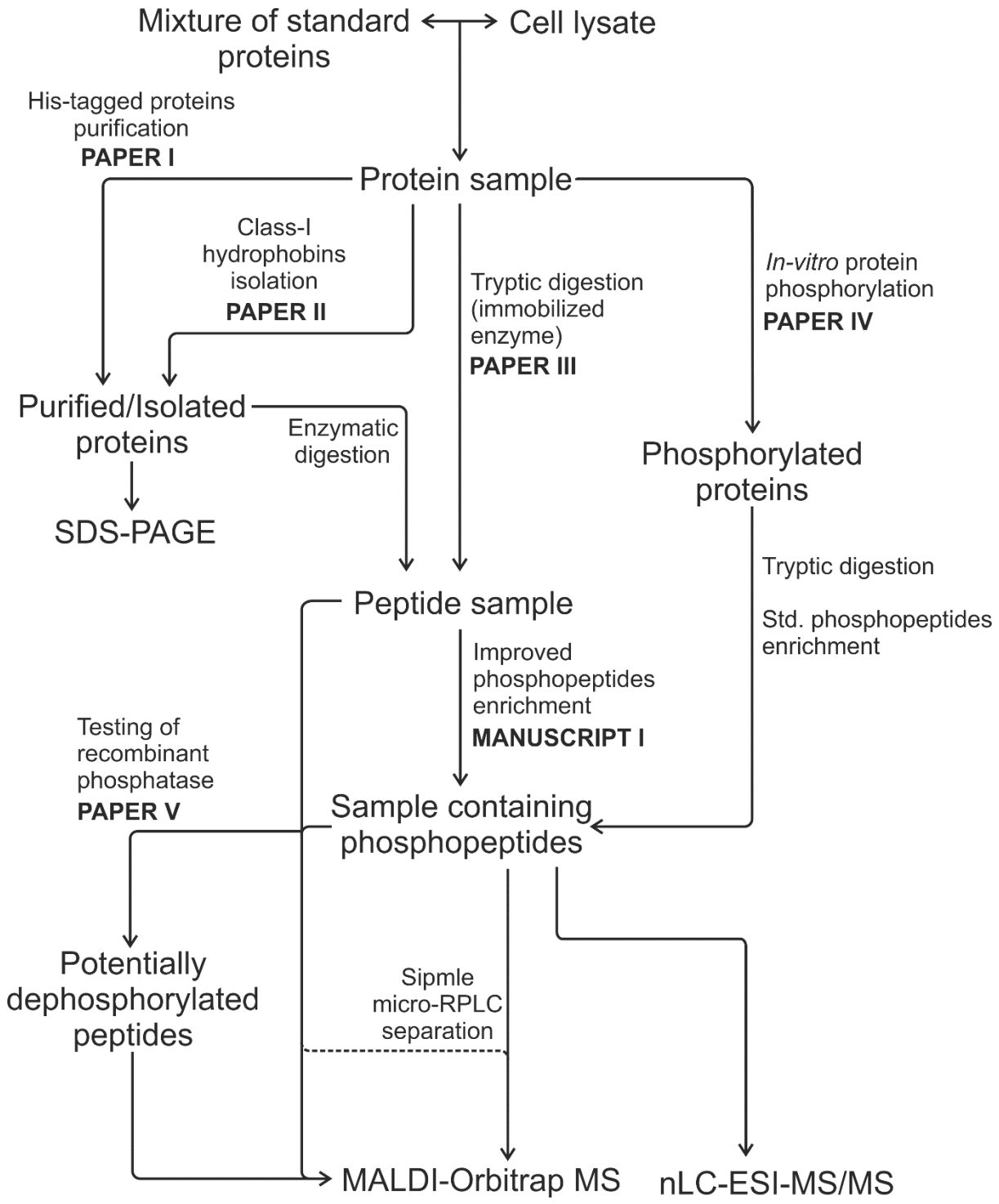

Figure 10.: An overview of selected part of proteomic sample preparation including the experimental design, methodology and materials used; papers addressing particular aspects of the overall analysis workflow are included.

