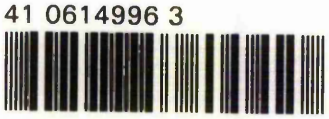

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CHARACTERISATION OF MHC CLASS I TUMOUR ANTIGENS

Jennie Rebecca Lill

A thesis submitted in partial fulfillment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

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Dedication

To Mum and Dad, the best parents in the world, thank you for everything.

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I thank Glen and my family for always loving me, supporting me and giving me encouragement over the last three years.

Finally I thank the Nottingham Trent University for financial support.

This thesis is, to the best of my knowledge, original except where due reference is made.

Jennie Rebecca Lill December 2000.

Abbreviations

AGC *Automatic Gain Control*

APC *Antigen Presenting Cell*

APCI *Atmospheric Pressure Chemical Ionisation*

BCG *Bacille Calmette Guerin*

β_2m *β_2-microglobulin*

CAD *Collision Activated Dissociation*

CD *Cluster of Differentiation*

CEA *Carcinoembryonic Antigen*

CI *Chemical Ionisation*

CID *Collision Induced Dissociation*

CML *Chronic Myelocytic Leukemia*

CTL *Cytotoxic T Lymphocyte*

DC *Dendritic Cell*

DNA *Deoxyribonucleic Acid*

DTH *Delayed Type Hypersensitivity*

EI *Electron Ionisation*

ESI *Electrospray Ionisation*

FAB *Fast Atom Bombardment*

GMCSF *Granulocyte Macrophage Colony Stimulating Factor*

HFBA *Heptafluorobutyric acid*

HIV *Human Immunodeficiency Virus*

HLA *Human Leukocyte Antigen*

HPLC *High Performance Liquid Chromatography*

HPV *Human Papilloma Virus*

HSV *Herpes Simplex Virus*

IEC *Ion Exchange Chromatography*

IFN *Interferon*

IL-2 *Interleukin-2*

IWF *Injection Wave Form*

KDa *Kilo-Daltons*

LCQ *Liquid Chromatography Quadrupole Ion trap*

Mab *Monoclonal Antibody*

MALDI-TOF *Matrix assisted laser desorption ionisation –Time of flight*

MCV *Melanoma Cell Vaccine*

MHC Major *Histocompatibility Complex*

mPC-CE-MS *Membrane Pre-concentration capillary electrophoresis mass spectrometry*

QITMS *Quadrupole Ion Trap Mass Spectrometry*

ODS *Octadecylsiloxane*

PEG *Polyethelyne Glycol*

PI *Isoelectric Point*

PSD *Post Source Decay*

PTFE *Polytetrafloroethene*

RP-HPLC *Reverse Phase HPLC*

TAA *Tumour Associated Antigen*

TCR *T cell receptor*

TFA *Trifluoroacetic acid*

TIL *Tumour Infiltrating Lymphocytes*

TMS *Trimethylsilane*

CONTENTS

CHAPTER 1: Introduction

CHAPTER 2: Extraction, biochemical clean-up and optimisation of mass spectrometric parameters for the characterisation of MHC class I associated peptides.

CHAPTER 4: Nano-electrospray and microbore liquid chromatography ion trap mass spectrometry studies of transition metal complexation with MHC restricted peptides

Summary

A protocol has been developed and optimised for the isolation and mass spectrometric characterisation of major histocompatibility complex (MHC) class I tumour associated antigens. The procedure involves the use of mild acid elution for the isolation of cell surface MHC class I associated peptides, through destabilisation of the MHC β_2-microglobulin, and protein precipitation with trichloroacetic acid. A two-dimensional chromatographic procedure using cation exchange and microbore reverse-phase high performance liquid chromatography (RP-HPLC) is employed for sample clean-up prior to mass spectrometric analysis. A nano-electrospray ionisation source has been constructed, and mass spectrometric conditions optimised for the detection and characterisation of low concentrations (fmol μl^{-1}) of cell surface MHC class I associated peptides. The protocol has been applied for the characterisation of peptides from transfected cell lines and cells derived from Chronic myeloid leukaemia patients. Peptides derived from the novel BCR-ABL fusion region of the Philadelphia chromosome have been characterised, which are specific to a translocation event unique to these haematopoietic malignancies. The mass spectrometric results correlate with T cell *in vitro* assays, suggesting that these peptides have potential for future tumour immunotherapeutics.

The formation of transition metal/peptide complex ions by nano-electrospray and microbore HPLC-electrospray mass spectrometry has been investigated for MHC class I and class II restricted peptides. Post-column addition of copper(II) acetate following microbore HPLC-MS separation was carried out using a mixing T-piece or *via* the sheath flow inlet of the electrospray source. Optimal analytical conditions for copper complex ion formation were determined by variation of copper concentration, pH, nebulization gas supply and spray voltage. Full scan mass spectrometry and tandem mass spectrometry of copper, silver, nickel, or a combination of these transition metals/peptide complex ions provides peptide sequence information and an insight into the peptide chelation sites.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

1.2 Tumour Immunology

Cancers are a major cause of death in the modern western world. Classical therapies for the treatment of malignancies include radiotherapy and chemotherapy, however "cancer vaccines" are being introduced as a more natural. method for the irradication of tumours. It was suggested that the immune system protects against tumour growth following studies by Coley in which he attempted to stimulate cancer patients' immune systems to eliminate tumour cells, by deliberately infecting the patients with viable, and then killed bacteria.[1] Although the results did not show a uniform rejection of all tumours, this work did however pave the way for further experiments that demonstrated that tumours could be eliminated by activation of the immune system. Bacille Calmette-Guerin (BCG) was used in vaccines to treat experimental animals against tumours, and more recently has been successfully applied to treat human bladder tumours and in therapies towards other tumours.[1] Tumour rejection following antigen-specific vaccination was demonstrated using synergic animal. tumours.[2] Antigens on tumours require both cytotoxic T cells (CTLs) and helper T cells for the recognition of tumour antigens and the destruction of the tumour.[3] The role of the immune system is to combat invading microorganisms, however it may also elude foreign tissue antigens such as those associated with malignancies *via* potential. reactions with tumour associated antigens. The different methods of immune surveillance are discussed below, and evidence of successful treatment of malignancies using "cancer vaccines" is demonstrated.

1.2.1 MHC Class I

Two of the main effector cells which recognize and kill tumour cells are cytotoxic T cells (CTLs) and natural. killer (NK) cells. The generation of adaptive immunity and memory is due to the processing and presentation of antigens by antigen presenting cells (APCs), for example dendritic cells, via the association of specific peptides with major histocompatibility complex (MHC) antigens. This complex system serves to distinguish 'self' from 'non-self' and al.lows the recognition of antigenic peptides resulting from necrotic cell death rather than apoptotic cell death.[4] MHC class I molecules are cell surface glycoproteins expressed on al.most all nucleated mammalian cells with the exception of those in immune privileged sites. MHC class I molecules consist of a 44-49 KDa polymorphic heavy chain that is non-covalently associated with the 12 KDa soluble light chain, β_2-microglobulin (β_2m) (Figure 1.1). The MHC class I molecules are 3 allelic series of peptide binding cell surface proteins Human Leukocyte Antigen (HLA) –A, -B &-C, the genes for which are located on chromosome 6. HLA-A are derived from 59 alleles, HLA- B from 118 and HLA – C from 36 alleles.[5] HLA-A2 is the most frequent class I allele being present in approximately 49% of the Caucasian population and HLA-A1 is the second most frequently expressed allele representing approximately 25%. The MHC class I molecule is composed of 4 domains, 3 domains are formed from the heavy chain and one from the β_2–microglobulin, the gene for which is located on chromosome 15, and which is non-covalently linked to the heavy chain. The α-domain of the heavy chain and β_2-microglobulin have a folded structure that is similar to that of immunoglobulin. The α1 and α2 domains of the heavy chain form 2 α helices that create a groove where peptides are presented, resting upon a sheet of

8 β-strands. This groove is occupied by peptides derived from self-proteins in the absence of foreign peptides. The residues in the heavy chain are composed of variable amino acids and it is the side chains of these peptides that form pockets within the peptide-binding groove; there are six pockets, designated A-F which hold or "anchor" the predominant amino acid side chains from the peptide in place. Size, hydrophobicity and charge play an important role in determining the composition of the bound peptides in the groove of MHC class I molecules.[3] Transcription of MHC class I heavy gene is governed by conserved DNA sequences in the promoter region of the gene. These sequences include enhancer A (enh A), the IFN-stimulated response element (IRSE), site α and enhancer B (enh B) and these play a crucial role in both the constitutive and cytokine induced regulation of transcription.[6]

Cluster of differentiation 8^+ ($CD8^+$) Cytotoxic T cells are MHC class I dependent and only recognise peptides that are presented in the groove of a self MHC class I molecule.[7] Antigen is processed intracellularly by proteases in the proteasome and then presented to the MHC antigen. Precursor proteins for MHC class I molecules appear to reside primarily in the cytosol, and their rates of synthesis and degradation regulate the concentration of processed peptides, with proteasomes being the main degradation machines of the cell.[8] Proteasomes are required for the processing of oligopeptides containing antigenic epitopes from 21 amino acids upwards in length, to cytotoxic T cells. Fragments of the antigen (8-11 amino acids) rather than the whole protein are transported from the proteasome by chaperone molecules and become associated with MHC class I molecules in the endoplasmic reticulum (ER) resulting in their expression on the cell surface. It is here that the MHC-peptide complex is recognized by antigen specific $CD8^+$ T cells *via* their T-cell receptor

CD3 complex. Both the heavy chain and the β_2-microglubulin are equipped with cell signal sequences that allow entry to the ER. Molecular chaperones and accessory molecules assist with the formation of the complete MHC class I molecule prior to peptide binding. Transporter associated with antigen processing 1 & 2 (TAP –1 & -2) are dimeric proteins specializing in the transportation of oligomeric peptides present in the cytosol to the lumen of the ER.

Antigenic peptides bind to MHC class I molecules by three types of interaction. Firstly by van de waals interaction of anchor side chain atoms to residues comprising the binding pockets of the MHC molecule. Secondly by hydrogen bonding of N and C termini to residues at the end of the binding groove and thirdly by hydrogen bonding of peptide backbone atoms to residues lining the binding groove.[9] Post-transitional modifications can greatly affect antigen presentation and it has been shown that glycosylated epitopes can be highly immunogenic,[10,11] with mannosylation being shown to have a particularly pronounced effect.[12,13] These post-translational modifications may actually enhance antigen presenting cells' (APCs) uptake of antigenic material.. Post-translational modifications are associated with problems in T cell recognition, in particular with cysteine residue modifications.[14] Although in general peptides presented by MHC class I molecules are derived exclusively from proteins synthesized by APCs themselves, it has been recently discovered that virally infected non-haematopoietic cells are unable to stimulate primary CTL-mediated immunity directly and instead bone marrow derived cells are required as the APCs. CTL immunity to virus infected non-haematopoietic cells requires presentation of exogenous antigen, hence proving that presentation of MHC class I antigen is not exclusive to one pathway.[15] Also

dendritic cells (DCs) have been shown to have the capability of picking up antigen from apoptotic cells and presenting these peptides *via* MHC antigens.[16]

1.2.2 MHC Class II

MHC class II molecules are 63 KDa heterodimeric glycoproteins (Figure 1.1). These molecules are composed of an intracellular and an extracellular domain, (α1,2 and β1,2) which are encoded by genes located on chromosome 6. Foreign bodies such as viruses and mutated or over expressed allo-proteins such as those derived from tumours may be presented by MHC class II molecules. Protein entering the endosomal. pathway is degraded by vesicular proteases into peptides. These peptides bind to MHC class II molecules that are then delivered to the cell surface for $CD4^+$ Helper T cell recognition. The environment for MHC class II assembly is of a much more acidic nature than that for class I, and assembly of MHC class II molecules is aided by the invariant chain (Ii) which trimerises with the α and β subunits. The newly synthesized MHC class II molecules travel to the cell surface in vesicles that then fuse with incoming endosomes that have an interior environment very similar to the MHC class II containing vesicles. Once within the endosome proteases cause the dissociation of Ii and it is at this stage that MHC class II molecules become competent to bind peptides.[25] The vesicle containing the degraded extracellular proteins fuses with the endosome and CLIP (class II associated invariant peptide bound to the peptide groove) is displaced from class II molecules by the binding peptide.

The class II molecules consist of the DP, DR and DQ al.leles and as with MHC class I antigens they are a highly polymorphic family of transmembrane

glycoproteins. Transcription of MHC class II, as for MHC class I, is controlled by a number of transcription factors which interact with a set of conserved *cis*-acting regulatory elements situated in the promoter region of the MHC class II genes. These sequences are important for both interferon γ- induced expression and constitutive expression and include the W/S, X1, X2 and Y-box elements.[6] The class I and class II genes are thought to be the most polymorphic gene families in the human genome.[8] Interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) are capable of inducing over expression of MHC antigen complexes on many cells.

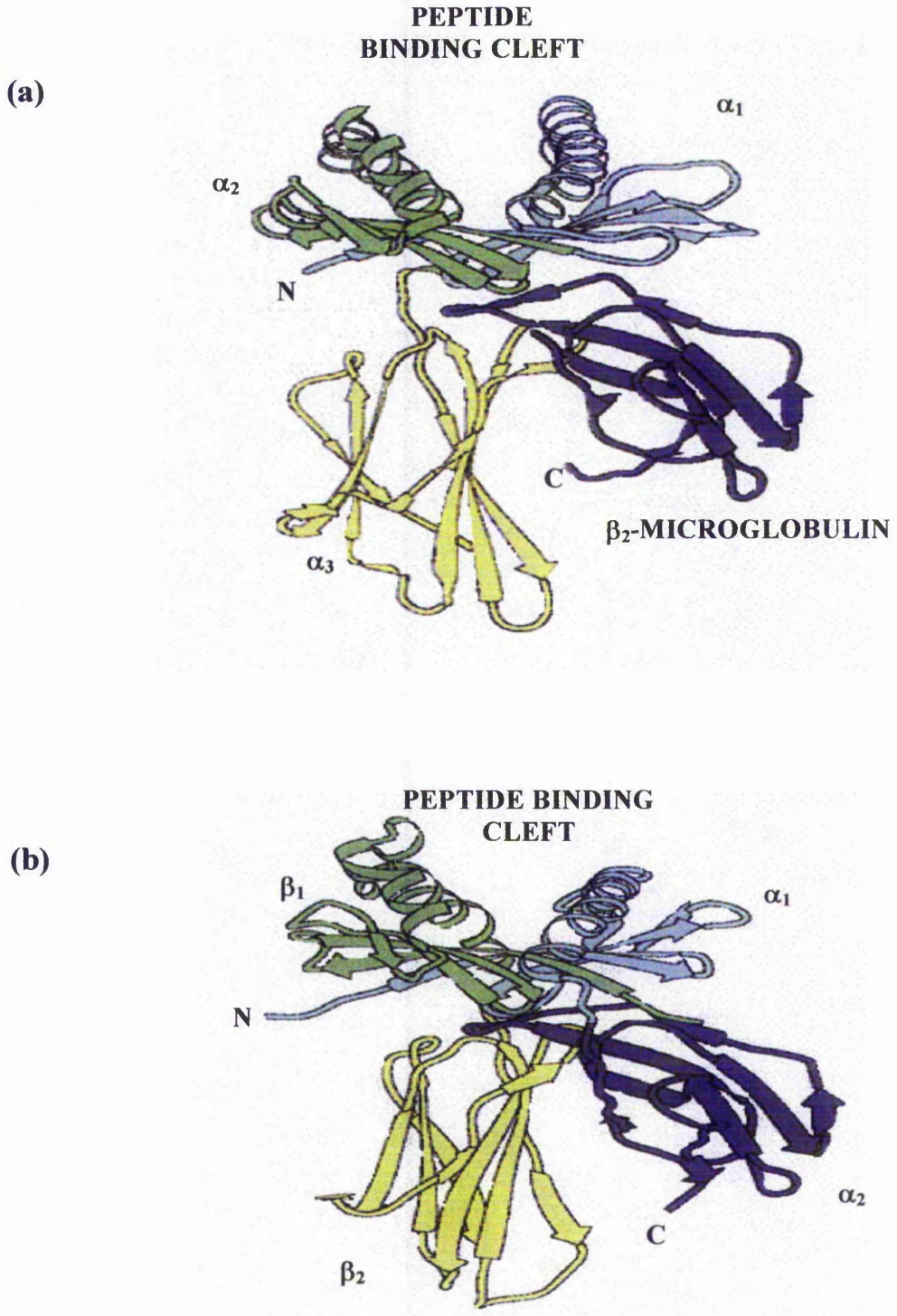


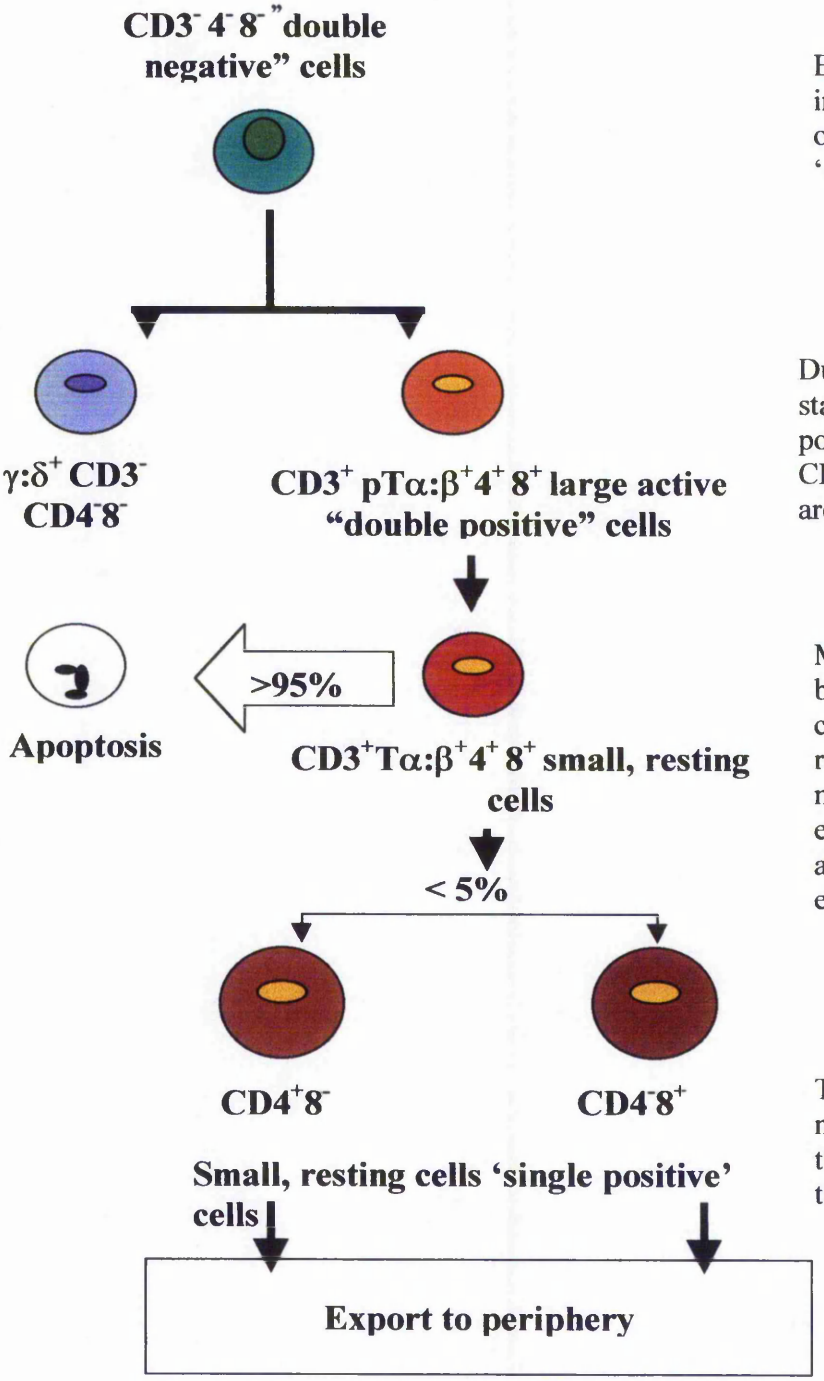
Figure 1.1: X-ray crystallographic structure of (a) MHC class I and (b) MHC class II antigens (Adapted from reference 25).

1.2.3 The T cell Receptor and T cell Activation

The T cell receptor is analogous to immunoglobulins on B cells, however unlike B cell receptors, TCR effector functions are dependent upon cell-cell contact. T-cells express more than 30,000 antigen receptor molecules on their surface.[17] Migration of T cells to the thymus, a lymphoid organ in the upper anterior thorax, occurs at a very early age and it is here that T-cell differentiation is carried out,[18] the production of T cells mostly occurs before puberty after which the thymus shrinks. During their maturation in the thymus, T cells are selected firstly to have self-MHC restriction and secondly self-tolerance. After rearrangement of antigen receptor genes and expression of the receptor on the cell surface of the immature T cells (thymocytes), screening occurs *via* positive selection for self-MHC restriction and negative selection for elimination of cells specific for self-peptides bound to self-MHC molecules.[19, 20] This leads to the development of a T cell repertoire that is principally involved in recognition of foreign antigens.

