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SIMPLIFIED SAMPLE HANDLING IN MASS SPECTROMETRY BASED PROTEIN RESEARCH –

Focus on Protein Phosphorylation

by

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TURUN YLIOPISTO
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Fall seven times, stand up eight.

-Japanese proverb-

Petri Kouvonen

Simplified sample handing in mass spectrometry based protein research - focus on protein phosphorylation

University of Turku, Faculty of Medicine, Department of Medical Biochemistry and Genetics and Turku Centre for Biotechnology, University of Turku and Åbo Akademi University

ABSTRACT

The human genome comprises roughly 20 000 protein coding genes. Proteins are the building material for cells and tissues, and proteins are functional compounds having an important role in many cellular responses, such as cell signalling.

In multicellular organisms such as humans, cells need to communicate with each other in order to maintain a normal function of the tissues within the body. This complex signalling between and within cells is transferred by proteins and their post-translational modifications, one of the most important being phosphorylation. The work presented here concerns the development and use of tools for phosphorylation analysis.

Mass spectrometers have become essential tools to study proteins and proteomes. In mass spectrometry oriented proteomics, proteins can be identified and their post-translational modifications can be studied. In this Ph.D. thesis the objectives were to improve the robustness of sample handling methods prior to mass spectrometry analysis for peptides and their phosphorylation status. The focus was to develop strategies that enable acquisition of more MS measurements per sample, higher quality MS spectra and simplified and rapid enrichment procedures for phosphopeptides. Furthermore, an objective was to apply these methods to characterize phosphorylation sites of phosphopeptides.

In these studies a new MALDI matrix was developed which allowed more homogenous, intense and durable signals to be acquired when compared to traditional CHCA matrix. This new matrix along with other matrices was subsequently used to develop a new method that combines multiple spectra from different matrises from identical peptides. With this approach it was possible to identify more phosphopeptides than with conventional LC/ESI-MS/MS methods, and to use 5 times less sample. Also, phosphopeptide affinity MALDI target was prepared to capture and immobilise phosphopeptides from a standard peptide mixture while maintaining their spatial orientation. In addition a new protocol utilizing commercially available conductive glass slides was developed that enabled fast and sensitive phosphopeptide purification. This protocol was applied to characterize the *in vivo* phosphorylation of a signalling protein, NFATc1. Evidence for 12 phosphorylation sites were found, and many of those were found in multiply phosphorylated peptides.

Keywords: Proteomics, mass spectrometry, MALDI, ESI, phosphopeptide purification, TiO_2, IMAC, *in situ* phosphopeptide purification

Petri Kouvonen

Pelkistetty näytteenkäsittely massaspektrometrikeskeisessä proteiinitutkimuksessa - fokus proteiinin fosforylaation tutkimisessa

Turun Yliopisto, Lääketieteellinen tiedekunta, Lääketieteellinen biokemia ja genetiikka ja Turun Biotekniikan keskus, Turun Yliopisto ja Åbo Akademi

TIIVISTELMÄ

Ihmisen genomi koostuu noin 20 000:sta proteiinia koodaavasta geenistä. Proteiinit ovat ihmiskehon toiminnallisia molekyylejä, jotka toimivat myös solujen ja niistä muodostuvien elinten rakennusmateriaaleina. Lisäksi niillä on erittäin merkittävä tehtävä solujen sisäisessä ja ulkoisessa tiedonvaihdossa, joka on ehdottoman välttämätöntä esim. immuunivasteen tehokkaalle toiminnalle.

Monisoluisessa organismissa solujen välinen kommunikaatio on tärkeää kudoksen normaalin toiminnan kannalta. Tämä monimutkainen signalointi solujen välillä ja sisällä välitetään proteiinien ja niiden translaation jälkeisten muokkausten eli modifikaatioiden avulla. Tärkein näistä modifikaatiosta on fosforylaatio, joka on myös yksi tutkituimmista modifikaatiosta eukaryoottisoluissa

Massaspektrometristä on tullut keskeinen työkalu proteiinien ja proteomien tutkimisessa. Massaspektrometrikeskeisessä proteomiikassa proteiinit voidaan tunnistaa ja niiden translaation jälkeisiä modifikaatioita voidaan tutkia. Tässä väitöskirjatyössä tavoitteena oli peptidi -ja fosforylaatioanalyysien näytekäsittelyn parantaminen ennen massaspektrometrianalyysiä. Tarkoitus oli keskittyä kehittämään strategioita, jotka mahdollistaisivat laadukkaan ja suuremman tietomäärän keräämisen näytteestä sekä yksinkertaistaa ja nopeuttaa fosfopeptidien rikastusta peptidiseoksesta. Lisäksi tarkoitus oli soveltaa näitä kehitettyjä metodeja fosfopeptidien karakterisointiin.

Tässä väitöskirjassa kehitettiin uusi MALDI-matriisi, joka mahdollistaa tasaisemman, intensiivisemmän sekä kestävämmän signaalin keräämisen massaspektrometrilla kuin mitä saavutetaan perinteisellä CHCA matriisilla. Tätä uutta matriisia käytettiin rinnan muiden perinteisten matriisien kanssa kehitettäessä menetelmää jossa yhdistetään saman peptidin spektrejä eri matriiseissa. Tällä menetelmällä voitiin havaita enemmän fosfopeptideitä kuin LC/ESI-MS/MS -menetelmällä käyttämällä vain 1/5 siitä näytemäärästä, joka vaadittiin LC/ESI-MS/MS -menetelmään. Tässä väitöskirjassa kehitettiin myös pinta, joka sitoo fosfopeptidejä. Tätä pintaa käytettiin ensin peptidistandardilla, jolloin rikastuspesun jälkeen peptidien avaruudellisen orientaation havaittiin säilyneen. Lisäksi kehitettiin nopeaan ja herkkään fosfopeptidirikastukseen uusi protokolla, jossa käytettiin kaupallista johtavaa lasilevyä. Tätä protokollaa käytettiin tutkimaan soluista puhdistettua fosforyloitunutta signaaliproteiinia, NFATc1:tä. Tässä karakterisoinnissa voitiin havaita merkkejä 12 fosforylaatiokohdasta, joista moni oli peptideissä, jotka sisälsivät useita eri fosforyhmiä.

Avainsanat: Proteomiikka, massaspektrometria, MALDI, ESI, fosfopeptidirikastus, TiO_2, IMAC, *in situ* fosfopeptidirikastus

CONTENTS

ABSTRACT **4**

TIIVISTELMÄ **5**

CONTENTS **6**

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **9**

1. INTRODUCTION **10**

2. REVIEW OF THE LITERATURE **11**

2.1. Mass spectrometers in proteomics studies 11
2.1.1. Ionisation methods for peptides 11
2.1.2. Protein identification 14
2.1.3. Sample preparation prior to MS analysis 16
2.1.4. LC-MALDI 17
2.1.5. Imaging Mass Spectrometry (IMS) 18
2.2. Protein phosphorylation 19
2.3. Phosphoprotein sample treatment 20
2.3.1. Phosphoprotein detection 20
2.3.2. Phosphopeptide purification methods 21
2.3.2.1. Chemical modifications 23
2.3.2.2. Ion exchange chromatography 23
2.3.2.3. Affinity chromatography 24
2.3.2.4. Combined phosphopeptide purification techniques 25
2.4. Phosphorylation: On-target purification 26
2.4.1. IMAC-based MALDI targets 27
2.4.2. Metal oxide affinity chromatography -based MALDI targets 28
2.4.3. Phosphopeptide fragmentation in MS 28

4. MATERIALS AND METHODS **31**

4.1. Publication (I) 31
4.1.1. Digestion 31
4.1.2. Matrix comparison 31
4.1.3. HPLC-MALDI 31
4.1.4. MS-analysis 32
4.1.5. Database search criteria 32
4.2. Publication (II) 32
4.2.1. TiO_2 coating 32
4.2.2. Thin film X-ray diffraction (XRD) measurement 32
4.2.3. Phosphopeptide enrichment from tryptic casein digests 32
4.2.4. Matrix coating 33
4.2.5. MALDI-based IMS 33
4.3. Publication (III) 33

4.3.1. Sample treatment 33
4.3.2. Mass spectrometry analysis and HPLC 33
4.3.3. Data analysis and database search 33
4.4. Publication (IV) 33
4.4.1. Chromatographic TiO_2-purification 33
4.4.2. Sample desalting 34
4.4.3. Optimized planar surface purification 34
4.4.4. PhosphorImager 34
4.4.5. MALDI-TOF (-TOF) analysis 34
4.4.6. Peak list generation, spectral processing and database search 34
4.4.7. Sensitivity test 34
4.5. Unpublished methods 35
4.5.1. On-tissue digestion 35

5. RESULTS AND DISCUSSION 36
5.1. Durable and sensitive MALDI-matrix for LC-MALDI (I) 36
5.1.1. Matrix comparison 36
5.1.2. Optimized matrix deposition 36
5.1.3. Durability of nitromatrix 37
5.2. Affinity imaging mass spectrometry, AIMS (II) 38
5.2.1. AIMS analysis of casein peptides 38
5.2.2. Trypsin digestion of the tissues 39
5.3. Phosphopeptide analysis without purification (III) 40
5.3.1. Phosphopeptide identifications 41
5.4. Phosphopeptide purification on planar surface (IV) 43
5.4.1. Optimization of sample loading and washing conditions 43
5.4.2. Sensitivity test 44
5.4.3. Application of the method to in vivo phosphorylated sample 44
5.5. Final remarks 45

6. SUMMARY 47

ACKNOWLEDGEMENTS 48

REFERENCES 50

ORIGINAL PUBLICATIONS 57

ABBREVIATIONS

AIMS	Affinity imaging mass spectrometry
ATP	Adenosine triphosphate
CID	Collision induced dissosiation
CRM	Charge residue model
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
IDA	Iminodiacetic acid
IEF	Isoelectric focusing
IEM	Ion evaporation model
IMAC	Immobilized metal affinity chromatography
IMS	Imaging mass spectrometry
ITO	Indium tin oxide
LC-MALDI	Liquid chromatography - MALDI
m/z	Mass-to-charge
MALDI	Matrix assisted laser desorption and ionization
MOAC	Metal oxide affinity chromatography
MS	Mass spectrometer
MS/MS	Tandem mass spectrometer
NTA	Nitrioacetic acid
PMF	Peptide mass fingerprint
PTM	Post-translational modification
RP	Reversed-phase
S/N	Signal-to-noise
SAM	Self-assembled monolayers
SAX	Strong anion exchange chromatography
SCX	Strong cation exchange chromatography
SELDI	Surface-enhanced laser desorption and ionization
SIMAC	Sequential elution from IMAC
SIMS	Secondary ion mass spectrometry
TFA	Trifluoroacetic acid

LIST OF ORIGINAL PUBLICATIONS

I. Kouvonen, Petri; McDonnell, Liam, A., Heeren, Ron. M.A., Corthals, Garry, L.: "Nitromatrix provides improved LC-MALDI signals and more protein identifications". *Proteomics. 2009, 9, 1662-1671.*

II. Imanishi, Susumu Y.; Kouvonen, Petri; Smatt, Jan-Henrik; Heikkila, Mikko; Peuhu, Emilia; Mikhailov, Andrey; Ritala, Mikko; Linden, Mika; Corthals, Garry L.; and Eriksson, John E.; "Phosphopeptide enrichment with stable spatial coordination on a titanium dioxide coated glass slide". *Rapid Commun Mass Spectrom. 2009; 23: 3661-3667.*

III. Kouvonen, Petri; Rainio, Eeva-Marja; Suni, Veronika; Koskinen, Päivi; Corthals, Garry, L.: " Data combination from multiple MALDI matrices: Opportunities and limitations for MALDI analysis" (*Rapid Commun Mass Spectrom. 2010; 24: 1-3, in press*)

IV. Kouvonen, Petri; Rainio, Eeva-Marja; Suni, Veronika; Koskinen, Päivi; Corthals, Garry, L.: "Enrichment and sequencing of femtomole amounts of phosphopeptides using indium tin oxide coated glass slides" (*Submitted*)

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1. INTRODUCTION

The human genome comprises roughly 20 000 protein coding genes (23739 consensus coding sequences representing 18173 genes, September 2, 2009)[1]. However, a single gene can encode multiple proteins by alternative splicing making the number of proteins far greater. Also, post-translational modifications (PTM) such as phosphorylation, glycosylation, ubiquitination and acetylation increase the number of distinct protein forms, thus magnifying the disproportion between the number of genes and proteins. For each type of PTM there is a related group of enzymes involved in protein modification processes. For example, for phosphorylation there are approximately 650 enzymes involved in the protein phosphorylation/dephosphorylation[2] process and for the ubiquitination/deubiquitination process approximately 700[3-4] meaning that 7 % of the entire genome is involved with only these two modifications, underlining the importance of PTM's.

Protein expression can be studied with many different techniques, but most often mass spectrometry (MS)[5-7] is used as it allows PTM definition in a relatively sensitive manner. The study of an organism/tissue/cell's proteome* is called proteomics[9]. MS-based proteomics is ubiquitously used life sciences and improves continuously through the independent development of several technologies, which include but are not limited to the following: soft macromolecular ionization techniques[10-14], gene and genome sequence databases, sophisticated computing and nano-flow liquid chromatography (nano-flow LC).

Protein phosphorylation is an important and therefore extensively studied PTM in eukaryotic cells. When a phospho-group is attached to a protein it can change protein's functional activity and thus contribute to signalling between and within cells. When the phospho-group is released, the proteins revert to their original state of activity. This reversible signalling is controlled by protein kinases[15] and phosphatases[16]. Any disruption in this mechanism disturbs normal cell and tissue homeostasis and might lead to diseases such as cancer[17-18].

In MS-based phosphoproteomics, phosphorylated proteins or peptides are usually isolated from the complex analyte mixture prior to MS analysis. This is due to the fact that phosphoproteins are usually of low abundance, making their detection extremely challenging. Highly specific purification methods have been developed to enable their selective enrichment thus allowing MS-based identification of thousands of phosphopeptides in a single study[19-21]. These identifications offer valuable information about *in vivo* phosphorylation of the proteins, which are identified and partially characterized. However, it is probable that many phosphoproteins remain undetected in these studies. One reason is because sample purification methods typically enrich the phosphoprotein fraction of the sample, but decrease the absolute amount of phosphopeptides in the sample. This will cause some of the phosphopeptides to fall below the detection limit.

