Development of Sensitive Proteomic Approaches for Protein Tyrosine Phosphorylation Detection

A thesis submitted for the degree of

Doctor of Philosophy

as a combination of conventional narrative and portfolio of publications by

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Abstract

The elucidation of the complex array of cell signalling cascades is imperative for a deeper understanding of cell biology in both physiological and patho-physiological states. Extensive biochemical characterisation of signalling networks has revealed the importance of post-translational modifications (PTMs), particularly phosphorylation. Signalling via protein phosphorylation occurs across homeostatic proliferative, differentiative and anti-apoptotic events. Dysregulation of the kinase signalling pathways as well as mutations in kinases involved in phosphorylation have been implicated in a number of pathologies such as cancer or immune deficiencies. While it is estimated that 50% of all proteins are phosphorylated during their lifetime, phosphorylated proteins are present in relatively low abundance compared to their non-phosphorylated counterparts. The rarity of phosphorylation, which occurs on serine, threonine and tyrosine residues, has prompted the development of sensitive approaches to improve phosphorylation characterisation. Proteomic-based strategies offer novel approaches to overcome the limitations of currently available strategies for phosphoprotein analysis. The research presented within describes the development of proteomic-based methodologies for phosphotyrosine identification, quantitation and characterisation. These methods utilise the anti-phosphotyrosine Antibody 4G10 along with other MS-compatible approaches for phosphotyrosine enrichment prior to MS analysis. Methods for more targeted phosphoprotein analyses involved coupling of 4G10 covalently to super para-magnetic beads or by affinity to super para-magnetic beads with protein G covalently attached. These 4G10-coupled beads successfully enriched tyrosine phosphopeptides derived from tryptic phosphoprotein digests for identification and characterisation of phosphopeptides using MALDI-TOF/TOF MS analysis.

The limited capacity of the magnetic bead approach for analysis of more complex samples necessetated the development of a more global proteomic strategy for tyrosine phosphorylation analysis. A global strategy that provides not only qualitative pTyr information but also shows quantitative changes that occur with pTyr signalling is imperative for detailed signalling cascade analyses. The global approach presented here utilised the 4G10 Ab/bead approach as well as Hydrophilic interaction chromatography (HILIC) for the enrichment of pTyr peptides from complex samples isotopically-labelled to quantify tyrosine phosphorylation after LC-MALDI-TOF/TOF MS analysis. Aspects of this approach were modified to improve phosphopeptide detection and characterisation, including the development of a novel optimised matrix-deposition strategy for LC-MALDI-TOF/TOF MS. The strategy, termed EZYprep LC, allowed the effective use of the atypical 2,5-

DHB matrix with phosphoric acid to improve phosphopeptide ionisation and subsequently identify and characterise more phosphorylation sites on phosphoprotein samples compared with LC-ESI-IT-MS/MS.

Another aspect of the global strategy was the development of a modified isotope protein coded label strategy (modified ICPL). The optimised ICPL approach ensured quantitative information from a larger sub-set of peptides after tryptic digest of complex samples. The improved ability to quantify using this approach was highlighted by a comparative analysis of complex cell lysates labelled using the conventional ICPL strategy and the modified ICPL strategy. The modified ICPL labelling strategy identified more proteins and provided more quantitative information that the conventional ICPL methodology. As such, the global phospho-tyrosine strategy, combined the modified ICPL labelling and 4G10 Ab/bead enrichment with peptide fractionation and MALDI-TOF/TOF MS analysis, was subsequently utilised to identify and quantify tyrosine phosphorylation occurring in insulin-stimulated insulin receptor A- and B-subtypes.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Mark Condina and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (as listed in the publications list below) resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Mark Condina

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"The larger the island of knowledge, the longer the shoreline of mystery." *Unknown author*

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Publications

Within Thesis:

Condina, M. R., Guthridge, M. A., McColl, S. R., Hoffmann, P., A sensitive magnetic bead method for the detection and identification of tyrosine phosphorylation in proteins by MALDI-TOF/TOF MS. *Proteomics* 2009, *9*, 3047-3057 – **Chapter 2**

Condina, M. R., Gustafsson, J. O. R., Klingler-Hoffmann, M., Bagley, C. J., McColl, S. R., Hoffmann, P., EZYprep LC-coupled MALDI-TOF/TOF MS: An improved matrix spray application for phosphopeptide characterisation. *Proteomics* 2010, 10, 2516-2530 – **Chapter 3**

Arising from Thesis:

Condina, M.R., Klingler-Hoffmann, M. and Hoffmann, P. Tyrosine Phosphorylation Enrichment and Subsequent Analysis by MALDI-TOF/TOF MS/MS and LC-ESI-IT-MS/MS. *Current Protocols in Protein Science* 2010, 62:13.11.1-13.11.26 – **Appendix 7B**

Commonly-Used Abbreviations

µL	Microlitre
µ-WPS	Micro-Well-plate-sampler
1-DE	one-dimensional poly-acrylamide gel electrophoresis
2-DE	two-dimensional poly-acrylamide gel electrophoresis
Å	Angstrom
AA	Amino acid
Ab	Antibody
Abs	Antibodies
AC	Affinity Chromatography
ACN	Acetonitrile
ACQA	Absolute quantitation
BS	Binding solution
BSA	Bovine Serum Albumin
C*	Carboxamidomeythl cycteine
CHCA	α-cyano-4-hydroxycinnamic acid
CHCA SMW	CHCA sample matrix wash
CID	Collision induced dissociation
Cov-P	Covalently-coupled 4G10 MB-covAC-Select
Da	Dalton
DD	Dried Droplet
DE	Delayed extraction
DHB	2,5-dihydroxybenzoic acid
DHB ML	DHB matrix layer
DIGE	Difference Gel Electrophoresis
DTT	Dithiothreitol
e	Elementary charge
ECD	Electron capture dissociation
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
E_k	Kinetic
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
FA	Formic acid
FACS	Fluorescence activated cell sorting

Fc	Fraction Collector
fmol	Femtomole
FTICR	Fourier-transform ion cyclotron resonance
G-250	Colloidal coomassie
GFPB	Glufibronopeptide B
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAP	Hydroxyapatite enrichment
HCl	Hydrochloric acid
HE	Hydrophobic eluate fraction
HFT	Hydrophobic flow through fraction
HIC	Hydrophobic interaction Chromatography
HIE	Hydrophilic eluate fraction
HIFT	Hydrophilic flow through fraction
HILIC	Hydrophilic Interaction Chromatography
HPLC	High performance liquid chromatography
I.D.	Inner diameter
IAA/IAM	Iodoacetamide
IC_{50}	The half maximal inhibitory concentration
ICAT	Isotope coded affinity tags
ICPL	Isotope coded protein labels
IDA	Iminodiacetic acid
IEX	Ion exchange Chromatography
IGF	Insulin-like growth factor
IGFBPs	IGF-binding proteins
IMAC	Immobilised metal affinity Chromatography
IP	Immuno-precipitation
IPA	Ingenuity pathways analysis
IR	Insulin Receptor
ISD	In-source decay
IT	Ion Trap
iTRAQ	Isotope tagging for relative and absolute quantitation
L	Length
LC	Liquid Chromatography
LDS	Lithium Dodecyl Sulphate
LDS	Lithium dodedyl sulphate
LIT	Linear Ion Trap
m	Mass

M*	Oxidised methionine
m/z	Mass-to-charge
MALDI	Matrix assisted laser desorption/ionisation
MAP	Mitogen-activated protein kinase
MAPK	MAP kinase
MB-covAC-Select	Magnetic bead based covalent affinity Chromatography for binding of freely selectable proteins
MB-IAC Prot G	Magnetic bead based immunoaffinity Chromatography on immobilised protein G
MB-IMAC Fe	Magnetic bead-IMAC Fe
MCP	Microchannel plate
MOAC	Metal oxide affinity Chromatography
Mr	Relative molecular mass
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS/MS	Tandem MS
NaF	Sodium fluoride
nLC	Nano-LC
NP-40	Nonidet P-40
NTA	Nitriloacetic acid
OVA	Ovalbumin
PA	Phosphoric acid
PAGE	poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
pI	Isoelectric point
PM1	Protein mix 1 (1 pmol BSA, 1 pmol OVA, 2 pmol β-cas)
pmol	Picomole
PMSF	Phenylmethanesulfonylfluoride
Pro-GP	4G10 affinity-coupled MB-IAC Prot G
PSD	Post-source decay
pSer	Phosphoserine
PTEN	Phosphatase and tensin homolog
pThr	Phosphothreonine
PTKs	Protein tyrosine kinases
PTMs	Post-translational modifications
PTPs	Protein tyrosine phosphatases
pTyr/pY	Phosphotyrosine
PVDF	Polyvinylidene fluoride

Q	Quadrupole
QQQ	Triple quadrupole
qTOF/QTOF	Quadrupole time of flight
Rf/RF	Radio frequency
RIPA	Radioimmunoprecipitation assay buffer
RP	Reverse Phase Chromatography
R^{-ve}	IGF-1R-deficient mouse embryo fibroblast
SA	sinapinic acid
SAX	Strong anion-exchange
SCX	Strong cation-exchange
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEC	Size exclusion Chromatography
SF1	Sheath fluid containing 0.05% PA/0.05% TFA/50% ACN
SILAC	Stable isotope labelling by amino acids in cell culture
SILE	Stable isotope labelling experiment
SIMAC	Sequential elution from IMAC
SRM	Single reaction monitoring
t	Time
TBST	Tris-buffered saline Tween-20
TED	Tris(carboxymethyl)ethylenediamine
TFA	Trifluoroacetic acid
TiO_2	Titanium Dioxide
TIS	Timed ion selector
TL	Thin-layer
TLC	Thin-layer Chromatography
TOF	Time-of-Flight
U	Voltage
v	Velocity
WAX	Weak anion-exchange
WCX	Weak cation-exchange
YAG	yttrium aluminium garnet
z	Charge
β-cas	β-casein
βic	β-intracellular domain of the granulocyte-macrophage colony-stimulating factor

1 Introduction

1.1 Proteomics

1.1.1 Proteomics

The term Proteomics, initially coined in 1996 by Wilkins [1] is the name given for identifying and characterising the proteome of an organism. The proteome consists of the entire protein complement expressed by the genome of a given cell at a given time. Identical organisms can have the same genome yet display an almost unrecognisable proteome. The completion of the human genome project saw the realisation that a deeper understanding of the proteins involved in normal function and pathology was required as a certain level of gene transcription will not represent the same level of protein translation. Compounding this complexity are the estimated 100 possible post-translational modifications involved in protein function and cell signalling. Perturbations in cell signalling are known to be imperative for a variety of pathologies, drastically altering cellular protein levels. The need for techniques that provide both qualitative and quantitative information is required to decipher the proteome. These can be categorised into four main aspects – protein separation, identification, characterisation and quantitation techniques.

1.2 Protein separation strategies

In terms of proteomic analysis, much emphasis has been placed on the development of strategies capable of resolving all proteins/peptides in a given proteome. At present, no one separation strategy has the capability of achieving this. This is due to the large protein concentration dynamic range of a given proteome. As a result, multidimensional strategies using a combination of separation techniques have been utilised to obtain complete resolution of proteins present in a complex mixture [2]. These separation strategies can be categorised into electrophoretic and chromatographic methods.

1.2.1 Two-Dimensional electrophoresis (2-DE)

Gel electrophoresis is considered as one of the most common of the separation strategies used in proteome analysis. This is due to its ability to separate a large component of the proteome in a reproducible and stable manner. In particular, high resolution two-dimensional poly-acrylamide gel electrophoresis (First described by [3], 2-DE) has been utilised for multiple proteomic analyses. It

involves the separation of proteins on the basis of isoelectric point (pI) in the first dimension, followed by molecular weight separation in the second dimension using poly-acrylamide gel electrophoresis (PAGE) [3, 4]. Using this method, one can simultaneously separate and resolve proteins over a wide isoelectric range (pH 3-11) and molecular weight range (from >10 kDa, <300 kDa). As an added advantage, separation using this technique provides a way to isolate proteins of interest in a manner that is compatible for downstream proteomic analyses. As such, it is a method used routinely to study global analyses of a variety of cellular processes including protein synthesis, glycolysis, gluconeogenesis, nucleotide and amino acid (AA) biosynthesis, lipid metabolism and stress responses [5-8]. Analyses of phosphorylation and other post-translational modifications for signal transduction pathways are also achievable with this separation strategy [7]. Phosphorylation, for example, changes the net charge of the protein and is often visualised on a 2-DE gel by a horizontal trail of protein spots [9]. In 1997, Unlu *et. al* [10] described Difference Gel Electrophoresis (DIGE), a method coupled to 2-DE that involves the labelling of two protein samples with spectrally resolvable fluorescent dyes referred to as Cy2, Cy3 and Cy5. The differentially-labelled protein samples (Cy3, Cy5) are combined with a pooled internal standard (equal amounts of both samples to be analysed that are labelled with Cy2) and simultaneously resolved using 2-DE. Running both the internal standard and protein samples on one gel removes experimental variation and as such, enhances the accuracy of measuring changes in protein regulation between the samples of interest (relative to the pooled standard). The subsequent analysis of differentially expressed protein spots from the gel using mass spectrometry (MS) enables researchers to obtain both qualitative and quantitative information on proteins of interest [11]. Despite the advantages of 2-DE DIGE for protein quantitation and identification, the method has some key limitations [7, 12, 13]. Very hydrophobic proteins (including membrane proteins) and basic proteins are unable to be sufficiently resolved, nor are low abundant proteins [14] or proteins that are of high molecular weight [15]. Despite aspects of the methodology being improved (e.g. the introduction of pre-cast gels) it is difficult to automate and hence may not be considered as a high throughput proteomic strategy [16]. Separation methods capable of addressing these limitations which would complement the robust 2-DE approach have been concurrently developed, including liquid chromatography [17].

1.2.1.1 High performance liquid chromatography (HPLC) overview

It is estimated that over 30% of signal transduction pathways involved in protein products of the human and mouse genome are mediated by receptors [18-20]. These pathways are initiated from direct physical contact between the receptors and their effector and regulatory molecular targets [18, 19]. These membrane proteins play vital roles in a multitude of signal transduction pathways, providing a key link between the external environment and internal cellular processes [21]. Changes in receptor

localisation, expression, and post-translational modifications (PTMs) have been shown to impact receptor functionality [22, 23]. Membrane proteins are difficult to resolve using 2-DE, mainly due to their increased hydrophobicity compared to cytosolic proteins. Fractionation of a proteome into its sub-cellular components can aid the overall resolution required for detailed analysis. The use of Liquid chromatography (LC), in particular HPLC, either separately or in combination with gel electrophoresis, has become the mainstream methodology for complex proteome analysis [2, 24]. LC strategies are advantageous as both proteins and peptides can be separated using a variety of column chemistries (Table 1.1), including Reverse Phase (RP), size exclusion, ion-exchange chromatography (IEX), and affinity chromatography, whilst always in the liquid phase. This is particularly important as sample loss is minimised by removal of protein/peptide extraction processes (from solid strategies – i.e. PAGE) and can be coupled directly to identification strategies, namely MS [24].

Property	Technique
Size	Size Exclusion chromatography (SEC), Gel Filtration
Charge	Ion-exchange chromatography (IEX), Chromatofocusing
Bio-recognition (ligand specificity)	Affinity chromatography (AC)
Hydrophobicity	RP chromatography, Hydrophobic Interaction chromatography (HIC), Hydrophilic Interaction Chromatography (HILIC)

Table 1.1: Protein chromatographic separation strategies (adapted from [25]).

The principle of protein/peptide LC separation involves the manipulation of either the mobile (solvent) phase or the stationary phase of the column. The chemistry used to separate complex mixtures is dependent on class of proteins/peptides to be separated.

1.2.1.2 Ion-exchange chromatography (IEX)

When considering potential separation strategies, it should be considered whether the strategy is compatible with down-stream methods, such as mass spectrometry (MS). IEX, which is able to effectively purify samples from undesirable solvents, concentrate diluted samples as well as separate components makes this strategy a popular first choice for protein LC separation [25]. In fact, the

combination of strong cation exchange chromatography (SCX) and RP is the most widely used strategy for complex peptide mixtures, providing orthogonal separation characteristics [26, 27].

IEX exploits the natural charge state characteristics of proteins, which are dependent on the AA side-chains in the sequence, as well as the carboxyl and amino termini, bound ions and PTMs. The nature of ion-exchange groups as well as the sorbent they attach to is the main parameter used to separate complex mixtures using IEX [28]. Taking advantage of the unique relationship of a protein's net surface charge and pH, reversible interactions between charged molecules and IEX media are used to enrich, separate and subsequently elute molecules of interest [25, 29]. Alteration of pH such that it is higher than the isoelectric point will result in a protein binding to positively-charged IEX media (anion exchange), while a pH lower than the isoelectric point will result in a net positive charge, thus binding to negatively-charged IEX media (cation exchange) [25, 28-30]. The stronger the net charge of the protein, the more strongly the protein will bind to the charged stationary phase. One can optimise conditions such that proteins of interest can be enriched, providing the pH of the mobile phase is set such that the protein has an overall net charge. Once bound, charged species of interest can be eluted using competing ions of the same charge in the mobile phase [25, 27, 29, 31].

A variety of stationary phases are currently utilised for IEX, depending on the type of separation and the aims of the research to be completed. Stationary groups whose charges are stable at any pH range, such as sulfonate groups (cation exchange) and tertiary or quaternary amines (anion exchange) are termed strong ion exchangers. Utilising strong cation-exchange (SCX) or strong anion-exchange (SAX) is advantageous as interaction mechanisms are simplified, minimising the number of parameters that need to be optimised. Both SCX and SAX have been successfully used for phosphopeptide enrichment [32-37]. However, the use of stationary phases whose charge state alters depending on pH, termed weak (e.g. weak cation-exchange [WCX], weak anion-exchange [WAX]) can be advantageous for improving retention and subsequent elution of particular proteins of interest [25-28, 31].

1.2.1.3 Size Exclusion Chromatography (SEC)

SEC is another separation strategy that has been employed for protein analysis in complex samples. It is particularly useful for samples that are sensitive to alterations in conditions such as pH or temperature, separating solely on the basis of size. In SEC, polymer beads with pores of different sizes are used to separate proteins. The smaller the protein, the longer the path length, as smaller proteins

will enter the pores of the beads. This results in the separation of proteins depending on size alone [25, 29, 38]. Although SEC has been successfully used in research strategies alone [39-41], for proteomics it is generally used as part of a multi-dimensional separation strategy for proteins [42-44]. This is due to the fact that samples are generally diluted over the course of the separation in mobile phases that are not compatible with downstream processes such as MS [25].

1.2.1.3 Affinity chromatography (AC)

Of the separation strategies that can be utilised, AC can ensure purification of molecules of interest from complex mixtures (as outlined in Figure 1.1). The method exploits selective interactions, both biological and chemical, between molecules attached to a stationary phase and its partner to be enriched, which can then be reversed to release analytes of interest [25, 45, 46]. The type of affinity matrix used will depend on the class of proteins or peptides to be separated, such as lectin columns for glycoproteins or streptavidin columns for biotin-labelled proteins or peptides.

NOTE:
This figure is included on page 5
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.1: An outline of AC utilising bound Ab for antigen (Ag) enrichment from a sample mixture [adapted from [47]].

This example of AC utilises bound Ab (on a solid support, in this case beads) to purify a targeted Ag that is contained in a complex mixture. The mixture is loaded into a column and allowed to run over the Ab-conjugated beads. Molecules that do not bind to the Ab pass through while the Ag of interest remains bound in the column. After washing, the Ag is eluted from the column into a new tube.

In terms of protein enrichment, it is possible to not only enrich proteins exclusively on the basis of biological function or structure, but also enrich on the basis of modifications of proteins, including biological post-translational modifications (e.g. phosphorylation) [34, 48-61], recombinant modifications (e.g. poly-histidine tag) [25, 62-64] and chemical modifications (e.g. Biotin addition) [64, 65]. Affinity matrices with covalently bound substrates (e.g. small peptides sequences that proteins of interest recognise) are also used to selectively purify proteins, providing the proteins of interest are in their biologically active conformation. The ability to conjugate antibodies (Abs) or ligands for Abs, termed immuno-affinity chromatography, has resulted in this separation strategy being a popular technique for protein purification and enrichment [25, 45]. The use of Abs in immuno-affinity chromatography for protein purification utilises affinity matrices that either recognise the Fc region of Abs (i.e. Protein A or G covalently attached to beads) or form direct covalent interactions through amide bonds with the Abs [46, 66, 67].

1.2.1.4 Reverse-Phase (RP) chromatography

The most common separation strategy used in proteomics, RP chromatography will separate protein and/or peptide mixtures on the basis of peptide hydrophobicity, where an increasing concentration of organic solvent in the mobile phase is used to separate proteins or peptides bound to a stationary hydrophobic phase (e.g. silica with carbon chains attached). A summary of the interaction that occurs in RP chromatography is shown in Figure 1.2

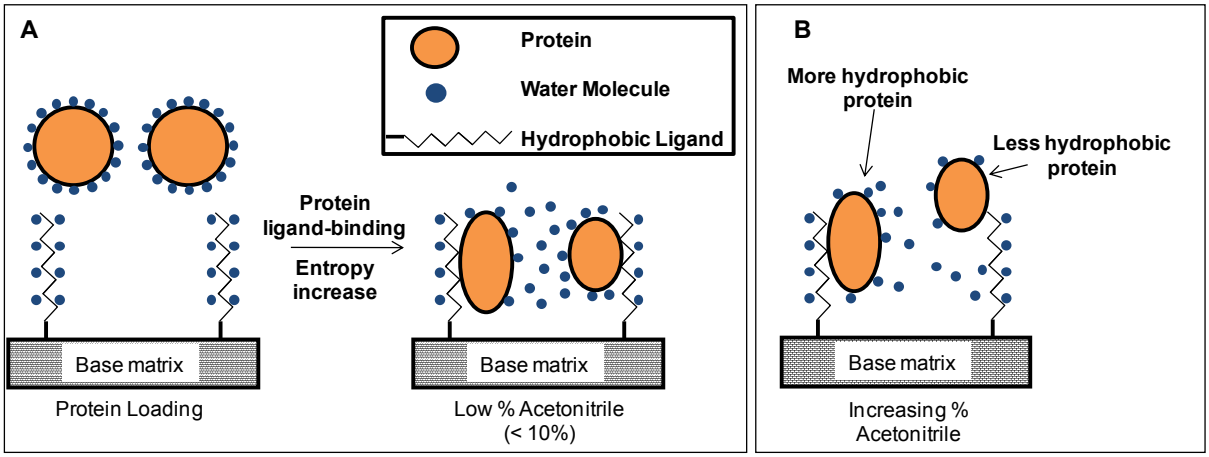

Figure 1.2: Schematic outlining the hydrophobic interaction between proteins with a hydrophobic ligand on a stationary support (A). Figure 1.2B outlines how proteins are separated using an increasing polar solvent gradient [Adapted from [25, 68]].

As Figure 1.2 illustrates, proteins and the hydrophobic ligand in aqueous conditions (binding conditions) are surrounded by molecules (from the aqueous mobile phase) in a structured way. Although the exact mechanism of RP is still debated, it is believed that when proteins bind to the immobilised hydrophobic ligand, the hydrophobic region of the protein becomes unprotected from water molecules. This reduction in the exposed hydrophobic region to water corresponds with a favourable increase in system entropy. In order to alter the polarity of the solvent and thus separate proteins with varying hydrophobicity, a gradient of increasing organic solvent, such as acetonitrile (ACN) is applied, resulting in the less hydrophobic proteins losing affinity for the immobilised ligand, thus being released [25, 68-71]. Multiple immobilised supports are available for hydrophobic separation of both protein and peptide. The stationary phase selected is dependent on whether separation is to occur on a protein or peptide level [25, 68]. In peptide separations stationary phases with pore sizes between 80-100 Angstroms are used, whereas in protein separation, the pore size of the stationary phase is larger, around 300 Angstroms.

The hydrophobicity of proteins and peptides is determined by a number of properties, including the AA sequence, and the tertiary and quaternary structure for proteins [68]. AAs are categorised into different groups based on their side-chain, namely acidic, basic, neutral, hydrophilic and hydrophobic [24]. The AA sequence of a peptide can subsequently be used to predict elution times of peptides during a gradient of organic solvent [24, 68]. The resolving ability of RP can be optimised by alteration of the mobile phase, namely in terms of ion-pairing reagents and pH. A lowering of pH (generally around pH 2) ensures a more unified charge state of a peptide or protein, increasing overall hydrophobicity of the

stationary phase when used in combination with an ion-pairing reagent [72-75]. The homologous series of volatile perfluorinated acids, such as TFA, are routinely used for RP-HPLC of peptide samples, particularly when coupled to MALDI-TOF/TOF MS [74, 76-78]. This is primarily due to its increased hydrophobicity over other ion-pairing reagents such as phosphoric acid (PA), thus not only acting as an effective ion-pairing reagent for basic residues but also improving peptide binding affinity to solid phase RP supports. The volatile nature and UV transparency of TFA at low concentrations are also advantageous. The high volatility does not interfere with peptide protein ionisation in MS and the UV transperancy improves data output (by minising the baseline interference of ion-pairing reagents) using UV traces to determine separation efficiency [73, 74, 77, 78]. In contrast, PA is more hydrophilic than TFA, yet has been shown to be as sensitive as TFA for peptide separation. In fact, its increased hydrophilicity has been shown to decrease overall peptide retention times; meaning better separation of complex samples [74, 77]. This is because slightly increasing the hydrophilicity of peptides will mean peptides will begin to elute earlier in the gradient, aiding effective separation of complex peptides using the same gradient [74].

In proteomics, large-scale analyses typically involve the use of two separation strategies prior to MS analysis, and here RP-HPLC is usually used as second strategy directly before MS analysis [26, 27, 79-83]. This is due to the fact that RP-HPLC is able to not only de-salt, concentrate and separate proteins or peptides, but able to do so in mobile phases suited for MS downstream analyses. Many proteomic strategies, therefore, involve RP-HPLC in their analysis of proteins or peptides prior to MS for identification of proteins/peptides in complex samples.

1.3 Mass spectrometry: Protein identification and characterisation

MS refers to the study of gas-phase ions and is used as an effective tool for the identification and characterisation of various molecules by measuring the mass-to-charge ratios (m/z, unit = Thompson [84]) of ions [25, 85-87]. These ions are formed as a result of a loss or gain of a charge from a neutral species, directed to a mass analyser via electrostatic forces, separated on the basis of m/z and subsequently detected [85, 86]. Since the development of the first mass spectrometer (then called a parabola spectrograph) by Sir J.J Thompson, a number of major breakthroughs in MS has enabled this technology to be utilised universally for both chemical and biological analyses [86]. Mass spectrometers

typically consist of three major components; an ionisation source, a mass analyser and a detector (Figure 1.3) and these ultimately determine the capabilities and limitations of mass spectrometers.

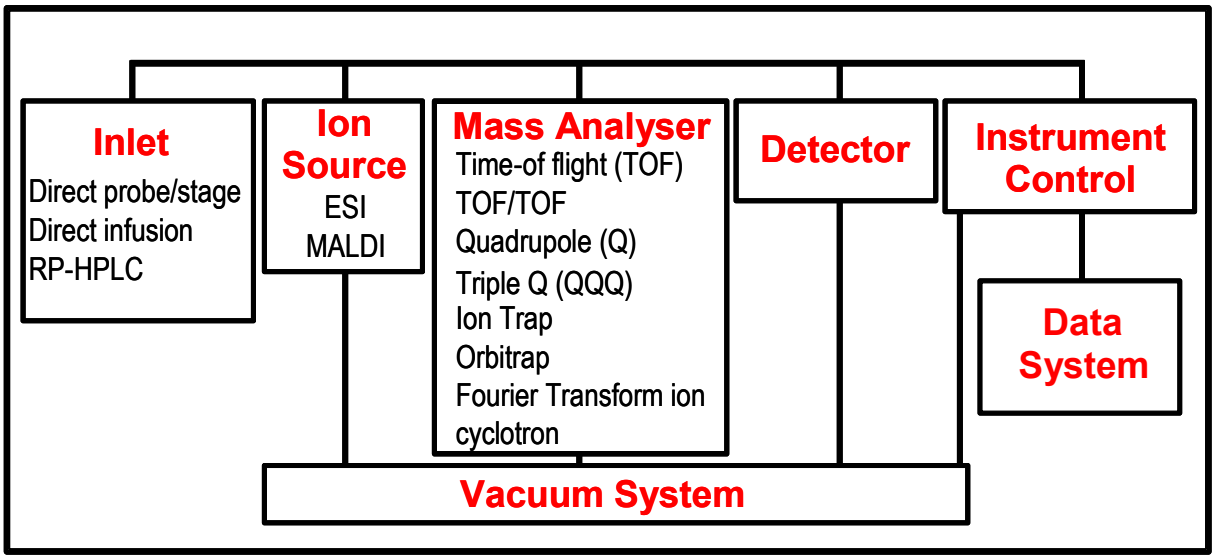

Figure 1.3: A diagram outlining the basic components of Mass Spectrometers [adapted from [25, 85, 86].

For the analysis of the proteome, MS has become the major hardware component, and this is due to the development of "soft-ionisation" MS techniques, referring to the conversion of intact analyte molecules (e.g. proteins and peptides) to corresponding ions [87]. Two techniques in particular, electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI) have developed into indispensable tools both in research facilities and pharmaceutical companies across the globe involved in analytical biochemistry [86, 88]. These ionisation processes coupled with various mass analysers has seen an explosion of the use of MS in biochemical studies. These analysers are based on magnetic and/or electric field interactions with the charged ions that are produced at the ion source [87]. Proteomic analyses typically utilise four types of mass analysers; quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear IT, LIT or LTQ), time-of-flight (TOF) mass analyser, and Fourier-transform ion cyclotron resonance (FTICR) mass analyser [89, 90]. In addition, hybrid instruments including QQQ, QQ-LIT, Q-TOF, TOF-TOF and LTQ-FTICR have been developed to combine advantageous capabilities, proving particularly useful for tandem MS analyses, commonly known as MS/MS [89, 90]. Discussing all mass spectrometer configurations is beyond the scope of this thesis. As a result, only ESI ion-trap (ESI-IT) and MALDI-TOF/TOF mass spectrometers will be discussed; the two configurations that account for a majority of proteomic data published [89].

1.3.1 ESI

ESI was originally conceived in 1968 but not developed for the analysis of biomolecules until almost two decades later by Yashamita and Fenn [86, 91, 92]. An outline of the ESI process is shown in Figure 1.4. ESI involves the spraying of analytes (e.g. peptides) dissolved in an acidic, aqueous solution, creating highly charged droplets in the presence of an electric field [25]. This occurs through a small diameter needle, which has a high positive charge applied to it, resulting in a spray formation of solvent. The positively charged droplets (which received protons from the solvent) move away from the positively charged needle toward the negatively charged (relative to the needle) entry of the mass spectrometer [25, 85, 86]. As this occurs, dry nebulising gas and/or heat are applied to the droplets under atmospheric pressure, resulting in the desolvation of charged ions. As the size of the droplets decreases, the Coulombic repulsion between the like-charges increases, such that it exceeds the forces of surface tension. This results in the expulsion of charged ions from the droplet, which subsequently enters the mass spectrometer [25, 85, 86].