Thymocytes pass through discrete phenotypic stages as they proliferate and differentiate. The thymus consists of a cortex and medulla and different stages of maturation occur within distinct thymic compartments. The medulla consists of a dense population of mature thymocytes, dendritic cells and macrophages.[21] Figure 1.2 shows the development of a T cell and shows the changes in cell-surface antigens associated with the stages of maturation. On first entering the thymus progenitor cells lack most cell surface molecules commonly associated with mature T cells and have not yet rearranged their receptor genes.

DEVELOPMENT OF A MATURE T CELL



Earliest cell population to be seen in the thymus do not express CD4 or CD8 and are therefore termed 'double negative" cells.

During maturation of α:β T cells a stage occurs called 'double positive' where both CD4 and CD8 as well as the T cell receptor are expressed on the same cell

Most of these die due to becoming small double positive cells. Some, however, have receptors which bind to self MHC molecules, and lose expression of either CD4 or CD8 and undergo an increase in T cell receptor expression.

The result is the production of mature 'single positive' T cells that go on to be exported from the thymus

Figure 1.2: Schematic of the development of T lymphocytes. (Adapted from reference 25).

Upon interaction with the thymic stroma the progenitor cells differentiate, proliferate and express the T cell specific surface antigen CD2. At this stage the thymocytes are "double negative" as they have not yet acquired CD4 and CD8 antigens and the immature T cells undergo positive and negative selection. These immature double negative cells form part of a small highly heterogeneous pool of cells of which 20% comprise cells that express genes encoding the γ: δ receptor, differing from α:β T-cells in their specificity, pattern of expression of CD4 and CD8 and their anatomical distribution. γ:δ T-cells can develop in the absence of the thymus; they are present in athymic nude mice [22] and are the first population to appear in murine development. A further 20% of al.l double negative T cells include cells bearing α:β TCR's that are limited in diversity. These are activated as part of the early response to microbial infections. The remaining 60% are committed to the α:β T cell lineage but have not yet rearranged their α:β TCR genes.

Thymocytes then become positive for CD44 and also acquire expression of the IL-2 receptor α chain CD25 and at this stage genes encoding the β chain of the TCR are not yet fully expressed. As expression of CD25 increases, CD44 expression is reduced and the cells become known as $CD44^{low}$, $CD25^{+}$ cells; cells undergo rearrangement of their TCR β chain genes and begin to lose expression of CD25 once more. The β chain now pairs with a pre T cell α chain (pTα) during the $CD44^{low}$ $CD25^{+}$ stage of development and T cell receptors acquire the CD3 molecule. At this point cell proliferation occurs followed by arrest of further rearrangements of the β chain genes. The T-cells are now termed "double positive" since they also express CD4 and CD8 antigens.[23] As cells become small double

positive cells they cease to proliferate and the α chain genes rearrange to produce a mature α:β T cell receptor; 98% of all thymocytes generated die during this process due to apoptosis, as a result of a strong selection process, the remaining cells are then rapidly exported from the thymus into the periphery.

Equilibrium of the T-cell repertoire is maintained keeping naive T cells alive and maintaining a low level of proliferation when required. Naive T cells may be kept alert due to low affinity interactions with self-peptide MHC, which is an ideal way for them to reaffirm their potential to respond to foreign peptides when higher affinity interactions occur. Following interaction with antigen some T cells differentiate into memory cells that respond rapidly upon subsequent encounter with specific antigen.[24]

The T cell receptor (TCR) (Fig. 1.3) consists of two disulfide bound chains, αβ, and the CD3 antigen complex and resides on the surface of T cells. Both T cell receptor chains consist of an amino terminal. variable hinge region possessing a cysteine residue forming an interchain disulfide bond. The chains span the lipid bilayer by a hydrophobic transmembrane and end in a cytoplasmic tail. The amino acids at the transmembrane region are positively charged and play an important role in the interaction of the two T cell receptor chains with oppositely charged polypeptides of the CD3, γ, δ and ε chains. It is this molecular complex that the MHC: peptide complex binds in order to initiate a T cell response. There are three CDR loops within the TCR that have sequence diversity, which results in the generation of a repertoire of T cells.[24] The γ, δ and ε chains are associated with transmembrane

kinase signaling to the interior of the cell upon association of the TCR with MHC:peptide.[25] The first signaling molecules in the T- cell activated cascade are two tyrosine kinases, ick and fyn. Fyn is associated with the ξ chains of the TCR:CD3, and ick with the CD4 (T helper cell) and 8 (cytotoxic T cell) molecules on the T cell surface. A signaling cascade is initiated *via* these two molecules and a CD45-linked kinase and leads to the production of kinase C and increases in intracellular calcium activating DNA binding proteins, which in turn activate transcription of the Interleukin-2 (IL-2) receptor. These series of events are essential. for promoting T-cell proliferation in an antigen-specific manner.

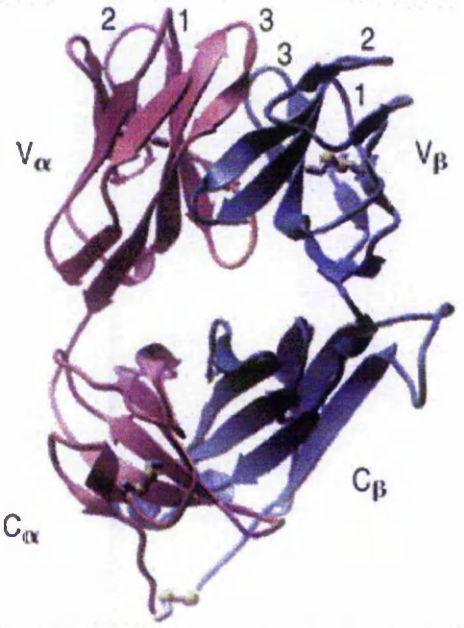


Figure 1.3: X-ray crystallographic structure of the T cell receptor.[25] Cα and C β are the constant domains and Vα and Vβ the variable domains of the TCR.

T-cells require an additional signal to cause clonal expansion and antigen specific T cell activation, which occurs via the interaction of the B7 molecule on the antigen-presenting cell (APC) with CD28 present on the T-cell. This generates another phase of intracellular signaling leading to the transcription of the IL-2 gene (important for T cell proliferation and activation). The IL-2 produced by helper T-cells activates CTLs that express the IL-2 receptor (the co-stimulation is provided by the APC via T cell help). T cells are activated resulting in the proliferation of large numbers of antigen specific T cells that recognize and kill cells expressing the nominated peptide presented in a restricted manner by MHC class I antigen. When T cells fail to receive the co-stimulatory signal. they are unable to respond to antigen and a condition known as anergy results. CD4 and CD8 molecules are required for the generation of a T-cell response, and bind to the non-polymorphic regions of class II and class I MHC antigens respectively. More than 10^4 identical MHC: peptide complexes are required to activate $CD4^+$ and $CD8^+$ T cells, thus showing the important role each cell-surface marker plays in the activation of a T cell immune response.[26] The interaction of T-cells with specific peptide:MHC, co-stimulatory molecules and several. sets of adhesion molecules is necessary to induce immunity e.g. CD40 /CD40 ligand (CD40L)[27] and OX-40/OX-40L. OX-40 and OX-40L are thought to be associated with T-cell APC interactions. Their interactions act after initial. activation events to enhance cytokine secretion and prolong clonal expansion.[28] OX-40L is a cell surface glycoprotein belonging to the tumour necrosis factor family, and is expressed primarily on activated helper T cells and may be involved in promoting long lived $CD4^+$ responses.[29] Expression of the OX-40 receptor has been located in tumour infiltrating lymphocytes, draining lymph node

cells but not peripheral blood lymphocytes. Figure 1.4 illustrates the immunocellular interactions associated with T cell activation.

1.2.4 Tumour Antigens

The first indication that human tumour cells expressed antigens which induced an immune response in the host was demonstrated by Boon *et al.* [1] They demonstrated that some cancer patients, most notably those bearing a malignant melanoma, had cytotoxic T lymphocytes (CTLs) which reacted specifically *in vitro* with autologous tumour cells. More recently autologous CTL clones were cultured *in vitro* from cancer patients and re-injected into the same cancer patient. These tumour reactive T cells were able to mediate tumour regression, thereby suggesting that CTLs have a potential role in controlling tumour growth *in vivo.*[30] It was shown that cancer cells show inflammatory infiltrates, and share a number of characteristics with pathogens which make them suitable candidates for elimination by the immune system.[31]

Human tumour antigens may be classified as tumour specific antigens (TSAs) or tumour associated antigens (TAAs). Tumour specific antigens are caused by a genetic change in the cell leading to the expression of a "new" antigen unique to that tumour, which may provoke an antibody or T cell response against the "new" protein in the host, depending on the cellular processing and presentation pathway utilized and the type of T-helper cell response (Th1 or Th2) generated. Point mutation of cell DNA occurs with reasonable frequency and can result in the aberrant expression of oncogenes or tumour suppresser genes which contribute to cell transformation to malignancy. One of the most common oncogenes, resulting from mutation, is the *ras* gene family which consists of three related genes: *ras H,*

ras K and ras N, that encode intracellular membrane bound proteins (p21 ras). Mutations in tumour cells are commonly found at residues 12/13 or 61 of the ras protein, which represent critical regulatory positions.[32] Alternatively, human tumour antigens may be classified as tumour associated antigens (TAA).[33]

Different types of human tumour-associated antigens can act as potential targets for tumour specific T cells, and can be classified as mutated gene products (*ras,* p53), recombinant fusion protein (bcr-abl) or non-mutated self-proteins e.g. oncofetal proteins and tissue specific and amplified proteins e.g. Her-2 neu, GA733-2 which are over-expressed in these cells.[34] Much research into tumour antigens has focused on the antigens expressed in malignant melanoma, these include tumour associated testis-specific antigens (MAGE, BAGE, GAGE, NY-ESO-1 and PRAME), melanocyte differentiation antigens (tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2) and mutated or aberrantly expressed antigens (MUM-1, CDK4, beta-catenin, gp100-in-4, p15 and N-acetylglucosaminyltransferase V).[31] Figure 1.4 shows the interaction of antigens with different molecules of the immune system.

Other Cancer Associated Antigens

Carcinoembryonic antigen (CEA), carbohydrate antigens 15-3, 19-9 and 72-4 (CA15-3, CA19-9 and CA72-4), cytokeratin 19 fragments (CYFRA 21-1), neuron-specific enolase (NSE), squamous cell carcinoma antigen (SCC)[35] and BAG-1,[36] can be used as diagnostic markers to indicate if malignancy is present and if so how far it has progressed.[37] Different combinations of these markers may be indicative of a certain type of cancer, and can be used in combination to detect even tumours where some antigens are not being expressed.

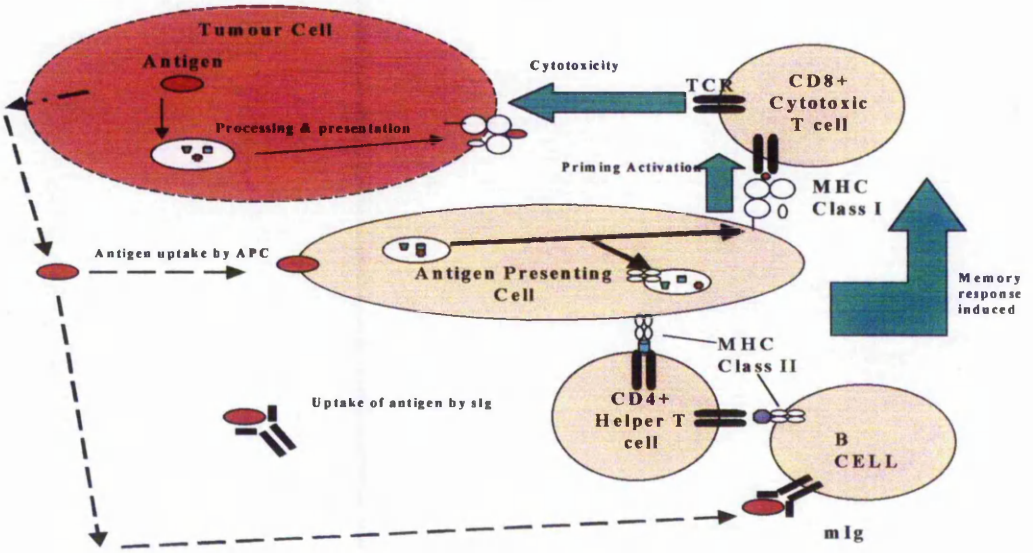


Figure 1.4: Interactions of the immune system with tumour antigens (Adapted from reference 25).

Surrogate antigens, not normally expressed in malignant cells, are often employed in experimental immunotherapy studies, since the gene encoding the antigen can be transfected into tumour cells to present immunogenic epitopes that are unique to the tumour, thus eliminating the possibility of recognition by T cells specific for self peptides. β-galactosidase and oval.bumin[26] are examples of proteins which have been exploited as surrogate antigens in murine tumour models.

Viral associated cancer antigens

Many viruses/viral gene products are associated with cellular transformation leading to the development of malignancies, and include Hepatitis B and C, Human

papilloma virus (HPV), Epstein Barr virus (EBV), SV40 virus and adenovirus. Hepatitis B and C viruses are causal agents of hepatocellular carcinoma and human papilloma viruses are associated with > 90% of all cancers of the cervix and with other genital malignancies. HPV are small DNA viruses and approximately 70 different types have been identified, with 8 members capable of infecting the lower genital tract of humans. The proteins E6 and E7 of HPV are potent oncogenes and are antigens found in HPV derived cancer.[38] EBV has a strong association with the formation of immunoblastic B-cell lymphoma, Burkitts lymphoma and nasopharangeal carcinoma. EBV derived peptides have been shown to illicit a class I restricted $CD8^+$ T cell immune response.[39, 40] Adenovirus induced tumours in mice have also been shown to be immunogenic in mice.[41]

Chronic Myeloid Leukemia

An example of a malignancy resulting from a chromosomal translocation is chronic myeloid leukemia (CML), a myeloproliferative disorder characterized by the occurrence of the Philadelphia (Ph) chromosome in greater than 95% of patients.[42] The Philadelphia chromosome is derived from the reciprocal translocation t(9;22)(q34;q11) whereby the downstream portion of the *ABL* protooncogene from chromosome 9 is fused with the upstream portion of the *BCR* gene on chromosome 22. This translocation event gives rise mainly to the chimeric *BCR-ABL* gene, although expression of the *ABL-BCR* variant protein occurs in approximately 40% of cases. The formation of the *BCR-ABL* gene product is one of the key events in the pathogenesis of CML, and is also present in other types of leukemia such as acute lymphoblastic leukemia and de novo acute myeloid leukemia[43] (Figure 1.5). CML patients may possess either a b3-a2 fusion or a b2-a2 fusion resulting from fusion of

different exons of the progenitor DNA between the *BCR* and *ABL* genes.[43] It is thought that the *BCR-ABL* fusion protein is involved in a defective β1-intergrin function which might induce abnormal circulation and proliferation of the Philadelphia chromosome positive progenitor cells. This novel fusion gene product is also thought to be involved in a number of signal transduction pathways including the activation of the *RAS* oncogene.[42] The *ABL-BCR* gene is known to be transcriptionally active and is thought to dysregulate GTPase activating protein (GAP) activity.[42]As the disease progresses it enters a phase known as 'Blast crisis'. At this stage many other karyotypic changes may occur including trisomy of chromosome 8, isochromosome (i 17q), trisomy 19, and an additional. Philadelphia chromosome (a condition known as double Ph). Many gene mutations also occur during the evolution of this disease including mutations to *p53, RBI, c-myc,* $p16^{INK4a}$, *ras and AML/EVI-1* a fusion protein resulting from translocation t(3;21)(q26;q22). P53 mutations are associated with apoptotic suppression and hence progression into the blast crisis.[43] CML has an incidence of 1 –2 cases per 100 000 population, with incidences increasing with age; the male: female ratio is 1.3:1.[43] The current methods of identification of this disease state are mainly through detecting karyotypic alterations by cytogenetic analyses. Polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR) and Southern blot analysis are commonly used to determine the exact breakpoints of the fusion genes and Western blot analysis is employed to demonstrate the presence of the BCR-ABL fusion protein. Currently therapy of CML involves stem-cell transplantation or interferon-α therapy. Both of these treatments are associated with risk; stem-cell transplantation has a high procedure related mortality rate, and interferon-α can produce a high incidence of neurotoxicity and other dangerous side effects in more

elderly patients.[43] Symptoms of this disease include fatigue, weight loss, abdominal fullness, bleeding, sweats, purpura and splenomegaly, however some patients are assymptomatic[43] and new therapies are currently being developed. These include the use of the plant alkaloid homoharringtonine,[44] the potent hypomethylating cytidine analogue 5-aza-2'-deoxycytidine,[45] the coupling of polyethylene glycol to interferon-α to enhance the half life of this cytokine,[46] the use of antisense oligonucleotides directed against *BCR-ABL* gene products,[47] tyrosine kinase inhibitors[48] and the implementation of adoptive immunotherapy.[49]

With the successful identification of MHC class I and class II restricted epitopes from many tumour antigens, attention is now focused on identifying those associated with CML. Mice have been successfully vaccinated with synthetic junction peptides resulting in the generation of peptide specific $CD4^+$ cells.[50] B3a2 derived peptides have been investigated for their ability to elicit MHC class I restricted CTLs, where 50% of the HLA-A3 positive donors tested showed a cytolytic response.[51] Also dendritic cells pulsed with *BCR-ABL* HLA-A3 restricted peptides can generate potent CTLs against CML cells.[52] This was shown for MHC class II restricted b3a2 specific epitopes which stimulated a $CD4^+$ T cell response.[53] Figure 1.5 shows the different MHC class I associated peptides that can be derived from the BCR-ABL fusion protein of the Philadelphia chromosome from chronic myeloid leukemia.

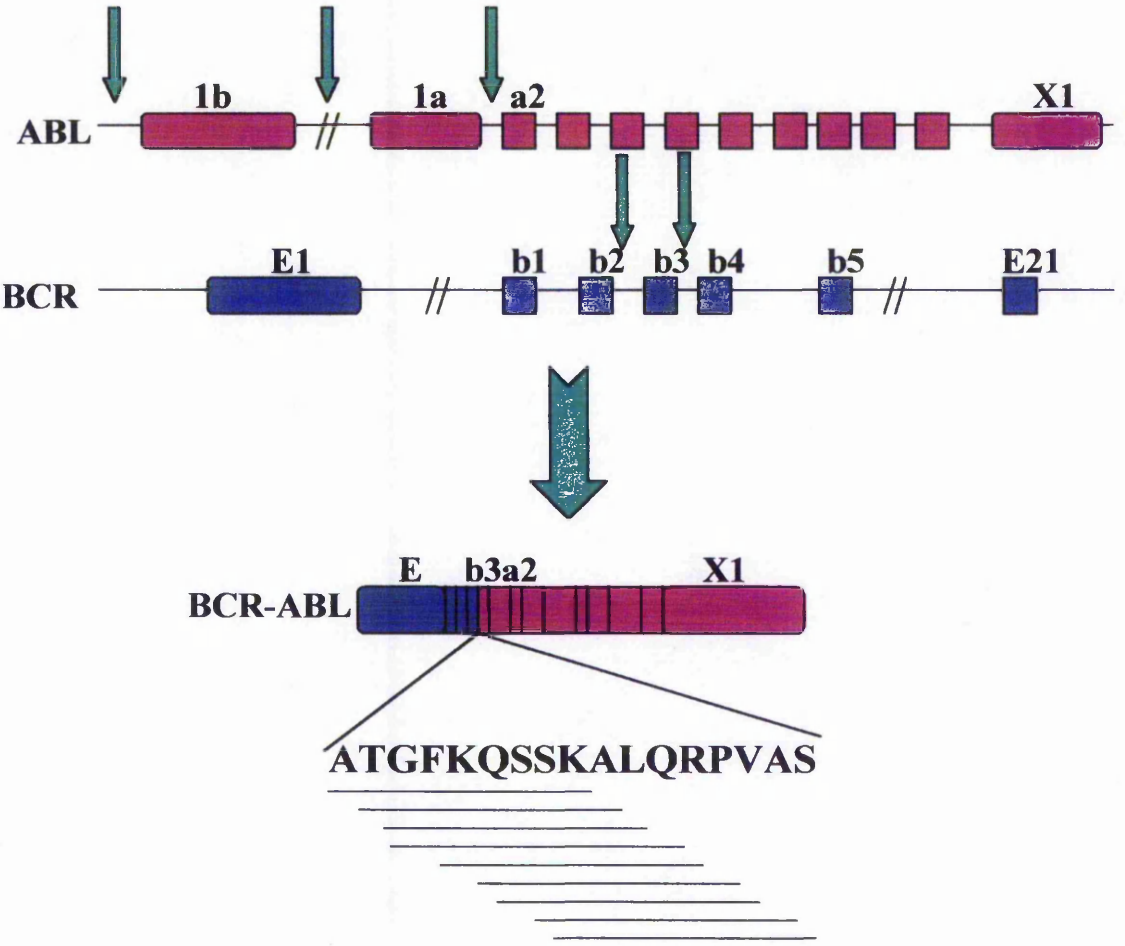


Figure 1.5: The BCR-ABL gene fusions in Philadelphia chromosomes and the resulting amino acid sequence generated from the junction region (Adapted from reference 61).