The aim of the work described in this thesis was to develop different methods for phosphopeptide analysis. The intention was to minimize the sample treatment in order to speed up the throughput, minimize losses, contaminations and errors. The focus was on phosphoprotein characterization on single protein samples for targeted analysis.

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* proteome = expressed proteins at a given time in a sample (cell/tissue/organism/biofluid)[8]

2. REVIEW OF THE LITERATURE

2.1. Mass spectrometers in proteomics studies

A mass spectrometer (MS) is an instrument that is used to measure the intensities of charged species. They are accelerated into the analyzer in the gas phase where they are separated according to their mass to charge ratio, (*m/z*) and detected. The first mass spectrometers were developed in the beginning of the 20th century[22] but it was the development of the soft ionization techniques (electrospray, ESI[10-12] and matrix assisted laser desorption/ionization, MALDI[13-14]) in the late 1980's that made the analysis of larger biomolecules, such as proteins possible. These ionization techniques are described as "soft" because they allow large molecules to be transferred to the gas-phase and ionized without significant fragmentation.

Mass spectrometers are extensively used in science. The following sections only cover the type of mass spectrometers and applications that are relevant for this thesis.

2.1.1. Ionisation methods for peptides

Mass spectrometers are capable of measuring only charged species (which will be referred as ions from here on); the ionization process is an extremely important step in the sample analysis workflow as it determines which molecules can be analyzed with the mass spectrometer. Two soft ionization techniques (ESI[10-12] and MALDI[13-14]) developed in late 1980's enable the analysis of large biological molecules, such as proteins and peptides by MS without significant fragmentation (shared Nobel Prize in Chemistry, 2002, http://nobelprize.org).

To perform ESI the sample is dissolved in a liquid that flows through an open tubular capillary or needle that is held at high potential (1-3 kV) immediately in front of a mass spectrometer, thus creating charged droplets (Figure 1). When the capillary is held in

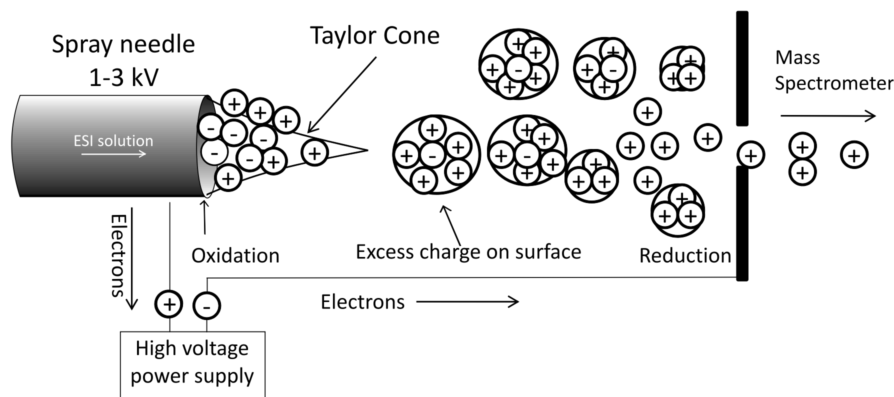


Figure 1: The illustration of the ESI process. The sample is pumped through the open capillary to which a high voltage is applied. A Taylor cone is formed when charges start to move towards the counter electrode (mass spectrometer). When the Rayleigh limit has reached droplets detach from the Taylor cone. When droplets move towards the mass spectrometer, ions are freed into the gas phase either entirely by evaporation of the solvent or by effects combined from evaporation of the solvent and coulomb fission.

positive potential it acts as a positive electrode and electrochemical oxidation reaction occurs.[23] The potential is directed to the liquid through metal junction or through conductive needle tip. This oxidation reaction supplies positive metal ions into the population of the other ions, such as analyte ions already present in the solution. When the capillary is set at positive potential the positive ions in the liquid move towards the counter electrode (mass spectrometer) and accumulate at the surface of the liquid at the tip. Liquid protrudes from the capillary tip forming a "Taylor cone[24]". When the point at which the repulsion of the same charges exceeds the surface tension of the solvent (Rayleigh limit[25]) droplets detach from the tip of the Taylor cone. As the droplets move towards the mass spectrometer, ions are freed into the gas phase. Two of the most accepted mechanisms for freeing ions into the gas phase are charged residue model (CRM)[26] and ion evaporation model (IEM)[26-28]. The CRM suggests that after a sequence of Rayleigh instabilities (Coulomb fission) together with solvent evaporation a droplet with only one molecule remains. That molecule is freed into the gas phase when the rest of the solvent evaporates. The IEM supports the same mechanism as CRM, differing only in the last step of the mechanism. Instead of solvent evaporation, IEM suggests that the ions are emitted into the gas phase from the small droplets[29]. Regardless of the mechanism the outcome is that the ions are transferred into the gas phase and the ions can be analyzed with mass spectrometer[30].

In MALDI, a two-step ionization mechanism is the most accepted model of ionization. First, the primary ions are formed after which ion-molecule reactions give rise to secondary ions. The cluster model (Figure 2) supports the suggestion where the matrix is mainly a medium in the desorption / ablation reaction.[31] According to this model the

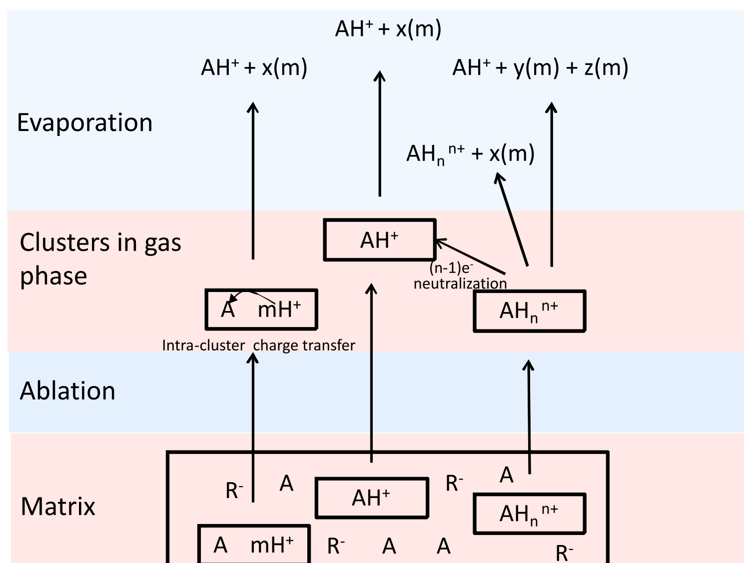


Figure 2: After the ablation in the "cluster model" of MALDI ionization, some clusters contain a net excess of positive charge(s), others a net negative excess (not shown). If the analyte is already charged, evaporation may free the ion (middle path). Charge might also need to migrate from the matrix to the analyte (left path). Additionally, during the evaporation, different free ions are formed from multiply charged analytes through neutralization of counter ions and electrons. A = analyte, m = matrix, R- = counter ion.

analytes are incorporated into the matrix crystals thus forming clusters. Their charge state is determined by the pH of the solution in which they have been deposited onto the MALDI target. During the laser irradiation the clusters are desorbed and the charged analyte is freed into the gas phase through sublimation of neutral matrix. In addition, some intra-cluster charge transfer and neutralization takes place. In the pooling model[32], described in Figure 3, a more active role is proposed for the matrix in ionizing the analyte. This model explains the pooling of the energy in the matrix molecules. Here the laser pulse excites two matrix molecules that concentrate energy more to one of the two molecules. This combined excitation is enough to transfer the matrix molecules' excitation stage to a higher level and ultimately produces matrix ions. Analyte ions are formed then from the primary matrix ions either by A) a proton, B) an electron or C) a cation transfer, producing singly charged analyte ions.

A) Proton transfer: $mH^+ + A \leftrightarrow m + AH^+$ and $(m\text{-}H)^- + A \leftrightarrow m + (A\text{-}H)^-$

B) Electron transfer: $m^+ + A \leftrightarrow m + A^+$ and $m^- + A \leftrightarrow m + A^-$

C) Cation transfer: e.g. $mNa^+ + A \leftrightarrow m + ANa^+$
m= matrix, A=analyte

ESI and MALDI have their own characteristics. MALDI ionization produces predominantly single charged ions, whereas ESI produces multiply charged ions[33]. Peptides carrying multiple charges can be more easily fragmented than singly charged peptides which require more energy for fragmentation[34]. The ESI and MALDI techniques favour the ionization of ions with different and at times exclusive efficiency, making these techniques somewhat complementary[35-36]. In addition, MALDI is more tolerant to salts and other contaminants from the sample handling[5].

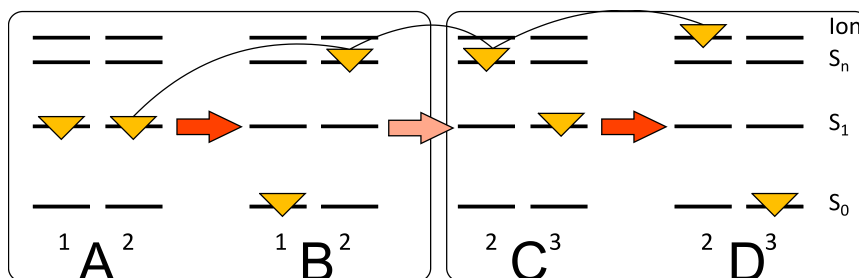


Figure 3: S1-S1 pooling (A and B) takes place when the laser excites matrix molecules by one photon energy and two neighbouring molecules (1 and 2) can transfer the energy entirely to another molecule exciting the third molecule from S1 to Sn. (S1-Sn) pooling (C and D) takes place when a molecule from S1-S1 pooling (2C) receives one photon energy from the another molecule (3C) thus forming a matrix ion (2D). After this primary ionization the secondary ionization of the analyte takes place through photon, electron or cation transfer (see text). S0 = electronic ground state, S1 = the first excited state, Sn = higher exited state, twice the energy of the S1, 1 and 3 = matrix molecules which donates the energy, 2 = matrix molecule which receives the energy

2.1.2. Protein identification

Proteins can be identified with a mass spectrometer using two different approaches. One approach is to analyze intact proteins (top-down approach) where a proteins mass can be determined and also protein sequence information is obtained due to protein fragmentation in the mass spectrometer[37]. Recently, the top-down approach has been used both with ESI[38] or MALDI[39] instruments to characterize proteins. These two studies were able to perform detailed characterization of the amino acid composition (100 % sequence coverage) of 15.2 kDa[38] and 13.6 kDa[39] proteins using top-down approach showing the power of this technique. Although this technique is beginning to become used more widely, the most common technique for protein characterization is based on peptides derived from proteins (aka, the bottom-up strategy). To facilitate identification proteins are usually digested into peptides using endoprotease specific enzymes or chemical reagents (Table 1). The different proteases produce different peptides, therefore their combined use has been reported in several studies enabling more complete protein or proteome characterization[40]. Identification and characterization is performed by correlating the peptide mass information (peptide mass fingerprint, PMF[41-45]) and peptide fragmentation information[46-47] with sequence databases[48], which has become the *de facto* method for protein identification nowadays. Several algorithms and software have been developed to match this information for protein identification. [43, 48-51] For MALDI based identification, PMF information is still complemented with peptide fragment information for more confident protein identification. The different types of spectra are generated in tandem MS (MS/MS) instruments, where first the peptide *m/z* is measured and a mass spectrum is generated, and secondly a peptide is selected for fragmentation and the *m/z* of the generated fragments are recorded in the second mass spectrum. During low energy dissociation, peptides are fragmented mainly along the peptide backbone generating specific fragment ions (Figure 4). The nomenclature for peptide fragments was first proposed by Roepstorff and Fohlmann[63] and later modified by Johnson *et al*.[64] The nomenclature divides fragments either deriving from N-terminus (a, b and c-ions) or from C-terminus (x, y and z-ions).

Several theories exist that describe the fragmentation process. One of the theories proposes that peptide fragmentation is mediated through "mobile proton"[65]. In this model any of the amide bonds in the peptide backbone can be protonated by the mobile proton making it more susceptible to fragmentation. Energy for this cleavage comes from collisions of the peptides with the inert gas molecules in the collision cell, and is referred to as collision induced dissociation (CID). Additionally, protons can be 'trapped' by basic amino acids preventing the proton from moving and as a result makes fragmentation more difficult. Therefore, during ESI, doubly charged peptides are more easily fragmented than singly charged peptides: If a first proton is trapped by a basic amino acid (in tryptic peptides C-terminus is basic arginine or lysine) the second proton is free to move across the peptide backbone to induce fragmentation. When the peptide backbone is fragmented in two positions, internal fragments are formed. Internal fragment with a single amino acid is called an immonium ion and they possess a characteristic mass[66] (see chapter 2.4.3).

Table 1: Proteolytic reagents used to fragment proteins. One-letter abbreviations for the amino acids are used with X denoting any amino acid. References are made to the publications utilizing these reagents (52-56 and 59-61) and to the publications first describing the enzyme (57-58).

	Cleavage site	Exception	Reference
A.Proteases			
Arg-C	RX		52
	Some KX	Some RX	
AspN	DX X-cysteic acid		53
	Some XE	-	
Chymotrypsin	FX, YX, WX, LX		54
	Some MX, IX, SX, TX, VX, HX, GX, AX.	XP	
Glu-C	EX, DX in phosphate buffers EX ammonium bicarbonate buffers	XP	55
Lys-C	KX		56
	Some NX		
Protease (V8)	EX		57-58
Trypsin	KX, RX	XP	59
B.Chemical reagents			
Cyanogen bromide	X-M		60
Hydoroxyalamine	N-G		61

Table has been modified from Corthals G.L. *et al.*[62]

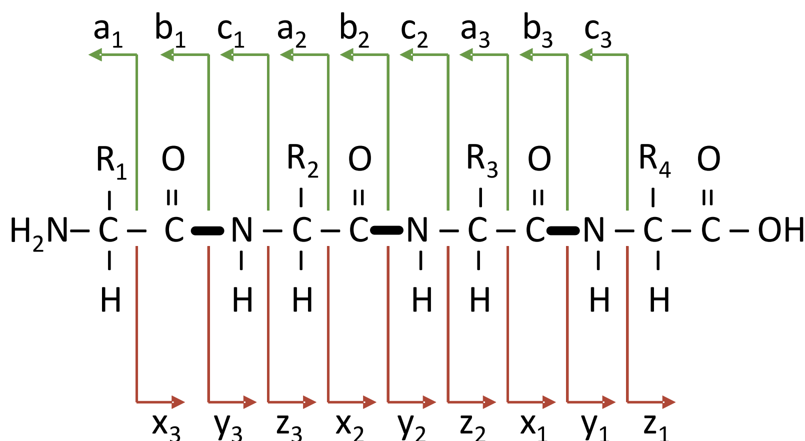


Figure 4: Peptide fragmentation nomenclature according to Roepstorff and Fohlmann. Green arrows indicate fragment ions deriving from N-terminus (a, b and c-ions) and red arrows show peptide fragments from C-terminus. Thickened lines between C and N represent peptide bonds.