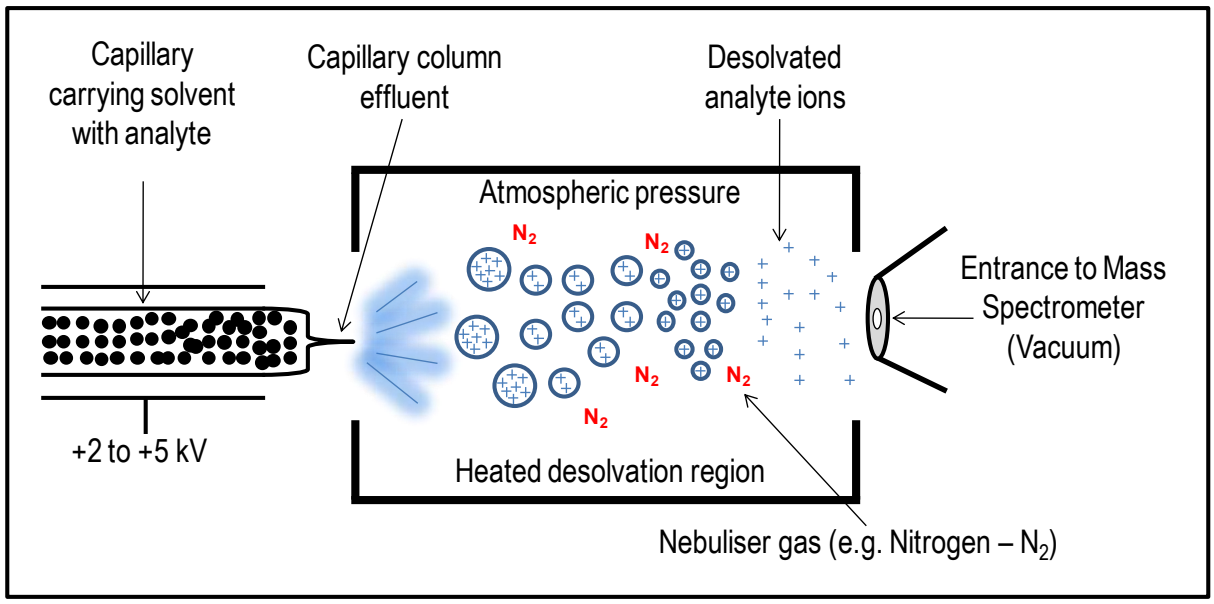

Figure 1.4: The formation of ions associated with ESI [adapted from [25, 85, 86]. In ESI, analytes dissolved in an acidified aqueous solvent are passed through a small diameter capillary, creating a mist of charged droplets. The charged droplets travel toward the entrance to the mass analyser due to the presence of an electric field. In this region, a dry nebulising gas and/or heat is applied to the droplets to desolvate the charged ions. As the droplet size decreases, the Coulombic force between like charges increases, resulting in the ejection of charged ions from droplets.

The ionisation of molecules using ESI results in the formation of ions with multiple charge states, due to the acidic conditions of the solvent [85]. For protein and peptide analysis in proteomic studies, this is particularly advantageous. The formation of multiply charged protein ions allows deduction of the molecular weight of proteins that are larger than the mass range of the mass spectrometer. For peptide analysis using ESI, the formation of multiply charged fragment ions using particular mass analysers for tandem MS/MS of peptides results in a larger amount of sequence information compared with only singly charged fragment ions. Additional advantages of ESI for proteomic analyses are its compatibility with HPLC (particularly RP-HPLC) and high ionisation efficiency resulting in a high sensitivity, typically down to the low femtomole to attomole range [85, 86, 93-96].

1.3.2 Ion Trap (IT) mass analyser

An IT (a three dimensional analogue of a quadrupole mass filter) consists of a ring electrode and two hyperbolic end caps, as outlined in Figure 1.5 [25, 85-87, 97-100].

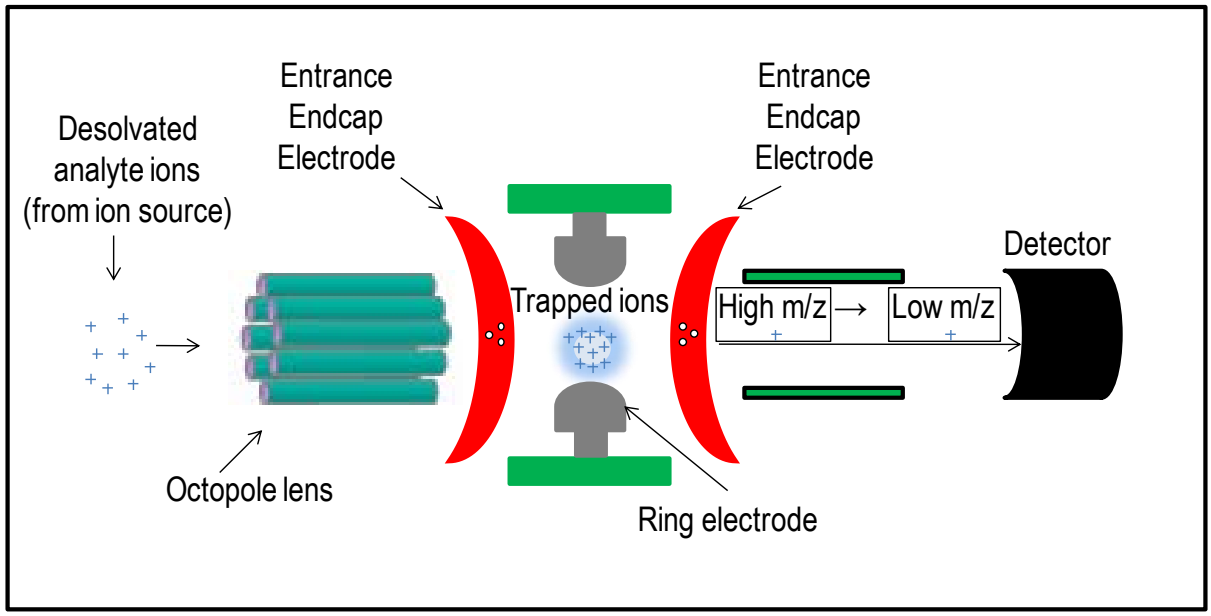

Figure 1.5: The ion trap mass analyser [adapted from [25, 85-87, 99]].

De-solvated ions from an ESI source enter the IT through a focusing device, shown in Figure 1.5 as an Octopole lens system. A radio frequency (rf) voltage is applied to the ring electrode, which creates a three-dimensional electric field. In addition, the inside of the IT is filled with helium. Entering ions begin to oscillate within the 3D field, and this oscillation is governed by both the rf voltage and the reduction in kinetic energy as a result of ions colliding with the helium gas [25, 85, 86, 99, 100]. The mass analysis

occurs by the scanning of the rf voltage to excite the trapped ions and increase the amplitude of oscillations, resulting in the ejection of the ions out the IT to the detector [100]. As the rf voltage is scanned to higher frequencies, ions with larger m/z become excited and subsequently exit the trap [86, 100]. This process is referred to as "mass-selective instability" as only ions with the selected m/z ratio are ejected from the IT [25, 85, 99, 100]. Mass Analysis using IT is particularly useful as it is possible to isolate one ion species by ejecting all others from the trap [86]. This is achieved by applying a waveform signal to the end cap electrodes. The isolated ion species (precursor ion) are fragmented by collisional activation with helium molecules after the application of a small voltage (rf pulse) across the end cap electrodes. The result of this collision between helium gas molecules and a selected ion species results in a conversion of kinetic energy to internal energy in the ion, resulting in fragmentation [85]. The fragments produced are then detected for analysis [25]. This fragmentation process, known as collision induced dissociation (CID), can be performed in real-time, allowing an extensive analysis of complex samples [25, 85, 86, 101]. Other advantageous properties of an IT analyser include its small size, its ability to be utilised for other MS/MS fragmentation strategies (e.g. MS^n and Electron transfer Dissociation – ETD) as well as its ability to accumulate ions of interest to ultimately provide better signal and therefore better fragment information for analyte identification and characterisation.

1.3.3 LC-ESI-MS/MS

LC-ESI-MS/MS consists of a HPLC system coupled directly to an ESI source for analyte ionisation, which subsequently enters a mass analyser, in particular an ion-trap (IT) mass analyser, for tandem MS analysis. This robust, high sensitivity as well as high throughput technology has seen LC-ESI-MS/MS, particularly nano-LC (nLC) ESI-MS/MS, become a powerful well established tool for proteomic research [102, 103]. Direct coupling of the LC to ESI-MS/MS allows analysis of complex biological samples, particularly protein samples digested with trypsin, that not only identify proteins and characterise the peptide sequences, but also supply quantitative information between sample types in a highly reproducible manner [102-105]. As shown in Table 1.2, the size of particle size of the stationary packing material, the column inner diameter (I.D.) in conjunction with the flow rate ultimately determines the sensitivity of the LC-MS/MS system. Taking this into consideration, nano-LC (nLC) systems produce the highest sensitivity when analysing small sample amounts compared with the other available formats and this, coupled with ESI-MS/MS, has proven to be most applicable for low abundant sample analysis. Despite the advantages of nLC-ESI-MS/MS for large-scale proteomic analyses, it does have some limitations, including a relatively low mass accuracy as a result of the limited number of ions that are able to be accumulated in the IT before distortion of ion distribution in the trap occurs due to space-charge effects [102]. In addition, as samples are analysed in real-time, collection of redundant data can

occur and due to the nature of ESI, sample is used up during the analysis [103]. A major problem facing nLC-ESI-MS/MS analysis of complex peptide samples is that the number of peptides eluted after chromatographic separation often exceeds the maximal scan rate at which mass spectrometers can acquire MS/MS data, which results in a number of ions of interest being excluded from MS/MS analysis [103, 106, 107]. However, the development of other ionisation sources, such as matrix assisted laser desorption/ionisation (MALDI), as well as mass analysers, like TOF mass analysers, provides researchers with multiple MS tools for complex sample identification and characterisation.

Scale	Column I.D. [µm]	Column volume (100 mm length) [µL]	Typical flow rate [µL/min]	Gain in sensitivity
Analytical	4600	1700	1000	1
Narrow	2100	350	200	5
Micro	1000	80	50	21
Capillary	300	7	1 to 4	237
Nano	75	0.5	0.2 – 0.5	3322

Table 1.2: Protein chromatographic separation methods (adapted from [108-110]).

1.3.4 MALDI

The "soft ionisation" technique, MALDI, was first described for proteins in 1988 by Hillenkamp, Karas and Tanaka [86, 88, 111-114] . Since then, it is now used for the analysis of proteins, peptides, and most other biomolecules, including carbohydrates, lipids and oligonucleotides [86]. The ionisation process revolves around the use of a small chemical laser absorbing organic molecule (termed matrix). The matrix and sample are co-crystallised (in excess over sample) onto a metal surface (MALDI target plate), as outlined in Figure 1.6. A laser (either an N_2 or a yttrium aluminium garnet [YAG] pulse laser) is used to vaporise the sample/matrix crystal resulting in a gaseous plume. Here, both sample and matrix are ionised for mass analysis (Figure 1.6).

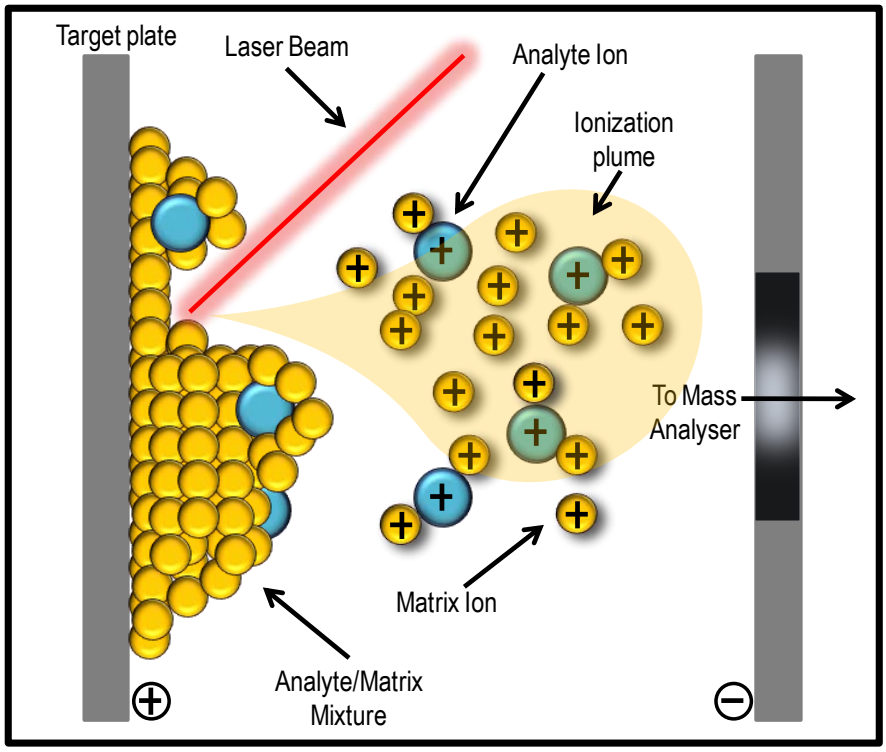

Figure 1.6: The MALDI Ion source [adapted from [86, 115]].
The MALDI process involves the vaporisation of analyte embedded within a solid matrix using a laser under vacuum. The result is the formation of a gaseous plume in which both analyte and matrix molecules are ionised. These ions are subsequently accelerated toward the mass analyser by electric fields.

Shown in figure 1.6 is an outline of the process of ionisation in a MALDI ion source. Despite the widespread applications of MALDI, the fundamental mechanisms that lead to the formation of charged matrix and analyte molecules remains poorly understood [88, 116, 117]. Models explaining MALDI ion formation have been proposed [116, 118-121], yet the most accepted model is that termed the "lucky survivor" model proposed by Karas [116]. In this model, proteins and peptides become charged as they are incorporated into the matrix. These charged species then undergo a process of re-neutralisation within desorbed matrix and analyte clusters [88, 116]. In the photoionisation process, electrons and protonated matrix species are formed. Conditions in the gaseous plume favour electron neutralisation and proton neutralisation of negative-ion species due to the high rate of reaction and high density of positive ions, respectively [116]. The result is a dominant neutralisation process in which the only lucky survivors are singly charged ion species as these have a high significant probability of surviving [111, 116]. This model explains the observation that mostly singly charged ions are detected following MALDI [88, 116, 122]. This model highlights the importance of the matrix not only for sample dilution and separation into solid phase, but also for absorbing the majority of the incident laser energy [88, 116]. Various matrices exist and their selection is dependent on the sample to be analysed. These include α-

cyano-4-hydroxycinnamic acid (CHCA) for peptides [123], sinapinic acid (SA) for proteins [124] and 2,5-dihydroxybenzoic acid (DHB) with phosphoric acid for phosphopeptides [59, 125]. Irrespective of the type of matrix used to embed sample for MALDI, the resultant singly charged ions are accelerated toward a mass analyser for detection (Figure 1.6).

1.3.5 MALDI-TOF/TOF MS

A widely adopted technique utilises a MALDI ion source coupled to either one or a series of TOF mass analysers (MALDI-TOF or MALDI-TOF/TOF MS) to provide a highly resolved measurement of ionised samples at high sensitivity (down to the attomole range) in a high throughput capacity [126-129]. The TOF mass analyser is particularly suited to MALDI ion sources because they produce ions in pulse cycles and of masses greater than 1 mega Dalton (MDa). TOF analysers are ideal as they are capable of measuring large ions [88]. TOF analysers determine the m/z ratio of ions by measuring the travel time through a field free drift region of the TOF tube. Ions with a smaller m/z have a higher kinetic energy than ions with a higher m/z, due to the acceleration of ions at the ion source by electric fields [88]. Large ions with high mass to charge ratios (m/z) have a greater TOF value and strike the detector later than small ions (Hillenkamp, Karas et al. 1991; Suckau, Resemann et al. 2003). Once through, the ion hits a detector, such as a microchannel plate (MCP). The MCP converts ion hits to the detector face into electrical pulses [88, 129]. As such, a digital storage oscilloscope is used to plot the ion current signal versus the ion arrival time. In axial TOF instruments, ions are accelerated by a given voltage [U] between the sample support and a nearby grid or open electrode, to a kinetic energy E_k before travelling down a flight path to the detector. Thus,

$E_k = zeU$, where z is charge and e is elementary charge.

Ions with the same E_k have different velocities [v], depending on their mass [m]. Therefore,

$E_k = 1/2mv^2$.

As v is defined by the length [L] of the drift region by the time [t] it takes the ion to travel through the drift region,

$1/2m(L/t)^2 = zeU$.

From this, the m/z of an ion can be determined;

$m/z = (2eU/L^2)t^2$ [88, 129].

MALDI-TOF instruments are configured in two modes, linear and reflectron mode (Figure 1.7). Linear mode is used for whole protein analysis and involves the measurement of ions across a linear drift region.

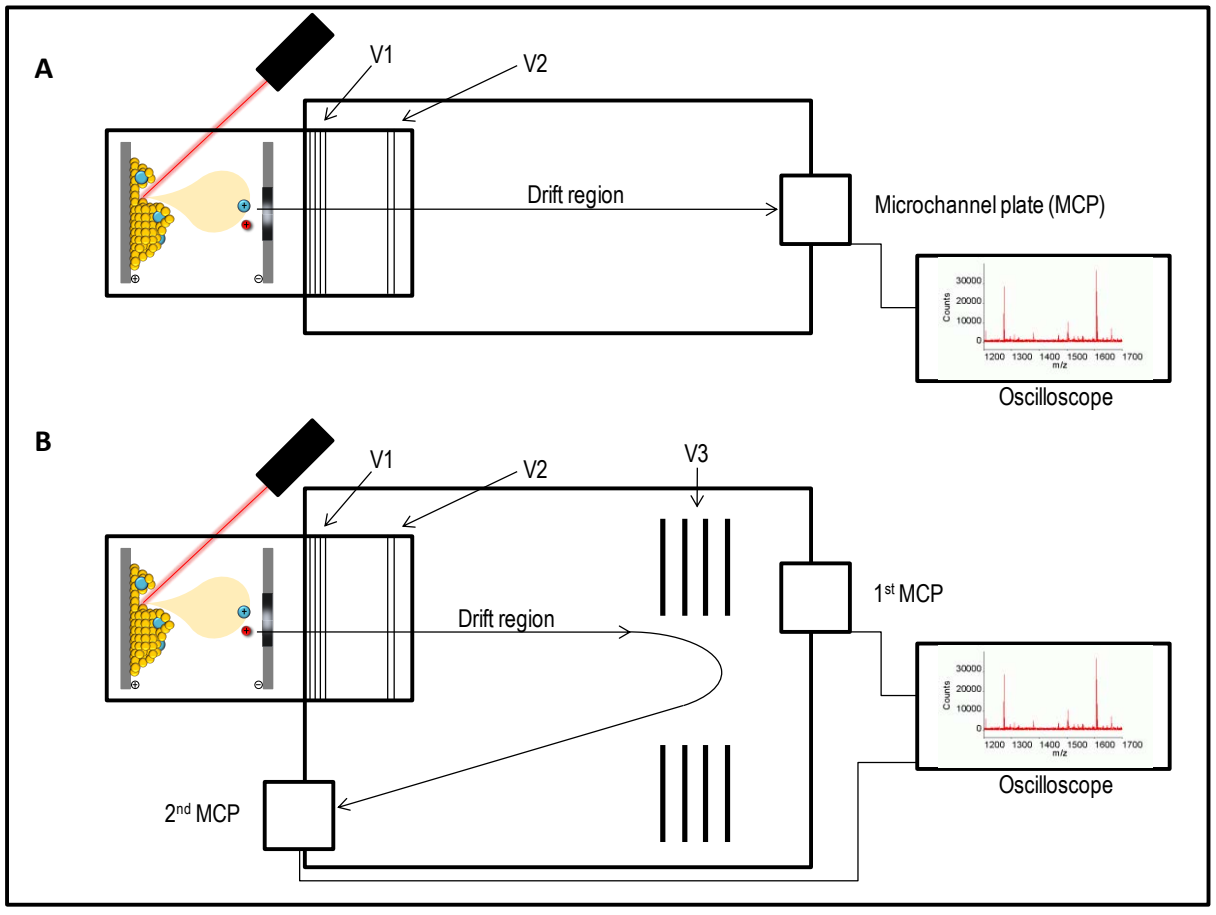

Figure 1.7: The two operational modes of a MALDI-TOF mass spectrometer. 1.6A shows linear mode and 1.6B shows reflectron mode [adapted from [25, 85, 88, 129]].

In linear mode (A), ions produced at the source are accelerated at V1 while a pulse voltage V2 is used to synchronise ions of the same mass leaving the source. The ions

travel over a linear flight path to the detector. In reflectron mode (B), variations in kinetic energies of ions produced at the source are compensated using the ion mirror, or reflectron (V3), deflecting ions such that they enter the second drift region at the same time and velocity.

Ions produced and subsequently analysed using MALDI-TOF usually have variations in their initial velocity. This is due not only to their position in the plume following desorption but also their position within the solid matrix/analyte crystal [88]. These velocity variations result in ions of the same mass reaching the detector at slightly different times, resulting in a loss of resolution. However, in linear mode, this is compensated by pulsed ion extraction, or delayed extraction (DE) [88, 130-133]. In DE, a pulsed voltage is applied after some time (typically nanoseconds) after ionisation. Ions with a greater initial velocity will travel further from the sample surface. The electrical field is then applied and this results in the faster ions having less electrical energy than ions with a lower initial velocity. This "compensation of energy spreads" between ions with different initial velocities ensures the ions will reach an end-point at the same time. Placing the detector at this end-point will result in an increase in resolution [88, 130-133]. Another option to compensate these kinetic energy variations is via the use of an ion mirror, or reflectron (Figure 1.7B), initially developed by Mamyrin [134]. The reflectron is a series of ring electrodes which create a constant electric field near their centre through a linear voltage gradient [88]. Ions with a higher velocity will penetrate the reflectron further than corresponding ions (same m/z) with lower velocities. These ions decelerate proportionally to their kinetic energies and leave the reflectron at the same time and velocity [88, 133].

During the ionisation process in MALDI, ions can undergo in-source decay (ISD) or post-source decay (PSD), generating fragments that are used to provide further information, such as the AA sequence of peptide [88, 133]. ISD ions are produced as a result of the desorption/ionisation event and undergo the same acceleration through the TOF tube to the detector, however the intensities of the ions are usually too low for practical purposes [88]. As such, PSD decay is routinely used in MALDI-TOF MS to generate fragment ions for MS/MS spectra. In PSD, precursor ions of interest undergo fragmentation within the drift-free region of the TOF tube [85, 88, 129, 135, 136]. This process is exploited for tandem MS analysis of ions. MALDI mass spectrometers with two TOF tubes are utilised to first select a precursor ion of interest (commonly referred to as a parent ion) using timed ion selectors (TIS), utilising deflecting voltages to isolate only ions of interest [129] which are also fragmented by PSD.

Metastable ion fragmentation is often unable to produce all fragment ions to effectively extract complete structural information of selected parent ions. However, this can be improved by increasing the internal vibrational energy of molecules to induce more fragmentation [88]. This is achieved by the use of a collision cell, which can be placed between two TOF tubes in a MALDI-TOF/TOF mass spectrometer. Here, neutral gas molecules can be introduced to induce fragmentation of parent ions that have been isolated following TOF m/z separation and TIS selection. This collision cell with fragment ions produced then acts as an ion source for the second TOF separation [88]. The effective generation of both MS and MS/MS data using MALDI-TOF/TOF MS has seen this approach become a powerful tool for proteomic analyses.

1.3.6 LC-MALDI-TOF/TOF MS

In proteomics, LC coupled on-line to ESI MS/MS has been used extensively for shot-gun proteomic strategies, providing both qualitative (protein identification) and relative quantitation of peptide mixtures using peptide labelling strategies for over 10 years [102-104, 137-147]. However, the development of automated offline LC strategies for coupling to MALDI-TOF/TOF MS has provided an alternative for proteomic analyses, particularly as this allows for more information to be achieved from each matrix position on a MALDI target. Although MALDI has certain advantages over ESI, including an increased dynamic range and higher resistance to salts, buffers and surfactants in samples [148], one critical factor of MALDI-TOF/TOF MS/MS analysis is the quality of the matrix/analyte spot [149]. For MALDI MS analysis, the smaller the number of peptides on one spot, the greater the increase in sensitivity of the analysis. Too many peptides on one spot results in ionisation suppression of less abundant peptides by highly abundant peptides [150, 151]. In addition, sample amount in each spot is finite, meaning only a limited number of MS/MS analyses can be acquired from the MALDI target as each desorption process accelerates sample loss [152]. The coupling of LC improves MALDI-TOF/TOF MS sensitivity by fractionating complex samples over a number of spots, increasing the number of peptides visible and subsequently improving MS/MS data for protein identification and characterisation. The offline approach also allows the archiving of samples and a decrease in generation of redundant MS/MS data, as it is possible to analyse MS results for a more strategic parent ion selection [104]. As a result, LC-coupled MALDI-TOF/TOF MS has been used extensively for proteomic analyses, both independently and in conjunction with LC-ESI-MS/MS [17, 103, 104, 125, 148, 153-160].

1.4 Quantitative LCMS-based proteomics

Quantitative proteomic analysis holds great promise for the detection of novel drug targets for the prevention and treatment of diseases as it is possible to elucidate changes in signal transduction between normal and diseased states [161]. 2-DE quantitative strategies, such as DIGE or silver stain-based gels have been invaluable for alterations in protein abundance between states however are unable to provide a complete representation of the proteome due to limitations in resolution and sensitivity. In order to complement these gel-based strategies and thus quantify parts of the proteome not compatible with PAGE, a number of LC-MS based quantitative methods have been developed [102, 161-165]. Generally protein or peptide quantitation using MS is performed either by label-free approaches or by stable isotope labelling [163, 166, 167]. In terms of protein-specific quantitation, methods involving the use of synthetic standards to quantify endogenous peptides derived from the protein during MS have been developed. Triple quadrupole (QQQ) mass spectrometers and multiple reaction monitoring (MRM) are particularly suitable tools [168]. For proteome-wide quantitation, many chemical labelling strategies have been employed due to the ability to utilise multiple MS configurations [169]. The developments now make it possible to label *in vivo* (i.e. stable isotope labelling by amino acids in cell culture – SILAC), chemically (e.g. isotope coded affinity tags – ICAT, isotope tagging for relative and absolute quantitation – iTRAQ, isotope-coded protein labels – ICPL) or enzymatically (e.g. $^{16}O/^{18}O$ labelling) [163].

1.4.1 Cysteine specific isotopic labelling - ICAT

Although MS has long been used in quantitative strategies for small molecule analyses, initial proteomic analyses utilised enzymatic modification by the incorporation of ^{18}O atoms at the C-termini of peptides [170-175]. However, the development of ICAT in 1999 by Gygi [140] and co-workers provided a method to simplify complex proteomic samples and concurrently provide quantitative information between control and test samples. The process involves the labelling of cysteine residues with a biotin-linked light or heavy isotopic label at either the protein or peptide level [140, 171, 176-178]. These labelled proteins and peptides can be selectively enriched using avidin affinity chromatography, thus reducing sample complexity approximately 10-fold [178, 179]. The remaining enriched labelled proteins are digested (for protein labelling) and labelled peptides are subsequently identified and quantified using LC-MS/MS [178, 179]. Although ICAT has been successfully used for quantitative analyses of the proteome [176, 177, 180], it does have some key limitations [171]. It has been previously reported that the avidin affinity purification steps for biotinylated peptides recover approximately 10% of the original peptides [181] and that not all cysteine-containing peptides with a biotin group could be captured [141].

Although non-specific binding to avidin columns and multiple reactions at the same site have been reported with ICAT [171], one major limitation is the rarity of cysteine residues compared with other residues in a given proteome, being found to be absent from some protein sequences [13, 21]. Also, the attached biotin group results in reduced ionisation of labelled peptides, which subsequently decreases the overall sensitivity of the method [17]. Modifications to the ICAT methodology such as an additional biotin cleavage step [17] as well as alterations in the labelling technique [182] have improved ICAT quantitation however the rarity of cysteine limits the amount of quantitative data achieved upon complex proteome analysis.

1.4.2 Amine specific isotopic labelling

Amine-specific labelling strategies, including iTRAQ, SILAC and ICPL labelling, overcomes the major limitation of ICAT, as these modify free amino groups on both lysine residues and free N-termini. This significantly increase the potential labelling sites that are available in protein sequences, resulting in higher sequence coverage and thus better quantitative information [13, 183].

1.4.2.1 Isobaric tags for relative and absolute quantitation (iTRAQ)

The iTRAQ labelling strategy involves the use of up to an 8-plex set of amine reactive isobaric tags to derivatise peptide digests at both the N-terminus and lysine residues [17, 144, 145]. After labelling, the peptide samples are combined to minimise experimental variance and analysed by MS. As the labelled peptides are isobaric, it is not possible to distinguish between labelled samples following MS analysis. Quantitation occurs at the MS/MS level via comparison of signature fragmentation ion (m/z = 112, 114-118, 119, 121) intensity [144, 145]. The iTRAQ tags contain a moderately strong basic group (*N*-methylpiperazine), which aids in improving peptide ionisation in positive ion mode MS analysis [17]. This is particularly useful for strategies utilising MALDI-MS, where it has been shown that peptides with a C-terminal arginine give stronger signal intensities compared with C-terminal lysine peptides [17, 184, 185]. This has been attributed to the basicity of the guanidino functionality of the arginine side chain [184-188], and iTRAQ labelling of the lysine containing peptides improves ionisation by a similar effect [17]. Although this strategy is a highly sensitive quantitation approach [17, 103, 104, 137-139, 143, 144, 146, 189-191], its key limitation, the poor detectability of the reporter ions in some types of mass spectrometers, has hampered its widespread use [163, 168, 192]. Compounding this is the fact that as quantitation can only be determined on the MS/MS level, MS/MS data must be acquired for all peptides in the sample. This is particularly detrimental for MALDI-MS strategies as MS/MS data are limited by the robustness of the sample/matrix spot. Thus, MS/MS analysis on low abundance peptides in the sample may not be possible after MS/MS of the highly abundance peptides has been acquired. Thus,

labelling strategies that reduce the number of MS/MS scans by providing quantitative information on the MS level are required for large-scale proteome analysis.

1.4.2.2 Stable isotope labelling of amino acids in cell culture (SILAC)

In order to overcome labelling reaction issues that can occur with iTRAQ and ICAT, Ong and colleagues developed SILAC [171]. This strategy, termed *in vivo* labelling, involves introducing isotopic variants of amino acids via protein synthesis of cells cultured in amino acid-deficient media. This approach removes affinity and chemical labelling steps and is compatible with most cell types, including primary cells [171]. For example, the test cells are cultured in medium containing arginine labelled with six ^{13}C, whilst control cells are cultured in media with normal arginine. As a result, all 'heavy' (^{13}C) arginine containing peptides are heavier than their normal 'light' (^{12}C) counterparts by 6 Da. Once incorporated, the proteome from both the control and test cells can be combined for any downstream processing prior to MS [171, 193-196]. During MS analysis of combined samples, relative peak intensities between labelled and unlabelled pairs are quantitative, enabling the selection of only labelled peptides for MS/MS to reduce redundancy and to evaluate the effect of stimuli on a range of proteins using one experiment [197, 198]. SILAC has become the method of choice for many strategies analysing both proteome and PTM changes in cell systems [162, 171, 194, 197-208]. As cell culture is a requirement for SILAC, it is not applicable for samples that cannot be obtained through cell culture. In addition, some cell types are unable to efficiently incorporate certain amino acids leading to incomplete labelling [193]. The SILAC process can also be quite time-consuming, involving a minimum of six cell passages to ensure complete incorporation [193]. The high number of passages may alter cell states, particularly for primary cells and care must be taken to minimise instances of interconversion between arginine and proline, which can affect quantitation efficiency [209]. The reliance of SILAC on cell culture has seen the development of an *in vitro* labelling strategy that is able to provide quantitative information at the MS level and is also suitable for virtually all sample types.

1.4.2.3 Isotope protein coded label (ICPL)

The non-isobaric technique ICPL, like iTRAQ, is an isotope-coded tag that is based on N-hydroxysuccinamide (NHS) chemistry that target primary amino groups (Figure 1.8), namely protein N-termini and lysine site chains [13, 163, 210-212].

