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Combining elemental and molecular mass spectrometry to study 3 types of biologically important compounds: DNA, phosphopeptides and anticancer drugs

Claire Louise Camp

LGC, Queens Road, Teddington, Middlesex, TW11 OLY

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COMBINING ELEMENTAL AND MOLECULAR MASS SPECTROMETRY TO
STUDY 3 TYPES OF BIOLOGICALLY IMPORTANT COMPOUNDS: DNA,
PHOSPHOPEPTIDES AND ANTICANCER DRUGS

By
Claire Louise Camp

A dissertation thesis submitted in partial fulfilment of the requirement for the award of the
degree Doctor of Engineering (EngD), at Loughborough University

January 2014

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# TABLE OF CONTENTS

1 BACKGROUND TO THE RESEARCH .......................................................... 1-1

1.1 INTRODUCTION .................................................................................. 1-1

1.2 BACKGROUND ....................................................................................... 1-1

1.3 THE INDUSTRIAL SPONSOR ................................................................. 1-2

1.4 THE CONTEXT OF THE RESEARCH ..................................................... 1-2

1.4.1 Quantitation of DNA ........................................................................ 1-2

1.4.2 Selective Analysis of Phosphopeptides ............................................. 1-6

1.4.3 Identification of proteins involved in toxicity caused by platinum based cancer drugs ..... 1-8

1.5 OVERARCHING AIM AND OBJECTIVES .............................................. 1-10

1.6 STRUCTURE OF THESIS ................................................................. 1-11

2 LITERATURE REVIEW ........................................................................... 2-1

2.1 INTRODUCTION .................................................................................. 2-1

2.2 DNA QUANTIFICATION ........................................................................ 2-1

2.3 PHOSPHOPEPTIDE ANALYSIS ........................................................... 2-10

2.3.1 Phosphopeptide Tagging Strategies .................................................. 2-11

2.3.2 Phosphopeptide Enrichment Strategies .......................................... 2-14

2.3.3 Phosphopeptide analysis by mass spectrometry ................................ 2-17

2.3.4 Phosphopeptide Analysis by ICP-MS ............................................... 2-18

2.3.5 Phosphopeptide Quantification ....................................................... 2-19
2.4 INTERACTION OF SMALL PEPTIDES WITH PLATINUM BASED ANTI-CANCER DRUGS ................................................................. 2-21

3 RESEARCH METHODOLOGY ......................................................................................... 3-1

3.1 INTRODUCTION ........................................................................................................ 3-1

3.2 OBJECTIVES OF RESEARCH .................................................................................. 3-1

3.3 TYPES OF RESEARCH ............................................................................................ 3-2

3.4 SCIENCE METHODOLOGY ...................................................................................... 3-3

3.4.1 Scientific Research Methods .............................................................................. 3-4

3.4.2 Formulating the research problem ..................................................................... 3-4

3.4.3 Developing working hypothesis ......................................................................... 3-5

3.4.4 Preparing the research design ............................................................................ 3-6

3.4.5 Collection and analysis of data .......................................................................... 3-6

3.4.6 Testing of hypothesis ......................................................................................... 3-7

3.4.7 Reporting results ............................................................................................... 3-7

4 INSTRUMENTATION ..................................................................................................... 4-1

4.1 INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)........ 4-1

4.1.1 High Resolution ICP-MS .................................................................................. 4-4

4.2 MOLECULAR MASS SPECTROMETRY – Linear Ion Trap Mass Spectrometer ... 4-6

4.2.1 Electrospray ionisation ...................................................................................... 4-9

4.2.2 Linear ion trap mass analyser ........................................................................... 4-12

4.2.3 Scan Modes ...................................................................................................... 4-16
6 CONCLUSIONS, IMPLICATIONS AND OPPORTUNITIES FOR FURTHER RESEARCH

6.1. INTRODUCTION

6.2. KEY FINDINGS OF THE RESEARCH
   6.2.1. Quantification of DNA
   6.2.2. Heterogeneous tagging of phosphopeptides with gallium
   6.2.3. Binding of carnosine and anserine with oxaliplatin

6.3. CRITICAL EVALUATION OF THE RESEARCH
   6.3.1. Contribution to knowledge
   6.3.2. Implications/impact on industrial sponsor
   6.3.3. Implications on wider community and industry

6.4. RECOMMENDATIONS

6.5. SUMMARY

7 REFERENCES
**TABLE OF FIGURES**

Figure 1: Polymerase chain reaction (PCR) mechanism of DNA replication. .................... 1-4

Figure 2: Mechanism of phosphorylation by ATP on a serine, threonine or tyrosine containing protein. ................................................................. 1-7

Figure 3: Proportion of platinum found in the different cellular components of various colorectal cancer cell lines. 10 ................................................................. 1-9

Figure 4: The classical linear science method. 87 ................................................................. 3-4

Figure 5: Basic principle of the mode of ICP-MS operation. ................................................. 4-2

Figure 6: Formation of the inductively coupled plasma, showing the different temperature regions. ......................................................................................... 4-3

Figure 7: Diagram of the reverse Nier Johnson magnetic sector instrument. 91 ................. 4-5

Figure 8: Schematic diagram of a linear ion trap mass spectrometer. ............................... 4-9

Figure 9: Electrospray ionisation source using a heated metal capillary for ion de-solvation and transfer........................................................................................................ 4-10

Figure 10: A - The ion evaporation process, an individual ion leaves the charged droplet. B - The charged residue model. A charged molecule reduces in size due to evaporation, a new Taylor cone forms from which smaller highly charged droplets are emitted. .............. 4-12

Figure 11: Linear trap, with slots in opposite rods for radial ion ejection to two detectors. 4-13

Figure 12: Overview of the research activities during the EngD programme. .................... 5-3
Figure 13: Proposed quantification of calf thymus DNA work-flow. .............................................. 5-5

Figure 14: Separation of all 4 dNMP’s on Phenomenex Gemini NX Column, Isocratic mobile phase - 15 mM TEAA, 2.5 % MeOH, Flow rate – 0.25 mL min⁻¹, Injection volume 20 µL, Column Temp 25 °C. ....................................................................................................................... 5-8

Figure 15: Digestion protocol for the digestions of calf thymus DNA........................................... 5-10

Figure 16: LC-SF-ICP-MS chromatograms of ³¹P, A: single monophosphate nucleotide standards, B: digested calf thymus DNA..................................................................................................................... 5-12

Figure 17: A typical calibration chart for nucleotide standards...................................................... 5-13

Figure 18: The formation of LysNTAGa tagged β-PDGF from LysNTA, Ga(III)Cl and β-PDGF. ................................................................................................................................. 5-17

Figure 19: Comparison of the found isotopic pattern for LysNTAGa (top) compared to the theoretical isotopic pattern generated by Xcalibur software (bottom)................................. 5-18

Figure 20: Formation of [LysNTAGa.H2O ]⁺; panel A experimental data, panel B theoretically predicted spectra by high resolution mass spectrometry performed on a Thermo Exactive Mass Spectrometer ........................................................................................................ 5-19

Figure 21: Structure for the Ga-LysNTA singly charged positive ions as calculated at the HF/STO3G and B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies (in kcal per mol) are indicated in parenthesis......... 5-20

Figure 22: Panel A experimental signal due to [LysNTAGa]⁺ adducts with H₂O and CH₃OH, panel B theoretically predicted composite spectrum of a 1:2 ratio of [LysNTAGa.2H₂O]⁺ and [LysNTAGa.CH₃OH]⁺........................................................................................................... 5-21
Figure 23: Full scan spectrum of reaction mixture showing evidence of [LysNTAGaPDGF]+ formation from Thermo LTQ Mass Spectrometer, showing the formation of LysNTAGaPDGF at m/z 1030.25, and the starting materials PDGF at m/z 702 and LysNTAGa at m/z 361.

Figure 24: Zoomed in scan showing the formation [LysNTAGaPDGF]+ at m/z 1030.

Figure 25: Product ion scan of [Ga-LysNTAPDG]+ at m/z 1030 from LTQ Ion Trap MS.

Figure 26: The lowest energy structure 2A calculated for the Ga-LysNTA-β-PDGF singly charged positive ions as calculated using the ONIOM method at B3LYP/LANL2DZ:PM6 level of theory. Bond lengths are in Angstroms.

Figure 27: LC-ICP-MS chromatograms of A – Unlabelled PDGF and B – PDGF labelled with LysNTAGa.

Figure 28: Molecular structure of oxaliplatin.

Figure 29: Full scan MS spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time.

Figure 30: Zoom in showing the isotope pattern of oxaliplatin obtained on the LTQ.

Figure 31: Zoom in of the isotope pattern resulting from the ion [carnosine+oxaliplatin+H]+ obtained on the LTQ.

Figure 32: MS2 scan of carnosine coupled to oxaliplatin obtained on the LTQ.
Figure 33: Zoomed in views of the daughter ions from carnosine:oxaliplatin with assignments, from the MS2 mass spectra obtained on the LTQ. ......................... 5-32

Figure 34: Structures for [Carnosine + OxPt + H]$^+$ ions in which a formal Pt coordination to carnosine is observed as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis. Italicized numbers are for solvated species. ............................................................. 5-33

Figure 35: Structure for the lowest energy [Carnosine + OxPt – CO$_2$ + H]$^+$ ion as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms. ....................... 5-35

Figure 36: Structure for the lowest energy [Carnosine – H + Pt(dach)]$^+$ ion as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms. ......................... 5-36
TABLE OF TABLES

Table 1: List of publications as a result of the research carried out as part of the EngD. ... 1-12

Table 2: Classification of the different types of research. 84 .................................................. 3-2

Table 3: pKa for bases in nucleosides and nucleotides at ~ 20 °C. 113 ..................................... 5-6

Table 4: Column testing conditions used in preliminary work for the separation of monophosphate nucleotides. ........................................................................................................... 5-6

Table 5: Recoveries of individual nucleotides using FI-ICP-MS. ............................................. 5-11

Table 6: Total peak areas for speciated nucleotide standards and digested NIST DNA and flow injection peak areas for nucleotide blend and undigested NIST DNA.......................... 5-15

Table 7: Proposed identification of the major ions found in the full spectrum scan of Ga-LysNTA-PDGF according to observed m/z values. ............................................................... 5-23
TABLE OF EQUATIONS

Equation 1: Rayleigh equation for the limiting charge. \(^{105,106}\) ............................................. 4-11

Equation 2: The resolution of two peaks is calculated from the difference in retention time of the two peaks divided by the combined widths of the eluted peaks. ........................................ 4-19

Equation 3: The equation used to determine the retention factor of an analytes based on its retention time and the time taken for the mobile phase to travel through the column. ........... 4-20

Equation 4: The number of plates can be calculated from the retention time and peak width. 4-20

Equation 5: The Van Deemter Equation describes the effects of band broadening in relation to the number of paths taken, the longitudinal diffusion and the rate of absorption and desorption. .......................................................... 4-20
ACKNOWLEDGEMENTS

I would like to acknowledge and express my appreciation to several people and institutions that have given me this opportunity and supported me through this journey.

Firstly, I would like to thank the EPSRC, LGC Ltd, CICE and the Department of Chemistry at Loughborough University for providing funding, equipment and facilities that allowed this project to be achieved.

I would like to say a very sincere thank you to my husband for his support over the last 6.5 years which have been a true test of our marriage. Thank you for being there for every midnight dash to the lab, the weekends of data processing, the missed family events and undone household chores and most importantly to accept my apologies and grant me your forgiveness.

I would like to thank my academic supervisors, Prof Barry Sharp and Dr Helen Reid, my industrial supervisor Dr Heidi Goenaga Infante and Dr. John Entwisle from LGC Ltd. Also a special thank you to Dr. Tamer Shoeib, without whom the majority of work in this thesis would not have been possible. His colleagues at the American University in Cairo for their assistance with the molecular modelling of compounds in this thesis. Finally to Dr Don Jones from Leicester University for the use of their mass spectrometers.

I would also like to thank Dr. Dhinesh Asogan, Dr. David Douglas and the support staff at the Department of Chemistry, Loughborough University for their help and support during this process.
ABSTRACT

Mass spectrometry was used to investigate three important biological molecules, deoxyribonucleic acid (DNA), phosphopeptides and oxaliplatin. The quantification of DNA is traditionally performed by UV spectroscopy; however the results can be affected greatly by the sample matrix. The method developed quantified phosphorus in digested calf thymus DNA and human DNA by high performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS). The method presented showed excellent baseline separation between all 4 DNA mono-nucleotides and 5'UMP. Column recoveries ranging from 95% to 99% for phosphorus resulted in a mass balance of 95% ± 0.5% for standard nucleotides, determined by LC-ICP-MS, compared to total DNA determined by flow injection coupled to ICP-MS (FI-ICP-MS). Protein phosphorylation and de-phosphorylation is one of the most common signalling pathways within cells, it is involved in regulating cellular processes, mediating enzyme inhibition, protein-protein recognition and protein degradation. A novel approach to the selective detection of phosphopeptides based on the incorporation of a metal tag, gallium–N,N-biscarboxymethyl lysine (Ga-LysNTA), in solution before separation and detection by liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS) was developed. Linear ion trap electrospray ionisation mass spectrometry (ESI-MS) was employed to study the interaction of the gallium tag with platelet derived growth factor beta receptor (β-PDGF), a small phosphopeptide. In addition molecular modelling was used to investigate the energetically favoured structures of both the Ga-LysNTA material and the β-PDGF-Ga-LysNTA complex. The complexation of the Pt-based anti-cancer drug oxaliplatin (OxPt) with biological ligands other than DNA is believed to be a major cellular sink for the drug reducing its therapeutic potential and acting as a potential cause of toxicity. The role of the naturally abundant cytoplasmic dipeptide ligand β-alanyl-L-histidine dipeptide (carnosine) in OxPt detoxification was investigated. Various mass spectrometry techniques employing electrospray ionization and chip nanospray were employed to study the interaction of oxaliplatin with carnosine as well as two of its derivatives β-alanyl-N-methylhistidine (anserine) and N-acetylcarnosine (NAC). Evidence of complexation between OxPt and each of the three ligands examined is presented. Most species observed were unambiguously assigned and compared to their theoretical isotopic patterns.
# LIST OF ABBREVIATIONS

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<tr>
<td>EngD</td>
<td>Engineering doctorate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRM</td>
<td>Certified reference material</td>
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<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>NMI</td>
<td>National Measurement Institute</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy N triphosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically modified</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>LC-ICP-MS</td>
<td>Liquid chromatography inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emission spectrometry</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>MCN</td>
<td>Micro-concentric nebuliser</td>
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<tr>
<td>DIHEN</td>
<td>Direct injection high efficiency nebuliser</td>
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<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
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ORS     Octopole reaction cell
FIA     Flow injection analysis
dAMP    Deoxyadenosine monophosphate
dCMP    Deoxycytidine monophosphate
dTMP    Deoxythymidine monophosphate
dGMP    Deoxyguanosine monophosphate
UMP     Uridine monophosphate
IMP     Inosine monophosphate
MALDI-TOF-MS  Matrix assisted laser desorption time of flight mass spectrometry
IDA     Isotope dilution analysis
SVP     Snake venom phosphatase
SAP     Shrimp alkaline phosphatase
BNPP    Bis -(4-nitro phenyl) hydrogen phosphate
PDE     Phosphodiesterases
NP1     Nuclease P1
AP      Alkaline phosphatase
NMR     Nuclear magnetic resonance
IMAC    Immobilised metal affinity chromatography
SILAC   Stable isotope labelling with amino acids in cell culture
SCX     Strong cation exchange
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>AML</td>
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<tr>
<td>iTRAQ</td>
<td>Isobaric tagging for relative and absolute quantitation</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>capLC-ICP-MS</td>
<td>Capillary liquid chromatography inductively coupled plasma mass spectrometry</td>
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<tr>
<td>HCD</td>
<td>High energy collision dissociation</td>
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<tr>
<td>MOA</td>
<td>Metal oxide affinity</td>
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<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
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<td>ETD</td>
<td>Electron transfer dissociation</td>
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<tr>
<td>MOAC</td>
<td>Metal oxide affinity capture</td>
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<td>Adenosine triphosphate</td>
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1 BACKGROUND TO THE RESEARCH

1.1 INTRODUCTION

This chapter provides an introduction to the overall theme of the EngD thesis; it outlines the different projects, giving background information on the importance of DNA quantitation and selective phosphopeptide determination in the wider community. The overall aim of the thesis and the individual objectives for each project are described, also the scope and justification for the work is included. This chapter also includes a summary of the papers published as a result of the EngD project, which should be read in conjunction with this thesis.

1.2 BACKGROUND

The first two projects of this thesis were designed to support the work performed by the UK National Measurement System, which is responsible for providing good measurement practice for the benefit of the nation. The National Chemical and Biological Metrology programme is responsible for new approaches to measurement and standardisation; the programme recognised the need for validated methods and certified reference materials (CRMs) for the measurement of DNA and proteins.

Recent research into protein and DNA quantification utilises the unique capabilities of Inductively Coupled Plasma Mass Spectrometry (ICP-MS), such as large dynamic range, excellent detection limits, near matrix independent analysis, and the capability to provide mass balanced separation when coupled with separation by High Performance Liquid Chromatography (HPLC). However, the research cannot be fully implemented in main
stream industry without robust and validated measurements and methods. As such the research performed during the EngD was designed to support the development of reference methods for the quantification and identification of phosphorus (P) containing compounds, for the characterisation of standard materials, clinical samples and pharmaceutical products.

1.3 THE INDUSTRIAL SPONSOR

LGC Ltd is an international leader in laboratory services, measurement standards, reference materials and proficiency testing, offering products and services in analytical and bioanalytical assays. The company's client base is diverse and extensive, with activities ranging from forensic science; pharmaceutical and biotechnology research, to those in the food chain and environmental surveillance and safety; life science and basic research. LGC Ltd is the UK's designated National Measurement Institute (NMI) for chemical and biochemical analysis and the National Reference Laboratory for a range of key areas including; added water in poultry, genetically modified organisms, and heavy metals and veterinary residues in products of animal origin. As the UK's designated NMI for chemical and biochemical measurements, LGC Ltd focuses on improving the accuracy and reliability of chemical and bio-measurements that are important to the UK's industrial competitiveness and quality of life.

1.4 THE CONTEXT OF THE RESEARCH

1.4.1 QUANTITATION OF DNA

Gene quantification is a vital technique which is commonly used in many analytical areas, such as the monitoring and labelling of genetically modified food stuffs, disease diagnosis and forensic analysis. The preferred method of gene quantification uses real-time polymerase chain reaction (PCR) technologies. The PCR technique is a method for the amplification of
DNA and RNA using single strand DNA and the enzyme DNA polymerase. The steps in PCR are outlined in Figure 1. Briefly the DNA is first denatured to unwind the DNA double helix; a primer is added to initiate the replication of the complimentary DNA strand. Deoxynucleotide triphosphates (dNTP’s), the building blocks of DNA, are added with the enzyme DNA polymerase and the amplification process begins. Real-time PCR is able to detect DNA and RNA concentration as the reaction is occurring compared to conventional PCR which is only able to quantify at the end-point of the reaction. It also has several other advantages over conventional PCR in that it has greater sensitivity and can be performed easily in closed-tube assays.
Background to the Research

Claire Camp

Figure 1: Polymerase chain reaction (PCR) mechanism of DNA replication, the template DNA strands A and B are replicated to give two new DNA strands.

Template DNA (strand A)

5’ → 3’

3’ ←

Template DNA (strand B)

5’ → 3’

Hybridisation of strand specific primers

Denaturing

Template DNA (strand A)

5’ → 3’

3’ ←

Primer B

Primer A

Template DNA (strand B)

5’ → 3’

Elongation of primers to form two daughter strands

Polymerase reaction

Template DNA (strand A)

5’ → 3’

3’ ←

Template DNA (strand B)

5’ → 3’

Extension, the process is repeated until the desired number of copies is reached.

Template DNA (strand A)

5’ → 3’

Copy strand A

Copy strand B

Template DNA (strand B)

5’ → 3’

3’ ←
Since the mapping of the human genome in 2003, the number of gene therapy treatments and tests has increased. Due to advances in PCR technology and with the whole of the human genome mapped, links between specific gene sequences and disease states has increased. As such, hundreds of specific tests have been designed to identify a patient’s susceptibility to a disease prior to any symptoms being displayed. However, there is concern that the accuracy of these tests varies greatly between institutions. The accurate quantification of DNA is of great importance to many areas of the research community. One of the most important is the identification of genetic defects resulting in disease. Limitations arise as large sample sizes are needed to identify small genetic defects and often the yield of DNA from patients is small, also the conservation of precious DNA samples is of great importance for clinical research in order to verify and validate findings. To prevent unnecessary DNA consumption for high-throughput genotyping, accurate and precise DNA quantification is needed. Genotyping is the method of comparing the genetic make-up of an individual to that of a reference sequence. Identifying single nucleotide polymorphisms (SNPs), a difference in one single nucleotide in the genome between members of the same species, by SNP genotyping can help predict a person’s susceptibility to disease by identifying relevant genes. It is believed that characteristic SNP profiles will be identified and DNA testing will be able to screen for common diseases by analysing a patients DNA for specific SNP patterns. Imprecise quantification of small amounts of patient DNA increases variability in the amount of DNA gained by polymerase chain reactions, and leads to lower confidence in scoring of genotypes.

Advances in technology and improved methodology have resulted in more laboratories having the ability to conduct detailed analyses of food composition. ² The development of genetically modified crops and public concerns over the consumption of these crops has led many countries to introduce strategies for accurate labelling and tracing of GM crops. This has resulted in the need for validating and standardising methods, certifying laboratories and having reliable standards and certified reference materials widely available. ² The availability of certified reference materials in the analysis of GM crops is especially important as all foodstuffs containing more than the threshold must be correctly labelled, this requires great accuracy, which is routinely measured against a CRM. ¹
To provide a complete picture of the genetic and compositional changes in food produced by either genetic engineering or conventional breeding, a targeted analysis using the tools of modern molecular biology should be used to provide information regarding the specific genetic changes that have occurred.

The research work described in this thesis was designed to produce a quantitative method that could be used to quantify small amounts of DNA based on the phosphorus content by ICP-MS.

1.4.2 SELECTIVE ANALYSIS OF PHOSPHOPEPTIDES

Protein structure and function is of great importance in the generation of new small-molecule drugs and in diagnosing and understanding disease processes. Many drugs have been developed as a direct result of understanding the signalling pathways in cells that cause proteins and other molecules within cells to be destroyed, exported or imported. Protein phosphorylation or de-phosphorylation is one of the most common signalling triggers within cells. The reversible, covalent attachment of a phosphate group to one or more amino acids is the most common posttranslational modification for regulating cellular processes, and it is estimated that as many as 30-50% of proteins are phosphorylated at any given time. Phosphorylation changes regions on a protein from hydrophobic to hydrophilic, and this can result in a conformational change of the whole protein. The phosphorylated amino acid can also form part of a structure recognized by the binding sites of other proteins, thus driving the regulated assembly and disassembly of protein complexes.

Kinases and phosphatases are the enzymes that phosphorylate and dephosphorylate proteins, respectively, on a timescale of seconds to minutes, the mechanism of phosphorylation is Shown in Figure 2. Many kinases are phosphoproteins themselves and are active only when phosphorylated. Cellular signalling is a cascade of reactions: phosphorylation activates kinase $x$, then $x$ phosphorylates kinase $y$, and so on. Disruptions of these cascades have been linked to several diseases, including cancer.
Figure 2: Mechanism of phosphorylation by ATP on a serine, threonine or tyrosine containing protein.

Compared with regular proteomics, phosphoproteomics poses some additional challenges. Phosphorylation is a dynamic, reversible process, and the ratio of phosphorylated to unphosphorylated proteins can be rather low in vivo. Phosphoproteomics experiments are more technically challenging because they require more initial separation and additional sensitivity. Moreover, the objectives for phosphoproteomics research are somewhat different from those of regular proteomics.

The research work described in this thesis was designed to produce a methodology that could be used to selectively detect slight changes in the degree of protein phosphorylation, in order to gain a greater understanding of the signalling pathways in cells, by the tagging of model phosphopeptides with gallium.
1.4.3 IDENTIFICATION OF PROTEINS INVOLVED IN TOXICITY CAUSED BY PLATINUM BASED CANCER DRUGS

In 2009, 41,000 people were diagnosed with colorectal cancer in the UK, in Europe it was estimated that there were over 300,000 new cases and 1.24 million new cases of colorectal cancer Worldwide in 2008. It is one of the most prolific cancers, accounting for approximately 13% of all cancer diagnosis in the UK. 3 Colorectal cancer develops via complex abnormalities that transform normal epithelium, the tissue that lines cavities within the body, such as the colon, into dysplastic epithelium which may lead to disease. Patients with colorectal cancer who are treated by the surgical removal of disease tissue are likely to suffer relapses and unfortunately die from this form of cancer, with the five year survival rate of patients with Stage II and Stage III tumours being only 80 and 60% respectively. 4

Cisplatin has been used to treat cancer for over 30 years 5, and when used in combination with other anti-cancer drugs, such as bleomycin and vinblastine, it has been shown to cure 80% of testicular cancers. 5 There are tens of platinum based anti-cancer drugs entered into clinical trials, but only four are approved world-wide, cisplatin, carboplatin, oxaliplatin and nedaplatin. 5, 6 However, as with all drugs, there are side-effects associated with all these drugs, including nephrotoxicity, nausea and vomiting, ototoxicity and peripheral neuropathy. 7, 8 Oxaliplatin is more commonly used in the treatment of colorectal cancer as it is better tolerated in terms of renal toxicity but its main disadvantage is that is has serious neurotoxicity side effects; including cold induced paresthesia, muscle spasms and muscle twitching. 4, 9 Neuropathy from oxaliplatin treatment ranges from acute sensory neuropathy immediately following oxaliplatin infusion, to chronic dose-limiting neuropathy, which only presents after several treatments. 8 Neurotoxicity is thought to be caused by the inhibition of voltage-gated sodium currents and by the presence of free oxalate ions acting as calcium chelators.

It has been found by the analysis of several cancer cell lines that the largest proportion of the drugs remains in the cytosol, see Figure 3. 10
The overall aim of this project was to identify which proteins and peptides in the cytosolic fraction of cancer cell lines bind to the platinum containing drug. Identification of these proteins may lead to the discovery of why patients suffer from neurotoxicity and to identify other complementary therapeutic agents which may increase the proportion of oxaliplatin reaching the nucleus, leading to lower doses. To simplify the project, peptides found in the cytosol at relatively high concentrations and which are known to be metal chelators were chosen to model the interaction of oxaliplatin with cytosolic peptides. The peptides identified as likely candidates for oxaliplatin scavenging were glutathione, carnosine and anserine, and their interactions with oxaliplatin were investigated by electrospray ionisation tandem mass spectrometry (ESI-MS<sup>n</sup>) and molecular modelling.
1.5 OVERARCHING AIM AND OBJECTIVES

To develop and validate a HPLC ICP-MS method for the quantification of trace levels of DNA nucleotides and phosphorylated peptides/proteins via their constant phosphorus content.

The specific objectives agreed by LGC and Loughborough University were:

1. To produce a literature survey of the current methods for DNA and phosphopeptide quantification
2. To produce a validated LC-ICP-MS method for the quantification of DNA based on the P content of DNA.
3. To produce a journal article based on the validated method stated in part 2.
4. To develop a validated LC-ICP-MS method for the selective detection of phosphopeptides.
5. To produce a journal article based on the validated method stated in part 4.

In addition to this the scope of the research was extended to include the identification of oxaliplatin bound peptides from cancer cell lines. The specific aims of this research were:

1. To identify peptides, or peptide motifs, likely to bind to oxaliplatin and investigate the structures of these adducts by ESI-MS<sup>®</sup>.
2. To produce a journal article based on the findings of part 1.
1.6 STRUCTURE OF THESIS

This thesis comprises five main chapters:

**Chapter 1** provides an overview of the three projects, the context of the research and the aims of each individual project.

**Chapter 2** is a literature review of the research done to date into each of the projects outlined in Chapter 1.

**Chapter 3** explains the research methodologies adopted to address the objectives of the EngD, and maps out the research activities.

**Chapter 4** outlines the instrumentation used for data collection in this thesis, outlining their basic principles, structure and mode of operation.

**Chapter 5** is a commentary of the work conducted to determine methods for quantifying DNA, selective detection of phosphopeptides by liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS) and investigation of the adducts of oxaliplatin with carnosine, anserine and glutathione by ESI-MS.

**Chapter 6** details the key findings of the project and the implications for the sponsor and wider industry, as well as presenting a critical review of the work and recommendation for future research related to the theme of this EngD.

**Appendices A to C** contain the full contents of the three papers referred to throughout this thesis, which were published as a result of the work undertaken. These papers should be read in conjunction with the thesis so that the link can be established between the detailed research work of the EngD, and the overall EngD theme.
<table>
<thead>
<tr>
<th>Paper ID</th>
<th>Name</th>
<th>Journal</th>
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<td>4</td>
<td>An isotope dilution strategy for the quantification of Pt-GG adducts in lung cancer cells exposed to carboplatin at doses relevant to the clinic (In preparation)</td>
<td>Intended Journal for publication: Metallomics</td>
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Table 1: List of publications as a result of the research carried out as part of the EngD.
2 LITERATURE REVIEW

2.1 INTRODUCTION

This chapter provides a detailed review of the existing literature on each of the 3 projects that form this thesis. The literature review is split into three sections; the quantification of DNA, the analysis of phosphopeptides and the interaction of platinum drugs with small peptides.