Identification of tumour antigens

The first human tumour antigens were successfully identified employing molecular techniques, cosmids transfected with cleaved tumour derived DNA.[54] These cosmids also carried a drug resistance gene as a selection marker. Transfected cells were exposed to T lymphocytes specific for the antigen and generated *in vitro* by stimulation of the patient's PBMC with tumour cells. CTLs cytotoxic against the

autologous tumour cells allowed the identification of genes encoding the MAGE-1 tumour antigen.

A more recent and alternative method for the identification of tumour antigens is *via* the use of SEREX (serological. identification of antigens by recombinant expression screening with autologous sera). Here a cDNA library is constructed from fresh tumour, tissues or cells and cloned into lambda phage expression vectors.[55]

Phages are used to transfect bacteria and the recombinant proteins expressed during lytic infection of the bacteria are transferred onto nitrocellulose membranes. These membranes are then reacted with diluted autologous patient sera. The clones reactive with high-titer IgG antibodies are identified with an enzyme conjugated to a secondary antibody specific for human IgG. The cDNA sequence can then be determined from single plaques derived from sub-cloning using reverse transcriptase polymerase chain reaction (RT-PCR). SEREX has been highly successful in the identification of both known and new tumour antigens.[56, 57] Tumour antigens such as New York Esophageal. antigen (NY-ESO-1), Hom-MEL-40 and SSX2 have been identified using this technique.[55]

1.2.5 T Cell Epitopes

T cell epitopes are immunogenic peptides presented by MHC class I and II antigens and recognized by CTL or helper T cells respectively. These have been successfully used as vaccines in immunotherapeutic trials of malignancies and the quest is now to identify tumour antigens and to characterize their MHC restricted epitopes. Experiments have been performed to determine whether the immunogenicity of

peptides presented naturally by MHC class I and class II antigens could be improved by substituting pre-defined amino acids. The ideal. situation is to generate high avidity CTLs and to induce a type I cytokine response, to heighten the anti-tumour immune response. This was demonstrated for the CAP-1 peptide YLSGAWLWC, an immunogenic HLA-$A2^+$ binding peptide derived from human carcinoembryonic antigen (CEA). By substituting amino acids at positions 8 and 9 to produce the sequence YLSGADLNL, Cap-1 specific CTLs are found to be sensitized 10^2- 10^3 times more efficiently than the original. epitope.[58] This was also demonstrated for a murine mutant Ras $CD8^+$ CTL restricted peptide epitope, where the amino acids naturally residing at the anchor residue positions were substituted with more common anchor residues. Again, this modification was found to enhance both *in vitro* and *in vivo* immunogenicity.[59] T cell epitopes have been characterised using a variety of methods including CTL assays, and mass spectrometry (discussed in section 1.3).

A good example of a malignancy related recombinant protein suitable, as a target for immunotherapy is the bcr-abl fusion protein expressed in chronic myeloid leukemia. The T cell epitopes presented from this protein have been of great interest as potential. CTL target peptides and have been characterized using *in vitro* binding efficiency studies for several. MHC restriction elements. The autoantigen repertoire of this leukemia has also been investigated to define epitopes from other proteins, to enable a strategy for vaccine development for this hematopoietic malignancy to be developed.[60] Figure 1.5 shows the possible nonamer non-self peptides that can result from *BCR-ABL* gene fusions.[61]

It is know that interferon γ upregulates MHC class I/peptide complex expression, and that some malignancies share peptide epitopes. This was demonstrated by Bernhard *et al.*. in that renal. cell carcinoma and malignant melanoma share an HLA-A2 restricted epitope.[62] T cell epitopes have also been identified for surrogate tumour antigens such as β-galactosidase where it was demonstrated that H2-k^b restricted peptide epitopes were indeed presented on the cell surface (for example, amino acid sequence $DAPIYTNV_{96-103}$). The H-$2L^d$ restricted peptide TPHPARIGL has also been identified from the β-gal. protein as a potent CTL cell target epitope.[63]

MHC class II restricted antigens have also been characterised and it has been demonstrated that the immune response against the tumour is far more potent if $CD4^+$ T cell help is available. These include a HLA-DR restricted T cell epitope from MAGE-3, $LLKYRAREPVTKAE_{121-134}$ which is a sequence also found in three other MAGE proteins, MAGE-1, -2 and –6, making this a potential candidate target peptide for anti-tumour immunotherapy.[64] When characterising epitopes from tumour antigens, it is important to define a range of HLA restrictions to cover the population.

1.2.6 Immunotherapy

The definition of a vaccine is "a preparation of micro-organisms or their antigenic components which can induce protective immunity against the appropriate pathogenic bacterium or virus but which does not itself cause disease".[65] Therefore vaccinations are classically a means of preventing disease rather than curing it. This strategy is feasible with many microorganisms because their genomes are relatively simple, so therefore they possess a limited number of defined antigens.[66] This is not

the case for cancer vaccines that have been implemented as a strategy to suppress cancers, since here the vaccine must induce an immune response at a late stage of the disease. It is generally agreed for immunotherapy to be successful the tumour load should first be reduced by surgery, irradiation or chemotherapy, since it is unlikely that the immune system will cope with and reject a large tumour mass. Cancer vaccines should therefore be targeted at residual metastases rather than the primary tumour.[67] Immunotherapy may include the use of monoclonal antibodies, various tumour vaccines (peptides, protein, DNA & anti-idiotype antibodies), adaptive transfer of lymphokine activation killer cells or tumour infiltrating lymphocytes (TIL).[68] There are two main types of immunotherapeutics currently under investigation for the treatment of malignant disease that involve the use of tumour antigens to stimulate a destructive anti-tumour response. Active immunization employs tumour antigens and T cell epitopes to stimulate an anti-tumour response *in vivo*, whereas adoptive immunization utilizes *in vitro* stimulation to aid destruction of the malignancy. Both active and adoptive immunization however, may be combined sequentially to achieve the best anti-tumour response.

1.2.6.1 Active Immunization

Cellular vaccination

Many immunotherapeutic strategies using the vaccination of tumour patients with one or more malignant cell lines have been explored. Single and polyvalent "whole cell' vaccines have been used to treat cancer patients with mixed results. Autologous and genetically modified whole cells, can be engineered to secrete interleukins e.g. (IL-2, IL-12) or GM-CSF, IFN-γ or IFN-α etc, thus helping the

immune response by providing extra immune stimulation. Hapten-modified cells, irradiated and non-irradiated autologous cell vaccines have also been investigated as potential anti-tumour treatment and many of these have been used in conjunction with other immunotherapeutic strategies (e.g. with BCG) for the same reason as above.[69] Maraveyas *et al.*, used active specific immunotherapy on patients with stage IV AJCC melanoma using a melanoma cell vaccine (MCV) allogeneic vaccine to greatly reduce the number of metastases' showing this type of immunotherapy can be extremely successful.[70]

Malignant melanoma patients were shown to produce an anti-tumour DTH and CTL response when vaccinated with autologous interleukin-2 secreting cells. Metastatic melanoma patients were vaccinated with irradiated autologous melanoma cells that had been genetically engineered to secrete IL-2.[71] Immunotherapy using autologous tumour cells engineered to express anti-tumour cytokines or immunostimulatory molecules shows great promise for the treatment of malignancies, particularly for secondary growths. Another innovative immunotherapeutic strategy was described by Kugler *et al.* whereby dendritic cells and renal carcinoma cells were fused to form a vaccine for the treatment of metastatic renal cancer. Cells were fused using PEG, an electrical pulse or other agents; they were then cultured, selected and used in the effective and non-toxic treatment of this renal mal.ignancy.[72]

Peptide vaccination

Peptide vaccines have come about *via* the "reductionist" view.[73] It is now recognized that the immune response does not recognize the whole protein/foreign body/tumour cell, but instead recognizes a short peptide that is usually 8-11 amino

acids in length. T cells and B cells recognize these short amino acid sequences. However, B cells recognize and elicit an antibody response against different epitopes whilst T cells recognise peptides presented in the groove of self-MHC molecules. Peptide vaccines representing T cell epitopes have been used for the treatment of a variety of pathogens and are now being investigated for use in tumour immunotherapy. Synthetic peptides representing MHC class I or class II presented peptides are safe, but there are some non-toxicological problems. For example peptides if administered on their own, do not cause a sufficient level of immunity in most cases, to irradicate tumours or viral infections. They are poor immunogens due to their small molecular size and have a very short half-life in the serum. Another problem seen with MHC class I peptide vaccines is that once parentally administered, they become internalized in endosomal compartments where they are prone to be extensively degraded or will fail to intersect with the MHC class I pathway. However, by coupling these peptides to other carrier molecules or by addition of an adjuvant to the vaccination protocol these problems can be overcome. Successful approaches include the use of recombinant foreign proteins expressing microbial. T cell epitopes, Chimeric viruses expressing T-cell epitopes, self-proteins expressing T cell epitopes, receptor linked peptide delivery systems and heat shock protein-peptide complexes.[74] Bacterial vectors expressing T cell epitopes from malignancies are currently proving successful, for example Listeria, and as well as being an ideal delivery system they may also provide a "danger" signal to activate APCs.[75]

Dendritic cells (DCs) have been shown to be the first cells of the immune system to respond to endogenous stress signals.[4] They are potent antigen presenting cells that

can be loaded with autologous or synthetic CTL specific tumour associated peptides to generate activated helper and cytotoxic T cells.[76] These are proving invaluable as immunotherapeutic tools.

Experimental validation has shown successful results in the use of peptide vaccines for MART-1 and Tyrosinase in malignant melanoma,[77] HER 2/neu antigens in ovarian and breast cancer[78] and also for a variety of antigens associated with hematopoietic malignancies.[79] T cell epitopes for the tumour suppresser p53 have been characterised and peptide pulsed dendritic cells (DCs) can induce a very effective CTL response.[80, 81] Peptide vaccines have the advantage economically over other vaccine preparations in that they are relatively cheap to produce and easy to transport. It has been suggested that protein/whole cell vaccines might be of preference for immunization over preselected peptides, because they offer a wider range of epitopes available to the immune system. However, Disis *et al.* [82] showed that by using both predefined peptides and whole proteins when immunizing rats against the HER-2/neu antigen that antigen specific cellular and humoral immunity was elicited when the rats were vaccinated with immunogenic peptides, but not when whole protein was administered. This also seems to be the case for MAGE peptides versus proteins in the latest clinical trials.

Adjuvants

An adjuvant is any substance that enhances the immunogenicity of antigens, and traditionally the best adjuvants in tumour immunotherapy are bacterial adjuvants such as BCG or *Corynebacterium parvum,* used in conjunction with reintroduction of surgically removed tumours. This approach was used mainly in the treatment of

melanoma, however the overall results were generally poor.[25] One of the most promising new adjuvants are heat shock proteins (Hsp) and a direct correlation between the Hsp expression in a malignancy and the patients' survival. has been shown. The prognosis of Osteosarcoma patients naturally over-expressing Hsp 72 is better after neoadjuvant chemotherapy than Hsp 72 negative cases,[83] also Hsp 90-α is usually associated with poor prognosis in breast cancer[84] and Hsp 25 is often associated with a strong down regulation of cellular proliferation of mal.ignancies.[84]

Heat shock proteins belong to a family of ubiquitous and abundant proteins that have now been used successfully in prophylactic and therapeutic vaccination against tumours.[85] They are naturally expressed in response to environmental stress factors such as pH, temperature, glucose starvation and chemical stress. Hypothermia and various types of chemotherapy are good examples of where chaperone hsp expression is induced; they were first discovered in Drosophila in response to elevated temperatures.[86] Hsp-peptide complexes have been shown to induce a potent antigen –specific immune response to both primary and metastatic tumours.[84] *In vivo* these Hsps are molecular chaperones which help damaged proteins to refold to their native conformation. They are particularly prevalent in tumours as malignant cells are in a particularly "stressful" environment, for example hypoxia, and are therefore susceptible to damage. Hsp 90 is necessary for stabilizing *src* and *raf* and other proto-oncogenic protein kinases and other Hsps are responsible for the folding of oncogenic viral proteins, e.g. Hepatitis B. Over expression of these chaperones protects the tumour and renders the cells more resistant against apoptosis. However, because of this over expression chaperone-peptide vaccination proves a very powerful technique.[84]

1.2.6.2 Adoptive Immunotherapy

Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) are a source of tumour specific T cells and these may be cultured with IL-2 *in vitro,* and used to induce anti-tumour activity. TILs have been used therapeutically and also for the identification and characterization of several melanoma associated antigens.[87, 88]

Cellular therapy can involve the modification of a patient's own tumour cells or CTLs *in vitro* and their replacement into the patient, or used *in vitro* to induce CTL by using appropriate cytokines (see below). Another approach is to non-specifically activate T-lymphocytes *via* the T cell receptor and re-introduce these back into the patient undergoing treatment.[89] Many cancer vaccination strategies have employed autologous and allogeneic tumour cells genetically modified to express a range of immunomodulatory genes including cytokines, tumour antigens and costimulatory molecules.[3]

Cytokines and other Immunostimulatory Molecules

One of the most successful experimental strategies to date is to engineer tumour cells that secrete granulocyte-macrophage colony stimulating factor (GM-CSF), hence attracting hematopoietic precursors to the tumour site, inducing their differentiation into the powerful APCs, dendritic cells (DCs). Co-stimulatory molecules such as B7 can also be engineered to be expressed in tumour cells to increase their immunogenicity[25] and up-regulation of immunostimulatory cytokines such as IL-2 and IL-12 rather than depletion of tumour enhancing cytokines is required as an immunotherapeutic strategy, preferentially inducing a Th1 response

to aid the development of CTLs. Inoculation/immunization with tumour cells engineered to express IL-2, IFN-γ, IL-4, TNF-α, IL-7 or IL-6 have been shown in mice to enhance anti-tumour activity, increasing the rejection of tumours and in some cases inducing a memory response.[90-96]

Gene therapy

Gene therapy has utilized a number of strategies to introduce cytokines, IFN-γ and other co-stimulatory molecules to the malignancy in order to promote tumour rejection. Gene therapy can also be used to introduce a tumour antigen for over expression, and many different gene delivery systems have been implemented. These include cationic and anionic liposome vector and viral gene delivery systems including the use of adenoviruses, retroviruses and vaccinia virus. Multi-epitope DNA vaccines are being investigated for their potential use against both pathogens and malignancies, with melanoma being the prime target for experimentation.[97]

1.2.7 Tumour Escape Mechanisms

One of the major hurdles still to be overcome in the field of tumour immunology is that of tumour escape from the immune response. Many mechanisms are now recognized that prevent tumours responding to immunotherapy. The tumour micro-environment has been shown to induce negative effects on the cells of the immune system, for example defects in T cell signals *via* down regulation of the ξ chain of the T cell receptor, possibly due to activation of intracellular peptidases activated by the tumour.[87] Often anergy is induced in T cells, especially $CD4^+$ cells, due to tumour induced cytokines such as IL-10, which can also have inhibitory effects on dendritic cell function.[97] T cells may also become naturally resistant to blockade by

the tumour and may therefore go on to reach replicative senescence without having served their purpose.[98] MHC or antigen down regulation may also influence the use of immunotherapeutics and strategies are being developed to transfect MHC and antigen processing machinery genes into tumour cells to overcome this defect.[99] One of the major concerns in the field of tumour immunology is the emergence of immunologically resistant tumour variants, which evolve under the selective pressure of vaccine immunization.[100]

1.3 Chromatography

1.3.1 Reverse phase –high performance liquid chromatography

Reverse-phase chromatography uses a polar mobile phase for elution of a column containing a non-polar stationary phase. The stationary phase is composed of silica particles (3-10 μm) with surface bonded groups, for example ocatadecylsiloxane (ODS). HPLC has the advantage over gas chromatography in that it is not limited in applicability by component volatility or thermal stability. This makes it an ideal analytical tool for the analysis of polymers, polar, ionic and thermally unstable materials.

A HPLC system consists of a set of pumps for the mobile phase, an injection loop, a column and a detector (Figure 1.6). There are several sizes of columns available, including the widely used 3 - 4.6 mm i.d. columns, small-bore 1mm i.d. columns and also microcapillary columns that can have internal diameters as small as 50μm. As a general rule, smaller columns are cheaper to run as they consume less solvent due to the low mobile phase flow rates and give an increase in sensitivity if a concentration sensitive detector such as electrospray is used. Several groups have emphasized that in such cases, the sensitivity of liquid chromatographic methods is a quadratic function of the LC column diameter. It can therefore be concluded that the replacement of a conventional 4.6 mm i.d. column by a 0.1 mm i.d. column can yield a theoretical increase in sensitivity by a factor of $(4.6/0.1)^2 = 2116$, providing equal sample volumes are injected when using micro and nanobore LC systems, limiting factors such as sample injection volume and injection speed apply.

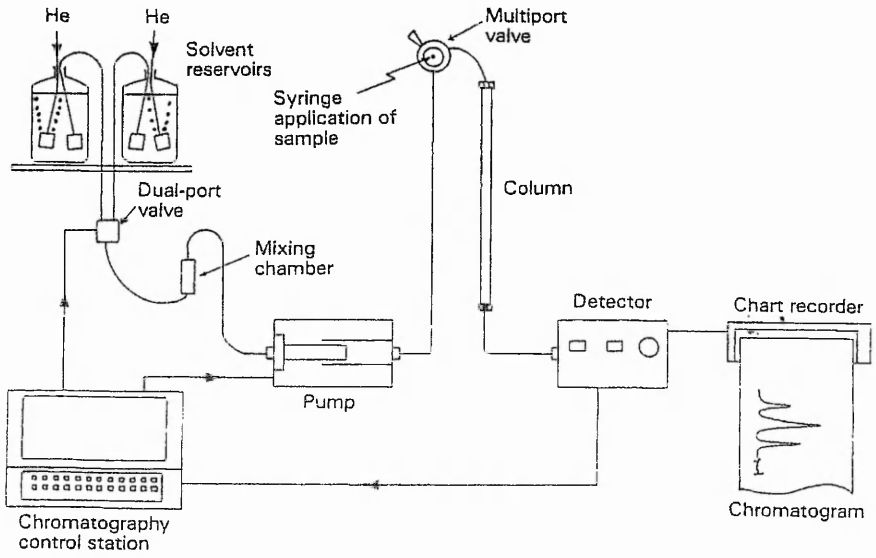


Figure 1.6 Schematic diagram of a HPLC system.

The simplest method of performing a separation by HPLC is using a single solvent to transport the analytes through the column. This is termed isocratic elution. However a gradient elution profile is more common practice, where two solvents that differ greatly in polarity are employed (e.g. acetonitrile and water). The reciprocating positive displacement design is the most commonly employed HPLC pump. It is composed of a piston situated in a low chamber, which alternatively draws up mobile phase from a reservoir and then flushes the pump volume into the column. The HPLC sample is injected in solution *via* an injection loop that is fitted to an injection valve. The injection loop is filled with analyte when in the load position, and the contents of the loop are flushed onto the analytical column when

the valve is switched to the inject position. The LC eluant may be detected by a variety of methods including UV and fluorescence detection. However for the characterisation of peptides, mass spectrometers have become the detectors of choice with full scan or tandem mass spectrometry (LC-MS and LC-MS/MS) being performed on-line.

1.3.2 HPLC theory

- ***Partition coefficient***

The retention characteristics for a solute are dependent upon its distribution between the mobile and stationary phase and the relative affinity towards each. The relative affinity of the solute for each phase is given by the partition coefficient K.

$$K = Cs/Cm \qquad \text{Eq. 1.1}$$

Cs = Concentration in stationary phase.

Cm = Concentration in mobile phase.

- ***Efficiency***

The efficiency of a column is defined by the number of theoretical plates (N) which relates chromatographic separation to the theory of distillation. Theoretical plates can be envisaged as a series of hypothetical layers situated in the column within which a solute is in an equilibrium process of adsorption-desorption between the two relevant phases.

The number of theoretical plates is given by

$$N=16(t_r/W)^2 \qquad \text{Eq. 1.2}$$

t_r = Retention time

W= Peak width at baseline (time)

- *Capacity Factor*

The capacity factor (k`) is a measure of the degree to which that component is retained by the column relative to an unretained component.

$$k` = (t_R-t_o)/t_o \qquad \text{Eq. 1.3}$$

t_R is the elution time of retained component and t_o is the elution time of the unretained component.