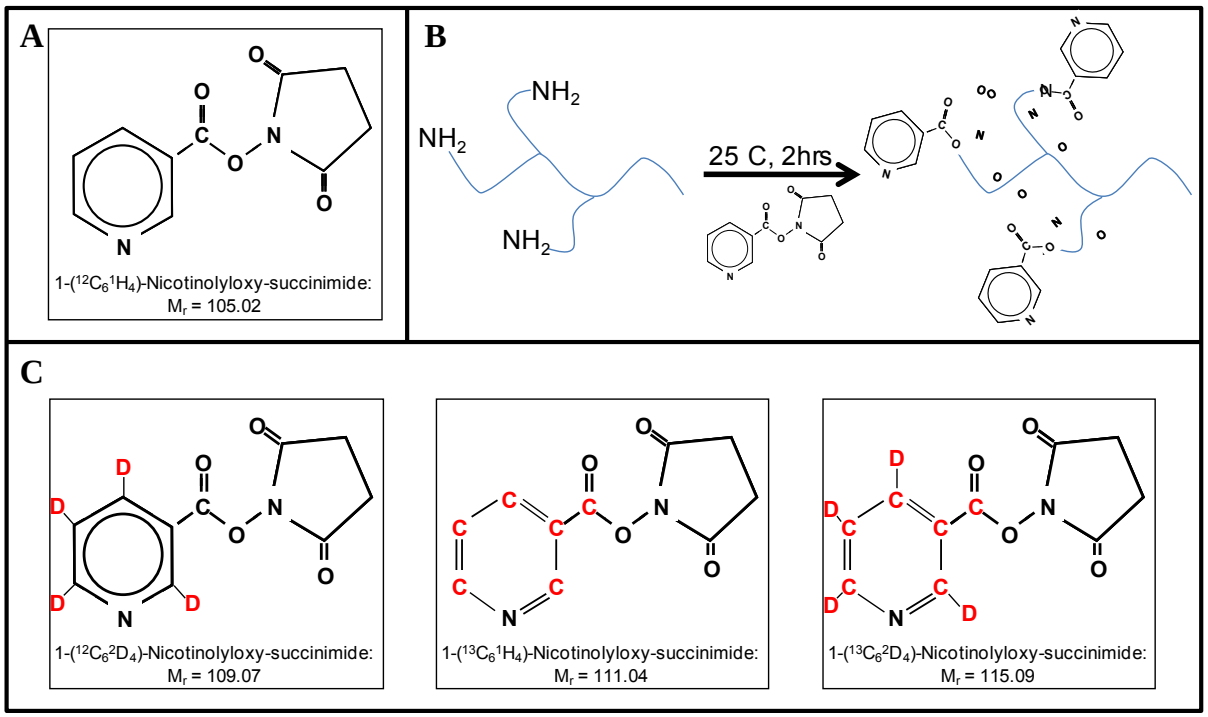

Figure 1.8: ICPL label molecular structure [Adapted from [13, 210, 213]].
The ICPL kit can contain up to four reagents. (A) outlines the 1-($^{12}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{12}C-Nic-Reagent (M_r = 105.0215 Da). (B) is a summary of the reaction scheme of ICPL labelling. (C) outlines the other ICPL reagents; 1-($^{12}C_6{}^2D_4$)-Nicotinoyloxy-succinamide ^{2}D-Nic-Reagent (M_r = 109.0715 Da), 1-($^{13}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{13}C-Nic-Reagent (M_r = 111.0419 Da) and 1-($^{13}C_6{}^2D_4$)-Nicotinoyloxy-succinamide $^{13}C^2$D-Nic-Reagent (M_r = 115.0919 Da). All four reagents can be used simultaneously for quantitation experiments.

A typical ICPL workflow will involve chemical labelling of proteins derived from control and test samples with 'light' and 'heavy' reagents, respectively. Once labelled, the protein samples are combined and either fractionated on the protein level to reduce complexity or digested in-solution for subsequent fractionation prior to MS. Since its introduction in 2005, different versions of ICPL reagent kits have been made available, enabling the quantitation of four samples simultaneously [214]. As the ICPL reagents are non-cleavable, a permanent shift in the labelled peptide mass occurs, resulting in the detection of peptide pairs (for duplex) or groups (for triplex or 4-plex) after MS acquisition. This method has been shown to improve the accuracy, sensitivity and dynamic range of existing methods [168, 210]. ICPL overcomes the disadvantages of other labelling strategies, including ICAT (low number of potentially labelable residues), iTRAQ (poor detectability of the reporter ions in several types of mass spectrometers) and SILAC (universally labels all proteins and does not require cell culture) [163]. Despite the adherent advantages, this particular labelling strategy has not been used as extensively as the other previously mentioned labelling techniques [13, 163, 168, 210, 211, 214]. The ability of this approach to effectively provide highly accurate and reproducible quantitation, detailed protein sequence

information, including PTMs and isoforms, as well as its compatibility with all commonly used protein and peptide separation techniques will see this strategy being used more extensively [13].

1.5 Phosphorylation

One of the most challenging aspects of proteomic research is the quantitation, identification and characterisation of PTMs of proteins in a biological sample. PTMs are enzymatic, covalent chemical modifications that occur after translation that potentially alter the physical or chemical properties of a protein. This alteration can result in conformational, stability and/or activity changes, and can even result in alterations in cellular location of a protein (e.g. the internalisation of a receptor into the cytoplasmic domain) [215]. PTMs can be used in a dynamic fashion, where reversible modification of proteins in signalling cascades is used to regulate cellular processes. Over 400 PTMs have been identified or are likely to be identified [215, 216].

Since the isolation of phosphoserine by Levene and Lipmann in 1932, phosphorylation has turned out to be one of the most biologically significant protein modifications [161]. As such, in proteomics, a large focus has been placed on the analysis of protein phosphorylation. Reversible phosphorylation is estimated to occur on 30% of the entire protein complement in mammalian cells at any point in time [217-219] and it has been speculated that 50% of all proteins are phosphorylated during their lifetime, yet phosphorylated proteins are present at relatively low abundance compared to their non-phosphorylated counterparts [220]. For example, a signal from the plasma membrane can be transmitted in the form of phosphorylation using a cascade of proteins until the signal reaches the nucleus. Phosphorylation by kinases and dephosphorylation by phosphatases are tightly regulated events that play an important role in many cellular processes, such as cell proliferation, differentiation, migration and the prevention of cell death [59, 221]. Genomic analysis has revealed that ~2-3% of all eukaryotic genes are likely to encode protein kinases (estimated to be 478), whilst over 100 human protein phosphatases have been predicted [222-224]. The phosphorylation of a protein may result for example in a change in its intracellular localisation, its catalytic activity and/or its interaction with other proteins. Over expression of so called oncogenes such as the epidermal growth factor receptor (EGFR) or loss of so called tumour suppressors like phosphatase and tensin homolog (PTEN) disturb the balance, which can lead to a number of pathologies such as cancer or immune deficiencies [225, 226](Condina *et. al.* In Press). In eukaryotic cells, phosphorylation occurs primarily on serine (pSer), threonine (pThr) and tyrosine (pTyr) residues, estimated to be in the ratio of 1800:200:1 [221, 225, 226], with phosphoserine and phosphothreonine being about 3000 times more abundant than

phosphotyrosine [221, 227]. It has been predicted that over 1 x 10^5 phosphorylation sites may exist in the human proteome [228]. Due to the critical role of this PTM in both normal cellular processes and perturbed signalling in multiple pathologies, the identification and characterisation of protein phosphorylation is of paramount importance [59, 161, 225, 229].

1.5.1 Phosphoprotein detection

To date, the identification of phosphorylation sites and evaluation of their function on proteins has been achieved through several standard procedures. These include radioactive labelling with ^{32}P-labelled ATP followed by 1D or 2D SDS-PAGE, thin-layer chromatography (TLC), HPLC or Edman Sequencing, site-directed mutagenesis or deletion experiments, chemical modification (particularly β-elimination of the phosphate moieties of serine/threonine phosphorylated proteins and peptides by alkali treatment and subsequent Michael-type addition of nucleophiles), Edman sequencing and the use of general phosphospecific antibodies (Abs), [59, 161, 220, 230-232]. The more recent development of phospho-specific stains, particularly Pro-Q Diamond, in conjunction with SDS-PAGE has facilitated phosphoprotein detection [233-240]. Although less sensitive than radioactive methods, their ease of handling is far more convenient [220]. Phosphoprotein visualisation can also be achieved by traditional western blot analysis of samples fractionated using 1D and/or 2D SDS-PAGE using phosphospecific Abs [241-245]. Western blot analysis can routinely detect very low amounts of phosphoprotein/s in a sample (low femtomole), however sensitivity is dependent on the Ab used [246]. Hundreds of commercially available phosphotyrosine Abs exist, such as 4G10, pY20, pTyr100, pY99 and pY66, all showing varying degrees of specificity and sensitivity [225]. However, these phosphotyrosine antibodies generally have low cross-reactivity to un-phosphorylated tyrosine or serine/threonine-phosphorylated residues [247, 248]. In contrast, Abs for phosphoserine and phosphothreonine are generally dependent on consensus sequences in addition to the phosphorylated residue, with steric hindrance decreasing effective Ab binding [220].

Analysis using a phospho-specific dye or western blot will only provide locations of phosphorylated proteins and not site-specific information. Many approaches analysing phosphorylation at the protein level will target either specific proteins of interest or the entire phosphoprotein complement using immuno-precipitation (IP) strategies to reduce sample complexity [241, 249-251], often using phosphospecific antibodies [252, 253]. Enriched phosphoproteins can then be characterised by MS, typically after proteolytic digestion. MS has thus become a powerful tool for the unbiased analysis of protein phosphorylation [102]. Providing the abundance of phosphopeptides is sufficient, successful characterisation of phospho-sites is achievable. However, this is seldom the case as phosphopeptides

are known to be difficult to analyse by MS, particularly when analysing a mixture of phosphorylated and non-phosphorylated peptides. This is due to a low ionisation efficiency of phosphopeptides (particularly multiply phosphorylated peptides) compared with non-phosphorylated peptides, resulting in ionisation suppression effects [59]. As phosphorylation site analysis remains a challenge for many laboratories, advances in phosphoproteomic technologies involving the enrichment of phosphopeptides have aided in the analysis of complex protein samples [161].

1.5.2 Phosphopeptide enrichment strategies

A summary of the proteomic-based methods that have been developed for phosphopeptide enrichment prior to MS analysis is outlined in Figure 1.9. Some methods outlined, particularly immobilised metal affinity chromatography (IMAC), SCX, SAX and immuno-affinity chromatography (e.g. using a phospho-specific Ab for pTyr) can enrich at both the protein and peptide level. Also, as shown, these methods can be used solely or in combination with other enrichment techniques to reduce sample complexity and thus maximise the probability of phosphopeptide detection.

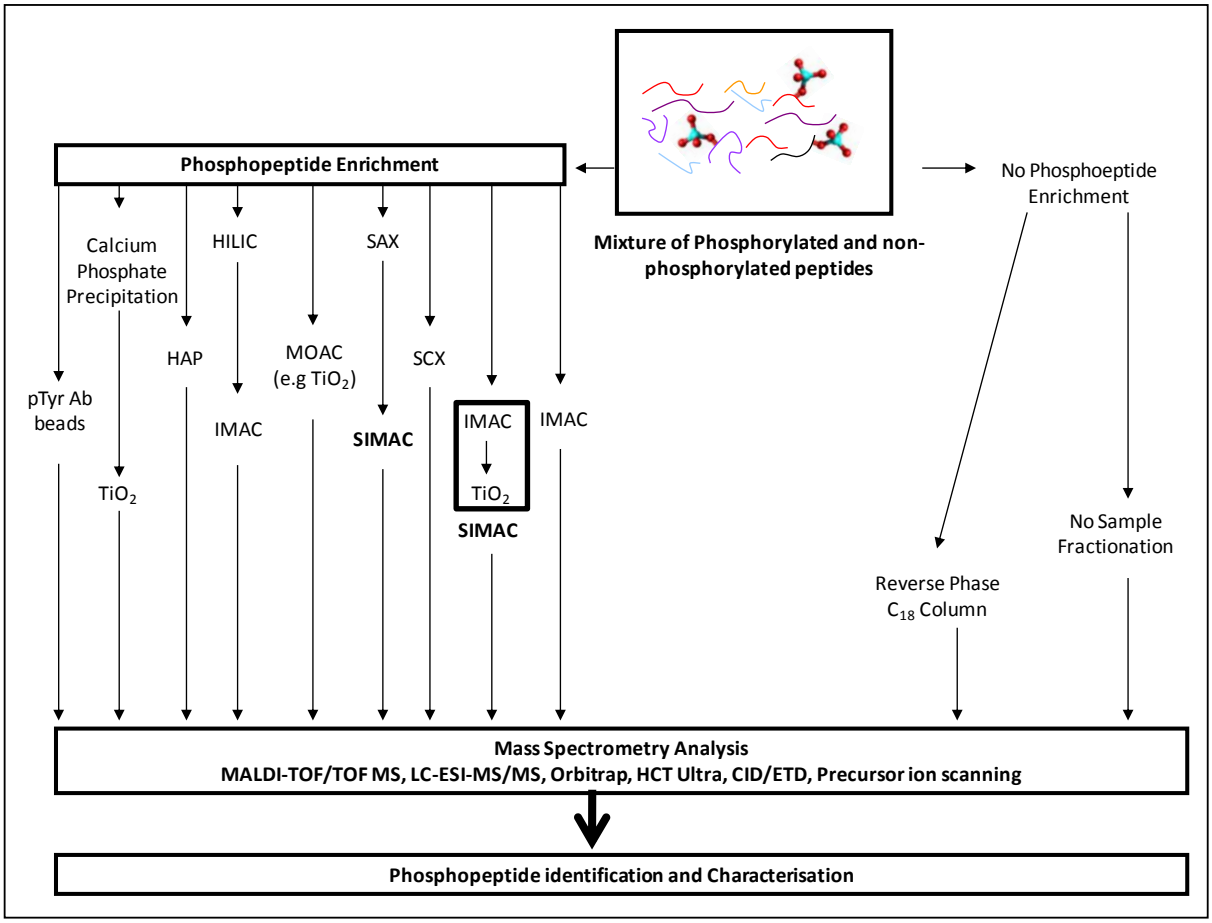

Figure 1.9: Enrichment strategies for phosphopeptides [Adapted from [59, 161, 220, 225, 254]].

A variety of proteomic based techniques have been developed an optimised for phosphopeptide enrichment to improve subsequent identification and characterisation by MS. These include IMAC, TiO_2 enrichment, MOAC enrichment, SCX or SAX, HILC with IMAC, calcium phosphate precipitation, pTyr Ab enrichment, Hydroxyapatite (HAP) enrichment and multi-dimensional enrichment strategies such as sequential elution from IMAC (SIMAC).

1.5.2.1 Immobilised metal affinity chromatography (IMAC)

IMAC, first introduced by Porath *et. al.* [255] for enrichment of histidine- and cysteine- containing proteins interacting with IMAC resin, has become one of the most frequently used phosphoprotein and phosphopeptide enrichment strategy [59, 61, 161, 220, 225, 254, 256]. The technique is based on metal ions (e.g. Fe^{3+}, Al^{3+}, Ga^{3+}, Ni^{2+}, Co^{2+}, Zr^{4+} or Mn^{2+}) immobilised on a chromatographic or bead support. These ions are chelated by either iminodiacetic acid (IDA) [255], nitriloacetic acid (NTA) [257] and Tris(carboxymethyl)ethylenediamine (TED) linkers [59, 61, 220, 225]. While divalent ions such as Ni^{2+}, Co^{2+} and Mn^{2+} are better for proteins with a high density of histidines, the trivalent ions Fe^{3+}, Al^{3+}, Ga^{3+} and more recently the tetravalent Zr^{4+} have been shown to be better for phosphorylated protein and peptide enrichment [59, 61]. The process of enrichment is seemingly straight-forward, involving column equibrilation, binding, washing and elution and has been successfully utilised in multiple strategies at both the protein and peptide levels [34, 48-58, 60, 80, 191, 201, 202, 205, 207, 258-264]. Despite its widespread use, the method as a stand alone approach can have some drawbacks, including non-specific binding of highly acidic peptides (peptides with aspartic acid or glutamic acid) and the strong binding of multiply-phosphorylated peptides. As IMAC is based largely on ionic interactions, peptides rich in glutamic and aspartic acid residues are often co-purified [240]. Reduction of non-specifically bound acidic peptides can be achieved by esterification of carboxylic acids to methyl esters [48, 50, 205, 259, 264]. However, care must be taken when performing this reaction to minimise side reactions, resulting in increased sample complexity [59, 220]. Once enriched, the isolated peptides can then be identified by MALDI-MS or LC-ESI-MS/MS as IMAC is compatible with MS. In one such study, peptides were obtained from yeast protein extract and methylated to reduce non-specific binding of acidic non-phosphorylated peptides to IMAC resin. This approach identified 216 phosphopeptides and 324 phosphorylation sites [50]. Other studies have combined IMAC with pre-fractionation protocols in order to enhance the effectiveness of phosphopeptide identification, such as using calcium phosphate precipitation of phosphopeptides prior to IMAC [53].

1.5.2.2 Titanium Dioxide (TiO2) phosphopeptide enrichment

An alternative strategy for phosphopeptide enrichment, TiO_2, is based on the selective interaction of water soluble phosphates with porous TiO_2 microspheres [59, 265, 266]. Phosphopeptides bind under acidic conditions are and subsequently eluted (after washing to remove non-phosphorylated peptides) using alkaline conditions. Although this is superior to IMAC in terms of non-specifically bound peptides, non-phosphorylated peptides often remain after TiO_2 enrichment. As such, recent studies have altered sample loading conditions to improve phosphopeptide binding and further reduce unspecifically bound peptides [261]. As with IMAC, this enrichment method is also compatible with MS and on-line columns have been developed to automate phosphopeptide enrichment, identification and characterisation [267, 268]. Further developments of metal oxide affinity chromatography (MOAC) [269-273], such as the use of $Al(OH)_3$-based technique by Wolschin *et. al.* [240] have aimed to further improve phosphopeptide binding and reduce non-specific binding.

1.5.2.3 HILIC

HILIC was first coined in 1990 to describe normal phase chromatography with mobile phases that are typically 10-40% aqueous [274, 275]. Although originally described in the 1970s for carbohydrate analysis, only in the last 15 years has it been applied in a number of analyses, mainly due to its versatility as well as the growing need for chromatographic materials that can fractionate polar compounds and are compatible with MS [26]. In HILIC, the stationary-phase material is sufficiently more polar than the mobile phase (*i.e.* compounds are loaded in a high organic mobile phase), resulting in the retention of polar compounds that are unable to be retained with conventional RP chromatography [26, 275]. The compounds bind by hydrogen bonding, which are subsequently disrupted by decreasing the organic environment in the mobile phase. Compounds subsequently elute according to their hydrophobicity, with the more hydrophobic peptides being eluted off first [26, 80]. HILIC is not only compatible with MS, it has shown to improve sensitivity for ESI-MS strategies [276, 277]. Furthermore, studies on the separation of unphosphorylated peptides using HILIC, SCX and RP-HPLC have indicated that a better orthogonal separation could occur between HILIC and RP-HPLC, making HILIC viable for the multi-dimensional separation of complex samples [278, 279]. As phosphorylated peptides are generally more polar than non-phosphorylated peptides, recent analyses of the phosphoproteome have utilised HILIC alone [275] or in conjunction with other separation strategies [58, 80].

1.5.2.4 Combined strategies for phosphopeptide enrichment

Despite the highly sensitive strategies reported here for phosphoproteome analysis, obtaining complete phosphoproteome characterisation remains a challenging task. In order to further reduce sample complexity and subsequently improve phosphopeptide identification and characterisation, a number of enrichment methods have been combined. For example, sequential elution IMAC (SIMAC), exploits the retention of multiply phosphorylated peptides by IMAC with the preference that TiO_2 has on mono-phosphorylated peptides [60]. The process involves the fractionation of multiply-phosphorylated peptides and mono-phosphorylated peptides by first enriching complex samples over IMAC. The unbound fraction (including unbound phosphopeptides) is collected for TiO_2 enrichment. The eluates from both the IMAC and TiO_2 are analysed separately to improve phosphopeptide characterisation [59, 60]. Other combined enrichment strategies involve the use of calcium phosphate precipitation, SCX, SAX, and HILIC prior to more specific phosphopeptide enrichment including IMAC, TiO_2, and SIMAC [34, 53, 58-60, 80, 202, 280].

1.6 Tyrosine phosphorylation

Although tyrosine phosphorylation (pTyr) makes up only a small percentage of the phosphoproteome, protein-tyrosine kinases (PTKs) are amongst the most important oncogenes known to date [281]. Of the more than 90 PTKs, more than half have been implicated in various types of cancers [282, 283]. This is due to the biological role of PTKs in cell proliferation, motility, actin re-organisation and chemotaxis [284]. For instance, unregulated tyrosine phosphorylation has been shown to drive malignancy and tumour growth due to its involvement in proliferative and survival signal transduction pathways [229, 282]. PTKs, Protein-tyrosine Phosphatases (PTPs) and their substrates have been associated with many normal physiological processes, and perturbation of these events is implicated in other disease states, including immune deficiencies [221, 226, 281, 285, 286]. The major role of tyrosine phosphorylation, particularly in cancer, has seen the development of novel proteomic and therapeutic approaches to identify, characterise and target unregulated PTKs and PTPs as well as the pathways involved in tumour growth and survival [226, 229, 284].

1.6.1 Proteomic methods for phosphotyrosine detection

The technical challenging nature of pTyr signalling analysis is attributed to the occurrence of tyrosine phosphorylation at an extremely low abundance within cells (often only a few hundred copy numbers per cell), the low stoichiometry for a given protein, the fact that pTyr represents only 0.05% of all phosphorylated amino acid content, and the labile nature of pTyr events during chemical processing

required for MS analysis [225, 280, 283, 287]. In addition, the rules of consensus do not work well with pTyr, with programs that utilise algorithms to predict pTyr sites not matching experimental outcomes [283].

Despite the rarity of phosphotyrosine in cellular protein extracts, a number of methods have been utilised to successfully characterise pTyr. The availability of broadly reactive Abs that recognize pTyr residues have aided in the identification and characterisation of many PTK substrates involved in growth factor and cytokine signalling [228, 288-292]. These Abs have been employed in immunoprecipitation as well as western blotting experiments [252]. The immunoprecipitated proteins are separated by gel electrophoresis, digested in-gel with a protease and analysed by MS. In general, these immunoprecipitated proteins are identified without mapping their tyrosine phosphorylation sites [229]. More recent pTyr analysis utilise protein specific Abs or pTyr specific Abs to batch purify prior to further enrichment using a phosphorylation-based strategy [208, 225, 259, 264, 283, 293-296]. Global strategies have generally selected one pTyr specific Ab in enrichment strategies [208, 229, 243, 297] however a combination of pTyr Abs have been used to improve subsequent identification [264, 288, 294]. To date, the majority of global pTyr analyses utilise pTyr-specific Abs at the protein level as after enrichment and digestion, both non-phosphorylated and phosphorylated peptides remain from the pTyr phosphoproteins. As most strategies follow a “bottom-up” approach (dealing with peptides derived from proteins) [298], the analysis of both phosphorylated and non-phosphorylated peptides improves protein identification. The use of pTyr Ab for protein IP can also enrich pTyr protein binding partners for identification, which aid in the characterisation of pTyr signalling cascades [225]. However, protein level enrichment will not distinguish between pTyr proteins and their binding partners without identification and quantitation of corresponding pTyr sites. Also, the presence of non-phosphorylated peptides can result in ionisation suppression effects on phosphopeptides, minimising pTyr site characterisation. As such, methods utilising pTyr specific Abs for direct pTyr enrichment at the peptide level [191, 229, 283, 299-301], improve sensitivity by avoiding ion suppression through non-phosphorylated peptides. Due to the extremely low abundance of pTyr in complex samples, various methodologies will utilise global phosphopeptide enrichment strategies either prior to or after pTyr peptide enrichment using immunoaffinity enrichment [225]. The combination of IMAC with pTyr Ab enrichment has been effectively used to characterise a larger set of pTyr sites [191, 264, 297, 301, 302].

1.6.2 Identification of phosphorylation sites by MS

Proteomic based methodologies, namely enrichment strategies coupled with MS analysis, have provided a number of methods that are beneficial in identifying, quantifying and characterising pTyr

events in cell systems [59, 161, 164, 220, 225, 254, 281]. The successful identification and characterisation of pTyr using MS is dependent on the consideration of phosphopeptide characteristics observed during the ionisation process. In addition to the low stoichiometry of phosphorylation compared with non-phosphorylated peptides in the sample, phosphopeptides generally have a lower ionisation efficiency (particularly in positive ion mode MS) and the labile nature of the phosphate group from a peptide minimises sufficient backbone fragmentation for phosphosite characterisation. Proteolytic digestions of proteins often produce phosphopeptides with more than one potential phosphorylation site, emphasising the need for full phosphosite characterisation [161]. As such, careful consideration must be taken when determining the MS strategy to be utilised in order to achieve sufficient information to identify and subsequently characterise pTyr sites.

Many strategies developed for phosphotyrosine identification utilise 1D or 2D LC coupled with tandem MS analysis, using ESI or MALDI ionisation [51, 59, 200, 225]. Nano-LC coupled online with ESI tandem MS (nLC-ESI-MS/MS) and LC coupled offline with MALDI tandem MS (LC-MALDI-MS/MS) provide simple, sensitive and automated analyses of complex peptide samples, with each method having advantages for phosphorylation analysis.

Despite the fragmentation suppression effects of the labile phosphate group, nLC-ESI-MS/MS is a technology capable of providing adequate phosphopeptide sequence coverage. In addition, its high sensitivity allows for the effective analysis of samples over a large dynamic range, which is particularly useful for phosphoproteome characterisation. The effectiveness of nLC-ESI-MS/MS for tyrosine phosphorylation analysis is dependent on the peak capacity of the gradient used for peptide separation and the electrospray ionisation efficiency. When separating complex peptide mixtures using LC, phosphopeptides and non-phosphorylated peptides often co-elute. As phosphopeptide ionisation is suppressed compared with non-phosphopeptides, these often are not selected for subsequent tandem MS analysis. Phosphotyrosine enrichment at a protein and/or peptide level minimises sample complexity (in comparison to direct analysis of the entire proteome) and, is often combined with nLC-ESI-MS/MS analysis. nLC-ESI-MS/MS is also used as a number of methods can be implemented to improve phosphopeptide identification, such as the use of precursor ion scanning for phosphopeptide selection as well as automated switching between fragmentation techniques (*i.e.* CID and ETD) [59, 161, 164, 220, 303-309].

CID fragmentation of phosphopeptides often results in the partial loss of phosphoric acid (H_3PO_4 – 98 Da loss) as a result gas-phase β-elimination of the phospho-ester bond, with pSer and pThr peptides

whilst with pTyr peptides, a partial loss of 80 Da (HPO_3) is more dominant. As a loss of the neutral molecule is thermodynamically preferred, further fragmentation of the amino acid backbone is rarely observed in CID of phosphopeptides. In order to overcome this inadequate phosphotyrosine peptide fragmentation, strategies which utilise characteristic phosphopeptide fragments in CID have been developed. An ion trap mass spectrometer and other MS^3-capable instruments are able to trigger a MS^3 analysis if the precursor ion is showing a neutral loss of 49 Da or 40 Da for doubly charged or 32.66 Da or 26.66 Da for triply charged ions. In this experiment, the fragmentation of the phosphopeptide after gas-phase β-elimination of the phospho-ester bond on pSer and pThr peptides and loss of HPO_3 for pTyr can provide peptide backbone fragmentation data which will elucidate the site of phosphorylation.

A strategy for pTyr includes the pTyr immonium ion at m/z = 216.043 Da as precursor selection criteria of peptides to be fragmented [231, 253, 310]. Precursor ion selection for the pTyr immonium ion increases the number of phosphotyrosine peptides that are selected for fragmentation, such that other co-eluting non-phosphorylated peptides do not interfere. Although this strategy for pTyr analysis has been successfully used previously, it does require the use of specific MS configurations with high-resolution and accuracy, such as a quadrupole TOF (qTOF) hybrid mass spectrometer, because other fragment ions such as b-ions NT or GS (both *m/z* = 216.098 Da) and y2-ion-NH3 DV (*m/z* = 216.069 Da), *etc.* are likely false positives [310]. Yet other fragmentation strategies have been developed that are applicable to various MS configurations, such as ECD and ETD fragmentation.

In 1998, McLafferty and co-workers introduced Electron Capture Dissociation (ECD) [311], a process where low energy electrons were reacted with peptide cations in the magnetic field of a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). This resulted in electron attachment to protonated peptides, forming cations with an additional electron. The odd-electron cation rearrangement is followed by subsequent dissociation of the N-Cα bond and not the peptide bond like in CID. The fragmentation of the N-Cα bond is independent of the amino acid itself and the modification of the amino acid. As such, unlike conventional CID, ECD does not cleave chemical modifications from the peptide (instead inducing random cleavage along the peptide backbone, keeping PTMs intact) and is indifferent to either peptide sequence or length [303, 304]. However, ECD requires an FT-ICR mass spectrometer in order to 'catch' electrons, instruments that are not readily available to many in the proteomics field.

In contrast, ETD utilises ion-ion chemistry. Originally reported in 2004 [312], this fragmentation strategy utilises an electron donor to generate charged-reduced peptide radical ions, which are then fragmented at alternative positions on the peptide backbone compared with CID [312, 313]. As with ECD, this fragmentation occurs solely on the peptide backbone (N-Cα bond cleavage) [59, 303, 304, 312, 314-318], keeping PTMs (such as phosphorylation) intact. In addition, this fragmentation strategy has been easily implemented in a number of MS configurations, making this an interesting strategy for phosphotyrosine peptide analysis [59, 161, 254, 305]. This is because ETD utilises a RF quadrupole ion trapping device instead of an FT-ICR-MS for ion trapping and detection [312], and as RF ion trap mass spectrometers are low-cost, low-maintenance, and widely accessible compared with FT-ICR-MS, this fragmentation strategy is more applicable in the proteomics field. Furthermore, the introduction of ETD on 3D ion trap mass spectrometers allows the collection of both the electron donor and peptide ions within the confines of the trap at the same time, improving charged-reduced peptide radical ion generation for subsequent ETD fragmentation of complex proteomic samples. The introduction of ETD in mass spectrometer configurations like nLC-ESI-3D-IT-MS/MS allows for complex peptide sample analysis where a multi-dimensional approach consisting of consecutive sample analyses using CID followed by ETD fragmentation is utilised, which improves overall protein identification and PTM characterisation, particularly for phosphopeptides [303-309].

Although nLC-ESI-MS/MS strategies have been successfully used for phosphotyrosine analysis, the combination of LC-coupled to MALDI-TOF/TOF MS/MS analysis can be advantageous for phosphopeptide identification. The developments of sample preparation methods for MALDI-TOF/TOF MS that enhance phosphopeptide ionisation make this a particularly useful tool for phosphoproteome analysis [200]. The added capability of this approach to characterise phosphorylation on large (>3 kDa) mono- and multi-phosphorylated peptides makes this an attractive and useful approach for global phosphorylation analysis [200, 319].

In order to enhance phosphopeptide ionisation using MALDI-TOF/TOF MS, matrix deposition strategies have been optimised, primarily using 2,5-dihydrobenzoic acid (DHB) with phosphoric acid (PA) added [51, 125, 320]. DHB has the added advantage of being more tolerant to salts/contaminants compared with other matrix strategies, such as α-cyano-4-hydroxycinnamic acid (CHCA). Yet one critical factor of MALDI-TOF/TOF MS/MS analysis is the quality of the matrix/analyte spot [149]. The offline approach also means the archiving of samples and a decrease in redundant MS/MS data, as it is possible to analyse MS results before MS/MS data have to be acquired, which make a more strategic parent ion

selection possible [104]. Both nLC-ESI-MS/MS and LC-MALDI-TOF/TOF MS analyses have specific advantages for pTyr characterisation. However, it has been shown that these two MS-based methods are complementary and have previously been used to provide more complete proteome coverage [103, 156, 321, 322].

1.6.3 Quantitation of tyrosine phosphorylation

Elucidation of perturbed signalling networks requires methods that can accurately quantify and characterise phosphorylation. Although traditional biochemistry approaches such as ^{32}P labelling, immunoblotting, and fluorescent labelling can be used to quantify changes in protein phosphorylation, MS-based approaches have increasingly become the method of choice [196, 225]. In regards to stable isotope labelling, a number of strategies have been adopted for pTyr quantitation analyses [191, 208, 264, 280, 294, 295, 297, 301, 323]. These approaches utilise a labelling strategy (at either the protein or peptide level) in combination with a pTyr enrichment strategy. In particular, SILAC [208, 280, 294, 323], iTRAQ [191, 295, 301] and ICAT [297] have had the most wide-spread use in conjunction with either protein [208, 294, 297, 323] or peptide [191, 295, 301] pTyr enrichment (using pTyr specific Abs), with some strategies additionally utilising IMAC [191, 295, 301] or TiO_2 [280] to further reduce sample complexity and thus improve pTyr quantitation and characterisation.