2.2 DNA QUANTIFICATION

Due to its importance in the human body the analysis and quantification of phosphorus in DNA is of great interest to food safety, bio-medical and pharmaceutical research. Nucleotides are the building blocks of DNA, nucleotide mutations are believed to be linked to cancer and genetic diseases. Analogues of nucleotides make effective drugs for the treatment of AIDS and cancer. In the food industry nucleotides can taint the taste of foods. Traditionally the quantification of phosphorus in DNA has been achieved by UV absorbance, fluorescence via the incorporation of dyes, or β radiation emission from radioisotope labelled $^{32}$P and $^{33}$P.

UV measurements have been used for the quantification of DNA in biological samples for over 60 years however, results acquired with spectrometric methods can be erroneous. UV absorbance methods are cheap and easy to perform; however, results are often compromised by impurities in the sample. The technique also requires relatively large sample volumes. DNA quantification by UV absorbance measures both double and single stranded DNA and is based on extinction coefficients, the measure of how strongly a chemical species absorbs light at a given wavelength. For 1 mg mL$^{-1}$ of DNA, the extinctions are generally accepted to be 20 m$^2$ mol$^{-1}$ at 260 nm and 10 at 280 nm. Proteins also absorb at 260 and 280 nm, at
concentrations of 1 mg mL$^{-1}$ extinction coefficients are accepted as 57 at 260 nm and 1 at 280 nm. The difference in extinction coefficients between DNA and protein at 280 nm, is a source of intrinsic error as a significant amount of protein can be hidden in a DNA preparation. $^{13}$

Fluorescent dyes such as Hoechst 33258 and Pico Green, are relatively selective to double stranded DNA and do not bind protein. Fluorescent dyes or probes bind to specific regions of the DNA, when bound, a photon of energy from an external source such as a laser or lamp is absorbed by the fluorophore and raises it to an excited state. In the excited state the fluorophore undergoes a conformational change, which helps to partially dissipate the energy in the higher energy state and allows secondary processes to occur such as collisional quenching, fluorescence resonance energy transfer and intersystem crossing. The excited state lasts for 1 – 10 nanoseconds, after which a photon of lower energy at a longer wavelength to that used to excite the fluorophore may be emitted. The difference in wavelength of the exciting and emitted photon, the Stokes Shift, generates the sensitivity of fluorescence analysis, as it allows emitted photons to be detected against low backgrounds. Fluorescence spectroscopy has good sensitivity and specificity however, the techniques relies strongly on light scatter, suffers from quenching of fluorescence by competing processes and the technique is affected greatly by physical factors such as temperature, pH, ionic strength and viscosity.

Differences in DNA quantification by spectroscopic and fluorescent methods were investigated by Holden et. al. $^{13}$ and English et. al. $^{14}$. The study by Holden et. al. $^{13}$ compared two methods for the quantification of DNA; UV absorbance and florescent (PicoGreen) analysis, whilst English et. al. compared four quantitative methods, two UV and two fluorescent techniques against each other and to ICP-OES. Holden et. al. $^{13}$ investigated the variation in results for the quantification of DNA extracted from plant tissue and found that quantification based on UV absorbance had good correlation to the quantification based on $^{31}$P content. $^{15}$ However, quantification based on fluorescence using PicoGreen did not agree with the P content, results from the fluorescent method were consistently lower than those from UV measurements. $^{15}$ This finding was supported by the results of English et. al. $^{14}$ which showed that PicoGreen gave a lower DNA concentration compared to the expected value. English et. al. $^{14}$ compared four different quantification methods, two UV absorbance methods and two different fluorescent probe techniques. Findings from each of the four
techniques were compared against each other and from an independent ICP-OES method. UV absorbance measurements were carried out using a conventional UV spectrometer and a NanoDrop spectrometer; the fluorescence dyes used were PicoGreen and Quant-iT. Results showed that PicoGreen gave the lowest DNA value compared to the value given by ICP-OES, due to the fact it only measured doubled stranded DNA, a limitation also noted by Holden et. al. Whilst the three remaining techniques reported higher values, by up to 33% more than the result reported by ICP-OES. Higher readings from spectroscopic methods were due to interference from non DNA molecules absorbing at 260 nm, which resulted in values of 28 and 33% higher than those obtained by ICP-OES. The results from the Quant-iT fluorescence dye gave best precision, but the uncertainty of the method was found to be large.

However, technological advances have been able to overcome the limitations of large sample volume; for example, the palmtop spectrometer as described by Qiu et. al, the NanoDrop spectrometer by ThermoScientific Inc., Wilmington, USA, the Pico100 and 200, (PicoDrop, United Kingdom) and highly automated systems such as the DopSense96 by Trinean, Belgium. Qiu et. al. developed a palmtop spectrometer that utilised a fibre transmission and a fibre reflection absorbance detection system, which was illuminated by either a UV-LED or deuterium lamp. The palmtop spectrometer achieved a sensitivity of 0.5 ng/µL and a wide measuring range of 0.5-2000 ng/µL for DNA, with sample consumption of only 1 µL for fibre reflection and 0.5 µL for fibre transmission detection.

The need for alternative and accurate methods for DNA quantification, lead to an increase in studies conducted using elemental mass spectrometry utilising the stoichiometric amount of $^{31}$P in DNA. The advantages of using elemental mass spectrometry to quantify DNA are listed below:

1. Specificity to $^{31}$P
2. Compound and matrix independent sensitivity
3. High elemental sensitivity
4. Robust
5. Ability to couple easily to liquid chromatography, capillary electrophoresis, gel electrophoresis and gas chromatography.
These aspects of elemental mass spectrometry were utilised by several groups to quantify DNA and to develop methods which could be used to develop reference materials. Whilst the traditional methods for DNA quantitation, such as UV absorbance and fluorescence detection are most applicable for routine analysis, these methods still need to be validated with standard reference materials. The generation of standard reference materials requires more accurate analytical techniques and must be traceable back to the International System of Units (SI Units). Several elemental mass spectrometry approaches have been investigated, including acid digestion of whole DNA to inorganic $^{31}$P, enzymatic digestion of DNA to single nucleotides and direct analysis of oligomers. To accomplish this, various separation techniques have been coupled to ICP spectrometers including gel electrophoresis, capillary electrophoresis and liquid chromatography. Whilst elemental mass spectrometry has many advantages for the quantification of DNA, one of the major disadvantages of detection by ICP-MS is that phosphorus has only one isotope, therefore isotope ratio analysis cannot be performed.

Relatively few reports have been published using gel-electrophoresis coupled to ICP-MS for the quantitation of DNA, possibly due to the fact that many separations require highly salted buffers often incompatible with ICP analysis. However, Brüchert and Bettmer $^{18}$ developed a quantification method for whole DNA by gel electrophoresis coupled to sector field ICP-MS. The use of whole DNA overcomes the problems of incomplete digestion of DNA to nucleotides. The elution of whole DNA from the gel is isocratic therefore there is no change in sensitivity to $^{31}$P therefore no internal standard was used to monitor baseline drift. Two different calibration methods were employed; the first used the commercially available QuantLadder standards and the second used the total phosphorus content from acidic digestions of whole DNA at equivalent concentrations to the QuantLadder standards analysed by flow injection ICP-MS (FI-ICP-MS). $^{18}$ The method was successful for the quantification of DNA fragments from 100-700 base-pairs (bp); however, larger fragments suffered from band broadening which prevented baseline separation from being achieved. $^{18}$ Additionally the method was unable to separate smaller DNA fragments from inorganic phosphates making quantitation of DNA fragments of less than 100 bp impossible. Good agreement in DNA concentration was reached between the manufacturer’s values and those for small fragments (100-300 bp) but a significant difference was noted for fragments of higher molecular masses. $^{18}$ The poor correlation was explained by degradation of the sample prior
to analysis; however, it is more likely that sample was lost either on the gel, through nebulisation or ionisation.

Whilst the coupling of capillary electrophoresis to ICP-MS/OES can prove to be difficult, coupling should allow a stable electrical connection to the outlet end of the capillary, prevent a laminar flow being introduced into the capillary and provide high sample transfer. 19 Electrical connections are achieved by the use of liquid junctions or metallic conductors. 19 Additionally the use of specialised nebulisers are required. The most commonly employed nebulisers in the coupling of capillary electrophoresis to ICP-MS are the micro-concentric nebulisers (MCNs), ultrasonic nebulisers and more recently the direct injection high efficiency nebulisers (DIHEN). These smaller volume nebulisers offer higher sample transfer efficiency whilst preventing the introduction of a laminar flow in the capillary, compromising the efficiency of the capillary separation which can occur when pneumatic nebulisers are employed. 19

The determination of $^{31}$P in deoxyribonucleotides has also been investigated by coupling capillary electrophoresis to ICP-MS, as reported by Pröfrock et al. 20, Yeh et. al. 21 and Fuji et. al. 22, 23. Profrock et. al. 20 coupled capillary electrophoresis (CE) and HPLC to an Octopole reaction system (ORS-ICP-MS). To overcome the problem of the negatively charged nucleotides moving in the opposite direction to the positively charged capillary outlet, a highly acidic mobile phase was used and coupling to the ICP-MS was achieved via a sheathed Teflon tube direct to the torch. 20 Results showed that the detection limits for coupling HPLC to ORS-ICP-MS were improved by an order of magnitude compared to those for CE-ORS-ICP-MS. Detection limits for $^{31}$P of 53 $\mu$g/L, equating to 0.6 pg $^{31}$P absolute, were achieved by CE-ICP-MS, whilst detection limits of 3 $\mu$g/L, giving 0.03 ng $^{31}$P absolute, were achieved with HPLC-ICP-MS. All four nucleotides from calf thymus DNA were identified, however not all sample peaks were identified using standards, positive identification being achieved with ESI-MS. The P content of the DNA was quantified using flow injection analysis (FIA) however, this technique was deemed to be in need of further optimisation to improve accuracy.
Fujii et al. 23 published a study describing the quantification of deoxynucleotides using the 31P signal by ICP-MS. Whilst the study by Profrock et al. 20 utilized a more commercially available coupling, Fujii et al. 23 developed a novel glass interface. The interface was developed to decrease the volume of sample needed to provide accurate quantification. The interface described improved robustness preventing choking and capillary breakages 23 and consisted of a custom made nebuliser (i.d. 200 µm) and a low-volume vaporising chamber, again custom-made. A polymer based fitting was used to connect the nebuliser to the nebuliser capillary (50 µm for HPLC coupling and 75 µm for CE coupling). 23 The set-up described was used for the separation of deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxycytosine monophosphate (dCMP) and deoxythymidine monophosphate (dTMP). Detection limits were reported as 0.77-6.5 ng/mL for dNMP’s by CE-ICP-MS and 4.0-6.5 ng/mL by μHPLC-ICP-MS. The absolute detection limit for phosphorus was reported as 0.012 pg for both the μHPLC-ICP-MS and the CE-ICP-MS experiments, 23 demonstrating the flexibility of the novel sample introduction system. Fujii et al. 23 reported better analytical results than previously reported for phosphorus within nucleotides, with detection limits two orders of magnitude lower with CE-ICP-MS.

Fujii et al. 22 later utilised the interface described above for the quantification of 31P in enzymatically digested DNA. All 4 dNMP’s were identified in the electropherograms as well as free phosphate and an unknown peak, 82Se was used as an internal standard and 133Cs to monitor the sheath liquid flow. 22 To establish the accuracy of the method, the DNA concentration was determined by fluorescence also, the average DNA concentration by CE-ICP-MS was reported as 28.6 µg/mL and by fluorescence 24.5 µg/mL, detection limits achieved by CE-ICP-MS were equal to that obtained by conventional fluorescence assay, which ranged from 3.1 – 26 ng/mL. 22

Yeh et al. 21 utilised a modified micro-concentric nebuliser to couple capillary electrophoresis to ICP-MS for the study of monophosphate nucleotides, AMP, GMP, uridine 5’-monophosphate (UMP) and inosine 5’-monophosphate (IMP). Detection limits achieved were in the range of 0.036-0.054 µg/mL for 31P, equivalent to 1.1-1.6 pg 31P.
The lack of suitable accurate quantitation standards for DNA measurements was tackled by Yang et al. The report concluded that the method developed for the quantitation of oligonucleotides could be applied to produce commercially available quantitation standards for DNA. The study highlighted the need for a metrologically sound quantification method for DNA. Their approach for the absolute quantification of DNA was based on the stoichiometric amount of P in the phosphodiester backbone of DNA. Oligomers underwent acid digestion to inorganic P and were measured by ICP-OES with yttrium as an internal standard. Moles of inorganic P were converted to moles of oligonucleotide and extremely reproducible results were obtained. The study outlined the problems which hinder the absolute quantification of DNA; complete recovery of P from acid digestion, removal of all P interfering components from DNA prior to analysis and the molecular purity of the oligonucleotide analyte. To confirm the molecular purity of the oligomer the group analysed the sample by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) with standard additions and to check the recovery of inorganic P from acid digestion of deoxymethyl thymidine (d'TMP). The concentration of the oligomer was accurately measured by ICP-OES and compared to the value obtained by UV analysis. The results of their studies indicated that an oligonucleotide could be certified and used as a reference material with uncertainties of less than 1%.

Quantification of DNA by liquid chromatography coupled to ICP-MS has been reported in numerous studies including one by Liang et al., who separated all 4 deoxyribonucleic acids by high performance liquid chromatography using ammonium acetate as a mobile phase. Limits of detection were reported as ranging from 0.173 – 0.225 µM and absolute $^{31}$P limits of 3.1 – 4.5 pM. The hyphenated technique was applied to the quantitation of enzymatically digested plasmid DNA, however this method was not validated against a reference material despite good correlation of the retention times from the digested DNA to the nucleotide standards.

Donald et al. reported an excellent study on the analysis of a 20 mer oligonucleotide by the coupling of liquid chromatography to molecular mass spectrometry and quantification by ICP-OES this work followed up their previous study on the quantitation of an oligomer by
LC-MS/MS with isotope dilution analysis (IDA) \(^{27}\) which was submitted as a proof of concept. The use of labelled nucleotide standards allowed the quantification of an oligonucleotide by enzymatic digestion into monomers and quantitation by LC-MS/MS-IDA, without the need for a pure oligonucleotide standard. \(^{27}\) However, they found that this method did not account for the end terminal nucleotide where the phosphate group was replaced with a hydroxyl group during the digest procedure, in their subsequent study a different enzyme was used to produce nucleosides. The approach taken was very much focused on quantitation by IDA by LC-MS/MS with confirmation of quantitation by ICP-OES and recovery of nucleotides and nucleosides by two different enzymatic digestion protocols. The most preferred enzyme digestion protocol involved digestion first to nucleotides with snake venom phosphodiesterase (SVP) followed by digestion to nucleosides with shrimp alkaline phosphatase (SAP), as this bypassed the problem of the terminal phosphate being converted to a hydroxyl during the digest with SVP only. \(^{26}\) By IDMS and confirmation by ICP-OES, they found good agreement with the oligomer concentration of 108 and 109 µg/g with good uncertainties at the 95% confidence levels.

The majority of work on \(^{31}\)P detection in DNA using ICP-MS has been in the detection of DNA adducts with the anti-cancer drugs cis-platin and melphalan and with the possible cancer causing adduct formation with styrene oxide. Edler \textit{et al.} \(^{28}\) utilised ICP-MS to quantify the formation of styrene oxide DNA adducts using bis (4-nitro phenyl) hydrogen phosphate (BNPP) as an internal standard. \(^{28}\) The group’s work was based on the earlier work by Siethoff \textit{et al.} \(^{29}\) with the improvement to that work being achieved by the use of hexapole and reaction cell ICP-MS. The advantages of using this technique compared to the use of a sector field ICP-MS is that spectral and non-spectral interferences were reduced and so direct quantification of the modified nucleotides could be achieved without any mathematical corrections for signal intensities when using gradient elution. \(^{28}\) Sar \textit{et al.} \(^{30}\) employed LC-ICP-MS for the speciation of both Pt and P in DNA adducts with cis-platin with structural characterisation by ESI-MS. Quantification of the 5 ’-monophosphates and adducts was obtained via the construction of a calibration curve with good correlation between the concentration of dGMP and \(^{31}\)P Peak area \((r^2 =0.999)\). \(^{30}\) It was demonstrated that they were able to separate the \(^{31}\)P containing nucleobases from the dGMP adduct using a narrow bore
column and noted that when using low flow rates (0.2 ml/min) very low backgrounds of polyatomic inferences were observed.  

Huang, Xi *et al.* 31 published a paper examining the efficiency and effectiveness of several enzymes and combinations of enzymes for DNA digestion. The study found that using DNase I at the beginning and phosphodiesterases (PDE) I and II the end of the DNA digestion processes, 8-oxo-dG can be much more efficiently released from DNA than when Nuclease P1 (NP1) and alkaline phosphatase (AP) are used. Their results showed that their modified DNA digestion conditions can significantly improve accuracy and reproducibility of their analyses.

Brennan *et. al.* 32 have recently published a paper for the quantification of DNA using high performance ICP-OES to detect phosphorus in acid digested nucleotides and DNA. The group employed a DIHEN which was used to reduce the volume of sample consumed during the analysis and to improve the robustness of the plasma. The use of this nebuliser decreased the sample uptake rate from 170 to 30 μL/min and the volume used from 10 to 2.4 mL. 32 The increased sensitivity observed with the DIHEN also meant that the mass of DNA required to perform the analysis could also be reduced from 300 μg for a 3 μg/g solution (P) to 12 μg for a 0.5 μg/g solution (P). 32 The phosphorus measurement was made using a germanium internal standard, this corrected for low-frequency noise, and to correct for any difference between the calibration standard and the sample. Brennan *et. al.* 32 reported a 4 fold increase in P sensitivity at 213.617 nm, allowing for a decrease in the P mass fraction (from 3 to 0.5 μg/g) analysed and the mass of DNA consumed. 32

Interest in accurately quantifying the amount of DNA in biological samples continues, with recent studies investigating the use of both MS and nuclear magnetic resonance (NMR) for this purpose. 15 To date the application of ICP-MS to the quantification of DNA has not been investigated to such an extent as the quantification of phosphoproteins. 18 The lack of a
suitable certified quantitative DNA reference material has contributed to the lack of research into this field; however several attempts have been made.

2.3 PHOSPHOPEPTIDE ANALYSIS

The phosphorylation or dephosphorylation of proteins, enzymes and receptors acts as a switch to “turn on” or “off” their activity by inducing a conformational change in the structure of these macromolecules. The phosphorylation process is controlled by the opposing action of two enzymes, kinase enzymes control phosphorylation and phosphatases control dephosphorylation. Only three amino acids undergo phosphorylation within the macromolecule, these being tyrosine, threonine and serine, as these amino acid residues contain a hydroxyl group. The addition of a phosphate group (PO$_4$) to the hydroxyl of one of these residues within a hydrophobic region can turn that region into a polar, extremely hydrophilic region, introducing a conformational change in the structure of the protein due to changes in the interactions with other hydrophobic and hydrophilic residues within the protein. The determination of phosphorylation sites within a protein of a given cell is extremely complicated as there are thousands of different proteins within a cell and phosphorylation may occur at more than one site on any given protein.

The analysis of phosphopeptides by mass spectrometry faces many challenges; there are several reasons for this.$^{34-38}$

- Phosphorylated proteins are found at very low concentrations in the body.$^{39-41}$
- The sub-stoichiometric levels at which phosphorylation takes place results in only a small portion of the actual protein under investigation being phosphorylated.$^{39-41}$
- The complexity of the phosphorylation and de-phosphorylation processes make analysis difficult, a single protein can be acted on by various kinases and phosphatases at different sites and at different times.$^{34,35,42}$
- During analysis, phosphorylated proteins may become de-phosphorylated unless precautions are taken to inhibit the action of the phosphatase enzymes. 36
- Post-translation modification bonds tend to be more labile than peptide amide bonds, and therefore become the preferred site of fragmentation, which can affect peptide identification by impeding efficient backbone fragmentation. 43

To improve selectivity, sensitivity and in some cases quantification, phosphopeptides and phosphoproteins are often labelled prior to analysis. Quantification strategies involve the inclusion of isotopic labelled agents, whilst sensitivity and selectivity can be improved by the inclusion of a metal labelled tag.

2.3.1 PHOSPHOPEPTIDE TAGGING STRATEGIES

Gruhler et. al. 43 incorporated both an enrichment procedure, namely immobilised metal affinity chromatography (IMAC) with a labelling strategy, and stable isotope labelling with amino acids in cell culture (SILAC), see Figure 4. The approach taken by Gruhler et. al. 43 was used to quantify phosphopeptides involved in the Yeast pheromone signalling pathway. SILAC encoding involved the incorporation of $[^{13}\text{C}_6]$ arginine and $[^{13}\text{C}_6]$ lysine in a double auxotroph yeast strain. To act as a control, pheromone-treated yeast cells were mixed with SILAC encoded cells, and after replication the cells were lysed and extracted proteins were digested with trypsin. Phosphopeptides were then enriched with a combination of strong cation exchange (SCX) and IMAC. A linear ion-trap Fourier transform ion cyclotron resonance mass spectrometer was used to analyse the phosphopeptide fractions by LC-MS. 43

More than 700 phosphopeptides were identified, 139 were identified as being up-regulated in response to mating pheromone by over 2-fold. The efficiency of the IMAC enrichment varied with the complexity of the sample. More complex samples gave poorer recovery than simple samples; therefore sub-fractionation was required prior to IMAC. Despite the large number of phosphopeptides identified, complete coverage was not achieved due to inefficiency in protein extraction, digestion and uneven sampling of peptides for fragmentation by mass spectrometry. 43
Cantin *et al.* reported that they combined SILAC with SCX and IMAC in a multidimensional protein identification tool, coined MudPIT to identify the epidermal growth factor (EGF) pathway in mammalian cells. Using this approach the group were able to identify 4470 unique phosphopeptides with 4729 phosphorylation sites. Light SILAC HeLa cell cultures were treated with EGF, whilst heavy HeLa cell cultures were left untreated. The cells were lysed, proteins extracted by dialysis, digested to peptides with trypsin and phosphopeptides were enriched by IMAC spin columns. Prior to analysis by MS and MS$^n$ on a Thermo LTQ Orbitrap, the phosphopeptides were separated by SCX and reverse phase liquid chromatography. Of the 4470 unique phosphopeptides found, 37% were quantified by Census software and of those 15% were found to be either up-regulated or down-regulated by 2-fold or more by the addition of EGF.
Weber et al. 46 used SILAC based MS to determine over 10 thousand distinct phosphorylation sites in the phosphoproteome of KG1 acute myeloid leukemia (AML) and the effect of treatment with two drugs; erlotinib and gefitinib. Less than 50 phosphorylation sites were identified as varying with drug treatment, which indicated specific interference with AML cell signalling. Of the changes observed a significant amount were associated with tyrosine phosphorylated protein network, including the Scr family kinases and the tyrosine kinases btk and Syk. 46

The isobaric tagging for relative and absolute quantitation (iTRAQ) technique employed by Wu et al. 47 in combination with high energy collision dissociation (HCD) in LTQ-Orbitrap allows the simultaneous identification and quantification of iTRAQ labelled phosphopeptides due to the combination of high mass accuracy MS/MS spectra and wide m/z scan range. An iTRAQ labelling workflow begins with the N-terminus of peptides being covalently bound to tags of varying mass. The labelled peptides are then pooled and analysed by mass spectrometry, see Figure 5.

Figure 5: iTRAQ labelling workflow with mass spectroscopy detection.
Sachon et al. \(^{48}\) applied a commercially available iTRAQ reagent to quantify phosphopeptides following in gel trypsin digests of proteins and phosphoproteins. Phosphopeptides were analysed by MALDI-MS after excess reagent was removed and phosphopeptides enriched by Fe(III)-IMAC. Abundance was determined by LC-MS/MS by either MALDI-MS or ESI-MS.

### 2.3.2 PHOSPHOPEPTIDE ENRICHMENT STRATEGIES

Wang et al. \(^{49}\) described a simple and effective immune-dot blot method for the detection of phosphopeptides by mass spectrometry. Initially three dot-blot immunoassays typically used for the detection of non-phosphorylated peptides were undertaken and tested with phosphopeptide standards. The first method tested utilised a pre-soaked polyvinylidene fluoride membrane (PVDF) in phosphate saline. \(^{49}\) The wet membrane was spotted with standard phosphopeptides and left to dry. In the second method phosphopeptide standards were directly spotted onto nitrocellulose membranes. \(^{49}\) In the final method, nitrocellulose membranes were treated with 1% glutaraldehyde for 10 minutes, prior to being spotted with the phosphopeptide standards. \(^{49}\) All three blotted membranes were probed with anti-phosphoserine antibody; of the three methods tested the membrane treated with glutaraldehyde gave the best signal to noise ratio by detection with tandem mass spectrometry. It was concluded that the greater retention was due to cross-linking between the peptide and the divalent cross-linker glutaraldehyde. \(^{49}\)

The limit of detection was determined for the glutaraldehyde method as 10 pmol of phosphopeptide with an anti-phosphoserine probe. To test the specificity for the anti-phosphoserine antibody for phosphopeptides, the phosphopeptides were treated with a phosphatase to remove the phosphate group. \(^{49}\) Three probes were tested, anti-phosphoserine, anti-phosphotyrosine and anti-phosphothreonine, all three gave positive responses for phosphopeptides and negative responses for phosphopeptides treated with phosphatase. The glutaraldehyde method developed was also tested with real biological samples. The biological samples tested were separated by gel electrophoresis, stained, excised and tryptically digested. Phosphopeptides from the digests were enriched using titanium dioxide affinity
chromatography. The results showed a positive result for the peptides eluted from the TiO$_2$ column, a negative result for the flow through from the TiO$_2$ column, and a much weaker response from the peptides not enriched by TiO$_2$.\textsuperscript{49} Eluted peptides were analysed by LTQ-Orbitrap mass spectrometer.