- *Separation Factor*

The selectivity parameter is denoted by α and is a measure of peak spacing and is expressed by:

$$\alpha= k_2`/k_1` = K_2/K_1 \qquad \text{Eq. 1. 4}$$

- *Resolution*

Resolution (Rs) is a measure of the ability of a column to separate two adjacent peaks:

$$Rs= \sqrt{N}/4\ (\alpha-1/\alpha)\ (k`/\ 1+k`) = 2(t_{r2}-t_{r1})/W_2+W_1 \qquad \text{Eq. 1.5}$$

Where k` is the average value for the two peaks.

1.3.3 Ion exchange chromatography

Chromatography is dependent upon interactions of different types of solute molecules with ligands immobilized on a chromatographic support. Ion exchange chromatography is one of the basic types of chromatography that works on the principle of reversible interaction of charged solute molecules with immobilized ion exchange groups of opposite charge. In addition to the main ion exchange effect, other weaker types of binding may also occur, for example van der Waals forces and non-polar interactions. Ion exchange columns can be categorized as anion exchangers that attract negatively charged ions, and cation exchangers that attract positively charged ions. Anion exchangers are typically composed of a gel with functional groups such as diethylaminoethyl (DEAE) or quaternary ammonium (Q), whereas cation exchange resins usually contain functional groups such as carboxymethyl (CM) or methyl sulphonate (S).

Ion exchange is a frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides and other charged biomolecules. An attractive feature of ion exchange is that biological activity is often preserved.[31] Ion exchange is therefore the ideal method for the purification of peptides, since virtually all these macromolecules have charged sites at an appropriate pH that allow their adsorption onto the solid ion exchanger. Ion exchange columns are composed of an insoluble polymeric matrix to which charged groups have been covalently bound. Peptides are positively charged at pH values below their pI and negatively charged at pH values above their pI. Ion exchange chromatography exploits this occurrence and allows the separation of peptides from other charged solutes (e.g. salts) by enabling the manipulation of pH, so the peptide

can stick to either type of ion-exchange column. If the pI of the peptide for analysis is known, the type of ion-exchanger, as well as the useful range of mobile phase pH can be predetermined. Ion exchange in conjunction with reverse-phase HPLC offers an orthogonal separation method for complex mixtures.

1.4 MASS SPECTROMETRY

Mass spectrometry has become one of the key techniques for the analysis of chemical and biochemical samples offering important information on the structure, composition and purity of analytes.[162] The first mass spectrometer was developed in 1912 by J.J.Thomson, the purpose of which was to demonstrate the existence of stable isotopes in elements such as neon.[163]

A mass spectrometer is an instrument that produces ions from a sample, separates them according to their mass-to-charge (m/z) ratio and records the relative abundance of each of the ions to obtain a mass spectrum. The component features of a mass spectrometer are discussed below.

1.4.1 Ionisation

- *Electrospray:*

Electrospray was first proposed as a source of gas-phase ions by Dole[165] and has proved to be one of the best methods for the ionization of macromolecular biochemical structures such as nucleotides, proteins and peptides.[164] In electrospray ionization (Figure. 1.7) a solution of analyte is delivered to the tip of a metal or metal coated glass capillary, which is maintained at a high potential (1-5kV). It is this field at the capillary tip that charges the surface of the emerging solvent/analyte flow producing a fine aerosol of charged droplets. As the droplets reduce in size through evaporation they reach the Rayleigh limit, which occurs when droplet surface charge density reaches the point when ion repulsion equals the liquid surface tension.[165] At this point the droplet breaks up as a result of electrostatic forces and smaller droplets are produced. The process is repeated until a further decrease in the

size of the droplets results in the formation of highly charged protonated or cationised molecular ion species. It is this desolvation at the molecular level that is essential to obtain high quality spectra when utilising the electrospray process.

One of the characteristics of an electrospray spectra is that multiply charged ions of the type $[M+nH]^{n+}$ can be produced as well as the $[M+H]^{+}$ species. This is an advantage when analysing the spectra of large biomolecules such as proteins, since multiply charged ions have m/z ratios which fall within the range of simple analysers such as quadrupoles (generally m/z ‹ 2000). There are two conflicting hypotheses on the mechanisms by which charged droplets produced by electrospray lead to gas phase ions; the ion evaporation model (IEM) and the charge residue model (CRM). The CRM proposes that after the Rayleigh limit has been reached, the molecule becomes a free gas phase ion, after several generations of droplet fission, by retaining some of its droplet's charge as the last of its solvent evaporates. The IEM however, suggests that before a droplet reaches the ultimate stage as described above, the field on its surface becomes strong enough to overcome solvation forces and lifts a solute ion from the droplet surface into the ambient gas.[166]

- *Nanospray*

A nanospray source is composed of a thin gold-coated glass capillary drawn to a fine tip (1-30μm) that is positioned about 0.5 - 2 mm away from the heated inlet orifice of the mass spectrometer. The main advantages of nanospray are the greater sensitivity that can be achieved compared to conventional electrospray, and the low flow rates (20-100 nl/min) that are possible with this ionization system.

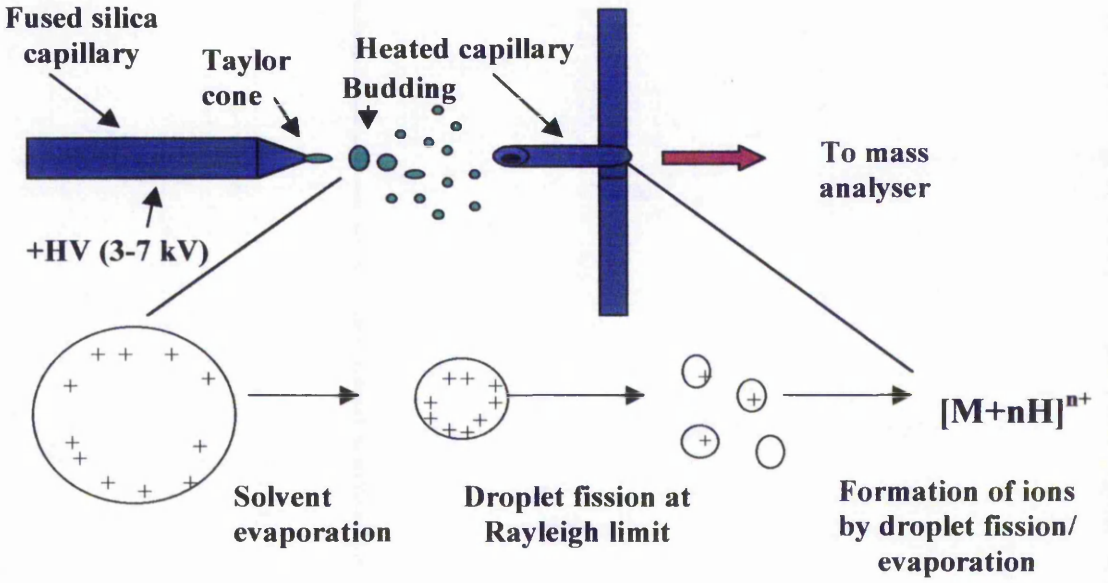


Figure 1.7: Droplet and ion formation in the electrospray interface.

The reduced sample consumption allows for longer analysis time for small samples, which can often prove important when performing tandem mass spectrometry in trace analysis. The higher sensitivity of nanospray is due partly to the size of the droplet formed, which is small in comparison to those produced in conventional electrospray.[167 168] The finer droplets produced have a larger effective surface area, which aids ionization. In addition, the nanospray tip can be positioned closer to the sampling orifice, because desolvation occurs rapidly for the smaller droplets allowing a greater proportion of the ions produced to be transferred into the mass spectrometer vacuum region. Nanospray therefore offers a more sensitive alternative to the conventional electrospray ionisation.[169]

- *Microspray*

Microspray is a term used to describe miniaturized electrospray where flow rates are generally low microlitre – high nanolitre per minute and spray voltages are between 2 –3kV. Many microspray devices have been described generally based on the use of narrow fused silica capillaries as the electrospray emitter.[170]

- *Fast Atom Bombardment*

Fast atom bombardment (FAB), also known as liquid secondary ion mass spectrometry (LSIMS) is a technique in which ionization is achieved by focusing a beam of neutral atoms, or ions onto a sample. The sample is dissolved in a non-volatile liquid matrix such as glycerol. A neutral atom beam may be produced by the ionization of an inert gas, usually argon or xenon. The ions formed are accelerated under the influence of an electric field (2-10kV) and are converted to fast neutrals by charge exchange with the inert gas. The resulting neutral ion beam is directed towards the surface of the sample, inducing a shock that ejects ions and molecules from the solution.[171]

- *Matrix Assisted Laser Desorption Ionization*

MALDI is an efficient ionization process in which the analyte is mixed with an excess of an organic matrix. The matrix is chosen to have a strong absorption at the laser wavelength (usually a pulsed nitrogen laser with emission at 337nm). Examples of common matrix materials are 2,5-dihydroxybenzoic acid, sinapinic acid and nicotinic acid. A solid solution of the sample is formed after the solvent has evaporated off, and it is the irradiation of this mixture by the laser which induces the

accumulation of a large amount of energy in the condensed phase through electronic excitation of the matrix molecules. A pulse of ablated sample is formed and proton transfer occurs between the photoexcited matrix and the analyte leading to the formation of $[M+H]^+$ ions that go on to be detected by the mass spectrometer.[172] The pulsed nature of the MALDI process makes it particularly suited to being combined with time-of-flight and ion trap analysers.

1.3.2 Mass Analysers

1.3.2.1 Linear Quadrupole and Quadrupole Ion Trap Mass Spectrometry

Ions formed in the ion source are separated according to their mass-to-charge ratio (m/z) in a mass analyser. There are three important characteristics of a mass analyser; the upper mass limit, which dictates the highest m/z that may be separated, the resolution, which is indicative of how effective the analyser is at yielding distinct signals for two ions that have a small mass difference, and the transmission which determines the sensitivity of the spectrometer. There are several types of mass analyzer including magnetic sector, time-of-flight, FT-ICR, linear quadrupole and quadrupole ion trap. The quadrupole ion trap mass spectrometer was employed in the work reported here, and a detailed description of this instrument and the related quadrupole analyzer are given below.

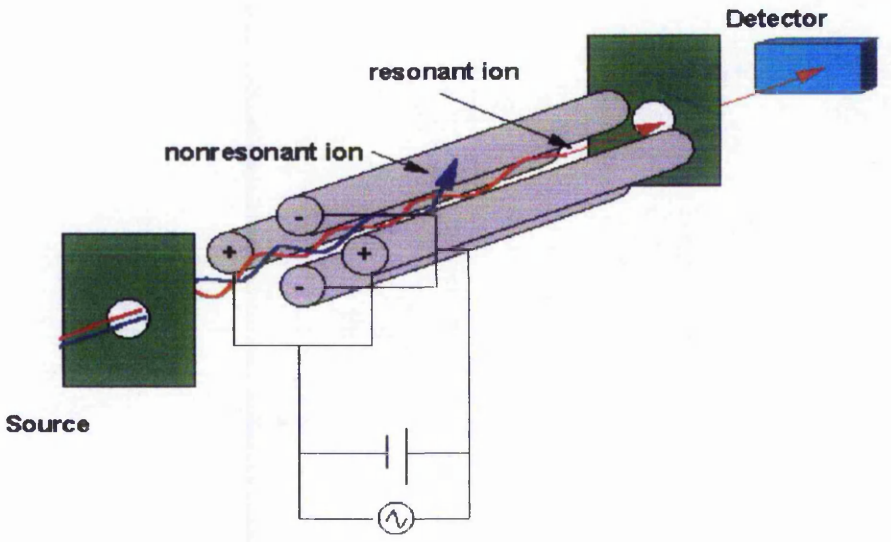


Figure 1.8: Schematic of a quadrupole mass filter.

The linear quadrupole mass filter, invented at the University of Bonn in 1956 by Paul and Steinwedel, gave rise to modern day quadrupole and quadrupole ion trap mass spectrometers.[173] This device has been applied to a wide range of analytical problems[174] and consists of four metal rods of hyperbolic or circular cross section, which are arranged as shown in Figure 1.8. The quadrupole rods are connected electrically in diagonally opposite pairs and superimposed DC and radiofrequency (RF) voltage components are applied to the rods creating a quadrupolar field within the analyser. The DC and RF potentials applied to each pair of rods are of equal magnitude, but the DC components are opposite in sign, and the RF component is shifted by 180°. Ions within the quadrupole mass spectrometer have complex trajectories, some of which are stable allowing ions to be transmitted through the device to the ion detection system. The equations for the movement of these ions

can be derived from Newton's second law (Force = mass x acceleration) to give second order differential equations similar to the Mathieu equation.

$$d^2u/d\xi^2 + (a_u - 2q\cos 2\xi)u = 0 \qquad \text{Eq. 1.6}$$

Where:

$u = x$ or y

$\xi = \pi ft$ (f = frequency, t = time)

and a and q are dimensionless constants, which for ions in a quadrupolar field are:

$$a_x = -a_y = 8eU/mr^2{}_0\Omega^2 \qquad q_x = -q_y = 4eV/mr^2{}_0\Omega^2 \qquad \text{Eq. 1.7}$$

Where m is the mass of a singly charged ion (e) traveling through a quadrupole of radius r_0, operating with RF and DC potentials V and U respectively and an RF drive angular frequency Ω ($=2\pi f$). When ions are assumed to have stable trajectories, and all experimental parameters remain constant, u depends only on **a** and **q** and conditions where ions are stable can therefore be represented on a stability diagram. The Mathieu stability diagram for linear quadrupole mass spectrometers is shown in Figure 1.9. Ions must be stable in both x and y directions in order to pass along a linear quadrupole, and thus be detected. That is to say that they must lie within the boundaries of the stability diagram.

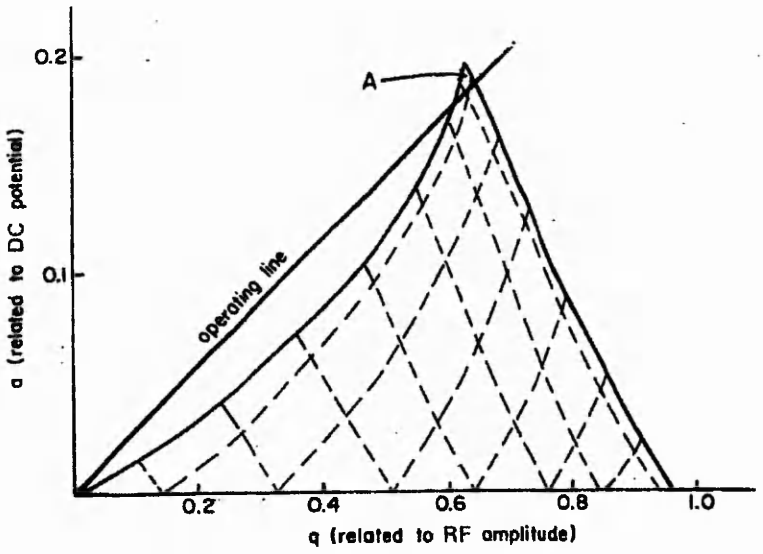


Figure 1.9: The stability diagram for the linear quadrupole.

The ions in this stable region are said to posses fundamental frequencies, Ω_0, where

$$\Omega_o = \beta\Omega/2 \qquad \text{Eq. 1.8}$$

β can take values between 0 and 1, and is a parameter directly related to the ion frequency. The same fundamental or "secular" frequency is possessed by all ions that lie on the marked iso-β lines. When the quadrupole voltages are scanned at a constant DC/RF voltage ratio, the working point of ions of increasing m/z ratio will in time have **a** and **q** values inside the top apex of the stability diagram. These ions will have stable trajectories, and will therefore pass along the analyser to the mass spectrometric detector, whilst ions with adjacent m/z ratios will have unstable trajectories and will not be transmitted to the detector.

The quadrupole ion trap mass spectrometer (QITMS) (Figure 1.10) is a three-dimensional analogue of the quadrupole mass filter. The ion trap consists of a central circular ring electrode and two end cap electrodes.

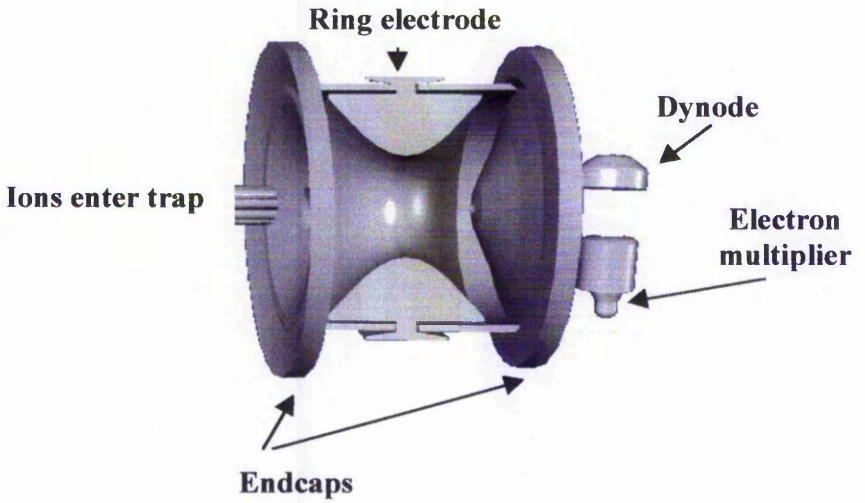


Figure 1.10: Generalized diagram of a quadrupole ion trap.

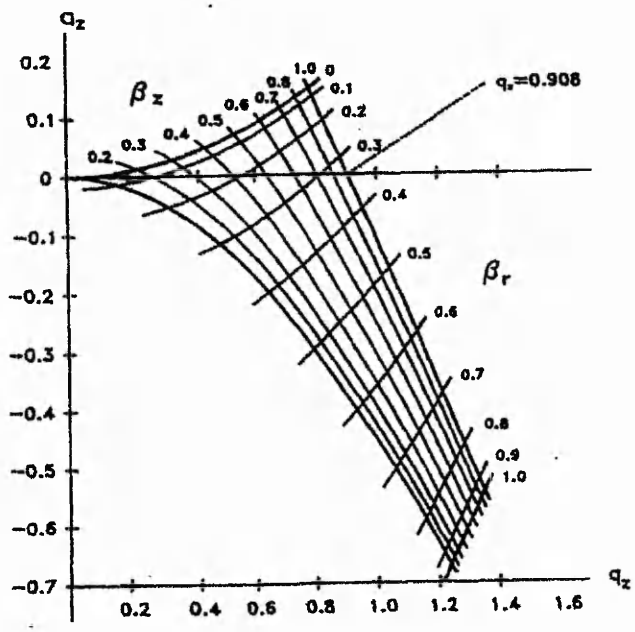


Figure 1.11: The ion trap stability diagram.

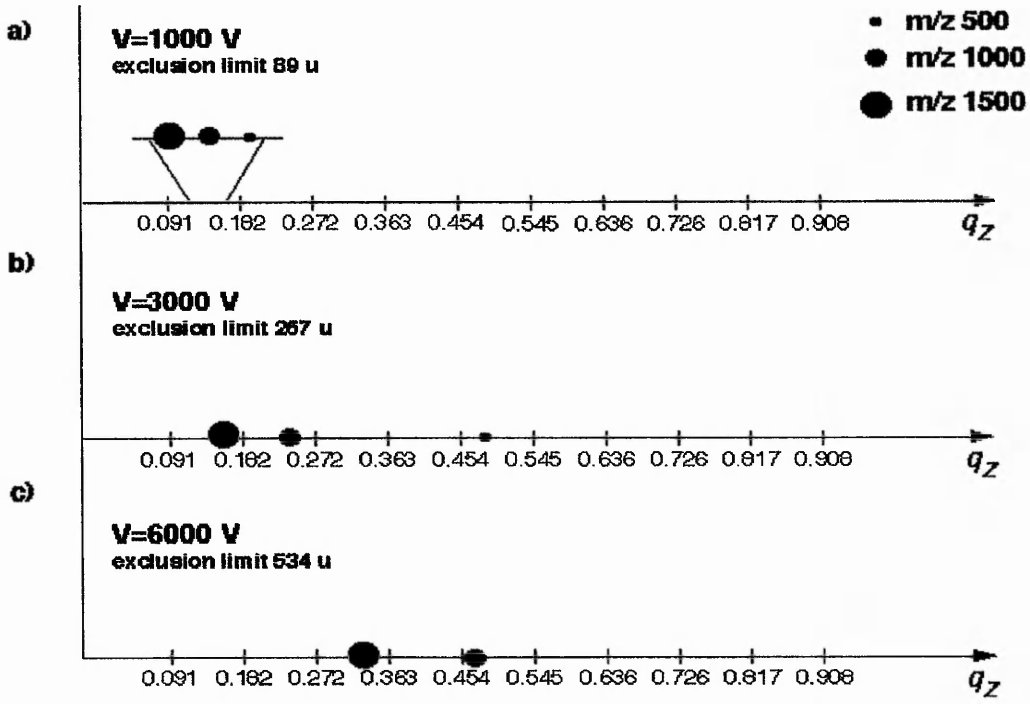


Figure 1.12: Simplified stability diagram of an ion trap operating in RF only mode (a_z= 0) at three *rf* voltage amplitudes.[175]

The stability diagram for the ion trap is shown in Figure 1.11. The area next to the origin of the stability diagram is where r and z stability regions overlap; here ions will have stable trajectories in all directions and are therefore trapped inside the device. The device is operated typically with a helium pressure of approximately 1 mTorr and collisions with the helium reduce the kinetic energy of the ions which serves to quickly contract trajectories of trapped ions towards the center of the ion trap.[174] Ions have secular frequencies in r or z directions which are determined by β_r and β_z at the working point of the ion. The simplest method for obtaining a spectrum is the mass-selective instability scan, devised by Stafford *et al.* where an RF voltage only is applied to the ring electrode and the end caps are earthed.[176] Under these conditions a_z is always equal to zero and all trapped ions have working points that lie along the q_z axis. High mass ions have a lower q value than low mass ions, and hence have working points to the left of the stability diagram. Figure 1.12. represents a simplified stability diagram of a trap operated in rf only mode, where q_z is a parameter derived from the Mathieu equation that for ion traps is defined as follows;

$$q_z = -2q_r = 4eV / mr_0^2 \Omega^2 \qquad \text{Eq. 1.9}$$

e = charge of an electron

m = mass of an ion

r_0 = radius of ring electrode

Ω = angular frequency of the fundamental rf

V = rf voltage amplitude

An ion therefore has a working point on the q_z line determined by its mass to charge ratio and the trap operating conditions (Figure 1.12).[175] Ions that have q_z values less than 0.908 are stable, whereas those with higher q_z values fall outside the stability region and are ejected from the trap along the z axis (i.e. towards the end caps). Increasing the amplitude of the rf voltage leads to the ejection of ions of increasing mass-to-charge ratio from the trap through a small aperture in the end cap electrode towards the detector, allowing a mass spectrum to be obtained.