Plain TiO_2 nanotubes (TiO_2NTs) and TiO_2 nanotubes decorated with Fe_3O_4 nanoparticles (TiO_2NTs@Fe_3O_4NPs) were prepared, tested and compared with new or traditional forms of

materials including magnetic nanoparticles and TiO_2 nanofibers. Nanotube-based materials were evaluated as an optimal choice for the purpose of phosphopeptides enrichment. TiO_2NTs with their amorphous structure are responsible for an increase in selectivity for phosphopeptide isolation. The material also allows to enrich multiphosphorylated peptides originating from cell lysate to the similar extent as commonly used commercial TiO_2 microspheres. Moreover, the same TiO_2 nanotubes further decorated with Fe_3O_4 nanoparticles bring another benefit to end-users in the form of fast and easy separation of this carrier due to the response of the material to magnetic field, without any significant loss of material selectivity. It was demonstrated that the developed materials achieved enhanced parameters as compared with commonly used materials. The technology is patented **(PATENT)** and after further experiments the application for phosphopeptide enrichment was submitted as **MANUSCRIPT I** (resubmitted after major revisions).

The main application of TiO_2NTs revealed in the testing experiments was isolation of phosphopeptides. On the other hand, TiO_2NTs@Fe_3O_4NPs showed us significantly different behavior for another specific type of biomolecules. The material can specifically isolate His-tagged recombinant proteins with high selectivity from complex cell lysates containing these fusion proteins. It was obvious that Fe_3O_4 nanoparticles on the surface of nanotubes played a key role for the observed performance difference. Hence, the properties of TiO_2NTs@Fe_3O_4NPs were further extensively tested and improved both for optimal material parameters as well as for isolation protocol. The material and technology were proven as a highly valuable tool for purification of recombinant proteins. The introduced technology is part of the **PATENT** and after further experimental confirmation and development was published as **PAPER I**.

Enrichment of phosphopeptides and recombinant protein purification are a constituent part of **PATENT**. In this work, the novel material in the form of TiO_2NTs@Fe_3O_4NPs was also further utilized for isolation of two other types of biomolecules: nucleic acids and cysteine containing peptides under specifically optimized conditions.

Another isolation technique extending the comprehensive overview of materials used for separation purposes was focused on isolation of extremely hydrophobic proteins. Hydrophobin SC3 of *Schizophyllum commune*, class I hydrophobin, is known for its self-assembly properties on hydrophobic surfaces and resistance to dissociation of self-assembled layer even in hot 2% sodium dodecyl sulfate (SDS) solution [288]. This feature was the cornerstone of development of protocol exploiting microcolumns packed with polytetrafluoroethylene microparticles for

selective isolation and analysis of hydrophobin SC3. Within this work, highly hydrophobic polytetrafluoroethylene (PTFE) microparticles were utilized as stationary phase to show the possibility of isolating extremely hydrophobic SC3 protein from the protein mixture. As the disulfide bonds are essential for stability of the hydrophobins, effective reduction of these bonds was important for enzymatic cleavage. Isolated protein can be analyzed with MS in its intact form or after specific fragmentation with a suitable proteolytic enzyme using optimized protocol as it is described in **PAPER II.**

Within the analysis of protein structure, endoproteinases are used for hydrolysis of peptide bonds at specific sites and the enzymes can be used in soluble or in immobilized forms. Trypsin, which cleaves peptide bonds predominantly at the carboxyl side of lysine and arginine, is by far the most common endoproteinase used. Due to this reason, trypsin was selected as the enzyme for immobilization on the new Fe_3O_4@SiO_2-NH_2 core/shell nanoparticles to create reusable biocatalytic unit. Such created nanoparticles improved tryptic digestion of proteins which was experimentally confirmed and described in **PAPER III.** These magnetic nanoparticles might be useful in many proteomic studies where specific digestion of proteins is crucial.