1.7 Thesis Synopsis

Despite the development of multiple strategies for phosphopeptide enrichment, quantitation and characterisation, as of 2003, fewer than 2000 phosphorylation sites were known of a possible 1×10^5 potential phosphorylation sites, with only a fraction being phosphotyrosine sites [228]. Despite advances in both pTyr enrichment and MS strategies, pTyr characterisation remains a challenging task. The difficulty of tyrosine phosphoproteome analysis highlights the need for methodologies that are sensitive enough to not only enrich for phosphotyrosine peptides, but also provide sufficient MS/MS data for accurate mapping the site of phosphorylation. The basis of my project involves the use of a phosphotyrosine specific Ab in conjunction with MALDI-TOF MS for the development of new methods to map tyrosine phosphorylation in biological samples. The coupling of this Ab to para-magnetic beads will be utilised as a targeted approach for direct enrichment of phosphopeptides from pTyr proteins. In order to map tyrosine phosphorylation in specific pathways in cell lines under stimulatory conditions, a more global approach is required. The comparative quantification of low-abundant tyrosine

phosphorylated proteins isolated from complex cell lysates will be analysed using isotopic labelling in conjunction with phosphopeptide enrichment strategies and LC-MALDI-TOF/TOF MS analysis.

1.8 Aims

The goal of the project was to develop and optimise sensitive proteomic based strategies for the analysis of the phosphoproteome, with a particular focus on tyrosine phosphorylation. The following aims were developed to achieve this goal:

- To develop a targeted proteomic approach for phosphotyrosine analysis from protein samples (Chapter 2)
- To develop an optimised LC-MALDI-TOF/TOF MS strategy for complex (global) sample analysis (Chapter 3)
- To quantify and characterise global pTyr phosphorylation for complex cell samples using a proteomics based strategy incorporating ICPL, HILIC and pTyr enrichment prior to LC-MALDI-TOF/TOF MS analysis (Chapter 4)

Chapter 2: Background

The rarity of pTyr phosphorylation coupled with the technical challenging nature of analysing this PTM has highlighted the need for more sensitive approaches for pTyr detection. Although a number of strategies have been developed to characterise the pTyr phosphoproteome, only a fraction of pTyr sites have been characterised. The aim of the study presented here was the development of optimised approaches that specifically target pTyr.

Many research strategies are focused on the characterisation of protein function and/or regulation in particular signalling cascades implicated in pathologies such as cancer. Often, western blot analysis, ^{32}P labelling and phosphospecific stains are used to show phosphorylation of proteins of interest during signalling. Although these methods can provide information as to what proteins are phosphorylated, they are unable to accurately characterise phosphorylation sites without further site-directed mutagenesis experiments. As such, the development of a target proteomic approach capable to identifying and characterising tyrosine phosphorylation on proteins of interest would aid signal transduction research.

The availability of broadly reactive pTyr specific Abs, such as 4G10, are able to be utilised to achieve adequate enrichment of pTyr proteins and peptides. Proteins of interest can be recombinantly produced and subsequently *in vitro* phosphorylated or are purified from complex samples (by IP using protein specific Ab or pTyr specific Ab) and fractionated by 1D or 2D SDS-PAGE. Bands corresponding to the protein of interest can then be excised and proteolytic in-gel digestion can be used prior to extraction of both phosphorylated and non-phosphorylated peptides.

Super para-magnetic beads specially designed for MALDI analysis has been commercialised (Bruker Daltonics, Bremen, Germany). The basis of my project involves the use of a phosphotyrosine specific Ab in conjunction with MALDI-TOF MS for the development of new methods to map tyrosine phosphorylation in biological samples. The coupling of this Ab to these para-magnetic beads will be utilised as a targeted approach for direct enrichment of phosphopeptides from phosphotyrosine proteins.

Chapter 2

A Sensitive Magnetic Bead Method for the Detection and Identification of Tyrosine Phosphorylation in proteins by MALDI-TOF/TOF mass spectrometry.

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Abbreviated Title: Tyrosine Phosphorylation Detection using Affinity Chromatography
Abbreviations: Ab, Antibody; **βic**, β-intracellular domain of the granucolyte-macrophage colony-stimulating factor receptor; **BS**, Binding solution; **Cov-P**, covalently-coupled 4G10 MB-CovAC-Select; **FA**, formic acid; **GM-CSF**, granulocyte-macrophage colony-stimulating factor receptor; **MB-covAC-Select**, magnetic bead based covalent affinity chromatography for binding of freely selectable proteins; **MB-IAC Prot G**, magnetic bead based immunoaffinity chromatography on immobilised protein G, **MB-IMAC Fe**, magnetic bead-IMAC, **Pro-GP**, 4G10 affinity-coupled MB-IAC Prot G; **PTK**, protein-tyrosine kinase; **PTP**, protein-tyrosine phosphatases

Key Words: Antibody/MALDI/MS/Phosphorylation/Proteomics Methods

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STATEMENT OF AUTHORSHIP FOR CHAPTER 2

A sensitive magnetic bead method for the detection and identification of tyrosine phosphorylation in proteins by MALDI-TOF/TOF MS. Proteomics 2009, 9, 3047-3057.

Mark R. Condina (Candidate)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Performed analysis on all samples, interpreted data, wrote manuscript

Certification that the statement of contribution is accurate

Signed.....Date.....

Mark A. Guthridge (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Data interpretation and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed.....Date.....

Shaun R. McColl (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Supervised development of work, helped in data interpretation and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed.....Date.....

Peter Hoffmann (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Supervised development of work, helped in data interpretation and manuscript evaluation and acted as corresponding author

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Chapter 3: Background

The development of the sensitive super para-magnetic bead approach utilising 4G10 provides a simple straight-forward targeted proteomic approach for pTyr characterisation on relatively simple (<4 protein) sample digests. The use of either Pro-GP and/or Cov-P beads in conjunction with more traditional biochemical techniques, namely IP and western blot analysis, will be able to identify proteins that have been phosphorylated after the implementation of test conditions as well as characterise the sites of pTyr without the need for site-directed mutatgenesis experiments or recombinant protein synthesis.

Despite the advantages of the Pro-GP/Cov-P method described in Chapter 1, the method is limited as a targeted proteomic approach. This is because the capacity of the 4G10 beads is small, requiring pre-fractionation (*e.g.* SDS-PAGE) or IP experiments so only a small number of proteins are digested for pTyr phosphopeptide enrichment. As such, if Pro-GP/Cov-P was to be used for more complex sample analysis, a serial enrichment strategy involving multiple enrichment steps would have to be implemented. However, this type of strategy would be time consuming and may not be successful in characterising total pTyr phosphorylation of complex samples. In addition, the substantial increase in sample handling prior to MS will increase pTyr loss and as pTyr abundance is low, the number of pTyr sites detected would be significantly decreased.

The complexity of cell lysates with the low abundance of pTyr has prompted the development of multiple strategies aiming to reduce sample complexity for successful phosphopeptide detection and characterisation. The coupling of HPLC and MALDI-TOF/TOF MS is one strategy that has been used successfully for phosphoproteomic analysis [156, 337-339]. Aspects of the approach can be modified to improve phosphopeptide recovery, detection and characterisation, particularly in regard to MALDI-TOF/TOF MS.

Prior to the analysis of a complex protein sample (*i.e.* cell lysates), the development of a LC-MALDI-TOF/TOF MS strategy that has been optimised for phosphoproteome analysis was required. Both the HPLC and MALDI MS aspects of the method would have to be optimised to ultimately minimise phosphopeptide loss during HPLC fractionation and improve phosphopeptide ionisation and subsequent detection in MALDI-TOF/TOF MS analysis.

Chapter 3

EZYprep LC - coupled MALDI-TOF/TOF MS: An improved matrix spray application for phosphopeptide characterisation.

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Abbreviated Title: Matrix Nebulisation for LC-MALDI-TOF/TOF MS
Abbreviations: β-casein: Beta-casein, **CHCA SMW:** CHCA sample, matrix, wash, **DHB ML:** DHB matrix layer, **EGF:** Epidermal growth factor, **EGFR:** Epidermal growth factor receptor, **ETD:** Electron transfer dissociation, **FA:** Formic acid, **OVA:** Ovalbumin, **PA:** Phosphoric acid, **Pro-GP:** 4G10 affinity-coupled MB-IAC Prot G, **TL:** Thin-layer.

Key Words: ESI-IT-MS/MS, LC, MALDI, Matrix Preparation, Phosphorylation, Technology

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STATEMENT OF AUTHORSHIP FOR CHAPTER 3

EZYprep LC-coupled MALDI-TOF/TOF MS: An improved matrix spray application for phosphopeptide characterisation. Proteomics 2010, 10, 2516-2530.

Mark R. Condina (Candidate)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Performed analysis on all samples, interpreted data, wrote manuscript

Certification that the statement of contribution is accurate

Signed.....Date.. .....

Johan O.R. Gustafsson (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Aided with matrix deposition using ImagePrep™ station, data interpretation and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Aided with immunoprecipitation of EGFR, data interpretation and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Aided with LC-ESI-IT-MS/MS using CID/ETD analysis, data interpretation and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Peter Hoffmann (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Supervised development of work, helped in data interpretation and manuscript evaluation and acted as corresponding author

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Chapter 4 Background:

The ability of the proposed EZYprep matrix deposition strategy to improve DHB matrix application for LC-MALDI-TOF/TOF MS as outlined in Chapter 3 now provides an optimised way to analyse the phosphoproteome of complex samples utilising the advantageous properties of DHB with PA. The low abundance of pTyr in complex samples highlights the importance of employing enrichment strategies as well as optimised MS approaches to improve pTyr characterisation. The development of proteomic methods capable of providing not only qualitative pTyr information but also show quantitative changes that occur with pTyr signalling are imperative to provide a complete overview of the phosphoproteome.

Although the method using Pro-GP/Cov-P for determination of the pTyr component of the phosphoproteome is very promising it is primarily a targeted approach in that the strategy has limited sample capacity making it less suitable for 'large-scale' (global) sample analysis. Tissue samples are often limited in both the amount and concentration of analytes, which can be attributed to a low number of cells present within the sample [127, 302, 372, 373]. As such, new methods, which display a higher stringency in terms of sensitivity and throughput, are essential to detect tyrosine phosphorylation sites [372].

The research presented aimed to utilise the 4G10 Ab/bead approach for the enrichment of pTyr peptides from complex samples. This enrichment step is subsequently combined with HILIC to improve phosphopeptide fractionation and resulting samples are analysed using the optimised LC-MALDI-TOF/TOF MS methodology outlined in Chapter 3. Experiments are designed to isolate and identify the presence and/or changes in phosphotyrosine sites between cell types cultured in the presence of specific stimuli. Quantitative analysis of pTyr between test samples is achieved by ICPL labelling of samples prior to pTyr enrichment. Both MS and subsequent MS/MS data will be used to characterise pTyr residues and quantify changes in phosphorylation between samples. This is potentially a highly sensitive approach that will not only identify phosphopeptides and the phosphorylation sites, but also will provide quantitative data relating to the level of expression.

Chapter 4

Phosphotyrosine quantitation and characterisation in insulin receptor signalling using ICPL and an optimised strategy for phosphoproteome analysis.

Abbreviated Title: Insulin receptor phosphorylation analysis using proteomics
Abbreviations: Ab, Antibody; **HILIC**, Hydrophilic Interaction Chromatography; **FA**, formic acid; **FACS**, Fluorescence activated cell sorting, **IGF**, insulin-like growth factor; **IGFBPs**, IGF-Binding proteins; **IR**, insulin receptor; **ICPL**, Isotope Protein coded Label; **MAP**, Mitogen-activated Protein, **PTK**, protein-tyrosine kinase; **PTP**, protein-tyrosine phosphatases

Key Words: ICPL/Insulin Receptor/LC-MALDI/MS/tyrosine phosphorylation

Abstract for Chapter 4

Despite the low abundance of phosphotyrosine in the mammalian proteome, the role of this PTM has been implicated in multiple signalling networks utilised in both normal and patho-physiological states. Its importance has resulted in research to not only identify and characterise tyrosine phosphorylation but also quantify expression levels in multiple cellular processes. Of the methodologies utilised, phosphotyrosine protein and/or peptide enrichment coupled with MS has shown the greatest promise to provide a comprehensive outline of the tyrosine phosphoproteome. The use of broadly-specific Abs for phosphotyrosine (e.g. 4G10) has been shown to enable sufficient enrichment of phosphoproteins and peptides for subsequent use in MS, as seen with the Pro-GP/Cov-P enrichment strategy previously described. Although suitable for low complexity samples (e.g. recombinant proteins), a more extensive enrichment strategy coupled with an optimised MS approach would be required for global analysis of the tyrosine phosphoproteome. As such, a multi-dimensional enrichment approach utilising HILIC coupled with phosphotyrosine peptide enrichment for subsequent analysis using EZYprep LC-MALDI-TOF/TOF MS analysis was utilised for the analysis of insulin-stimulated cell lysate to characterise phosphorylation for both IR-A and IR-B insulin receptor subtypes. In addition, an optimised ICPL labelling strategy was utilised to quantify phosphorylation levels of the insulin receptor subtypes.

4.1 Introduction

The insulin-like growth factor (IGF) system is composed of three ligands (insulin, IGF-I and IGF-II), their cognate receptors (IGF-IR, IGF-IIR and the insulin receptor isoforms A and B; IR-A, IR-B), and six high-affinity IGF-binding proteins (IGFBPs) [374, 375]. The IGF system is critical in normal foetal and post-natal growth and development, and has been implicated in multiple pathologies including cancer and diabetes due to its role in cell survival, metabolism and mitogenesis [376-381]. The critical role played by circulating IGFs in the development of cancer has resulted in a significant focus on characterisation of signal regulation in this system for the development of novel therapeutics.

The insulin receptor (IR), a member of the tyrosine kinase growth factor receptor family, consists of two alpha and two beta subunits linked by disulfide bonds [382]. The extracellular alpha subunits contain the ligand-binding domain, whilst the transmembrane beta subunits contain an intracellular tyrosine kinase domain [383]. The two demonstrated isoforms of IRs differ as a result of alternative splicing of exon 11, with IR-A lacking the 12 amino acids normally encoded by exon 11 [376, 384, 385]. In normal cell pathology, the IR-B isoform is predominant in a number of insulin-responsive tissues, primarily mediating the metabolic effects of insulin [376, 384-386]. In contrast, IR-A is the predominant isoform in the foetus and is responsible for regulation of growth and development [376, 387]. However, it has also been shown to be the dominant receptor sub-type expressed in a variety of cancer cell types, and activation of this receptor isoform by IGF-II plays a significant role in cell proliferation, survival and migration [387-391].

The binding of insulin to either IR-A and IR-B results in the activation of two downstream signalling pathways, the Akt/PKB and MAPK pathways. Both metabolic (predominantly Akt/PKB) and mitogenic (predominantly MAPK) signalling can be induced by insulin upon binding to both isoforms, however, IR-A activation on cancer cells has been shown to lead to mitogenic responses including survival and growth [387, 389-391]. The over-expression of insulin receptors in cancer could have important implications in the progression of the disease, highlighting the importance of a deeper understanding of IR signalling.

The activation of either metabolic or mitogenic signalling is dependent on not only the signalling pathway activated by the particular IR, but also contacts that are made between the ligand and the

receptor [392, 393]. Upon receptor ligation, auto-phosphorylation of the receptor occurs on tyrosine residues at specific regions of the receptor, including the catalytic domain and the C-terminal tail [394-397]. Auto-phosphorylation of specific tyrosine residues results in a conformational change which triggers the activation of intracellular signalling pathways [382]. It has been hypothesised that the activation of downstream pathways is dependent on the phosphorylation of specific residues upon ligand binding [398]. IR-B binds insulin with a 2-fold higher affinity (based on inhibition of europium-labelled insulin; IC_{50} 1.4 ± 0.1 nM) than IR-A, but binds both IGFs with low affinity (366 ± 15 IGF-1, 68 ± 11 nM IGF-II) [399]. In contrast, IR-A binds both IGF-1 (3-fold) and IGF-II (3.7-fold) with an affinity that is greater than the B-isoform (IC_{50} 120.4 ± 34.1 nM and 18.2 ± 2.4 nM for IGF-I and IGF-II, respectively), highlighting the influence of the twelve amino acids encoded by exon 11 on ligand binding [399-402]. Despite this difference in insulin binding affinity, the IR-B subtype undergoes greater auto-phosphorylation and has a higher level of tyrosine kinase activity than IR-A [399, 403]. The differences between IR-A and IR-B in terms of expression, binding affinity to ligands and activation of signalling pathways suggest that the two isoforms perform different functions [401, 402].

To date, analysis of phosphorylation of the IR and substrates has been performed using 1D and 2D SDS-PAGE and peptide sequencing [392, 395-397, 404-407]. The developments in MS for phosphoproteome identification and quantitation has seen a number of strategies focus on the effects of ligands and inhibitors on phosphorylation of the IR and downstream signalling targets [203, 244, 283, 301, 377, 408, 409]. Recent research on receptor tyrosine kinase phosphorylation has shown the activation of specific signalling pathways is dependent on the ordered phosphorylation of the receptor, which may be similar for all tyrosine kinases, including IR [398, 404, 410]. Therefore, the key differences in IR-A and IR-B signalling may be attributed to alterations in not only the sites of phosphorylation but also the duration and order that residues are phosphorylated. A sensitive proteomic-based strategy capable of quantifying and characterising phosphorylation (particularly pTyr phosphorylation) may identify the key regulators of IR signalling, depending on which IR isoform is activated.

Here a global phosphoproteomic strategy that utilises isotopic labelling combined with phosphopeptide enrichment and LC-MALDI-TOF/TOF MS analysis for the comparative analysis of IR activation upon insulin binding is described. Mouse IGF-1R-negative fibroblasts over-expressing the human IR-A (R^{-ve}IR-A) or IR-B (R^{-ve}IR-B) cells [399] were stimulated with insulin prior to lysate collection. Lysates from both cell types were labelled using an optimised ICPL strategy. The digested samples were combined

and the phosphopeptides were enriched using HILIC and a 4G10 Ab column. Both flow-through and eluted fractions were subsequently analysed using LC-MALDI-TOF/TOF MS, utilising EZYprep DHB matrix deposition for phosphopeptide fraction analysis. This global phosphoproteomic strategy was undertaken to identify quantitative differences in tyrosine phosphorylation of the IR-A and IR-B receptor subtypes, and on tyrosine phosphorylation events in signalling cascades downstream of the receptors upon stimulation with insulin.

4.2 Experimental

4.2.1 Materials

Formic acid (FA, p.a., >98% purity) and Phosphoric Acid (PA, 50% w/w) were obtained from Fluka Chemie GmbH (Neu-Ulm, Germany). DTT and iodoacetamide (IAM) were purchased from Sigma-Aldrich (Castle Hill, Australia). TFA was obtained from Applied Biosystems (Warrington, UK), whilst LC grade ACN was obtained from Merck (Darmstadt, Germany). Ammonium bicarbonate was from BDH (Poole, UK). For enzymatic digestion, modified porcine Trypsin was purchased from Promega (Madison, WI, USA). CHCA and DHB matrices were purchased from Bruker Daltonics (Bremen, Germany). All aqueous solutions were prepared using deionised water with a resistance of less than 18.2 MΩ. All other reagents and solvents were of highest-grade analytical quality and were used without further purification. Protein A (Prot A) sepharose beads were purchased from GE Healthcare, while anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. The SERVA ICPL™ kit was obtained from Bruker Daltonics.

4.2.2 R^{-ve}IR-A/ R^{-ve}IR-B Cell lines and culture

IGF-IR-deficient (R^{-ve}) mouse embryo fibroblast lines engineered to express similar levels of human IR-A and IR-B have been previously described [399, 411]. 5.3 x 10^6 cells/T175 flask were serum starved for 4 hours in serum-free medium before being treated with 100 nM insulin for 10 mins at 37°C, 5% CO_2. Following stimulation, cells were washed twice with ice-cold PBS before being lysed in 1 mL lysis buffer containing 1% (vol/vol) Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM NaF and protease inhibitor cocktail (Sigma-Aldrich). Flasks were incubated for 1 hour at 4°C and lysates were centrifuged at 12000 x g for 10 mins at 4°C. Each supernatant was transferred to a fresh tube. Lysates were then either stored at -80°C for LDS-PAGE or a 5-fold excess of 100% ice-cold acetone was added and precipitated lysates stored at -80°C.

4.2.3 LDS-PAGE and Western blotting

Protein concentration was determined using EZQ quantitation kit (Invitrogen). 100 µg lysate from stimulated and control R^{-ve}IR-A and R^{-ve}IR-B cells was concentrated using vivaspin centrifugal concentrators (GE Healthcare) with a 10 kDa filter and added to 4x lithium dodecyl sulphate sample buffer (Invitrogen, Carlsbad, California, USA), and 0.5 M DTT in a 65:25:10 volume ratio, respectively.

Samples were heated at 95°C for 10 mins and loaded onto NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen). Novex pre-stained molecular weight markers (Invitrogen) were loaded on each gel. Electrophoresis was performed between 125 V to 200 V using 50 mM MOPS, 50 mM Tris base, 3.5 mM SDS, 1mM EDTA, pH 7.7 as running buffer in XCell SureLock™ 1D gel tanks (Invitrogen, Carlsbad, California, USA). The resulting gels transferred to a nitrocellulose membrane using the wet transfer blotting system (Invitrogen) for 2 hours according to manufacturers' instructions. After transfer, the membrane was blocked in Tris-Buffered Saline Tween-20 (TBST) solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 0.5% bovine serum albumin (BSA) overnight at 4°C with agitation. The blots were then probed with either 4G10 Ab (1 µg/mL) in TBST containing 3% skim milk powder (3% MLK-TBST), phopsho-IR /IGF-1R [$Y^{1158/1162/1163}$] in TBST containing 3% BSA (Biosource International, Camarillo, CA), CT-1 anti-IR (a gift from Professor K. Siddle, Cambridge, UK) in TBST or the PathScan® Multiplex Western Cocktail 1 (Cell Signalling) Ab for 2 hours at room temperature with agitation.

After washing 5x with TBST for 5 mins each, the blots were probed with the relevant secondary Abs using donkey anti-mouse horseradish peroxidise (Rockland Immunochemicals, Gilbertsville, USA) at 1/5000 dilution in 3% MLK-TBST for 1 hour at room temperature with agitation. Following three washes with TBST for 10 mins each, the blot was developed using Chemiluminescent Western Blotting Kit (Sigma-Aldrich) for 5 mins.

4.2.4 Flow Cytometry - Fluorescence activated cell sorting (FACS)

Cultured cells were serum starved for 4 hours in serum-free medium before being disrupted using cell disruption buffer (40 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, filter sterilised). Cell counts were performed using trypan blue staining and cells re-suspended at 4 x 10^6 cells/mL in 1 x phosphate buffered saline (PBS)/1% BSA/0.01% sodium azide. Fc receptors were blocked using murine gamma globulin for 20 mins on ice and the cells were subsequently treated with IgG1 for negative control or the monoclonal anti-IR antibody 83-7 (a gift from Professor K. Siddle, Cambridge, UK) for 1 hr on ice in the dark. After washing twice with 1 x PBS/1% BSA/0.01% sodium azide, Fluorescein isothiocyanate (*FITC*) conjugated secondary Ab (anti-IgG) was added to the cells and incubated for 30 mins on ice in the dark. Cells were washed three times using 1 x PBS/1% BSA/0.01% sodium azide, and fixed in 1/10 dilution of 37% Formaldehyde (3.7%) in PBS for 20 mins on ice in the dark prior to storing cells in PBS with 1% formaldehyde in the dark. IR expression was determined by FACS analysis using the BD FACSCanto Flow Cytometer (BD Biosciences, San Jose, CA) controlled using FACSdiva software (Version 5.0.3, firmware version 1.14).

4.2.5 ICPL™ protein labelling

Precipitated protein was re-dissolved in ICPL lysis buffer (6M Guanidine-HCl/0.1M HEPES, pH 8.5) and protein concentration was determined as described in section 3.2.3. Protein lysates were labelled using the ICPL™ reagent kit (Serva Electrophoresis, Heidelberg, Germany) according to the manufacturers' instructions. A more detailed description of the protocol is described elsewhere [13, 210, 211]. After labelling and precipitation with acetone (according to the ICPL protocol), lysates were prepared for in-solution digests using trypsin.

4.2.6 In-solution digestion with trypsin

Following reduction and alkylation according to the ICPL protocol (for both protein and peptide labelling - [13, 210, 211]), trypsin in 50 mM acetic acid was added to a final ratio of substrate: enzyme of 50:1. The sample was incubated overnight at 37°C; the reaction was stopped using 5 µL 10% TFA or prepared for ICPL peptide labelling. For protein labelling experiments, 50 mM ammonium bicarbonate (NH_4HCO_3) was used as the digestion buffer.

4.2.7 ICPL™ peptide labelling

ICPL peptide labelling was carried out as per manufacturers' instructions with modifications to improve protein recovery. For control labelling experiments, lysate from R^{-ve}IR-A cells was dissolved in ICPL lysis buffer. Proteins were reduced and alkylated as described by the ICPL protocol [13, 210, 211]. Subsequently the labelled protein was diluted with six volumes of deionised water to bring the concentration of guanidine-HCl to 1M. Labelled proteins were digested in-solution as described above. Following digestion, the pH of the peptide mixture was adjusted to 8.5 using 2N NaOH and HCl for ICPL labelling. After labelling, the peptide mixture was either analysed by LC-MALDI-TOF/TOF MS or desalted using columns (Alltech) packed in-house using zorbax-C18 packing material (Agilent Technologies). After desalting, samples were concentrated by lyophilisation for HILIC analysis.

4.2.8 HILIC

An Agilent 1100 series binary LC system was used for LC-MALDI-TOF/TOF MS applications. The system was operated using binary gradients of solvent A (98% water with 0.1% TFA) and solvent B (98% ACN with 0.1% TFA) was utilised. A 4.6 x 250mm TSK-gel Amide-80 HILIC column packed with 5 µm, particle column (TOSOH Biosciences) was used for separation. Additional LC components included

a degasser system (G1379A) coupled to a quaternary pump (G1311A), multi-wave detector (G1365B), fraction collector (G1364C) and automated sampler (G1329A). The TSK-gel Amide-80 column used a flow rate of 0.5 mL per minute and 10 µL of peptide sample was loaded in 90% solvent B. Loaded samples were eluted using an inverse gradient of 90% B to 85% B in 5 minutes, followed by 85% B to 70% B in 40 minutes and finally a steep gradient to 0% B in 5 minutes. Twenty-three 1 mL fractions were collected throughout the gradient. HILIC fractions were pooled according to their retention times such that two fractions; hydrophobic and hydrophilic fractions remained. After lyophilisation, samples were re-dissovled in 30% ACN in ultrapure water followed by 10 mM NH_4HCO_3 and pH was adjusted to 8.5 using 2N NaOH for pTyr peptide enrichment.

4.2.9 4G10 column generation

Protein A sepharose™ beads (GE healthcare) were loaded into Micro Bio-Spin™ chromatography columns (Bio-Rad, California, USA) and the column was equibrilated with 10x bed volumes of 50 mM Tris-HCl, pH 8.0. 4G10 Ab in 50 mM Tris-HCl was loaded at a ratio of 1:1 (1 µg beads: 1 µg 4G10) and incubated for 1 hour at room temperature with mixing. After incubation, the column was washed with 10x bed volumes of 50 mM Tris-HCl and subsequently stored in 50 mM Tris-HCl, pH 8.0/0.01% sodium azide. Flow-through and wash fractions were collected at binding efficieny was determined using absorbance at 280 nm using the SmartSpec™ 3000 (Bio-Rad, California, USA).

4.2.10 Tyrosine phosphopeptide enrichment

4G10 Ab columns were equibrilated using 10x bed volumes of 10 mM NH_4HCO_3. Peptide samples (in 10 mM NH_4HCO_3) were loaded and column was incubated for 3 hours at 4°C with mixing. After incubation, flow-through fractions were collected (for unbound non-phosphorylated peptides as well as serine and threonine phosphopeptides) and the column was washed with 10x bed volumes 10 mM NH_4HCO_3. Bound peptides were eluted with 10 mM NH_4HCO_3/150 mM phenylphosphate. All flow-though and eluted fractions were dried down and prepared for LC-MALDI-TOF/TOF MS analysis.

4.2.11 Capillary HPLC for MALDI-TOF/TOF MS

An Agilent 1100 capillary HPLC (LC) system was used for LC-MALDI applications. The system was operated using binary gradients of solvent A (0.1% TFA in 5% ACN) in ultrapure water and solvent B (0.1% TFA in 95% ACN) was utilised. A 0.18 x 150mm Acclaim Pepmap100™ reverse-phase (RP) column packed with 3 µm, porous (100 Å) C-18-coated silica beads (LC Packings Dionex) was used for separation. Additional LC components included a degasser system (G1379A) coupled to a capillary

pump (G1376A) and micro-Well-plate-sampler (μ-WPS) automated sampler (G1377A). The Acclaim Pepmap100 column used a flow rate of 1 μL per minute and 5 μL of peptide sample was loaded in 0% solvent B. After loading for 5 minutes, peptides were eluted with a gradient of 0% B to 12% B in five minutes followed by 12% B to 48% B in 36 minutes, then 48% B to 60% B for five minutes and finally a steep gradient to 80% B for 4.5 minutes before returning to initial conditions (0% B). Fractions were spotted from 7 minutes onwards to ensure collection of any peptides that did not bind to the column.

4.2.12 LC Fraction Collecting

Eluted peptides were collected directly from the post-column capillary using a Proteineer Fraction Collector™ (Bruker Daltonics). The fraction collector (Fc) was used to deposit chromatographic fractions directly onto a MTP 384 MALDI 600 μm AnchorChip™ target (Bruker Daltonics) every 15 seconds (192 fractions) over the course of the gradient. With each fraction, a sheath solution (0.5 μL) of 50% ACN/0.05% PA/0.05% TFA in water was deposited using the matrix deposition function of the Proteineer Fc. A 1:100 dilution of Bruker standard peptide calibrant (Bruker Daltonics) dissolved in 0.1% TFA in water was deposited manually in 1 μL amounts onto each calibration spot.

4.2.13 Matrix deposition strategies

Following fraction collection, target sample spots were allowed to dry completely at room temperature. Two matrix deposition strategies were utilised; CHCA SMW application, as described previously [152], was used for ICPL labelling optimisation experiments and analysis of non-phosphorylated samples. For all other LC-MALDI-TOF/TOF MS experiments, EZYprep matrix deposition with DHB matrix was utilised as described previously [306], in which a total of 80 layers were applied to effectively cover all raster positions on the MALDI target.

4.2.14 MALDI-TOF MS

MALDI TOF mass spectra were acquired using a Bruker ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in reflectron positive ion mode. FlexControl (version 3.1, Bruker Daltonics, Bremen, Germany) software was used to control the analysis. Auto-execute acquisition (high-throughput automatic collection) of mass spectra of LC-fractions was controlled using WARP-LC software (version 3.2, Bruker Daltonics, Bremen, Germany) in SILE mode interfaced with FlexControl. MALDI laser intensity was selected by the operator to provide optimal intensity and resolution of acquired mass spectra. External calibration was performed using

peptide standards (Bruker Daltonics, Bremen, Germany) which were analysed under identical conditions. Peak masses and intensities of TOF and MS/MS spectra were detected with FlexAnalysis (Version 3.1, Bruker Daltonics, Bremen, Germany) using the SNAP algorithm.