A problem with the ion trap is that peak broadening can sometimes occur due to overloading of the trap. The ions repel each other so that ions of the same m/z occupy different working points on the stability diagram and are ejected at slightly different times during the analytical scan. To overcome this problem a software modification known as "Automatic Gain Control" (AGC) was devised which performs a short "pre-scan" before the main scan thus measuring the number of ions being allowed into the trap. The ionization time can therefore be calculated so that an overload of ions does not occur, preventing this space-charging effect.

1.4.3 Tandem mass spectrometry

The technique of tandem mass spectrometry (MS/MS) may be used to obtain structural information about a selected precursor ion retained in the ion trap. In the case of peptide analysis, tandem mass spectrometry allows the full or partial constituent amino acid sequence of the sample to be determined. MS/MS may be performed easily using a QITMS, and can even be extended to multistage tandem experiments (MS^n). The sample is first ionized, or ions are introduced from an external source, and then all ions except for those of a selected m/z ratio, are ejected

from the trap. The selected precursor ion is excited by the application of an auxiliary rf voltage to the end caps, which is tuned to the secular frequency of the trapped ion. This leads to increased collisions with the helium buffer gas, fragmentation and product ion formation. The product ions may then be ejected and mass analysed using a mass-selective instability scan, or a product ion may be selectivity retained in the trap for further stages of tandem mass spectrometry.

Collision activated dissociation (CAD) was the first and remains the most popular ion dissociation method used in tandem mass spectrometry.[177, 178] High energy CAD is possible in magnetic sector instruments where E_{lab} (the laboratory frame of reference energy) is in the KeV range and usually the ion undergoing CAD will only experience a single collision. Low energy CAD occurs in quadrupole and ion trap instruments where E_{lab} values are in the low eV range (<100 eV) and multiple collisions can be experienced by the ion. Tandem mass spectrometry not only gives sequence information but also lowers the limit of detection by enhancing the signal: noise ratio.

CAD in tandem mass spectrometry can be described by the following process:

Ion activation:

$$M_1^+ + N \rightarrow M^*_1 + N \qquad \text{Eq. 1.10}$$

Ion dissociation:

$$M_1^* \rightarrow M_2^+ + M_3$$

Where;

M_1^+ is the accelerated precursor ion with high translational energy

N is the neutral target gas (e.g. helium, nitrogen or argon)

M^*_1 is the activated ion ($E>E_0$) and

M_2^+ is the resultant fragment ion.

Collision complexes are not favoured because of the high relative velocities of the ion and the neutral target gas. The internal energy of an activated ion leads to fragmentation and the maximum kinetic energy converted to internal energy per collision is given by the collision energy in the centre of mass frame of reference, E_{cm} .

$$E_{cm} = E_{lab}\, mt/(mt + mp) \qquad \text{Eq. 1.11}$$

Where;

mt = mass of target gas

mp = mass of precursor ion

E_{lab} = energy in laboratory frame of reference

1.5 Mass spectrometric analysis of peptides

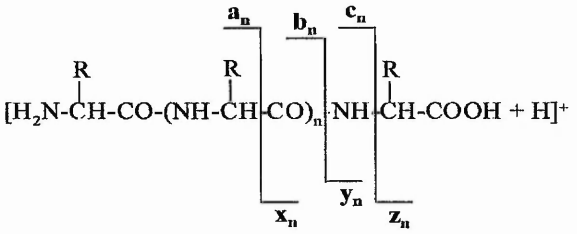
Many different techniques have been employed for the sequencing of peptides and proteins over the past thirty years. Edman degradation was first employed[179] to sequence peptides by removing one amino acid at a time from the amino terminus using chemical reagents, followed by the analysis of the released amino acid derivative. The use of mass spectrometric methods for the sequencing of peptides can be traced back over 30 years. Vilkas *et al.* developed a procedure for the derivatization of peptides to allow them to be stable, both in terms of thermicity and volatility, thus enabling their introduction into an electron impact source of a mass spectrometer using a solid probe. Unfortunately using this technique high nmol quantities of peptide were required for any sequence information to be obtained.[182] Following the development of appropriate ionization techniques, mass spectrometry began to be used for the sequencing of unidentified peptides. Sequences were identified from the mass-to-charge ratios (m/z) of enzymatically cleaved peptide fragment ions. The m/z ratios of the peptides detected were used to search a protein database to match a set of peptide masses predicted for this enzymatic digestion[180]. However, problems commonly arose when using both of these two sequencing techniques due to a lack of homogeneity in peptide content and ambiguity in assigning correct amino acid sequences, so these approaches are not well suited to the sequencing of peptides derived from complex mixtures.

Tandem mass spectrometry (**1.4.3**) differs from the above techniques in that a mixture of peptides can be analysed, the reason for this being that the first stage of MS/MS acts as a separation technique to select the peptide ion of choice from a mixture of ions. After selection, the precursor ion is activated. This is done typically

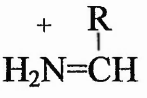
via collisions with gas molecules (collisionaly activated dissociation), by collisions with surfaces (surface activation) or by absorption of a photon (photodissociation).[181] By performing tandem mass spectrometry detection limits have now been lowered to sub-femtomole/μl levels[183]. Tandem mass spectrometry can be used on both linear and cyclic peptides (such as gramicidin S) to elucidate the amino acid sequence. When peptides are subjected to CAD, the precursor peptide ions mostly fragment at the amide bonds along the backbone, generating a ladder of sequence ions.[184] The most frequently observed ions are the N-terminal type *b* acylium ions (NH_2-CHR_1-CO.....$NHCHR_nCO^+$) or the C terminal ions, *y* ions, (NH_3^+-CHR_n-CO.....$NHCHR_1$-CO_2H) formed by H rearrangement on the carboxyl terminus (Figure 1.13) A series of one type of fragment ion allows the amino acid sequence to be determined by the differences in the masses of adjacent sequence ions.[180] Other fragment ions arise from dissociation of the peptide at bonds other than the amide bond. If the break occurs one carbon towards the N or C terminus of the amide bond, and the charge is retained on the amino terminus, an *a* or *c* ion is formed. Similarly, *x* and *z* ions can be formed in this way too when the charge is retained on the carboxy terminus.[185, 186] Low m/z ions that correspond to the structure (R)$CH=NH_2^+$ (immonium ions) are also common in the MS/MS spectra of peptides and convey important information about the amino acid content of the peptide. Immonium ions are frequently observed when the amino acids Met, His, Trp, Tyr or Phe are present in the peptide[185]. All these fragmentation events may occur under both high and low energy, although immonium and internal fragment ions are more commonly seen with high energy CAD. Figure 1.13 shows the different types of ions resulting from cleavage of the various bonds within a peptide sequence after it has undergone CAD. Table 1 lists the common types of

internal fragment ions that can be observed under low mass collision activated dissociation. Table 1.2 shows the less common fragment ions resulting from high-energy collision activated dissociation. These d, v and w ions are more readily seen on dissociation of peptides in mass spectrometers such as magnetic sector instruments.

[a]

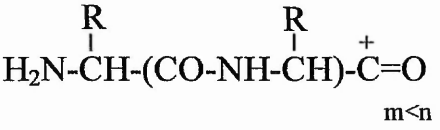


[b]



immonium ion

[c]



internal fragment ion

Figure 1.13: The different fragment ions that can be produced when peptide ions are subjected to collision activated dissociation; (a) a peptide containing three amino acids and the typical fragment ions produced as a result of low energy collision activated dissociation, (b) an immonium ion and (c) an internal fragment ion.

Table 1.1: Common peptide fragment ions resulting from low energy collision activated dissociation.

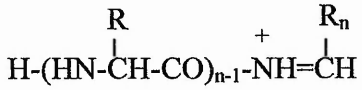
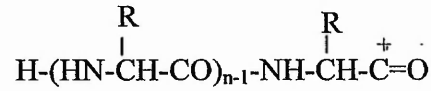
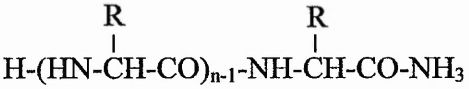
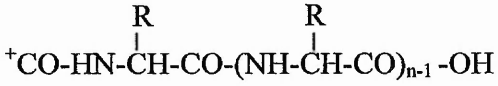
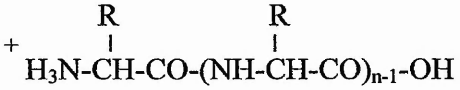
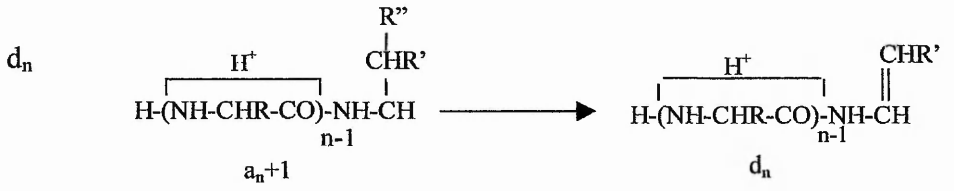
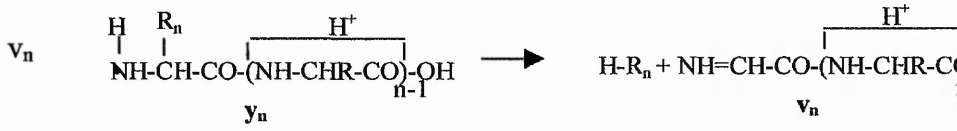
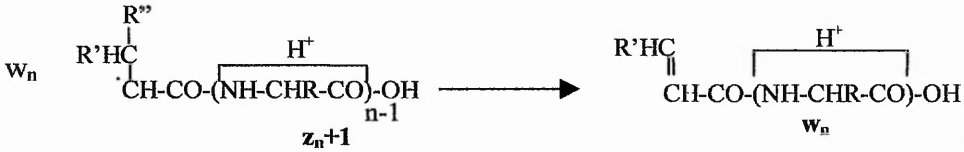
Fragment ion type	Structure of Fragment Ion
a_n	
b_n	
c_n	
x_n	
y_n	
z_n	

Table 1.2: Fragment ions observed more commonly in high energy peptide ion fragmentation.

Fragment ion type	Formation and Structure of High Energy CAD Fragment ions
d_n	
v_n	
w_n	

Low energy fragmentation of doubly protonated peptides that incorporate arginine and cysteic acid residues has been investigated by Summerfield *et al.*[187] These studies revealed that unexpected product ions corresponding to *d* type ions are produced due to cleavage of the side chain of cysteic acid. When a high energy system is employed new fragments that require the cleavage of the peptidic chain and of the amino acid lateral chain can be seen, which can allow the isomers Leu and Ile to be distinguished[188]. Due to the difficulty in determining whether a set of ions correspond to *b* or *y* fragments, Qin *et al.* devised a method for distinguishing between these two types of fragment ion by using ^{18}O labeling of the C-terminus of tryptic peptides using 50% ^{18}O water during trypsin digestion. C-terminal ions are

readily identified by the presence of O^{18} incorporated into the isotope distribution, whilst *b* ions are recognised by their normal isotope distribution.[189] This makes the characterisation of peptides derived from enzymatically cleaved proteins easier, it does not however aid the simplification of sequencing peptides from naturally derived short length peptides (e.g. cellular epitopes).

Problems can arise in detecting useful fragment ions depending on which constituent amino acids are present in the peptide and where these are situated in the sequence. For example, it has been shown that the position of basic amino acid residues, such as arginine and lysine, on the peptide backbone dictates which type of sequence ion will form. *b* type ions dominate MS/MS spectra of peptides containing arginine or lysine at the N-terminus, and *y* ions if these amino acids are positioned at the C-terminus. The spectrum can increase in complexity if these basic amino acids are situated in the middle of the peptide backbone.[190] One approach to overcoming problems associated with basic amino acids has been reported by Wang *et al.* in which Arg was converted to ornithine (Orn), thus lowering the gas-phase basicity, leading to a lower charge state (i.e. doubly rather than triply charged species).[191] It has also been observed that protons can attach to the side chains of the amino acids in a peptide in preference to the back bone, therefore occasionally no structural information can be gained through tandem mass spectrometric analysis.[192] One group has proposed that the fragment ion abundance and product ion spectral reproducibility can be increased by altering the acidity of collision gas when employing hybrid tandem mass spectrometry such as in triple quadrupole mass spectrometry.[193]

One major obstacle in the assignment of amino acids within a peptide sequence is the capacity to distinguish between the isomers leucine (L) and isoleucine (I), and the isobaric lysine (K) and glutamine (Q). Several methods have been reported that overcome this problem including high energy CAD to generate C-terminal *w* and *v* ions, allowing L and I to be distinguished, and chemical derivatization.[194] Common derivatization procedures include N-succunimidyl-2 (3-pyridyl) acetate (SPA) derivatization,[195, 196] acetylacetone condensation of Arg[197] and esterification of acidic residues[198]. SPA reacts in aqueous media with N-terminal amino acid groups and with the side chain amino group of lysine. Alternatively K can be distinguished from Q by acetylation, where a mass shift of 84 Da is observed. Hydrogen/deuterium (H/D) exchange has been employed to distinguish between isobaric K/Q residues when using post source decay (PSD) to sequence peptides, using a Q-TOF analyser. Other modifications can be applied to aid the characterisation of MS/MS spectra such as the incorporation of pyridylacetate (PA) group to promote the formation of *b*-type ions, thus allowing full sequence information to be obtained.

To determine where along the peptide chain different fragment ions are derived, Vachet *et al.* used stored waveform inverse Fourier transform (SWIFT) and double resonance techniques in combination with a quadrupole ion trap. An arbitrary waveform generator output was applied to the endcaps at frequencies corresponding to the secular frequency of a selected product ion. This was applied simultaneously to the application of the resonance excitation signal in the trap and had the effect of resonantly ejecting the product ion as it was formed. The product ions could

therefore be individually selected and ejected from the trap and the intensities of the remaining ions measured.[199]

Multiply charged species are formed when using ESI and the application of MS/MS on doubly, triply and quadruply charged peptides has also been investigated extensively.[200, 201] Downard and Biemann [201] performed a systematic comparison of the high-energy CAD spectra of the singly and doubly charged ions for several basic peptides of similar primary structure. Their studies demonstrated that the dissociation behavior of doubly charged ions is strongly influenced by the location of the basic residues within the peptide. Also they showed that the type of fragment ions produced might differ from the dissociation of the corresponding singly charged precursor ion, although this is not always the case.

Once tandem mass spectrometric analysis has been performed, computerized search algorithms can be employed for data analysis, e.g. Sequest[189] and Genpept[180]. It is now possible to identify peptides that contain post-translational modifications, such as acetylation and phosphorylation and also non-standard amino acid residues such as hydroxyproline and hydroxylysine.[202] With hundreds of gene products being discovered and entered into databases daily, the characterization of peptides through tandem mass spectrometry has become an invaluable analytical tool.

1.6 Analytical procedures for the Characterisation of MHC class I bound antigens.

Characterisation of peptides binding to MHC class I and class II allows the identification of CTL and helper T cell epitopes for developing synthetic peptide-based T cell vaccines, MHC binding prediction confirmation and a better understanding of the mechanisms of processing pathways[119, 140-160, 194]

Human antigen presenting cells (APCs) are thought to bear 10^5 to 10^6 gene products of each MHC class I or II loci on their surfaces. With peptide binding being essential for cell surface expression, it is estimated that the peptide density on an individual APC is 10^{-19} to 10^{-18} per MHC type. Each MHC type is estimated to comprise over 10,000 different peptides with the copy rate for individual peptides being estimated at 10-100 complexes per cell.[119] Abundant peptides (i.e. from infected/transfected or over expressing cell lines) are thought to present > 1,000 to 10,000 copies per cell. It is estimated that generally between 100-500 copies of an individual peptide are required for T cell recognition although cases of one peptide/MHC complex per target cell have recently been reported. The peptide repertoire from MHC class II elution samples are thought to be even more complicated than those from class I elutions. Quantitatively, a single copy of antigen peptide eluted from 10^9 cells will yield 1.6 fmol.[194]

1.7 Extraction of MHC class I & II bound peptides

There are three methods for the extraction of MHC class I molecules from cells;

- Acid extraction of total cellular peptides with TFA
- Acid elution of peptides from immunoaffinity purified class I or class II complexes
- Elution with mild bufffer.

The first two methods above both require a high number of cells to obtain a sufficient amount of MHC class I bound peptides for detection by RP-HPLC and mass spectrometric analysis (10^9 – 10^{11} cells as compared $\approx 10^9$ for the citrate phosphate buffer elution).[101] There are several problems associated with using TFA for the extraction of total cellular peptides. Firstly, treatment with TFA at pH 2 results in total cell cytolysis and the release of all cytosolic peptides. This cytosolic material contains proteolytic peptidases that can alter or destroy peptides of potential interest. To overcome this problem protease inhibitors have to be added to minimize peptide degradation. Also, because of the total cell lysis, only a small percentage of the peptides released are actually those bound to MHC class I molecules, which presents difficulties for the extraction of MHC class I or class II bound peptides.

Isolation of MHC class I or class II bound peptides by immunoaffinity is a more efficient method than TFA cell lysis. Monoclonal antibodies (mabs) are raised against individual class I allotypes and these can then be used to immunopurify class I complexes. Again the cell has to be lysed either by employing a detergent or by lowering the pH, revealing total cellular content and, as above, protease

inhibitors have to be added to minimize the degradation and damage of cellular components. However, by using specific mabs, the MHC class I molecule of choice can be selected, and the peptide bound to this molecule can be dissociated by acid elution. There are two main ways of exploiting mabs for the isolation of MHC class I (and indeed class II) bound peptides. The first is to construct an immunoaffinity column packed with mabs bound to a suitable support. The alternative method is to use immunoprecitation, where the materials are essentially the same, but the procedure is performed in an eppendorf, instead of elution through a column.[102-160] Although specific, one of the main problems with this technique is that MHC molecules may also bind to peptides released from within the cell interior by cytolysis, which would not normally go on to be expressed at the cell surface. These peptides are therefore not presented to the immune system, and CTL responses do not occur against these particular peptides. Hence it would be not be useful to concentrate on these peptides as CTL targets for immunotherapy.

For class II bound peptides the method of immunoaffinity purification is the only currently employed method for isolation of MHC class II peptide complexes.[161] After the collection of MHC class I or class II peptides, the eluates are subjected to biochemical clean-up, separation using RP-HPLC followed by sequencing using mass spectrometric analysis.

In 1993 Storkus *et al.* [101] described a new method for the extraction of MHC class I bound peptides, which involved treating cells with pH 3.3 citrate phosphate buffer for periods as short as 15 seconds. After this treatment cells were found to become phenotypically class I deficient and this was confirmed by serological tests and also

by the incapacity of acid-treated cells to be lysed by cytotoxic T lymphocytes. An experiment using flow cytometry revealed the absence of the β_2- microglobulin light chain, but the class I heavy chain was shown to still be associated with the cell membrane. This loss of the β_2-microglobulin suggested that the MHC class I bound peptide was also released. The other advantage of this method was that class II antigen serological reactivity was unaffected by pH 3.3 treatment allowing repeated harvesting of MHC class I bound peptides from the same cell culture. This method is the only one currently that can ensure that only cell surface MHC bound peptides are detectable.