For deciphering of molecular mechanisms of protein phosphorylation for structural and interaction changes, preparation of *in-vitro* phosphorylated forms of studied proteins is beneficial. Kinases, enzymes that catalyze the transfer of phosphate groups to specific substrates, are suited to be immobilized as they are expensive and therefore a reusable system could reduce costs significantly. The second benefit could be in sequential introduction in biochemical reactors and the easy removal of these enzymes from reaction system at the right point (e.g. with regard to possible cross-inhibition reaction). In our work, selected kinases were immobilized to the commercial sub-micro particles. The other aim was also to describe the spectrum of peptide substrates by monitoring the differences before and after phosphorylation reaction and to optimize the protocol for an effective tau protein phosphorylation. For this purpose, several magnetic particle types and methods for kinase immobilization were tested together with a proper strategy for phosphopeptide enrichment. Comparison of different approaches to create kinase-immobilized beads and analysis of their reaction products is described in **PAPER IV**.

Protein immobilization on microparticles may also be crucial for structural and activity stabilization of proteins. Many proteins are unstable out of their natural environment inside cells, or recombinant proteins can be unstable either due to lack of posttranslational

modifications not acquired from expression system or due to the presence of unwanted artificially added sequence. In these cases, it could be beneficial to immobilize such isolated or synthesized proteins as it is shown in **PAPER V**, where recombinant phosphatases were immobilized on IMAC resin and allowed to identify appropriate substrates and reaction conditions for these newly prepared enzyme-carriers.

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 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-SDS-PAGE [289] or Tricine-SDS-PAGE [290] – 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.

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 RP-LC 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 ACN content. Peptides eluted from RP microcolumn were directly applied onto the MALDI plate with subsequent mixing with MALDI matrix solution [291-293].

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 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 PAPER I: New Interface for Purification of Proteins: 1D TiO_2 Nanotubes Decorated by Fe_3O_4 nanoparticles

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To produce highly pure recombinant proteins and to facilitate the purification process, suitable affinity tags are commonly added to target protein. Commonly used specific sequence is so-called His-tag formed by 6–8 histidines attached at the N- or C-terminus of the recombinant protein. For the isolation of such modified recombinant proteins IMAC has been the most widely employed technique. However, there are still several known limitations of this technology, such as insufficient purity of separated proteins, gradual release of toxic metal ions during separation or poor mechanical and chemical stability of the IMAC carrier.

To address these limitations, 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 to 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) was ineffective for this purpose. It was found that key component of elution solution was sodium phosphate, optimally in the presence of imidazole. 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 for an improved reduction of non-specific protein binding.

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. 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.

This article was published in 2017; ACS Applied Materials & Interfaces – **IF (2017): 8.097.**

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

Kupčík R.[1], Zelená M.[2], Řehulka P.[3], Bílková Z.[1], and Česlová L.[2]

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Hydrophobins are small surface-active proteins produced by filamentous fungi with highly conserved eight cysteine residues ordered in a characteristic pattern that is strongly stabilized by four disulfide bonds. Although several isolation techniques for both classes of hydrophobins were previously reported, a highly selective and efficient technique for specific isolation of class I hydrophobins has not been described yet.

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 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, 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, 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. 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 (DTT) and tris(2-carboxyethyl)phosphine (TCEP) 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.

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.

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

This article was published in 2016; Journal of Separation Science – **IF (2016): 2.557.**

2.2.3 PAPER III: Application of trypsin $Fe_3O_4@SiO_2$ core/shell nanoparticles for protein digestion

Slováková M.[1], Sedlák M.[2], Křížková B.[1], **Kupčík R.[1],** Bulánek R.[3], Korecká L.[1], Drašar Č.[4], Bílková Z.[1]

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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. 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.