\textit{Niklew et. al.}\textsuperscript{50} also employed titanium dioxide as an enrichment material but as a nanocrystalline film rather than as support material. These films were used as Affinity MALDI Targets for the analysis of proteolytic digests of $\alpha$ and $\beta$–casein.\textsuperscript{50} The titanium dioxide films were synthesised via the sol-gel route for producing mesoporous film, these films have an increased internal surface area compared to a flat surface of the same diameter, increasing the available binding sites for phosphopeptides.\textsuperscript{50} $\beta$-casein was digested using trypsin, and the digest was applied to the film and incubated for up to 45 minutes. On application of the digest to the film a colour change was observed indicating that the matrix molecules had bound to the Ti metal centres.\textsuperscript{50} In order to release the phosphopeptides from the metal, the film was washed with NH$_4$H$_2$PO$_4$, the inorganic phosphate displaces all other ligands from the Ti metal centres. Three major peaks were observed corresponding to the phosphopeptides expected from $\beta$-casein, to further prove the effectiveness of the enrichment procedure, the bound phosphopeptides were treated with phosphatase prior to desorption of the phosphopeptides, in order to improve the ionisation efficiency of the phosphopeptides in positive mode.\textsuperscript{50}

\textit{Choi et. al.}\textsuperscript{51} used both magnetic iron oxide particles (Fe$_3$O$_4$) and titanium dioxide (TiO$_2$) particles to selective enrich phosphopeptides from bovine $\alpha$-casein. The enriched phosphopeptides were eluted from the metal oxide particles and combined, the resulting mixture was then separated and analysed by LC-MS/MS. The results from the sequential enrichment process support the findings of others that Fe$_3$O$_4$ particles retain more multi-phosphorylated peptides than TiO$_2$ particles but are not as good as TiO$_2$ particles in retaining mono-phosphorylated peptides.\textsuperscript{51}
Tang et. al. 52 developed mesoporous TiO₂ microspheres for the enrichment of phosphopeptides. The microspheres synthesised by a simple hydrothermal reaction had a higher surface area to volume ratio than commercially available TiO₂ microspheres. 52 Compared to smooth TiO₂ microspheres, mesoporous TiO₂ microspheres have a surface area of approximately 25 times larger. They were successfully applied to the analysis of casein phosphopeptides and to a real biological sample obtained from rat brain. Phosphopeptides from a tryptic digest of β-casein were enriched using both smooth and mesoporous TiO₂, and the results for both types of material were similar. The experiment was then repeated with a mixture containing tryptic digests of both bovine serum albumin (BSA) and β-casein in a 1:1 and a 10:1 ratio. 52 With a constant concentration of β-casein and in identical experimental conditions, the mesoporous TiO₂ microspheres performed better than the smooth microspheres, in that the signals corresponding to the phosphopeptides were enhanced compared to those from unphosphorylated peptides in the mass spectra resulting from the enrichment by mesoporous microspheres. 52 It was concluded that the mesoporous TiO₂ microspheres have a much larger binding capacity for phosphate groups as well as much higher capture efficiency for phosphopeptides than smooth TiO₂ microspheres. 52

Zhao et. al. 53 reported an automated metal-free multiple column nanoLC method for high throughput analysis of phosphopeptides with MS detection. The method used a combination of online solid phase extraction columns and a capillary C₁₈ column connected via an automatic switching valve. A limit of detection of 0.4 fmol was obtained using a linear ion trap tandem mass spectrometer. 53

Leitner et. al. 54 reported on the use of tin dioxide as an enrichment material for phosphopeptides. Tin dioxide (SnO₂) microspheres were produced by a nanocasting technique using silica of different morphology to give microspheres that varied in their particle size and porosity. 54 The nanocasting process used, utilised porous silica spheres as a template for the generation of metal oxide replicas in the pores of the silica material. 54 The SnO₂ particles were produced by impregnating the SiO₂ particles with SnCl₂ solution, heated in air to decompose the salt to SnO₂, silica was then removed by leaching with HF or NaOH.
HCl was used for activation of SnO$_2$ microspheres, the SnO$_2$ material was refluxed overnight with HCl, collected by filtration, washed with water and dried in vacuo. $^{54}$

The efficiency of the enrichment process was analysed using LC-MS, higher specificity for phosphopeptides was observed when NaOH was used for leaching compared to HF. It was also observed that when dilute HCl was used for activation the microspheres produced had increased performance. $^{54}$ To confirm these early conclusions, microspheres were produced from a single starting material, one half of the starting material was treated with HF and the other half treated with NaOH. These two portions were then split in half again to give four portions, one half of the silica treated with HF was treated with dilute HCl, and one half of the silica treated with NaOH was treated with dilute HCl. $^{54}$ From the data obtained from LC-MS, the amount of unspecific binding was lowest for the SnO$_2$ microspheres produced by treatment of silica with NaOH and HCl. One of the synthesised peptides used in the study, Glu-fibrinopeptide B, exhibited the strongest unspecific binding due to its acidic nature. 37% and 14% of the total amount of peptides was bound to SnO$_2$ microspheres treated with HF and NaOH respectively, whilst those treated with HCl had only 15% and 9% bound to SnO$_2$ microspheres, leached with HF and NaOH respectively. $^{54}$

2.3.3 PHOSPHOPEPTIDE ANALYSIS BY MASS SPECTROMETRY

McLachlin and Chait $^{55}$ described the improvements in mass spectrometry technology and the benefits these improvements made to the analysis in phosphopeptide and phosphorylation site identification. In the crudest form Mass Spectrometry can accurately predict the molecular mass of the phosphorylated protein, comparison with the molecular mass of the un-modified phosphoprotein or the phosphoprotein treated with phosphatase can give the average number of phosphorylation sites. $^{55}$
2.3.4 PHOSPHOPEPTIDE ANALYSIS BY ICP-MS

Navaza et. al. investigated peptide dephosphorylation by coupling capillary HPLC (capLC) to ICP-MS, the lower flow rates allowed the use of higher percentages of organic modifier than would normally be tolerated by the ICP-MS. The group also utilised a post-column sheath flow to compensate the gradient conditions used during the capLC separation as gradient flows affect the sensitivity of the ICP-MS signal. To quantify the amount of phosphopeptides present, bis(4-nitro-phenyl) phosphate (BNPP) was spiked into each sample to act as a P-standard. The sensitivity factor for BNPP was calculated and used to quantify the amount of each phosphopeptide. Model phosphopeptide solutions were prepared and treated with phosphatase enzymes and analysed at different time periods.

The technique employed allowed even small differences in phosphorylation degrees to be detected, due to the high precision and accuracy associated with the absolute phosphopeptide and phosphorylation degree measurements, reported as 0.6 - 4.1% RSD and from 1.5 - 4.3% RSD respectively. The absolute quantitative data for one of the model phosphopeptides at each time point was calculated as 0.2 – 2.4% RSD over the dephosphorylation process. This approach developed by Navasa et.al. allows phosphorylation dynamics to be studied; its ability to quantify the absolute amount of each separated phosphopeptide at any given time may have significance for clinical applications.

CapLC-ICP-MS was also used by Ellis et. al. to study protein phosphorylation in low molecular weight cerebral spinal fluid fractions. Detection limits for $^{31}$P by capLC-ICP-MS were 5µg/L for the synthetic peptide Pp60 c-src and 10µg/L for P60 c-src, using a 1µL injection. Following $^{31}$P screening by capLC-ICP-MS fractions were collected off-line and analysed by nano-LC chip ion trap mass spectrometry to provide confirmation of protein and peptide molecular weight and sequence. The paper showed that the sensitivity achieved with capLC-ICP-MS in combination with nanoLC-CHIP/ITMS is a very powerful technique for characterising phosphopeptides in complex matrices such as cerebral spinal fluid.
Bettmer et al.\textsuperscript{58} have recently published a review outlining the role of ICP-MS in proteomic analysis. The review states the reasons for using ICP-MS for proteomic studies, including:

1. Its specificity for the heteroatom
2. Non-species dependent detection sensitivity
3. Direct isotope information, allowing quantification by IDMS and metabolic studies
4. Robust sample preparation
5. Variability in sample introduction.\textsuperscript{58}

The use of ICP-MS for the fast screening of complex peptide mixtures for phosphopeptides was also outlined, the specificity of the ICP-MS allows the exact elution time for the phosphopeptides to be found, therefore allowing fraction collection and subsequent identification via ESI-MS.\textsuperscript{58} This decreases the complexity of the sample for molecular spectrometry, which is often a barrier to analysis of phosphopeptides by this technique.

### 2.3.5 PHOSPHOPEPTIDE QUANTIFICATION

Wu et al.\textsuperscript{47} produced a comprehensive study of HeLa cell lysates, in which they identified 3557 distinct phosphopeptides and were able to quantify 2709 of those from 5 mg of the HeLa cell lysate. Separation of the phosphopeptides from the lysate was achieved by trapping the phosphopeptides on a titania packed column, labelling with iTRAQ reagents and fractionation using a SCX column. Identification and relative quantification was achieved by high-energy collision dissociation (HCD) and using the iTRAQ reporter ions.\textsuperscript{47}

Wu et al.\textsuperscript{47} compared two different enrichment strategies. In the first method fractionation was achieved by strong cation exchange at pH 2.7, the resulting fractions were then incubated with titania beads\textsuperscript{47} and analysed by LC-MS/MS on an Orbitrap instrument.\textsuperscript{47} The second method used a titania bead packed column to enrich the phosphopeptides from the unfractionated cell lysate digest. Phosphopeptides were eluted from the column in ammonia solution and fractionated using a strong cation exchange column, the resulting fractions were analysed by an Orbitrap instrument using the same conditions as in the first method.\textsuperscript{47}
The first method using the SCX-TiO$_2$ protocol, repeated in parallel, identified 3106 and 2838 distinct peptides, with a false discovery rate of $\sim$2.0%\textsuperscript{47}, the second method using the TiO$_2$-SCX protocol identified 4845 and 4340 distinct peptides under the same conditions. It was reported that on average the TiO$_2$-SCX protocol led to the identification of 54.5% more phosphopeptides than the traditional method of SCX-TiO$_2$ enrichment.\textsuperscript{47} The overlap of identified peptides between method replicates was also investigated; the overlaps observed were relatively low, 58% and 51% for TiO$_2$-SCX and SCX-TiO$_2$ workflows respectively.\textsuperscript{47} It was concluded that the error came from irreproducible sampling of the mass spectrometer in data-dependent scan mode as an overlap of 60% was observed when running two replicate LC-MS/MS analyses of the same TiO$_2$-SCX sample.\textsuperscript{47}

Metal oxide affinity (MOA) chromatography for the enrichment of phosphopeptides was reviewed by Leitner.\textsuperscript{59} In the review Leitner reported on improvements in technology that have aided the advancements in phosphopeptide analysis, strategies to improve enrichment protocols and significant advancements in characterising phosphopeptides using MOA materials.\textsuperscript{59} Improvements in MS technologies have increased the number of phosphorylation sites characterised. Techniques such as electron-capture dissociation (ECD) and electron-transfer dissociation (ETD) allowed simpler mass spectra to be obtained, as fragmentation can be achieved without the neutral loss of phosphoric acid.

The use of helium as a collision gas was first applied to studies of DNA but has been expanded to the analysis of phosphorus in phosphorylated proteins.\textsuperscript{20, 60, 61} Profrock \textit{et al.}\textsuperscript{20} found that using helium as a collision gas and “kinetic energy discrimination” achieved by the octopole and quadrupole bias settings, decreased background at the $^{31}\text{P}$ mass by approximately 2 fold.\textsuperscript{20} In standard H$_2$ mode with standard cell conditions background equivalent concentration (BEC) could not be achieved but by switching to He mode a BEC of 4 µg/L was achieved. It is also possible to reduce the polyatomic background by reducing the amount of solvent that reaches the plasma\textsuperscript{60} and by introducing the liquid aerosol at a lower flow rate by using micro or capillary nebulisers.\textsuperscript{60} The polyatomic background is created by dissolved atmospheric gasses giving rise to $^{15}\text{N}^{16}\text{O}^+$, $^{14}\text{N}^{16}\text{O}^{1}\text{H}^+$, $^{14}\text{N}^{14}\text{N}^{1}\text{H}^+$, $^{14}\text{N}^{17}\text{O}^+$, $^{13}\text{C}^{18}\text{O}^+$, $^{12}\text{C}^{18}\text{O}^{1}\text{H}^+$ ions which overlap with the $^{31}\text{P}^+$ signal.
Leitner commented that one of the most powerful approaches to phosphopeptide analysis was that of metal oxide affinity capture (MOAC). The most popular theory explaining the binding mechanism of metal oxides to phosphate groups is by the bidentate binding between the phosphate anion and monosubstituted phosphates to the metal oxide surface. Metal oxides act as Lewis acids, interacting with the phosphate anion.

2.4 INTERACTION OF SMALL PEPTIDES WITH PLATINUM BASED ANTI-CANCER DRUGS

Out of the tens of platinum based anti-cancer drugs entered into clinical trials, only four are approved world-wide, cisplatin, carboplatin, oxaliplatin and nedaplatin. The barrier to effective treatment of cancer with platinum based drugs remains adverse side effects including nephrotoxicity, renotoxicity, nausea and vomiting, ototoxicity and peripheral neuropathy and the development of chemoresistance.

In the search for new platinum based anti-cancer drugs, the focus has changed from identifying the most cytotoxic to identifying those with sufficient cytotoxicity, fewer side effects and reduced chemoresistance. This has also been reflected in the increase in interest of both in-silico and in-vitro experiments where the interaction between cisplatin and intra and inter-cellular proteins, peptides, enzymes and phospholipids has been investigated. This line of enquiry is supported by the fact that less than 5% of the total drug administered reaches the target DNA in the nucleus, a similar situation occurs with other platinum based drugs such as oxaliplatin demonstrating that improvement of the inefficient transport of these drugs to the target is the key to improving their efficacy.

The transport and interactions of cisplatin and other platinum based drugs into and within cells are still not fully understood. However, based on previous work we can assume that the majority of the administered drug, once it has entered the cell, is found in the cell cytosol.
Prior to entering the cell it is estimated that anywhere between 60-95% of the drug is involved in quasi-reversible binding to human serum albumin (HSA). The most simplistic route from blood to nuclear DNA involves the passive diffusion of cisplatin into the cell from the extracellular fluid, where the Cl\(^-\) concentration is high (> 100 mM), into the cytoplasm, where the Cl\(^-\) concentration is low (4-20 mM). The low Cl\(^-\) concentration in the cytoplasm results in the solvation of cisplatin to its active forms \([\text{Pt}(\text{NH}_3)_2(\text{OH}_2)\text{Cl}]^+\) and \([\text{Pt}(\text{NH}_3)_2(\text{OH}_2)_2]^{2+}\) which bind to nuclear DNA forming crosslinks between the purine bases forming Pt-DNA adducts, see Figure 6. The solvation of cisplatin has been investigated both experimentally and theoretically indicating that the strong electrostatic interactions resulted in cisplatin being a strong donor and acceptor of hydrogen bonds and that when cisplatin interacts with water it behaves in an aliphatic manner, as it can form strong hydrogen bonds as both a donor and acceptor.
In its activated solvated form, cisplatin interacts with a plethora of non-DNA ligands; it is thought that the most likely sites for cisplatin interaction are the thiol groups of intracellular peptides and proteins; it is these interactions that are thought to be responsible for chemoresistance and many of the side effects.\textsuperscript{67, 68} It is widely thought that the inefficient transport of cisplatin to the nucleus is attributed to the two most abundant sulfur containing peptides in the cell cytosol, glutathione (GSH) and methionine, and it has been estimated that approximately 60\% of the total cellular platinum concentration is associated with GSH binding.\textsuperscript{69} Recent studies have shown that the activated form of oxaliplatin forms very stable complexes with glutathione (GSH).\textsuperscript{68, 70}

**Figure 6:** A) The solvation of cisplatin in the cell, B) the interaction of cisplatin with GG bases and AG bases

Cisplatin interacts with N7 of guanine and N7 of adenine (not shown)

Cisplatin forms interstrand crosslinks with guanine

Cisplatin forms interstrand crosslinks with guanine and adenine
Despite a large catalogue of evidence to suggest that cisplatin binds selectively to sulfur containing peptides within the cell cytosol, there has been very little investigation into the mechanisms by which this occurs and whether these complexes are energetically favourable. Whether glutathione plays a key role in increased sensitivity to cisplatin is still in debate with early studies showing that cancer cell lines resistant to cisplatin showed elevated levels of GSH, suggesting that GSH plays a protective role against the genotoxic effect of cisplatin. Whilst other work has reported no correlation between the cellular levels of GSH and the cytotoxicity of platinum based drugs. One possible mode of action for the decrease
in cisplatin cytotoxicity due to increased GSH levels is the inhibition of reactive oxygen species (ROS) generation and mitogen activated protein kinases (MAPK) activation, which are involved in cell apoptosis, proliferation, survival and differentiation. 76 Nephrotoxicity is believed to be caused by the production of glutathione S-conjugates with platinum, catalysed by gamma-glutamyl transpeptidase (GGT) and cysteine S-conjugate beta lyase. 77-79

The use of carnosine as an anti-cancer therapeutic agent has been discussed by Gaunitz and Hipkiss. 80 The discussion focussed on carnosine as an anti-neoplastic agent in the treatment of brain tumours. The study was conceived on the evidence that carnosine inhibited the growth of glioblastoma-derived cells; this fact prompted an alternative route of administration, intranasal, which avoided bio-transformation of carnosine by carnosinase in the plasma. 80 Also, the blood brain barrier is often damaged in patients suffering from glioblastomas and it is debated whether carnosine is able to penetrate the blood brain barrier through uptake by PepT2. 80 Despite no experimental data being presented, the argument that carnosine would be a potential drug for glioblastoma treatment was well presented and considered, side-effects, dosage and bio-availability were discussed in detail.

Iovine et. al. 81 presented a study which described the effect of carnosine on the growth of Kirsten rat sarcoma viral oncogene homolog (KRAS)-mediated hematocrit (HCT) 116 colorectal cancer cells. They found that the administered 50-100 mM of carnosine decreased the concentration of adenosine triphosphate (ATP) and ROS, and the KRAS-mediated colorectal cells increased the level of mitochondrial ROS, responsible for cell proliferation. Levels of ATP were measured by bioluminescence as carnosine levels were increased from 5 to 300 mM, the results showed a decrease by 50% in the level of ATP detected at 100 mM of added carnosine compared to the control, this result was representative of the results found by cell growth of the HCT116 cells. Cell proliferation measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) analysis, a colorimetric analysis, was found to decrease by 50% to 60% with 50 and 100 mM administered carnosine respectively. 81 The reduction in cell proliferation was concluded to be due to the inhibitory effect of carnosine on glycolysis, which is vital for tumour proliferation and survival. 81
Horii et al.\textsuperscript{82} conducted a similar study to Iovine et al.\textsuperscript{81}, in which Horii et al. investigated the effect of carnosine on splenic sympathetic nerve activity and tumour proliferation.\textsuperscript{82} They found that intraduodenal injections of 3.3 mg/Kg of body weight of carnosine into urethane-anaesthetised rats significantly decreased splenic sympathetic nerve activity; they also found that 1 mg/mL carnosine solution inhibited tumour proliferation in rats.\textsuperscript{82}
3 RESEARCH METHODOLOGY

3.1 INTRODUCTION

This chapter details the research methodology applied in this thesis. In this thesis research is defined as a structured enquiry that utilises acceptable scientific methodology to solve problems or generate new knowledge that adds to the existing body of knowledge. The scientific methodology employed was designed to ascertain and discover facts, by generating and finding evidence through experiments in the laboratory setting and reporting theories based upon the evidence generated to the wider scientific community.

3.2 OBJECTIVES OF RESEARCH

The main objective of research is to uncover answers to questions which were previously unknown through the application of scientific procedures. Although each research thesis has its own specific research objectives, the objectives can be generalised into four distinct categories:

1. To gain familiarity with a phenomenon or to achieve new insights into the phenomenon.
2. To test for causal relationships between variables.
3. To portray accurately the characteristics of a particular situation, individual or group.
4. To determine the frequency with which something occurs or with which it is associated with something else.
3.3 TYPES OF RESEARCH

There are many different ways to classify research, but by identifying the main characteristics of the different types of research it simplifies the classification. Table 2 outlines the classification of research types based upon the criteria outlined above. ⁸⁴

<table>
<thead>
<tr>
<th>Type of Research</th>
<th>Basis of Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploratory, descriptive, analytical or predictive research</td>
<td>Purpose of the research</td>
</tr>
<tr>
<td>Quantitative or qualitative research</td>
<td>Process of the research</td>
</tr>
<tr>
<td>Applied or basic research</td>
<td>Outcome of the research</td>
</tr>
<tr>
<td>Deductive or inductive research</td>
<td>Logic of the research</td>
</tr>
</tbody>
</table>

Table 2: Classification of the different types of research. ⁸⁴

As this thesis focuses on the outcome of the research, the type of research undertaken in this thesis can be classified as applied research and not basic research. The development of a new theory or law is classified as basic research, whereas applied research assesses the relevance of new or existing theories under certain circumstances. Applied research is also employed to find an immediate solution to problems being faced by society, an entity or organisation. The central aim of applied research is to identify and find a solution to a pressing practical problem, whereas basic research aims to find new knowledge or information that adds to the already existing body of scientific knowledge. ⁸³
3.4 SCIENCE METHODOLOGY

This section will describe scientific research methodology as a whole. Research methodology is defined as the investigation of research methods, determining which research methods are relevant and why.

Despite the controversial belief of a few notable scientists including the Nobel laureate, Sir Peter Medawar, that there is no such thing as ‘The Scientific Method’, to neglect the general principles of scientific methods can be extremely detrimental to the progression of research. The principles of the scientific method are not widely taught; Sir Peter Medawar commented that

“If the purpose of scientific methodology is to prescribe or expound a system of enquiry or even a code of practice for scientific behaviour, then scientists seem to be able to get along very well without it. Most scientists receive no tuition in scientific method, but those who have been instructed perform no better as scientists than those who have not.”

However, it is believed that understanding the principles of scientific method enhances scientific productivity and technological progress.
3.4.1 SCIENTIFIC RESEARCH METHODS

In this thesis scientific research method refers to all the methods/techniques used for conducting the research:

1. Methods used for selection of samples and collection of data.
2. Statistical techniques used for establishing relationships between relevant variables.
3. Methods used for evaluating the accuracy of the results obtained.

A classical scientific approach was employed in order to conduct the research; this process is outlined in Figure 7.

Figure 7: The classical linear science method. 87

3.4.2 FORMULATING THE RESEARCH PROBLEM

In order to formulate the research problem, the problem needs to be thoroughly understood and translated into a meaningful task or series of tasks from an analytical point of view. This was achieved by undertaking a thorough survey of the literature and the MSc short course ‘Atomic Spectroscopy’ was completed. Also during this initial stage a series of objectives were drawn up, these are summarised below:
1. Review current advances in analytical methods for the measurement of phosphorus-containing bio-molecules by inductively coupled plasma mass spectrometry (ICP-MS).

2. Develop capabilities for the accurate quantification of phosphorus at low ppb levels in biological matrices by ICP-MS in transient signal mode.

3. Investigate the performance of methods used for phosphorus quantification in biological matrices.

4. Develop a micro or capillary high performance liquid chromatography HPLC method for the separation of DNA nucleotides assisted by ultra-violet-visible (UV-VIS) detection.

5. Develop a HPLC-ICP-MS method for the accurate quantification of P-containing bio-molecules.

### 3.4.3 DEVELOPING WORKING HYPOTHESIS

The working hypothesis is formulated to draw out and test the logical or empirical consequences of the research. It serves to sharpen the understanding of the problem and focus attention to the important aspects of the problem. It also helps to identify the type of data required and which methods of data analysis are appropriate. An ideal hypothesis is extremely specific and limited to the research problem so that it can be readily tested. Three hypotheses were generated related to the three areas of enquiry. These were:

1. Accurate quantification of DNA can be achieved by the digestion of whole DNA into its individual nucleotides and analysed by LC-ICP-MS.

2. Quantification of a wide range of phosphopeptides can be achieved by tagging the phosphopeptides with lysine nitrilotriacetic acid gallium and analysing by LC-ICP-MS.

3. The binding of oxaliplatin to proteins within the cell is partly through the nitrogen rich amino acid residues within that protein.
3.4.4 PREPARING THE RESEARCH DESIGN

Preparing the research design refers to the process used to determine the methods and techniques used to collect data, evidence and information and the methods used to analyse and evaluate the data. The techniques used to collect data are explained in more detail in Chapter 4, but briefly the techniques used to collect data were, HPLC coupled to both molecular mass and elemental mass spectrometers and fragmentation mass spectrometry. It was proposed that the data collected would be compliant with the requirements of standard statistical methods.

3.4.5 COLLECTION AND ANALYSIS OF DATA

There are 3 common methods of data collection used in collection of data for a research thesis; these are qualitative methods, quantitative methods and mixed-mode methods. A quantitative approach is one in which the researcher primarily uses measurement and observations to test theories. The researcher typically uses experiments and surveys to collect data on predetermined instruments that yield statistical data. A qualitative approach is one in which the researcher makes a conclusion based upon individual or multiple experiences with the intent of developing a theory, pattern or perspective. Methods used in a qualitative approach include case studies, narratives, ethnologies and grounded theory studies. The data collected tends to be open-ended with the primary intent of developing themes from the data. A mixed mode approach is one in which the researcher makes a conclusion based on pragmatic grounds. In the mixed mode approach both numeric information, from instruments and text information and from interviews is collected simultaneously or sequentially in order to understand the research problem.

In this thesis a quantitative approach was used to gather data to answer the research problem. Following data collection, statistical and mathematical techniques including correlation, regression and analysis of variance (ANOVA) analysis were applied to support the outcomes of the research.
3.4.6 TESTING OF HYPOTHESIS

Once the data were collected and analysed the initial hypotheses drafted in the early stages of the research were tested. The question asked was ‘do the findings support the hypothesis or are they contrary to it?’

3.4.7 REPORTING RESULTS

The real value in research lies in the ability to interpret the findings, which may lead to new theories, laws or principles in a particular context and to report these to the wider community. There are several ways of reporting research findings, the findings of the research conducted in this thesis were reported in scientific posters at an international conference ‘The Winter Plasma Conference’, Graz, 2009, a national conference ‘The 5th Nucleic Acids Forum’, Bristol, 2009 and on the National Measurement Institute website. It has been reported at a scientific conference as a verbal presentation, the Biennial National Atomic Spectroscopy Symposium, Brighton, 2008. The research has also been reported in the three journal papers summarised in Chapter 1 and a 4th, that draws on some of the methodology developed in this thesis is in preparation for publication.
4 INSTRUMENTATION

4.1 INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

ICP-MS is a mass spectrometry technique used in the analysis of elements at ultra-trace levels. This analytical technique can provide multi-element analysis, high sample throughput and isotope ratios, it is highly sensitive, selective and quantitative over a large linear range. \(^{89-91}\) In addition to these characteristics ICP-MS is compatible with several sampling techniques including solution sampling, laser ablation and chromatographic techniques. \(^{92-94}\) ICP-MS generates positive ions from a solution of an analyte(s) by a high-temperature plasma, which provides efficient ionisation and atomisation of the analyte. These positive ions are detected by various methods depending upon the design of the mass spectrometer. The main principles of operation are summarised in Figure 8.
Analyte solution, typically in a mineral acid, in the ppt-ppb range. Analyte solution converted to fine aerosol by nebuliser. Larger aerosol droplets filtered out by spray chamber.

Analyte atomised and ionised by plasma. Ions selected by mass-to-charge ratio by various methods depending on instrument. Ions detected and signal intensity reported.

Figure 8: Basic principle of the mode of ICP-MS operation.

The sample solution containing the metal(s) or non-metal(s) of interest is drawn into a nebuliser, where it is converted to a fine aerosol with argon gas. The most widely used nebuliser is the concentric nebuliser which has a central capillary through which the sample is drawn. A stream of argon gas is introduced concentrically to the sample solution which breaks the sample into droplets due to the dynamic action of the gas as it meets the sample. Larger aerosol droplets are separated from the finer droplets by a spray chamber; the aerosol is introduced into the plasma torch by an injector. The plasma is a partially or fully formed ionised gas, generated at atmospheric pressure by the interaction of an intense alternating current gives rise to a magnetic field on a flow of argon gas. The magnetic field is generated by a radiofrequency wave passing through a copper coil, the radio frequency (RF)
generator is operated between 1100-1500 W, which ionises the gas which is seeded with electrons from an electrical spark and generates the high-temperature plasma. The high-temperature (6000-7000 K in the central channel) plasma generates high numbers of positively charged ions by ionising and atomising the metal/non-metal atoms in the sample aerosol.

Figure 9 shows the different region of the plasma in which the sample aerosol is dried, vaporised, atomised and ionised.

![Figure 9: Formation of the inductively coupled plasma, showing the different temperature regions.](image)

Once the ion stream is generated in the plasma, ions are directed into the mass spectrometer through a sampler and skimmer cone that form the interface region, maintained at 1-2 mbar by a roughing pump. The interface region allows the ions to be transported efficiently from the plasma at atmospheric pressure to the mass spectrometer at typically $7 \times 10^{-6}$ bar. The ion optics direct the positive ion beam towards the mass spectrometer, these electrostatic lenses focus the ion beam and stop photons and neutral atoms reaching the detector. The ion beam then enters the mass spectrometer region, maintained at a vacuum of around $10^{-7}$ mbar by a turbomolecular pump. The most commonly used mass spectrometers are quadrupoles, magnetic sectors and time of flight spectrometers. They all work in different ways and have
their own advantages and disadvantages, but their function is to allow ions of a particular mass-to-charge (m/z) ratio through to the detector.

Finally the ion beam is converted into an electrical signal by the detector. The most common detector is an electron multiplier, which consists of a series of metal dynodes the length of the detector. Ions from the mass spectrometer collide with the first dynode and converted to electrons; the electrons are attracted to the next dynode and so on along the length of the detector. This results in a very high pulse of electrons being emitted from the final dynode which is transformed into an electrical signal and processed into an analyte concentration.

### 4.1.1 HIGH RESOLUTION ICP-MS

Whilst quadrupole instruments typically have a resolution of 300, high resolution mass spectrometers have mass resolution capabilities in the region of 10,000. The improved resolution allows signal’s from elements to be separated from those resulting from polyatomic interferences. As a high resolution sector field mass spectrometer was the primary instrument used it will be discussed in further detail. The reverse Nier-Johnson design (shown in Figure 10) consists of two analysers, a traditional electromagnetic analyser and an electrostatic analyser (ESA). Ions are accelerated to a few kilovolts (kV) in the ion optic region before entering the magnet. Ions are focused in the magnetic field, which disperses ions based on their momentum and mass. The ion beam then enters the ESA, which is dispersive with respect to ion energy. The ions are focused onto the exit slit, where the electron multiplier detector is positioned. When the energy dispersion of the magnet and the ESA are equal in magnitude but in opposite directions, the ion beam is focused in both ion angle and ion energy. Ions of certain m/z are selected when the electrical field is changed to the opposite direction during the cycle time of the magnet. Once a certain magnetic field strength is passed, the electric field strength returns to its original value and the next mass is frozen. Scan times are slower than quadrupole instruments, but time can be saved by only scanning the mass peaks of interest by varying the voltage.
There are two main scan functions used with double-focusing mass spectrometers, a voltage scan (E scan) and a magnet scan (B scan). An E scan is a fast scan where the magnet field strength is constant at a fixed magnet mass and the voltage varied across a limited portion of the mass range. The magnetic scan (B scan) is the opposite where the accelerating voltage is kept constant and the magnet jumps between masses by changing the current in the magnet coils. It is a slower scan than the E scan but is not limited in range. A combination of the two modes are used to make routine scans; all analysis (across a peak) and mass selections (mass jumps) are made by changing the accelerating voltage unless the measured mass lies outside the 30% range at which point the magnet moves to and settles at a new magnet mass and the electric scan begins again. Polyatomic interferences can be removed by filtering.
ions through optics and slits to achieve variable resolutions from 300 to 10,000 resolving power.