Traditionally MHC class I bound peptides were characterised using CTL recognition assays of targets expressing exogenously added synthetic peptides.[140-147] In this case synthetic peptides are constructed and allowed to bind in culture with MHC processing deficient mutant cell lines such as T2, which is HLA-A2 positive, but may be transfected with other HLA genes of choice. In order for a MHC molecule to become stable it is necessary for it to have a peptide bound within its groove. By cell lysis, usually using a detergent, followed by immunoprecipitation it is possible to isolate and characterize these MHC class I bound peptides. One method is to derive a cell eluate, fractionate by RP-HPLC and test the resulting fractions to determine whether they are capable of stimulating a cytotoxic T cell response. The sequence of the peptides from this fraction can then be obtained *via* Edman degradation, overlapping synthetic peptide HPLC and CTL studies, or characterized by tandem mass spectrometry. Parallel analysis of MHC eluates permits a much more precise selection of the sensitizing peptide, as this may co-elute with hundreds of other peptides. Many groups practice this method of parallel

CTL/LC-MS studies,[119] by using a post-column in-line splitter to direct one part of the HPLC eluant into the ESI source and the remaining part into small wells for CTL analysis. The ratio of the split is dependent on the sensitivity of the mass spectrometer. Usually the majority of the sample is directed to the mass spectrometer as CTL assays are generally more sensitive.

Many groups have adopted methodologies to elucidate the sequences of naturally expressed peptides bound to MHC class I molecules (i.e. those that have been produced by the proteasome of the cell). In the majority of studies peptides are obtained following cell lysis and immunoprecipitation although an alternative approach is to use cell surface eluted MHC class I presented peptides such as that described by Storkus *et al.* [203, 204, 215] (see section 1.6). Denaturation followed by fractionation according to molecular weight, using either centrifugation or a molecular sieve is then used and the low molecular weight material is subjected to RP-HPLC.[147] This method is ideal for studies involving infected/transfected cell lines versus non-infected/transfected cells. The difference between RP-HPLC chromatographic profiles are identified and, by making sequentially overlapping synthetic peptides from the epitope/ protein of interest, it is possible to compare these to the CTL and HPLC profiles of the naturally eluted peptides. This technique has proved popular when examining the CTL epitopes of viruses that have relatively simple genomes compared to eukaryotic systems. Subtractive analysis is an attractive solution to identifying unknown MHC class I presented peptides when CTLs are not available for immunological assays. It can however be rather time consuming, especially using electrospray ionization as all charge states have to be taken into consideration. Also retention times from RP-HPLC have to be very

reproducible as even a small shift can cause ambiguity in deciphering results. Other groups[141] have adopted a similar technique, but employing Edman degradation to sequence the eluted peptides rather than the more complex overlapping synthetic peptide technique. This allows peptides of unknown origin to be identified. Peptide analysis has been used in conjunction with amino acid prediction assays to assess which epitope of the protein will be expressed and recognized by CTLs.[205, 206] These predictions rely on peptide sequences being dictated by dominant amino acids located at key anchor residues that facilitate peptide binding to the MHC class I groove.

In the early 1990`s mass spectrometry became a more reliable method for determining the sequence of MHC bound peptides. After peptide extraction/immunoprecipitation and separation of high and low molecular weight material, RP-HPLC on a 4.6-mm reverse phase column was employed to produce crude fractions. These fractions were then either tested for their capability to produce a CTL response *in vitro* as in the previously described assays, lyophilized, or subjected to further rounds of HPLC on narrower diameter columns to concentrate the MHC class I peptides into smaller more manageable fractions. These fractions were then analysed using mass spectrometry and the peptides characterized by performing tandem mass spectrometry to gain fragment ions and sequence information (see section 1.4.3). These fragment ions can then be entered into a peptide database and the protein of origin identified. One group of researchers has adopted membrane pre-concentration-capillary electrophoresis mass spectrometry (mPC-CE-MS) as a clean up and concentration strategy and have successfully identified several T cell epitopes.[132-134, 207-209] MHC class II peptides

are of a much longer chain length than class I peptides and are more difficult to sequence with CAD alone, although several groups have been successful in identifying MHC class II associated peptides using mass spectrometric analysis.[210-212] Some of these MHC class II restricted epitopes[213] were found to be tumour specific whereas others have been identified as self-peptides which are over expressed on tumour cells.

1.8 MHC class I antigens characterised by mass spectrometry.

Two main ionization and detection techniques are employed for the analysis and identification of MHC bound peptides. Many groups employ MALDI-TOF as their primary analytical technique, often followed by ESI-with tandem mass spectrometry to obtain sequence information.[150-159] Other groups have employed electrospray ionization tandem quadrupole mass spectrometry without prior MALDI analysis.[102-124, 214] Nanospray is becoming increasingly popular as an ionisation technique for the mass spectrometric analysis of MHC class I and class II derived peptides due to its enhanced sensitivity and extended analysis time compared to conventional electrospray ionisation.[128, 136, 215]

The MALDI-TOF/MS spectrum produced during these analyses is generally less complicated because the ionization process favours the formation of singly charged ions. This can be advantageous when performing a comparison of spectra from complicated samples such as MHC eluted peptides. ESI-MS however can be easily interfaced to separation techniques such as HPLC and CE-MS. Fast Atom Bombardment (FAB) is also a suitable ionization technique for the analysis of peptides, however only one group has employed this as their technique of choice for

the analysis of MHC presented antigens.[149] A novel design of ionization interface to analyze the functional expression and recognition of the nonclassical MHC class $IT10^b$ has been described using a triple quadrupole mass spectrometer equipped with an APCI source interfaced to a polyimide-coated fused silica microcapillary HPLC column.[216]

It is necessary to determine the individual amino acids and the order in which they are linked to gain peptide sequence information (see section 1.5). Reflectron MALDI-TOF can be employed to identify fragment ions by a technique known as post-source decay (PSD). PSD analysis is capable of presenting complete sequence information generated from daughter ion fragmentation patterns. These PSD fragmentation patterns are, however, different to those obtained when using other tandem mass spectrometric techniques with collision activated dissociation. Low energy CAD is performed in triple quadrupole and ion trap mass spectrometers to form a series of characteristic fragmentation ions. When choosing an instrument for molecular ion fragmentation, the ion trap has many advantageous characteristics for analysing and characterizing MHC bound peptides, such as ion accumulation storage and to achieve high sensitivity. The main advantages of the ion trap are its capability to isolate a given ion of interest while ejecting all nonselected ions from the instrument thus allowing multiple fragmentation experiments (MS^n) to be performed on a given ion and also its high sensitivity.[217]

A unique method for determining MHC class I peptide sequence information has been conducted by Woods *et al.* [218] Here the sequence of a murine MHC class I restricted peptide derived from the NP protein of influenza virus was deduced by

performing MALDI on an on-slide exopeptidase digested peptide. By using carboxy- and amino- peptidases, sequence information could be deduced.

The first group to employ mass spectrometry for the analysis of MHC class I bound peptides was Rotzscheke *et al.* [137] who compared the HPLC profiles of virally infected and non-infected murine cells. Those fractions showing a difference in HPLC profile were investigated for their CTL inducing properties. Synthetic peptides representing overlapping regions of the viral protein under investigation were selected and compared with the results obtained from natural peptides HPLC profiles and CTL inducing properties. These peptides were then characterized by ion spray tandem mass spectrometry. The first peptides to be characterised in this way were found not to have natural T cell responses against them, and were therefore described as ligands rather than epitopes as they may be immunologically irrelevant.

Hunt *et al.* [214] used LC-ESI with a triple quadrupole mass spectrometer to identify intracellular epitopes presented as peptides by HLA-A2.1. They adopted an approach involving fractionation on a 4.6 mm RP-HPLC column followed by narrow bore and microcapillary LC-ESI MS, with consecutive HPLC runs used to concentrate the peptides with minimum sample loss. This procedure was employed to overcome the problems that were previously encountered with the use of HPLC and Edman degradation. HPLC was unable to completely resolve the complex mixture, therefore this technique could only be applied to the few fractions that contained one or two dominant peptides. Edman degradation is also confined to picomole amounts of peptide whereas sub-femtomole amounts have been sequenced using tandem mass spectrometry. Immunoprecipitation was used to isolate HLA-

A2.1 bound peptides from the human B lymphoblastoid cell line CIR-A2.1 and these were compared to a mock extract that contained an equal number of HLA-A or –B negative cells. Hunt used 10^8 cells that gave approximately 20 pmol of material. These results provided an insight into the benefits of using mass spectrometric analysis over conventional methods. It was shown that the peptides characterized were nonamers and had certain dominant amino acid residues at position 2. This correlated with Ramensees[137] MHC class I anchor residue binding prediction method for the determination of MHC class I peptides. Synthetic versions of the peptides were obtained to confirm that HPLC elution properties and mass spectrometric fragmentation patterns were similar to those obtained from the MHC class I molecules. More recently this group has modified their analytical approach by employing nanoflow liquid chromatography ESI on a FTMS, to identify peptides present in biologically active fractions from second dimension HPLC fractions, with sequence information being derived using CAD on an ion trap mass spectrometer.[219] This approach was used successfully to identify a new source of HY epitopes and also provided an insight into the influence of post-translational modifications on MHC class I peptides on T cell recognition. This paper also describes the use of a marker peptide used to determine where the peptides elute during RP-HPLC analysis.

1.8.1 T Cell epitopes derived from infectious agents

Viral epitopes have been identified using the methods previously described.[151, 214, 220, 221] HIV-1 epitope sequences from HLA-A2.1 were found by adopting subtractive analysis of peptides using narrow bore and microcapillary HPLC in conjunction with ESI-MS.[220] Other T cell epitopes of infectious origin identified

include sequences from Listeria monocytogenes in mice, using ESI-MS,[108] Human T lymphotrophic leukemia virus –1 (HTLV-1) epitopes detected using MALDI-MS[150] and Influenza-A epitopes using MALDI-MS.[151, 221] Protozoan T cell epitopes have also been characterized from the intracellular protozoan parasite *Trypanosoma cruzi*, using ESI-MS on a triple quadrupole mass spectrometer. For this analysis, hearts from infected mice were subjected to acid extraction followed by hydrophobic interaction chromatography clean up, HPLC and mass spectrometric analysis.[222] Some viruses integrate into the genome of the diseased individual (see section 1.1.4), and are the causal agents of the malignancy. Characterising T cell epitopes from such transformed cells therefore offers a clear insight into immunotherapeutic strategies for the irradication of such virally induced tumours.

1.8.2 T Cell epitopes derived from malignancies

T cell epitopes from malignant disease have also been characterized by mass spectrometry. These include melanoma antigens discovered employing both ESI-MS[119] and narrow bore and capillary RP-HPLC with subsequent spotting on polyvinylidene difluoride membranes for MALDI-MS and Edman microsequencing.[160] The CTL epitopes displayed by murine colon tumour cells were investigated[111] by collecting HPLC fractions and testing their capability to produce a CTL response *in vitro*. The bioactive fractions were then re-chromatographed and subjected to separation by microcapillary chromatography and analysis on-line by MS^2 using ESI-triple quadrupole mass spectrometry. The peptide identified was derived from an endogenous ecotropic murine leukemia provirus glycoprotein called gp70.

Fisk *et al.* determined the sequence of ovarian derived tumour-associated $CD8^+$ CTL epitopes. A HLA-A2.1 transfected ovarian tumour cell line over expressing HER-2 was used and the peptides derived compared to CIR.A2 cells that had also been transfected in an identical manner.[129] One of the most recent uses of mass spectrometry to determine the MHC class I bound peptides was that associated with mutant p53, a protein which is either mutated or over-expressed in over 50% of malignancies. Citrate phosphate buffer was used to extract cell surface MHC class I bound peptides, which were then subjected to RP-HPLC, followed by ESI-MS/MS and the identified sequences were compared to synthetic analogues of these peptides.[215]

A colon cancer cell line of human origin was investigated by Sovoie *et al.*[126] The primary purpose of their studies was not to determine the epitope for use as a vaccine, as outlined above, but to demonstrate different MHC class I presented peptide profiles expressed by different human cell lines. The data from this analysis confirmed large differences in the natural peptide repertoires from colon cancer cells compared to B cells. Immunoaffinity precipitation was used to extract the MHC class I bound peptides, which were characterized by LC-ESI-MS in a triple quadrupole mass spectrometer.

1.8.3 Self T cell epitopes

Mass spectrometry has been used not only for the identification or confirmation of tumour/infectious agent antigens, but also to explore the repertoire of peptides presented amongst the highly polymorphic MHC class I molecules. Prilliman *et al.* [215] used nanospray on RP-HPLC fractions to investigate alpha-2 domain

polymorphism and HLA class I peptide loading. Their studies confirmed that polymorphisms in α-2 vary from subtle to extreme in the way in which they moderate ligand presentation. Zappacosta *et al.* [130] have also used mass spectrometry in this manner to explore the functions of different amino acids as anchor/auxiliary anchor residues.

Some HLA-B27 alleles are thought to have a strong association with susceptibility to spondoarthropathies (AS) and are also associated to other diseases. Through mass spectrometric analysis of self-peptides expressed by different HLA-B27 isoforms, it was determined that some C-terminal residues are more prominent than others. All B-27 subtypes were found to have a strongly conserved preference for peptides having an Arg residue at position 2. The C-terminal however displays a much broader specificity. Some isotypes (e.g. B2705) seem to be largely restricted to a Tyr C-terminus, whereas B2709 molecules appear to accept only hydrophobic C-terminal residues. Investigative analyses such as these may offer a better understanding into the mechanisms involved in susceptibility to disease.[104, 223] Other related MHC queries have been answered through mass spectrometric analysis including evidence that positive selection generates a repertoire of T cells that are weakly self-reactive and cross-reactive. Wang *et al.* [224] found that the MHC class I self expressed epitope YLDPAQQNL, which is derived from an unknown member of the zinc finger protein family, is presented by HLA-A*0201. This data suggests that allogeneic responses are directed against epitopes that are present even at low abundance.

One interesting observation made by Luckey *et al.* [225]was that proteasomes may actually destroy epitopes that may otherwise be presented by MHC class I molecules, suggesting that some epitopes must be generated by nonproteosomal proteases in the cytosol. By using two different proteasome inhibitors to investigate the role of proteasomes in the generation of peptides associated with HLA-A*0201, mass spectrometric analysis was performed on aliquots of proteasome digests which were separated on-line with a microcapillary column and introduced into an ESI source fitted to a triple quadrupole mass spectrometer. A synthetic nonamer peptide was spiked into each aliquot for standardization. Mass spectrometric techniques may therefore be employed not only to identify T cell epitopes for malignant and infectious disease, but also to gain a better understanding of the cellular machinery.

1.8.4 Identification of minor histocompatibility antigens

When HLA identical individuals undergo organ grafts, minor histocompatibility antigens can still cause rejection. After a graft has been performed, immunocompetent T cells may launch a response that is usually weaker than alloreactivity against MHC-incompatibility due to a reaction against MHC presented peptides derived from polymorphic gene products that differ between donor and recipient. These are termed minor histocompatibility antigens (MiHag) and they may act as "transplantation barriers" between HLA matched individuals.[226] The male specific minor histocompatibility complex antigens that cause rejection of organs in females after transplantation have been investigated using mass spectrometry in both human and murine cell lines.[135, 136, 150, 227] Human H-Y antigens were first discovered using mass spectrometry by de Haan *et al.* in collaboration with Hunt,[227] the analysis being performed using parallel LC/MS and

LC/CTL assays. An 11mer human MiHag SPSVDKARAEC, derived from SMCY bound by HLA-B7, was found using a variety of analytical techniques including 2-D HPLC fractionation with CTL prescreening, followed by parallel LC-ESI/MS and LC-CTL assay of positive fractions using an on-line post-column splitting device (80% going to the mass spectrometer).[191] Chemical derivatization (see section 1.5) for the conversion of Arg to Orn for ease of analysis was followed by CAD MS/MS to characterize these peptides, which were estimated to be at a level of approximately 20 fmol. H-Y antigens have also been characterized by Gaskell *et al.* [136] using a Q-TOF. HLA-B27 presented antigens were collected by immunoprecipitation, subjected to RP-HPLC followed by CTL screening, and RP-HPLC MS. Sequencing was performed using a Q-TOF fitted with a low flow ESI source and acetylation was employed to distinguish between K and Q.

1.8.5 Post translational modifications of T cell epitopes

It is now recognized that post translational modifications have a significant effect on T cell recognition (see section 1.1.5).[228] Pierce *et al.* identified an antigen containing a cysteinylated cysteine residue derived from a HLA-A*0101 restricted HY minor histocompatibility antigen which originates from DFFRY. Care was taken during the biochemical clean up to avoid *in vitro* modifications to the amino acid residues. Iodoacetamide was omitted from their protease inhibitor mix in order to avoid amidocarboxymethylation of free cysteine residues. The peptide of interest represented by the A1-YY epitope was XVDC*XTEM^{OXY} (X = Ile or Leu), where C* is a cysteine residue covalently modified by the attachment of a cysteine residue *via* a disulfide linkage and M^{OXY} is representative of methionine which has had the sulfur oxidized to a sulfoxide.[219] Several peptides with different combinations of

oxydation and cysteinylation have been synthesized and evaluated for their ability to represent the T cell epitope.[219] This was found to be a variation of the peptide where the cysteine was cysteinylated, but with a non-oxidized methionine. The analysis by tandem mass spectra of peptides carrying post-translational modifications is not an easy task and this is one area of mass spectrometric analysis of T cell epitopes that has only recently been explored.

In a publication by Hunt *et al* on the mass spectrometric evaluation of HLA-A*0201-associated peptides from MART-1 and gp100, the authors employed citrate phosphate buffer for the elution of cell surface peptides.[203] They highlighted the possibilities of amino acid/peptide structural modifications that may occur due to the experimental conditions. Acidic conditions used during peptide extraction may cause modifications of amino acid residues in naturally processed peptides, resulting in changes in the mass of the target peptide, hence causing difficulty in mass spectrometric interpretations. For example glutamine (Q) and glutamic acid (E) are both prone to cyclization, especially when located at the amino terminus. Under conditions such as those imposed by citrate phosphate buffer, the side chain of glutamine or glutamic acid reacts with the NH_3 group on the amino terminus and forms a ring. This cyclization event could have important effects on the mass spectrometric interpretation of cell surface eluted MHC class I peptides.[203]

Although much work has been performed on the isolation and characterization of MHC class I and class II restricted peptides, there are still many HLA haplotypes in malignancy and disease conditions for which T cell epitopes are not known. Refinements such as nanospray, microspray, nanoscale liquid chromatography,

capillary electrophoresis and capillary zone electrophoresis have made the analysis of MHC class I antigens by mass spectrometry far more sensitive and routine. The science of characterising T cell epitopes is at an early stage and deserves further research in order to gain a fuller understanding of the behavior of MHC associated antigens and the immune response.

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CHAPTER TWO

EXTRACTION, BIOCHEMICAL CLEAN-UP AND OPTIMISATION OF MASS SPECTROMETRIC PARAMETERS FOR THE CHARACTERISATION OF MHC CLASS I ASSOCIATED PEPTIDES

2.1 Introduction

Many different methods have been reported for the isolation, separation and detection of MHC class I and class II associated peptides including the use of affinity chromatography,[1] ion exchange chromatography,[2] size exclusion chromatography[3], and reverse-phase high performance liquid chromatography.[1-4] These are discussed in detail in section 1.3. The objective of the research reported in this chapter was to develop a novel strategy for the isolation and biochemical clean-up of cell-surface MHC class I associated peptides. The method developed includes the use of mild acid elution, trichloroacetic acid protein precipitation, cation exchange chromatography and RP-HPLC, offering an orthogonal chromatographic separation. Nano-electrospray ion trap/tandem mass spectrometric analysis was performed for the characterisation of the eluted peptides. This chapter also describes the development of the nano-electrospray ionisation source, and the optimization of mass spectrometric parameters to allow detection of low level (fmol μl^{-1}) cell surface eluted peptides.

2.2 Experimental

2.2.1 Extraction and biochemical clean-up of cell surface MHC class I associated peptides

For all the experimental procedures described below the glassware was prepared by acid washing (chromic acid, 1M) or by washing in organic solvents, including dichloromethane, hexane, acetonitrile, water + 5% v/v acetic acid. After washing the glass-wear was treated with a silanizing agent (Sigmacote, Sigma, UK) and then rinsed twice in with water.

2.2.1.1 Mild acid elution of cellular material

Adherent cell lines were maintained in ISCOVES medium at 37°c with 5% CO_2. When confluent they were passaged using EDTA + trypsin twice each week. Mild acid elution was performed according to the method of Storkus *et al*[5] to destabilize the β_2-microglobulin and release the MHC class I bound peptide with the following modifications. Citrate phosphate buffer was prepared from 0.131 M citric acid (2.52g in 100 ml dd H_2O) + 0.066 M sodium phosphate (0.94 g in 100 ml dd H_2O) adjusted to pH 3.3 with sodium hydroxide (1 M). The cells were washed with the buffer (20 ml) for four minutes and trypsinized, spun and re-suspended and a cell count was performed using a haemocytometer. FACS[6] was carried out on approximately $5x10^5$ cells using monoclonal antibodies raised against the β_2-m and the HLA groups –A, -B and –C to estimate the destabilization of the β_2-microglobulin, which leads to the release of the MHC class I bound peptide. Acid eluted and non-acid treated cells were subjected to FACS analysis. A slot blot[7] was carried out to determine whether β_2-m from the cell surface was present in the eluate.