Sometimes the protein sequence in question is not in the database, because the appropriate database is not available or the protein contains unpredicted modifications or mutations in its amino-acid sequence. In these cases protein identification using database search is not possible and *de novo* sequencing has to be conducted. *De novo* sequencing refers to manual interpretation of the spectrum[67], even though some specially designed software tools are beginning to merge[68].

2.1.3. Sample preparation prior to MS analysis

Due to the wide concentration range of proteins in many biological samples and different ionization efficiencies of peptides, many proteins will remain undetected when analyzed with a mass spectrometer. The peptides present in the sample are under sampled because most automated MS/MS analyses select the most intense peptides for fragmentation. In addition, many more peptides will not be analyzed during the time when the mass spectrometer is switching between MS and MS/MS mode (duty cycle). Here, some of the techniques that try to address under sampling will be discussed.

There are a number of different steps that can be undertaken during sample preparation, before a protein is analysed by MS, and these steps can increase the number of protein identifications. Depending on the sample origin and the biological questions, these might include subcellular fractionation, protein fractionation, protein digestion, peptide fractionation and desalting. Often additional handling steps are required when post-translational modifications (PTM) such as protein phosphorylation are under investigation. Then, the sample containing phospho-proteins or -peptides are subjected to additional purification step(s) to selectively enrich phosphorylated proteins or peptides (discussed more detailed in section 2.3.).

After protein extraction, further sample treatment is often required to reduce the sample complexity prior to MS analysis. For MALDI based work where a protein centred approach is adopted the separation occurs at the protein level after which the peptides are generated and analyzed by MS (Figure 5). The most common example is two-

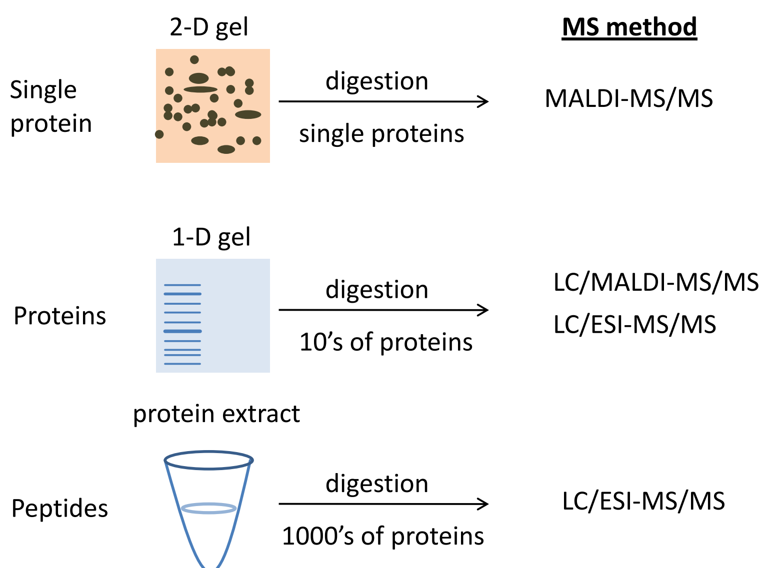


Figure 5: In protein centric approaches, proteins are separated prior to digestion and MS/MS. Samples derived from 2-DE are usually analyzed by MALDI-MS/MS. With the use of 1-DE further separation of peptides is achieved by LC prior MALDI-MS/MS or ESI-MS/MS. In the peptide centric approach the sample is digested into peptides without prior separation at the protein level. Here the peptides are further separated by LC-(LC) prior to ESI-MS/MS

dimensional gel electrophoresis (2-DE) where proteins are first separated according to their isoelectric point, and secondly by separation according to their size[69-70]. With this approach a representation of the sample is visualized as a distribution of spots. The spots containing proteins can be subjected to enzymatic digestion and MS analysed[71]. Running the gel only in one dimension, separating the proteins solely according to their mass (SDS-PAGE)[72] will reduce complexity, but requires additional peptide separation techniques to reduce the complexity of the peptide sample analyzed by MS. Therefore, samples derived by 1-DE are usually analyzed with a MS coupled to nanoflow-HPLC system. Following digestion a LC system is used to separate the peptides, after which they will be introduced into the MS for MS/MS. For LC-MS characterization, peptide separation is mostly performed using reverse phase (RP) chromatography. Increasing concentration of the organic solvent has the effect that peptides sequentially elute with increasing hydrophobicity. In the peptide centred approach the entire sample is digested into the peptides and separated into fractions in the first dimension either by chromatography or IEF and then each fraction is introduced into the MS through a nanoflow-HPLC system.

The purpose of these treatments is to make the sample less complex in order to increase proteome coverage. However, when dividing the sample into multiple fractions excessive sample treatment is likely to introduce artefacts, both systematic and human errors. The Association for Biomolecular resource facilities (ABRF, www.abrf.org) web page lists 353 post-translational modifications (PTM's) in proteins and Unimod (Protein modifications for mass spectrometry, http://www.unimod.org/modifications_list.php) in turn lists 636 protein modifications. Subtracting PTM's and all the intentional modifications, such as isobaric labels from the 636 modifications, we still end up with hundreds of modifications. For unattended modifications, artefacts are mostly caused by sample handling and therefore are not desired nor expected. Without prior knowledge of these artificial modifications protein identification can become more difficult or even impossible.

2.1.4. LC-MALDI

For the analysis of complex proteome samples liquid chromatography is usually incorporated into the sample treatment pipeline[5-6]. Sample injection and analysis is made efficient using automated sample injection, column switching unit and LC than off-line analysis (see below). The final, peptide separation technique is generally reversed phase chromatography due to its solvent compatibility with mass spectrometers (organic solvent, no salt). This connection can be either direct into an ESI-based mass spectrometer *i.e.* on-line, or the chromatographic fraction can be collected for later analysis (offline). On-line analysis is always conducted with ESI and off-line analysis with MALDI. The major difference in these two methods is that with the on-line workflow the sample is consumed in the time frame of the LC-MS separation/analysis whereas in off-line analysis the sample is partially retained and can be reanalyzed.

With the LC-MALDI workflow, the peptides are separated in the RP column and the LC fractions are spotted on to a MALDI target with a MALDI matrix. Without the duty cycle limitations (see first paragraph 2.1.3) this allows for time independent analysis of the

sample. After the database search all the non-identified peptides can be re-analyzed from the same sample. Also, an exclusion list, which comprises from previously identified peptides, can be generated from the same sample to avoid redundant peptide analysis, thus potentially yielding more information from the sample when reanalyzed. With ESI the sample is injected in to the instrument and cannot be recovered so any reanalysis would require new sample.

Replicate LC-MALDI analyses can be performed to analyze highly complex samples[73-74]. Replicate analysis using exclusion lists to avoid redundant protein identifications in sequential MS analysis was successfully used by Chen *et al.*[75] They analyzed *Escherichia coli* lysate and showed a 20% increase in unique protein identification by excluding the confident identifications from the previous run. Without the exclusion strategy the same 20% increase in protein identification was only achieved after five repetitions. These consecutive analyses were made from five sequential LC runs. Ideally, however, these repeated analyses would be made from the same LC run avoiding the need of specific alignment tools. Alignment of the retention times is needed for exclusion list generation.

The use of a matrix which would allow multiple analysis of the same sample could circumvent the need of sequential LC runs like described in previous paragraph. In MALDI analysis laser ablation consumes the sample-matrix crystals from the target. This happens particularly when automated data acquisition is used and laser power settings have been preset and are not controlled for individual peptides. For some MALDI instruments, when acquiring MS/MS, the laser power is set slightly higher than when for MS, because peptide fragmentation requires more energy to produce good quality MS/MS than MS from intact peptides.

In publication I, a matrix was developed for which the peptides could be ionized with less laser power than other tested matrices in the study. Additionally, the derived signal was homogeneous, generating even peptide intensities throughout the sample. These features in combination with physical properties of the matrix allowed ten repeat MS analyses from the same LC eluent spotted onto a MALDI target. Results from this study are discussed in more detail in section 5.1.

2.1.5. Imaging Mass Spectrometry (IMS)

Imaging Mass spectrometry (IMS) is a technique where spatial origin of the signal can be retained and overlaid with the microscopic image. Preliminary work for imaging by mass spectrometry was conducted by Slodzian and Castaing in the 50's and early 60's[76] utilizing secondary ion emission[77]. Initial applications were on mineral samples and the first time biological samples were analyzed was on the 70's[76, 78-79].

At present, the most commonly used techniques for IMS for tissue samples are MALDI and secondary ion mass spectrometry (SIMS)[80]. Due to their different ionization mechanisms they can be used to analyze different molecules. MALDI is used to record the signals from intact proteins and peptides whereas secondary ion mass spectrometry (SIMS) is used for smaller molecules, such as lipids and vitamins[81]. MALDI can be used to analyze high mass molecules with 25µm or more spatial resolution whereas SIMS offers sub-micron resolution for analytes below 1000 m/z[81]

IMS-technology was used in our study (II) where the locality of the phosphopeptides was visualized after purification wash. Results from this study are discussed in more detail in section 5.2.

2.2. Protein phosphorylation

Protein phosphorylation is an important post-translational modification that is involved in regulating many cellular processes in eukaryotic cells (phosphorylation may also be found in prokaryotes[82-83]). Pioneering work in this field was done by Edmond Fisher and Edwin Krebs who were the first ones to describe the reversible protein phosphorylation[84]. For their contribution to this field they shared the Nobel Prize in Physiology or Medicine 1992 “for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism”[85].

Protein phosphorylation is reversibly controlled by protein kinases[15] and phosphatases[16], which means that proteins revert to their original state of (in)activity (Figure 6). In a multicellular organism cells need to communicate with each other to maintain the normal function of the tissue. This complex signalling between and within the cells is performed by proteins and regulated by post-translational modification, such as phosphorylation. Phosphorylation of a protein changes a protein’s folding and functional properties *e.g.* enzymatic activities[86] . Protein phosphorylation is involved in many functions and so proteins can be phosphorylated in many different ways. Four different classes of phosphorylation occur (O-, N-, S- and acyl-phosphorylation) in several different amino acids (serine, threonine, tyrosine, arginine, histidine, lysine, cysteine, aspartate

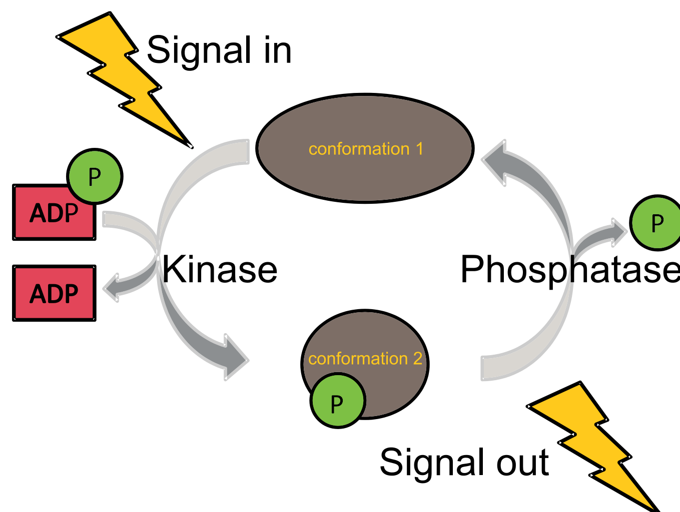


Figure 6: Protein phosphorylation is triggered by a stimulus inside or outside the cell. The protein kinase enzyme transfers the phospho-group from adenosine triphosphate (ADP+P = ATP) to the protein. Attaching the phospho-group to the protein will change its conformation and thus changes its activity. Usually, the protein is inactive without phosphorylation and will be activated by addition of the phospho-group. The protein reverses its conformation using a protein phosphatase, which de-phosphorylates the protein.

and glutamate)[87-88]. The most typical and the most studied phosphates in eukaryotes are O-phosphates on serine, threonine and tyrosine (pSer, pThr and pTyr) residues. N-phosphates attach mostly to histidine and lysine, S-phosphates to cysteine and acyl-phosphatases to aspartic and glutamic acid residues.

Analysis of phosphoproteins is considered technically difficult due to the following reasons. Many phosphoproteins have relatively low concentration and stoichiometry.[89] In addition, the phosphorylation-dephosphorylation cycle can be extremely fast. It has been reported that there are more than 100 000 phosphorylation sites in the human proteome[90] controlled by about 500 kinases and 100 phosphatases[2]. Abnormalities in this complex (de)phosphorylation process have been shown to be related to many diseases, including cancer[17-18] and diabetes[91]. Identifying phosphorylation sites related to these and other diseases might help to understand the origin, progression and hopefully the termination of these diseases.

Due to the reasons mentioned above (low copy number and stoichiometry, transient nature) the analysis of phosphoproteins and the resulting phospho-peptides is difficult. The confirmation of phosphoprotein identification is challenging since the analysis is usually based on peptides. Consequently, sample purification has become an essential step (Figure 7) for the characterization of protein phosphorylation events, as it offers a reduction in complexity of the sample and thus tries to address problems described above. In addition, there is a pressing interest for validation of identified phosphopeptides since the antibodies to their parent proteins have known limitations of cross reactivity and availability. Also, the epitopes are often not known so one does not know which part of the phosphoprotein one is characterizing. Due to recent advances in phosphopeptide purification and improved MS capabilities, thousands of phosphopeptides have been reported from single studies[19-21]. Some of the most commonly used purification methods are discussed below.

2.3. Phosphoprotein sample treatment

The essential part of sample preparation for phosphoprotein analysis from intact cells is to limit enzymatic activity following cell lysis, in particular phosphatase and kinase activity. Cell lysis frees all the phosphatases and the proteases from normal cellular regulation and thus affecting phosphoproteins stoichiometry and number (proteases dephosphorylates and proteases digest low copy number signalling proteins). Inadvertent activities can be inhibited by adding a cocktail of phosphatase and protease inhibitors. Microwaves[92] and rapid heating[93] of samples have been used for inactivating kinases and phosphatases.