4.1.2. COLLISION/REACTION CELL ICP-MS

The collision reaction cell technology of the Agilent 7500ce removes interferences by using differences in the kinetic energy of small charged ions and larger charged molecules. A single set of cell conditions is capable of removing interferences on multiple elements. Removal of interferences takes place in a pressurised octopole reaction system (ORS), the cell is pressurised with a collision gas such as helium that reacts with the interference. Removal of the interfering species occurs through one of several processes depending on the reaction gas and interference. In the helium collision mode, polyatomic interferences are removed due to their size rather than their relative reactivity with a reactive gas. As all polyatomic interferences are larger in size than the analyte ion of similar mass they will collide more often with the collision gas. These collisions lead to a decrease in energy of the polyatomic ions as they move through the collision cell. When the polyatomic ions reach the cell exit they have significantly lower ion energy than the analyte ions and can be prevented from leaving the cell by applying a stopping voltage. This type of separation is called kinetic energy discrimination (KED). The advantages of this type of interference removal is that the collision gas is inert therefore does not create any new interferences, no signal loss occurs as helium does not react with the analyte ions and several interferences can be removed from the analyte ion(s) at the same time. Other advantages include ease of use, there are no cell voltages to set up or optimise and the same cell conditions can be applied to every sample, every matrix with little being known about the sample matrix. In order for the collision cell to work effectively in He mode, the energy spread of the ions entering the cell must be small and the polyatomic ions must have a sufficient number of collisions to differentiate them.
from analyte ions. The first of these two conditions is realised by having a physically grounded torch shield rather than an electrically grounded one. The second condition is realised by having an octopole cell, octopoles have a smaller diameter this allows the cell to operate a higher pressure than would be possible with a quadrupole or hexapole cell due to smaller entrance and exit cell aperture’s. Octopoles also have better focusing efficiencies than hexapoles and quadrupoles.
4.2 ELECTROSPRAY MASS SPECTROMETRY – LINEAR ION TRAP
MASS SPECTROMETER

Molecular mass spectrometry offers complementary information to that gained during ICP-MS analysis as structural information is retained. Linear ion-trap instruments have been used widely in the analysis of peptides and nucleic acid sequences. Figure 11 shows a schematic diagram of a typical ion-trap instrument used as the primary molecular mass spectrometer in this research. As shown in the schematic the mass spectrometry used in this research consisted of an electrospray ionisation source, ion optics and a mass analyser to isolate ions of interest and a photomultiplier detector system. The following processes are carried out by the mass spectrometer:

1. Production of ions from the sample in the ionisation source.
2. Separation of these ions according to their mass-to-charge ratio in the mass analyser.
3. Fragmentation of the selected ions and analysis of the fragments in a second analyser.
4. Detection of the emergent ions from the last analyser and measurement of their abundance to convert into an electrical signal.
5. Control of the instrument by feedback from the computer based on the signal transmitted from the detector.
4.2.1 ELECTROSPRAY IONISATION

Electrospray ionisation (ESI) is widely used for the ionisation of bio-molecules that are often highly charged and polar and was the ionisation source used in this research. A strong electric field at atmospheric pressure is applied to a liquid passing through a capillary tube in order to create the electrospray. At the capillary end a charge accumulation is induced at the liquid surface by the electric field, which causes highly charged droplets to break from the liquid surface. A Taylor Cone is formed when the charge accumulation at the surface is great enough to breach the surface tension. Dispersion is controlled by a heated coaxial gas stream at a low flow rate. The droplets then pass through a heated capillary to remove the last of the solvent. A schematic is shown in Figure 12.
The process by which charged droplets generate ions is the central question in electrospray theory. There have been two theories presented; one by Thomson and Iribarne known as the ion evaporation mechanism (IEM) in which small highly charged droplets are formed from the explosion of larger droplets due to Coulombic forces. The second theory is known as the charge residue model (CRM) put forward by Dole.

In the ion evaporation model, droplets produced at the capillary tip shrink due to evaporation of the solvent, when the field strength at the droplet surface is large enough solvated ions can be expelled. Energy required to enlarge the droplet at the surface and to expel a solvated ion is provided by the strong electric field at the surface. In the charge residue model, the highly charged droplet shrinks due to solvent evaporation until the field strength at the greatest point of curvature is large enough to cause the formation of a new Taylor Cone. At the tip of this new Taylor Cone smaller droplets are emitted, this process is repeated until droplets are formed that contain a single analyte molecule. Larger molecules such as proteins will not desorb but are freed as the solvent is evaporated and are often multiple charged if they have more than one ionisable site. The generation of multiple charged ions is advantageous with improved sensitivity at the detector and allowing the detection of larger molecules with mass spectrometers of lower nominal mass limit.
The Rayleigh equation is used to describe the liquid droplet’s structural integrity once airborne in relation to its surface tension and the electrostatic repulsion from the solvated ion. Due to evaporation the Rayleigh limit is reached as the charges become closer together increasing the repulsion between them. When the Rayleigh limit is reached the droplet undergoes Coulombic explosion splitting into daughter droplets and the process begins again.

Figure 13, shows the difference in droplet formation by the two different models described above.

\[ q^2 = 8\pi^2 \varepsilon_0 \gamma D^3 \]

Equation 1: Rayleigh equation for the limiting charge.\(^\text{105, 106}\)

q = Rayleigh limit

\(\varepsilon_0\) = permittivity of vacuum (F m\(^{-1}\))

\(\gamma\) = liquid surface tension (N m\(^{-1}\))

D = Diameter of the droplet (m)
Figure 13: A - The ion evaporation process, an individual ion leaves the charged droplet. B - The charged residue model. A charged molecule reduces in size due to evaporation, a new Taylor cone forms from which smaller highly charged droplets are emitted.

4.2.2 LINEAR ION TRAP MASS ANALYSER

Ions are transmitted via the heated transfer capillary to the mass analyser. The ions are focussed by the tube lens and then pass through the skimmer cone, which helps step down the pressure between the interface and the mass analyser. After passing through the skimmer cone the ions travel through a series of ion guides and lenses guided by two quadrupoles and an Octopole to the mass analyser. The first two quadrupoles consist of four square profile rods with DC and RF voltages applied. Ions with a stable path pass through the first quadrupole, and then are guided to the second quadrupole by a lens. The ions are finally guided to the Octopole by the gate lens to the mass analyser. It is the gate lens that controls the number of ions injected into the mass analyser.
The linear ion trap confines ions by using an oscillating electric field, it uses a two-dimensional radio frequency field to trap ions and reflect the ions back into the trap axially by a stopping potential applied to electrodes.\textsuperscript{99,108} Collisions with an inert gas cool the ions which travel along the $z$-axis between the end electrodes whilst oscillating in the $xy$ plane due to the RF potential.\textsuperscript{99} Linear ion-traps have several advantages over 3D Paul ion traps, including higher ion trapping capacity and higher ion volume capacity. Space charge effects result in the repulsion of ions of like charges and result in broad peaks, loss of resolution and reduced sensitivity.\textsuperscript{109} Collisions with He gas reduce axial dispersion giving greater injection efficiencies than their three dimensional quadrupole trap counterparts.\textsuperscript{109} These attributes increase both the sensitivity and dynamic range of the mass analyser.\textsuperscript{99,108} The Thermo LTQ trap ejects ions radially through two parallel slots (see Figure 14) into two detectors which further increases the sensitivity.

Figure 14: Linear trap, with slots in opposite rods for radial ion ejection to two detectors.
Segmented quadrupoles are used rather than trapping by end electrodes to avoid producing fringe fields, the rods are cut into three sections, two smaller end sections and a longer central section, the ions are trapped by applying a DC voltage to the end sections. Ions are trapped in the central section which has a lower potential than the two end sections. A large potential well is created during the analysis in the central quadrupole section by an increased potential of +20 V on the two end sections. Negative ion storage is obtained by reversing the potentials.\textsuperscript{107} Ions are ejected in a radial direction by applying an AC voltage between the two rods.\textsuperscript{107} The AC voltage remains constant, in the radio frequency range, the amplitude is varied to store and eject ions. To store ions, they are kept stable by making the main RF voltage low; during analysis the ions become unstable as the RF potential is ramped up at a constant rate. Once the ions become very unstable they are ejected through the exit rods. The voltage at which an ion of defined m/z is ejected is known as the resonance voltage. The ejected ions are then directed to the electron multiplier detectors.\textsuperscript{107}

The storage, isolation and fragmentation of ions and scan events are outlined below.

i) Ion storage
   - DC voltages applied to all three sections of the rods the central section has lower voltage than the ends to create a potential well and trap ions in the axial (z) direction.
   - The main RF amplitude is low; the ions are kept stable in the radial (xy) direction and confined in the mass analyser.\textsuperscript{107}

ii) Ion isolation
   - Used for single ion monitoring (SIM), selected reaction monitoring (SRM), consecutive reaction monitoring (CRM) and general tandem MS.
   - An AC voltage is applied to the exit rod (ion isolation waveform).\textsuperscript{107}
   - Ion of selected m/z is trapped by the main RF and ion isolation waveform; all other ions are ejected from the trap.

iii) Collision/Fragmentation
   - Used for SRM, CRM and MS\textsuperscript{8}.
   - Ion movement in the radial direction is controlled by applied voltages (resonance excitation voltages).\textsuperscript{107}
• This voltage causes ions to collide with He, but not sufficient for ions to be ejected.
• The parent ion fragments into daughter ions due to the collisions with He.

iv) Ion Scan Out
• Ions are ejected due to the applied resonance ejection RF voltage to the exit rods. 107
• The amplitude of the resonance ejection RF voltage is ramped up to resonate the ions. 107
• During resonance the ion moves further away from the centre of the trap. The further away from the centre, the smaller the space charging effects, also the RF voltage is stronger which causes the ions to be ejected. 107

The ion-trap allows ions with defined m/z to be stored, fragmented and then transmitted to the detector. Structure elucidation is achieved due to the collision of the analyte ions with He gas, unlike ICP-MS where fragmentation and secondary reactions are avoided. 99 MS/MS experiments involve two stages of ion selection and fragmentation followed by the analysis of the daughter ions. However, these stages can be repeated n times in MS^n in order to gain detailed structural information. He gas also induces collisional focusing; the kinetic energy of the injected ions is reduced in the ion trap preventing them from leaving the trap, but keeping them near the centre of the quadrupole. 99, 107 This has the effect of increasing sensitivity.
4.2.3 SCAN MODES

The mass spectrometer used in this research can obtain information on a particular ion by several different scan modes including, full scan mode, single ion monitoring (SIM), selected reaction monitoring (SRM), consecutive reaction monitoring (CRM) and zoom scan.

Full scan mode detects all ions within a set m/z range; all parent ions are sequentially ejected out of the ion trap and detected. This mode is useful for gaining a lot of information about the sample but lacks sensitivity and is slower than other scan types. 107

In a SIM scan only ions of a selected m/z are detected resulting in faster acquisition and increased sensitivity compared to full scan mode. Parent ions of interest are stored in the ion trap and all other ions are scanned out of the mass analyser. SIM has poor specificity as if another compound other than the analyte of interest forms a parent ion of same m/z as the analyte they will be detected alongside the analyte. 107

In a SRM scan parent ions are stored in the ion trap; whilst all other ions are scanned out, the parent ion of interest undergoes fragmentation with He gas to form daughter ions. The daughter ions of interest are then stored in the trap whilst all other daughter ions are ejected. A selected reaction monitoring mass spectrum is then generated by scanning out the daughter ions of interest. A SRM scan is more specific than a SIM scan as it is highly unlikely that an unwanted parent ion will form daughter ions of the same m/z as those belonging to the analyte. 99, 107

A consecutive reaction monitoring (CRM) scan is similar to both a SIM and SRM but involves more MS steps provided MSn can be achieved. As with SIM and SRM parent ions are stored in the trap whilst all other ions are scanned out of the mass analyser. The parent ion of interest then undergoes fragmentation with He gas to form daughter ions. The daughter ions of interest are then stored in the trap whilst all other daughter ions are ejected from the trap. The selected daughter then becomes the parent ion which is further fragmented to give new daughter ions; this process is repeated up to MS10 and as such has increased specificity. A CRM mass spectrum is only generated after the final mass analysis step, where the selected daughter ions are scanned out of the ion trap. 107
A high resolution zoom scan enables the molecular weight and charge state of an ion to be determined by analysing $^{12}\text{C}/^{13}\text{C}$ isotope separations. This scan type can allow the molecular weight of an unknown compound to be calculated.

4.2.4 EXPERIMENT MODES

As with scan types there are several different experimental modes available with this type of mass spectrometer, including general MS and MS$^\text{n}$, data dependent, ion mapping and ion tree. In general MS experiments only the mass(es) of the parent ion(s) need to be known whereas for MS$^\text{n}$ experiments the masses of both the parent and daughter ions need to be known. The greater the number of stages of mass analysis the more information about the analyte is found.\(^{107}\)

When very little is known about the analyte, very detailed structural information can be gained from a data dependent experiment. The most abundant parent ions are selected by the ion trap whilst minor parent ions are ejected. The trapped parent ions are then fragmented and the most predominant daughter ions can then be trapped and fragmented further, within a defined mass range.\(^{107}\) Parent ion masses can be specified and the ions selected and fragmented to give product ion mass spectra whilst unwanted parent ions can be specified and ignored. This type of experiment gathers detailed MS information on each mass analysis stage.\(^{107}\)

Ion tree experiments can be data dependent where the instrument decides the next stage of the experiment by collecting and analysing the data, or the parent ion of interest can be specified for MS$^\text{n}$.\(^{107}\) A set number of MS experiments is performed by the instrument before moving onto the next most intense parent ion and performing the defined number of MS experiments on that ion, then onto the next most intense ion and so on, this is known as ‘depth focus’. Breadth focus will perform MS on all the specified parent ions and then move onto the next level of MS and analyse the product ions.\(^{107}\)

Ion mapping experiments enable unknown compounds in a mixture to be analysed and characterised in detail. There are three main types of ion mapping experiments; total ion map,
parent ion map and neutral loss. In a total ion map experiment, product ions scans are acquired, so that which parent ions were fragmented in order to produce specific product ions can be determined. In a parent ion map experiment the parent ions that produced specific product ions are identified. A neutral ion loss experiment provides evidence that a neutral fragment loss has occurred. As neither ion mapping nor ion tree experiments were carried out during this research the specific details have been omitted but can be found elsewhere.
4.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography refers to the method for separating compounds based upon the interactions of each analyte within the liquid mobile phase with the stationary porous phase. The interaction of the analytes with the stationary phase is dependent on their structure, namely their size and polarity due to the presence of charges, polar groups or moieties. In the case of reverse phase high performance liquid chromatography (HPLC) the mobile phase is a liquid usually consisting of a polar (water) phase and a less polar phase (methanol or acetonitrile) and the stationary phase is a column filled with porous particles that are predominantly non-polar. The components that make up a HPLC system are as follows; an injection port, a pump which delivers the mobile phase, a stationary phase where the separation occurs and a detector. Various detectors can be coupled to the HPLC system including UV, fluorescence, refractive index, evaporative light scattering and electrochemical detectors as well as mass spectrometers. The choice of stationary phase is dependent on the polarity of the analytes for separation, where the polarity of the stationary phase is roughly matched to that of the analytes and the mobile phase is different to both the analyte and stationary phase. The ability of the column to separate two analytes is measured by the resolution ($R_s$), which is given by the relationship between the retention time ($t_R$) and the width (W) of two consecutive peaks from two different species A and B, as shown by Equation 2, where retention time is defined as the time from injection of the analyte onto the column to detection at the detector. The rate of migration for an analyte through the column is referred to as the retention factor, $k'$ and is given by Equation 3, where $t_M$ is the time taken for the mobile phase to travel through the column.

$$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

Equation 2: The resolution of two peaks is calculated from the difference in retention time of the two peaks divided by the combined widths of the eluted peaks.
\[ K'_A = \frac{t_R - t_M}{t_M} \]

Equation 3: The equation used to determine the retention factor of an analyte based on its retention time and the time taken for the mobile phase to travel through the column.

Resolution can be improved by lengthening the column therefore increasing the number of theoretical plates but this causes an increase in the retention time. Theoretical plates are used to describe the efficiency (N) of the column which is a measure of the peak width. The sample equilibrates between the mobile phase and each individual plate as it moves down the column. The number of theoretical plates, N, in a column is determined experimentally by examining the chromatographic peak, its peak width and retention time. N is given by Equation 4, where \( w_{1/2} \) is the peak width at half height.

\[ N = \frac{5.55 t_R^2}{w_{1/2}^2} \]

Equation 4: The number of plates can be calculated from the retention time and peak width.

The rate at which an analyte moves through the column is determined by the rate at which it equilibrates between the mobile and stationary phases, and as such the resulting shape of the chromatographic peak is related to the rate of elution. The peak shape is also affected by the path through the column; these effects cause the peak to broaden in an effect known as band broadening. The Van Deemter equation (Equation 5) describes the effect of band broadening on the theoretical plate height (HETP).

\[ HETP = A + \frac{B}{u} + Cu \]

Equation 5: The Van Deemter Equation describes the effects of band broadening in relation to the number of paths taken, the longitudinal diffusion and the rate of absorption and desorption.
Where A is the eddy diffusion, B is the longitudinal diffusion, C is the resistance to mass transfer and $u$ is the average velocity of the mobile phase. The different paths the analyte molecules can take through the column result in different path lengths which gives rise to eddy diffusion. B describes the difference between the concentrations at the end and centre of the band. The concentration of the analyte at the end of the band is less than it is at the centre of the band and the longer the analyte spends on the column the greater the width of the band will become. Mass transfer refers to the time taken for the analyte to equilibrate between the stationary and mobile phase. If the analyte has a strong affinity for the stationary phase and the mobile phase is moving at a high rate then the analyte in the mobile phase will move past the analyte in the stationary phase, resulting in band broadening. 110
5 RESEARCH UNDERTAKEN AND KEY FINDINGS

5.1 INTRODUCTION

This section describes the main activities over the five years of the research programme and the findings of the research. The process is described in terms of experiments carried out in each theme, findings and outputs. The chapter should be read in conjunction with the published papers referenced throughout the text (see appended papers A1-A3).

5.2 OVERVIEW

The first four months of the research were spent gaining a broad understanding of the subject area by conducting an in-depth literature review on the quantification of DNA by ICP-MS and the enrichment and analysis of phosphopeptides.

During the first two years of the research preliminary experiments were carried out on the enrichment of phosphopeptides using commercially available enrichment kits and tips, HPLC method development for the separation of nucleotides and completing MSc modules (60 credits) in Engineering Management and Innovation as part of the EngD programme. This meant that approximately 20% of the researcher’s time was spent at Loughborough University, undertaking different MSc modules, coursework and exams. The modules selected facilitated a better understanding of bio-analytical chemistry, atomic spectroscopy, management techniques and people skills, management and professional development and research innovation and communication skills. During this time period the Research Engineer
(RE) supervised several project students, providing training, and mentoring as part of the RE’s continued professional development. Two conferences were attended where the RE presented research results orally (BNASS 2008, Brighton) and in poster format (Winter Plasma Conference, 2009, Graz).

The third year and the first part of the fourth year of the research was spent developing and validating the method for the quantification of DNA by ICP-MS, resulting in the first publication at the end of the fourth year. Also during this time period a further two project students were supervised by the RE and the RE presented research findings in poster format at the Nucleic acid forum (Bristol, 2010). The second half of the fourth year was spent developing the labelling of phosphopeptides with gallium, the method was finalised in the first half of the fifth year, and research findings were sent for publication at the end of the fifth year. Also during this time period an in-depth literature review of the interaction of proteins and peptides with anti-cancer drugs was conducted and data was collected for the interaction of the anti-cancer drugs; cis-platin, oxaliplatin and carboplatin with the endogenous peptides carnosine and anserine. The research activities are summarised in Figure 15.
Research Undertaken and Key Findings

Claire Camp

2008 -2010  Research activities and preliminary work

- Completion of in-depth literature review
- Completion of taught EngD programme
- Preliminary experiments for research themes 1 and 2
- Research findings reported at two conferences

2010-2012  Method development

- Separation of nucleotides by HPLC-ICPMS achieved and validated
- Tagging of phosphopeptides with galium preliminary work completed
- Research findings reported at one conference

2012-2013  Research finalisation and publication

- In depth literature review for research theme 3
- Data collection for research themes 2 and 3
- Publication of research findings in academic journals

Figure 15: Overview of the research activities during the EngD programme.
5.3 QUANTIFICATION OF DNA BY LC-ICPMS

5.3.1 INTRODUCTION

The aim of the research was to develop a LC-ICP-MS method for the quantification of DNA from an enzymatic digest of whole DNA to monophosphate nucleotides. An assay for the quantification of DNA would benefit many clinical diagnosis and forensic and biological studies. Quantification of DNA is also important in the labelling of foodstuffs; these measurements are needed in the determination of transgenic material in animal feed. European law requires labelling of imported foodstuffs containing more than 0.9% transgenic material. This is important for America which is the world’s largest exporter of agricultural wares and prepared foodstuffs. The increase in genetically modified crops grown in America has increased the demand for a reliable analysis technique for a nucleic acid certified reference material. Currently these measurements are made using real time PCR measurements. This work was carried out in collaboration with Dr Heidi Goenaga-Infante and Dr. John Entwistle from the sponsoring company LGC Ltd.
5.3.2 RESEARCH UNDERTAKEN