2.2.1.2 TCA precipitation

Trichloroacetic acid (TCA) precipitation was carried out to separate high molecular weight proteins, such as the β_2- microglobulin, from the MHC class I bound peptides. A TCA solution (72% w/v) was added to the acid elution buffer extract in a ratio of 1:10. Three experiments were performed in triplicate: (a) the TCA was added and the eluant was spun at 15,000g 10 min^{-1} at 4°C, after which the supernatant was collected. (b) The procedure was repeated, but the sample was

placed in the ultrasonic bath for ten minutes prior to centrifugation, followed by collection of the supernatant and (c) two ultrasonication steps (10 minutes each) were introduced into the procedure, one prior to and the other after centrifugation, before the supernatant was removed carefully without disturbing the pellet. The whole procedure was kept on ice and the sample stored at 4°C until cation exchange or further experimentation was performed.

An experiment was performed to determine the recovery of peptides through the TCA protein precipitation process. A standard was prepared consisting of 50:50 acetonitrile + 5% v/v acetic acid: water (800 μl), that contained gp70 (184 pmol μl^{-1}), A3, (250 pmol μl^{-1}) and β-gal (87 pmol μl^{-1}) peptides. A sample of the above peptides was also prepared to which bovine serum albumin (200 μg) was added. These samples were subjected to the protocol outlined above. The recovered supernatants had volumes of ≈ 400 μl, to which acetonitrile was added to a final volume of 800 μl. The mass spectrometric response following TCA precipitation were compared to the standard by injecting 5 μl, in triplicate, into a flow of 200 μl min^{-1} of 50:50 acetonitrile + 5% v/v acetic acid using the LCQ injection port. For analysis in full scan MS, the capillary temperature was set at 250°C, sheath gas flow at 80 (arb units), auxiliary flow at 30 (arb units), total microscans at 3 and maximum injection time at 200 ms.

The suppression effect of trichloroacetic acid on the peptide response during the electrospray ionization mass spectrometric analysis was investigated. Two mixtures were prepared composed of (i) 50:50 acetonitrile + 5% v/v acetic acid: water (800

μl) and (ii) trichloroacetic acid (72% w/v), that each contained a standard mixture of peptides (gp70, 184 pmol μl^{-1}; A3, 250 pmol μl^{-1}; and β-gal, 87 pmol μl^{-1}). The mixtures were injected (5 μl) in triplicate, into the LCQ injection port into a flow of 50:50 acetonitrile + 5% v/v acetic acid (200 $\mu l\ min^{-1}$).

2.2.1.3 Removal of elution buffer salts using RP-HPLC

The removal of elution buffer salts was investigated initially using RP-HPLC, with the HPLC eluate tested for sodium ion concentration by flame photometry. Citrate phosphate buffer (20 ml) was loaded onto an ODS guard column or ODS guard column + analytical column (Beckman Ultrasphere 4.6 mm x 150mm) *via* the HPLC pump at 1 ml min^{-1}. The standard salt solution (citrate phosphate buffer, 10μl) was diluted to 10 ml with water. Samples of the HPLC eluate (10 μl) were collected at five minute intervals and diluted to 10 ml. The sodium ion concentration of the samples was measured using emission from the flame photometer (Coring Flame Photometer 410). The 1/1000 original sample was used to prepare a calibration curve by making up 1/10 serial dilutions for emission measurement. The same procedure was performed for measuring the salt concentration eluting from the combined guard column and analytical column after cation exchange eluates were washed through following adjustment to pH 8 with HCl (0.1M). These experiments were performed with NaOH (0.1M) as the eluting buffer for the cation exchange chromatography.

2.2.1.4 Ion exchange chromatography

Ion exchange chromatography was investigated using both anion and cation exchange resins. For cation chromatography a 1 ml Econo S cartridge (Bio-Rad, Hemel Hempstead, UK) was conditioned by injecting NaOH (0.1 M, 10ml) followed by HCl (0.1M, 15 ml). Citrate phosphate buffer (20ml; pH 1-2) spiked with the A3 peptide (500 nmol) was then injected on to the column. The column was washed with 0.1 M HCl (30ml, 1 ml min^{-1}) and the peptide eluted with NaOH (0.1M, 6ml). Fractions (1 ml) were collected into 3 ml silanized glass bottles. For anion chromatography a 1 ml Econo Q cartridge (Bio-Rad, Hemel Hempstead, UK) was conditioned by injecting HCl (0.1 M, 10ml) followed by NaOH (0.1M, 15 ml). Citrate phosphate buffer (20ml; pH 11-12) spiked with the A3 peptide (500 nmol) was then injected on to the column. The column was washed with 0.1 M NaOH (30ml, 1 ml min^{-1}) and the peptide eluted with 0.1M HCl (6 ml). Fractions (1 ml) were collected into 3 ml silanized glass bottles. The pH of each of the anion and cation eluate fractions was measured using a pH meter (HANNA-Instruments, UK). The 1 ml fractions for both the cation and anion exchange eluates were place into quartz cuvettes. A blank was prepared using NaOH (0.1M) for the cation eluates or HCl (0.1M) for the anion eluates respectively. The absorbance of each fraction was then measured at 214 nm with a deuteranium lamp using spectrophotometry (Perkin Elmer 5515 UV/VIS spectrophotometer, UK).

2.2.1.5 Cation Exchange and RP-HPLC

A 1 ml Econo S cartridge (Bio-Rad, Hemel Hempstead, UK) was conditioned by injecting NaOH (0.1 M, 10ml) followed by HCl (0.1M, 15 ml). Citrate phosphate

buffer (20ml; pH 1-2) spiked with the A3 peptide (500 nmol) and the β-gal peptide (65 nmol) was then loaded onto the column. The column was washed with 0.1 M HCl (30 ml) and the peptide eluted with 0.1M NaOH (8 ml). Fractions (2 ml) were collected into 3 ml silanized glass bottles. The pH of each fraction was measured using a pH meter (HANNA-Instruments, UK). Fractions above pH 12 were adjusted to pH 8 and introduced onto the Beckman Ultrapshere (4.6 x 150 mm I.D.) column. The RP-HPLC column was then washed with water (pH 8, 20 ml at 1 ml min^{-1}). Elution of the peptides was performed by introducing a gradient from 0-100% acetonitrile (HiPersolv, BDH, UK) +5% v/v glacial acetic acid (Fisher Scientific, Loughborough, UK) and 0.01% TFA (Aldrich, Milwaukee, WI, USA) over 40 minutes. HPLC eluate fractions (2ml) were collected into 3 ml silanized glass bottles from 19% acetonitrile onwards.

A standard was prepared by adding A3 peptide (500 nmol) and β-gal peptide (65 nmol) to 25:75 acetonitrile: water + 5% v/v acetic acid (2 ml). The peptides were analysed by injecting 5 μl of each sample in triplicate into the LCQ injection port into 50:50 acetonitrile: water + 5% v/v acetic acid (200 μl min^{-1}). A blank consisting of mobile phase was also injected in triplicate in between each sample. Mass spectrometric analysis of the peptide ions was performed under the following conditions; heated capillary 250°C, sheath gas flow 80 (arb units), auxiliary gas flow 30 (arb units), total microscans = 3 and maximum injection time 200 ms. For MS/MS the $[M+H]^+$ ion for the A3 peptide, m/z 1045.6 and for the β-gal peptide m/z 961.2 were selected with an isolation width of 3 u and CAD was performed with a relative collision energy of 35%.

2.2.1.6 Preconcentration

The effect of lyophilization was determined by subjecting peptides (A3, 72 nmol; p18, 2.86 nmol; β-gal, 22.8 nmol and gramicidin S, 3.7 nmol) to cation exchange and reverse phase chromatography as described previously (section 2.2.1.5). RP-HPLC fractions (2 ml) were collected into 3 ml silanised glass bottles and stored at 4°C until needed. An aliquot from each sample (1 ml) was placed into a 1.1 ml silanised tapered glass vial with a teflon seal. These were then frozen in liquid nitrogen and placed in the freeze dryer for 3 hr or until near dryness. The remaining 1 ml of HPLC eluate was added and the drying down process repeated. The residue was made up to 60 µl with 50:50 acetonitrile 5% v/v acetic acid: water. The samples were vortexed for 1 min and then placed in the ultrasonic bath for five minutes before injection into the mass spectrometer. A standard was made up in 50:50 acetonitrile + 5% acetic: water (60 µl) containing the same peptide mixture (A3, 72 nmol; p18, 2.86 nmol; β-gal, 22.8 nmol and Gram S, 3.7 nmol). Three samples were also made up with the same amount of added peptide but to 2ml of 50:50 acetonitrile 5% v/v acetic: water. These three samples were lyophilized in the same manner as described above.

Loop injections (5 µl) of the standard and samples were performed in triplicate with a blank consisting of mobile phase ran between each sample. These were injected into a flow of 50:50 acetonitrile + 5% acetic: water (200 µl min^{-1}). The analysis was

performed by mass spectrometry using the same mass spectrometric conditions as described in section 2.2.1.4.

2.2.1.7 Microbore LC-MS

A microbore LC system consisting of a pre-column and an analytical column connected by a 6-port switching valve was constructed to allow on-line sample preconcentration and switching between the two columns (Figure 2.1). Peptides were loaded *via* an injection loop (3 ml, Rheodyne 7010) onto a RP-HPLC microbore pre-column (Jupiter, Phenomonex, ODS, 1 x 30mm; 200 μl min^{-1}) connected on-line to an analytical RP-HPLC microbore column (Jupiter, Phenomonex, ODS, 1 x 150mm) *via* a six port switching valve. (The pre-column was washed with 0.1% v/v TFA (200 μl min^{-1}) and the column effluent, containing the ion exchange salts, was directed to waste. After 20 minutes washing the 6-port valve was switched and the peptide fraction eluted onto the analytical column using 20:80% solvent B (acetonitrile + 5% v/v acetic acid + 0.01% v/v TFA): solvent C (water + 5% v/v acetic acid + 0.01% v/v TFA) at 200 μl min^{-1}. The analytical column was eluted using a gradient of 20-90% B:C over 45 minutes at a reduced flow rate of 20 μl min^{-1} *via* a microflow splitter (SGE, UK). Mass spectrometric analysis of the eluant was either performed on-line using the LCQ ESI-MS source or RP-HPLC fractions were collected every 20 seconds (1-4 μl) after 35 % solvent B for analysis by nano-electrospray MS and MS^n. For the analysis of mock or cellular derived eluates containing MHC class I eluted peptides, the cation exchange eluate had an ion pairing reagent (TFA 70 μl) added to adjust the pH to < 2 to encourage efficient binding of the peptides to the reversed phase packing.

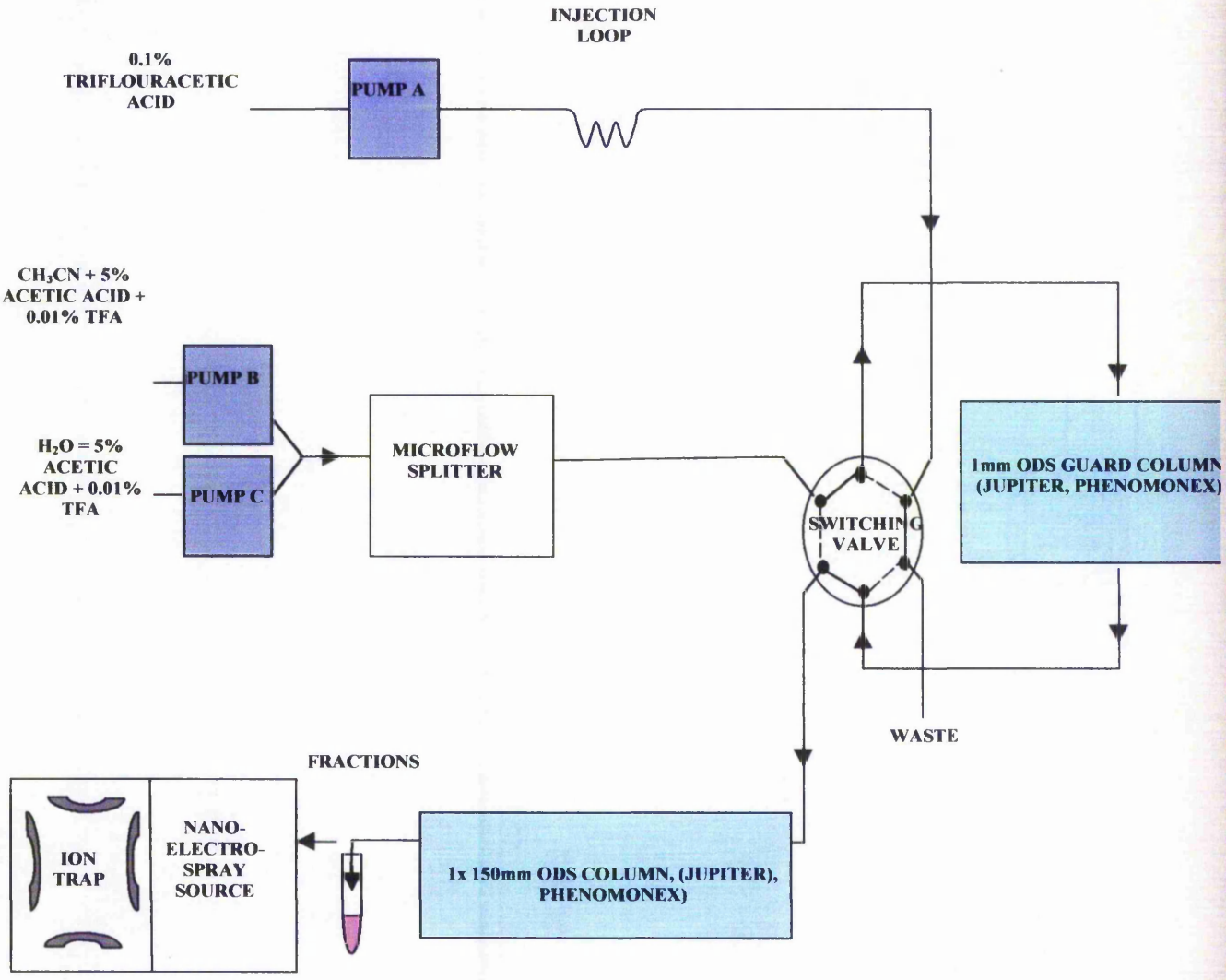


Figure 2.1: RP-HPLC configuration with 6-port switching valve for the biochemical clean-up of MHC class I cell eluates.

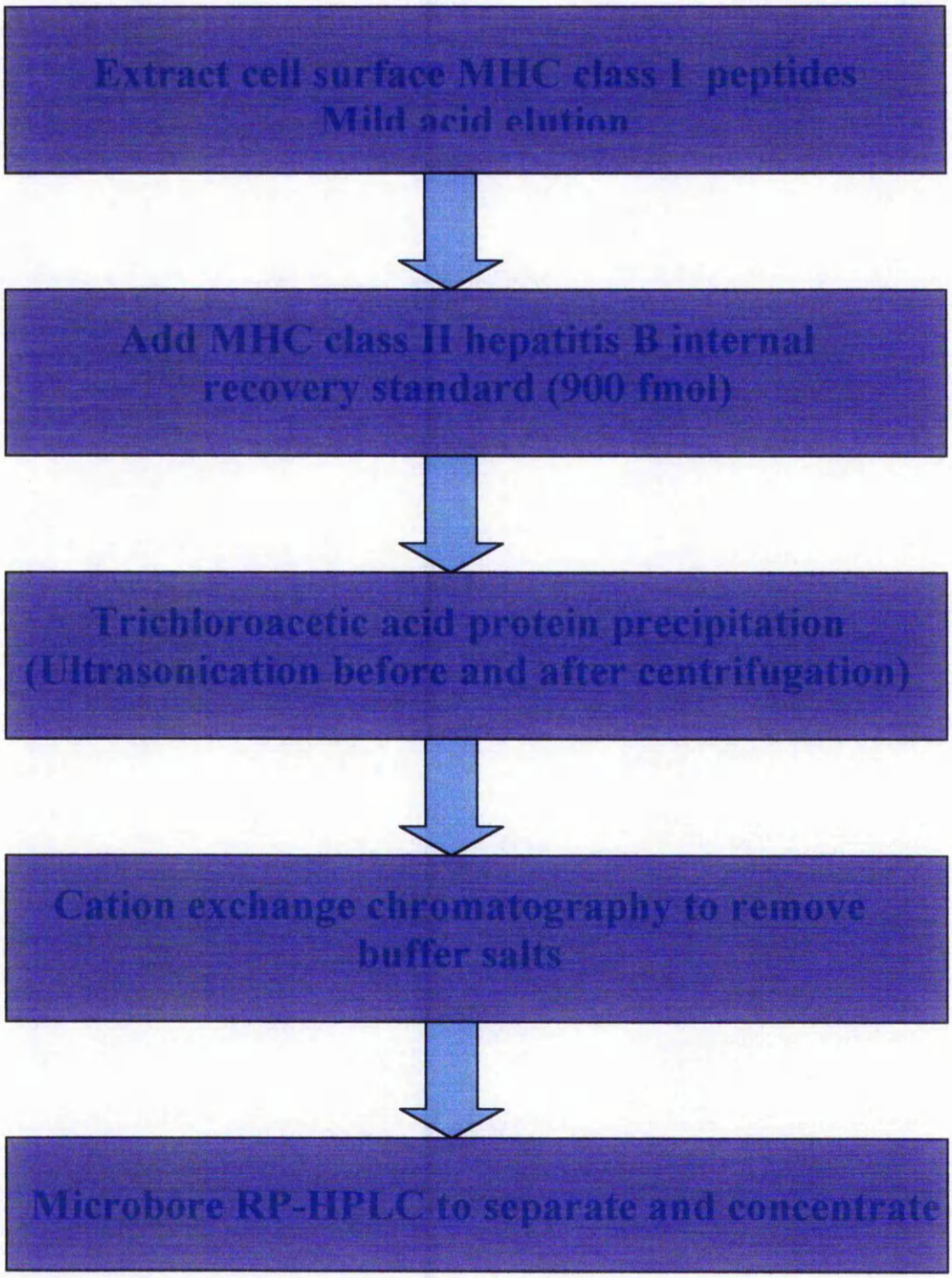


Figure 2.2 Flow diagram summarising the proposed analytical protocol for the extraction and biochemical clean-up of cellular derived MHC class I peptides.

2.2.1.8 Recovery of model MHC class I and class II restricted peptides

The proposed analytical protocol for the extraction and clean-up of cellular derived peptides developed from the experiments described above are summarised in Figure 2.2. The efficiency of the proposed protocol for the isolation, biochemical clean-up, separation and analysis of cell surface MHC associated peptides was tested using synthetic peptides. Synthetic MHC class II Hep B peptide (5.25 nmol) was added to citrate phosphate buffer (20 ml), which was then subjected to the methodology outlined below. Trichloroacetic acid (2 ml) was added to the buffer, and the resulting suspension was placed in an ultrasonic bath for 10 mins, centrifuged (15,000g, 10 min, 4°C) and then returned to the ultrasonic bath for a further 10 minutes. The supernatant was removed and loaded onto a cation exchange column (1 ml Econo S catridge, BioRad, UK) pre-equilibriated in HCl (0.1 M, 20 ml), at a rate of 1ml min^{-1}. The column was washed with HCl (30 ml) and the peptide was eluted using NaOH (0.1 M) with the eluant (2.5 ml) collected into a 10 ml silanized glass bottle after the pH had risen to > pH 2. Triflouroacetic acid (TFA, 70 µl) was added and the eluant was loaded on to the HPLC system described in section 2.2.1.7 (Figure 2.1) with RP-HPLC fractions collected every 5 minutes. The volume of these fractions was made up to 130 µl with acetonitrile + 5% v/v acetic acid. A standard was then prepared consisting of Hep B (5.25 nmol) in an equal volume of mobile phase (130 µl, 50:50 acetonitrile: water + 5% v/v acetic acid + 0.01 TFA). Loop injections into a flow of 50:50 acetonitrile: water + 5% v/v acetic acid + 0.01% v/v TFA (200 µl min^{-1}) were performed in triplicate with a blank run in between each sample. The analysis was performed in full scan MS under the following mass spectrometric conditions; heated capillary 200 °C, capillary voltage

39V, tube lens offset 30 V, spray voltage 4.2 kV, sheath gas 80 arb units and the auxiliary gas 20 arb units.