2.3.1. Phosphoprotein detection

In 2-DE phosphoproteins can appear as a line of spots since the attached phospho group changes the proteins' pI[94] and alters its mass by 80 Da per added phospho group. The result is that a shift in pI is usually detected, but the mass change is often not noticed. The addition of phospho-groups to a protein will shift the pI to become more acidic, but the total effect on the pI will depend on all modifications and protein sequence itself[95].

Also, specific stains directed towards the phospho-group itself can be used to detect phophoproteins[96]. Ideally only proteins carrying phospho groups would be visible in the gel after the staining.

Phosphoproteins can also be detected by radioisotope[97]. Radioisotope labelling of the proteins can be done using commercially available radioactive isotopes such as ^{32}P and ^{33}P. Higher emitting energy and lower cost could be reasons why ^{32}P is more often used in life-sciences for protein labelling than ^{33}P. The radioactivity of the sample can be measured using liquid scintillation counting[98]. Due to differences in the emitting energies ^{32}P can be measured directly using Cerenkov counting[99] where as ^{33}P requires a fluorescent compound to be in the solvent to mediate the energy as emitting light.

In vivo, radioactive ortho-phosphate can be added to a cell culture, the endogenous kinases of the cells will incorporate radioactive phosphate groups in the phosphoproteins. With *in vitro* phosphorylation experiments the purified proteins and ATP in which the gamma-phosphate is radioactive, are incubated with specific kinase(s) that incorporate the radioactive phosphate to its specific amino acid. Even though the method can detect the phosphorylation in the cell the method does not give any information about the locality of the phosphorylation in the protein.

Immuno-blotting is another way of visualizing phosphoproteins[100]. In this technique phosphospecific antibodies are used to recognize pSer, pThr and pTyr residues. The specificity and the sensitivity of this technique is heavily dependent on the antibodies used[101]. In this method the sample is separated by 1-DE or 2-DE, after which the proteins are transferred to a protein binding membrane (nitrocellulose, Teflon or Polyvinlidene Fluoride). After blocking the membrane from unspecific binding the primary antibody is introduced. Then, the unbound primary antibody is washed and the secondary antibody with a reporter enzyme is incubated with the membrane. Usually, the luminescence is measured from the secondary antibody which displays the locality and the quantity of the phosphoproteins from the gel[102]. This method offers quick and relatively inexpensive way of detecting and quantifying phosphoproteins. However, the disadvantages are the requirement of specific antibody, which might not offer enough specificity for reliable detection. This same principle can be used also to selectively isolate phosphoproteins and peptides[103] .

2.3.2. **Phosphopeptide purification methods**

Due to the complexity of the biological questions as well as the diverse array of available MS techniques, numerous different phosphopeptide purification methods have been described[104-114]**.** The need to purify the sample from non-phosphorylated peptides has already been discussed. In addition to previously mentioned reasons, an ion suppression effect has also been suggested as a reason for low ionization efficiency of phosphopeptides in the presence of higher abundant non-phosphorylated peptides[108, 115-116] . However, Steen *et al.*[89] have argued that there was no obvious loss in phosphopeptide signal intensity in the presence of a large excess of nonphosphorylated peptides. They tested this by mixing 1000-fold excess of tryptic BSA digest with peptide/phosphopeptide pairs thus generating saturated ionization conditions. In these conditions the total number of charges is limited thus forcing the analytes to compete

for the available charges. No selective suppression of phosphopeptides was observed as compared to their non-phosphorylated counterparts. However, the authors conclude that all peptides with low concentration (*i.e.* phosphopeptides) will suffer from unspecific suppression under saturated ionization conditions. Therefore prefractionation and phosphopeptide purification are necessary when analyzing complex sampled. The most commonly used prefractionation and phosphopeptide purification methods are chemical modifications, ion exchange chromatography, affinity chromatography and combinations of these (Figure 7). These techniques will be described in the following sections.

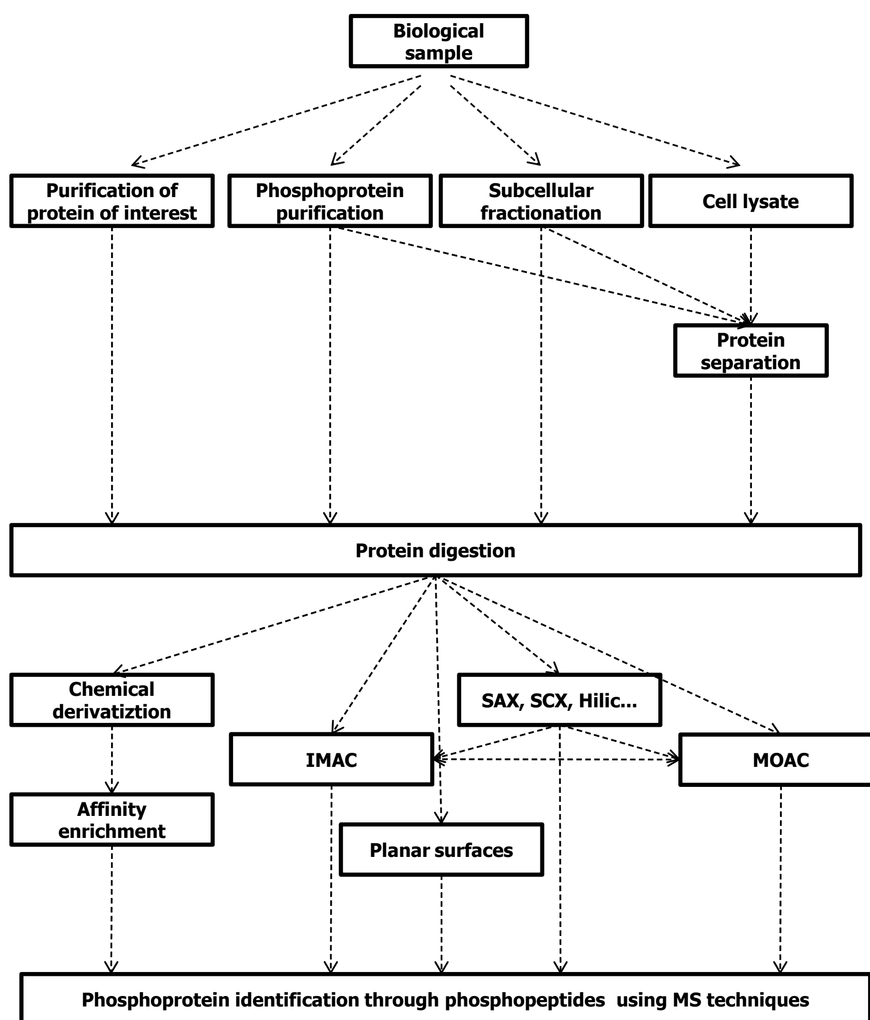


Figure 7: Commonly used pre-fractionation strategies for phosphoprotein containing samples. SAX = strong anion exchange chromatography; SCX= strong cation exchange chromatography; MOAC = metal oxide affinity chromatography; IMAC = immobilized metal affinity chromatography; Hilic= hydrophilic interaction liquid chromatography. Chart modified from Pinkse and Heck (2006)112

2.3.2.1. Chemical modifications

Chemically modified phosphopeptide/-protein purification based on β-elimination was first published by Oda *et al.* [104]. They used the labile nature of phosphate ester bonds to their advantage by substituting the phosphate with a biotin tag. Under strongly alkaline conditions the phosphate group undergoes β-elimination exposing a reactive dehydroanalyl residue[117]. The biotin affinity tag is then coupled to the residue with a nucleophile ethanedithiol. Peptides possessing a biotin tag can then be specifically purified using avidin affinity chromatography. This reaction works only for pSer and pThr since they undergo β-elimination whereas pTyr does not, owing to its aromatic ring[118] .

Another derivatization method was developed by Zhou *et al.*[105]. In this method cystamine is added to the phospho-group enabling phosphopeptide purification by immobilized iodoacetyl groups. After washing away non-phosphopeptides, the phosphopeptides can be eluted by cleaving the phosphoramidate bonds with TFA. The advantage of this method is that it works for pSer, pThr, and pTyr-containing peptides. For this method there have been two follow-up studies[119-120] where the method has been further optimized for better yield. The optimized method was used in parallel with two other enrichment methods[121] (immobilized metal affinity chromatography, IMAC and titanium dioxide, TiO_2. See below). Each method isolated different, but partly overlapping phosphopeptides with similar specificity. However, the chemical modification requires many additional sample handling steps which make this method extremely laborious. The higher risk of introducing sample-handling artefacts using cystamine is the principal reason why IMAC and TiO_2 are now the methods of choice.

2.3.2.2. Ion exchange chromatography

The separation of phosphopeptides according to their charge state was first introduced by Gygi and co-workers 2004[106]. They used strong cation exchange (SCX) chromatography to separate phosphopeptides from the nuclear fraction of HeLa cell lysate. They reported 2002 phosphorylation sites deriving from 967 proteins, the largest dataset of phosphorylation sites at that time. Separation of peptides by SCX chromatography is accomplished on the basis of peptide charge. At low pH tryptic peptides carry two positive charges (at the amino-terminus and C-terminal lysine or arginine). A phosphate group maintains its negative charge at low pH giving the phosphopeptide a net charge of +1 (Figure 8). Due to an extra negative charge, phosphopeptides have weaker interactions with an SCX column and therefore elute in earlier fractions than non-phosphorylated peptides.

In strong anion exchange (SAX) chromatography the separation of the analytes is based on net negative charges. Since the phosphopeptides carry a negative charge in the phospho-group they have stronger retention on SAX than non-phosphorylated counterparts and thus will elute later from the SAX column. This approach was investigated by Han *et al.* [107] in their study of human liver cancer. From the non cancerous liver tissue they reported 274 unique phosphorylation sites. They compared their study with the SCX study of Gygi's group [106] on the basis of the ratio of MS^3 to MS^2 experiments. This ratio represents the efficiency of the purification since MS^3 is automatically triggered by the neutral

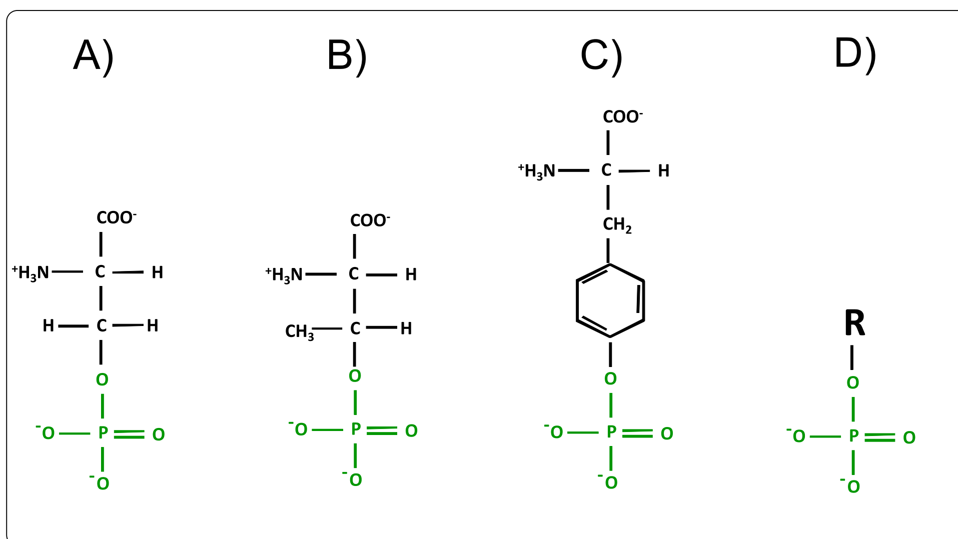


Figure 8: A) phosphoserine; B) phosphothreonine, C) phosphotyrosine and D) inorganic phosphogroup. Phospo-amino acids are formed by addition of phosphate group to an amino acid residue by replacing the hydrogen from the amino acid or OH -group in the case of tyrosine. pKa1 for phosphoric acid is 2.12. However, it has been reported that in organic environment pKa1 for phosphoric acid is as low as 1.1 (Saha, et al.).

loss of the phosphate group from phosphopeptides. The ratios were 4.41% for Han *et al.* [107] and 2.29% for Beausoleil *et al.*[106]. From this finding the authors concluded that SAX was more selective than SCX as a phosphopeptide purification method. However, a comparison using these parameters is difficult due to the different criteria in the MS-methods for triggering the MS^3-event.

2.3.2.3. Affinity chromatography

Immobilized metal affinity chromatography (IMAC) was first introduced by Porath *et al.*[122] for purification of His-tagged proteins. Currently it is the most frequently used method for phosphopeptide enrichment (PubMed, http://www.ncbi.nlm.nih.gov/pubmed). In IMAC the retention of phosphopeptides is based on interactions between the metal ion and the phosphate group. The metal ions (Fe^{3+}, GA^{3+}, Zr^{4+} or Al^{3+})[123] are bound to a column by iminodiacetic acid (IDA) or nitrioacetic acid (NTA) linkers [124]. The sample loading conditions for IMAC are acidic to reduce nonspecific binding from non-phosphorylated peptides. However, peptides containing acidic residues (glutamic acid and aspartic acid) are also known to bind to the IMAC resin. This problem has been circumvented by converting the carboxyl groups of the amino acids residues to methyl esters [125] or by using glu-C as an enzyme to digest the peptides [126]. Glu-C digests the protein at the C-terminus of glutamic and aspartic acid, leaving only one acidic amino acid per peptide and thus reducing its acidity.

Metal oxide affinity chromatography (MOAC) is based on the same chemistry as IMAC. However, the difference is that in IMAC the metal ions are bound to a substrate to form a column whereas in MOAC the column is formed directly from metal oxide particles. This

makes MOAC columns more resistant to the highly acidic loading solution and highly basic elution solution, without the risk of leaching the metal ions from the column[127]. These physical properties enable phosphopeptides to be purified with high selectivity and without the need of methyl esterification.