The overall work-flow for the quantification of DNA is shown in Figure 16.

```
<table>
<thead>
<tr>
<th>Purified DNA</th>
<th>Quantify whole DNA by ICP-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease P1</td>
<td>Mass balance calculation</td>
</tr>
<tr>
<td>Digested Nucleotides</td>
<td>Quantify by LC-ICP-MS</td>
</tr>
<tr>
<td></td>
<td>using external calibration</td>
</tr>
</tbody>
</table>
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Figure 16: Proposed quantification of calf thymus DNA work-flow.

Each stage in the work-flow was developed and verified using standard analytical methodology. These stages were:

1. HPLC method development for the separation of mono-phosphate nucleotides.
2. Compete digestion of calf thymus DNA to mono-phosphate nucleotides.
3. Verification of digestion products by molecular LC-MS.
4. Determination of the method limit of detection for LC-ICP-MS.
6. Method validation against a CRM.

7. Quantification of calf thymus DNA.

Method development for the separation of nucleotides began with the search for the most appropriate column, mobile phase, column temperature and pH. The three columns initially selected for evaluation were: Supelco R16 Amide column, Agilent Zorbax RX column and the Phenomenex Gemini NX. The method chosen for the separation was ion-pair chromatography and the ion-pair reagents initially tested were tetra-methyl ammonium hydroxide (TMAH), tri-ethyl ammonium acetate (TEAA) and ammonium acetate. The pKa’s for the four nucleotides are given in Table 3, the pH range for separation was from 5.5 to 7.0.

<table>
<thead>
<tr>
<th></th>
<th>Nucleoside pKa</th>
<th>5’ Nucleotide pKa</th>
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<tr>
<td>Adenosine (N1)</td>
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</tbody>
</table>

Table 3: pKa for bases in nucleosides and nucleotides at ~ 20 °C.  \[113\]

Column testing was carried out by HPLC with UV detection, conditions for column tests are given in Table 4.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Temperature (°C)</td>
<td>25</td>
</tr>
<tr>
<td>Injection Volume (µL)</td>
<td>5- 25</td>
</tr>
<tr>
<td>Flow Rate (mL/min)</td>
<td>Recommended flow rate of column</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>5 - 25 mM ion-pair reagent and 5- 25% methanol</td>
</tr>
</tbody>
</table>

Table 4: Column testing conditions used in preliminary work for the separation of monophosphate nucleotides.
Once the optimum separation conditions were determined, the method was used to separate the components of a DNA digest mixture. Several conditions were used to optimise the digestion of calf thymus DNA, including varying the temperature at which the digestion was performed, concentrations of the reagents and enzymes used. The digestion was also performed by microwave digestion. Each digest mixture was analysed by HPLC to compare against the chromatograms for the nucleotide standards.

To further confirm that the enzymatic digest of calf thymus DNA produced all four monophosphate nucleotides, the digest was analysed by LC-ESI-MS. Comparison of retention times and mass spectra between the calf thymus DNA digest and nucleotide standards were made. Injections of each single nucleotide were made in order to record their retention time and parent ion m/z, after digestion of calf thymus DNA the digest mix was injected and its mass spectra recorded.

Following the optimisation for the separation of both the nucleotide standards and the DNA digest mix, quantification by LC-ICP-MS was started. Firstly the limit of detection for phosphorus was determined by:

1. direct nebulisation of an inorganic phosphate standard and 5’AMP,
2. FIA of an inorganic phosphate standard,
3. LC-ICP-MS of five nucleotide standards,
4. blank inspection by direct nebulisation, FIA and LC-ICP-MS.

To determine the completeness of digestion and degree of loss on the column for the nucleotides, the recovery for each nucleotide was calculated by flow injection analysis ICP-MS (FIA-ICP-MS). Similarly the mass balance for phosphorus was achieved by analysis of whole DNA by FIA-ICP-MS. The flow injection conditions used for the recovery of each nucleotide and for the mass balance of phosphorus in DNA are given in Table 6. The quantification of calf thymus DNA was performed on two separation systems, the first consisted of a Thermo Surveyor HPLC coupled to a Thermo Element 2 XR ICP-MS, the
second consisted of an Agilent 1100 HPLC system coupled to an Agilent 7500 ce ICP-MS. Prior to analysis the system suitability was checked by performing 6 repeat injections of d’CMP at a phosphorus concentration of 250 ppb. A calibration blend of the 4 monophosphate nucleotides at concentrations of phosphorus ranging from 50 to 500 ng/g were run followed by several blanks and aliquots of the calf thymus DNA digest mixture. The column was then removed and the same experiment was repeated in order to determine the total phosphorus concentration of the digest mix in order to determine the mass balance.

5.3.3. RESEARCH FINDINGS AND DISCUSSION

The separation of nucleotides by HPLC is difficult due to their inherent amphoteric nature and the loss of phosphate groups due to either acidic or basic hydrolysis. At the operating pH of silica based columns, pH 2-8, 5’ monophosphate nucleotides are ionic molecules and so are poorly retained on non-polar stationary phases. As such ion-pair agents are needed to increase retention on non-polar columns. It has been documented that quaternary ammonium alkylates are suitable ion-pair reagents for 5’monophosphate nucleotides giving rapid separation of polar water soluble compounds on non-polar stationary phases. Of the 4 ion pair reagents tested, TEAA was found to give the best separation of mono-phosphate nucleotides, a column temperature of 25°C and a flow rate of 0.25 mL/min gave the best results as shown in Figure 17.

![Figure 17: Separation of all 4 dNMP's on Phenomenex Gemini NX Column, Isocratic mobile phase - 15 mM TEAA, 2.5 % MeOH, Flow rate – 0.25 mL min⁻¹, Injection volume 20 µL, Column Temp 25 °C.](image-url)
A conventional digest procedure was found to be the best strategy for the digestion of calf thymus DNA, the protocol was based on that by Crain and is outlined below in Figure 18.

![Digestion protocol for the digestion of calf thymus DNA.](image)

To assess the suitability of the LC-ICP-MS method for the quantification of DNA, standard nucleotides were analysed several times on different days. Three calibration sets were run on the same day in order to make a comparison based on repeatability, two further calibration sets were run on two separate days to compare reproducibility. Application of one-way ANOVA showed there was no statistical difference (n=4, p=0.95) between the slopes for the 4 different nucleotides. The relative standard deviation (RSD) of the slopes for these calibration curves were 3.6% for the intra-assay and 6.5% for the inter-assay. Repeatability of injection was calculated as 0.2% RSD (n=6).
Column recoveries were determined for each nucleotide standard from the injection of 5 µL of the nucleotide standard with and without the column in place, the recoveries ranged from 95 to 98%, as shown in Table 5.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>d’CMP</td>
<td>98.7 ± 0.5</td>
</tr>
<tr>
<td>d’TMP</td>
<td>97.8 ± 1.1</td>
</tr>
<tr>
<td>d’GMP</td>
<td>99.1 ± 1.2</td>
</tr>
<tr>
<td>d’AMP</td>
<td>98.4 ± 1.2</td>
</tr>
<tr>
<td>UMP</td>
<td>98.0 ± 3.1</td>
</tr>
</tbody>
</table>

Table 5: Recoveries of individual nucleotides using FI-ICP-MS.

Calibration blends of standard monophosphate nucleotides were prepared in mobile phase (20 mM triethylammonium acetate (TEAA at pH 6.4) at $^{31}$P concentrations of 50 - 500 ng/g. Two calibration runs were made, followed by three repeats of each digested DNA sample and a final calibration set, three blanks were run between each calibration set and sample run. Figure 19 shows typical chromatograms obtained from both the calibration blend and a digested DNA sample analysed by LC-ICP-MS.
Figure 19 compares the separation of a mixture of mono-nucleotide standards and digested calf thymus DNA. The slight change in the observed retention times was due to the variation in the mobile phase pH, from non-consecutive chromatograms, however, the peaks were positively identified by LC-MS. The unknown peak observed in both the nucleotide standards and the DNA digest is thought to be the result of phosphate buffers used in the purification process. This peak was found to vary in magnitude reflecting some variability in the phosphate levels in the standards. The presence of these buffers did not affect the quantification of DNA when speciation was used, because the first peak can be ignored, but would affect an assay based on total P where it would be included. This was proven by
fraction collecting the individual nucleotide peaks from the Sigma standards, digesting to phosphate and quantifying by direct nebulisation into the ICP-MS. This experiment was also performed on the DNA digest. Chromatograms run including UMP in the mixture showed the UMP baseline separated with a retention time of approximately 4.8 minutes.

Appropriate calibration curves, shown in Figure 20, were plotted, from which the $^{31}$P concentration for each monophosphate nucleotide was calculated. The molecular weight of each nucleotide was used to calculate the nucleotide concentration; these were then summed and back calculated to give the DNA concentration.

![Figure 20: A typical calibration chart for nucleotide standards.](image)
At the end of the calibration and sample sequence, whole DNA was analysed by FIA-ICP-MS in order to calculate the $^{31}$P mass balance. Four repeat injections of undigested DNA were run and the total peak area was used to calculate mass balance by comparing it to the combined peak areas of the digested samples analysed by LC-ICP-MS. Measuring total P as an indicator of DNA concentration is problematic as even in highly purified and well characterised samples, such as the National Institute of Standards and Technology (NIST) standard, there are likely to be P-containing residues from the purification procedure.

The LC-ICP-MS results show that a mass balance of 97.2% ± 0.5% was achieved by this experiment, which was expected due to the high column recoveries observed. The mass balance calculation data is shown in Table 6 for both NIST SRM DNA and nucleotide standards. Errors were calculated from the standard error of the mean for each peak in the digest and for the repeated flow injection peaks, and then treated with the propagation of errors formula.
Table 6: Total peak areas for speciated nucleotide standards and digested NIST DNA and flow injection peak areas for nucleotide blend and undigested NIST DNA.

5.3.6. CONCLUSIONS
The phosphorus content of the 4 nucleotides was calculated by external calibration with standard nucleotides from Sigma as; d’CMP 11.2 ±0.1 ng/µL, d’GMP 13.1±0.1 ng/µL, TMP 13.6 ±0.1 ng/µL and d’AMP 14.2 ±0.1 ng/µL, giving a total of 52.1 ng/µL. The stated DNA concentration for component B out the four different NIST DNA components was 53.6 ±0.4 ng/µL, giving a recovery of 97.2%.
5.4 TAGGING OF PHOSPHOPEPTIDES WITH GALLIUM

5.4.1 INTRODUCTION

The aim of the research was to develop an LC-ICP-MS method for the quantification of phosphopeptides from an enzymatic digest of whole phosphoproteins by labelling the phosphate group with a metal ion, namely gallium. This proved to be more difficult than first thought and the quantification of phosphopeptides was not achieved; however, labelling of phosphopeptides with gallium was achieved. The quantitation of the proteome is of increasing importance as it is the amount of particular proteins or changes in their relative abundance that reflect a change in a biological system. Relative quantification of phosphopeptides and proteins has been achieved by the incorporation of various isotopic labelling agents such as SILAC and ICAT. Alternatively naturally occurring tags such as phosphorus and sulfur can be utilised for relative quantification by ICP-MS analysis. However these elements are not efficiently ionised by the plasma and suffer from interferences, labelling with a metal tag would enable improved detection limits. Labelling of the phosphate moiety with gallium was achieved and investigated using ESI-MS and molecular modelling. This work was carried out in collaboration with Dr Tamer Shoeib and Eslam Moustafa from the American University in Cairo, who carried out the molecular modelling experiments and calculations.

5.4.2 RESEARCH UNDERTAKEN

The first stage in the tagging of phosphopeptides with gallium was the generation and characterisation of the ligand \( \text{N,N-bis(carboxymethyl)lysine gallium (LysNTAGa)} \). The ligand was based on the typical support medium found in a classic IMAC column. The starting materials, N,N-bis(carboxymethyl)lysine (LysNTA) and gallium(III) chloride were purchased (Sigma Aldrich, UK) and combined in a 1:2 molar ratio to form the ligand LysNTAGa, see Figure 17. To confirm that the ligand formation was successful, a series of mass spectrometry experiments were carried out, including \( \text{MS}^n \) and LC-MS. Excess cations were removed with ion exchange solid phase exchange cartridges prior to mass spectrometry analysis. A small phosphopeptide was chosen as a test molecule for the tagging process, platelet derived growth factor \( \beta \)-receptor (\( \beta \)-PDGF) which is a phosphopeptide that regulates
Research Undertaken and Key Findings
Claire Camp

Cell growth and division. β-PDGF is a pentapeptide phosphorylated on the terminal tyrosine. Prior to tagging β-PDGF with LysNTAGa, the pentapeptide was characterised by MS, MS^n, and LC-MS. LysNTAGa was combined with β-PDGF in a 2:1 molar ratio to ensure complete tagging of the phosphopeptide, see Figure 17. The tagged phosphopeptide was characterised by MS and LC-CP-MS. To confirm that the tagged phosphopeptide is a thermodynamically possible molecule, the most energetically stable conformer was found by molecular modelling.

Figure 21: The formation of LysNTAGa tagged β-PDGF from LysNTA, Ga(III)Cl and β-PDGF.
5.4.3. RESEARCH FINDINGS

PDGF and LysNTA were investigated using molecular mass spectroscopy, the molecular ions were identified, these molecular ions were trapped and fragmented to give daughter ion spectra. Following the characterisation of the two starting materials mentioned above, the gallium tagging reagent Ga-LysNTA was formed and investigated by MS. The complete formation of LysNTA-Ga was shown by the absence of free LysNTA+H+ ion (m/z 263) in the ESI-MS spectrum of the metal ligand complex. The synthesis of the ligand was confirmed by comparing the isotopic pattern from experimentation to that generated by the Xcalibur software, this was performed on both the linear trap MS (Thermo LTQ) and the high resolution mass spectrometer (Thermo Xactive). See Figure 22 and Figure 23 for the comparison spectra.

Figure 22: Comparison of the found isotopic pattern for LysNTAGa (top) compared to the theoretical isotopic pattern generated by Xcalibur software (bottom).
Research Undertaken and Key Findings

Figure 23: Formation of [LysNTAGa.H2O]+; panel A experimental data, panel B theoretically predicted spectra by high resolution mass spectrometry performed on a Thermo Exactive Mass Spectrometer.

Molecular modelling was employed to determine the lowest energy conformation of Ga-LysNTA and is shown in Figure 24. This structure shows that the gallium atom is bound to three carbonyl oxygen atoms and one nitrogen atom from the lysine backbone. This structure helps to disperse the highly positive charge on the metal atom and forms three distinct five-membered heterocyclic rings with the gallium metal atom at the centre. A strong hydrogen
bond is also formed between the terminal amino group on lysine and the closest carbonyl group forming a pseudo nine membered ring. The large number of rings formed in the conformation shown in Figure 24 is responsible for this being the lowest entropy structure found from molecular modelling experiments.

Figure 24: Structure for the Ga-LysNTA singly charged positive ions as calculated at the HF/STO3G and B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies (in kcal per mol) are indicated in parenthesis.

On examination of the daughter ions, the presence of a water adduct to Ga-LysNTA was identified and confirmed by comparison of the experimental signal with the theoretical isotope pattern model and was found to be in excellent agreement with an average mass accuracy of 0.08ppm. The mass spectra of the water:LysNTAGa adduct is shown in Figure 25. An interesting cluster was observed at m/z 361-370, which is proposed to be due to the signals of LystNTAGa•CH$_3$OH and Ga-LysNTA•2H$_2$O adducts. Closer inspection of this cluster and matching to modelled theoretical isotope patterns indicated that it consisted of a 2:1 ratio of [Ga-LysNTA•MeOH]$^+$: [Ga-LysNTA•2H$_2$O]$^+$. This suggests that the two acidic carbonyl groups have lost a hydrogen atom in order to form two ionic bonds to the gallium.
Hypothetical coordination in the Ga-LysNTA complex; therefore the Ga-LysNTA is unlikely to accept lone pairs from remaining water molecules in the gas phase.

Figure 25: Panel A experimental signal due to [LysNTAGa]⁺ adducts with H₂O and CH₃OH, panel B theoretically predicted composite spectrum of a 1:2 ratio of [LysNTAGa.2H₂O]⁺ and [LysNTAGa.CH₃OH]⁺.
The formation of Ga-LysNTA with the phosphopeptide PDGF β-receptor, which has a primary structure of H-pTyr-Val-Pro-Met-Leu-OH, to form the Ga-LysNTA-β-PDGF adduct at m/z of 1030.3 alongside the Ga-LysNTA and β-PDGF reactants were observed in the mass spectrum shown in Figure 26. The isotopic pattern of Ga-LysNTA-β-PDGF is shown in Figure 27, this was confirmed by comparison with the theoretical isotope pattern and by accurate mass FT-MS with an average mass accuracy of 4 ppm (see Paper 2, Table 1). Several other species which were observed in the mass spectrum were identified and summarised in Table 7.

Figure 26: Full scan spectrum of reaction mixture showing evidence of [LysNTAGaPDGF]+ formation from Thermo LTQ Mass Spectrometer, showing the formation of LysNTAGaPDGF at m/z 1030.25, and the starting materials PDGF at m/z 702 and LysNTAGa at m/z 361.
<table>
<thead>
<tr>
<th>m/z or cluster</th>
<th>Proposed assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>263</td>
<td>[LysNTA]^+</td>
</tr>
<tr>
<td>329</td>
<td>[LysNTAGa]^+</td>
</tr>
<tr>
<td>361</td>
<td>[LysNTAGa 2H2O]^+[LysNTAGa CH3OH]^+</td>
</tr>
<tr>
<td>515</td>
<td>[PDGF[LysNTAGa]^2+</td>
</tr>
<tr>
<td>591</td>
<td>[LysNTAGaProLeu CH3OH]^+</td>
</tr>
<tr>
<td>677</td>
<td>[(LysNTAGa)2 H2O]^+</td>
</tr>
<tr>
<td>702</td>
<td>[PDGF]^+</td>
</tr>
<tr>
<td>730</td>
<td>[(LysNTAGa)2·4H2O]^+</td>
</tr>
<tr>
<td>1030</td>
<td>[PDGF[LysNTAGa]^+</td>
</tr>
</tbody>
</table>

Table 7: Proposed identification of the major ions found in the full spectrum scan of Ga-LysNTA-PDGF according to observed m/z values.

Figure 27: Zoomed in scan showing the formation [LysNTAGaPDGF]^+ at m/z 1030.
To further confirm the formation of Ga-LysNTA-β-PDGF, the resulting entire isotopic pattern was selected at an isolation width of 8 Da and fragmented at collision energy of 15 eV, resulting in the MS/MS spectrum shown in Figure 28. Fragmentation of the complex resulted in daughter ions representing β-PDGF and the cluster at m/z 361-370 as described above.

![Figure 28: Product ion scan of [Ga-LysNTAPDGF]+ at m/z 1030 from LTQ Ion Trap MS.](image)

To confirm the structure of Ga-LysNTA-PDGF further molecular modelling was employed, this was used to confirm that the structure was energetically possible. The structure that was most favourable is shown in Figure 29. Due to the complexity of the molecule a two layer experiment was performed using an ONIOM calculation, which separated the study into real and model regions. The real region contains all the atoms in the molecule whereas the model region contains the reactive centre. The results of the ONIOM calculation showed that gallium is penta-coordinated to two carbonyl oxygen atoms from the LysNTA substrate and two carbonyl oxygen atoms from valine and methionine from the β-PDGF backbone, the final coordination bond was found to be to one of the phosphate oxygen atoms.
The viability of this procedure as a tagging method for phosphopeptide analysis was investigated using LC-ICP-MS. Separation was achieved with gradient elution and post column dilution. Organic phase dilution was needed to reduce the effect of carbon on signal intensity and reduce carbon loading on the ICP-MS cones. The binding of PDGF to Ga in the form of LysNTAGa was investigated by LC-ICP-MS, using conditions as used for the separation of tryptic peptides from β-casein. The resulting chromatograms are shown in Figure 30. The first shows untagged PDGF with a high Ga background removed after column cleaning, the second is the $^{31}$P trace of tagged PDGF showing the shift in retention time of the PDGF peak, and the third shows both the $^{69}$Ga and $^{31}$P peaks co-eluting indicating that binding was successful.
Figure 30: LC-ICP-MS chromatograms of A – Unlabelled PDGF showing the $^{31}\text{P}$ and $^{68}\text{Ga}$ signals, B – PDGF labelled with LysNTAGa, showing the $^{31}\text{P}$ signal only to highlight the unbound PDGF at the same retention time as in A and C – PDGF labelled with LysNTAGa showing both the $^{31}\text{P}$ and $^{68}\text{Ga}$ signals co-eluting.
5.4.4. CONCLUSIONS

The successful labelling of β-PDGF with gallium was demonstrated in this work, the labelling with gallium allowed detection through LC-ICP-MS. LC-ICP-MS data showed the co-elution of the $^{31}$P of the phosphorylated peptide and the $^{69}$Ga of the label, indicating successful Ga-LysNTA-β-PDGF formation. This was supported by identification of the labelled complex through ESI-MS$^a$ and accurate mass FT-MS data. Molecular modelling supported the MS data and revealed that just one of the phosphate oxygen atoms was targeted by the Ga centre, the other analyte derived coordination sites being provided by electron donating groups on the amino acid residues. This suggests that complex stability will vary from peptide to peptide and may go some way to explaining why there are diverse and sometimes contradictory reports in the literature regarding the efficiency and selectivity of metal affinity techniques.

5.5 INTERACTION OF OXALIPLATIN WITH CARNOSINE AND ANSERINE

5.5.1 INTRODUCTION

The aim of the research was to investigate the binding of endogenous peptides, namely carnosine and anserine with platinum anti-cancer drugs. The structure of oxaliplatin is shown in Figure 31.
The interaction of anti-cancer drugs with DNA has been of interest to this research group and the work by Zayed et. al. 10 produced significant data to show that the majority of the administered drug is found in the cell cytosol. Recent studies have shown that the activated form of oxaliplatin forms very stable complexes with GSH. 70 However, work by this group 116 and other research groups suggests that Pt binding to intracellular peptides through their terminal amino nitrogen atoms and carbonyl oxygen atoms are possible and energetically competitive to sulfur attachment. Due to these findings in research it was decided that investigating the binding between carnosine, anserine and oxaliplatin would be of significant interest as these peptides are small enough to allow in-depth investigation by mass spectrometry and molecular modelling and are found at high concentrations in cells.

5.5.2. RESEARCH UNDERTAKEN

To begin this investigation, carnosine and oxaliplatin were characterised by molecular mass spectrometry. The formation of the oxaliplatin:carnosine adduct was also characterised by mass spectrometry, the molecular ion was identified and subsequent daughter ions were investigated in order to determine the structure of the adduct. The exact m/z value of the parent ion and daughter ions were determined by high resolution mass spectrometry. This was performed to confirm the m/z of the daughter ions compared to theoretically calculated m/z values. Structures were elucidated from the m/z values, and from losses in mass due to collision induced dissociations. To further confirm that the carnosine:oxaliplatin adduct is a thermodynamically possible molecule, the most energetically stable conformer was found by molecular modelling. The in vitro effect of carnosine on oxaliplatin cytotoxicity was investigated by treating liver hepatocellular carcinoma (HepG2) cells, a perpetual carcinoma cell line. The viability of the cells was investigated by colleagues at The American University in Cairo. Finally density functional calculations were used to obtain structural information about the complex formed and starting materials.
5.5.3. RESEARCH FINDINGS

Carnosine, anserine and N-acetyl-carnosine with oxaliplatin experiments were performed on four different mass spectrometers, a Thermo linear ion trap (LTQ), a Thermo high resolution Exactive mass spectrometer, a Thermo Orbitrap and a Waters XEVO TQ tandem quadrupole mass spectrometer. The starting materials were first investigated and their mass spectra recorded. A mixture of oxaliplatin with carnosine, anserine and N-acetyl-carnosine were electrosprayed in the positive ion mode generating the mass spectra. However, only the results for the interaction between carnosine and oxaliplatin are discussed in detail in this thesis as the pattern for interaction is believed to be common between all three dipeptides. The mass spectrum of oxaliplatin bound to carnosine is shown in Figure 32.

Figure 32: Full scan MS spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time.
Research Undertaken and Key Findings

Claire Camp

Figure 33: Zoom in showing the isotope pattern of oxaliplatin obtained on the LTQ.

Figure 34: Zoom in of the isotope pattern resulting from the ion [carnosine+oxaliplatin+H]^+ obtained on the LTQ.

The cluster shown at m/z 624 is the parent ion [Carnosine+OxPt+H]^+, also seen in the mass spectra were ions belonging to oxaliplatin, m/z 398, shown in Figure 33 and carnosine, m/z 227 and the carnosine dimer, m/z 453. A MS2 mass spectrum is shown in Figure 35.
Figure 35: MS2 scan of carnosine coupled to oxaliplatin obtained on the LTQ.

The ion clusters shown in Figure 35 are expanded and shown in detail in Figure 36. The loss of carbon dioxide produced the first cluster at m/z 580, loss of the six membered ring leaving just diaminocyclohexane (dach) resulted in the second cluster at m/z 534, protonated oxaliplatin and the loss of carbon dioxide from oxaliplatin resulted in the last two clusters at m/z 398 and 354, respectively.
Molecular modelling experiments can be divided in two categories involving either direct Pt bonding to carnosine or electrostatic interactions between protonated carnosine and OxPt. In the first category, bonding involves the exchange of a single Pt-oxygen to a formal Pt-coordination to one of the electron rich sites such as one of the imidazole nitrogen atoms, a carbonyl oxygen or the terminal amino nitrogen of the dipeptide. The electrostatic category involves electrostatic interactions between the oxaliplatin molecule and protonated carnosine through hydrogen bonding which are responsible for keeping the resulting $[\text{Carnosine} + \text{OxPt} + \text{H}]^+$. The found structures from these two categories of experiments can be found in
Appendix C, Figure S12. The global minimum on the gas phase potential energy surface is structure 1A as shown in Figure 37, this structure is internally stabilised by four strong hydrogen bonds. In the gas phase structure 1A, the hydrogen bond between one of the NH hydrogen atoms of the dach ligand and the carboxylic oxygen of the terminal carboxylate group is replaced by external solvation in solution. The strongest hydrogen bonds in structure 1A, are between each of the hydrogen atoms of the protonated oxalate moieties and their respective terminal amino nitrogen atom of each of the carnosine dipeptides.

Figure 37: Structures for [Carnosine + OxPt + H]^+ ions in which a formal Pt coordination to carnosine is observed as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis. Italicized numbers are for solvated species.
2A is the lowest energy structure calculated for the [Carnosine + OxPt – CO$_2$ + H]$^+$ species observed as the m/z cluster around 580 in the MS$^2$ spectra shown in Figure 36. Structure 2A is stabilised by three internal hydrogen bonds and is shown in Figure 38.
Figure 38: Structure for the lowest energy \([\text{Carnosine} + \text{OxPt} – \text{CO}_2 + \text{H}]^+\) ion as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms.

Structure 3A is calculated to be the lowest structure for the \([\text{Carnosine} – \text{H} + \text{Pt(dach)}]^+\) species assigned as the cluster centered on \(m/z\) 534 in Figure 36. This assignment was further confirmed by the isotopic pattern obtained on the high resolution Q-Exactive FT-MS shown in S10 of Appendix C. The formation of the \([\text{Carnosine} – \text{H} + \text{Pt(dach)}]^+\) species from the \([\text{Carnosine} + \text{OxPt} – \text{CO}_2 + \text{H}]^+\) precursor is supported by the MS\(^3\) spectrum in Figure S7 clearly showing the cluster centred on \(m/z\) 534 being the dominant fragment due to the dissociation of the\([\text{Carnosine} + \text{OxPt} – \text{CO}_2 + \text{H}]^+\) precursor.
Figure 39: Structure for the lowest energy [Carnosine – H + Pt(dach)]$^+$ ion as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms.

The *in-vitro* investigation between carnosine and oxaliplatin, was carried out by colleagues at The American University in Cairo. The carcinoma HepG2 cells showed decreased viability with exposure to increased concentrations of OxPt. To examine the *in vitro* effect of carnosine on OxPt cytotoxicity, several solutions were prepared at a fixed OxPt concentration while varying the molar equivalent of carnosine. The results of these experiments indicated a positive correlation between cell viability and increasing molar equivalence of carnosine relative to OxPt. This suggested that carnosine can interact with the OxPt drug to form complexes that reduce the drug’s action.
To investigate the ability of carnosine to be passively diffused through cells, the same set of experiments were repeated. However the cells were first incubated with various concentrations of carnosine for 24 hours, after this time the media containing carnosine was discarded and replaced with carnosine free media and incubated for a further 24 hours. The results of these *in vitro* experiments indicate slightly increased cell viability upon the exposure to carnosine. The much less pronounced effect shown in Figure 3 relative to that shown in Figure 2 is most likely due to the inability of carnosine to effectively passively diffuse through the cells. The results obtained by the *in-vitro* experiments indicated that carnosine has the ability to interact with OxPt most likely through the chelation of the platinum metal.

5.5.4. CONCLUSIONS

The research carried out to investigate the interaction of oxaliplatin and carnosine suggested that the dipeptide carnosine may inhibit the cytotoxic action of OxPt, through the formation of complexes that are less cytotoxic than OxPt alone. Mass spectrometry with both electrospray ionization and chip nanospray were employed to study the interaction of oxaliplatin with carnosine and two of its derivatives, anserine and N-acetylcarnosine. The formation of a complex between oxaliplatin and the three dipeptides investigated was shown in this chapter and in Appendices 3 and 4. The majority of species observed were unambiguously assigned and compared to their theoretical isotopic patterns. It was found that the three complexes formed followed a common fragmentation pattern due to the collisionally-activated protonated complexes of each of the ligands examined with OxPt. The common fragmentation products were; \([\text{OxPt} - \text{CO}_2 + \text{H}]^+, [\text{OxPt} + \text{H}]^+, [\text{M} - \text{H} + \text{Pt(dach)}]^+, [\text{M} + \text{OxPt} - \text{CO}_2 + \text{H}]^+\) and \([\text{M} + \text{H}]^+\). Density functional calculations were used to obtain structural information and relative free energies of different isomers of the observed precursor \([\text{Carnosine} + \text{OxPt} + \text{H}]^+\) and the fragments produced. Data presented showed several binding modes between electron rich sites such as N and O centers of Carnosine and the Pt metal of OxPt.
5.6 SUMMARY

The aim of this chapter was to describe the key results of the research undertaken. The research undertaken was able to produce findings in three important bio-analytical areas by mass spectrometry. The research undertaken has demonstrated a reliable, accurate and sensitive method for the quantification of DNA, utilising the $^{31}$P label by LC-ICP-MS. Phosphate was further investigated as a binding moiety for gallium in a novel heterogeneous metal affinity tagging scheme, from which the binding strategy was shown to be mono-dentate with respect to phosphate as opposed to bi-dentate. Metal-peptide interactions were further investigated between platinum based anti-cancer drugs and small peptides; carnosine anserine and N-acetylcarnosine. The research illustrated the structures formed between the three small peptides and oxaliplatin by mass spectrometry with confirmation by molecular modelling.
6 CONCLUSIONS, IMPLICATIONS AND OPPORTUNITIES FOR FURTHER RESEARCH

6.1. INTRODUCTION

This chapter concludes this thesis by discussing the implications of the research on both the industrial supervisor and the wider industry. A critical evaluation of the overall research is also provided, together with the contribution offered by this research and recommendations for further improvements. The research conducted has been diverse, although under the common theme of mass spectrometry of small endogenous biomolecules. Findings and conclusions can be drawn under three themes, namely:

- Quantification of DNA
- Heterogeneous tagging of phosphopeptides with gallium
- Interaction of endogenous peptides with anti-cancer drugs