4.2.15 MALDI-TOF/TOF MS/MS analysis and database search

Following completion of auto-execute MS collection, a WARP-LC method was used for calculation of a list of labelled peptide pairs for MS/MS analysis, based upon a pre-set LC-MALDI SILE experimental set up, known as a WARP-LC method. The criteria for this automatic method were the following: MS tolerance set to 100 ppm, compounds separated by less than 5 HPLC fractions were combined and peaks appearing in more than 50% of spectra were defined as background. Signal to noise (S/N) thresholds for MS/MS acquisitions were between 15 and 30 depending on the calculated compound list. Auto execute data files from WARP-LC containing combined peptide MS and MS/MS data were exported to BioTools (Version 3.1, Bruker Daltonics, Bremen, Germany) and submitted to the in-house Mascot database search engine (Matrix Science). The specifications were the following: (i) enzyme: Trypsin, (ii) fixed modifications: Carbamidomethyl (C*), (iii) variable modifications: Oxidation (M), Phosphorylation (ST) and (Y), (iv) mass tolerance MS: 100 ppm, (v) MS/MS tolerance: 0.8 Da, (vi) missed cleavages: 2, (vii) Taxonomy: Mus. The MOWSE and probability scores were used as the criterion for ion annotation in the spectra. Alternatively, MS/MS spectra matching to precursor ions of potential phosphotyrosine peptides were automatically annotated using Biotools and/or manually using FlexAnalysis and different positions of phosphorylation were compared. For replicate analyses of the insulin stimulated R^{-ve}IR-A and R^{-ve}IR-B lysates, the individual search results were combined into protein compilations using the ProteinExtractor functionality of Proteinscape 2.1.0 573 (Bruker Daltonics, Bremen, Germany), which conserved the individual peptides and their scores, while combining them to identify proteins with much higher significance than achievable using individual searches. In order to exclude false positive identifications, peptides with Mascot scores below 25 (which was chosen on the basis of manual evaluation of the MS/MS data of peptides with scores below this number) were rejected, unless part of a peptide pair in which one peptide pair had a score above 25. In addition, any protein with a score of 30 or less was instantly rejected.

4.2.16 Ingenuity Pathways Analysis (IPA)

Identified proteins from the insulin stimulated ICPL labelled R^{-ve}IR-A/R^{-ve}IR-B lysate with regulation values deemed significant were searched in the Ingenuity Pathways Analysis® (IPA) software (Ingenuity System Inc, USA). The “Core Analysis” function of IPA was used to identify the biological functions and

diseases that most significantly correlated with the proteomic data set submitted. Fischer's exact test was utilised for p-value calculation to determine the probability that each biological function and/or disease assigned to the regulated proteins were by chance alone. Networks of the regulated proteins were algorithmically generated based on their connectivity and assigned a score, which takes into account the number of proteins in the network and size of the network to determine the relevance to the selected regulated proteins.

4.3 Results

The study presented here aims to quantify phosphorylation (with a particular focus on tyrosine phosphorylation) of both the IR isoforms upon insulin stimulation as well as tyrosine phosphorylation of proteins involved in activated downstream signalling pathways. In order to achieve this, the implementation of an optimised sample preparation method, which was compatible with MS analysis and minimised phosphorylation loss, was utilised. The low abundance of phosphoproteins compared with non-phosphoproteins in the proteome emphasises the requirement for enrichment prior to MS analysis. The use of ICPL for quantitation coupled directly with HILIC and pTyr enrichment prior to MS sought to provide effective fractionation of the sample such that pTyr enriched fractions, pSer/pThr enriched fractions and non-phosphorylated fractions could be analysed independently using an optimised LC-MALDI-TOF/TOF MS analysis.

4.3.1 ICPL labelling optimisation for peptide quantitation

The use of stable isotope labelling in conjunction with MS shows promise to effectively quantify and characterise the phosphoproteome of complex protein samples. In particular, ICPL, with its ability to improve peptide ionisation and quantify peptide abundance at the MS level minimises data acquisition at the MS/MS level, thereby minimising redundancy and subsequently improving characterisation of only differentially-expressed proteins.

In order to utilise ICPL labelling for quantitation of protein phosphorylation, the ICPL labelling methodology required alteration such that all peptides generated from protease digestion of complex samples were labelled. This was achieved by labelling samples after protease digestion (using trypsin), which results in at least one free amino group (labelling site) per peptide. The efficiency of ICPL peptide labelling was determined using lysate collected from R^{-ve}IR-A cells, which was split at a 1:1:1:1 ratio in four vials. Two lysate samples were directly labelled according to the conventional ICPL approach (for protein labelling [13]), whilst the other two lysate samples were labelled using a modified ICPL peptide approach, involving digestion of protein samples with trypsin prior to labelling (Figure 4.1). Upon labelling, light and heavy samples from the protein and peptide samples were combined and 2µg lysate was analysed using LC-MALDI-TOF/TOF MS.

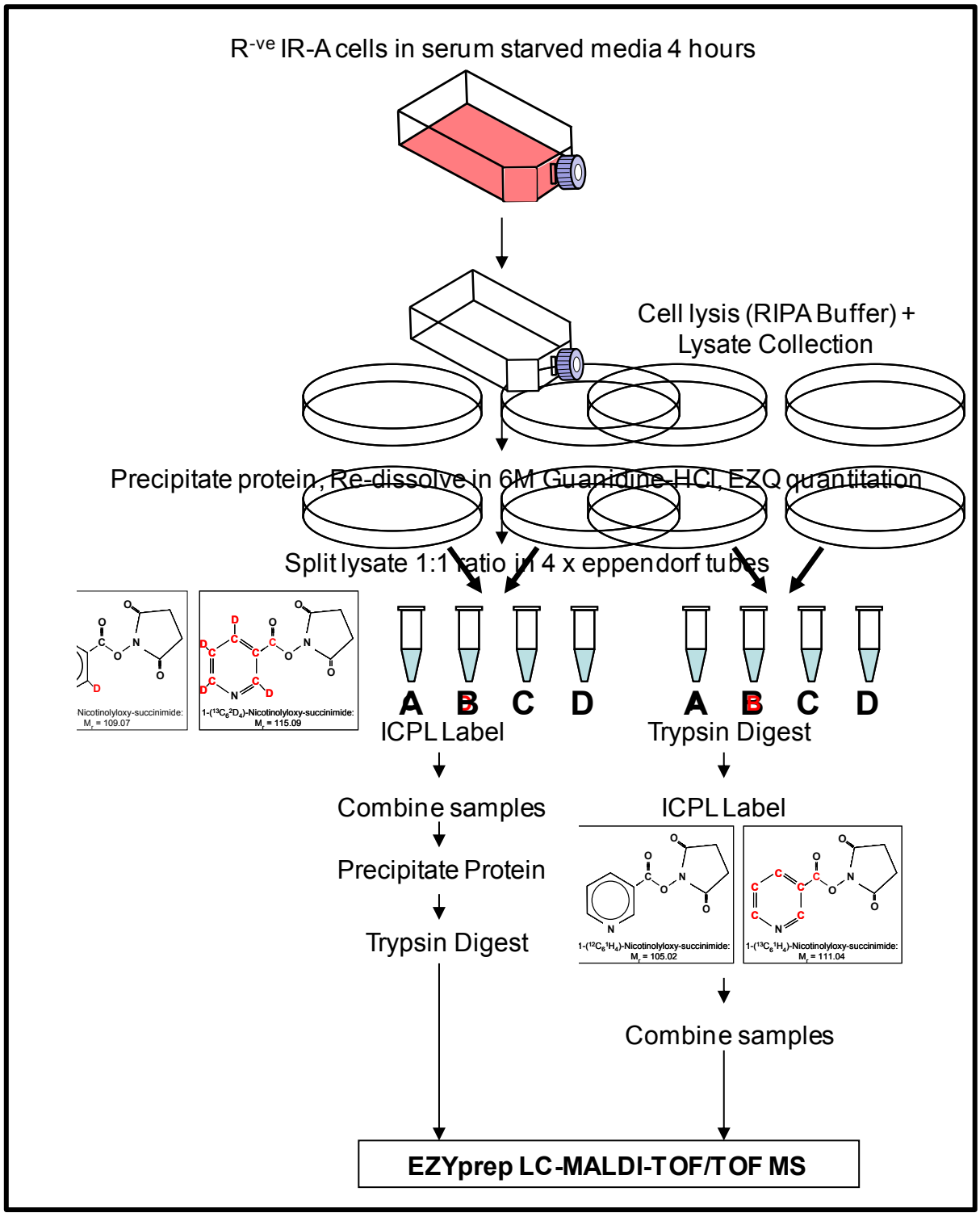


Figure 4.1: ICPL protein versus peptide labelling
Diagram of the experimental workflow used for the comparative analysis of ICPL protein versus peptide labelling. R-veIR-A lysate is collected and split evenly across four tubes (1:1:1:1). Protein is labelled according to the standard ICPL protocol; the samples are precipitated and subsequently digested with trypsin for LC-MALDI-TOF/TOF MS analysis. Alternatively, protein is digested prior to labelling with ICPL. Once labelled, peptide samples are combined and 2µg of lysate prepared for LC-MALDI-TOF/TOF MS analysis.

A summary of proteins identified following LC-MALDI-TOF/TOF MS analysis of R^{-ve}IR-A lysate labelled on either a protein or peptide level is shown in Table 4.1. LC-MALDI-TOF/TOF MS analysis of 2µg R^{-ve}IR-A lysate labelled with ICPL on the protein level identified eighteen proteins, providing quantitative information for ten proteins. Using ICPL protein labelling strategy, 4 proteins were quantified by one labelled peptide pair, 4 proteins quantified by 2 labelled peptide pairs, 1 protein quantified by three labelled peptide pairs and only 1 protein quantified by 4 labelled peptide pairs. In contrast, twenty-five proteins were identified following LC-MALDI-TOF/TOF MS analysis of lysate labelled with ICPL at the peptide level (Table 4.1). Significantly, quantitative information was obtained for all twenty-five proteins. Using ICPL peptide labelling, 6 proteins were quantified by one labelled peptide pair, 7 proteins by two labelled peptide pairs and the remaining (12) proteins quantified by more than two labelled peptide pairs. In the whole analysis of the ICPL peptide labelled sample, only one un-labelled peptide could be identified. Nine proteins were identified and quantified solely using ICPL peptide labelling. This is in contrast to only two proteins being identified solely with ICPL protein labelling; protein S100-A4 and ubiquitin.

The sequence coverage of all observable peptides, such as proteins such as Pyruvate kinase isozymes M1/M2 (total MS/MS ICPL peptide labelling – 19%; total MS/MS ICPL protein labelling – 17.3%) and Tubulin beta-5 chain (total MS/MS ICPL peptide labelling – 13.7%; total MS/MS ICPL protein labelling – 15.7%) was comparable between the two strategies (ICPL protein labelling and ICPL peptide labellling, Table 4.1). However, the sequence coverage of only ICPL labelled peptides was, not surprisingly, higher in the ICPL peptide labelling approach, such as with Actin cytoplasmic 1 protein (ICPL peptide labelling strategy = 32.3% MS/MS coverage, ICPL protein labelling strategy = 19.2% MS/MS coverage) and Tubulin beta-5 chain (ICPL peptide labelling strategy = 13.7% MS/MS coverage, ICPL protein labelling strategy = 3.6% MS/MS coverage). The results from Table 4.1 suggest an improvement in quantitative data acquisition using the optimised ICPL peptide labelling, which was achieved because all observable peptides were labelled.

In an ICPL label experiment, one should consider determining the regulation window of the experimental variation by combining identical amounts of differentially labelled samples (*i.e.* labelled lysate combined at a 1:1 ratio) and quantify the peptide pairs. In a situation without experimental variability, all ratios obtained would be 1. Previous analyses on ICPL-labelled lysate samples combined at a 1:1 ratio (using the conventional ICPL protein labelling strategy) have determined that ICPL H/L ratio values outside 0.73 and 1.39 can be considered as regulated [163]. Other research analyses

Protein*	Accession	No. Peptide Pairs ICPL PEPTIDE LABELLING (n=3)[a]	No. Unlabelled peptides (n=3)[a]	Av. Seq. Cov. MS (n=3)[a]	Av. Seq. Cov. MS/MS (n=3)[a]	Ratio L/H (Av. ± SD)[b]	Av. Seq. Cov. MS/MS Labelled peptides (n=3)[a]	No. Peptide Pairs ICPL PROTEIN LABELLING (n=3)[a]	No. Unlabelled peptides (n=3)[a]	Av. Seq. Cov. MS (n=3)[a] Arg-C	Av. Seq. Cov. MS/MS (n=3)[a]	Ratio L/H (Av. ± SD)[b]	Av. Seq. Cov. MS/MS Labelled peptides (n=3)[a]
Heat shock cognate 71 kDa protein	**HSP7C_MOUSE**	9	-	79.3%	21.7%	1.04 ± 0.19	21.7%	-	5	22.3%	16.7%	-	0
Actin, cytoplasmic 1	**ACTB_MOUSE**	8	-	63.7%	32.3%	0.99 ± 0.08	32.3%	4	8	34.6%	40.3%	1.54 ± 0.19	19.2%
Pyruvate kinase isozymes M1/M2	**KPYM_MOUSE**	5	-	78.7%	19.0%	0.99 ± 0.04	19.0%	1	3	27.7%	17.3%	1.46 ± 0.37	3.1%
Heat shock protein HSP 90-beta	**HS90B_MOUSE**	5	-	70.7%	9.7%	1.01 ± 0.13	9.7%	-	5	**n/a**	9.0%	-	0
Alpha-enolase	**ENOA_MOUSE**	4	-	82.0% (n=1)	11.3%	1.03 ± 0.09	11.3%	2	2	22.7%	13.7%	1.61 ± 0.09	8.9%
Tubulin alpha-1B chain	**TBA1B_MOUSE**	2	-	50.7%	14.3%	0.97 ± 0.05	14.3%	2	4	34.0%	23.0%	1.53 ± 0.13	8.2%
Tubulin beta-5 chain	**TBB5_MOUSE**	4	-	56.0%	13.7%	1.07 ± 0.08	13.7%	2	4	18.0%	15.7%	1.34 ± 0.44	3.6%
Serum albumin	**ALBU_MOUSE**	4	1	72.3%	8.0%	0.98 ± 0.09	7.0%	-	2	**n/a**	5.7%	-	0
Annexin A2	**ANXA2_MOUSE**	4	-	92.5% (n=2)	16.7%	0.90 ± 0.12	16.7%	-	4	33.3%	22.0%	-	0
Elongation factor 2	**EF2_MOUSE**	2	-	69.0% (n=1)	7.0%	0.86 ± 0.04	7.0%	-	2	24.5% (n=2)	5.0%	-	0
Annexin A5	**ANXA5_MOUSE**	4	-	81.0% (n=1)	14.7%	1.67 ± 0.17	14.7%	-	4	**n/a**	17.3%	-	0
Annexin A1	**ANXA1_MOUSE**	3	-	95.3%	11.3%	0.85 ± 0.16	11.3%	-	3	**n/a**	11.0%	-	0
ATP synthase subunit beta, mitochondrial	**ATPB_MOUSE**	2	-	64.5% (n=2)	7.0%	0.93 ± 0.06	7.0%	-	2	13.7%	7.3%	-	0
Galectin-1	**LEG1_MOUSE**	3	-	73.0% (n=2)	25.0%	1.02 ± 0.07	25.0%	1	1	**n/a**	17.0%	1.32 ± 0.02	8.9%

Peptidyl-prolyl cis-trans isomerase A	**PPIA_MOUSE**	2	-	83.0% (n=1)	23.7%	1.11 ± 0.07	23.7%	3	1	49.0%	32.0%	1.84 ± 0.19	22.0%
Nucleoside diphosphate kinase B	**NDKB_MOUSE**	3	-	**n/a**	22.0%	0.99 ± 0.11	22.0%						
Tropomyosin alpha-3 chain	**TPM3_MOUSE**	2	-	**n/a**	9.7%	0.99 ± 0.19	9.7%						
Elongation factor 1-alpha 1	**EF1A1_MOUSE**	2	-	**n/a**	6.0%	1.73 ± 0.19	6.0%	1	2	**n/a**	6.0%	1.8 ± 0.02	4.1%
Glyceraldehyde-3-phosphate dehydrogenase	**G3P_MOUSE**	2	-	**n/a**	11.3%	1.08 ± 0.07	11.3%						
Protein S100-A4	**S10A4_MOUSE**							1	1	**n/a**	33.0%	1.88 ± 0.18	18.8%
Protein S100-A6	**S10A6_MOUSE**	1	-	**n/a**	8.0%	1.04 ± 0.04	8.0%						
60 kDa heat shock protein, mitochondrial	**CH60_MOUSE**	1	-	**n/a**	4.3%	0.91 ± 0.05	4.3%						
Eukaryotic initiation factor 4A-I	**IF4A1_MOUSE**	1	-	**n/a**	5.3%	0.85 ± 0.10	5.3%						
Ubiquitin	**UBIQ_MOUSE**							2	-	**n/a**	44.7%	1.48 ± 0.21	44.7%
60S ribosomal protein L14	**RL14_MOUSE**	1	-	99.3%	5.0%	0.96 ± 0.04	5.0%						
Annexin A3	**ANXA3_MOUSE**	1	-	**n/a**	4.0%	0.64 ± 0.02	4.0%						
Heterogeneous nuclear ribonucleoprotein H	**HNRH1_MOUSE**	1	-	**n/a**	6.3%	1.01 ± 0.07	6.3%						

n/a: No peptide matches based on combined MS and MS/MS data

* Proteins selected based on being a minimum of two unique peptides above the identity threshold and being present across triplicate analyses

[a] Values are obtained from the average of triplicate analyses unless otherwise stated

[b] The ratios of all isotopic pairs were calculated from the peak intensity in the MALDI-MS spectrum and averaged.

Table 4.1: Proteins identified from ICPL peptide and/or protein labelling.

(utilising alternative SILE labelling such as SILAC) have adopted a regulation window of 0.66 to 1.5 [197] or the use of a normalised expression difference of 1 standard deviation (1SD) as exclusion criteria [211]. In order to determine an appropriate regulation window for proteins at a 1:1 ratio, a preliminary assessment of variance was performed using 50 randomly selected peptide pairs from a R^{-ve}IR-A ICPL peptide labelling experiment. The intensity ratio of heavy to light peptides in each pair was log transformed, mean and standard deviation were calculated and these values were converted back to ratios using an exponential function (data not shown). A regulation ratio of 1.2 was calculated for one standard deviation [1.45 for two standard deviations]. Thus, 95% of all control regulated peptides should fall within the range 0.689 and 1.452.

Of the twenty-five proteins identified using the optimised ICPL peptide labelling strategy, only three proteins (Annexin A3, A5 and Elongation Factor 1-alpha) produced ratios outside the regulation window (0.64 ± 0.02, 1.67 ± 0.17 and 1.73 ± 0.19, respectively). The reasons why these ratios were obtained were unclear; however one possible explanation is that ICPL-labelled peptides may co-elute with other peptides with almost identical m/z, thereby increasing the MS intensity obtained. Despite the presence of proteins with H/L ratios outside the regulation window, the majority (22 out of 25) of ratios obtained were close to 1 (Table 4.1).

In contrast, only two (tubulin beta-5 chain and Galectin-1) of the ten proteins from the conventional ICPL protein labelling strategy that yielded quantitative information were within the regulation window of 0.689 to 1.452 (Table 4.1). Our laboratory has previously determined a regulation window (using a different lysate) for ICPL protein labelling to be between 0.67 and 1.5 (Gustafsson *et. al*. unpublished). This regulation window is almost identical to the window obtained with the optimised ICPL peptide labelling approach. Using the previously reported regulation window, the number of ratios obtained from the conventional ICPL protein labelling strategy of R^{-ve}IR-A lysate increases to four (tubulin beta-5 chain, Galectin-1, Pyruvate kinase isozymes M1/M2 and Ubiquitin, Table 4.1). All L/H ratios obtained from ICPL protein labelling were between 1.32 and 1.88, suggesting a systematic error with the heavy label used in ICPL protein labelling experiments.

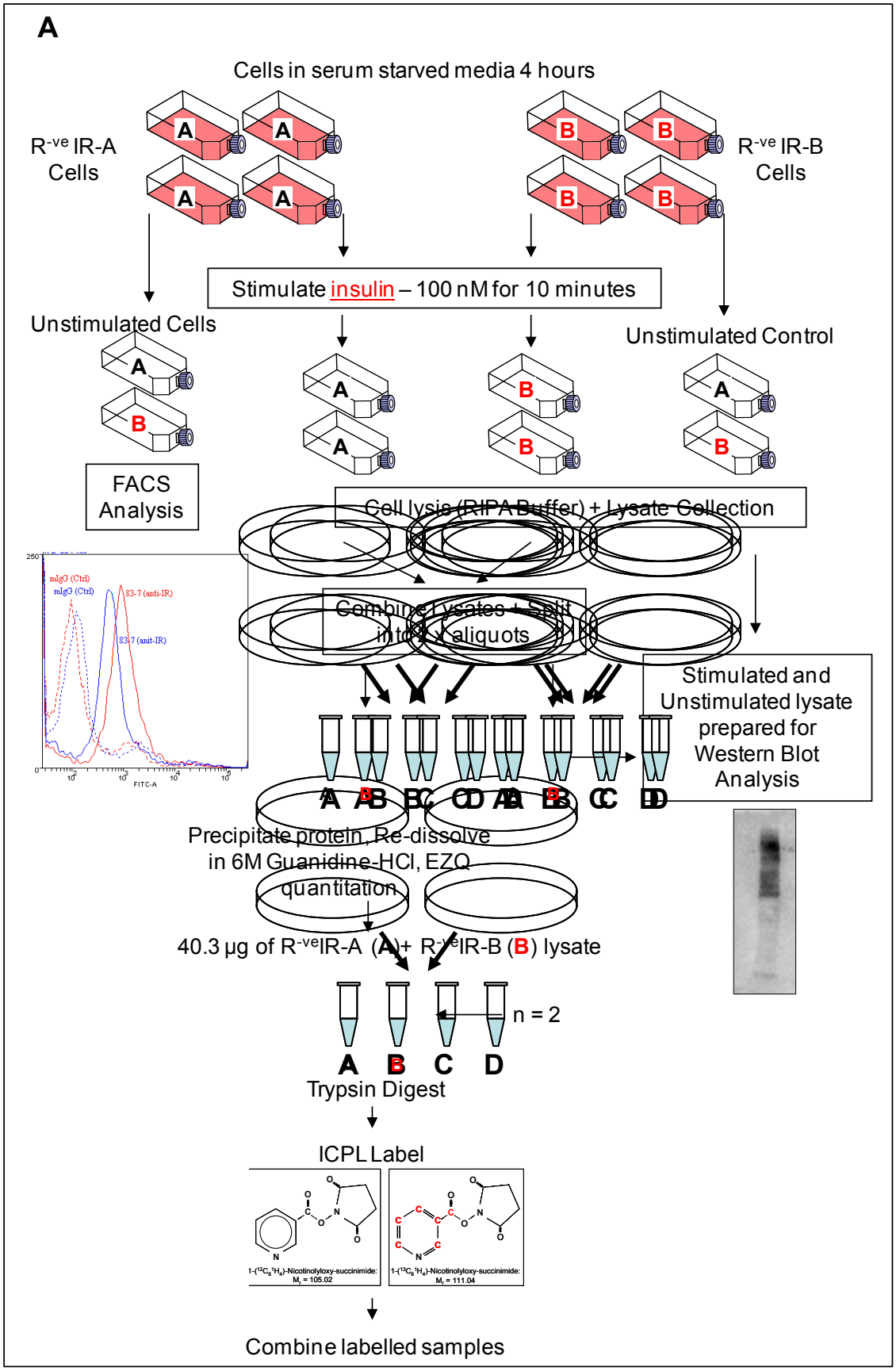
4.3.2 Quantitative analysis of the tyrosine phosphoproteome after IR stimulation with insulin

The results from 4.3.1 presented here clearly showed that labelling of complex samples using the optimised ICPL peptide labelling strategy increased the number of proteins identified compared with the conventional ICPL labelling strategy. Furthermore, the ICPL peptide labelling provided more quantitative information of these proteins identified as it ensured the observed peptides contained at least one label for ratio determination. The labelling of every observable peptide with at least one ICPL label is important for bottom-up PTM analysis, such as phosphorylation, as phosphopeptides produced after trypsin digestion of protein samples may not contain an ICPL labelling site (lysine residue or an N-terminal fragment). As such, in order to quantify changes in tyrosine phosphorylation after insulin stimulation of lysate IR-A and IR-B isoforms and downstream signalling proteins, the ICPL optimised peptide labelling strategy was combined with HILIC and pTyr enrichment (by affinity chromatography) for analysis using EZYprep LC-MALDI-TOF/TOF MS analysis.

Figure 4.2 outlines the proteomic-based approach taken for global tyrosine phosphorylation analysis. The protocol utilises multiple techniques in order to provide information on changes in pTyr depending on which IR isoform was activated. For insulin stimulation, a concentration of 100 nM was chosen. Although this concentration is higher than the typical insulin concentration of ≥ 10 nM used in many insulin signalling studies [412-418], it has been consistently used to stimulate phosphorylation of insulin-activated pathways [412]. Interestingly, insulin stimulation at physiological concentrations (between 100-500 pM) has been shown to stimulate phosphorylation of IR-B whereas higher physiological and pharmacological concentrations do not increase IR-B phosphorylation signal intensity [412]. However, the 100 nM concentration strongly stimulates phosphorylation of downstream signalling proteins, including Akt and eNOS [412]. As such, higher insulin concentrations have been used, particularly in proteomic-based strategies analysing the phosphoproteome of insulin signalling [203, 283].

Lysate was collected from unstimulated and stimulated cells for downstream western blot analyses or ICPL labelling followed by pTyr enrichment (utilising HILIC followed by a pTyr-specific 4G10 Ab column) and analysis using LC-MALDI-TOF/TOF MS (Figure 4.1). In addition, FACS analysis was employed to determine IR receptor expression of both R^{-ve}IR-A cells and R^{-ve}IR-B cells. Here, unstimulated R^{-ve}IR-A and R^{-ve}IR-B cells were collected and probed using the 83-7 Ab (specific for the IR) or an IgG negative control. Using flow cytometry, cell populations for both the negative control and IR^{+} cells were defined

based on the negative control for both R^{-ve}IR-A and R^{-ve}IR-B cells. Figure 4.3 shows the overlayed FACS graphs of R^{-ve}IR-A and R^{-ve}IR-B cells. As shown in the graph, the IR^{+} populations from both R^{-ve}IR-A and R^{-ve}IR-B cells are similar; meaning that the cells are expressing similar levels of receptor (slightly higher levels on the R^{-ve}IR-A cells) and a direct comparison of these cell types can be achieved with confidence.


A
Cells in serum starved media 4 hours
R-ve IR-A Cells
R-ve IR-B Cells
Stimulate insulin – 100 nM for 10 minutes
Unstimulated Cells
Unstimulated Control
FACS Analysis
Cell lysis (RIPA Buffer) + Lysate Collection
Combine Lysates + Split into 2 x aliquots
Stimulated and Unstimulated lysate prepared for Western Blot Analysis
Precipitate protein, Re-dissolve in 6M Guanidine-HCl, EZQ quantitation
40.3 µg of R-veIR-A (A)+ R-veIR-B (B) lysate
n = 2
A B C D
Trypsin Digest
ICPL Label
Combine labelled samples

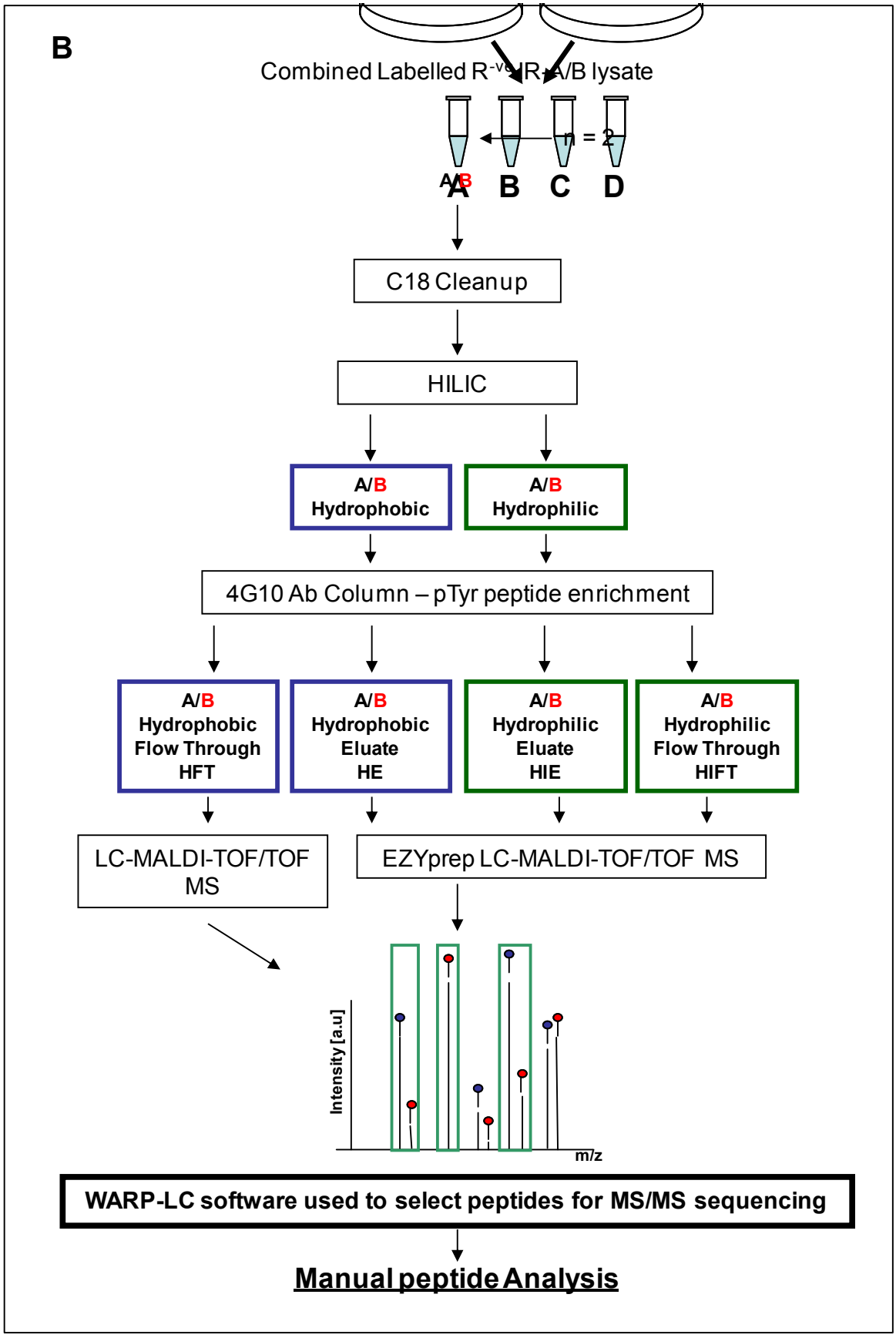


Figure 4.2: Proposed global pTyr phosphoproteome approach

Diagram of the experimental workflow used for the quantitative and qualitative analysis of pTyr of a complex protein sample (cell lysate). (A): R^{-ve}IR-A and R^{-ve}IR-B cells are placed in serum starved media for four hours (4 x technical replicates). Cells are stimulated with 100 nM insulin for 10 minutes prior to cell lysis using RIPA buffer. Unstimulated cells are also lysed as a control for western blot analysis or collected for FACS analysis. Lysate is split equally and one lot is used for western blot analysis (along with lysate from the unstimulated control), whilst protein from the other lot is precipitated and re-dissolved in ICPL lysis buffer. After protein concentration determination, identical sample amounts (40.3 µg) from R^{-ve}IR-A and R^{-ve}IR-B lysates are digested with trypsin prior to labelling with the light and heavy ICPL label, repectively. (B) After labelling, samples are combined and desalted prior to HILIC fractionation to separate hydrophobic and hydrophilic peptides. The pTyr component of the two (4) resulting HILIC fractions (Hydrophobic and Hydrophilic) are enriched using 4G10 Ab Protein A column to produce four (4) fractions; Hydrophobic Flow Though (HFT), Hydrophobic Eluate (HE), Hydrophilic Flow Through (HIFT) and Hydrophilic Eluate (HIE). The fractions were then analysed using LC-MALDI-TOF/TOF MS analysis with CHCA SMW for HFT and EZYprep DHB matrix deposition for HE, HIE and HIFT.