Using TiO_2 is arguably the most popular MOAC-method for purifying phosphopeptides. At a low pH TiO_2 is positively charged (pKa for TiO_2 = 4.4)[128] and amino acid residues are protonated, preventing them from binding to TiO_2. Phosphate groups attached to a peptide will remain in the dissociated form (negatively charged) due to the strong acidic nature of the phosphate group making conditions favourable for phosphopeptide binding to TiO_2. A high pH solution is used to elute bound phosphopeptides from TiO_2: the high pH changes the charge of TiO_2 from positive to negative, releasing the bound phosphopeptides. After its discovery as a useful packing material for HPLC -column in 1989 [129-130] and its ability to capture organic phosphates, about one year later [131], it took several years to develop the first automated LC systems for phosphopeptide purification, for both off-line MS analysis[132-133] and hyphenated LC-MS[20].

Other metal oxides have also been reported to have an affinity to phospho-group. Zirconium dioxide (ZrO_2)[134] has drawn attention as an alternative metal oxide for TiO_2 due to its similar phosphopeptide purification performance[135]. Also the use of aluminium hydroxide ($Al(OH)_3$), niobium oxide (Nb_2O_5), and tin dioxide (SnO_2) [136] have been reported.

2.3.2.4. Combined phosphopeptide purification techniques

The comparison of different phosphopeptide purification methods has given different results quantitatively and in qualitatively[105, 121]. A comparison of the phosphopeptides identified using the different purification methods has revealed that only small fractions of their phosphopetides overlap. However, the complementarity might not be from the success, but rather from the lack of it. Due to poor efficiency of the methods there is little overlap, which results in this complementarity. Some groups have been able to exploit these deficiencies in enrichment to our benefit.

The method termed sequential elution from IMAC (SIMAC)[109] takes advantage of the different affinities of singly and multiply phosphorylated peptides to further fractionate the phosphopeptides from a complex sample. The method uses IMAC - and TiO_2-purification in sequence. After loading the sample onto the IMAC column at low pH, the flow-through from the loading and from the acidic washing are collected. This fraction contains non-phosphorylated peptides and some singly phosphorylated peptides. Non-phosphorylated peptides are protonated and do not bind to the positively charged IMAC column. Singly phosphorylated peptides have less affinity towards the IMAC column than multiply phosphorylated peptides due to fewer negative charges in the peptide (phospho-group). This flow-through fraction is subjected to a TiO_2-purification to capture the singly phosphorylated peptides. The multiply phosphorylated peptides are eluted from the IMAC column under alkaline conditions.

In another study the use of SCX chromatography fractionation prior to IMAC was reported[21, 137-138] and another study reported the use of SCX and SAX chromatography in tandem[110]. A second form of phosphopeptide purification method enables further enrichment; in the study by Dai *et al.*[110], the flow-through from the SCX loading was collected and loaded onto SAX column for additional fractionation. The authors reported that the SCX fractions contained 659 unique phosphopeptides and SAX fractions (flow-through from SCX) contained an additional 210 phosphopeptides. The ratios for acquired spectra per phosphopeptide were 2.95 for SCX and 1.59 for SAX indicating higher phosphopeptide intensity in the SAX fraction.

In hydrophilic interaction chromatography (HILIC) the interaction is through hydrogen bonding between peptides and the hydrophilic stationary phase[139]. The increasing polarity of a peptide (hydrophilicity) increases its retention on the column making the phosphopeptides elute later than nonphosphorylated peptides[111]. McNulty and Annan[111] have demonstrated that when a dual IMAC and HILIC strategy is used for phosphopeptide purification the order of the separation steps are crucial: using IMAC purification prior to HILIC they were able to identify 899 peptides, 60% of which were phosphorylated (545). When performing the HILIC fractionation first, 820 peptides were identified of which 99% were phosphorylated (814).

2.4. Phosphorylation: On-target purification

Chromatography based phosphopeptide enrichment methods such as IMAC and MOAC involve numerous sample handling steps such as column preparation, sample dilution, loading and a number of sample washing and elution steps. After the washes, the phosphopeptides are eluted from the TiO_2-column. Usually following the enrichment step reversed phase chromatography (separation prior MS analysis or desalting) is performed. For the desalting step another column preparation is required followed by sample loading, washing, elution and drying. IMAC protocols follow a similar workflow. Kokubu *et al.*[140] reported a 10-15% loss of phosphopeptides during the washing step, 10-20% did not elute from the column and another 10-20% was lost during the desalting after the enrichment. Similar results were reported by Corthals *et al*[141] where they reported 70% recovery from the Fe(III)-IMAC column.

A distinct advantage of on-target purification of phosphopeptides over traditional chromatography based techniques is that it requires only a few sample-handling steps. Phosphopeptide containing sample is purified on a same planar surface from where it is analyzed. This particular feature means that the on-target purification can be faster, reduces the risk of sample-handling modifications, and enable smaller sample volume processing. For these reasons several groups have investigated surfaces for phosphopeptide sample purification [142-151].

2.4.1. IMAC-based MALDI targets

The first affinity surfaces for desorption of intact macromolecules was developed by Hutchens and Yip[152]. After affinity adsorption of the analyte on the surface, the slide was introduced into a MALDI instrument for ionization and mass analysis. This concept

was later commercialized by Ciphergen Biosystem Inc. as The ProteinChip Biology System[153]. Qiagen also offers commercial chips for phosphopeptide purification and both of these systems have been used for phosphopeptide purification[151, 154]. Even though the relative enrichment could be seen in both studies, the recoveries reported[151] were only 30%.

A number of research groups have prepared custom MALDI targets with IMAC properties using self-assembled monolayers (SAMs)[149-150], polymer modification[151] or coating the plate with a zirconium phosphonate film[148]. Here the MALDI target is coated with a "sandwich" of molecules: the first compound acts as an immobilized ligand that is used bind a second molecule to the MALDI target. The second molecule has an affinity for phosphopeptides. The structure of the IMAC surface on the MALDI target is analogous to the IMAC column used in chromatographic purification (described in section 2.3.2.3). Shen *et al.* [149] used nitrilotriacetate, (NTA) as a ligand and Ga(III) as an affinity metal ion to form GA(III)-NTA-SAM modified plate for phosphopeptide enrichment. The study showed that the relative intensity of the phosphopeptides in a MALDI spectrum could be enhanced by using this plate. Xu *et al.*[150] fabricated a MALDI target plate with Fe(III)-iminodiacetate (IDA) as an affinity metal ion-ligand complex bound to porous silicon. From a 300 fmol loading of *β*-casein they were able to detect phosphopeptides without nonspecific binding from the other peptides. The limit of detection was achieved by showing the phosphopeptide signal after 10 fmol of loading. However, the signal-to-noise level for those peaks was ≤ 1.

Dunn *et al*[151] prepared (2-hydroxyethyl methacrylate) brushes modified with Fe(III)-NTA complexes (Fe(III)-PHEMA-NTA) for phosphopeptide purification. They reported a 15 fmol detection limit with ~70% recovery. However, the authors reported significant decrease in the recovery in the presence of digest reagents (such as urea) suggesting the need for sample purification prior to enrichment. This in turn would increase the sample handling steps and might lead to significant sample losses. Another group[148] manufactured zirconium phosphonate-silicon surface showing 2 fmol sensitivity with a *β*-casein digest.

Hoang *et al.*[144] prepared a functionalized MALDI target plate for phosphopeptide enrichment using immobilized zirconium on a phosphonate self-assembled monolayer (SAM)[144]. The plate was structured so that hydrophobic areas outside the sample spotting area enabled concomitant concentration of the sample[155]. The plate enabled larger sample volumes to be used for the method, thereby reducing sample loading errors. High sensitivity was reported with the detection of phosphopeptides from a 1 fmol loading of *β*-casein.

2.4.2. Metal oxide affinity chromatography -based MALDI targets

Blacken *et al.* modified a MALDI target by embedding a ZrO_2-coated stainless steel plate into the target[142-143]. First they established the suitability of this new surface for phosphopeptide enrichment[142], and later performed comparisons with titanium, zirconium and hafnium (IVB metals from periodic table of elements), and evaluated the effect of the surface thickness and preparation[143]. They concluded that of the three tested metals zirconium produced the best results.

TiO_2 surfaces have been widely used in various on-target purification approaches. In one study a MALDI target was covered with an array of sintered TiO_2 nanoparticle spots[145] and in two more recent studies a TiO_2 film has been used[146,147]. Niklew *et al.* described the preparation of a TiO_2 film on top of conductive glass using a sol-gel technique[146]. This study reported 300 fmol sensitivity after their purification steps using a single 2062 *m/z* peak from a digest of *β*-casein as a standard. In another study, high sensitivity phosphopeptide purification and MS detection was shown using a planar surface for which pulsed laser deposition was used to generate a TiO_2 film onto the MALDI-target[147]. The sensitivity of the method was demonstrated by analyzing 25 fmol of *β*-casein digest where after washing two phosphopeptides were detected, one of which could be identified by MS/MS.

Major drawbacks for the existing methods for on-target purification of phosphorylated peptides/proteins is that either their use is limited to specific instrument (SELDI) or the manufacturing of the functionalized MALDI-target is a complex procedure and not commercially available.

2.4.3. Phosphopeptide fragmentation in MS

As discussed earlier (section 2.1.2), peptide fragmentation occurs through charge-directed dissociation, where the amide nitrogen becomes protonated and thus weakens the amide bond making the fragmentation of the peptide possible together with CID. The bond between the peptide and the phospho-group is labile which makes it favourable for fragmentation. Therefore, phospho-group detaches from the peptide easily generating an intense neutral loss ion of 98 Da or 80 Da lower than the measured peptide. These neutral losses represents the losses of H_3PO_4 (98 Da) and HPO_3 (80Da). The loss of 98 Da is observed from the pSer and pThr where as the loss of 80 Da originates from pTyr (and to some extent from pSer and pThr) [118]. Different mechanisms for neutral loss from pSer and pThr have been proposed[118, 156] differing in the model for removal of the hydrogen by the departing phosphate group. The charge-remote loss of H_3PO_4 (98 Da), know as *β*-elimination (Figure 9), suggests that the hydrogen originates from the *α*-carbon[118], whereas the charge-directed loss theory proposes that the hydrogen is the mobile proton[123]. A neutral loss from pTyr produces the loss of HPO_3 (80 Da)[118]. The H_3PO_4 cannot be lost since the bond between the carbon in aromatic ring and the

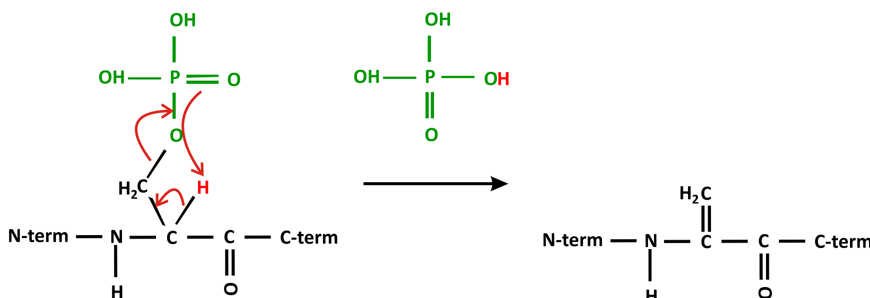


Figure 9: In β-elimination, the hydrogen from α-carbon of the phosphorylated residue is transferred to the phosphate group. The transferred hydrogen is marked red in the figure.

oxygen in the phospho-group is stronger than the corresponding bond in pSer and pThr due to stabilization by the aromatic ring[118] (Figure 8). The bond in the phospho-group between oxygen and phosphate is weaker so the loss of HPO_3 occurs instead of H_3PO_4.

Steen *et al*[157]. developed a mass spectrometry based method for detection of Tyr phosphorylated peptides where they used immonium ion of pTyr. In this method the MS instrument is set to scan *m/z* 216.043 (immonium ion of tyrosine[66],136 Da and phospho-group, 80 Da = 216 Da) which has been reported to be a characteristic fragment ion for pTyr containing peptides[158].

3. AIMS OF THE STUDY

The overall objectives of this Ph.D. thesis was to improve the robustness of MALDI based MS methods for peptide and phosphorylation analysis. The focus was to develop strategies that enable 1) acquisition of more MS measurements per sample; 2) higher quality MS spectra, and 3) simplified and rapid enrichment procedures for phosphopeptides. Furthermore an objective was to apply these methods to characterize phosphorylation sites of phosphopeptides. The specific aims of the research projects in this Ph.D. thesis were:

1. Application of MALDI matrices and procedures for improved peptide ionization and fragmentation of phosphopeptides, that provide higher quality MS information relevant for unambiguous phosphopeptide identification (I)
2. Develop new methods that efficiently enrich phosphopeptides and -proteins and minimize sample treatment, focusing on using the MALDI target plate as a single-step enrichment surface (II, IV)
3. Develop new methods that enable phosphopeptide characterization without purification procedures ensuring that most phosphopeptide remain in the sample at the time of analysis (III)
4. Integration of all above mentioned aims and their application to phosphoproteomics projects (to be done)

4. MATERIALS AND METHODS

This section describes methods used in the separate publications and that are not already mentioned in the published details. Also methods not previously published are represented and commented here (section 4.5.)

4.1. Publication (I)

4.1.1. Digestion

Proteins were reduced using 5 µl Reducing Reagent (200 mM ditriothreitol in 25 mM ammonium bicarbonate) and a 1 hour incubation at 37 °C after which proteins were alkylated by adding 20 µl Alkylating Reagent (200 mM iodoacetamide in 25 mM ammonium bicarbonate) and incubated in the dark for 1 hour (at RT). 20 µl of Reducing Reagent was added to consume any remaining alkylating agent to prevent trypsin from being alkylated. 900 µl of ammonium bicarbonate solution was added to dilute the urea to 0.6 M before adding the trypsin (1:30). Trypsin digestion was carried out at 37 °C overnight.

4.1.2. Matrix comparison

Tryptic peptides from BSA and matrix were manually deposited onto an AnchorChip MALDI target (four repeats for each matrix). Nitromatrix (nitrocellulose and CHCA) was compared to: i) CHCA; ii) the pre-spotted anchor-chip (PAC™) protocol following the manufacturer's instructions (Bruker Daltonics Product information, Prespotted AnchorChip, version 05-01-20); and iii) ammonium monobasic phosphate.