6.2. KEY FINDINGS OF THE RESEARCH

The key findings of the research by theme are discussed below.

6.2.1. QUANTIFICATION OF DNA

A successful methodology for the separations of all 5 common nucleotides was developed and used to quantify calf thymus DNA and validated against the SRM (NIST 2372). Limits of detection for phosphorus within individual nucleotides were calculated at comparable levels in other studies and mass balance for monophosphate nucleotides is possible. The research provided the sponsoring company with a method that could quantify DNA at low concentrations by measuring the total P signal from DNA by FIA-ICP-MS and by the summation of the P signal from the individual nucleotides following a simple digestion protocol of whole DNA and analysis by LC-ICP-MS.

6.2.2. HETEROGENEOUS TAGGING OF PHOSPHOPEPTIDES WITH GALLIUM

The research conducted showed that the metal ligand LysNTAGa successfully bound the phosphopeptide β-PDGF. Due to the nature of ICP-MS, the research demonstrated that Ga-LysNTA is a sensitive and highly selective label for the detection and analysis of phosphopeptides by LC-ICP-MS. Confirmation of the complex was achieved with ESI-MS and accurate mass FT-MS data and by comparison with theoretical spectra. Molecular modelling supported the MS data and revealed that just one of the phosphate oxygen atoms was targeted by the Ga centre, the other analyte derived coordination sites being provided by electron donating groups on the amino acid residues. This suggests that complex stability will vary from peptide to peptide and may go some way to explaining why there are diverse and sometimes contradictory reports in the literature regarding the efficiency and selectivity of metal affinity techniques.
6.2.3. BINDING OF CARNOSINE AND ANSERINE WITH OXALIPLATIN

The research showed that oxaliplatin does interact with both anserine and carnosine, which may be significant in determining why 80% of the administered drug remains in the cytosolic portion of cancer cells. This hypothesis was supported by the in-vitro experiment involving oxaliplatin and carnosine which suggested that carnosine may inhibit the cytotoxic action of oxaliplatin, through the formation of complexes that are less cytotoxic than oxaliplatin alone. The various mass spectrometry experiments between carnosine and oxaliplatin showed that there several binding modes between the electron rich sites such as N and O of Carnosine and the Pt metal of oxaliplatin.

6.3. CRITICAL EVALUATION OF THE RESEARCH

The aim of this research was to investigate the interaction of small bio-molecules with metals, in the form of tagging agents and drugs and also to quantify DNA. The above, coupled with the limited timeframe available for the EngD project, mean that there are certain limitations associated with this research, in addition to the many contributions and benefits to the sponsoring company and the research community, as discussed below.

6.3.1. CONTRIBUTION TO KNOWLEDGE

The quantification of DNA discussed in this thesis (Section 5.3) was the first that showed the conservation of mass by analysing the total $^{31}$P signal from single nucleotides peaks following digestion of calf thymus DNA and comparing it to whole DNA. It also provided the first quantification of DNA by LC-ICP-MS using a sector field instrument without the need for a specialised introduction system such as DIHEN.
The binding of the phosphate to gallium was shown by this research to be mono-dentate, previous studies have thought that the binding was bi-dentate through two oxygen atoms of the phosphate group. Many enrichment protocols for use in the analysis of phosphopeptides are compromised by non-selective binding by non-phosphopeptides that contain carboxylic acid groups in the peptide chain, the molecular modelling carried out in conjunction with this research suggests that the metal preferably binds to carboxylate groups. The research conducted which shows that the binding between the phosphate group and gallium is only mono-dentate could explain why there is so much variation in the enrichment strategies employed by other research groups. The benefit of this to other research groups is that it indicates other strategies for enrichment of phosphopeptides is needed and that combinations of enrichment strategies are likely to give a greater coverage of the phosphoproteome. Although this knowledge was already shown through studies using combinations of strategies, the reason why this was the case was not fully understood.

Other studies conducted in this research group showed that 75-78% of the total administered oxaliplatin drug remains in the cytosolic fraction of the cancer cell. Therefore the interactions of oxaliplatin with the peptides and proteins that make up the cytosol are of great importance. The research carried out in this thesis is the first time that the interaction between oxaliplatin and carnosine has been investigated.

6.3.2. IMPLICATIONS/IMPACT ON INDUSTRIAL SPONSOR

LGC Ltd’s main objective for sponsoring this research was to develop assays in line with their role as a National Measurement Institute. In view of this, the EngD project has not made as much of a positive contribution to the sponsoring company as hoped due to time limitations and instrument issues. However, the sponsor has been able to apply the methodology developed to another assay in order to improve the quality of the results, which facilitated the publication of a further research paper. This research paper quantifies oxaliplatin-nucleotide adducts by LC-ICP-MS with isotopically labelled oxaliplatin, the LC separation and the DNA digest conditions used in this work were based upon the findings from preliminary work carried out in this thesis.
6.3.3. IMPLICATIONS ON WIDER COMMUNITY AND INDUSTRY

The quantification of phosphopeptides is important as it allows the assessment of cellular phosphorylation due to the activity of kinases and phosphatases and cellular signal transduction pathways which is an important aspect of cell dynamics. Current methodologies for the quantitation of phosphopeptides are suited to analysis by molecular mass spectrometry by the inclusion of heavy isotopes, $^{13}$C, $^2$H, $^{15}$N. The advantages of a quantitation method for phosphopeptides by ICP-MS is that quantification can be achieved with inorganic standards as ICP-MS is non-species specific, which negates the need to create phosphopeptide calibration standards. Improvements in the heterogeneous tagging of phosphopeptides with a metal such as gallium could lead to a simple absolute quantitation method by LC-ICP-MS. The research described in this thesis is a basis from which this goal can be built on, by showing that the LysNTAGa tagging method is successful and that it can be used for LC-ICP-MS analysis of phosphopeptides.

Due to the work started in this thesis, the interaction of several small peptides and their sulfur analogues with oxaliplatin is being investigated by ESI-MS by the research group. These interactions are hypothesised to be of significance due to being N-rich, therefore acting as binding sites for oxaliplatin. It is thought that oxaliplatin is bound by a wide range of peptides and proteins in the cytosol and not just by a few select ‘sulfur sinks’ such as methionine.

6.4. RECOMMENDATIONS

As a result of the EngD research the following recommendations are made:

1. Apply methodology developed for the quantification of DNA to clinical DNA sample, to establish if the method is robust enough for clinical samples and not affected by extraneous sources of phosphate from DNA extraction methods.
2. Digest DNA to phosphate with acid digestion and compare to whole DNA $^{31}$P concentration by FIA-ICP-MS and enzymatically digested DNA $^{31}$P concentration. The method for digesting DNA to phosphate by acid digestion has been used in studies
estimating the number of nucleotide bases per Pt atom in cancer studies. This experiment would prove if the digestion of patient DNA to phosphate is a true indicator for DNA concentration.

3. Determine structure of the LysNTAGa-PDGF complex by NMR and X-ray crystallography, this will confirm the structure predicted by computational methods.

4. Tag different phosphopeptides with LysNTAGa and analyse by ESI-MS and compare the binding with non-phosphopeptides containing several carboxylic acid groups. More experiments to compare the binding efficiencies are needed to see if competition from carboxylate groups would compete with phosphopeptides.

5. Apply LysNTAGa tag to tryptic digest of β-casein and other phosphoproteins in a whole cell digest to assess how effective the tag is in a complex sample.

6. Develop LC-ICP-MS and LC-MS method for the analysis of carnosine-oxPt and anserine-oxPt this will allow these adducts to be identified and quantified from an enzymatic digest of cancer cell cytosol.

7. Identify specific binding points between oxaliplatin and carnosine by NMR and X-ray crystallography.

6.5. SUMMARY

This research project has investigated several small bio-molecules by both molecular and elemental mass spectrometry and their interactions with metals. Experiments have been performed to look at how to quantify these molecules and how they interact with metals, the findings show that bio-molecules can be quantified by LC-IC-MS with good accuracy, repeatability and not affected by extraneous sources of phosphate including RNA.
7 REFERENCES


References


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ANALYSIS OF MONO-PHOSPHATE NUCLEOTIDES AS A POTENTIAL METHOD FOR QUANTIFICATION OF DNA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY

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Keywords

Quantification, DNA, ICP-MS, Mass Balance, Nucleotides.
Abstract

The determination of total deoxyribonucleic acid (DNA) concentration is of great importance in many biological and bio-medical analyses. The quantification of DNA is traditionally performed by UV spectroscopy; however the results can be affected greatly by the sample matrix. The proposed method quantifies phosphorus in digested calf thymus DNA and human DNA by high performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS). The method presented showed excellent baseline separation between all 4 DNA mono-nucleotides and 5’UMP. Column recoveries ranging from 95% to 99% for phosphorus resulted in a mass balance of 95% ± 0.5% for standard nucleotides, determined by LC-ICP-MS, compared to total DNA determined by flow injection coupled to ICP-MS (FI-ICP-MS). The ability of LC-ICP-MS to act as an internal check that only DNA derived phosphorus was counted in the assay was demonstrated by establishing a mass balance between the total phosphorous signal from undigested DNA and that from the speciated DNA. The method for quantification was evaluated by analysis of NIST SRM 2372; a total speciated DNA recovery of 52.1 ng/µL, compared with an expected value of 53.6 ng/µL, was determined by external calibration. From repeat measurements a mass balance of 97% ± 0.5% for NIST DNA was achieved. The method limits of detection for individual nucleotides were determined between 0.8 to 1.7 µg L\(^{-1}\) (\(^{31}\)P) for individual nucleotides by LC-ICP-MS, and 360 ng L\(^{-1}\) for 5’AMP by direct nebulisation.
Introduction

As one of the most important life elements, phosphorus is an integral part of a wide range of biochemical functions and structures, most notably protein phosphorylation and as the backbone of DNA. The backbone of both RNA and DNA is formed from phosphodiester linkages and due to the fixed stoichiometry of phosphorus; the quantification of such macromolecules can be obtained via the $^{31}$P signal by ICP-MS. Accurate quantification of DNA is of great importance for many biological, clinical and microbiological studies. Traditionally the quantification of phosphorus in bio-molecules has been achieved by UV absorbance, fluorescence via the incorporation of dyes, or by counting $\beta$ radiation emission from radioisotope labelled $^{32}$P and $^{33}$P. Results acquired with spectrometric methods can be compromised by impurities in the sample, from RNA and metabolic molecules such as adenosine triphosphate (ATP) and require relatively large sample volumes [1]. The method presented here is able to distinguish contaminants of the DNA sample by differentiating between retention times. Recently, $^{31}$P, determined by ICP-MS, was used to estimate DNA yields in samples from patients undergoing Pt-based chemotherapy [2]. A robust and more accurate analytical method based on LC-ICP-MS could improve the reliability of such results.

Over the last ten years elemental mass spectrometry has been increasingly used for the quantification of metals and heteroatoms within biomolecules, including the quantitation of phosphorus within nucleotides, oligonucleotides and DNA. Elemental analysis is preferred over other techniques as it offers lower detection limits, a larger dynamic range, a response that is to a first approximation independent of molecular form, and potentially more accurate quantification at low concentrations by isotope dilution analysis. Both ICP-MS and ICP-OES have been utilised for the quantification of nucleotides, DNA, oligonucleotides and DNA adducts [3-15]. The quantification of oligonucleotides has been achieved by two different methods by Yang et al. [4] and Donald et al. [7], with the latter group digesting the oligonucleotides to mono-nucleosides and quantifying by isotope dilution electrospray mass spectrometry (ES-ID-MS), and comparing that value to the total $^{31}$P signal obtained from ICP-OES and the gravimetric value. Styrene oxideate and mephalen DNA adducts have been investigated by elemental mass spectrometry, by Edler et al. [12, 13] and styrene -7,8-oxide adducts by Siethoff et al. [11].
One of the most attractive characteristics of quantification by ICP-MS is the ability to mass balance the element of interest before and after speciation, however, because of the many different species of phosphorus within cells, quantifying total cell phosphorus content prior to separation is of limited value, as mass balance would not be achieved. Additionally, the quantification of phosphorus containing bio-molecules is hindered by the lack of suitable molecular internal standards and significant levels of background phosphorus within biological buffers. Furthermore, the detection of phosphorus by ICP-MS is difficult due to its high first ionisation potential (10.5 eV), resulting in only ~33% of the element being ionised, and polyatomic interferences caused by $^{14}\text{N}^{16}\text{O}^+1\text{H}^+$ and $^{15}\text{N}^{16}\text{O}^+$. These molecular interferences can be removed in a collision/reaction cell by the addition of a collision/reaction gas such as He or H$_2$, alternatively the element of interest can be reacted, e.g with O$_2$, so that the product ion is in an area of less interference, in the case of phosphorus by the formation of $^{31}\text{P}^{16}\text{O}$. The preferred solution is to use a sector field mass spectrometer that can resolve the spectral interferences and provides superior sensitivity.

Despite several publications citing the analysis of phosphorus from enzymatic digests of nucleic acid, the quantification of each nucleotide within such a digest had not been established until recently by Fujii et al [16] who employed capillary electrophoresis coupled to ICP-MS. Accurate quantification of phosphate within each nucleotide from a DNA digest requires complete and reproducible enzymatic digestion, under conditions compatible with the separation and detection method chosen. This work demonstrates for the first time that mass balance can be achieved for total DNA and the speciation of all 4 nucleotide standards by LC-ICP-MS.

**Experimental Section**

**Instrumental**

Two ICP-MS instruments were used in this study, the first being an Agilent 7500 ce (Agilent Technologies, Berkshire, UK) equipped with an octopole reaction cell system (ORS) and the second a Thermo Element 2 XR, sector field instrument (Thermo Scientific, Hemel
Appendix A

Claire Camp

Hempstead, UK); operating conditions are given in Table 1. The Agilent 7500ce was optimized with a standard solution of 1 ng mL\(^{-1}\) Be, Co, In, Pb, and U in normal mode, and further optimized in ORS mode for the flow rate of the reaction gases. The Element 2 XR was optimised with a standard solution of 1 ng mL\(^{-1}\) containing In, Li and Pb. An LTQ linear ion trap mass spectrometer was used in this study (Thermo Scientific, San Jose, USA) equipped with an electrospray ionisation (ESI) source operated in positive mode at 5.5 kV and 280 °C. N\(_2\) was used as the nebulisation gas with sheath, auxiliary and sweep gas flows set at 50, 10, and 10 arbitrary units, respectively.

<table>
<thead>
<tr>
<th>ICP-MS system</th>
<th>Thermo-Scientific Element 2 XR</th>
<th>Agilent 7500ce</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (kW)</td>
<td>1.25</td>
<td>1.57</td>
</tr>
<tr>
<td>Gas Flows (L min(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>15.5</td>
<td>15.00 (Make-up – 0.27)</td>
</tr>
<tr>
<td>Auxiliary</td>
<td>0.80</td>
<td>1.00</td>
</tr>
<tr>
<td>Nebuliser</td>
<td>1.13</td>
<td>0.87 (carrier)</td>
</tr>
<tr>
<td>Reaction gas (ORS mode)</td>
<td>N/A</td>
<td>Helium (3.1 mL min(^{-1}))</td>
</tr>
<tr>
<td>Resolution</td>
<td>Medium</td>
<td>O(_2) (25% in Argon, 3.1 mL min(^{-1}))</td>
</tr>
</tbody>
</table>

Table 1: Instrumental parameters for the elemental mass spectrometers

LC-ICP-MS was conducted using an Agilent 1100 system connected to the Agilent 7500 ce using a micro-flow nebuliser (Agilent), with a cooled Scott double pass spray chamber. The Element 2 XR was connected to an HPLC system, consisting of a solvent delivery pump and micro auto-sampler (Surveyor, Thermo Scientific, Hemel Hempstead, UK), via a 0.005"" peek tube, a cyclonic spray chamber (ESI) and poly(fluoroalkoxy) (PFA) nebuliser (Elemental Scientific, Omaha, USA).
Reagents

Ultra-pure water (> 18 MΩ) was obtained from a Milli Q Element system (Millipore, Herts, UK). Mobile phases were prepared by diluting triethylamine acetate (TEAA, HPLC grade, Fluka, Dorset, UK) in ultra-pure water; the pH was monitored using a Jenway pH meter. All other reagents and standards were purchased from Sigma Aldrich (Dorset, UK) including thymidine-5'-monophosphate (5'TMP > 98-100%), cytidine-5'-monophosphate sodium salt (5’CMPNa ≥ 99%), guanosine-5'-monophosphate sodium salt (5’GMPNa ≥ 97%), uridine-5'-monophosphate disodium salt (5’UMPNa₂ ≥ 97%), adenosine-5'-monophosphate (5’AMP Na ≥ 98%), Nuclease S₁ and calf thymus DNA.

Optimisation of separation conditions: The chromatographic method was developed from that of Profrock et al. [5], by altering the conditions to improve separation and changing the mobile phase from ammonium acetate to triethylamine acetate to improve compatibility with ICP-MS and using a C₁₈ column, with high mechanical strength due to a unique organo-silica grafting process making it compatible with high aqueous loading. All separations were made using a Phenomenex Gemini NX column (150x2.1mm, 5µm) at a flow rate of 0.20 mL/min and the column was kept at a constant 25°C. Isocratic separations were made using TEAA (5mM, pH 6.5) as the mobile phase and samples were injected onto the column at a volume of 2µL.

Calf thymus DNA enzymolysis. After parametric optimization, a total of 20 µg Plasmid DNA was heated to 80 °C for 5 min at 200W, cooled on ice and was then incubated at 50 °C for a further 5 minutes with 20 U µg⁻¹ Nuclease P₁ at 55W.

Quantification Strategy

This strategy exploited the compound independent signal sensitivity of ICP-MS which allows quantification of complex organic molecules by calibration with inorganic metal salts. Thus prior to speciation, the flow-injected signal magnitude (area under curve) measured by ICP-MS should be equal to the total signal magnitude after speciation, provided that there is
efficient recovery of species from the separation column. The proposed strategy for the quantification of calf thymus DNA is detailed in Figure 1.

Figure 1: Proposed quantification of calf thymus DNA work-flow
Results and Discussion

**Linearity and Robustness**: The data presented here were collected using LC-SF-ICP-MS, calibration curves were prepared for all 5 mono-phosphate nucleotides in the range of 5-15 ng P/g for inter-assay (n=3) and intra-assay (n=3). Figure 2 shows typical calibration data collected for d’AMP, d’CMP, d’GMP and TMP exhibiting good linearity with correlation coefficients of greater than 0.997. Application of one-way ANOVA showed there was no statistical difference (p=0.95) between the slopes for the 4 different nucleotides. The relative standard deviation (RSD) of the slopes for these calibration curves were 3.6% for the intra-assay and 6.5% for the inter-assay. Repeatability of injection was calculated as 0.2% RSD (n=6).

**Limits of Detection**: LOD’s for phosphorus for the standard nucleotides were calculated using the standard error of the regression data, as ranging from 0.8 to 1.7 µg/L, by LC-SF-ICP-MS, which are similar to results reported by Fujii et al. [17] from µLC-ICP-MS. A limit of detection of 20 pg of P on column was achieved. This is significantly higher than that reported by Fujii et al. [18], but if the method reported here were to be scaled down, further improvement in the absolute limits of detection would be obtained. Limits of detection were calculated as ranging from 2.4 to 9.1 µg/L, by ORS-ICP-MS with oxygen addition to the reaction cell, and 2.0 to 3.1 µg/L with helium addition. LOD’s ranging from 0.3 to 2.7 µg/L were calculated from the standard deviation of the blank, with helium addition to the octopole reaction cell. Whilst it was expected that the LOD’s obtained by the SF-ICP-MS instrument would be up to an order of magnitude lower than those obtained with the ORS-ICP-MS instrument, it was not observed as such. The high blank concentrations of phosphorus negated the better signal to noise ratio of sector field instrument.
Figure 2: A typical calibration chart for nucleotide standards

**Column recoveries:** These were determined for each nucleotide standard from the injection of 5 µL of the nucleotide standard with and without the column in place, the recoveries ranged from 95 to 98%, as shown in Table 2.

**Table 2: Recoveries of individual nucleotides using FI-ICP-MS**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>d’CMP</td>
<td>98.7 ± 0.5</td>
</tr>
<tr>
<td>d’TMP</td>
<td>97.8 ± 1.1</td>
</tr>
<tr>
<td>d’GMP</td>
<td>99.1 ± 1.2</td>
</tr>
<tr>
<td>d’AMP</td>
<td>98.4 ± 1.2</td>
</tr>
<tr>
<td>UMP</td>
<td>98.0 ± 3.1</td>
</tr>
</tbody>
</table>
Mass Balance

A solution containing 200 ng/g of each nucleotide was prepared in 20 mM TEAA at pH 6.4 and analysed by LC-ICP-MS using the conditions stated in Table 1. The total peak areas for the individual nucleotide standards and that of the void volume peak were summed and compared to the peak area of a flow injection peak for the same standard solution in order to calculate the mass balance. The LC-ICP-MS results show that a mass balance of $97.2\% \pm 0.5\%$ was achieved by this experiment, which was expected due to the high column recoveries observed. The mass balance calculation data is shown in Table 3 for both NIST SRM DNA and nucleotide standards. Errors were calculated from the standard error of the mean for each peak in the digest and for the repeated flow injection peaks, and then treated with the propagation of errors formula.
Table 3: Total peak areas for speciated nucleotide standards and digested NIST DNA and flow injection peak areas for nucleotide blend and undigested NIST DNA.

<table>
<thead>
<tr>
<th>NIST DNA</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Run</td>
<td>Total Peak Area / counts</td>
<td>Run</td>
</tr>
<tr>
<td>1</td>
<td>165538.4</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>160938.8</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>163914.6</td>
<td>3</td>
</tr>
<tr>
<td>Average</td>
<td>163463.9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mass Balance</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleotide Standards</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>Total Peak Area / counts</td>
<td>Run</td>
</tr>
<tr>
<td>1</td>
<td>11065522</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>11155169</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>11348798</td>
<td>3</td>
</tr>
<tr>
<td>Average</td>
<td>11189830</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mass Balance</td>
<td></td>
</tr>
</tbody>
</table>
Measuring total P as an indicator of DNA concentration is problematic as even in highly purified and well characterised samples, such as the NIST standard, there are likely to be P-containing residues from the purification procedure.

Figure 3 compares the separation of a mixture of mono-nucleotide standards and digested calf thymus DNA. The slight change in the observed retention times was due to the variation in the mobile phase pH, from non consecutive chromatograms, however, the peaks were positively identified by LC-MS. The unknown peak observed in both the nucleotide standards

A-12
and the DNA digest is thought to be the result of phosphate buffers used in the purification process. The presence of these buffers did not affect the quantification of DNA when speciation was used, because the first peak can be ignored, but would affect an assay based on total P where it would be included. This was proven by fraction collecting the individual nucleotide peaks from the Sigma standards, digesting to phosphate and quantifying by direct nebulisation into the ICP-MS. This experiment was also performed on the DNA digest. Chromatograms run including UMP in the mixture showed the UMP baseline separated with a retention time of approximately 4.8 minutes.

**LC-ESI-MS**, using the same separation conditions as for LC-ICP-MS, was used to positively identify each peak by analysis of a mixture containing all 4 DNA nucleotide standards as seen in Table 4. Comparison of parent ions from the standard run and that of the digest allowed the identification of 4 out of the 5 peaks in the DNA digest LC-ICP-MS chromatogram. As indicated above, it is believed that the unknown peak observed in both the standards and the digest are phosphates from biological buffers.

<table>
<thead>
<tr>
<th>Table 4: DNA digest positive peak identification by LC-ESI-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleotide Standards</strong></td>
</tr>
<tr>
<td>m/z</td>
</tr>
<tr>
<td><strong>Peak 1</strong></td>
</tr>
<tr>
<td><strong>Peak 3</strong></td>
</tr>
<tr>
<td><strong>Peak 5</strong></td>
</tr>
</tbody>
</table>

**Accuracy** of the quantification method was evaluated by the analysis of NIST SRM 2372, component B. Component B was digested using the procedure outlined above, diluted to a
final volume of 65 µL and analysed by LC-ICP-MS. The phosphorus content of the 4 nucleotides was calculated by external calibration with standard nucleotides from Sigma as; d’CMP 11.2 ±0.1 ng/µL, d’GMP 13.1±0.1 ng/µL, TMP 13.6 ±0.1 ng/µL and d’AMP 14.2 ±0.1 ng/µL, giving a total of 52.1 ng/µL. The stated DNA concentration for component B was 53.6 ±0.4 ng/µL, giving a recovery of 97.2%. To further confirm the mass balance, 3 repeat flow injection analyses and two repeats digest analyses (n=6) were performed. The summation of the peak areas of the digested DNA were compared to the peak area of undigested NIST DNA analysed by FI-ICP-MS which yielded a mass balance of 97 ± 0.5%. The unknown phosphate peak observed in the digest was included in the mass balance calculation.

Conclusions

Successful separation and P-selective detection of all 5 common DNA nucleotides within a timeframe of 20 minutes was achieved using reversed phase HPLC with ICP-MS detection. Limits of detection for phosphorus associated with individual nucleotides were found to be comparable with previously reported values, ranging between 0.8 to 1.7 µg L⁻¹. Furthermore, it has been shown that a full mass balance for monophosphate nucleotides with the newly developed method is achievable. The method was validated against NIST SRM 2372 with recoveries of 97.2 %. The results presented here have demonstrated that DNA can be quantified at low ng/g concentrations by measuring the total P concentration from DNA using FI-ICP-MS versus the summation of the P signal peak area from the individual nucleotides following a simple digestion of whole DNA and HPLC-ICP-MS analysis.

Acknowledgements

The authors would like to thank LGC, Teddington, UK for their support and DIUS and EPSRC for funding the project.
References

ICP-MS, ESI-MS and molecular modelling investigation of homogeneous gallium affinity tagging (HMAT) of phosphopeptides

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Abstract

Protein phosphorylation and de-phosphorylation is one of the most common signalling pathways within cells, it is involved in regulating cellular processes, mediating enzyme inhibition, protein-protein recognition and protein degradation. Compared with normal proteomics, phosphoproteomics poses some additional challenges requiring more initial separation and additional sensitivity to detect and quantify potentially ultra-low abundance species. In this work, a novel approach to the selective detection of phosphopeptides is described based on the incorporation of a metal tag, gallium–N,N-biscarboxymethyl lysine (Ga-LysNTA), in solution before separation and detection by liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS). Linear ion trap electrospray ionisation mass spectrometry (ESI-MS) was employed to study the interaction of the gallium tag with platelet derived growth factor beta receptor (β-PDGF), a small phosphopeptide. In addition high resolution Fourier transform mass spectrometry (FT-MS) was used for accurate mass determination and multistage tandem mass spectrometry of the gallium-β-PDGF complex identified the fragmentation pathway. Finally, molecular modelling was used to investigate the energetically favoured structures of both the Ga-LysNTA material and the β-PDGF-Ga-LysNTA complex.

Keywords: Phosphopeptides, metal tagging, ICP-MS, Ion-trap ESI-MS, DFT, ONIOM
Introduction

Protein phosphorylation is an important post-translational modification and plays an important role in the regulation of many systems within organisms. Irregular protein phosphorylation can result in diseases such as chronic myelomonocytic leukaemia and papillary renal cancer.[1] The characterisation of phosphorylation sites has been widely investigated by the use of tandem mass spectroscopy.[2-11] A typical phosphoproteome work-flow involves the purification of the protein of interest by gel electrophoresis or reversed phase chromatography, trypptic digestion, enrichment of phosphopeptides followed by analysis using liquid chromatography coupled to tandem mass spectrometry. Phosphopeptide identification by triple quadrupole mass spectrometry is achieved by two methods; neutral loss scans that identify phosphopeptides from the loss of $\text{H}_3\text{PO}_4$ or $\text{HPO}_3$ on collisional activation from phosphorylated serine and threonine residues or parent ion scans for the common product ions $\text{H}_2\text{PO}_4^-$ or $\text{PO}_3^-$. [12-14]

It is often necessary to enrich phosphopeptides following digestion of the target protein, due to their low concentrations and poor ionisation efficiencies in positive mode mass spectrometry.[15-29] Many different analytical techniques have been developed to specifically enrich phosphopeptides including Immobilised Metal Affinity Chromatography (IMAC), utilizing iron, gallium, and more recently titanium and zirconium. [15, 18, 29-46] The efficiency of each of these metals in selectively binding phosphopeptides has been extensively investigated many times with varying success. This has been attributed to the non-specific binding of the acidic amino acid residues asparagine and glutamine to the metal ions, the binding of hydrophobic residues to the gel bed and the elution of multiply phosphorylated peptides and impurities in the metal oxide material. [47,48] Research suggests that selectivity can also be dependent on the combination of resin bed and metal ion, with several studies showing that nitrilotriacetic acid (NTA) combined with Fe$^{3+}$ gave higher specificity for phosphopeptides than iminodiacetic acid (IDA) with Fe$^{3+}$ [49,50] however, when Ga$^{3+}$ was used the reverse was found to be true and IDA gave better specificity than NTA, [51] although Ga$^{3+}$ was found to have better selectivity overall. [51-54] Selectivity can also be improved by blocking acidic amino acid residues by methyl-esterification, [55] however there were two problems reported with this approach these being incomplete esterification [56] and increased complexity due to side reactions. [39] Additionally, selectivity for mono- and multiply-phosphorylated peptides is dependent on both the metal
ion and the pH of the loading and elution buffers. Reports have shown that Ti$^{2+}$ and Fe$^{3+}$ have a higher selectivity for mono-phosphorylated peptides. [57, 58] Peptides containing phosphorylated serine and threonine residues can be enriched and modified by methods based on Isotope Coded Affinity Tags, [10, 59] in which the phosphate group is eliminated by base hydrolysis and a biotin or mercaptoethanol group is introduced by Michael addition, then selectively detected by capture/elution with streptavidin or with sulphur specific antibodies. [12]