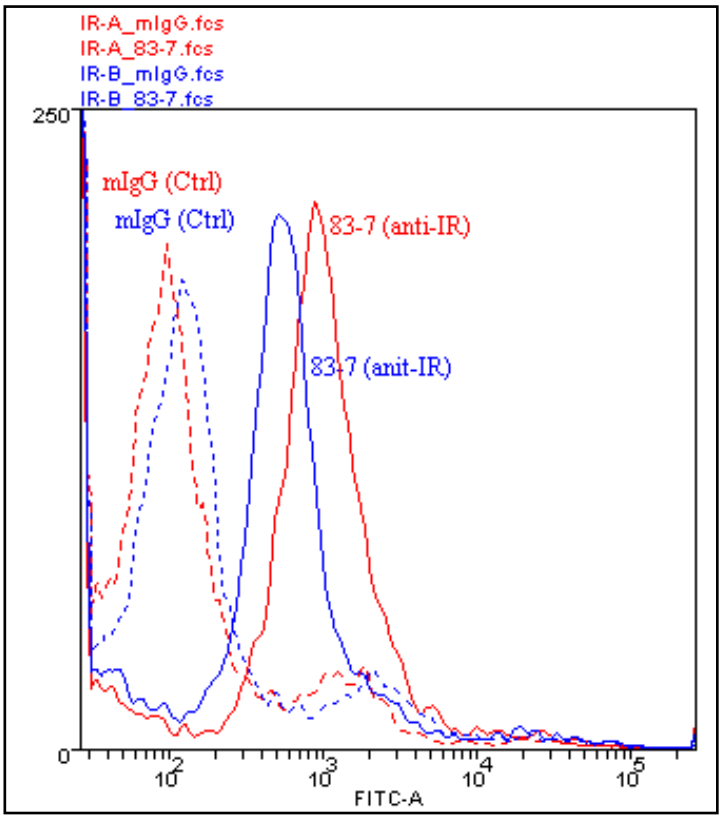


Figure 4.3: FACS analysis of R^{-}IR-A and R^{-}IR-B cells

To determine whether the IR expression of R^{-ve}IR-A and R^{-ve}IR-B cells were similar, R^{-ve}IR-A and R^{-ve}IR-B cells were harvested and probed with the IR specific Ab 83-7 according to the material and methods and analysed by flow cytometry. Graphical representation of the data produced from the analysis of both R^{-ve}IR-A and R^{-ve}IR-B cells show a similar level of IR expressed in each cell type. This ensures that direct comparison of IR phosphorylation in both cell types can be achieved with a high confidence as similar IR numbers will be stimulated.

To confirm this observation as well as determine similar downstream activation upon insulin stimulation of the IR of both cell types, western blot analyses were performed (as described in the material and methods 1.2.3 LDS-PAGE and Western Blotting). Lysate collected from unstimulated and stimulated R-^{ve}IR-A and R-^{ve}IR-B cells were assayed using four western blots; utilising CT-1 (IR specific Ab), 4G10 (pTyr specific Ab), phopsho-IR /IGF-1R [$Y^{1158/1162/1163}$] Ab and the the PathScan® Multiplex Western Cocktail 1 (which assays activation of multiple pathways by detection of phosphor-p90RSK, phosphor-Akt, phosphor-p44/42, MAPK (Erk 1/2), phosphor-S6 Ribosomal protein as well as eIF4E detection for a loading control). Shown in Figure 4.4 are the western blot analyses obtained from unstimulated and stimulated lysates collected from R-^{ve}IR-A and R-^{ve}IR-B cells.

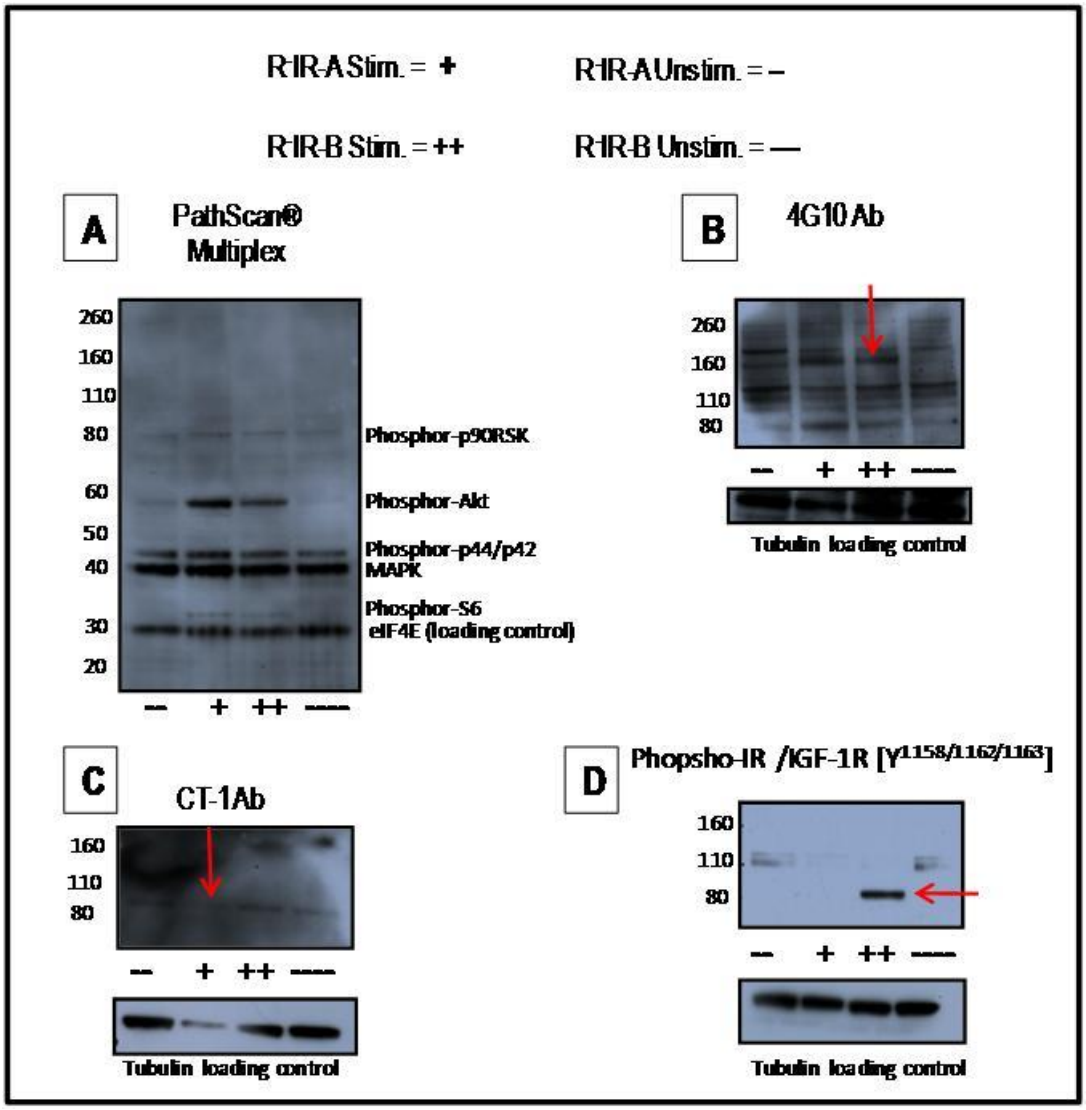


Figure 4.4: Western blot analysis of R^{-ve}IR-A and R^{-ve}IR-B stimulated and unstimulated lysate

To confirm IR activation and subsequent activation of downstream signalling cascades, lysates collected from unstimulated and stimulated R^{-ve}IR-A and R^{-ve}IR-B cells were fractionated using LDS-PAGE and subjected to western blot analysis as described in the materials and methods. **A.** Western analysis probing with the PathScan® Multiplex assay, containing Abs detecting downstream activation of known signalling pathways, including phospho-p90RSK, phospho-Akt, phospho-p44/p42, MAP Kinase (MAPK), and phospho-S6 ribosomal protein. A loading control eIF4E is also included. An increase in the expression of both phospho-Akt and phospho-S6 ribosomal protein is seen in the stimulated lanes. **B.** Western analysis probing with the pTyr specific Ab 4G10. Although multiple bands across all lanes can be seen (which may be due to non-specific binding of the Ab), definitive differences between stimulated and non-stimulated bands are apparent, such as a band ~160 kDa (highlighted). **C.** After probing with the CT-1 Ab, a band at ~95 kDa, representing the β-region of the IR can be seen across all lanes, however the band in the stimulated R^{-ve}IR-A lysate (highlighted) lane is less intense compared with the band from the stimulated R^{-ve}IR-B lysate lane. The re-probing with tubulin (as a loading control) verifies a lower concentration being loaded in the stimulated R^{-ve}IR-A lysate lane compared with the stimulated R^{-ve}IR-B lane. **D.** Western blot analysis probing with the Ab specific for the 3-Y residues known to be phosphorylated in IR and IGF-1R shows an intense band only in the lane corresponding to the stimulated R^{-ve}IR-B lysate (highlighted). Analysis of the tubulin loading control shows no differences in loading material, suggesting the presence of the triply phosphorylated region in the stimulated R^{-ve}IR-B only.

The analysis of the western blot showed clear differences between unstimulated and stimulated lysates when probed with 4G10, which is indicative of different phosphotyrosine abundances (Figure 4.4B). Further western blot analysis on unstimulated and stimulated R^{-ve}IR-A and R^{-ve}IR-B lysates showed that although similar activation with respect to known downstream proteins (including phosphor-Akt and phosphor-S6 ribosomal protein from the PathScan® Multiplex assay) occurred, a clear difference in the expression of the phosphorylated 3Y region of the IR receptor can be seen between insulin stimulated R^{-ve}IR-A and R^{-ve}IR-B cells (Figure 4.4D). This observation correlates with previous reports showing that insulin binding to the IR-B subtype results in greater auto-phosphorylation (and has a higher tyrosine kinase activity) than IR-A, despite IR-A having a greater binding affinity than IR-B [403, 419]. The inability to detect this phosphorylated 3Y region may be due to the levels being lower than the detection threshold of the western blot analysis. Interestingly, this difference in auto-phosphorylation between IR-A and IR-B did not affect qualitatively the activation of downstream signalling proteins, such as Akt (as shown in Figure 4.4A) at this time-point, yet this is based on visual inspection only. As such, there may be quantitative differences in downstream phosphorylation, which would indicate that the difference in auto-phosphorylation seen between the receptors did affect the activation of signalling pathways. Any differences in phosphorylation of these downstream proteins would be identified and characterised using the proposed quantitative proteomic strategy.

The FACS analysis indicated similar IR expression levels in both R^{-ve}IR-A and R^{-ve}IR-B cells, while the western blot analyses showed phosphorylation (particularly tyrosine and serine) of proteins following insulin stimulation of both the IR-A and IR-B receptors. As such, the remaining lysates collected from insulin stimulated R^{-ve}IR-A and R^{-ve}IR-B cells were ICPL-labelled using the optimised ICPL peptide labelling strategy (R^{-ve}IR-A with the light isotope and R^{-ve}IR-B with the heavy isotope), combined and fractionated using HILIC to produce a Hydrophobic and Hydrophilic fraction. These fractions were then subjected to pTyr peptide enrichment using 4G10 (as outlined in Figure 4.2). Following HILIC and pTyr enrichment, four fractions, Hydrophobic Flow-Through (HFT), Hydrophobic eluate (HE), Hydrophilic eluate (HIE) and Hydrophilic Flow-Through (HIFT) were subjected to LC-MALDI-TOF/TOF MS analysis. The EZYprep method was utilised as the matrix preparation for LC-MALDI-TOF/TOF MS analysis of the HE, HIE and HFT fractions as phosphopeptides were anticipated to be localised in these after phosphopeptide enrichment.

Table 4.2 outlines the proteins identified from each fraction, the % sequence coverage (MS/MS data) obtained for each hit, the number and sites of any phosphorylation on the identified proteins, the average regulation (H/L) obtained from each proteins and the number of ICPL-labelled peptide pairs used to identify the proteins. In order to minimise false positives, only proteins with at least one peptide above a mascot score >25 were considered as being present.

Protein*	Accession	Sequence Coverage %	No. of phosphopeptides	Site of phos.	Average Ratio H/L	No. ICPL Peptide Pairs
HFT						
Actin, cytoplasmic 1	ACTB_MOUSE	20.0	-	-	0.98	5
Heat shock protein HSP 90-beta	HS90B_MOUSE	7.3	-	-	1.04	5
Annexin A2	ANXA2_MOUSE	14.5	-	-	1.23	4
Heat shock cognate 71	HSP7C_MOUSE	9.9	-	-	0.97	3

kDa protein						
Annexin A1	ANXA1_MOUSE	12.7	-	-	1.36	2
Alpha-enolase	ENOA_MOUSE	8.3	-	-	0.94	2
Tubulin alpha-1C chain	TBA1C_MOUSE	6.7	-	-	1.18	2
Serum albumin	ALBU_MOUSE	4.3	-	-	1.16	1
Annexin A5	ANXA5_MOUSE	10.3	-	-	1.20	2
Tubulin beta-5 chain	TBB5_MOUSE	10.6	-	-	1.55	2
Nucleoside diphosphate kinase B	NDKB_MOUSE	26.3	-	-	1.36	2
60S ribosomal protein L6	RL6_MOUSE	8.4	-	-	0.98	2
Tropomyosin alpha-4 chain	TPM4_MOUSE	6.9	-	-	1.07	2
Pyruvate kinase isozymes M1/M2	KPYM_MOUSE	5.6	-	-	1.00	2
Annexin A3	ANXA3_MOUSE	5.0	-	-	1.29	1
Rho GDP-dissociation inhibitor 1	GDIR1_MOUSE	15.7	-	-	1.72	2
60S ribosomal protein L14	RL14_MOUSE	8.3	-	-	1.17	1
40S ribosomal protein S8	RS8_MOUSE	7.2	-	-	1.03	1
Galectin-1	LEG1_MOUSE	8.9	-	-	1.24	1
Serpin B6	SPB6_MOUSE	5.0	-	-	1.73	1
Filamin-A	FLNA_MOUSE	0.9	-	-	1.53	2

Alpha-actinin-1	ACTN1_MOUSE	2.4	-	-	1.38	1
40S ribosomal protein S3	RS3_MOUSE	11.1	-	-	1.20	1
Putative RNA-binding protein 3	RBM3_MOUSE	9.2	-	-	1.21	1
Serpin H1	SERPH_MOUSE	3.6	-	-	1.56	1
HE						
Serum albumin	ALBU_MOUSE	2.1	-	-	1.25	1
HIE						
Actin, cytoplasmic 1	ACTB_MOUSE	7.7	-	-	1.00	2
HIFT						
Actin, cytoplasmic 1	ACTB_MOUSE	20.0	-	-	1.20	7
Heat shock cognate 71 kDa protein	HSP7C_MOUSE	12.2	-	-	1.11	4
Myristoylated alanine-rich C-kinase substrate	MARCS_MOUSE	10.7	-	-	1.05	1
Galectin-1	LEG1_MOUSE	27.4	-	-	1.24	3
Filamin-A	FLNA_MOUSE	1.5	-	-	1.77	2
Reticulon-4	RTN4_MOUSE	3.8	-	-	1.04	2
Annexin A2	ANXA2_MOUSE	8.0	-	-	1.43	1
Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_MOUSE	4.2	-	-	0.99	1

Table 4.2: Proteins identified from HFT, HE, HIE and HIFT fractions of insulin stimulated R^-IR-A/R^-IR-B lysate.

Table 4.2 shows that the majority of proteins identified (25) were in the HFT fraction, with only one protein positively identified in the HE (serum albumin) and HIE (Actin, cytoplasmic 1) fractions. Additionally, the HIFT fraction identified 8 proteins, all with H/L ratios within the regulation window set in control ICPL experiments. Of the 25 proteins positively identified in the HFT fraction, 5 proteins were shown to be significantly regulated (i.e. outside the determined 0.689 – 1.452 regulation window), including tubulin-beta-5 chain (1.55), Rho GDP-dissociation Inhibitor (1.72), Serpin B6 (1.73), Filamin-A (1.53) and Serpin H1 (1.56). These proteins were entered into the IPA database to determine their function/s, their connectivity in characterised biological networks, and whether these proteins in the networks have been previously characterised in biological disorders. Filamin-A, Serpin-H1 and tubulin-beta-5 chain were categorised as being involved in filament organisation (p-value 6.82 e^{-6}) and individual searches of the proteins identified their primary functions to be increasing actin filament organisation, organisation of collagen fibrils and decreasing microtubule organisation, respectively. Similarly, Rho GDP-dissociation inhibitor was identified as being involved in actin reorganization. Serpin B6 has numerous functions, such as serine-type endopeptidase inhibitor activity, protein binding and peptidase inhibitor activity. As anticipated, no phosphorylation sites were identified in this fraction, which can be attributed to it being obtained from the hydrophobic fraction (after HILIC enrichment) and also run through the 4G10 column (to enrich remaining pTyr peptides). In addition, the majority of the digested lysate would be contained in this fraction (after HILIC and pTyr). As such, LC-MALDI fractionation over a half-target will be limited in its capacity to effectively fractionate a sample of this complexity, which is evident by the low number of proteins identified. The complexity of the HFT fraction also minimises the possibility of identification of any remaining hydrophobic pSer and pThr peptides, primarily due to low abundance of phosphopeptides compared with non-phosphorylated peptides as well as ion suppression effects.

Table 4.2 shows that despite HILIC fractionation coupled with 4G10 pTyr enrichment, no phosphopeptides were successfully identified and characterised in any of the eluted fractions (*i.e.* HE and HIE) after EZYprep LC-MALDI-TOF/TOF MS analysis. Only a small number of peptides were matched to proteins present in the sample, namely serum albumin in the HE fraction and actin cytoplasmic, 1 in the HIE fraction. The identification of these proteins reflects non-specific binding of high abundance peptides/proteins remaining despite multiple phosphopeptide enrichment strategies. EZYprep LC-MALDI-TOF/TOF MS analysis of the HIFT fraction identified 8 proteins, with Filamin-A once again having a regulation (1.77) outside the pre-defined window. As with the other fractions, no phosphopeptides were identified and characterised, suggesting that phosphorylation in the samples was

at a level below the sensitivity threshold of the proposed HILIC/pTyr specific Ab EZYprep LC-MALDI-TOF/TOF MS analysis.

5 Discussion

5.1 Discussion for Chapter 2:

The studies presented in this chapter sought to develop a sensitive approach for pTyr characterisation on targeted proteins of interest by exploiting the availability of pTyr-specific Abs and MALDI TOF/TOF MS. Utilising pTyr-specific Abs for pTyr protein identification is not a new concept, as these Abs have been used for IP and western blot analysis and, more recently, these Abs have been used prior to MS analysis to improve pTyr protein identification, particularly for global pTyr analysis [191, 229, 231, 283, 294-297, 301, 323]. However, research on individual proteins will commonly utilise more traditional biochemistry techniques (IP and/or western blot), mainly as these methods are straight-forward, cost effective and of high sensitivity. Despite their ability to provide phosphoprotein identification and to a lesser extent, quantitation, these methods will not provide phosphosite information without the use of site-directed mutagenesis experiments or MS [229, 252]. Furthermore, because of the low abundance of phosphoproteins and peptides compared with non-phosphorylated proteins or peptides, the success of MS is limited. For the analysis of proteins of interest where tyrosine phosphorylation is known to occur but the site has not been elucidated, there is a need for methods that enrich and isolate phosphopeptides to improve subsequent MS identification and characterisation.

The Pro-GP/Cov-P approach exploits the availability of super para-magnetic beads that are compatible with MS. These beads can be removed from enriched samples using a magnet so they do not interfere with MS analysis. Proteins can be isolated or separated from complex samples by a number of different methods such as IP or SDS-PAGE. Alternatively, recombinant proteins, generated for protein structure and function analyses, can be phosphorylated *in vitro* using known interacting phosphotyrosine kinases. Following in-gel or in-solution digestion with proteases such as trypsin, phosphopeptides generated from the protein of interest can be subjected to MS analysis or enriched using various strategies targeting the phosphoproteome, including IMAC, TiO_2, Pro-GP/Cov-P, HILIC, SCX or a combination of these strategies. Magnetic beads compatible with MS are of particular importance as multiple surface chemistries can be exploited to enrich for proteins, peptides and PTMs of interest.

The Pro-GP/Cov-P approach described here combines pTyr-specific Ab enrichment with these magnetic beads to enrich pTyr peptides for direct analysis by MALDI-TOF/TOF MS analysis. Specifically targeting pTyr phosphopeptides improves pTyr enrichment in comparison to methods targeting the

entire phosphoproteome. Many strategies targeting the tyrosine phosphoproteome will utilise a combination of pTyr-specific Ab enrichment in combination with a more global phosphopeptide enrichment strategy such as IMAC or TiO_2 [191, 264, 295, 301, 420]. Previous strategies solely utilising pTyr Ab-conjugated beads for phosphopeptide enrichment have been focused on global phosphoproteome mapping of signalling networks [191, 229, 301]. Although it is possible to utilise these methods for less complex sample analyses, an optimised strategy developed for pTyr analysis is required as a straight-forward, cost-effective and reproducible alternative to conventional biochemistry techniques. The Pro-GP/Cov-P protocol was developed primarily for low complexity samples. The ability to directly analyse eluates without the requirement for additional sample preparations (e.g. de-salting) minimises sample loss (including pTyr-peptides), which is important for analysis of extremely low abundant phosphopeptides. The direct analysis coupled with the use of optimised DHB matrix deposition strategies further enhances MALDI-TOF/TOF MS sensitivity.

Often, tyrosine phosphopeptides generated from proteins will be at a significantly lower abundance than non-phosphorylated peptides from the same protein. This is because only a small percentage of a protein may be tyrosine phosphorylated after a specific stimulation time. Hence, if only 1% of the protein of interest is tyrosine phosphorylated, then only 1% of the peptides generated from this protein will be phosphorylated on tyrosine. This, in conjunction with ion-suppression effects of non-phosphorylated peptides, makes it extremely difficult to detect those phosphopeptides and it is imperative to be able to visualise low abundant peptides in order to ensure adequate sequence coverage, particularly on areas of proteins known or thought to be phosphorylated. As shown in Chapter 2 (Figures 2.3 and 2.4), the Pro-GP/Cov-P approach is able to successfully enrich phosphopeptides that are at a 200:1 ratio compared with non-phosphorylated peptides present in a sample.

An important factor of the Pro-GP/Cov-P approach is that it targets solely the pTyr phosphoproteome. Although other strategies, such as IMAC and TiO_2, are commonly promoted phosphopeptide enrichment strategies, they are only utilised for pTyr research in combination with pTyr protein or peptide enrichment with pTyr Abs [191, 264, 295, 301, 420]. As shown in Chapter 2 (Figure 2.2), the Pro-GP/Cov-P approach was necessary to identify three pTyr peptides added to a BSA tryptic digest at 1:10 ratio. Enrichment using these strategies ensured the phosphopeptides were amongst the top five most intense peptide ions, and these could subsequently be selected for MS/MS characterisation if the sample was unknown. In contrast, although the IMAC enrichment approach was able to detect two of

the three phosphopeptides, neither of these constituted the top five most intense peptide ions. As such, these peptides would not be selected for MS/MS analysis unless prior sequence information of the protein/s of interest was known. These results show the need to couple IMAC with pTyr protein or peptide enrichment to ensure detection of low abundant pTyr peptides.

The ability to use the Pro-GP/Cov-P strategy as a sole method for phosphotyrosine characterisation is particularly useful for laboratories that are unable to access sensitive LC-MS/MS equipment for proteomic analyses. The high set-up costs associated with LC systems means many laboratories are limited to more traditional biochemical techniques, such as western blot analysis. The Pro-GP/Cov-P approach allows for researchers to undertake pTyr characterisation without the need for more expensive LC-MS systems using MALDI-TOF/TOF MS instruments.

The Pro-GP/Cov-P strategy can also be utilised for the analysis of more complex samples. This is because the super para-magnetic beads utilised form part of the ClinProt™ magnetic bead system developed by Bruker for large-scale automation of magnetic bead enrichment strategies. Multiple Pro-GP/Cov-P beads can be loaded into wells for a serial enrichment of complex samples. Serial enrichment is necessary due to the capacity limitations of the beads. The manufacturers' instructions suggest a loading concentration of 100 pmol or less. Large sample analysis would therefore require a serial enrichment strategy where non-bound peptides (sample flow through fraction) would be re-enriched over the following Pro-GP/Cov-P beads. This process is automated using the robotic system supplied by Bruker Daltonics.

Additionally, the Pro-GP/Cov-P beads can be combined with more conventional SDS-PAGE for sample analysis. In chapter 2, 1D SDS-PAGE and Pro-GP/Cov-P was used to characterise pTyr phosphorylation on the βic recombinant protein after *in vitro* phosphorylation (Figure 5 and 6). For more complex analysis, 2-DE could be used for more effective separation of proteins. Replicate gels can be used for western blot analysis of the 2-DE using 4G10 to identify the location of tyrosine phosphorylated proteins. These spots can be excised from replicate coomassie stained gels and proteins digested in-gel for subsequent Pro-GP/Cov-P enrichment. Alternatively, proteins can be extracted off the western blot membrane itself to ensure accurate excision of protein spots identified. After extraction and digestion, Pro-GP/Cov-P enrichment of pTyr peptides can be conducted.

An appealing component of the current Pro-GP/Cov-P protocol is that any pTyr Ab can be used. It is known that pTyr specific Abs have varying affinities to different ligands [225, 244, 421], which is due to the fact that particular Abs are generated to target specific antigens [294]. In order to account for varying affinities of pTyr specific Abs to ligands, alteration of the protocol to conjugate a combination of pTyr specific Abs will improve coverage of the tyr phosphoproteome (overall tyrosine phosphopeptide recovery). Abs raised against specific pSer and pThr epitopes can also be conjugated to Pro-G/Cov-P magnetic beads to enrich the relevant phosphopeptides and confirm the site of phosphorylation by MS. These Abs specific to pSer/pThr recognise multiple consensus sequences (up to ten residues either side of the phosphorylation site to form epitopes), which improve pSer/pThr enrichment and subsequent identification by MS.

5.2 Discussion for chapter 3:

5.2.1 Matrix deposition for phosphopeptide analysis

Improvement of phosphopeptide detection is critical in order to increase the ability to characterise phosphorylation in both targeted and global approaches. Research on phosphoproteome method development focuses on optimisation of both the enrichment strategies as well as MS sample preparations. Optimisation of phosphoproteomic strategies, such as methylation prior to IMAC [50] or the implementation of a DHB conditioning step with TiO_2 enrichment [261] have both improved phosphopeptide recovery. Similarly, the results from Chapter 2 showed that alteration of the protocols developed for MB-IAC Prot G and MB-CovAC-Select beads to develop Pro-GP/Cov-P enrichment (namely the use of a matrix solution for phosphopeptide elution) increased detection sensitivity.

Despite improvements in proteomic enrichment methods, the low stoichiometry of phosphorylation coupled with the ionisation suppression effects of phosphorylation [256, 422] still pose a challenge for phosphosite characterisation. To aid phosphopeptide enrichment strategies and subsequent detection using MS, a number of optimised sample preparation methods have been developed. In terms of MALDI-TOF-MS analysis of phosphopeptides, several methods have been employed, including alternating polarity (from positive-ion mode to negative-ion mode) to aide in phosphopeptide selection for MS/MS [333, 423-427]. Analysing in negative-ion mode favours negatively charged ions and as the

phosphate group carries a negative charge, analysis in this mode has been shown to improve phosphopeptide ionisation [333, 423-427]. Other methods have employed the use of ammonium salts as an additive to matrices such as diammonium hydrogen citrate (DAHC) [428-431], ammonium acetate [428] and monoammonium dihydrogenphosphate [426]. These salts improve ionisation efficiency of phosphopeptides crystallised with matrices in an ionic form. As each phosphate group can carry up to two charges, if a multiply charged analyte is co-crystallised in matrix in ionic form, insufficient energy is available for analyte desorption. The higher the number of phosphate groups attached, the higher the negative charge state of the peptide. As predominately singly-charged ions are favoured in MALDI [116], a low energy chemical pathway to allow a lowering of charge state is required [428]. The addition of ammonium cations ($NH4^+$) forms complexes with negatively-charged groups, including phosphate groups. During the desorption/ionisation process, NH_3 is lost, leaving the charged group in a neutral form with a proton attached [428, 432].

More recent strategies have combined the use of matrix additives with particular matrix types to improve phosphopeptide ionisation and characterisation. In particular, the advantages of the combination of DHB with phosphoric acid have been well documented and utilised in a number of phosphoproteomic analyses [51, 125, 200, 320]. The benefits of DHB/PA for phosphopeptide ionisation and detection are attributable to a number of observations. The DHB matrix is considered a 'cold' matrix as it leads to the formation of ions with a low internal energy [125, 433]. As such, these ions remain intact during the mass analysis. The use of this matrix for phosphopeptide analysis improves peptide stability, minimising loss of the phosphate group during ionisation. In contrast, the use of CHCA (considered a 'hot' matrix) leads to significant decomposition of the phosphopeptides, resulting in the loss of the phosphate group or phosphoric acid (80 and 98 Da, respectively) [125]. The addition of between 0.1 – 1% PA to the matrix has shown to further improve ionisation of co-crystallised phosphopeptides. Previous analyses have shown that the rate of matrix/analyte crystallisation affects the incorporation ratio of hydrophilic peptides [434]. It has been speculated that the slow evaporation helps drive phosphopeptides and phosphoric acid into the matrix crystals, improving recovery [51, 125]. Additionally, PA is thought to incorporate phosphopeptides with higher charge states, due to previous analyses that have observed the presence of both doubly-charged ions and molecular cluster ions of phosphopeptides but not of non-phosphorylated peptides in the crystalline analyte/matrix deposit. As such, it has been hypothesised that PA creates a "salting out" of phosphopeptides, leading to more efficient incorporation into the matrix crystals [51, 125]. Finally, PA has been observed to reduce the number of sodium adducted peptide ions in mass spectra, which may be due to it acting as a "sodium sink" to capture sodium ions, minimising sample complexity [125].

In our hands, comparative analysis of a tryptic digest of β-casein using either CHCA or DHB/PA clearly shows and increase in phosphopeptide ionisation, particularly for multiply-phosphorylated peptides (as shown in Figure 5.1).

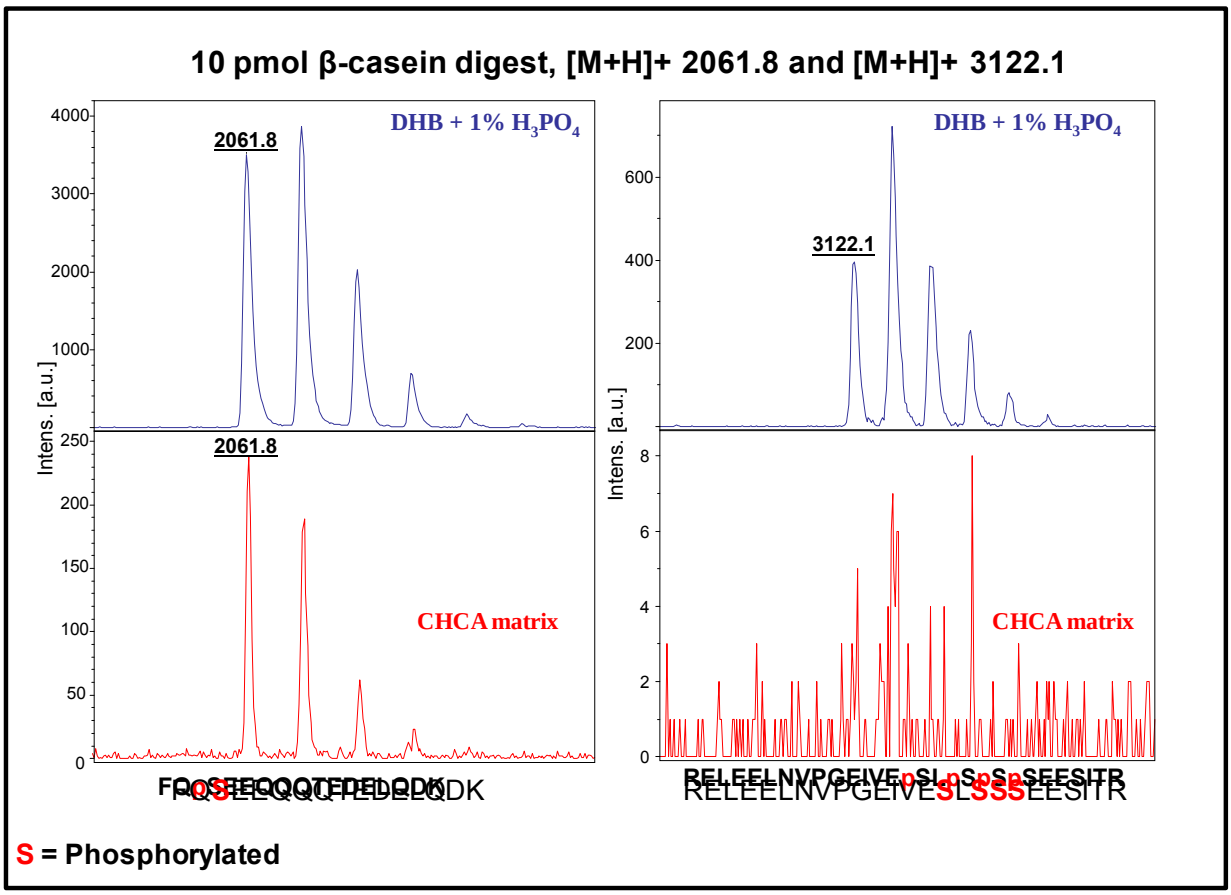


Figure 5.1: The analysis of two phosphopeptides generated from a tryptic digest of β-casein using MALDI-TOF MS analysis with either DHB/PA matrix or CHCA matrix.
A 10 pmol β-casein digest mixture was spotted with either 5 mg/mL DHB/1% PA or with CHCA Thin-layer (CHCA TL) method. As shown, the intensity of two common phosphopeptides produced are more intense in spectra obtained when DHB/PA was used compared with CHCA. This is particularly apparent with the multiply phosphorylated peptide, which was not detectable with CHCA matrix.