4.1.3. HPLC-MALDI

HPLC was performed using an UltiMate™ nanoLC with a homemade reversed-phase LC-column (Magic C-18 75 µm x 150 mm, 5 µm, 100 Å, 200 nl/min). Two types of durability tests were performed: 1) to test nitromatrix's durability by repeated MALDI analysis and 2) to compare durability between CHCA and nitromatrix using imaging features of the instrument following an LC-MALDI analysis. In both cases the sample was 0.1 µg of enzymatically digested yeast mitochondrial proteins. Peptides were HPLC separated and spotted on to a MALDI target with nitromatrix. For repeated MALDI analyses the same sample was sequentially analysed ten times, in total 76.5 hours of continuous analysis. Peptides were separated and 30 s LC-fractions were collected using a Probot™ fraction collector and deposited onto an AnchorChip plate with 800 µm anchor size. The matrix was continually added at 1.3 µl min^{-1} to the column eluent via a separate liquid line, consequently the sample and matrix were mixed at the column exit and on the MALDI target.

4.1.4. MS-analysis

All MS-analysis were performed on a Bruker Ultraflex II equipped with a 200 Hz Smartbeam^{TM} laser system. Data was acquired in fully automatic manner using either WARP-LC version 1.1 (LC-MALDI experiment; figure 2), BioTools version 3.0 (matrix comparison; figures 1 and 4) or FlexImaging (imaging experiment; figure 3). For the LC-MALDI experiments compounds with a signal to noise ratio exceeding 25 were selected for MS/MS acquisition. 300 and 1000 laser shots were used to acquire the MS and MS/MS experiments, respectively. For all other experiments the spectra from 600 laser shots were accumulated. The laser power was 2-3% above ionization threshold.

4.1.5. Database search criteria

MASCOT version 2.1 (Matrix Science, London, UK) was used to search the SwissProt database (a) version 48.9 for comparison of matrix preparation and b) version 50.6 for matrix durability test for protein identification. The following search settings were used: mass error tolerance for parent ion and fragment ions: 0.2 Da and 0.6 Da respectively; fixed modification: cysteine carbamidomethylation; variable modification: methione oxidation; enzyme: trypsin; number of missed cleavages: one. For comparison of matrix preparations the mass tolerance settings were 0.1 (parent) and 0.3 Da (fragment).

4.2. Publication (II)

4.2.1. TiO_2 coating

Glass slides (75 x 25 mm) coated with an indium tin oxide (ITO) layer, purchased from Bruker Daltonics (Bremen, Germany), were used as substrates for the TiO_2 thin films. The dipping solution was prepared with a molar ratio of $TiCl_4$:EtOH:H2O:THF:F127 = 1:250:10:20:0.001 (F127=The block co-polymer Pluronic) dip coating procedure was performed three times to ensure a complete coverage of the substrate and between each dipping step the deposited films were heated directly at 400 ˚C for 3 min on a hot plate. Finally, the films were heated at 400 °C for 3 min or 450 °C for 30 min to obtain the desired crystalline phase.

4.2.2. Thin film X-ray diffraction (XRD) measurement

X-ray diffractograms were recorded with PANalytical X’Pert PRO MPD diffractometer. Grazing incidence configuration with 1° incident angle was used for all the measurements. The data was acquired from an angular range of 24-50° 2θ using 0.04° 2θ step size.

4.2.3. Phosphopeptide enrichment from tryptic casein digests

Commercially available phosphoproteins bovine α- and β-caseins were separately digested with modified trypsin at 37 °C for 20 h. The tryptic digests were acidified with trifluoroacetic acid (TFA). Equal amounts of the digests were then mixed together. The mixture (500 fmol/μL or 50 fmol/μL) was loaded onto the metal oxide-coated slides, and then dried. The slides were washed three times with 500 μL of 6% TFA/80% acetonitrile (ACN), and then dried.

4.2.4. **Matrix coating**

All the tested matrices were applied using a standard airbrush. Working pressure for the airbrush was set to 1 bar and 60 iterative spraying cycles from 30-40 cm distance were carried out. The quality of the matrix layer was confirmed using Zeiss SteREO Lumar. V12 microscope.

4.2.5. **MALDI-based IMS**

IMS was performed on a Bruker Daltonics Ultraflex II MALDI-TOF/TOF MS equipped with 200 Hz repetition rate Smartbeam laser technology. Mass spectra were collected from 200 x 200 µm raster in positive reflector mode. Mass filter settings for visualizing the peptide ion signals were set to theoretical *m/z* ± 3.

4.3. Publication (III)

4.3.1. **Sample treatment**

NFATc1 was phosphorylated *in vitro*, in-gel digested with trypsin after SDS PAGE and either analyzed directly with a mass spectrometer or subjected to TiO_2-retention to enrich for phosphopeptides.

4.3.2. **Mass spectrometry analysis and HPLC**

MS analysis was performed either with a MALDI-TOF/TOF instrument (Ultraflex II) from Bruker Daltonics or a q-TOF instrument (Q-STAR Pulsar) from Applied Biosystems. Chromatographic equipment (Ultimate Plus gradient pump, Famos autosampler and Switchos column switching unit) were from LC-Packings. All MALDI analyses were conducted on an AnchorChip (800µm) MALDI target.

4.3.3. **Data analysis and database search**

Data was acquired using Bruker's FlexControl (3.0) software for the Ultraflex and Analyst QS 1.0 for the Q-STAR.. For database searching a custom database was searched using Mascot 2.2 (Matrix Science). The custom database contained modified NFAT sequence with GST and linker with PIM and PKA kinase sequences.

4.4. Publication (IV)

4.4.1. **Chromatographic TiO_2-purification**

Approximately 3 mm TiO_2 micro columns were prepared on top of a 3M C-8 Empore Disk, after which the diluted sample was loaded into the column. The sample was washed once with 10 µl of loading solution, once with 20 µl of washing solution (80% ACN, 1% TFA) and once with 5µl of MQ-H_2O. The sample was then eluted from the column with 20µl of ammonium hydroxide-solution (NH_4OH, pH 10.5) followed by 2 µl of 30% ACN to elute the phosphopeptides bound to the C-8-disk. The eluted fraction was acidified with 2.5 µl of 100 % formic acid (FA) prior to the desalting step.

4.4.2. Sample desalting

Samples from TiO_2-purification were desalted using C-18 micro columns. The column was equilibrated with 10 µl of 0.1% TFA and the acidified sample was applied into the column using gentle air pressure. The column was washed with 10 µl of 0.1% TFA and the retained peptides were eluted directly to the MALDI target using 2 µl of 80% ACN, 0.1% TFA.

4.4.3. Optimized planar surface purification

Two microliters of loading buffer (80% ACN, 1% TFA) was incubated on top of indium tin oxide (ITO) coated glass slides (Bruker Daltonics, Bremen, Germany) prior to sample application. One microliter of the sample was deposited on to the ITO-coated glass slide and mixed with the loading buffer on target. After sample loading the glass slide was placed into a desiccator to dry. Next the sample was washed with 50 µl of 80% ACN, 1% TFA, incubated with NH_4OH (pH 11) for approximately 5 minutes after which 2µl of MALDI-matrix was added (20 g/l of DHB in 50% ACN, 1% PA, 0.1% TFA) and left to dry in the desiccator.

4.4.4. PhosphorImager

Peptides from radioactively labelled NFATc1 were spotted on top of ITO-coated glass slides and washed with different concentrations of TFA (1-6% TFA) in 80% ACN. The photo-stimulated luminescence (PSL) was measured using Fujifilm BAS-1800 PhosphoImager before and after the washes.

4.4.5. MALDI-TOF (-TOF) analysis

All MS analysis was performed using an Ultraflex II from Bruker Daltonics equipped with a 200 Hz Smartbeam laser system. For more details, see original publication.

4.4.6. Peak list generation, spectral processing and database search

See original publication for details.

4.4.7. Sensitivity test

A dilution series of monophosphopeptide from *β*-casein was prepared containing 200, 100, 50, 25, 12 and 6 fmol/µl monophosphopeptide in 10% ACN, 0.1% TFA. One microliter of each dilution was spotted onto the ITO-coated glass slide (x3), purified according to our optimized planar surface enrichment protocol and measured by MS. In parallel, an equivalent sample was subjected to chromatographic TiO_2-purification after which the sample was desalted using a C-18 microcolumn and spotted directly on to a ground steel MALDI target (Bruker Daltonics).

For additional materials and methods (plasmids, protein production and purification, cell culture and immunoprecipitations, i*n vitro* kinase assays, gel electrophoresis and radioactivity measurements, in gel digestion of proteins and alkaline phosphatase treatment) see original publication's supporting information.

4.5. Unpublished methods

4.5.1. On-tissue digestion

Tissues were washed by gently agitating the plates in two successive 30 s baths of 25 ml of 70:30% v/v ethanol/water followed by a 15 s wash in 25 ml of ethanol. Excess solvent on the tissue was removed by a gentle flow of nitrogen gas. The sample was placed in the desiccator prior to trypsin digestion (protocol modified from Aerni *et al.*)[159].

A 32 µg/µl stock solution of trypsin (Sigma T1426-250mg, TPCK treated, from bovine pancreas) was made using 100 mM ammonium bicarbonate. A dilution series was made by diluting the previous sample (32, 16, 8, 4, 2 and 1 µg/µl) and 2 x 0.5 µl was pipetted on to the tissues. The tissues were placed in a Petri dish containing a moist tissue and incubated at 37 °C for 3 hours. 25 mg/ml of DHB in 1:1 methanol/0.5% TFA (aq.) was the spotted on top of the tissue in 0.5 µl portions to cover the entire tissue.

5. RESULTS AND DISCUSSION

5.1. Durable and sensitive MALDI-matrix for LC-MALDI (I)

Several parameters were optimized to enhance the durability and sensitivity for LC-MALDI-MS. As reported in publication I (Publication I, Table 1), several matrix protocols and compounds were evaluated, and nitromatrix was found to provide the best results. First, signal intensities were monitored by the signal-to-noise (S/N) ratios (Publication I, Figure 4). Other features relevant for database searches such as the number of peptides, sequence coverage and Mascot scores (Publication I, Figure 1) were also monitored. Nitromatrix provided the best performance for each of the tested parameters. Subsequently the nitromatrix was optimized for its durability (Publication I, Figures 2 and 3) and used in LC-MALDI-MS experiments (Publication I, Figure 5). The results from these experiments will be shortly presented and discussed.

5.1.1. Matrix comparison

For the matrix comparison four different sample preparations were compared. The sample was trypsin digested BSA and the monitored values were signal-to-noise, sequence coverage, number of peptides identified and Mascot score (Publication I, Figures 1 and 4). Nitromatrix outperformed three other protocols having the highest value in all the measured parameters.

5.1.2. Optimized matrix deposition

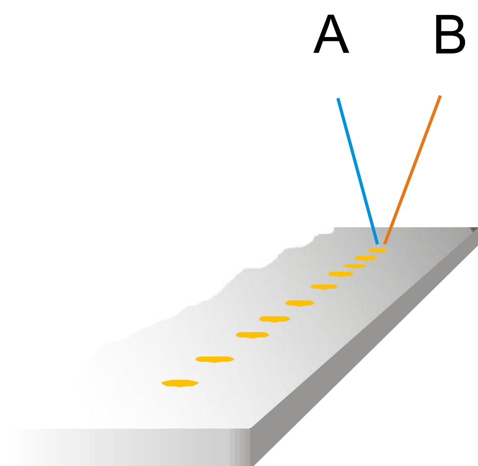


Figure 10: The LC flow from the column (A) was separated from the matrix flow

Inconsistencies were observed with matrix deposition using a spotting robot's factory configuration (Probot, LC-Packings, The Netherlands). The vendor's setup uses a T-piece to mix the eluting LC and matrix solutions prior to spotting on a MALDI target. Due to the different pressures in the connected tubings the spotting was not reproducible. Separating these two lines and mixing the matrix and the LC eluent at the exit of the silica capillaries improved the situation (Figure 10).

The nitromatrix was tested against commonly used sample preparation techniques and found to be superior for its performance in protein identification. The durability of the matrix was also demonstrated by analyzing a single LC-MALDI sample ten times (0.1 µg of trypsin digested proteins extracted from yeast mitochondria). However, the initial purpose of the study was not to find as many yeast proteins as possible but to test the durability of the matrix and also the merits of using peptide exclusion

lists. Exhaustive automated data dependent analysis on the same LC spotted gradient was conducted ten times, after which protein identification was still possible without substantial decrease in signal quality or the number of protein identifications. As expected, the total number of unique identifications plateaued after several repeat analyses. If mass spectrometry vendors would release software that could include reiterative analyses with the incorporation of exclusion lists based on previously identified peptide masses this would be a significant advance for this application.

5.1.3. Durability of nitromatrix

Post-acquisition imaging of peptide signals was applied to investigate the signal distribution of the LC-MALDI samples. Two LC runs with two different matrices (CHCA and nitromatrix) were spotted onto an AnchorChip plate and analyzed by MS. After the MS analysis the IMS was performed to investigate the signal distribution of the remaining peptides. The peptide intensity images recorded using CHCA as a matrix show an dark circle at the centre of the image, which corresponds to no peptide signal (Figure 11; also Publication I, Figure 3). This loss of signal is caused by laser ablation of the sample-matrix complex from the target during the previous LC-MALDI analysis of the sample. In contrast the image obtained when using the nitromatrix showed an even distribution of signal across the sample, even after 10 reiterative analyses. In subsequent experiments it was observed that the nitromatrix required less laser power to generate peptide ions, ultimately allowing more laser shots per sample. One could argue that the “film” formed by the nitrocellulose on top of the CHCA contributes to the durability of the matrix (Figure 12), although this has not been investigated systematically. On the other hand, it should be noted that nitrocellulose has an affinity for proteins. Therefore, the nitrocellulose could act as an affinity film for peptides during solvent evaporation and thus help concentrate the peptides at the surface of the matrix. In conclusion one could speculate that these two features, (1) a protective film against laser ablation and (2) affinity towards peptides contributes to the durability of the nitromatrix (Figure 13).

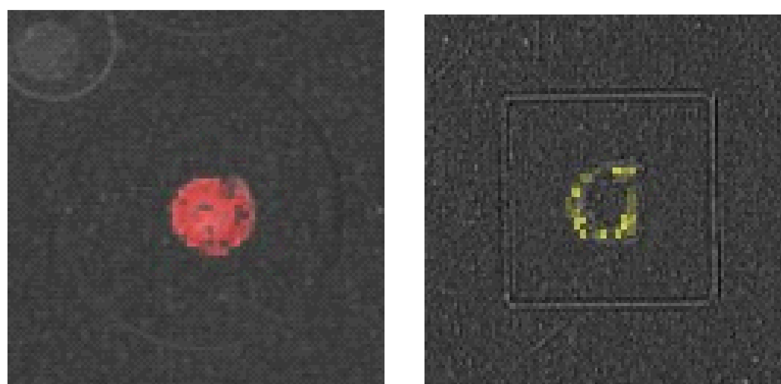


Figure 11: Red (left) indicates signal from nitromatrix after LC-MALDI analysis (m/z 2818 +/- 3). Yellow represents signal from CHCA (m/z 2001 +/- 3). After the first LC-MALDI analysis the signal is still uniformly observed from the nitromatrix where as CHCA has almost disappeared.