This article was published in 2015; Process Biochemistry – **IF (2016): 2.529.**

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

Hromadkova L.[1,2,3], **Kupcik R.[1],** Vajrychova M.[4,5], Prikryl P.[6], Charvatova A.[1], Jankovicova B.[1], Ripova D.[2], Bilkova Z.[1], Slovakova M.[1]

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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. These immobilized systems were 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.

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.

This article was published in 2015; Analyst – **IF (2017): 3.864.**

2.2.5 PAPER V: *PHO15* genes of *Candida albicans* and *Candida parapsilosis* encode HAD-type phosphatases dephosphorylating 2-phosphoglycolate

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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 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.

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.

This article was published in 2019; FEMS Yeast Research – **IF (2017): 2.609**

2.2.6 MANUSCRIPT I: Amorphous TiO_2 Nanotubes as a Platform for Highly Selective Phosphopeptide Enrichment

Kupcik R.[1], Macak J. M.[2,3], Rehulkova H.[4], Sopha H.[2,3], Fabrik I.[4], V.C. Anitha [2], Klimentova J.[3], Murasova P.[1], Bilkova Z.[1]. Rehulka P.[4],

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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 to minimum level. 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.

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.

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.

Author's contribution: 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.

This article was submitted in 2019 to ACS Omega.

2.2.7 PATENT: Method for separation of biopolymer molecules and a carrier for application of this method

Inventors: **Kupčík R.,** Bílková Z., Řehulka P., Macák J.

Applicant: **University of Pardubice**; Studentská 95, 532 10 Pardubice

Publ. No.: WO/2016/015690 A1 (04. 02. 2016); CZ305599 (18. 11. 2015)

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 the current state.

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.

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.

This patent was applied for in the year 2014 to the Industrial Property Office (Czech Republic) and Czech patent was issued in 2015 as **CZ305599.** In parallel, international PCT application was applied for as PCT/CZ2015/000081 with the following Pub. No.: WO/2016/015690 A1 published in 2016.

3. CONCLUSIONS AND PERSPECTIVES

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, polytetrafluoroethylene 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.

4. PUBLICATION ACTIVITY

Author or co-author of 7 papers in international scientific journals with IF (2.222 – 8.097) and 2 papers in peer-reviewed scientific journals. Co-inventor of 1 national patent with PCT application and 1 patent pending.

Research articles published in scientific journals with IF:

Simple Fabrication of Structured Magnetic Metallic Nano-Platelets for Bio-Analytical Applications

Novotny J., Juskova P., **Kupcik R.,** Bilkova Z., Foret F.

Micromachines 10 (2019), 106.

(IF in 2017: 2.222)

PHO15 Genes of Candida Albicans and Candida Parapsilosis Encode HAD-Type Phosphatases Dephosphorylating 2-Phosphoglycolate

Kročová E., Neradová S., **Kupcik R.,** Janovská S., Bílková Z., Heidingsfeld O.

FEMS Yeast Research 19 (2019), foy112.

(IF in 2017: 2.609)

Kinase-loaded magnetic beads for sequential *in vitro* phosphorylation of peptides and proteins

Hromadkova L., **Kupcik R.,** Vajrychova M., Prikryl P., Charvatova A., Jankovicova B., Ripova D., Bilkova Z., Slovakova M.

Analyst 143 (2018), 466–474.

(IF in 2017: 3.864)

New Interface for Purification of Proteins: 1D TiO_2 Nanotubes Decorated by Fe_3O_4 nanoparticles

Kupcik R., Rehulka P., Bilkova Z., Sopha H., and Macak J. M.

ACS Applied Materials & Interfaces 9 (2017), 28233–28242.

(IF in 2017: 8.097)

Difficulties associated with the structural analysis of proteins susceptible to form aggregates: The case of Tau protein as a biomarker of Alzheimer's disease

Hromadkova L., **Kupcik R.,** Jankovicova B., Rousar T., Ripova D. Bilkova Z.

Journal of Separation Science 39 (2016), 799–807.