Blacken et al. [12, 58] reported the use of post separation column tagging of phosphopeptides with N,N-biscarboxymethyl-lysine ligand (Ga-LysNTA, C$_{10}$H$_{16}$N$_{2}$O$_{6}$Ga) prior to analysis by tandem mass spectrometry. We hypothesized that this tag could be used homogeneously in solution to selectively tag phosphopeptides followed by separation and analysis employing the very high selectivity and sensitivity of ICP-MS to quantify the Ga. For this to work as a viable analytical method the requirement is for a stable stoichiometry between the Ga and the phosphopeptide (notionally a 1:1 complex) and high selectivity to the phosphate group. In the original papers it was claimed that binding to the phosphate group was independent of the amino acid to which the phosphate group is bound. Because of the potential complexity of working with mixtures of phosphopeptides derived from tryptic digests, a model system was chosen that would lend itself to careful analysis by mass spectrometry supported by molecular modelling. We did not here consider using conventional structural analysis techniques such as XRD or NMR because in the analytical context this method has to work at the nmol level if it is to be useful and therefore we have restricted the experimental work to techniques that can function at that level.

The feasibility of using this homogeneous metal affinity tagging ‘HMAT’ of phosphopeptides in solution was investigated. HPLC coupled to high resolution, sector-field ICP-MS was used for the separation of the resultant complexes and detection of both $^{69}$Ga and $^{31}$P signals. Elemental mass spectrometry is preferred over other techniques as it offers lower detection limits, a larger dynamic range, a response that is to a first approximation independent of molecular form, and potentially more accurate quantification at low concentrations by isotope dilution analysis. Linear ion trap electrospray ionisation mass
spectrometry (ESI-MS) was employed to study the interaction of the gallium tag with platelet derived growth factor beta-receptor (β-PDGF) which is a cell surface receptor that is important in the regulation of cell proliferation, growth and development and is linked to many disease states including cancer. In addition high resolution Fourier transform mass spectrometry (FT-MS) was used for accurate mass determination and multistage tandem mass spectrometry of the gallium-β-PDGF complex formation and determination of the fragment pathway. Finally, molecular modelling was used to complement the experimental results and to obtain more detailed structural and thermodynamic data for the complexes formed.

Materials and methods

Instrumentation

Electrospray-MS. An LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, USA) and a high resolution Q-Exactive Fourier transform mass spectrometer (Thermo Scientific, San Jose, USA) were used in this study for mass spectrometric determinations. The LTQ-MS was equipped with an electrospray ionisation (ESI) source operated in positive mode at 5.5 kV and 280 ºC for the capillary temperature. Nitrogen was used as the nebulising gas with sheath, auxiliary and sweep gas flows set at 50, 10, and 10 arbitrary units, respectively. The Q-Exactive was equipped with a Tri Versa NanoMate ESI chip nanospray (Advion, New York, USA). Both instruments were calibrated using Ultramark® 1621, caffeine and Met-Arg-Phe-Ala (MFRA) in accordance with the manufacturer’s recommendations.

ICP-MS. Thermo Element 2 XR (Thermo Scientific, Hemel Hempstead, UK), with operating conditions as follows, an operating power of 1.25 kW, plasma gas flow of 15.50 L min⁻¹, nebuliser gas flow of 1.18 L min⁻¹ and an auxiliary flow of 0.85 L min⁻¹. The instrument was optimised with a standard solution containing 1 ng mL⁻¹ of In, Li and Pb. For LC-ICP-MS analysis, the column outlet was directly connected via a 0.005” peek tube to a poly(fluoroalkoxy), (PFA) LC nebuliser (Elemental Scientific, Omaha, USA). A cyclonic spray chamber (Elemental Scientific, Omaha, USA) was used throughout all analyses.
**Reversed-Phase HPLC system.** Reversed-phase separations were carried out using a Thermo Surveyor MS Pump Plus (San Jose, CA, USA) and sample injections were performed by a Thermo Micro AS (San Jose, CA, USA). The HPLC column used for the separation was a Supelco Ascentis Express Peptide C18 column (150 x 2.1mm, 2.7 µm).

**Materials**

\( \text{N}_\text{a}, \text{N}_\text{a}^- \text{Bis(carboxymethyl)-L-lysine hydrate (LysNTA, C}_{10}\text{H}_{18}\text{N}_{2}\text{O}_{6} \cdot \text{xH}_2\text{O} > 97\%}, \) Gallium (III) chloride (anhydrous, 99.99% trace metals free), ammonium acetate (99.99% trace metals free) and acetic acid were purchased from Sigma Aldrich (Dorset, England). Trypsin Gold (mass spectrometry grade) was purchased from Promega (Southampton, UK). Methanol (HPLC grade) was sourced from Thermo Fisher (Loughborough, UK). Water was purified to 18 MΩ using an Elix and Element System (Millipore, Billerica, Massachusetts, USA).

**Sample Preparation and Procedures**

**Formation of \( \beta\text{-PDGF-Ga-LysNTA} \):** LysNTA (5.2 mg), Ga\(_2\text{Cl}_6\) (4.7 mg) and \( \beta\text{-PDGF} \) (0.5 mg) were dissolved in 5 mL of 50:50 (v/v) methanol:water solution to give a final concentration of 0.1 mg mL\(^{-1}\) of \( \beta\text{-PDGF-Ga-LysNTA} \) with a 10 fold excess of LysNTA and Ga\(_2\text{Cl}_6\). For ESI-MS\(^{n}\) analysis further dilution was not necessary, for LC-ICP-MS analysis the sample was diluted in 50:50 (v/v) methanol:water solution to a concentration of 10 µg mL\(^{-1}\) of \( \beta\text{-PDGF-Ga-LysNTA} \) assuming a 100% tagging efficiency.

**Confirmation of \( \beta\text{-PDGF-Ga-LysNTA} \) formation by ESI-MS\(^{n}\).** To optimise signal intensity for the ions of interest, the LTQ Ion Max auto-tune optimisation was performed to set the values for the lens, quadrupole and Octopole voltages. Helium gas was used as the collision gas for collision-induced dissociation (CID) with the ion-trap held at a pressure of approximately 10\(^{-3}\) Torr. Fragmentation experiments were conducted by selecting the ion of interest with an isolation width of 8 Daltons, collisional activation was applied by setting the activation amplitude (the amplitude of the radiofrequency (RF)) at 15 – 25 % of the maximum voltage available (determined empirically) and an activation Q set at 0.25 units.
Sample solutions were infused at a flow rate of 10 µL min\(^{-1}\) through an electrospray probe using nitrogen as the nebulising gas, with sheath gas flows set to 10 arbitrary units.

**Confirmation of β-PDGF-Ga-LysNTA formation by LC-ICP-MS.** To confirm successful binding of Ga-LysNTA to β-PDGF, HPLC separation coupled to ICP-MS detection was performed employing a linear gradient over 15 mins from 5 % aqueous (Eluent A: 100 % H\(_2\)O with 0.05 % acetic acid) to 70 % organic (Eluent B: 100 % CH\(_3\)OH with 0.05 % acetic acid) at a total flow rate of 100 µL min\(^{-1}\). The column was allowed to re-equilibrate for 5 minutes between injections. Both mobile phases were spiked with 500 ppt of Mo to act as an internal standard to monitor changes in sensitivity due to the increase in organic solvent. The sample injection volume was 5 µL, which gave 50 ng of β-PDGF-Ga-LysNTA (34 ng of β-PDGF) on column. The column was heated to 30 °C for the length of the run. To decrease the concentration of organic solvent in the plasma, an additional flow of 700 µl min\(^{-1}\) of H\(_2\)O was added post-column. For the ICP-MS analysis; the power was set to 1.25 kW, nebuliser, auxiliary and plasma gases to 1.18, 0.85 and 15.50 L min\(^{-1}\) respectively and operated in ‘speed mode’ in medium resolution.

**Computational Methods**

All calculations were performed with Gaussian 09. [60] Initially structures were optimized without symmetry constraints by means of Hartree–Fock (HF) calculations using the STO3G basis set. These initial optimisations were then used as starting geometries for higher level calculations employing density functional theory (DFT) at the B3LYP level, [61] in conjunction with the electron core potential (ECP) double-zeta LANL2DZ basis set. [62, 63] All critical points were characterized by harmonic frequency calculations and were shown to be at minima. Total energies, zero point energies, thermal corrections and entropies are given in supplementary material. The relative computational tractability of this theoretical method makes it suitable for calculations of systems containing relatively large numbers of heavy atoms such as those under study here. Much more importantly however are the results of DFT calculations in systems involving transition metals, which are in many cases in better agreement with experimental data than those obtained from Hartree–Fock (HF) calculations.
A two-layer ONIOM (Quantum Mechanical (QM):Semi-Empirical (SE)) calculation at the (B3LYP/LANL2DZ:PM6) level was conducted on the lowest energy species found for the β-PDGF-Ga-LysNTA complex. In a two-layered calculation the complex under study is separated into model and real regions. The real region contains all the atoms of the complex and is thus calculated only at the lower level being the PM6 semi-empirical method. The PM6 method was chosen here as it has been shown to have suitable parameterization for biochemical systems and the capabilities of carrying out calculations on molecules containing transition metals such as the complex investigated here. [65] The model region on the other hand contains the reactive centre and any other part of the system that is of particular interest. Both higher level of theory employing the B3LYP/LANL2DZ method and lower level calculations using PM6 were carried out for the model region. The total energy of this system was obtained from these three independent calculations:

\[ E^{\text{ONIOM}} = E^{\text{real,SE}} + E^{\text{model,QM}} - E^{\text{model,SE}} \]  

(eq. 1)

When dividing the complex into the real and model regions care was exercised not to cut through multiple covalent bonds or aromatic systems. However, because there are single covalently bonded interactions between the two regions, hydrogen link atoms were chosen to saturate the open valence created.

Results and Discussion

Evidence for Ga-LysNTA tag formation

Evidence for the formation of Ga-LysNTA can be observed in the experimental data shown in Panel A of Figure 1. This experimental isotopic pattern compares very favourably with the theoretical isotopic pattern of Ga-LysNTA shown in Panel B of Figure 1. The formation of Ga-LysNTA was further confirmed by means of accurate mass FT-MS where the average
mass deviation was only 0.08 ppm (See Table 1) (as calculated according to equation 2 shown below) for the observed isotopic peaks that make up the experimental pattern for Ga-LysNTA.

\[ ppm = \left( \frac{M_{\text{exp}} - M_{\text{calc}}}{M_{\text{calc}}} \right) \times 10^6 \quad (eq. 2) \]

Calculations on the Ga-LysNTA\textsuperscript{+} species were initially performed at HF/STO3G, the lowest five energy species from these calculations were re-optimised at the B3LYP/LANL2DZ level of theory. The lowest energy conformation calculated for this species is structure 1A as shown in Figure 2. In this structure the gallium metal is tetra-coordinated, binding to three carbonyl oxygen atoms as well as to the backbone nitrogen of the LysNTA substrate. This binding arrangement, while serving to dissipate the large positive charge on the metal centre, also results in the formation of three distinct five membered heterocyclic rings with the gallium metal being at their intersection. Highlighted in Structure 1A is a strong hydrogen bond (1.595 Å as calculated at B3LYP/LANL2DZ) where the hydrogen atom formally resides on the terminal amino nitrogen and is shared with the closest carbonyl oxygen atom. This hydrogen bonding results in the formation of a pseudo-nine membered ring along the LysNTA backbone. The large number of rings that result due to this conformational geometry is responsible for one of the lowest entropy terms calculated out of all conformations considered for the Ga-LysNTA species as listed in supplementary materials (see Table S1). The next lowest energy species on this surface is Structure 1B being 10.3 kcal mol\textsuperscript{-1} higher in free energy, in this structure the gallium centre is also tetra-coordinated and is also at the intersection of three distinct heterocyclic rings. The coordinating sites to the gallium metal in Structure 1B are the terminal amino nitrogen, the backbone nitrogen as well as two of the carbonyl oxygen atoms of the LysNTA substrate. This geometric arrangement results in the formation of two of the smaller five membered heterocyclic rings and one larger eight membered heterocyclic ring. Structure 1C is calculated to be 37.6 kcal mol\textsuperscript{-1} higher in free energy on the potential energy surface relative to Structure 1A. This higher energy species shows a similar gallium metal coordination as that of Structure 1A being tetra-coordinated and binding to three carbonyl oxygen atoms as well as to the backbone nitrogen of the LysNTA substrate. The lack of formal coordination and the absence of any hydrogen bonding at the amino nitrogen in Structure 1C allows for a much
more extended backbone arrangement relative to that of Structure 1A. This is evident in the larger entropy term listed in supplementary materials for Structure 1C. Structure 1D is nearly identical to Structure 1C with the former having a gallium hydroxyl oxygen coordination substituting for a stronger coordination of the metal to one of carbonyl oxygen atoms of the LysNTA substrate in the latter. This difference in coordination makes Structure 1D seven kcal mol\(^{-1}\) higher in free energy relative to Structure 1C. The lowest energy structure calculated on this surface is Structure 1E, where the gallium metal is tri-coordinated, the metal centre binding to two carbonyl oxygen atoms and the backbone nitrogen of the LysNTA substrate. The gallium-carbonyl oxygen bonds in Structure 1E are calculated to be 1.758 and 1.754 Å which are much shorter and thus stronger than those found in Structure 1A being 1.787, 1.794 and 1.841 Å respectively. Structure 1E also features a strong hydrogen bond (1.493 Å as calculated at B3LYP/LANL2DZ) between a hydrogen atom which formally resides on the terminal amino nitrogen and a carbonyl oxygen atom. This hydrogen bonding helps to stabilise Structure 1E, however, the lower coordination number of gallium in this structure makes it nearly 60 kcal mol\(^{-1}\) higher in energy relative to Structure 1A. The remaining structures 1F, 1G and 1H shown in Figure 2 were the highest energy species calculated at HF/STO3G being 48.8, 57.0 and 127.3 kcal mol\(^{-1}\) higher in free energy respectively relative to Structure 1A and were thus not considered at the higher level of theory.

The presence of a water adduct to Ga-LysNTA was identified and confirmed by comparison of the experimental signal with the theoretical isotope pattern model and was found to be in excellent agreement with an average mass accuracy of 0.08 ppm (see Figure 3 and Table 1). An interesting cluster was observed at m/z 361-370, which is proposed to be due to the signals of LystNTAGa•CH\(_3\)OH and Ga-LysNTA•2H\(_2\)O adducts (see Figure 4). Closer inspection of this cluster and matching to modelled theoretical isotope patterns indicated that it consisted of a 2:1 ratio of [Ga-LysNTA•MeOH]\(^+\):[Ga-LysNTA•2H\(_2\)O]\(^+\). This suggests that the two acidic carbonyl groups have lost a hydrogen atom in order to form two ionic bonds to the gallium cation in the Ga-LysNTA complex; therefore the Ga-LysNTA is unlikely to accept lone pairs from remaining water molecules in the gas phase.
Evidence for $\beta$-PDGF Ga-LysNTA complex formation

The complexation of Ga-LysNTA with the phosphopeptide PDGF $\beta$-receptor, which has a primary structure of H-pTyr-Val-Pro-Met-Leu-OH, to form the Ga-LysNTA-$\beta$-PDGF adduct at m/z of 1030.3, was confirmed by comparison with the theoretical isotope pattern (see Figure 5) and by accurate mass FT-MS with an average mass accuracy of 4 ppm (see Table 1). The Ga-LysNTA and $\beta$-PDGF reactants are also visible in the mass spectrum (see Figure 5). Several other species which were observed in the mass spectrum were identified and summarised in Table 2. To further confirm the formation of Ga-LysNTA-$\beta$-PDGF, the resulting entire isotopic pattern was selected at an isolation width of 8 Da and fragmented at collision energy of 15 eV, resulting in the MS/MS spectrum shown in Figure 6. Fragmentation of the complex resulted in daughter ions representing $\beta$-PDGF and the cluster at m/z 361-370 as described above.

Complementary evidence for the formation of Ga-LysNTA-$\beta$-PDGF was obtained by means of HPLC-ICP-MS, using the conditions outlined in the methods section. The chromatogram obtained shows a single peak in the $^{31}$P signal trace at a retention time of 3.89 mins which corresponded to an exact retention time match to that of the complexed $^{69}$Ga signal peak, confirming the formation of Ga-LysNTA-$\beta$-PDGF, see Figure 7. The earlier eluting $^{69}$Ga peak at 3.13 mins, was most likely due to unbound Ga-LysNTA as confirmed from a separate LC-ICP-MS run of Ga-LysNTA in the absence of $\beta$-PDGF (data not shown). The sample introduction system was not optimised for sensitivity and so an 8 fold post column dilution was employed to reduce plasma instability due to the high concentration of methanol in the mobile phase. The detection limit for Ga (calculated from three times the standard error in the curve) was 0.5 ng ml$^{-1}$ which for a 10 µl injection equates to 5 pg Ga or ~50 pg of PDGF. The $^{98}$Mo signal remained constant throughout the chromatographic run as would be expected given that the gradient eluent was diluted 8 fold post column.

Computational modelling of this complex between the Ga-LysNTA species and the phosphopeptide PDGF $\beta$-receptor was performed in order to gain insight into the structure. Three conformations of this complex were calculated at HF/STO3G while only two of these
structures were calculated at the higher B3LYP/LANL2DZ level (see the results listed in Table S1). As observed before for the Ga-LysNTA species calculated in this work, the relative free energy ordering of the conformers calculated did not change from the lower HF method to the higher level of theory employed here. The most favoured of the three structures energetically is Structure 2A as shown in Figure 8 in which the gallium metal is a penta-coordinate centre. In this structure the gallium centre binds to two carbonyl oxygen atoms of the LysNTA substrate which does not exhibit any formal binding or hydrogen bonding at its terminal amino nitrogen resulting in an extended open arrangement for backbone of the LysNTA substrate in the formed complex. Structure 2A also shows the gallium centre binding to the two carbonyl oxygen atoms of the valine and methionine residues of the phosphopeptide PDGF β-receptor while the last coordinating site to the gallium metal is one of the oxygen atoms of the phosphate moiety which replaces the hydrogen of the hydroxyl group of the tyrosine residue. This observation is extremely important as it throws some light on why the literature offers diverse and sometimes contradictory information about the efficiency and selectivity of IMAC (and in this case the related HMAT) techniques. Whilst the Ga certainly does target the phosphate group, it only uses one of the phosphate oxygens with the other analyte derived coordination sites being provided by electron donating centres on the amino acid residues of the peptide. The inference here (which would take a much larger study to verify) is that Ga tagging complexes, such as the one described here, can successfully target the phosphate group, but that the stability of the complex will also depend on the residue sequence of the peptide. It is also possible, though not investigated here, that weaker complexes could form with peptides that do not contain phosphate. This would be revealed by an increased complexity in the elemental chromatogram, but could be controlled by varying the experimental conditions or chemical blocking of competing groups. A group of five internal hydrogen bonds help not only to stabilize Structure 2A but also to result in a somewhat constricted configuration. Although we expect 6 coordination for Ga, efforts to obtain structures with higher coordination at the gallium centre were not successful; in fact, the other two structures calculated for this complex each involved either a tri- or a tetra-coordinated gallium metal and were shown (see HF data listed in Table S1) not to be very energetically competitive. This is most likely due to steric crowding given the bulky ligands that are surrounding the Ga. The complex in which the gallium metal is tetra-coordinated was calculated at the higher level of theory employed here. This structure 2B (not shown, see Table S1) featured a near
tetrahedral gallium centre binding to two carbonyl oxygen atoms of the LysNTA ligand and two of the oxygen atoms of the phosphate group on the PDGF β-receptor. It also features six internal hydrogen bonds making for a very compact geometry and was calculated to be 27.5 kcal mol\(^{-1}\) higher in free energy relative to Structure 2A. Finally, a two layer ONIOM method was used to model Structure 2A shown in Figure 8, predominantly in order to determine the feasibility of this method for examining larger transition metal containing complexes to be investigated in future works. The selection of the high layer, modelled at B3LYP/LANL2DZ, included the active portion of the system the gallium centre and its coordinating sites, the phosphate group as well as all atoms involved in hydrogen bonding, the remainder of the complex on the other hand was treated using semi-empirical techniques employing the PM6 method. Of major interest are the ONIOM obtained geometries which were shown in Figure 8 to be very similar to those resulting from the B3LYP/LANL2DZ calculations. This provides us with good indications that such two layered calculations may be used in the future to obtain relative energies of different conformers of larger transition metal-containing complexes provided that adequate care is taken to include exactly the same numbers and types of atoms in the high layer in each of the conformers examined.

**Conclusions**

This work demonstrates that Ga-LysNTA can be used to tag the phosphate moiety in a model phosphopeptide thereby enabling the detection of the peptide through the Ga atom by ICP-MS. LC-ICP-MS data showed the co-elution of the \(^{31}\)P of the phosphorylated peptide and the \(^{69}\)Ga of the label, indicating successful Ga-LysNTA-β-PDGF formation. Unambiguous identification of the labelled complex was achieved through ESI-MS\(^n\) and accurate mass FT-MS data. Excellent correlation with the theoretically predicted spectra were shown (see Figure 5). Molecular modelling supported the MS data and revealed that just one of the phosphate oxygen atoms was targeted by the Ga centre, the other analyte derived coordination sites being provided by electron donating groups on the amino acid residues. This suggests that complex stability will vary from peptide to peptide and may go some way to explaining why there are diverse and sometimes contradictory reports in the literature regarding the efficiency and selectivity of metal affinity techniques.
Future work will aim to investigate further the issue of selectivity using different peptide sequences and tryptic digests of phosphopeptides. The ultimate aim is to quantify phosphopeptides by elemental mass spectrometry using isotope dilution analysis with spiked gallium.

Acknowledgements

The authors would like to thank the EPSRC, LGC ltd., the Centre for Analytical Science at Loughborough University, the American University in Cairo and the Centre for Innovative and Collaborative Engineering at Loughborough University for the funding sponsorship and provision of resources for the project.
Figure 1: Signal due to Ga-LysNTA tag from LTQ Ion Trap MS; Panel A experimental data, Panel B theoretically predicted spectrum at a resolving power of 1500 m/Δm.
Figure 2: Structures for the Ga-LysNTA singly charged positive ions as calculated at the HF/STO3G and B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies (in kcal per mol) are indicated in parenthesis.
Figure 3: Formation of [Ga-LysNTA.H$_2$O]$^+$ from Q-Exactive FTMS; Panel A experimental data, Panel B theoretically predicted spectra.
Figure 4: Panel A experimental signal due to [Ga-LysNTA]^+ adducts with H$_2$O and CH$_3$OH from LTQ Ion Trap MS, Panel B theoretically predicted composite spectrum of a 1:2 ratio of [Ga-LysNTA.2H$_2$O]^+ and [Ga-LysNTA.CH$_3$OH]^+ at a resolving power of 1000 m/Δm.
Figure 5: Full scan spectra of reaction mixture from LTQ Ion Trap MS showing evidence of 
[Ga-LysNTAPDGF]^+ formation (main figure, insert A experimental data, insert B 
theoretically predicted spectra) at a resolving power of 1700 m/Δm.
Figure 6: Product ion scan of [Ga-LysNTAPDGF]$^+$ at m/z 1030 from LTQ Ion Trap MS.
Figure 7: LC-ICP-MS chromatogram of [Ga-LysNTAPDGFl$^+$ from Element 2XR; Panel A signal from $^{31}$P, Panel B signal from $^{69}$Ga.
Figure 8: the lowest energy structure 2A calculated for the Ga-LysNTA-β-PDGF singly charged positive ions as calculated using the ONIOM method at B3LYP/LANL2DZ:PM6 and the B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.
Table S1: Electronic energies, Zero-Point Vibrational Energies, Thermal Energies, Entropies and relative free energies for species calculated at HF/STO3G and B3LYP/LANL2DZ (italicized values). ONIOM values for species 2A are listed in bold within and underneath the table.

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### Appendix B

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**ONIOM: PM6 model system energy:** -0.612107102528

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**ONIOM: PM6 real system energy:** -1.082139387972

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Table 1: Summary of the mass accuracy for all proposed assignments of LysNTAGa with methanol and water adducts and PDGF.

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Table 2: Summary of the proposed assignments of LysNTAGa-PDGF full mass spectra scan, showing both starting materials.

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References


Oxaliplatin Complexes with Carnosine and its Derivatives: *In Vitro* cytotoxicity, Mass Spectrometric and Computational Studies with a focus on Complex Fragmentation.

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Abstract

The complexation of the Pt-based anti-cancer drug oxaliplatin (OxPt) with biological ligands other than DNA is believed to be a major cellular sink for the drug reducing its therapeutic potential and acting as a potential cause of toxicity. In this paper, an in vitro study on hepatocellular carcinoma HepG2 cells suggests that the naturally abundant cytoplasmic dipeptide ligand β-alanyl-L-histidine dipeptide (carnosine) may inhibit the cytotoxic action of OxPt most likely through the formation of complexes that are less cytotoxic than OxPt alone. Evidence is provided to suggest that pre-exposure of HepG2 cells to elevated levels of carnosine appears to have a lasting effect on reducing the cytotoxicity of OxPt even after the removal of the carnosine. This effect, however, is shown to be under kinetic control as its magnitude was shown not to vary significantly with the level of carnosine exposure within the concentration range used in this study. Various mass spectrometry techniques employing electrospray ionization and chip nanospray were employed to study the interaction of oxaliplatin with carnosine as well as two of its derivatives being β-alanyl-N-methylhistidine (anserine) and N-Acetylcarnosine (NAC). Evidence of complexation between OxPt and each of the three ligands examined is presented. Most species observed were unambiguously assigned and compared to their theoretical isotopic patterns. Common fragmentation products due to the collisionally-activated protonated complexes of each of the ligands examined with OxPt, [M + OxPt + H]^+ where M= carnosine, anserine or NAC were reported. Density functional calculations at B3LYP/LANL2DZ were used to obtain structural information and relative free energies of different isomers of the observed precursor [Carnosine + OxPt + H]^+ both in the gas phase and in solution as well as to probe its fragmentation, highlighting plausible fragmentation mechanisms that account for all the experimental results.

Data are presented to show several binding modes between electron rich sites such as N and O centers of carnosine and the Pt metal of OxPt. Calculations were also employed to obtain proton affinities and free energies of key reactions. The proton affinities of carnosine, Anserine and NAC at 298 K were calculated to be 254.4, 255.9 and 250.2 kcal mol\(^{-1}\) respectively. To the best of our knowledge the proton affinities of anserine and N-acetyl-carnosine are the first reported values in the literature.

Introduction
Oxaliplatin (OxPt) is a third generation chemotherapeutic drug used in the treatment of colorectal cancer and cisplatin resistant cancers, [1-13] it was developed to overcome the nephrotoxicity of cisplatin and myelosuppression with carboplatin treatment. [14] Oxaliplatin has a broader range of activity than cisplatin and increased cytotoxicity over cis and carboplatin due to increased inhibition of DNA synthesis and greater efficiency in DNA repair. [13, 15, 16] The amine groups of cisplatin are replaced by diaminocyclohexane (dach) to form [Pt(dach)oxalate], oxaliplatin. The dach ligand is not recognised by mismatch repair complexes, which may explain its effectiveness in the treatment of cisplatin and carboplatin resistant cancers. [15]

The transport and interactions of oxaliplatin within cells is still not fully understood however, the simplistic route from blood to nuclear DNA involves the passive diffusion of oxaliplatin into the cell from the extracellular fluid where the Cl\(^-\) concentration is high (> 100 mM) into the cytoplasm, where the Cl\(^-\) concentration is low (4-20 mM). [17] The low Cl\(^-\) concentration in the cytoplasm results in the solvation of oxaliplatin, to its active forms [Pt(OH\(_2\))Cl(dach)]\(^+\) and [Pt(OH\(_2\))\(_2\)(dach)]\(^2+\)[11,13], shown in Figure 1 and can interact with a plethora of non-DNA ligands. Based on previous work by Zayed et. al.[18] we can assume that the majority of the administered drug is found in the cell cytosol. It is thought that the most likely sites for oxaliplatin interaction are the thiol groups of intercellular peptides and proteins. Recent studies have shown that the activated form of oxaliplatin forms very stable complexes with glutathione, being the most abundant low molecular weight thiol containing molecule in human cells. [19-21] However, work by this group [21, 22] and elsewhere [22, 23] suggests that Pt binding to intercellular peptides through their terminal amino nitrogen atoms and carbonyl oxygen atoms are possible and energetically competitive to sulphur attachment. This in combination with the large excess of these binding sites over those of sulphur raises the possibility of Pt adduction to non-thiol containing ligands in the proteome. All such non-DNA interactions are responsible for chemo-resistance and many of the side effects; including sensory-motor neuropathy, myelosuppression, gastrointestinal toxicity, idiosyncratic and hypersensitivity reactions. [24]

The endogenous dipeptide L-carnosine (β-alanyl-L-histidine) was discovered by Gulewitsch and Amiradzibi [25] from muscle tissue extracts in 1900 [26, 27] and is found in several organs including the brain (olfactory bulb and hippocampus) [28], cardiac muscle, kidney and stomach.[29, 30] In the skeletal muscle and nerve cells it is found at concentrations of up to...
20 mM.[31, 32] Since its discovery there have been many investigations into its biological function and it is now known that carnosine acts as an anti-oxidant, scavenger of free radicals and active oxygen species [30], as a biological buffer, a source for histidine, an immuno-stimulant and transition metal ion chelator, especially for Zn^{2+} and Cu^{2+} [33] and heavy metals. [34] The ability of carnosine to bind Zn^{2+} helps modulate neuronal excitability by preventing the Zn^{2+} inhibition of neurotransmitter receptors. [35] Carnosine is also effective in the prevention and partial reversal of cataracts [36] and in the treatment of Wilsons disease [37] and Alzheimers due to its metal chelating and free radical scavenging ability and also acting as a β-amyloid toxicity inhibitor. [35, 38] It has also been shown that carnosine acts as a neuroprotector [39, 40] and can retard tumour growth in mouse models. [41] More recently it has been reported that carnosine can prevent cell proliferation in colon cancer cells. [47, 48] The related dipeptides N-acetylcarnosine and anserine share many of the beneficial characteristics of carnosine. The N-acetylated form of carnosine, N-acetyl-carnosine has been shown to be effective in the treatment of stomach ulcers [42] and similarly to carnosine has been used in the treatment of cataracts. [43, 44] Anserine on the other hand, acts a chelator for copper and as an antioxidant as well as enhances the anti-tumour activity of doxorubicin. [45] Due to its presence in high abundance in the cytosolic fractions of cells [30] and its ability to scavenge transition metal elements we believe that the interactions between carnosine and oxaliplatin are of important scientific value. The investigation of the coordination chemistry of carnosine and oxaliplatin may provide new understanding in the mechanism of the cellular response to these drugs. Given that there are 1-5 Pt adducts per 10^6 nucleotides in patient white blood cells which represents approximately 10% of the total administered drug. [18] Assuming all nuclear Pt is bound to DNA, representing approximately 10^5 Pt atoms per cell, the majority of the administered drug is available to bind to other ligands such as proteins and peptides within the cell. With a large concentration of proteins per cell, approximately 10^6-10^7, [46] many with multiple binding sites, the potential for a wide range of Pt adducts to exist within the cell rather than specific Pt targets is a possible scenario. The interactions of carnosine and the related dipeptides anserine and N-acetylcarnosine with Oxaliplatin are therefore of great interest. By using a simple highly abundant dipeptide, such as carnosine to act as a simplified model of the broader Pt-meallome, we present detailed structural analysis of carnosine and the hydrolysed forms of oxaliplatin.