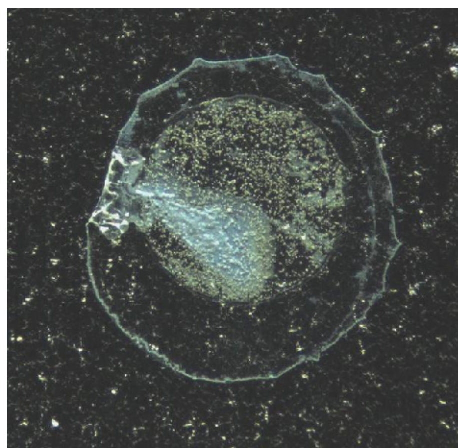


Figure 12: In nitromatrix the nitrocellulose formed a thin film on top of CHCA possibly contributing its durability against laser ablation.

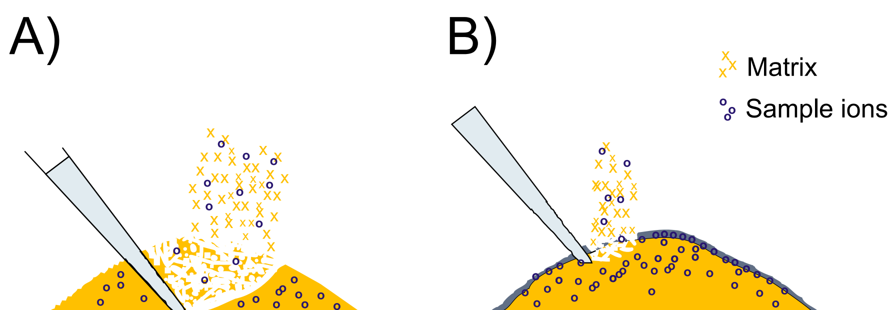


Figure 13: The laser penetrates into the normal CHCA matrix and ablates most of the matrix (A). During matrix crystallization the nitrocellulose forms a film on top of the matrix and concentrates peptides to the surface. Therefore, nitrocellulose offers mechanical protection against the laser, but also due to the concentration effect less laser power is needed for ionizing sample (B).

5.2. Affinity imaging mass spectrometry, AIMS (II)

The second publication (II) addresses the development of a MALDI affinity target for use in IMS applications for the selective enrichment of classes of peptides and proteins. Of particular interest was an ability to capture phosphopeptides. Thus, the aim of the study was to establish a platform where phosphopeptide enrichment was achieved while preserving the spatial orientation of the molecules. To achieve affinity for phosphopeptides TiO_2-coated glass slides were manufactured. After developing the surface and matrix optimization it was possible to immobilize phosphopeptides onto the TiO_2 coated glass slides. Subsequent extraction and immobilization of phosphopeptides from tissue was attempted. The results from these previously unpublished attempts are presented and discussed below.

5.2.1. AIMS analysis of casein peptides

The AIMS study demonstrated that a self made TiO_2 -surface retained phosphopeptides maintaining the spatial orientation (Publication II). The proof of principle was established

using a casein peptide mixture. Peptides deriving from these proteins were spotted manually to form a letter “P” on to the TiO_2-coated glass slide, washed, covered with matrix and analyzed. After IMS analysis the letter “P” was clearly visible by imaging of the phosphopeptides (Publication II, Figure 4).

5.2.2. Trypsin digestion of the tissues

To visualize phosphorylation of proteins in tissues, each protein’s phosphopeptides need to be generated by enzymatic digestion. For tissue digestion a thin film of agarose soaked with trypsin and electroblotting with an immobilized trypsin membrane[160] were tested. Neither of these approaches was successful (data not shown). Subsequent development was continued by optimizing the amount of trypsin that would be required for digestion directly on the tissue. While direct pipetting may result in peptide movement within the tissue, enrichment on the plate surface was still attempted. Prior to trypsin addition the tissues were washed and dried. Different amounts of trypsin (1, 2, 4, 8, 16 and 32 µg) were pipetted on top of six tissue slides incubated, covered with matrix and measured (Figure 14).

It was observed that 1 µg of trypsin was sufficient to produce peptide signals. The spectral complexity did not increase with the amount of trypsin. The most abundant peptides were subjected to MS/MS and two proteins were identified: myelin from peptides *m/z* 1460 and 2141; and tubulin from *m/z* 1621 and 2798. After the measurement the tissue

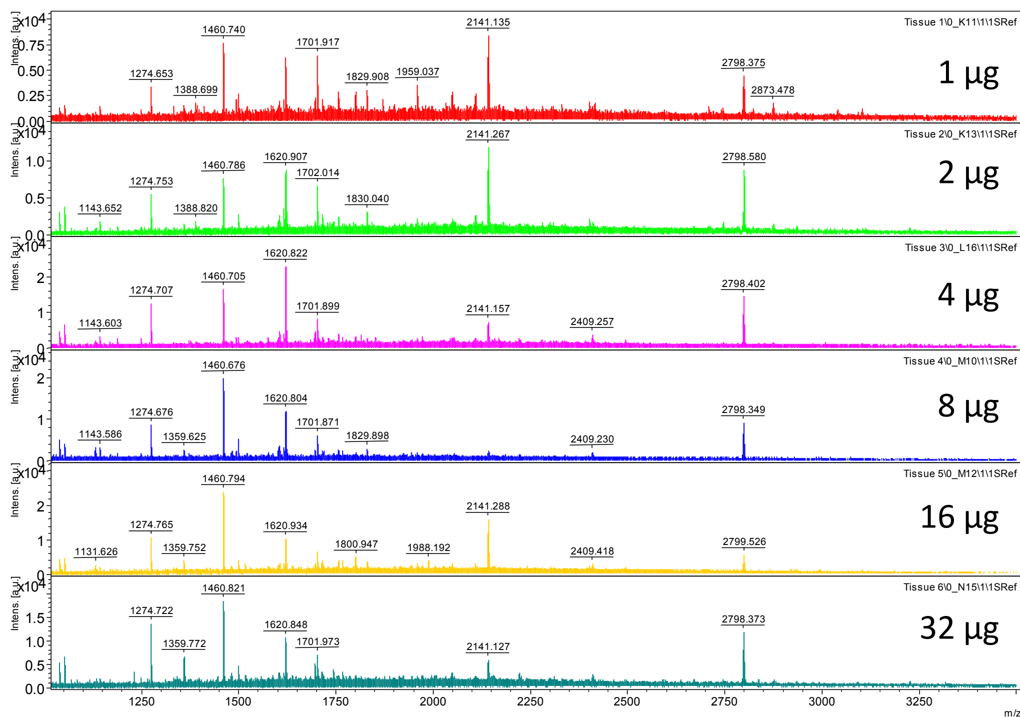


Figure 14: Optimization of trypsin amount used for on-tissue digestion. There was no significant increase of the amount of peptides when the trypsin amount was raised from 1 µg to 32 µg on tissue. The ions m/z 1460 and 2141 were identified as myelin and the ions m/z 1621 and 2798 as tubulin when MS/MS was performed on the tissue.

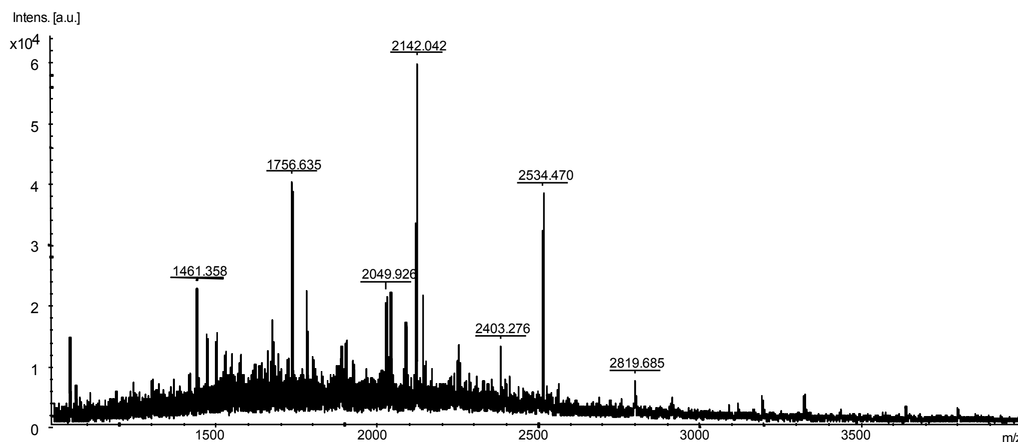


Figure 15: MS spectrum after trypsin digestion, tissue removal and matrix addition. In addition to already identified peptides (m/z 1460, 2141, 1620 and 2798) additional peaks were detected. However, none of the peptides were identified when MS/MS was performed.

was mechanically removed from the glass slide, washed for phosphopeptide enrichment, covered with matrix and measured again.

It was possible to record a peptide signal indicating that the trypsin was able to penetrate through the tissue (Figure 15). However, many of the peptides were the same as observed prior to tissue removal, indicating their high abundance in the tissue. Along with the already identified peptides some new and intense signals were also present (m/z 1756 and 2534) but it was not possible to identify these new peptides by MS/MS.

As discussed in the publication (II) the application of this technique to tissues is challenging. Even though the electroblotting of a tissue through an immobilized trypsin membrane was not successful, revisiting this approach should be done. The major obstacle, I believe, was that the electroblotting cannot be done onto a solid surface. Therefore, another type of surface could be made which might capture phosphopeptides and could also be used in electroblotting. Since nitrocellulose is already used in electroblotting proteins from SDS-PAGE and exhibits longevity features as a MALDI matrix (discussed above), a phosphopeptide capture membrane based on nitrocellulose may be useful for capturing and image phosphopeptides.

5.3. Phosphopeptide analysis without purification (III)

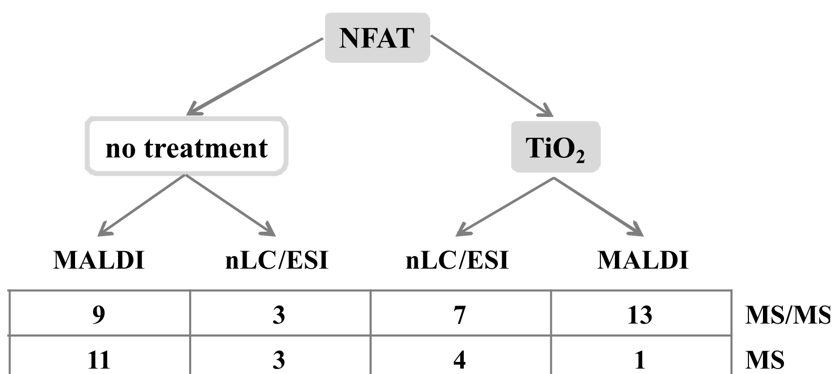
Most of the phosphopeptide purification methods use liquid chromatography to purify the sample. Connected to an on-line system these methods offer high throughput and up to thousands of phosphopeptide identifications. The purpose of this publication (III) was to investigate the capacity of phosphopeptide analysis from planar surfaces for, thereby circumventing elaborate purification methods. As it is well documented that different matrices generate both unique and redundant ions, this study also investigated if a combination of spectra generated by different matrices (from the same sample) could be used for to improve confidence in peptide identification. Accordingly the same sample was analyzed by MALDI-TOF/TOF where four different matrices were used.

As a control experiment each sample was enriched for phosphopeptides by TiO_2 and subsequently analyzed by ESI-qTOF. After combining the results from four different matrices the results were compared with the samples analyzed by LC/ESI-qTOF MS/MS. Using only 20 % of the sample that was consumed for ESI the MALDI measurements identified more phosphopeptides.

5.3.1. Phosphopeptide identifications

After analysis of a simple phosphopeptide mixture in positive mode it was observed that MALDI-MS/MS offered more information than using nano-flow LC/ESI-qTOF-MS (Schema 1). Even though the MALDI analysis was performed with four different matrices the total amount of sample consumed for each MALDI analysis was only 20% of that used for nano-flow LC/ESI-qTOF-MS/MS. Without purification MALDI identified (MS/MS) 9 phosphopeptides whereas ESI only 3 were identified. After purification 13 and 7, were respectively identified. Peptide masses of the phosphopeptides, detected in the MS spectra were as follows: before purification MALDI 11 and ESI 3. After the purification 1 and 4, respectively. The results are summarized in Schema 1.

As expected the results from this work suggest that by using multiple matrices one obtains more phosphorylation sites than using any single matrix alone. Furthermore this approach enabled confirmation of phosphorylation sites because the different matrices generated a disparate ion series. This work showed that by combining two or more ‘low quality’ MS (peptide) spectra, protein identification and subsequent phosphopeptide identification became possible. Similarly combining the spectra at the MS/MS level would assist in protein and phosphopeptide identification by virtue of the complementary information found for the different matrices from the fragment ions. Also, analysis of the same peptide multiple times adds more statistical confidence in protein identification and phosphorylation site determination.