In addition to the advantageous observations from previous analyses focusing on DHB/PA, we have observed that DHB/PA displays a higher tolerance to sample contaminants compared with CHCA, as outlined in Chapter 2. Another property of DHB/PA matrix that has increased its use for phosphopeptide analysis is its compatibility with phosphopeptide enrichment strategies, particularly

IMAC. It can be used as an elution solution, minimising sample handling prior to MS. The use of DHB/PA to elute phosphopeptides bound to IMAC resin was shown to improve recovery of both singly- and multiply-phosphorylated residues [51]. Interestingly, the use of CHCA/PA as an elution solution resulted in poor peptide recovery from IMAC, indicating that PA alone is not enough to elute bound phosphopeptides. A similar result was also noticed during optimisation of the Pro-GP protocol, where no phosphopeptides were obtained when CHCA was used as the elution solution, but were obtained when DHB/PA was used (data not shown). Although the CHCA did not contain PA, it did contain TFA (0.1%), which should be sufficient to denature the 4G10 Ab as the pH is low (2.0). The results obtained from these Pro-GP optimisation experiments are more likely due to the DHB/PA matrix being more tolerant to residual immobilisation and wash buffers remaining in the sample after elution.

Although the advantages of DHB/PA matrix for phosphopeptide ionisation have been well documented, the heterogeneity of the DHB crystals have hampered it widespread use. The generation of non-homogeneous sample preparations often results in hot-spot formation, making it difficult to utilise this matrix for more complex sample analysis, which typically utilises automated data collection, including LC-MALDI-TOF/TOF MS analysis [430, 433]. In order to overcome DHB crystal heterogeneity, the ImagePrep™ station (Bruker Daltonics), a device developed to apply homogenous matrix layers over tissue sections for MALDI imaging, was utilised to generate a DHB matrix 'mist' for matrix application onto MALDI targets for subsequent MALDI-TOF/TOF MS analysis. The use of this instrument as a DHB deposition strategy was based on the improved homogeneity of DHB crystals formed on tissue sections, as shown in Figure 5.2.

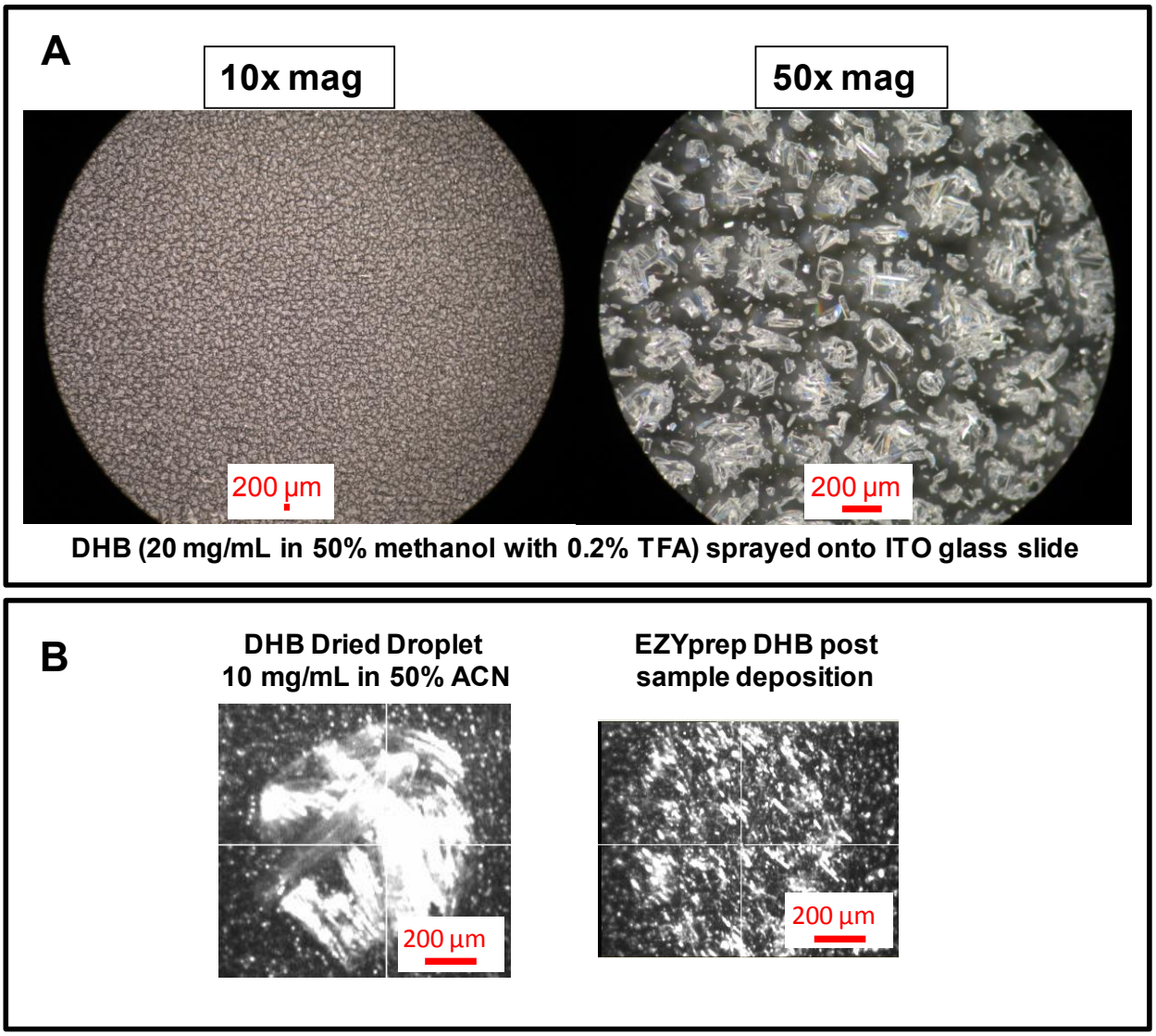


Figure 5.2: (A) DHB crystallisation after ImagePrep station deposition onto a tissue section slide. (B) MALDI CCTV shots of raster positions highlighting crystal formation using DHB DD deposition or EZYprep (using ImagePrep Station) DHB post sample matrix deposition.

As shown in Figure 5.2B (and Table 3.2 from Chapter 3), crystals generated using the EZYprep approach (using the ImagePrep station) have a higher degree of homogeneity than crystals generated from conventional DHB DD matrix deposition. In addition, the results from Chapter 3 clearly show that the EZYprep approach substantially improved LC-MALDI-TOF/TOF MS analysis results for peptide digest mixture PM1 compared with results obtained using the DHB DD approach. This highlights the improvement of automated acquisition (by EZYprep approach) on homogenous analyte/matrix crystal preparation, which ensures that spectra can be obtained at more positions on the raster. Also, improved crystal homogeneity minimises spatial distribution of analyte on the raster position. This is important as large differences in spatial distribution of analytes results in variations in initial kinetic

energies after desorption/ionisation, which can decrease mass accuracy [88]. In contrast, although DHB DD preparation has similar advantages in terms of phosphopeptide ionisation, the numbers of proteins identified as well as phosphopeptides detected for PM1 were lower compared with EZYprep DHB deposition. The crystal heterogeneity of the DHB DD method can cause slight mass shifts between individual spectra across the raster position, which decreases the mass accuracy of peptides obtained. Furthermore, the large crystals formed in DHB DD deposition could be missed when utilising automated acquisition, which is required for LC-MALDI-TOF/TOF MS analysis, and this decreases spectra quality and intensity.

As shown in Chapter 3, the protein identification and phosphopeptide detection results between EZYprep DHB and CHCA SMW were comparable. This can be attributed to the improvement of analyte/DHB crystallisation using EZYprep. Consistent with previous analyses comparing phosphopeptide intensity obtained with DHB/PA with other matrix types, a statistically significant increase in phosphopeptide intensity was observed when using EZYprep DHB instead of CHCA (Chapter 2). Although this was not beneficial for the analysis of phosphopeptides in PM1, this increase in phosphopeptide intensity will aid in the detection of phosphopeptides from more complex sample analyses. This is because a higher number of non-phosphorylated peptides will co-elute with phosphopeptides, despite LC fractionation. The increase in non-phosphorylated peptides will increase ion suppression effects on phosphopeptides in the same fraction. As such, phosphopeptides with a low intensity may no longer be detectable. Improving phosphopeptide intensity using EZYprep DHB will decrease instances where phosphopeptides succumb to ion suppression effects.

5.2.3 LC-MALDI-TOF/TOF MS versus LC-ESI-IT-MS/MS

The EZYprep DHB approach is suitable for the analysis of the phosphoproteome of complex samples as it is coupled to LC sample fractionation prior to MALDI-TOF/TOF MS analysis. The use of this separation strategy has particular advantages over LC-ESI-IT-MS/MS, which has been the method of choice to date for phosphoproteome analysis [200]. Samples fractionated onto MALDI targets become stable, essentially 'frozen in time,' and repeated analysis can be conducted should more information from the sample be required. In addition, MS/MS data acquisition is not restricted to any time constraints (like with LC-ESI-IT-MS/MS), meaning more time can be spent on MS/MS data collection on peptides of interest that may not constitute the most intense ions in the spectra. This allows manual intervention at any stage of the acquisition and ultimately reduces the number of acquired MS/MS

spectra as it minimises the collection of redundant MS/MS spectra. Moreover, LC-MALDI-TOF/TOF MS is more tolerant to sample contaminants and/or additives compared with LC-ESI-IT-MS/MS. The main disadvantage of LC-MALDI-TOF/TOF MS is the longer time required to analyse samples, making it less suited for high through-put analyses. As such, LC-ESI-IT-MS/MS is ideal for routine sample analysis where MS data alone is insufficient, whilst LC-MALDI-TOF/TOF MS can be utilised for the analysis of more precious samples. Previous analyses comparing LC-MALDI-TOF/TOF-MS and LC-ESI-MS/MS have found that although a degree of overlap occurs in regards to results, the two approaches can be complementary [321, 435-437].

In order to gauge the sensitivity of the proposed EZYprep LC-MALDI-TOF/TOF MS approach and evaluate the complementarity of LC-MALDI-TOF/TOF MS with LC-ESI-IT-MS/MS, both methods were utilised to characterise EGF-stimulated EGFR phosphorylation over 5 minutes. In addition, the Pro-GP protocol was also evaluated using this sample to highlight the capacity of this approach for pTyr characterisation. Analysis of EGFR phosphorylation using the three approaches successfully identified 16 unique phosphopeptides over the time-course analysed, as outlined in Chapter 3 (Figure 3.2, Table 3.4) and in Table 5.1.

Process	**Number of phosphopeptides from EGFR identified and characterized**			
	Time 0 min	**Time 1 min**	**Time 3 min**	**Time 5 min**
Pro-GP (pTyr Only)	0	6	7	5
EZYprep LC-MALDI	**5**	**9**	**13**	**13**
LC-ESI-IT-CID/ETD	2	4	4	6
Unique EGFR phosphopeptides	**5**	**10**	**14**	**15**

Table 5.1: Overview of the phosphopeptides identified from chapter 2 using Pro-GP, EZYprep LC-MALDI-TOF/TOF MS and LC-ESI-IT-MS/MS.

At each time point, EZYprep LC-MALDI-TOF/TOF MS analysis detected the most phosphopeptides, which highlights the improved sensitivity of the approach. After automated MS acquisition for each time point using Pro-GP bead enrichment or EZYprep LC-MALDI-TOF/TOF MS, the spectra were evaluated manually and ions whose m/z corresponded to potentially phosphorylated peptides (generated from an

in silico digest of the EGFR) were selected for MS/MS analysis. In contrast, an inclusion list (also generated from an *in silico* digest of the EGFR) was analysed using the software controlling automated LC-ESI-IT-MS/MS acquisition. Although the same inclusion list was used for all approaches, only in LC-MALDI-TOF/TOF MS was the collection of MS/MS data controlled such that potential phosphopeptides were the primary choice for MS/MS acquisition. With LC-ESI-IT-MS/MS, potential phosphopeptides were selected (even if they did not constitute the top 3 ions). However, the time constraints on MS/MS acquisition may have hampered adequate MS/MS data. In addition, ion-suppression effects (due to the high abundance of co-eluting non-phosphorylated peptides) may have hampered detection. Although ion suppression effects also hamper phosphopeptide detection using LC-MALDI-TOF/TOF MS analysis, the ability to load fractionated samples across multiple raster positions on a MALDI target improved the chances of phosphopeptide detection. It is important to mention that less starting material (1 µL) was used for LC-ESI-IT-MS/MS compared with LC-MALDI-TOF/TOF MS (3.5 µL). This was due to capacity limitations of the trapping column used for LC-ESI-IT-MS/MS. Despite this, the approach allowed identification and characterisation of many of the phosphopeptides at each time point, highlighting the superior sensitivity over LC-MALDI-TOF/TOF MS. However, the venn diagrams generated show that whilst some phosphopeptides were detected using both approaches, many were detected solely by LC-MALDI-TOF/TOF MS. This may be attributable to different ionisation efficiencies of peptides with MALDI and not just because a larger sample amount was used.

One highlight was the ability of the Pro-GP approach to identify and characterise phosphopeptides without the need for LC separation. As shown in the venn diagrams in chapter 3 (Figure 3.2), the Pro-GP approach identified many of the phosphopeptides identified using EZYprep DHB LC-MALDI-TOF/TOF MS at each time point, with the exception at time 0 min. The Pro-GP failed to detect any of the phosphopeptides detected at time 0, even at later time points (Chapter 3, Table 3.4). This is due to the fact that all but one phosphopeptide was found to be a phosphorylated at either serine or threonine, which would not be enriched using the Pro-GP approach utilising 4G10 pTyr specific Ab. Moreover, the inability for the Pro-GP approach to detect the pTyr peptide at time 0 may be due to the varying affinity of the 4G10 Ab to pTyr, as previously reported in Chapter 2 and elsewhere [225, 244]. Despite the inability of the Pro-GP to detect phosphopeptides at time 0, these results show the effectiveness of the magnetic bead strategy as an alternative to LC-based strategies, successfully providing phosphopeptide information, albeit providing less sites than the more sensitive alternatives. Moreover, Pro-GP enrichment identified and characterised one phosphopeptide $[M+H]^+$ 1644.688, that was not detected using the other approaches.

Despite the EZYprep DHB LC-MALDI-TOF/TOF MS approach identifying the majority of phosphopeptides, none of the approaches analysed was able to provide total phosphosite characterisation of EGFR phosphorylation, emphasizing the potential importance of using both LC-MALDI and LC-ESI-MS/MS approaches to obtain a more complete overview of the phosphoproteome. However, further improvement of either strategy could increase phosphopeptide identification and characterisation. Particular emphasis on LC separation of phosphopeptides using alternative or varying combinations of ion-paring reagents has the potential to improve phosphopeptide binding to the RP column. For example, the addition of PA in the analyte solution has been shown to enhance detection of multiply-phosphorylated peptides in LC-ESI-MS/MS [438]. Alternatively, PA can be added to the mobile phase, which can improve peptide separation as it increases the hydrophilicity of peptides, meaning an earlier elution in the gradient [74] which means elution occurs over a longer period of time. Interestingly, preliminary LC-MALDI-TOF/TOF MS analyses of PM1 were conducted using 0.05% PA in the mobile phase instead of TFA. On analysis of chromatographic peak shape and retention time of both phosphorylated and non-phosphorylated peptides present in both tests, no discernable differences were noted. Chromatographic peak width did vary randomly between tests. However, retention times of the peptides analysed were identical (data not shown). Furthermore, as PA can be destructive not only on surfaces in the LC system but on the AnchorChip™ target, the buffer system with 0.1% TFA was used for all future analyses.

Without the introduction of phosphopeptide enrichment (*e.g.* online coupling of TiO_2 with RP-HPLC), modifying phosphopeptide separation (by utilising lower flow rates and/or a more gentle gradient) may improve phosphopeptide detection using EZYprep LC-MALDI-TOF/TOF MS. A higher number of fractions would consequently be collected, which would improve separation. However, a more extensive fractionation would increase the chromatographic peak width of peptides, resulting in peptide dilution across fractions, which is not ideal for the analysis of low abundance phosphopeptides. In addition, because of the design of the ImagePrep™ station, the AnchorChip™ target does not sit flat in the chamber. As such, it is difficult and time consuming to apply matrix using EZYprep, particularly over the entire target surface. As a more extensive LC separation would utilise more fractions on the AnchorChip™ target, a modified ImagePrep with a chamber suitable for the target would be useful. Alternatively, the use of an AnchorChip™ target with a higher number of raster positions (*e.g.* AnchorChip™ with 1536 raster positions) would be better suited for EZYprep matrix deposition.

However, it is important to consider that no one method can provide a complete 'snapshot' of the phosphoproteome and as a result, the use of multiple approaches will be necessary.

5.3 Discussion for Chapter 4:

5.3.1 ICPL Labelling Optimisation for Phosphopeptide analysis

Isotopic labelling of samples prior to MS analysis has proved to be a versatile strategy to identify, quantify and characterise complex proteomic samples. Of the various labelling strategies available, the non-isobaric strategy ICPL has great potential, particularly in regards to the analysis of post-translational modifications. This is because of the specific advantages this strategy has over other labelling strategies, namely ICAT (low label site number), iTRAQ (quantitation only on the MS/MS level and poor detectability of the reporter ions in several types of mass spectrometers) and SILAC (developed solely for *in vivo* labelling and applicable to certain cell types only) [163]. Additionally, the ICPL reaction labelling technique has been found to be very specific and efficient, with no apparent by-products or undesired side reactions [13, 163].

ICPL is a protein labelling strategy, designed to label free amino groups both on lysine residue side-chains and protein N-termini. Once samples (e.g. Control and Test samples) are labelled, they are combined for subsequent fractionation (most commonly 1 or 2-DE), digested in-gel and finally analysed using LC-MALDI-TOF/TOF MS analysis. After MS analysis, intensity ratios between labelled peptide pairs are used to quantify expression levels of the associated proteins and these pairs are subsequently selected for MS/MS analysis to identify the differentially-expressed proteins. The selection of only the differentially-regulated pairs for MS/MS data minimises redundancy and ultimately can improve the quality of the data. The use of ICPL as a labelling strategy in proteomics is beginning to gain momentum, with more laboratories utilising it as the preferred approach [163, 168, 214, 439, 440].

The advantages of ICPL (namely the improvement of peptide ionisation as well as the ability to quantify on the MS level) made it the labelling strategy of choice to be combined with pTyr enrichment and EZYprep LC-MALDI-TOF/TOF MS analysis for phosphopeptide identification, quantitation, and characterisation. However, in order to successfully quantify post-translational modifications in proteins

using ICPL, it must be considered that ICPL will not label all peptides when used according to the original methodology. This is because ICPL has been developed for labelling at the protein level of samples from cell or tissue lysates. As such, labelling occurs only on free amino groups. The free amino groups on lysine residues and protein N-termini ensure that the majority of proteins will be labelled, however, upon digestion, only peptides with lysine (or the N-terminal peptide) will be able to be used for quantitation. As a result, comparative analysis of phosphopeptides that do not contain labelled residues is not possible. Optimisation of the protocol to increase the number of labelled peptides per protein is imperative, particularly for PTM analysis [163]. This is achieved by alteration of the ICPL protocol such that labelling occurs at the peptide level, ensuring a label on either lysine (if present) or the free N-terminus of a peptide.

In the present study, R^{-ve}IR-A lysate was divided into four fractions equally (1:1:1:1) and labelled at either a protein or peptide level using the four isotopes available in the ICPL 4-plex kit (i.e. the 1-($^{12}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{12}C-Nic-Reagent [M_r = 105.0215 Da] and 1-($^{13}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{13}C-Nic-Reagent [M_r = 111.0419 Da] for peptide labelling, 1-($^{12}C_6{}^2D_4$)-Nicotinoyloxy-succinamide ^{2}D-Nic-Reagent [M_r = 109.0715 Da] and 1-($^{13}C_6{}^2D_4$)-Nicotinoyloxy-succinamide $^{13}C^2$D-Nic-Reagent [M_r = 115.0919 Da] for protein labelling). Upon labelling, differentially labelled samples were combined and either analysed directly using LC-MALDI-TOF/TOF MS analysis (i.e. ICPL peptide labelled samples) or subjected to clean-up steps (*i.e.* the protein precipitation step for ICPL protein labelling) followed by LC-MALDI-TOF/TOF MS analysis. Eluted peptides were fractionated over half a target (192 fractions), which was chosen on the basis that this LC-MALDI-TOF/TOF MS approach would be subsequently used for the analysis of pTyr-purified lysate. Triplicate analyses over half-target (192 fractions) of both ICPL protein- and peptide-labelled lysate identified eighteen and twenty-five proteins, respectively. The number of positively identified proteins from this analysis is low when considering the complexity of cellular lysate, which is attributable to utilising a gradient optimised for samples that have been pre-fractionated. The SurveyViewer (Bruker Daltonics) shows simulated 2-D maps (Figure 5.3), outlining the peptide chromatographic separation of representative ICPL protein and peptide runs. The sample fractionation has allowed the detection of peptides that would otherwise be suppressed by the ionisation of the more abundant/better ionisable peptides. Yet, many fractions (matrix spots) contain multiple peptides, meaning a greater fractionation (e.g. over an entire target) would further improve peptide identification. Although this was anticipated, the fractionation over a half-target (192 fractions) was utilised as this level of fractionation was deemed adequate for the analysis of pTyr purified fractions, as the phosphotyrosine component of the phosphoproteome is considered to be less than 1%. These control ICPL labelling experiments served to determine both the most efficient

ICPL strategy (i.e. ICPL protein labelling versus ICPL peptide labelling) as well as the level of fractionation.

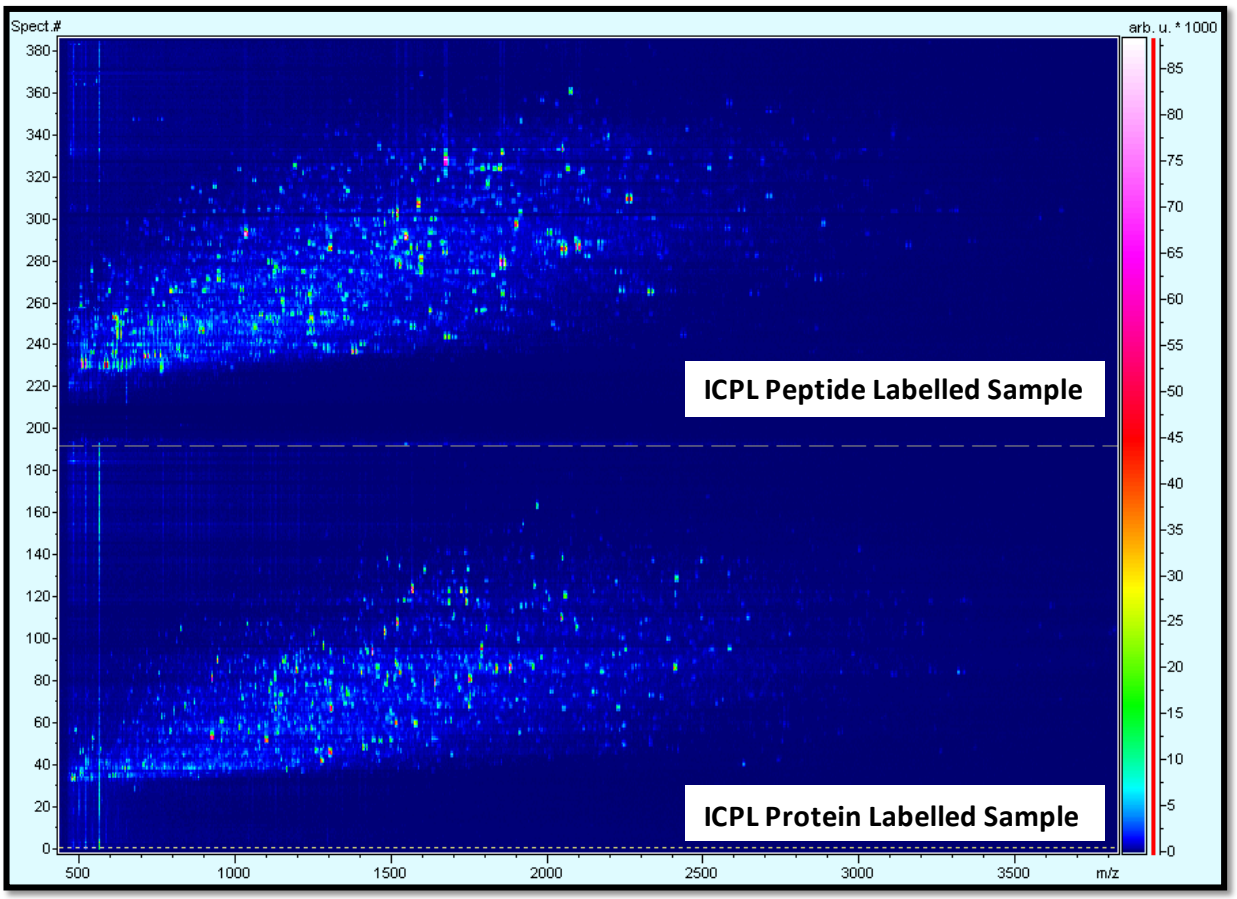


Figure 5.3: The SurveyViewer (version 1.1, Bruker Daltonics) generated 2-D maps outlining the peptide chromatographic separation for each ICPL labelled (peptide or protein) sample after LC-MALDI-TOF/TOF MS analysis.

The results from Table 4.1 showed that modifying the ICPL protocol such that all peptides are labelled not only increases the number of proteins identified, but also provided more information regarding protein expression levels between two samples, which improved the quality of the quantitation data obtained. A recent publication utilising an ICPL post-digest approach also showed an improvement in the quantitation and number of proteins identified in both standard protein samples as well as complex bacterial samples [440]. This is because the addition of the label to each peptide improves MS ionisation due to the addition of nicotinic acid (up to 100-fold) as described previously [13, 441]. Also, the increase in peptide pairs identified provides more information about the level of protein expression, and this is clearly shown in Table 4.1, where the known 1:1 ratio of R^{-ve}IR-A lysate was deduced with a higher degree of accuracy with ICPL peptide labelling (88% of proteins [22 out of 25] obtained a

regulation within the pre-determined window of 0.689 to 1.452). Three proteins (Annexin A3, A5 and Elongation Factor 1-alpha) produced ratios outside the regulation window (0.64 ± 0.02, 1.67 ± 0.17 and 1.73 ± 0.19, respectively). This may have been due to the co-elution of ICPL-labelled peptides with other peptides with an almost identical m/z, increasing the MS intensity obtained. Although this has been reported previously [163], it may not explain the observations here as multiple peptide pairs for two proteins (4 peptide pairs for Annexin-A5 and 2 peptide pairs for Elongation Factor 1-alpha) were used to obtain the average ratio. A combination of factors may be responsible, such as co-elution issues coupled with the relative abundance of the proteins. Previous analyses determining the labelling efficiency of the ICPL technique have reported effective labelling at protein concentrations of 5 fmol [13]. However, this was analysed solely with ICPL protein labelling. As such, the increase in sample complexity by trypsin digestion of lysates may produce peptides at varying abundances (depending on the digest conditions of the samples to be labelled) and this may account for variances.

Utilising the conventional ICPL protein labelling strategy resulted in only two of the ten (20%) proteins having expression ratios within the regulation window. It should be noted that the regulation discrepancy seen with the ICPL protein labelling strategy was consistent, with the heavy labelled lysate being lower. The labelling efficiencies for all four available isotopes should be identical; however this systematic error in protein expression seen with the 1-($^{13}C_6{}^2D_4$)-Nicotinoyloxy-succinamide $^{13}C^2D$-Nic-Reagent [M_r = 115.0919 Da] may be indicative of incomplete labelling by the heaviest tag in the ICPL protein labelled samples. Recent analysis of the ICPL reagents for the development of novel ICPL-based software has identified that a few percent of the ICPL reagents do not exhibit the complete isotopic labelling, particularly with the ^{13}C containing labels [214]. Around 20% of the 1-($^{13}C_6{}^2D_4$)-Nicotinoyloxy-succinamide $^{13}C^2D$-Nic-Reagent [M_r = 115.0919 Da] has been shown to display this impurity, which would not have been accounted for in the WARP-LC software used to determine the ratios. If a 20% variance is considered, seven (7) of the ten ratios obtained can be considered to be within the regulation window obtained for this study (0.689 to 1.452). Of the peptides used for identification and quantitation, only one unlabelled peptide was characterised using ICPL peptide labelling (1 peptide from serum albumin). In contrast, unlabelled peptides were identified consistently in seventeen of the eighteen proteins identified with ICPL protein labelling, with labelled peptides being identified in ten.

The results here clearly highlight the advantage of labelling with ICPL on a peptide level with respect to quantitation (as more labelled proteins are identified), however it is important to consider that the

preferred ICPL workflow originally described in [13] describes a fractionation step after protein labelling, such as 1- or 2-DE or LC. In contrast to this recommendation, lysate ICPL labelled on the protein level was combined and digested in-solution, without any pre-fractionation. Although this protocol is complementary with the described protocol, the complexity of the labelled lysate will result in a decrease in the number of identified proteins compared with multiple digests of pre-fractionated protein bands. The ability to pre-fractionate ICPL protein-labelled samples to improve subsequent identification in complex samples is advantageous over ICPL peptide labelling, as pre-fractionation prior to labelling will introduce new sources of error. These observations show that an ideal strategy to provide both increased identification of proteins as well as quantitative data for not only proteins but also post-translational modifications may be to undertake a comparative analysis of a complex sample split for both ICPL peptide and protein labelling. This combined approach will allow protein fractionation (utilising 1D or 2D PAGE with ICPL protein labelled samples) to increase the number of proteins identified by undertaking a more exhaustive analysis (albeit increasing the number of LC-MALDI-TOF/TOF MS analyses) as well as to provide quantitative information on PTMs, in which the ICPL peptide labelled samples can be coupled with enrichment techniques (as all peptides are now labelled) prior to MS analysis.

5.3.2 Global pTyr analysis of R-IR-A/R-IR-B stimulated lysate

The improvement in quantitation data as a result of utilising the optimised ICPL peptide labelling strategy provides a way to assess global pTyr changes in signalling cascades when combined with other proteomic-based strategies for pTyr enrichment. Strategies, like the use of 4G10-coupled stationary phase for affinity purification and HILIC, with EZYprep DHB coupled with LC-MALDI-TOF/TOF MS analysis can be coupled with the ICPL peptide labelling strategy to provide the opportunity for a comprehensive global pTyr analysis of complex protein samples.