Appendix C

Claire Camp

**Instrumentation**

An LTQ linear ion trap mass spectrometer with an electrospray source and a high resolution Q-Exactive Fourier transform mass spectrometer (Thermo Electron, San Jose, CA, USA) were used. Both instruments were calibrated using Ultramark® 1621, caffeine and Met-Arg-Phe-Ala (MFRA) in accordance with the manufacturer’s recommendations. The Q-Exactive was equipped with a Tri Versa NanoMate ESI chip nanospray (Advion, New York, USA). For the LTQ Resolving powers achieved were in the order of 1500 while the upper instrumental error limit in measurements was 0.2 m/z units. The LTQ auto-tune routine was used to obtain lens, quadrupole and Octopole voltages for maximum transmission of the ions of interest. Helium gas, admitted into the ion trap at a maintained pressure of approximately $10^{-3}$ Torr, was used as the buffer gas to improve the trapping efficiency and as the collision gas for collision-induced dissociation (CID) experiments performed here at 15 eV in the lab frame. Experiments designed to elucidate ion structures or fragmentation pathways on the LTQ were performed as follows: the ion of interest was selected then collisionally activated by setting the activation amplitude at 25–35% of the maximum voltage available (determined empirically), and the activation Q setting (used to adjust the frequency of the RF excitation voltage) was set at 0.25 units. Sample solutions were continuously infused at a flow rate of 5 µl min$^{-1}$ into the pneumatically assisted electrospray probe using dry nitrogen as the nebulising gas. Auxiliary and sheath gases were tuned daily for maximum signal transmission. Two tandem quadrupole mass spectrometers, An Acquity TQ and A XEVO TQ (Waters, MA, USA) both equipped with electrospray ionisation interface were also used in this work. Both triple quadrupoles were operated in positive ion mode, with typical values of cone, capillary and extractor voltages set to 30, 2500, and 3 respectively. The desolvation gas was usually set at a flow of 250 L h$^{-1}$ and a temperature of 150 °C. Argon was used as the collision gas at a typical flow rate of 0.15 ml min$^{-1}$. Tandem mass spectra were obtained at collision energies in the range 5-25 eV in the lab frame having both Q1 and Q3 operated at unit resolution with typical a dwell time of 25 millisecond per transition.
Reagents

Carnosine, Anserine, HPLC-grade water and methanol were all purchased from Sigma-Aldrich, UK. N-Acetyl carnosine was purchased from Nanjing Gemsen international, China while oxaliplatin was obtained from Sanofi-Synthelabo Limited (Guildford, UK).

*In Vitro* cell viability assays

To determine the *in vitro* effect of carnosine, the hepatocellular carcinoma cell line HepG2 was exposed to varying concentrations of OxPt, as well as several solution mixtures of OxPt and carnosine. The viability of HepG2 was assessed through MTT assay, which utilizes the conversion of the tetrazolium salt MTT (dimethylthiazol diphenyl tetrazolium bromide purchased from Serva, Germany) to formazan by dehydrogenase enzymes in living cells. [49] In brief, cells were cultured in 96-well plate (30,000 cells/well) at 37 °C humidified with 5% CO₂ in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FBS (Fetal bovine serum) and 5% Penicillin-Streptomycin mixture (Lonza, Switzerland). Cell counting was performed using Countess® Automated cell counter (Invitrogen). Solutions of OxPt at 8, 12, 16, 20, 24 and 28 μg ml⁻¹, as well as solution mixtures at varying molar ratios of carnosine at a fixed OxPt concentration of 24 μg ml⁻¹ were made in the culture media. Each of the sample solutions made was incubated with the HepG2 cells for 24 hrs, wells containing cells treated only with media served as controls. After incubation the media was discarded, 20 μl MTT (5 mg/ml) and 100 μl fresh media were added to each well and incubated for 3 hours, all media was then discarded, and the purple formazan crystals formed were solubilized via the addition of 100 μl DMSO (Sigma-Aldrich, USA). The absorbance of each well was measured at 595 nm using a microplate reader FLUOstar OPTIMA (BMG LabTech, Germany). Cell viability was determined by calculating the absorbance of the test wells as a percentage of the control wells.

Results and discussion

HepG2 cells showed decreased viability with exposure to increased concentrations of OxPt as shown in Figure 1. The most significant changes in cell viability were observed at exposure to OxPt concentrations of 16 μg ml⁻¹ and greater as indicated by *P* values < 0.00001. To
examine the *in vitro* effect of carnosine on OxPt cytotoxicity, several solutions were prepared at a fixed OxPt concentration of 24 μg ml\(^{-1}\) while varying the molar equivalent of carnosine. While the choice of OxPt concentration in these experiments was arbitrary, a value was chosen that corresponds to relatively low cell viability in order to easily observe the effects of subsequent addition of carnosine. It is also important to note that the OxPt concentration value chosen for these set of experiments was one that brought the most statistically significant decrease in the viability of the treated HepG2 cells as indicated by *P* values < 0.00001 (see Figure 1). The results of these experiments is shown in Figure 2 which points to a positive correlation between cell viability and increasing molar equivalence of carnosine relative to OxPt. These differences were much less pronounced past the initial incremental addition of carnosine where a significant increase in cell viability was shown due to the exposure of a 1:1 molar ratio of carnosine to OxPt relative to cells only exposed to OxPt at the same concentration and for the same incubation time. This was shown to be statistically significant with *P* value < 0.00001 at all non-zero molar ratios of carnosine to OxPt used. This result suggests that carnosine can interact with the OxPt drug forming complexes that reduce the drug’s action.

In order to investigate the ability of carnosine to be passively diffused through cells, the same set of experiments were repeated but now cells were first incubated for 24 hours with carnosine at various concentrations. After this incubation period, the media containing carnosine was discarded and fresh media containing 24 μg ml\(^{-1}\) of OxPt were added and allowed to incubate with the cells for a further 24 hour period. The results of these last set of *in vitro* experiment shown in Figure 3 indicate slight increased cell viability upon the exposure to carnosine. The much less pronounced effect shown in Figure 3 relative to that shown in Figure 2 is most likely due to the inability of carnosine to effectively passively diffuse through the cells. This also suggests that the process is kinetically controlled and is consistent with the near constant cell viability data obtained whenever the carnosine was present irrespective of the concentration at which it was allowed to incubate with the cells. While no claim is made that the *in vitro* data presented here replicates biological conditions in which OxPt and carnosine might interact, they do however indicate that the dipeptide carnosine has the ability to interact with OxPt most likely through the chelation of the platinum metal and thus if such interaction were present it may reduce the cytotoxic action of OxPt and thus warrants the investigation of such complexes.
To study the complexes of each of carnosine and its derivatives, anserine and N-acetyl-carnosine with oxaliplatin experiments were performed on four different mass spectrometers as detailed in the Instrumentation section. For the interest of conciseness, only selected representative results are shown here, however, all data are available in supplementary material. All sample solutions employed in this work were a 2 : 1 mM mixtures of either carnosine, anserine or N-acetyl-carnosine with oxaliplatin in a 1 : 1 (v/v) water/methanol solutions. Electrospraying each of these three solutions in the positive ion mode generated the mass spectra shown in Figures 4-6 (also see Figures S1 and S2 in supplementary material). In all cases, irrespective of the instrument used, the base peaks obtained were due to [M + H]^+ ions where M = carnosine, anserine or N-acetyl-carnosine. These ions corresponded to the signals at m/z values of 227, 241 and 269 shown in Figures 4, 5 and 6 (also see Figures S1 and S2) respectively. Three other common ions were observed in each of the full scan MS spectra of the three solution mixtures examined here. Signals due to protonated oxaliplatin, [OxPt + H]^+, were always present as evidenced by the clusters observed in all full scan MS spectra obtained at around m/z 398. The lowest energy structure calculated for this protonated species was previously reported to have the external proton located on the ‘inside’ position of one of the oxalate carbonyl oxygen atoms. [21] Protonated ligand dimers, [M2 + H]^+ ions, were also observed at m/z 453, 481 and 537 as seen in Figures 4, 5 and 6 (also see Figures S1 and S2). The third ion observed in all full scan MS spectra obtained was due to the [M + OxPt + H]^+ species as seen in the clusters around m/z 624, 637 and 666 in Figures 4 (also see Figure S1), 5 (also see Figure S2), and 6.

The assignments of these ions were confirmed by comparing the observed isotopic patterns to those theoretically modelled for each of the proposed species. For further confirmation the isotopic patterns for the proposed [Carnosine + OxPt + H]^+ and [Anserine + OxPt + H]^+ species being the focus of this study were obtained on the Q-Exactive FT-MS at a resolving power of about 60,000 and compared to their theoretical isotopic patterns at an equivalent resolution, the average error obtained over all the isotopic peaks observed were 2.13 and 1.48 ppm for [Carnosine + OxPt + H]^+ and [Anserine + OxPt + H]^+ respectively. This mass accuracy obtained without the use of lock masses provided unequivocal identifications as seen in Figures S3 and S4 of the supplementary material.

The significantly lower signal intensities of the [OxPt + H]^+ ion cluster centered at m/z 398 relative to the [M + H]^+ ions observed here as the base peaks in Figures 4-6 (also see Figures
S1 and S2) was also observed previously where similar conditions were used but where M was glutathione. [21] Several explanations were given for this observations including the difference in proton affinities of OxPt and M. [21] The proton affinity of OxPt at 298 K was previously reported to be 233.5 kcal mol\(^{-1}\) [21] whereas the proton affinities for Carnosine, anserine and N-acetyl-carnosine is calculated here to be 254.4, 255.9 and 250.2 kcal mol\(^{-1}\) respectively. To the best of our knowledge the proton affinities of anserine and N-acetyl-carnosine are the first reported values in the literature while three previously calculated values for the proton affinities of carnosine were given at 256.5 (at 0 K), [50] 245.4, [51] and 232.98, [52] kcal mol\(^{-1}\). The latter of these values most likely needs to be revisited as it is significantly lower than all other calculated values and the only available experimental value for the proton affinity of carnosine of 244.59 kcal mol\(^{-1}\) obtained by the Graham Cooks method [51]. The proton affinities for Carnosine, anserine and N-acetyl-carnosine calculated here are in agreement with the lower \([\text{OxPt} + \text{H}]^+\) signal intensity relative to each of their respective protonated \([\text{M} + \text{H}]^+\) ions observed here.

Figures 7–9 and S5 of the supplementary material show the MS\(^2\) spectra obtained by the mass selection followed by the subsequent fragmentation of each of the three \([\text{M} + \text{OxPt} + \text{H}]^+\) species, where M= carnosine, anserine and N-acetyl-carnosine. These spectra indicate common fragmentation pathways leading to the generation of several common ions being \([\text{M} + \text{H}]^+, \ [\text{OxPt} + \text{H}]^+, \ [\text{M} + \text{OxPt} – \text{CO}_2 + \text{H}]^+, \ [\text{M} – \text{H} + \text{Pt(dach)}]^+\) where M= carnosine, anserine and N-acetyl-carnosine; OxPt is oxaliplatin and dach is diaminocyclohexane as indicated earlier in the text.

The precursor ion \([\text{Carnosine} + \text{OxPt} + \text{H}]^+\) were theoretically examined in great detail in the gas phase and in solution as this complex is most likely formed initially in the solution mixture used here. The potential energy surfaces of this complex in the gas phase and in solution were found to be relatively flat with several low lying minima as listed in Table 1 and shown in Figures 10 and 11. The calculated structures on these potential energy surfaces can be divided in two categories involving either direct Pt bonding to carnosine or electrostatic interactions between protonated carnosine and OxPt. The bonding category involves the substitution of a single Pt-oxygen bond of the oxalate moiety of OxPt by a formal Pt-coordination to an electron rich site on the carnosine substrate; this typically being one of the imidazole nitrogen atoms, a carbonyl oxygen or the terminal amino nitrogen of the dipeptide as shown in Figure 11. The electrostatic category on the other hand involves
electrostatic interactions between the oxaliplatin molecule and protonated carnosine through hydrogen bonding which are responsible for keeping the resulting [Carnosine + OxPt + H]^+ complex together as shown in Figure 11. It is interesting to note that all the structures constituting this category involve the formal protonation of the carnosine ligand as opposed to OxPt. This is consistent with the higher calculated proton affinity of carnosine relative to Oxpt as discussed earlier. Structures in the first and latter of these categories calculated to be within about 15 kcal mol\(^{-1}\) in free energy of both the gas phase and solution global minima found are shown in Figures 10 and 11 respectively, while all structures calculated on this potential energy surface are listed in Table S1 in supplementary material. Seven of the eleven structures shown in Figure 11 involve Pt coordination to either the \textit{pros} or \textit{tele} nitrogen atoms of the histidine ring of carnosine. Structures 1A, 1B and 1D in Figure 10, all resulting in the cluster centred on m/z 624 as seen in Figure 4 and Figures S1 and S3 of the supplementary material, comprise the two lowest energy conformers in the gas phase and the lowest two energy conformers in solution on these potential energy surfaces. These three structures all involve Pt coordination to the \textit{pros} nitrogen atoms of the histidine ring of carnosine and formal protonation on one of the carbonyl oxygen atoms of the oxalate moiety of oxaliplatin. This is consistent with an earlier study examining the silver ion binding energies of all 20 α-amino acids, showing that histidine had the third highest silver ion binding energy being lower than that of arginine and only slightly lower than that of lysine. [53] The lowest energy conformer of the Ag\(^{+}\)-histidine complex in that perious study also showed Pt-coordination to the \textit{pros} nitrogen atoms of the histidine ring. [53] Here, the global minimum on the gas phase potential energy surface is structure 1A as shown in Figure 10, this structure is internally stabilised by four strong hydrogen bonds making it 3.4 kcal mol\(^{-1}\) lower in energy in the gas phase relative to 1D and only 0.8 kcal mol\(^{-1}\) removed from the lowest energy structure calculated on the solution phase potential energy surface being structure 1D as shown in Figure 10. Structure 1D is also stabilised by two internal hydrogen bonds. It is interesting to note that in the gas phase structure 1A the hydrogen bond between one of the NH hydrogen atoms of the dach ligand and the carboxylic oxygen of the terminal carboxylate group being at 1.886 Å becomes none existent in solution (distance of 5.297 Å as shown in Figure 10) as it is replaced by external solvation. The strongest hydrogen bonds in either the two gas phase structures, 1A and 1D, is between each of the hydrogen atoms of the protonated oxalate moieties and their respective terminal amino nitrogen atom of each of the carnosine dipeptides. This is determined based on the shortest hydrogen bonding distance calculated in
either gas phase structure being 1.315 and 1.328 Å for structures 1A and 1D respectively as well as on the resulting hydrogen bonding angles being closest to the ideal linear arrangement calculated to be 166° for the N-H-O in both cases.

In the solution phase on the other hand structures 1A and 1D both have their terminal amino nitrogen atoms protonated while forming strong hydrogen bonds to a carboxylic oxygen atom of the oxalate moiety in each case at 1.509 and 1.496 Å respectively. If the solution phase structures 1A or 1D being the two lowest energy structures on that potential energy surface are transferred to the gas phase by solvent evaporation through the soft electrospray ionisation technique while maintaining the protonation on the terminal amino nitrogen atom a structure 1K or 1I would result, this structure is similar to 1C with the noted difference that structure 1C involves Pt coordination to the tele nitrogen of the histidine ring. Structure 1K is calculated to be energetically competitive in the gas phase and in solution being only 8.7 and 2.7 kcal mol\(^{-1}\) removed from either global minima respectively. Alternatively, the gas-phase transfer of the hydrogen atom of the protonated oxalate moiety to the terminal amino nitrogen atom in either gas phase structure 1A or 1D while maintaining the Pt coordination to the pros nitrogen of histidine would also results in the formation of structure 1K or 1I. The loss of CO\(_2\) from the deprotonated oxalate moiety of this resulting structure, 1K or 1I, followed by a proton transfer from the formally protonated NH\(_3^+\) group of the carnosine dipeptide to the carbon atom of the remaining CO\(_2\) group of the oxalate moiety of results in the formation of structure 2A shown in Figure 12. Structure 2A is the lowest energy structure calculated for the [Carnosine + OxPt – CO\(_2\) + H]\(^+\) species observed as the m/z cluster around 580 in the MS\(^2\) spectra shown in Figure 4 and as the signals at m/z 579, 580 and 581 for \(^{194}\)Pt, \(^{195}\)Pt and \(^{196}\)Pt as shown in panels A, B and C of Figure S5 respectively. Structure 2A is shown to contain three internal hydrogen bonds which help stabilise it. It is interesting to note that the lowest three energy structures calculated for the [Carnosine + OxPt – CO\(_2\) + H]\(^+\) species, assigned as the cluster centred on m/z 580 as seen in Figure 7 and resulting in the signals at m/z 579, 580 and 581 in panels A, B and C respectively of Figure S5 of the supplementary material also all involve Pt-coordination to the Pros nitrogen atom of the hystidine ring of the carnosine dipeptide. Structures 2B and 2C are calculated to be only 2.6 and 5.9 kcal mol\(^{-1}\) higher in free energy relative to structure 2A respectively. The gas phase inter-conversion between these three structures is expected to be rapid and well within the energies available under the collision induced dissociation conditions employed here as they involve simple rotations about single bonds. Interestingly, structure 2C involves a hydrogen
bond at 1.765 Å between the OH hydrogen of the carboxylate group of the carnosine dipeptide and the Pt-bound oxygen atom of the remaining HCO₂ group of the oxalate moiety. The transfer of this hydrogen atom from the OH group to the carboxylate group of carnosine followed by the neutral loss of the resulting H₂CO₂ group and the subsequent binding of the resulting carboxylic oxygen to the Pt center results in structure 3A shown in Figure 13. Structure 3A is calculated to be the lowest structure for the [Carnosine – H + Pt(dach)]⁺ species assigned as the cluster centered on mz/534 in Figure 7 and the signals at m/z 533, 534 and 535 in panels A, B and D respectively in Figure S5 of the supplementary material. This assignment was further confirmed by the isotopic pattern obtained on the high resolution Q-Exactive FT-MS shown in Figure S6 of the supplementary material. The formation of the [Carnosine – H + Pt(dach)]⁺ species from the [Carnosine + OxPt – CO₂ + H]⁺ precursor is supported by the MS³ spectrum in Figure S7 clearly showing the cluster centred on m/z 534 being the dominant fragment due to the dissociation the[Carnosine + OxPt – CO₂ + H]⁺ precursor.

The two other signals observed at significantly lower intensities in Figure S7 are the clusters centred on m/z 517 and 354. These signals were also weak signals observed in the MS² spectrum of [Carnosine + OxPt + H]⁺ shown in Figures 7 and S5 of the supplementary material and were assigned as [Carnosine – NH₃ – H + Pt(dach)]⁺ and [OxPt – CO₂ + H]⁺ respectively. The latter of these species is most likely produced via the neutral loss of carnosine from [Carnosine + OxPt – CO₂ + H]⁺ species clustered on m/z 580 generating the ion [OxPt – CO₂ + H]⁺, Structures 5A and 5B in Table 1. The lowest energy of these two structures contains a Pt centre that is dicoordinated to the dach ligand while forming two other symmetrical bonds to either oxygen atom of the COOH moiety.

The species observed as a cluster centred on m/z 517 assigned as [Carnosine – NH₃ – H + Pt(dach)]⁺ is most likely due to the loss of NH₃ from [Carnosine – H + Pt(dach)]⁺ as confirmed by MS⁴ data (not shown). Here, a 1,3 hydride shift to the terminal amino nitrogen atom of the lowest energy species on the [Carnosine – H + Pt(dach)]⁺ surface, Structure 3A, and the subsequent loss of the resulting NH₃ group results in the formation of the lowest energy structure calculated for the [Carnosine – NH₃ – H + Pt(dach)]⁺ species, structure 4A, being the lowest energy species on this surface as shown in Figure 14.

Conclusions
In this paper, *in vitro* studies on hepatocellular carcinoma HepG2 cells suggest that the dipeptide carnosine may inhibit the cytotoxic action of OxPt most likely through the formation of complexes that are less cytotoxic than OxPt alone. Evidence was provided to suggest that pre-exposure of HepG2 cells to elevated levels of carnosine appears to have a lasting effect on reducing the cytotoxicity of OxPt even after the removal of the carnosine. This effect, however, was shown to be under kinetic control as its magnitude was shown not to vary significantly with the level of carnosine exposure within the concentration range used in this study. Various mass spectrometry techniques employing electrospray ionization and chip nanospray were employed to study the interaction of oxaliplatin with the naturally abundant cytoplasmic dipeptide ligand β-alanyl-L-histidine (carnosine) as well as two of its derivatives being β-alanyl-ε-methylhistidine (anserine) and N-Acetylcarnosine (NAC). Evidence of complexation between OxPt and each of the three ligands examined is presented. Most species observed were unambiguously assigned and compared to their theoretical isotopic patterns. Common fragmentation products due to the collisionally-activated protonated complexes of each of the ligands examined with OxPt, [M + OxPt + H]^+ where M= carnosine, anserine or NAC were shown to be [OxPt – CO₂ + H]^+, [OxPt + H]^+, [M – H + Pt(dach)]^+, [M + OxPt – CO₂ + H]^+ and [M +H]^+. Density functional calculations at B3LYP/LANL2DZ were used to obtain structural information and relative free energies of different isomers of the observed precursor [Carnosine + OxPt + H]^+ and the fragments produced highlighting plausible fragmentation mechanisms that account for all the experimental results. Data presented showed several binding modes between electron rich sites such as N and O centers of Carnosine and the Pt metal of OxPt. Values for proton affinities of carnosine, anserine and NAC at 298 K were calculated to be 254.4, 255.9 and 250.2 kcal mol⁻¹ respectively.

**Acknowledgements**

The authors would like to thank the EPSRC, NSCCS, LGC Ltd., the Centre for Analytical Science at Loughborough University, the American University in Cairo and the Centre for Innovative and Collaborative Engineering at Loughborough University for the funding sponsorship and provision of resources for the project. Samir Nabhan of the Department of Chemistry as well as Aya Youssef and Mariam Rizkallah of the Department of Biology both at the American University in Cairo are acknowledged for technical assistance. We are
grateful for Dr. Mehmet Ozturk from the Department of Molecular Biology and Genetics, Bilkent University for providing us with the HepGe cell line.
Figure 1. Percent viability of HepG2 cells upon exposure to varying concentrations of Oxaliplatin for 24 hours. Each data point within an experiment is the average of three separate runs. The data presented here in boxplot format are the average of three separate experiments. The $P$ values for statistical significance were computed using the Tukey honest significant differences test, * for $P < 0.01$, ** for $P < 0.0001$ and *** for $P < 0.00001$.
Figure 2. Percent viability of HepG2 cells upon exposure to increasing molar ratios of carnosine in the presence of 24μg ml⁻¹ oxaliplatin for 24 hours. Values at (0:1, 1:1, 1.5:1, 2:1 and 2.5:1) refer to the molar ratios of carnosine to oxaliplatin. Each data point within an experiment is the average of three separate runs. The data presented here in boxplot format are the average of three separate experiments. The P values for statistical significance were computed using the Tukey honest significant differences test for the solution mixtures relative to 24 μg/ml Oxaliplatin and is represented by * for $P < 0.001$, ** for $P < 0.0001$ and *** for $P < 0.00001$.
Figure 3. Percent viability of HepG2 cells upon the initial exposure to increasing carnosine concentrations followed by the exposure to 24μg ml⁻¹ oxaliplatin for 24 hours. Values at (0.5:1, 1:1, 1.5:1, 2:1 and 2.5:1) refer to the molar ratios of carnosine to oxaliplatin. Each data point within an experiment is the average of three separate runs. The data presented here in boxplot format are the average of three separate experiments. The P values for statistical significance were computed using the Tukey honest significant differences test, * for $P < 0.01$, ** for $P < 0.0001$ and *** for $P < 0.00001$. 

C-17
Figure 4: Full scan MS spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time. The sections of the spectrum shown under “x10” signify the magnification of the signal by 10 fold for clarity. This magnification means that for example the intensity of the ion at m/z 453.0 is about 3% of the base peak. The signals assigned as [OxPt + H]^+ and [Carnosine + OxPt + H]^+ are each expanded and normalized to 100% in inserts A and B respectively for clarity.
Figure 5: MS² spectrum of the entire isotopic envelope of the ion \([\text{Carnosine + OxPt + H}⁺]\) generated at 15 eV in the lab frame and isolated from the full scan spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time. The sections of the spectrum shown under “x10” and “x50” signify the magnification of the signal by 10 and 50 fold respectively for clarity. The signals assigned as \([\text{OxPt - CO₂ + H}⁺]\), \([\text{OxPt + H}⁺]\), \([\text{Carnosine - H + Pt(dach)}⁺]\) and \([\text{Carnosine + OxPt - CO₂ + H}⁺]\) are each expanded and normalized to 100% in inserts A through D respectively for clarity.
Figure 6: Structures for [Camosine + OxPt – CO₂ + H⁺] ions as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.
Figure 7: Structures for [Carnosine – H + Pt(dach)]⁺ ions as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.
Figure S1: Full scan MS spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Acquity TQ without allowing for incubation time. The signals assigned as [OxPt + H]^+ and [Carnosine + OxPt + H]^+ are each expanded and normalized to 100% in inserts A and B respectively for clarity.
Figure S2: Full scan MS spectrum of a (2:1) molar mixture of Anserine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the XEVO TQ without allowing for incubation time. The section of the spectrum shown under “x10” signify the magnification of the signal by 10 fold for clarity. This magnification means that for example the intensity of the ion at m/z 637.2 is about 4% of the base peak.
Figure S3: Full scan MS spectrum of a (2:1) molar mixture of Anserine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time. The sections of the spectrum shown under “x10” signify the magnification of the signal by 10 fold for clarity. This magnification means that for example the intensity of the ion at m/z 481.0 is about 2% of the base peak. The signals assigned as [OxPt + H]^+ and [Anserine + OxPt + H]^+ are each expanded and normalized to 100% in inserts A and B respectively for clarity.
Figure S4: Full scan MS spectrum of a (2:1) molar mixture of N-acetyl-carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Acquity TQ without allowing for incubation time. The signals assigned as [OxPt + H]^+ and [N-acetyl-carnosine + OxPt + H]^+ are each expanded and normalized to 100% in inserts A and B respectively for clarity.
Figure S5: Full scan MS spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Q-Exactive FT-MS without allowing for incubation time showing the isotopic pattern of [Carnosine + OxPt + H]^+. The sections of the spectrum shown under “x5” signify the magnification of the signal by 5 fold for clarity. Panel A shows the experimental data while Panel B shows the theoretically modeled spectrum using the Thermo Xcalibur software. Errors in ppm are listed next to each of the experimental isotopic peaks observed.
Figure S6: Full scan MS spectrum of a (2:1) molar mixture of Anserine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Q-Exactive FT-MS without allowing for incubation time showing the isotopic pattern of [Anserine + OxPt +H]⁺. The sections of the spectrum shown under “x10” signify the magnification of the signal by 10 fold for clarity. Panel A shows the experimental data while Panel B shows the theoretically modeled spectrum using the Thermo Xcalibur software. Errors in ppm are listed next to each of the experimental isotopic peaks observed.
Figure S7: MS² spectrum of the ion [Carnosine + OxPt + H]⁺ generated at 25 eV in the lab frame and isolated from the full scan spectrum of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Acquity TQ without allowing for incubation time. Panels A, B and C show the CID patterns obtained due to the isotopes ¹⁸¹Pt, ¹⁹⁵Pt and ¹⁹⁶Pt of [Carnosine + OxPt + H]⁺ respectively.
Figure S8: MS² spectrum of the entire isotopic envelope of the ion [Anserine + OxPt + H]⁺ generated at 25 eV in the lab frame and isolated from the full scan spectrum of a (2:1) molar mixture of Anserine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ without allowing for incubation time. The sections of the spectrum shown under “x50” signify the magnification of the signal by 50 fold for clarity. The signals assigned as [OxPt – CO₂ + H]⁺, [OxPt + H]⁺, [Anserine – H + Pt(dach)]⁺ and [Anserine + OxPt – CO₂ + H]⁺ are each expanded and normalized to 100% in inserts A through D respectively for clarity.
Figure S9: MS² spectrum of the ion \([\text{N-acetyl-carnosine + OxPt + H\textsuperscript{+}}]\) generated at 20 eV in the lab frame and isolated from the full scan spectrum of a (2:1) molar mixture of N-acetyl-carnosine (NAC in this Figure) and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Acquity TQ without allowing for incubation time. Panels A, B and C show the CID patterns obtained due to the isotopes \(^{194}\text{Pt}, ^{195}\text{Pt}\) and \(^{196}\text{Pt}\) of \([\text{N-acetyl-carnosine + OxPt + H\textsuperscript{+}}]\) respectively.
Figure S10: Tandem mass spectra of a (2:1) molar mixture of Carnosine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the Q-Exactive FT-MS without allowing for incubation time showing the isotopic pattern of [Carnosine – H + Pt(dach)]⁺. Panel A shows the experimental data while Panel B shows the theoretically modeled spectrum using the Thermo Xcalibur software. Errors in ppm are listed next to each of the experimental isotopic peaks observed. Errors in ppm are listed next to each of the experimental isotopic peaks observed.
Figure S11: Tandem mass spectra of the fragment ion cluster centered around m/z 580 generated and isolated from the CID of [Camidine + OxPt + H]^+ at 30 eV in the lab frame which is in turn isolated from the full scan spectrum of a (2:1) molar mixture of Camidine and OxPt in a (1:1) (v/v) water/methanol solution as obtained on the LTQ.
Figure S12: Structures for [Carnosine + OxPt + H]^+ ions in which a formal Pt coordination to carnosine is observed as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis. Italicized numbers are for solvated species.
Figure S13: Structures for [Carnosine + OxyPt + H]^+ ions in which no formal Pt coordination to carnosine is observed as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis. Italicized numbers are for solvated species.
Figure S14: Structures for [Carnosine – NH$_2$ – H + Pt(dach)]$^+$ ions as calculated at the B3LYP/LANL2DZ level of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.
Table S1: Electronic energies, Zero-Point Vibrational Energies, Thermal Energies, Entropies and relative free energies for species calculated at B3LYP/LANL2DZ. Italicized values are for PCM calculations in a water solvent. Structures 1A-1U refer to \([\text{Carnosine} + \text{OxPt} + \text{H}]^+\) in which direct Pt bonding to carnosine is observed. Structures 1V-1Y refer to \([\text{Carnosine} + \text{OxPt} + \text{H}]^+\) in which no direct Pt bonding to carnosine is observed. Structures 2A-2N, 3A-3G, 4A-4M and 5A-5B refer to \([\text{Carnosine} + \text{OxPt} – \text{CO}_2 + \text{H}]^+\), \([\text{Carnosine} – \text{H} + \text{Pt(dach)}]^+\), \([\text{Carnosine} – \text{NH}_3 – \text{H} + \text{Pt(dach)}]^+\) and \([\text{OxPt} – \text{CO}_2 + \text{H}]^+\) respectively.

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References

51. Cheng, M. K. Mass spectrometric and theoretical studies on protonated and potassium cationized biological molecules in the gas phase, Hong Kong Polytechnic University (Hong Kong), Hong Kong, 2007.