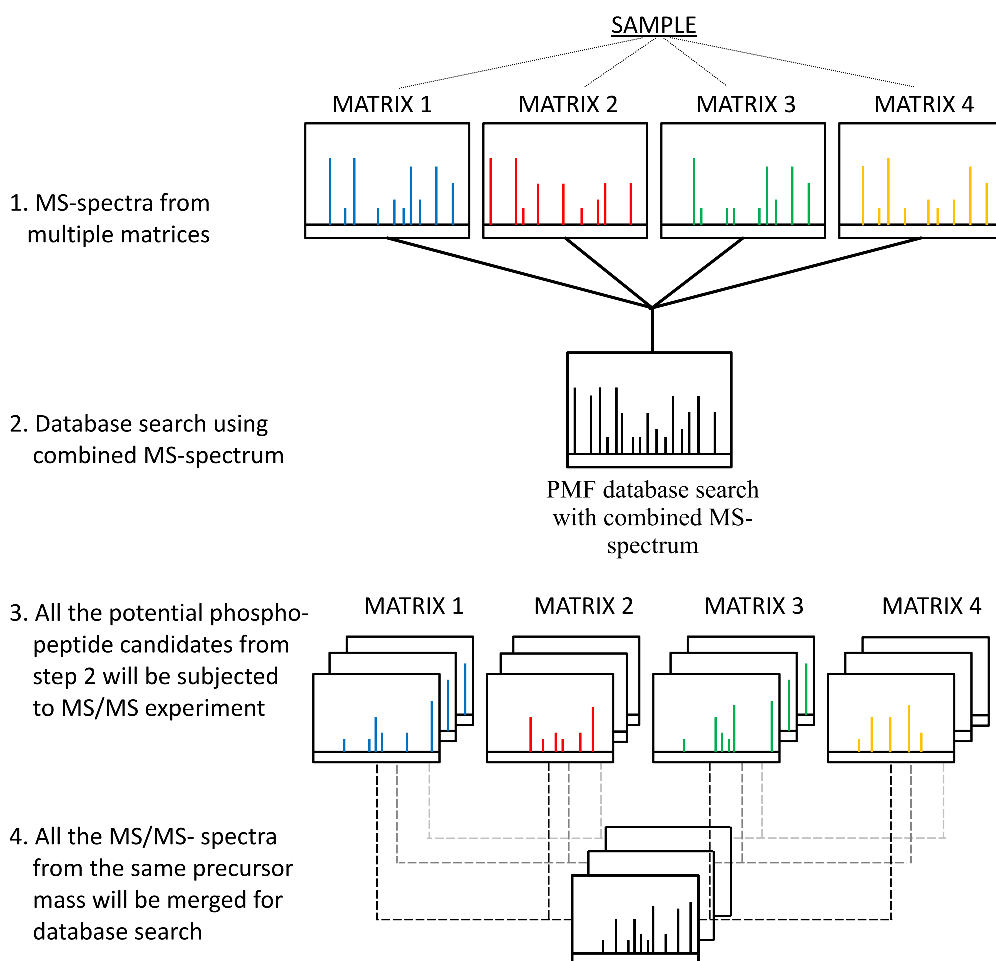


MALDI	nLC/ESI	nLC/ESI	MALDI	
9	3	7	13	MS/MS
11	3	4	1	MS

Schema 1: The NFATc1 was enzymatically digested with trypsin. and analyzed before and after TiO2-purification by MALDI-TOF and ESI-qTOF. Before the purification the MALDI method identified nine phosphopeptides where as ESI only three. After the purification the figures were 13 and 7, respectively. MS information (peptide mass) from phosphopeptides was acquired as follows: before purification MALDI 11 and ESI 3. After purification 1 and 4, respectively. Modified from Publication III.

Using the multimatrix approach we were able to produce similar results to TiO_2-enrichment (III). However, many peptides which matched to a peptide mass + phospho-group(s) were not identified because of only few peaks in tandem mass spectrum. This problem could be circumvented by combining multiple spectra from the same peptide but from a different MALDI matrix, with a slightly different information content. This could help in the identification of phospho-peptide assignment. This hypothesis was partly proven to be true in the publication, although testing this argument in a systematic manner would require specifically developed software by instrument vendors. For this we suggest the following workflow (Schema 2).

In the multimatrix approach the sample is analyzed using multiple MALDI matrices. The different MS-data (peptide masses) are collected from the same sample using several different MALDI matrices. This data is then merged and subjected to a database



Schema 2: 1) The sample is analyzed in a MALDI-TOF/TOF with different matrices. 2) Spectra from different matrices are combined and the sum spectrum is subjected to a database search. 3) Phosphopeptides assigned by PMF database search are subjected to MS/MS analysis. 4) All the MS/MS spectra with the same precursor mass are merged for database searching.

search. From the PMF-data potential phospho-peptides can then be subjected to MS/MS. The data is then collected from all matrices regardless from where the phospho-peptide was originally detected. This is due to the fact that even though these peptides cannot be detected from every matrix, the detection from any one of the matrices is proof that the molecule exists. Thus while a MS spectrum may not be present an MS/MS spectra might be generated without the detection of the precursor ion. This has been reported previously, although in a different framework, by Chait and colleagues[161]. In the experiment discussed here, once the MS/MS spectra have been collected from all the matrices, the data is merged and subjected to database search.

5.4. Phosphopeptide purification on planar surface (IV)

Previous work on enrichment of phosphopeptides led to the evaluation of various surfaces for enrichment and trapping of phosphopeptides on planar surfaces. Amongst the surfaces that showed interesting results were ceramic slides, stainless steel, TiO_2 coating and indium tin oxide (ITO) coating. An interesting difference that ITO slides have to the others is that they are commercially available from several sources, and therefore do not require elaborate procedure for production before use. Therefore the capabilities of ITO slides as a platform for phosphopeptide analysis by MALDI-MS were investigated further. This study (IV) broadened our understanding of ITO-coated glass slides' capability to selectively retain phosphopeptides from simple protein samples. The purpose of this study was therefore to systematically evaluate the characteristics of the ITO coated glass slide and to develop a simple, fast and sensitive method for phosphopeptide purification. With our method we are able to purify 15 samples in an hour with a limit of identification (LOI) of 6 fmol.

5.4.1. Optimization of sample loading and washing conditions

In established chromatographic phosphopeptide purification methods utilizing metal oxide affinity (MOAC), low pH and high organic solvents are used for sample loading and washing. For phosphopeptide elution a high pH is used[108, 162]. The rationale behind useage of these solvents is that the high organic content in the loading step solution hinders the unspecific binding of non-phosphorylated peptides and the low pH sets the correct ionic states for the phospho-group to bind (more exclusively) to the metal oxides. When eluting the phosphopeptides from the column, the high pH reverts the charge stage of the metal oxide so that the phosphopeptide is released from the column. Since ITO is a mixture of two metal oxides, optimization was started with the same solvents that are used in MOAC.

First, the sample loading conditions were evaluated (Publication IV, Figure 1). The effects of the sample loading solution as preconditioner of the glass slide prior to sample loading were tested as well as the effect of a high pH solution incubation on the sample prior to matrix addition was tested. The results clearly showed that preconditioning of the surface reduced the non-specific binding and the sample incubation with ammonia before the matrix addition released more phosphopeptides from the surface. Subsequently the amount of TFA in the washing solution (Publication IV, Figure 2) was optimized. In initial experiments (sample loading optimization) 6% TFA was used. However, on the

basis of the optimized conditions reported for TiO_2-MOAC by Imanishi *et al.*[162-163] these were re-evaluated for this study. The radioactivity of the *in vitro* phosphorylated NFATc1 was measured for varying percentages of TFA in the washing solution. The percentage of the total peptide signals from to phosphopeptides was also calculated, which enabled the results of the radiolabelling experiments to be compared with those obtained using MALDI-MS. Radiolabelling provides a direct measurement of the total phosphopeptide amount, since the radioactivity can be measured without influence from the matrix or other peptides. This is not case in MS, as the signal is affected by the matrix and the complexity of the sample.

When the relative amount of phosphopeptides in the sample was measured, the highest amount was monitored for washing solutions containing 1-3% TFA. The radioactivity diminished as the TFA percentage was increased. The measured radioactivity was highest at 1% TFA, indicating that this concentration retained the most phosphopeptides, so a 1% TFA washing solution was used in the flowing experiments.

5.4.2. Sensitivity test

To evaluate the sensitivity of the method a monophosphorylated peptide from β-casein was used as a standard. Although this standard represents only one phosphopeptide, and the results would not apply for all phosphopeptides, it was chosen as it would allow a comparison between our method and previously published methods.

Hence, a dilution series of β-casein was prepared and analyzed using the planar surface purification method and with TiO_2-MOAC. After TiO_2-purification the sample was desalted and applied to a regular stainless steel target, where three replicates were analyzed. Confident peptide identification from all three replicates was achieved using 100 fmol of the peptide, and from one replicate using 50 fmol of the peptide. When the samples were purified using the ITO-coated glass slide, confident identification was achieved for all three replicates when the starting amount was 12 fmol, and one replicate identified the peptide using 6 fmol of the phosphopeptide.

5.4.3. Application of the method to in vivo phosphorylated sample

After optimization of the method it was used for analyzing phosphorylation of the protein NFATc1. After 1-DE NFATc1 was trypsin digested and the peptides were deposited on to the ITO-coated glass slide, purified and measured by MALDI-MS/MS (Publication IV, Figure 4 and table 2). Phosphopeptides with one, three and four phosphorylation sites were detected indicating this method's applicability to purify multiply phosphorylated peptides.

Previous reports of NFATc1 phosphorylation report only four phosphorylation sites. [Protein Knowledgebase (UniProtKB) www.uniprot.org; A database for S/T/Y phosphorylation sites (PhosphoELM), phospho.elm.eu.org; Posttranslational modification database (PHOSIDA) 141.61.102.18/phosida/index.aspx]. These sites were Ser-245[164] , Ser-269[164], Ser-294[165] and Ser-233[166] of which only Ser-233 had been detected by MS. Two other studies used Edman degradation for phosphopeptide sequencing. In this publication (IV) Ser-233 and Ser-245 were detected, but not Ser-269

or Ser-294. Altogether 5 different phosphorylation sites (Ser- 233, 245, 278, 282 and 359; Publication IV, Table 2) were confirmed and 7 other potential phosphorylation sites were observed that could not be assigned with high confidence (Ser 229, 237, 239, 241, 286, 290 and Thr 284).

In a study by Mohammed *et al.*[166] they also reported phosphorylation in Ser-233. What is interesting in their study is the finding of only one phosphopeptide, and is likely to be a trend that is seen elsewhere. In this study a whole cell lysate was digested by 3 different enzymes (Lys-C, Lys-N and trypsin) and fractionated by SCX (40 fractions /digest) after which all the fractions (3x40) were analyzed using an LTQ-Orbitrap MS/MS mass spectrometer coupled to nanoRP-HPLC system. Using this experimental setup they were able to identify 5036 nonredundant phosphopeptides of which only one originated from NFATc1 (Ser-233). This is four less than in our study, which was optimized for single protein analysis. This illustrates the fact that while shotgun methods produce enormous amount of data, methods that allow comprehensive analysis of specific proteins of interest can provide a more complete analysis of that protein's phosphorylation state. Therefore targeted approaches are needed alongside large-scale studies.

5.5. Final remarks

LC-MALDI (I) provides a robust platform for off-line sample fractionation. When the sample is deposited onto a MALDI target with the matrix it provides time-independent MS analysis. This in turn allows the sample to be re-analyzed after a database search, either for more protein identifications as well as characterization of specific peptides. For this approach to be successful I believe that we need a MALDI matrix that will not be consumed from the MALDI target after the first round of analysis. When combining this technique with surface affinity enrichment for phosphopeptides (Publications II, IV), analysis of more complex samples, than single proteins, with planar surface enrichment becomes possible. This in turn opens up more interesting prospects for testing and analyzing samples with LC-MALDI, with or without phosphopeptide enrichment, using multiple matrices (III). A number of different chromatography techniques, which have already been mentioned in this thesis (HILIC, SCX, SAX, IMAC), could be used in addition to reversed phase chromatography. However, different software tools should be available to adequately measure and report the performance and findings. Firstly, non-redundant analysis should be available by using exclusion lists from the repeated analyses. Secondly, combining the spectra in the MS level and also in MS/MS level before the database search should be implemented into the overall workflow. Combining spectra from multiple matrices at the MS level could guide the MS/MS analysis, however this is not possible currently.

All the information is already in a cell and the scientists are trying to find the way to visualize and detect that information. This is done by developing different sample treatments and different sample handling methods, and includes the use of different enzymes, purification methods, ionization techniques, mass spectrometers and different mass spectrometry methods. I have started to address some areas that could be further developed. This would allow us to further exploit MALDI-MS capabilities. The trend in MS is currently toward faster repetition rate, higher accuracy and resolution. I

believe that with the points addressed in this work, MALDI can be developed in an application driven manner, as well as the technology driven approach pursued by MS companies.

6. SUMMARY

In this Ph.D. thesis, different methods for mass spectrometry based proteomics have been developed. The scope was to develop methods that would enable the systematic qualitative characterization of protein phosphorylation states and to apply these methods to phosphoproteomics projects. These aims were party fulfilled, partly not.

The first aim listed in "Aims of the study" (section 3) was to develop a specific MALDI matrix that would provide higher quality MS information for improved protein identification. It was indeed possible to develop such a matrix in publication I, which was durable and provided superior quality data compared to other matrices and sample preparation techniques. Nitromatrix was used in study (III) where it added valuable and unique information for improved phosphopeptide identification and characterization.

The second aim was to develop new methods to enrich phosphopeptides and proteins in an efficient manner using only minimal sample treatment steps. This aim was achieved first in publication (II) and again in publication (IV). In publication (II) a phosphopeptide affinity MALDI target was prepared by coating it with a material that is known to have affinity for the phospho-group of phosphopeptides. In the same study expansion of the application of the method by capturing phosphopeptides directly from tissue was attempted. As discussed previously, applying the technique to tissues was extremely challenging. Publication (IV) continued to evaluate on-target purification of phosphopeptides from simple peptide mixtures. In this study commercially available ITO coated MALDI targets were used, similar to those that are used in IMS. A new protocol was developed that enabled fast and sensitive phosphopeptide purification, which was ultimately applied to characterize an *in vivo* phosphorylated signalling protein, NFATc1. Three new phosphorylation sites were identified in the protein.

In publication (III) methods to identify phosphorylation sites without any purification methods as developed. Different matrices were used to analyze the same protein, after which the MS data was combind. This approach identified more phosphopeptides than conventional LCESI-MS/MS methods, and used 5 times less sample. However, a complete evaluation of the method is hindered by lack of efficient software tools.

The last aim was the integration of all the above tools and their application in phosphoproteomics projects. The aim was not completely fulfilled due to 1) unsolved problems in surface chemistry when applying high organic solvents on a phosphopeptide purification surface (discussed in publication IV), and 2) a deficit in targeted software tools to handle the suggested analysis pipeline. These obstacles are now under investigation. Nevertheless, the presented techniques can be used individually and can be further optimized. The optimization will be continued at the same time as their integration into a unified analytical workflow.

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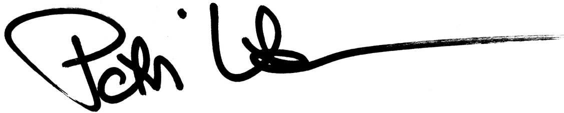

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