(IF in 2016: 2.557)

Selective isolation of hydrophobin SC3 by solid-phase extraction with polytetrafluoroethylene microparticles and subsequent mass spectrometric analysis

Kupčík R., Zelená M., Řehulka P., Bílková Z., Česlová L.

Journal of Separation Science 39 (2016), 717–724.

(IF in 2016: 2.557)

Application of trypsin Fe_3O_4@SiO_2 core/shell nanoparticles for protein digestion

Slováková M., Sedlák M., Křížková B., **Kupčík R.,** Bulánek R., Korecká L., Drašar Č., Bílková Z.

Process Biochemistry 50 (2015), 2088–2098.

(IF in 2015: 2.529)

Manuscript submitted in a scientific journal peer-reviewed scientific journal without IF:

Amorphous TiO_2 Nanotubes as a Platform for Highly Selective Phosphopeptide Enrichment

Kupcik R., Macak J. M., Rehulkova H., Sopha H., Fabrik I., V.C. Anitha, Klimentova J., Murasova P., Bilkova Z., Rehulka P.

Submitted in ACS Omega, 28. 2. 2019. (resubmitted after major revisions)

Research articles published in peer-reviewed scientific journals without IF:

Benefits of Immunomagnetic Separation for Epitope Identification in Clinically Important Protein Antigens: A Case Study Using Ovalbumin, Carbonic Anhydrase I and Tau Protein.

Jankovicova B., Svobodova Z., Hromadkova L., **Kupcik R.,** Ripova D., Bilkova Z.

Universal Journal of Biomedical Engineering 3 (2015), 1–8.

Comparison of Monoclonal Antibodies Suitable for Immunomagnetic Purification of Native Tau Protein. Jankovičová B., Hromádková L., **Kupčík R.,** Kašparová J., Řípová D. and Bílková Z., *Scientific Papers of the University of Pardubice, Series A 20* (2014), 145–161.

Patent:

Patent CZ305599 – Method for separation of biopolymer molecules and a carrier for application of this method.

Applicant: University of Pardubice.

Inventors: **Kupčík R.,** Bílková Z., Řehulka P., Macák J.

CZ patent granted (18. 11. 2015) – issued in bulletin 52/2015. PCT application – Publ. no. WO2016015690 A1 (4. 2. 2016).

Patent application:

Method for preparation of iron oxide-based magnetic composite carrier for separation of biomolecules

Applicant: University of Pardubice.

Inventors: Macák M., Bílková Z., **Kupčík R.**

Applied as PV2018-43. Priority date: 29. 1. 2018. Application has been filed and is still pending.

Presentation at conferences

Oral presentations

Kupčík R., Macák J., Řehulka P., Bílková Z., New highly selective interface for phosphoproteome profiling, BIOSPOT 3rd Annual Conference 2018, 1. 3. 2018, Prague, Czech Republic.

Kupčík R., Macák J., Řehulka P., Sopha H., Bílková Z., TiO_2 Nanotubes Decorated by Fe_3O_4 Nanoparticles: A Tool to Selectively Isolate Biomolecules. Advances in Chromatography and Electrophoresis & Chiranal 2018, Olomouc, Czech Republic, 29. 1. – 1. 2. 2018; pp. 52 – 53

Kupčík R., Řehulka P., Stulík J., Bílková Z., Phosvitin as a standard protein for optimization of enrichment of multiply phosphorylated proteins. 7th Central and Eastern European Proteomics Conference on Proteomics Driven Discovery and Applications; Jena, Germany; 13. – 16. 10. 2013; pp. 47 – 48.

Kupcik R., Rehulka P., Jankovicova B., Svobodova Z., Hernychova L., Klimentova J., Stulik J. and Bilkova Z., Microchip isolation combined with MS-identification of phosphoproteins from Francisella tularensis. CECE Junior 2012, Brno, 31. 10. 2012; pp. 69 – 73, ISBN: 978-80-904959-1-3.

Poster presentations

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