To highlight the capability of this proposed global pTyr approach, the insulin-like growth factor system was selected, based on both the role the system has in developmental and patho-physiological states (including cancer and diabetes) and the existing knowledge regarding receptor and downstream tyrosine phosphorylation. In regards to insulin binding to the insulin receptor, previous research has shown that insulin binding to either IR-A or IR-B results in the activation of two signalling pathways (Akt/PKB and MAPK pathways), however under certain conditions such as cancer, insulin binding to IR-A has been shown to improve cell survival and promote growth [387, 389-391]. Differences in insulin

binding affinity, activation of signalling cascades under certain physiological and patho-physiological sates and protein expression have been observed between IR-A and IR-B, indicating that the IR isoforms can be utilised for different cellular functions [401, 402]. As the IR receptor is a tyrosine kinase receptor, the initiation of different signalling cascades by these receptor isoforms may be due to the alterations in pTyr signalling, which emphasises the need for methods to provide quantitative and qualitative information regarding phosphorylation of the receptor and downstream signalling cascades.

In order to determine pTyr signalling differences between insulin stimulated IR-A and IR-B, lysates collected from insulin-stimulated IGF-IR-deficient (R^{-ve}) mouse embryo fibroblast lines engineered to express similar levels of human IR-A and IR-B (R^{-ve}IR-A and R^{-ve}IR-B), were digested and labelled with ICPL reagents 1-($^{12}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{12}C-Nic-Reagent [M_r = 105.0215 Da] for R^{-ve}IR-A and 1-($^{13}C_6{}^1H_4$)-Nicotinoyloxy-succinamide ^{13}C-Nic-Reagent [M_r = 111.0419 Da] for R^{-ve}IR-B. Both FACS and western blot analysis were utilised to confirm similar receptor copy number as well as similar activation of receptor and downstream signalling cascades, respectively. Although the FACS analysis showed similar receptor expression levels numbers for both R^{-ve}IR-A and R^{-ve}IR-B cells, differences in the level of receptor phosphorylation were noted (Figure 4.4D), with the IR-B receptor showing a higher level of receptor tyrosine phosphorylation. This is consistent with previously reported *in vitro* kinase activity of this receptor isoform [399, 403, 419, 442]. Visual inspection of western blot analysis of key downstream signalling proteins (phospho-p90RSK, phospho-Akt, phospho-p44/p42, MAP Kinase [MAPK], and phospho-S6 ribosomal protein) show similar levels of downstream activation, irrespective of whether IR-A or IR-B is activated (Figure 4.4A). However, although IR-B is known to show a higher level of phosphorylation, the results from Figure 4.4D are quite striking, with no evidence of IR-A phosphorylation on the 3Y region of the IR receptor. As levels of phosphorylation of the IR-A are known to be lower than IR-B after insulin stimulation, the possibility of incomplete inhibition of tyrosine protein phosphatases may account for the dramatic difference seen in Figure 4.4D. As the same lysis buffer (and consequently, the same phosphatase inhibitors) were utilised for R^{-ve}IR-A and R^{-ve}IR-B cells, the loss of pTyr in both lysates may have resulted in complete loss of signal in R^{-ve}IR-A and only partial loss in the R^{-ve}IR-B lysate, as the initial pTyr levels would have been higher with IR-B. Incomplete phosphatase inhibition may also account for the minor differences in levels of downstream signalling proteins between IR-A and IR-B. Despite the differences in receptor phosphorylation observed, the results from Figure 4.4A and Figure 4.4B (pTyr specific blot using 4G10) provided sufficient evidence of stimulation (resulting in an increase in tyrosine phosphorylation) upon insulin binding compared with the unstimulated control for both R^{-ve}IR-A and R^{-ve}IR-B lysate.

Upon labelling, ICPL labelled peptides from protein digests of lysates were combined and pTyr peptides enriched using HILIC followed by 4G10 affinity purification prior to EZYprep DHB LC-MALDI-TOF/TOF MS analysis. After HILIC and pTyr enrichment using 4G10, the remaining four fractions, HFT (Hydrophobic Flow Through), HE (Hydrophobic Eluate), HIE (Hydrophilic Eluate) and HIFT (Hydrophilic Flow Through) were analysed for labelled phosphopeptides. As HFT was comprised of flow-through (or non-bound) fractions after 4G10 pTyr enrichment, it was anticipated that the majority of non-phosphorylated peptides would be identified in this fraction. In contrast, it was anticipated that pTyr peptides would be found in the pTyr enriched fractions, HE and HIE. Also, despite the increased sample complexity of the HIFT (Hydrophilic flow-through) compared with the HE HIE (Eluted hydrophilic fraction), the fraction was expected to provide pSer and pThr phosphopeptides.

Unfortunately, despite fractionation to enrich pTyr phosphopeptides, no conclusive MS/MS data could be obtained from any fractions to identify and characterise phosphopeptides and thus provide any information regarding pTyr expression. IPA database analysis of the proteins identified and shown to be significantly regulated were involved in cytoskeleton re-organisation, particularly actin and filament organisation. These proteins were only identified in the HFT and HIFT fractions. Many of the proteins identified, such as actin cytoplasmic 1, Heat shock proteins and annexins were also identified in the previous analyses carried out for the ICPL labelling optimisation experiments (Table 4.1). The identification of these proteins in the ICPL control labelled samples (5.3.1) and the HFT and HIFT fractions indicate these proteins to be the most abundant proteins in the sample. The high complexity of these flow-through fractions, particularly HFT, meant the effectiveness of phosphorylated peptide identification would be low, particularly compared with the pTyr enriched fractions HE and HIE.

The results in Table 4.2 show that the proposed phosphoproteomic method for the global quantitation, identification and characterisation of pTyr was unsuccessful in conclusively identifying and characterising pSer, pThr or pTyr in insulin stimulated lysate. In light of this, a number of aspects to the method can be optimised and/or modified to improve phosphopeptide recovery for subsequent analysis using MS. Improvements in the sample preparation (including labelling strategy), phosphopeptide enrichment and subsequent MS analysis will ensure phosphopeptide identification and characterisation. Of the alterations proposed, the most imperative of these involves optimisation of the sample preparation prior to MS analysis.

Unless sufficient precautions are taken, a substantial component of the phosphoproteome is susceptible to loss upon cell lysis, which is due to the activation of various protein proteases and phosphatases. The addition of protease and phosphatase inhibitors as well as sample handling at low temperatures (*i.e.* 4°C) minimises the degradation effects, particularly if used in all initial steps until complete protein inactivation (e.g. reduction and alkylation followed by digestion or treatment with denaturing buffers such as SDS following reduction) [59, 220]. The addition of protein kinase inhibitors to minimise phosphorylation of sites with no biological significance and lysis buffers compatible with downstream processing must also be considered. The western blot analysis in Figure 3.4D showed a striking difference between phosphorylation at three well-characterised tyrosine residues of the IR-A and IR-B isoforms, which may be due to incomplete inhibition of protein tyrosine phosphatases. As such, an increased concentration of freshly made inhibitors may minimise loss due to sample handling.

Improvements in sample handling prior to MS analysis will minimise instances of phosphorylated proteins/peptides being lost, to subsequently increase phosphorylation site characterisation. However, certain aspects of the proposed pTyr global approach that requires significant optimisation concerns both the enrichment and quantitation strategies utilised. The enrichment strategies utilised in this study, both HILIC and pTyr enrichment using a 4G10 column, were chosen as both have been utilised successfully in phosphoproteome analyses [58, 80, 229, 306, 358]. HILIC, when coupled with IMAC, was shown to improve phosphopeptide selectivity when utilised as a first-stage enrichment tool [58, 80]. This is due to multiple aspects, including the compatibility of buffers used for HILIC and IMAC as well as the improvement of the binding efficiencies of phosphorylated peptides to the IMAC resin after HILIC enrichment. Although the HILIC-IMAC strategy enriches all phosphorylation (pSer, pThr and pTyr), the low abundance of pTyr compared with pSer and pThr provided a rationale to replace the IMAC enrichment with an enrichment strategy based solely on pTyr enrichment to improve pTyr proteome coverage. The ability to pre-fractionate phosphopeptides from non-phosphorylated peptides (on the basis that phosphopeptides would be more hydrophilic than the majority of non-phosphorylated peptides) prior to 4G10 enrichment was thought to minimise non-specific binding of non-phosphorylated peptides. This is important as the non-specificity of pTyr Abs in general as well as 4G10 for peptides with a higher number of tyrosine residues has been observed previously [225, 358].

The inability to identify any phosphopeptides after HILIC-4G10 enrichment can be attributed to the low amount of starting material and multiple sample handling steps prior to MS analysis. Many previously reported strategies for pTyr as well as global phosphoproteome analyses (including the strategies involving HILIC enrichment) commenced with large starting material, in some cases processing miligrams at a time [58, 80, 191, 229, 243, 264, 294, 443-445]. In contrast, the global strategy proposed here was utilised for the analysis of pTyr from a low amount of starting material (40.3 µg from digested R^{-ve}IR-A and R^{-ve}IR-B stimulated lysates), which after ICPL peptide labelling was combined (resulting in 80.6 µg starting material), was desalted using RP-cleanup and pTyr phosphopeptides were enriched using the HILIC-4G10 method. This starting amount was determined on the basis of the modified ICPL peptide labelling requirements, such that increasing the amount of sample to be labelled would significantly increase the costs associated with the process (as more label would be required). Label-based strategies can be limited by cost and although pre-fractionation or sample down-scaling can improve label quantity issues, detection and adequate quantification of low abundant proteins/peptides (such as pTyr proteins/peptides) can become compromised [169, 209, 445]. Despite the drawbacks of label-based starting material limitations, a recent study successfully identified, quantified, and characterised phosphorylation using similar starting materials coupled with ICPL peptide labelling and phosphopeptide enrichment [439]. In that study, 50 µg of protein extracts were digested with trypsin, peptides were labelled using ICPL and 40 µg were desalted using RP-cleanup and phosphopeptides enriched using TiO_2 enrichment. However, of the phosphopeptides reported in the supplementary data in that study, none were pTyr peptides, emphasising the need for larger amounts of starting material to improve pTyr recovery and identification. The HILIC-4G10 enrichment method utilised in the proposed global strategy may be beneficial for larger amounts of starting material (or batch processing) to compensate for sample losses that occurs with increased sample handling. Additionally, the use of a mixture of pTyr specific-Abs (instead of solely 4G10) may improve pTyr recovery as other pTyr-specific Abs will likely cover more of the pTyr proteome. However, increasing the starting material will increase the costs associated if a label-based quantitation strategy is utilised. As such, alteration of the quantitation strategy will allow for increased sample amounts to be analysed.

In terms of protein quantitation in proteomics, label-based and label-free techniques have been simultaneously developed, as outlined in Figure 5.4. Quantitation using MS is achieved by measurement of an intact ion in the MS spectrum or the signal of one or several fragment ions in the MS/MS signal. Quantities determined by MS (using either the MS or MS/MS signal) are always based on signal comparisons [169, 209].

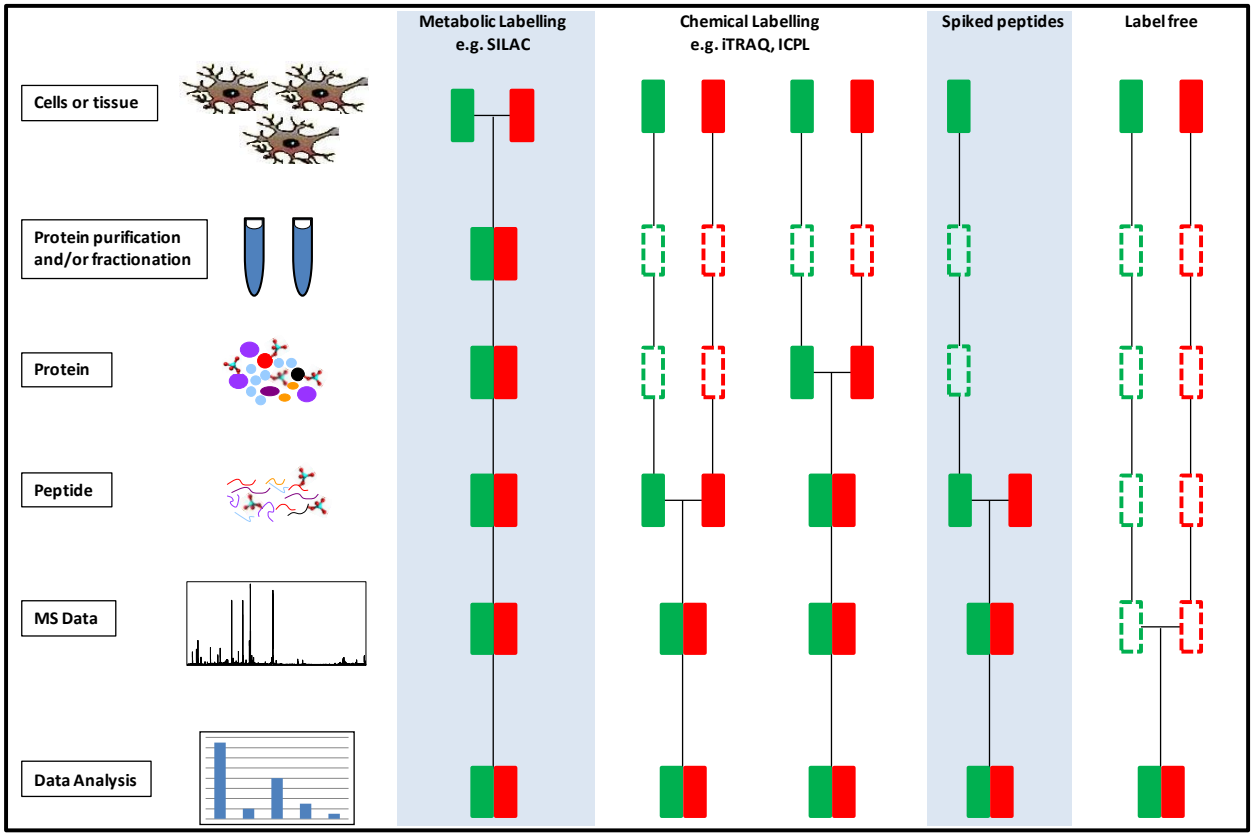


Figure 5.4: A summary of the proteomic workflows that can be utilised for protein and/or PTM quantitation [Adapted from [446, 447]].
The green and red box corresponds to two biological states and the horizontal line indicates the point at which the samples are combined. The boxes that are dashed indicate potential sources of experimental error, which affects quantitation read-outs.

Label-based strategies utilise isotopic references (the measurement of each peptide related to an isotopically labelled peptide with the same molecular structure), whilst label-free strategies utilise global references, which require the total ion count of peptides is used to derive quantitative information using appropriate normalisations [169, 448]. All label-free based strategies involve the following fundamental steps: sample preparation (including extraction and digestion), sample separation by 1D or 2D LC and analysis (typically by ESI based MS instruments) [449]. Quantitation in label-free strategies is based on the spectral count of a particular protein or comparison of peak intensity of the same peptide [169, 449]. The spectral count approach, which is one of the early methods to determine relative protein abundance, involves comparing the number of identified MS/MS spectra from the same protein in each of the multiple LC-MS/MS analyses [449, 450]. The approach is based on the premise that an increase in protein abundance typically results in an increase of the number of detected (and subsequently identified) peptides from that protein. The approach has been widely applied in a number of proteomic analyses, including biomarker discovery in human saliva from patients with type-2 diabetes and

screening of phosphotyrosine-binding proteins in mammalian cells [451, 452]. Despite the effectiveness of the approach, the availability of software and high resolution MS instruments allowing the quantitation based on the total ion intensity of the peptides has seen a shift away from the spectral counting method [169].

Label-free quantitation software typically involves data normalisation of the entire LC chromatographic run using global internal standards or normalisation of the sum of all ion intensities, time alignment, peak detection, peak quantitation based on ion intensity, peak matching, identification and statistical analysis [169, 449]. Label-free quantitation methods are particularly attractive as they require only minimal sample modification and handling and are therefore the most compatible method for biological experiments [169]. Both label-based and label-free techniques have their own advantages and disadvantages (for an excellent table summary review, refer to [169]). As shown in Figure 5.4, different strategies have varying levels of potential sources of experimental variation (leading to quantitation errors downstream), as highlighted by the dashed boxes for each method. It should be noted that although the metabolic labelling strategy (*e.g.* SILAC) has no dashed boxes, cell culture conditions must be monitored to prevent inter-conversion between arginine and proline, which could negatively affect quantification accuracy [209]. As such, no proteomics-based quantitation strategy is immune from experimental errors.

Label-free techniques offer clear advantages over label-based strategies in terms of decreased sample handling, acquisition times, sensitivity thresholds and have been shown to cover a larger dynamic range, which may be more applicable to the analysis of tyrosine phosphorylation as it makes up an extremely small component of the proteome [209, 221, 225, 227, 330, 358, 439]. However, label-free techniques are limited to particular high-resolution MS configurations (including Orbitrap or Q-TOF MS) and require appropriate software, as well as stable HPLC systems (routinely UHPLC systems) for high reproducibility between replicate analyses [169]. MS configurations, like QQQ, Q-TOF and also 3D IT can be utilised for specific mass spectrometric scanning modes used for quantitation, such as multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). MRM allows selective detection and quantification of targeted molecules in complex mixtures [169, 453, 454]. It involves the application of m/z filters for a specific parent and one or several fragment ions from all incoming ions. The use of these filters produces spectra only from ions that match the expected parent m/z as well the unique fragment ions to that parent ion. This MRM approach has been applied for the investigation of the temporal dynamics of 226 phosphorylation sites at different time points following EGF stimulation of

HMECs. As only pre-selected phosphopeptides are monitored in MRM mode, the reproducibility in phosphopeptide identification improved significantly between exploratory mode and monitoring mode [455]. The major disadvantage of the MRM approach is that this scanning mode needs to be established for every new targeted molecule [169]. Although a database that records peptides that provide unique fragment ions to allow detection using MRM has been developed [456], the MRM strategy is not the method of choice for exploratory quantitative proteomics.

In regards to quantitation by sample ion intensity, in order to ensure that ion intensity is reflective of the molecular concentration of a peptide in a sample, the ionisation process must be stable and reproducible [169, 457, 458]. In ESI instruments, at flow rates higher than 1 µl/min, hydrophobic peptides have a higher desolvation efficiency than hydrophilic peptides and ion suppression effects are more pronounced [459]. This results in irregular ion intensities that do not reflect molecular concentration [169]. As such, the majority of LC-MS/MS configurations will utilise LC flow rates less than 0.5 µL/min, as spectral intensities have been shown to correlate well with sample molecular concentration [169]. The effects of the ionisation process on signal intensity is even more pronounced using MALDI due to the variable ionisation efficiency of peptides within a sample. This is due to the ionisation process in MALDI, involving proton transfer from the matrix to the analyte [169]. As the number of protons available for ionising compounds is limited to the amount of matrix present, compounds with a high proton affinity will pull protons away from other compounds present in the sample, resulting in pronounced ion-suppression effects, particularly for phosphopeptides [112, 116, 150, 169, 256]. Compounding these known ion suppression of samples using MALDI is the fact that poor matrix heterogeneity of analyte/matrix crystallisation results in poor peak intensity reproducibility [457, 458, 460, 461]. Despite the negative effects that MALDI can have on compound signal intensity, recent label-free strategies have successfully utilised LC-MALDI-TOF/TOF MS analysis as a quantitative strategy [458, 460-462]. To overcome potential ion suppression and poor intensity reproducibility, label-free quantitation using LC-MALDI requires well controlled signal acquisition conditions, such as increasing the shot count per raster (up to 5000 shots over the raster position) [461]. This resulted in the determination of differentially expressed proteins between analysed samples [461]. Another study evaluating the quantitative reproducibility of MALDI signal intensity found a significant correlation between spiked peptide concentration and intensity in the range of 5 to 20 fmol/µL [458]. Yet, despite this, the known ion suppression effects of phosphopeptides will further complicate the ability to successfully quantify phosphorylation using label-free LC-MALDI. The improvements in peptide ionisation when labelled with ICPL will minimise this ion suppression and ensure phosphopeptide intensity will be reflective of molecular concentration between samples. Furthermore, an increase in

shot number per raster position will significantly decrease MS/MS information that can be obtained from a LC-MALDI analysis, as the sample amount is finite. Multiple LC-MALDI-TOF/TOF MS analyses would be required; one for label-free quantitation and one for peptide identification. However, this approach increases sample acquisition times compared with LC-ESI-MS/MS analysis.

Although the implementation of integrated LC and MS systems with the appropriate software for label-free quantitation becoming more affordable and mainstream, a large number of laboratories are not equipped with both the hardware and software required for label-free protein quantitation, including our laboratory at the time of writing. As such, label-based strategies are utilised as they can provide effective quantitation using multiple MS configurations. In addition, comparative analyses of samples using label-based strategies (such as iTRAQ and ICPL) and label-free analyses have shown significant correlation between the quantitative strategies [439, 463], highlighting label-based techniques as an appropriate quantitation strategy for proteomic analyses. Although the developments and availability of label-free quantitation over recent times in terms of both hardware (*e.g.* the development of next generation MS configurations with high resolution and mass accuracy and UHPLC systems) and software has improved, quantitative phosphoproteomic strategies being reported utilise label-based, label-free or a combination of both strategies [439, 464-468]. As with the complementary nature of ESI and MALDI MS methods for phosphopeptide identification and characterisation, at the present time no one quantitative strategy is capable for providing global expression changes. The proposed ICPL labelling strategy coupled with pTyr enrichment followed by EZYprep LC-MALDI-TOF/TOF MS analysis still offers the potential to be an effective global phosphoproteomic approach for pTyr identification, quantitation and characterisation.

Chapter 5: Concluding Remarks:

The study presented in this thesis targeted the limitations associated with tyrosine phosphoproteome identification, quantitation and characterisation. The methods developed in this thesis were designed to provide both a targeted and global pTyr enrichment strategy, allowing the analysis of pTyr samples across multiple MS platforms, with a particular focus on MALDI-TOF/TOF MS analysis. The development of the Pro-GP/Cov-P approach provides an easy targeted pTyr strategy to phosphosite characterisation on proteins of interest. The low degree of sample handling coupled with the compatibility of the approach with both ESI and MALDI MS makes this an attractive, cost effective strategy for most laboratories. The ability of this approach to identify and characterise pTyr phosphorylation on EGF-stimulated EGFR highlighted the approach as an alternative strategy to the more sensitive LC-MALDI-TOF/TOF MS and LC-ESI-IT-MS/MS analyses.

A drawback of the Pro-GP approach is the limited capacity of the Pro-GP/Cov-P beads for pTyr enrichment of complex samples. This point highlighted the requirement for the development of a global pTyr method that is able to provide quantitative as well as qualitative information. The EZYprep DHB method takes advantage of the atypical matrix DHB and PA for MALDI phosphopeptide ionisation to provide homogeneous sample/matrix crystallisation for coupling off-line to LC-MALDI-TOF/TOF MS. This approach improved pTyr identification and characterisation of EGF-stimulated EGFR compared with Pro-GP due to the ability to pre-fractionate the complex sample over multiple raster positions. Furthermore, comparison with LC-ESI-IT-MS/MS using CID and ETD fragmentation showed that EZYprep DHB LC-MALDI-TOF/TOF MS identified more phosphopeptides than LC-ESI-IT-MS/MS analysis. However, the comparison also highlighted the complimentarily between EZYprep DHB LC-MALDI-TOF/TOF MS and LC-ESI-IT-MS/MS, which adds further support to the use a multi-dimensional strategy to provide complete coverage of the pTyr phosphoproteome.

The advantages of the EZYprep DHB LC-MALDI-TOF/TOF MS approach for phosphopeptide identification and characterisation made the approach suitable for use as part of a global pTyr methodology. This global analysis coupled ICPL labelling, phosphoproteome enrichment, namely HILIC and pTyr enrichment using 4G10 Ab, with EZYprep LC-MALDI-TOF/TOF MS analysis. The optimisation of the ICPL labelling such that all peptides generated from complex protein sample digests improved overall quantitation efficiency, providing more accurate quantitation per protein identified. Despite this, limitations at the level of starting material hindered the ability of the global HILIC/pTyr 4G10 Ab

enrichment to conclusively identify, quantify and characterise the pTyr phosphoproteome involved in insulin signalling through either the IR-A or IR-B receptor isoforms and downstream cascades. In order for this approach to be successful for exhaustive pTyr phosphorylation analysis of complex protein samples, an increase in the amount of starting material and/or alteration of the enrichment protocols used (*e.g.* TiO_2 as a replacement to HILIC) should be considered.

These targeted and global pTyr methods have joined the explosive growth of pTyr phosphoproteomic-based methods for quantitation, identification and characterisation of phosphorylation sites on cellular proteins, as highlighted on the timeline (Figure 5.5).

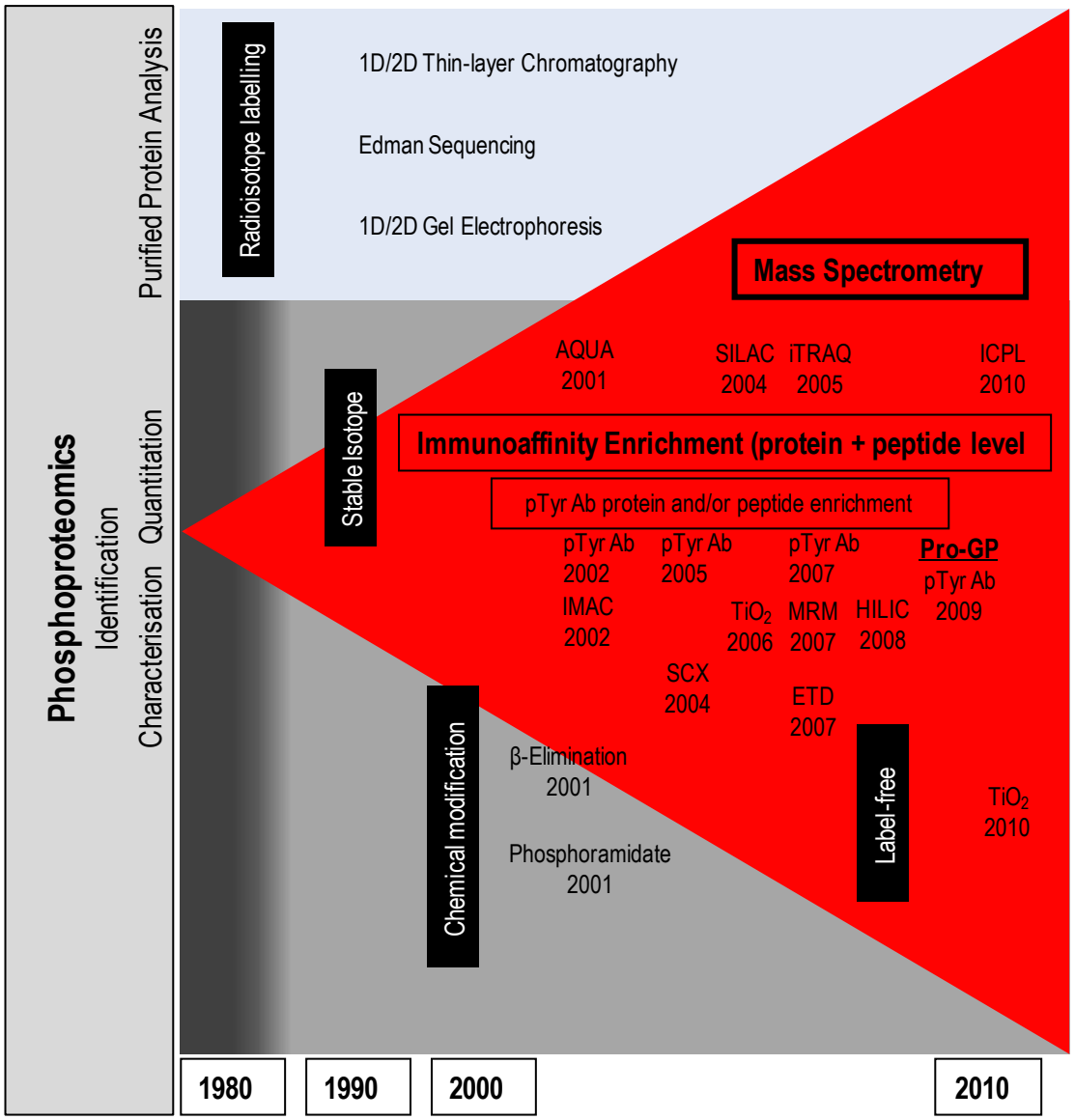


Figure 5.5: A Timeline of key milestones in the analysis of the phosphoproteome [adapted from [209, 443, 469-471]].

The past decade has seen an explosion in the types of methods for phopshoproteome analysis from the more conventional 1- and 2-DE and Edman sequencing. These new methods (particularly the global strategies) utilise MS for identification, quantitation and characterisation. These include β-elimination [472] or phosphoamidate modification [473], the use of pTyr Abs for protein and/or peptide [229, 253, 358, 444], IMAC [50], SCX [33], TiO_2 [280] and HILIC [58, 80] enrichment strategies to quantitative strategies in addition to enrichment, including Absolute Quantitation (AQUA) [474, 475], SILAC [294], iTRAQ [191], ICPL [439] and multiple reaction monitoring (MRM) [455]. Advancements in MS have additionally seen the use of ETD [305] and label-free analyses [465, 467] for phosphoproteome quantitation and characterisation.

Currently, phosphoproteome method development is in a state of flux, with the number of reported global phosphoproteomic-based methods increasing significantly since 2005 [209]. This can be attributed to the inability of any one method to fully characterise the phosphoproteome, despite the increasing knowledge base of phosphoproteome signalling. This was highlighted with the comparative analysis of the EGFR using EZYprep MALDI-TOF/TOF MS and LC-ESI-IT-MS/MS, which was shown to be complementary. Furthermore, this complementarity extends to the varying quantitative strategies such as differences between label-based and label-free quantitative results. In regards to pTyr analysis, the low abundance of this PTM extends the dynamic range required to be detected for global characterisation, which would require the use of multiple MS configurations. A greater understanding of the dynamic range of tyrosine phosphorylation along with more effective enrichment strategies will ultimately improve our understanding. Methods successfully characterising the phosphoproteome will most likely use label-free strategies as it reduces the issues with low abundance pTyr quantitation.

The onset of the large number of phosphoproteomic methods has resulted in an increase in the identification and characterisation of phosphorylation sites throughout the mammalian proteome. Further analysis of these sites to confirm their presence in *in situ* and *in vivo* settings as well as the determination of the function of these modifications in cellular signalling is imperative in order to gain a full understanding of transduction pathways in normal and patho-physiological states. Method development for the quantitation of low abundance pTyr must remain a focus to ensure absolute and reliable quantitation in pTyr proteins in cells. Methods such as the use of high resolution capillary isoelectric focusing to resolve (and subsequently immobilise) protein isoforms and individual phosphorylation forms, which is followed by probing with phospho-specific Abs, has been shown to quantify cell signalling in a small number of cells (<25) [476]. High resolution methods such as this one coupled with MS-based strategies will provide the selectivity and sensitivity required for pTyr characterisation.

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Appendix 7A

PROTEOMICS

Supporting Information for Proteomics

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EZYprep LC-coupled MALDI-TOF/TOF MS: An improved matrix spray application for phosphopeptide characterisation

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Appendix 7B

Tyrosine Phosphorylation Enrichment and Subsequent Analysis by MALDI-TOF/TOF MS/MS and LC-ESI-IT-MS/MS.

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STATEMENT OF AUTHORSHIP FOR APPENDIX 7B

Tyrosine Phosphorylation Enrichment and Subsequent Analysis by MALDI-TOF/TOF MS/MS and LC-ESI-IT-MS/MS. Current Protocols in Protein Science 2010, 62:13.11.1-13.11.26.

Mark R. Condina (Candidate)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Designed experimental methods, interpreted data, wrote manuscript

Certification that the statement of contribution is accurate

Signed.....Date.....

Manuela Klingler-Hoffmann (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Aided in method parameter suggestions and manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed.....Date.....

Peter Hoffmann (co-author)

Statement of contribution (in terms of the conceptualization of the work, its realisation and its documentation)

Supervised development of methods, helped in data interpretation and manuscript evaluation and acted as corresponding author

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed.....Date.. .....

Condina, M.R., Klingler-Hoffmann, M. and Hoffmann, P. (2010) Tyrosine Phosphorylation Enrichment and Subsequent Analysis by MALDI-TOF/TOF MS/MS and LC-ESI-IT-MS/MS.
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