2.2.2 Optimisation of mass spectrometric conditions for the detection of MHC class I associated peptides.

Mass spectrometric conditions must be optimized if low concentrations (fmol μl^{-1}) of peptides such as those derived from MHC class I molecules are to be detected and sequenced. This section describes the steps taken to enhance ionisation and detection procedures for the ion trap mass spectrometer used in this work. To gain ultimate sensitivity for the detection of MHC class I derived tumour antigens a miniaturized electrospray ionization source offering lower flow rates and higher sensitivity than conventional ESI sources was constructed and optimized, and the effect on the transmission of ions of several mass spectrometric parameters were investigated. These include the voltage and temperatures applied to the heated capillary, the voltages applied to the skimmers and octopolar lenses, ionization times and a close monitoring of the number ions entering the ion trap for all types of scan routine functions.

2.2.2.1 Construction and Optimization of a nano-electrospray ionization source.

All experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Finnigan, Hemel Hempstead, UK). The nano-electrospray ionization source was constructed by holding a commercial nanospray tip in a low dead volume fitting (SGE, UK) using a graphite ferrule which was supported in a PTFE holder. This

PTFE was mounted on a X, Y, Z adjustable platform to allow precise movement of the nanospray tip with respect to the mass spectrometer heated capillary. The conventional ESI source was removed from the LCQ spectrometer and the nano-electrospray platform was attached to the front plate of the instrument. The high voltage power connector from the LCQ was used to apply a voltage (0.9-1.3 kV) to the nanospray tip *via* connection to a piece of teflon coated conductive wire.

The following synthetic human or murine derived MHC associated peptides were supplied by the Queens Medical Centre, Nottingham: HLA-A3 restricted *bcr-abl* fusion peptide, $KQSSKALQR_{33-41}$ (A3); Tumour antigen p53, $LLGRNSFEV_{264-272}$ (P53); H2-kd restricted murine glycoprotein, $SPSYVYHQF_{454-463}$ (gp70); H2-kd restricted murine β-galactosidase, $TPHPARIGL_{377-386}$ (β-gal) and the MHC class II restricted Hepatitis B HBVc, $TPPAYRPPNAPIL_{128-140}$, (Hep B).

Two different brands of nanospray tips were compared in this investigation; fused silica gold coated 5±1 μm I.D picotips™ (New Objective, Inc., MA, USA) (Figure 2.3) and borosilicate Au/Pd coated nanospray tips (Protana, Denmark) (Figure 2.4). The stability of ion signal, sensitivity, flow rate and droplet sizes were all compared for the two types of tip.

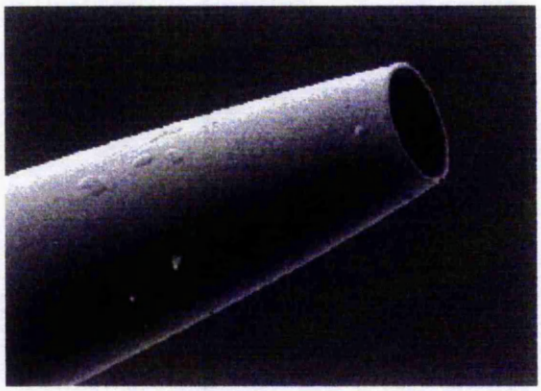


Figure 2.3: SEM of a 10 μm ID PicoTip fabricated from 360 μm diameter Fused-silica tubing.

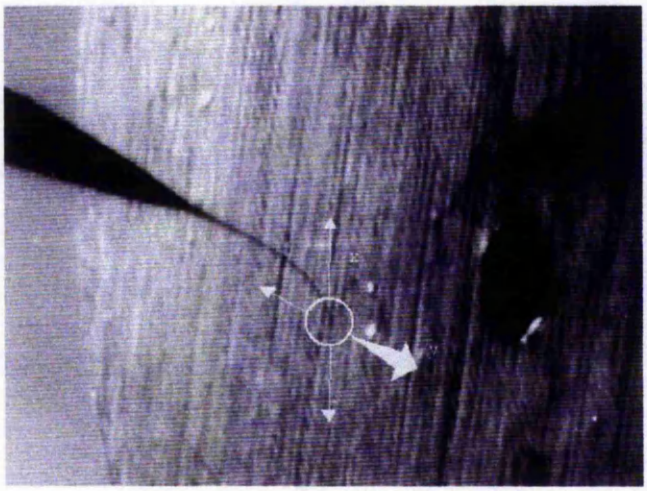


Figure 2.4: Au/Pd Coated borosilicate Nano-ES tips from Protana.

- *Scanning electron microscopy*

Scanning electron microscopy (SEM) was performed to measure the diameter of the spraying orifice of both the New Objective 5 ±1 μm i.d. tips and the Protana tips after breaking. Three tips an unbroken tip, a tip which gave rise to high flow rates, and one which gave rise to low flow rates were taken and examined under SEM. The tips were mounted onto a carbon disk and the uncoated silica was painted with carbon paint to allow conductivity.

- *Ion stability*

The stability of the nanospray signal for each type of tip was investigated by monitoring the TIC observed for a run time of > 1 hour. The signal intensities of the ions in the MS/MS spectra of the selected test peptides were investigated to ensure that signal quality was not deteriorating after prolonged analysis time.

- *Sensitivity*

Standard solutions of three peptides, A3 (KQSSKALQR), gp70 (SPSYVYHQF) and Hep B (TPPAYRPPNAPIL) were prepared at sub pmol μl^{-1} concentrations in 50:50 water: acetonitrile + 5% acetic acid. Limits of detection were determined by performing MS/MS under optimized conditions for each particular peptide. CAD was performed on the $[M+3H]^{3+}$ ion, m/z 349.5, for the A3 peptide, the $[M+H]^{+}$ ion, m/z 1127.5 for gp70 and the $[M+2H]^{2+}$ ion, m/z 481, for Hep B. For MS/MS experiments the spray voltage was held at 1.3 kV, an isolation width of 3u was selected and between 35 and 40% relative collision energy was applied. The

automatic gain control (AGC) was turned off and the injection wave form (IWF) set at 1500 msec.

- *Flow rate/ droplet size*

The Protana tips were loaded with 1 µl of peptide solution using a gas chromatography syringe (SGE, UK). The tips were broken and the peptide sample allowed to run to completion. The effect of spray voltage on flow rate was also investigated for the Protana tips by applying voltages in the range of 1.1 to 1.3 kV, which is the optimum voltage for the New Objective tips.

- *The Matrix Effect*

A bovine serum albumin (BSA) tryptic digest was performed by incubating BSA ($1x10^{-7}$ mol) in water (10 ml) + trypsin (500 µg) for 14 hours at 37°C. TCA precipitation was carried out by placing the digest in the ultrasonic bath for 10 min, followed by the addition of trichloroacetic acid (0.5 ml, 72% v/v). The solution was centrifuged at 4°C at 15,000g for 10 min. The digest was then placed in the ultrasonic bath for a further ten minutes and the supernatant removed.

Cation exchange was performed on an S type catridge (1 ml) (Bio Rad, Alfred Nobel, CA). This was conditioned with 0.1 M HCl (10 ml). The supernatant was then added to the column at a rate of approximately 1 ml min^{-1}, followed by 0.1 M HCl (30 ml). The peptides were eluted with 0.1 M NaOH at 1 ml min^{-1} and 2 ml fractions were collected. The pH of these fractions was adjusted to pH 8 with 0.1 M HCl, and 100 µl of the second fraction was loaded at 50 µl min^{-1} onto an ODS

reverse phase column (Jupiter, Phenomonex 1x150 mm). The elution program at 50 $\mu l\ min^{-1}$ consisted of 5 min water (pH 8) followed by 0-100% acetonitrile (BDH, UK) + 5% glacial acetic acid (Fisher, Loughborough, UK) + 0.01 % TFA (Sigma, UK) over 60 min. Fractions (5 min) were collected between 25 and 45 minutes, and these were analysed by nano-electrospray ionisation using the Protana tips. The fraction collected between 30 and 35 minutes contained peptides recognised by a database as being derived from a BSA tryptic digest.

An MHC peptide mixture was prepared at the following concentrations in 50:50 acetonitrile + 5% acetic acid: water; p53, 160 fmol μl^{-1}; gp70, 188 fmol μl^{-1}; A2, 240 fmol μl^{-1} and β-gal, 208 fmol μl^{-1}. 10 μl of the 30-35 minute HPLC fraction of the BSA digest was mixed with 10 μl of the peptide spike to give a 2 fold dilution of the MHC peptides in the BSA digest. Concentrations of 0, 0.05, 0.5, 5, and 50 pmol μl^{-1} of BSA digest were also prepared each spiked with a final concentration of p53, 80 fmol μl^{-1}; gp70, 94 fmol μl^{-1}; A3, 120 fmol μl^{-1} and β-gal, 104 fmol μl^{-1}. These samples were analysed by nanospray using the Protana tips with a spray voltage of 1.1 kV, heated capillary temperature of 100°C, tube lens offset voltage at –30 V, and an ion injection time of 1500 ms. After performing tandem mass spectrometry on the $[M+2H]^{2+}$ of each of the four peptides, the signal to noise ratios for selected fragment ions were measured and compared.

2.2.2.2 Investigation into the effect of mass spectrometric parameters.

All experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Finnigan, Hemel Hempstead, UK) with the automatic gain control set at $5x10^7$ ions. For the nano-electrospray experiments the standard electrospray source was removed and replaced with the in-house constructed nano-electrospray source using Au/Pd coated nanospray tip (Nano-ES tip, Protana, Denmark). The nano-electrospray tip was maintained at voltages in the range of 0.9-1.1 kV by connection to the instrumental high voltage power supply.

The MHC class I restricted peptide A3 was chosen because it contains a high number of basic residues and readily forms an $[M+3H]^{3+}$ species. The A3 peptide (5 µl, 72 pmol μl^{-1} in 50:50 acetonitrile: water + 5% acetic acid + 0.01% TFA) was analysed by nano-electrospray in full scan mode with the heated capillary set at temperatures ranging from 50-300°C and the tube lens offset voltage set at -24 volts. The effect of tube lens offset voltage was investigated by maintaining the heated capillary temperature at either 100 or 200 °C, and adjusting the offset voltage to values ranging from –80 to +120 volts. This experimental procedure was repeated for another two synthetic MHC associated peptides, using both nano-electrospray, and conventional electrospray with a spray voltage of 4.2 kV, the sheath gas at 30 (arb units) and a flow rate of 5 µl min^{-1}. A solution of the protein cytochrome C (20 pmol μl^{-1} 50:50 acetonitrile: water + 5 % acetic acid) was also investigated using the conventional electrospray source under the above conditions. The temperature of the

heated capillary was set at either 100 or 250°C and the tube lens offset voltage varied between –60 and +40 V.

HPLC fractions (10μl) were collected in regions where common contaminant ions corresponding to plastisicers and other polymeric materials are typically observed during a LC-MS chromatographic run of a cell eluate. These were spiked with gp70 to a final concentration of 61 pmol μl^{-1}. The samples were then subjected to variations in tube lens offset voltage at capillary temperatures of 100°C and 200°C.

2.3 Results & Discussion

2.3.1 Extraction and biochemical clean-up of cell surface MHC class I associated peptides.

The objective of the investigations described in this chapter was to develop a robust and sensitive method for the extraction and identification of MHC class I bound cell surface peptides. In order to achieve the required sensitivity and selectivity a multistage clean-up procedure was developed involving mild acid elution of peptides from the cell surface, TCA precipitation of co-extracted proteins, removal of buffer salts by cation exchange and separation of peptides by RP-HPLC. The method is summarised in Figure 2.2. The following sections describe the optimization and efficiency of each of these stages.

2.3.1.1 Mild-acid elution of cellular peptides

Our objective was to develop a method to extract and analyse MHC class I associated peptides presented only on the cell surface, since intracellular MHC/peptide complexes may not necessarily continue to the cell to become a immunological targets. The majority of research in the area of MHC restricted peptides employs total cell lysis followed by immunoprecitation,[4] which releases all intracellular and cell surface peptides, however mild acid elution of cell surface MHC class I associated peptides has been explored recently.[1, 5] The method developed for the mild acid extraction of cell surface peptides in this work was based on that reported by Storkus *et al.* using citrate phosphate buffer at pH 3.3.[5] Flow cytometry using antibodies raised against the β_2-microglobulin showed that it

was efficiently destabilized by mild acid treatment of cells, indicating that the MHC class I bound peptides are released from the cell surface by this procedure. Analysis of the acid elution by slot blot confirmed that the β_2- microglobulin was present in the acid eluate. Trypan blue was used to see if any cell lysis had occurred during the elution process and results showed that the cellular membrane had remained intact. Hence, cell surface MHC class I associated peptides may be eluted without cellular lysis and therefore presented peptides could be immunologically relevant *in vivo*.

2.3.1.2 Trichloroacetic acid protein precipitation

Trichloroacetic acid protein precipitation was employed to separate the β_2-microblobulin and other high molecular weight material derived from the cell surface, from the low molecular weight MHC class I restricted peptides. Other research groups have employed the use of membrane bound molecular sieves[2] to separate MHC class I related proteins from the 8-11 aa length peptides. However, with a sample volume of 20 ml after the mild acid elution it was not feasible to use these molecular sieves due to their low capacity and flow rates.

The procedure for determining the recoveries of three model peptides, using the TCA precipitation procedure is described in section 2.1 are shown in the Figure 2.6. The precipitation protocol was investigated with and without incorporation of ultrasonic dispersion. The results are shown in Figure 2.6. In the absence of ultrasonic dispersion of precipitated proteins and sample cooling during the precipitation, peptide recoveries were < 20% (Figure 2.6(a)). The recoveries of the

peptide mixture when an ultrasonication stage was introduced prior to centrifugation and when ultrasonication was employed both prior to and after centrifugation are shown in Figures 2.6 (b) and (c) respectively. The observed recoveries varied significantly for each of the methods investigated and also depending on peptide sequence. However, TCA precipitation gave a recovery rate of > 55 %, with an average recovery of 82%.

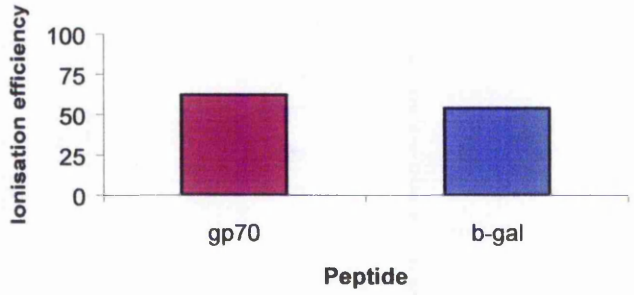
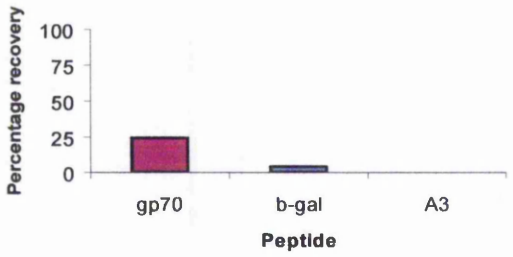


Figure 2.5: TCA precipitation recovery

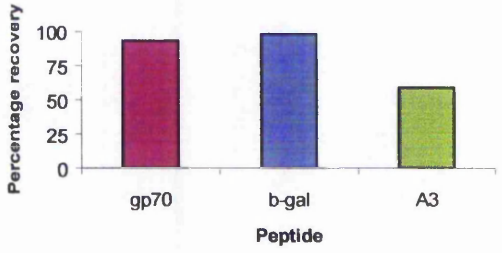
Therefore the experimental protocol containing two stages of ultrasonication was used in the TCA protein precipitation method.

It is known that some acids, may cause ion suppression during electrospray ionization. An experiment was therefore conducted to investigate the suppression effects of triflouroacetic acid (TFA) on the ionisation of model peptides. Figure 2.5 compares the ionization of a mixture of synthetic peptides in TCA (72% w/v) with a mixture prepared in 50:50 acetonitrile: water + 5% v/v acetic acid when injected into a flow of 50:50 acetonitrile: water + 5% v/v acetic acid.

(a)



(b)



(c)

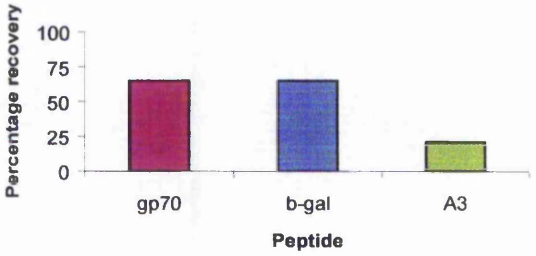


Figure 2.6: Recovery of peptide mixture using trichloroacetic acid protein precipitation; (a) no ultrasonication step, (b) with ultrasonication (10 min) carried out prior to centrifugation and (c) with ultrasonication (10 min) both before and after centrifugation.

Significant ion suppression was observed in the presence of TCA. Buffer salts remaining from the mild acid elution and also resulting from the TCA protein precipitation therefore needed to be removed before electrospray ionisation of the eluted peptides can take place. Both ion exchange and reverse phase chromatography columns were investigated for their potential to retain peptides while removing the majority of buffer salts.

2.3.1.3 Ion exchange chromatography

Several methods have previously been described for the desalting of biological matrixes. These include the use of C_4 or C_{18} resins to form a slurry in the presence of methanol in an eppendorf to concentrate the peptides, or by employing the use of on-line trap cartridges.[8, 9] Recently Link *et al* reported the application of orthogonal two-dimensional separation of a complex mixture of proteins employing a strong cation exchange (SCX) chromatography column along with traditional RP-HPLC chromatographic columns.[10] Cation and anion exchange columns were therefore compared for their ability to retain peptides whilst washing the buffer salts from the citrate phosphate buffer to waste. Figures 2.7(a) and (b) below show the results of the UV spectrophotometric analysis of fractions collected after elution from both types of ion exchange column, after loading with the synthetic MHC class I associated A3 peptide in citrate phosphate buffer. The A3 peptide was shown to elute from the anion exchange column in several fractions. For the cation exchange however, the A3 peptide was observed to elute principally in the second fraction (≈ 80 %). These experiments were then repeated with other synthetic MHC class I peptides and similar high recoveries were obtained. Therefore it was decided to

employ cation exchange as the method for the separation of the peptides from the buffer and other salts.

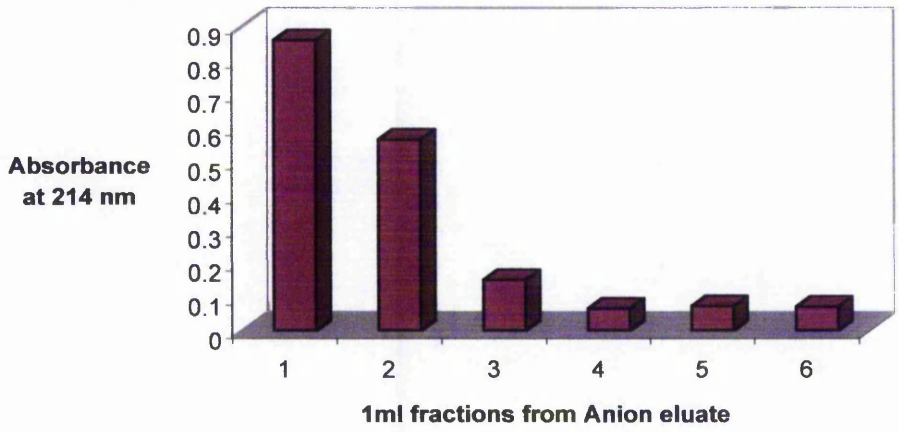


Figure 2.7(a) Absorbance of fractions (1 ml) collected from the anion exchange column measured using spectrophotometry (214 nm).

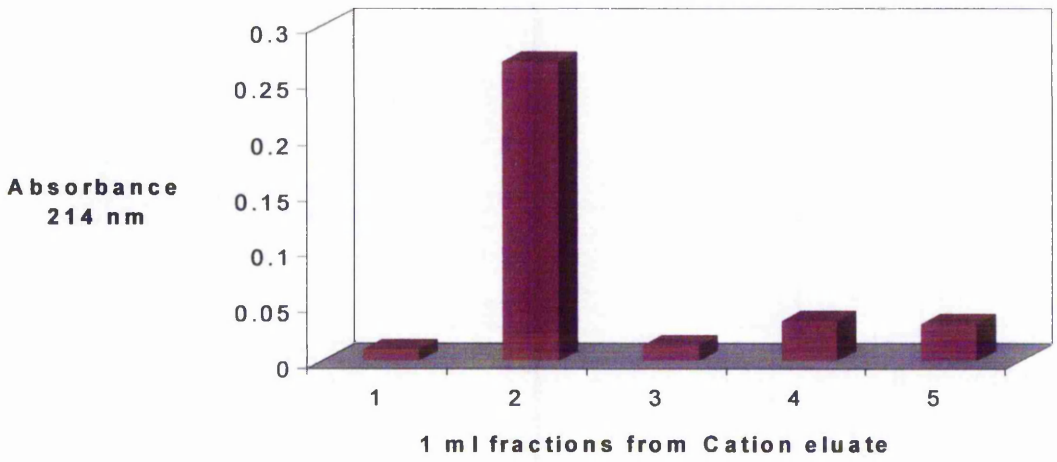


Figure 2.7(b) Absorbance of fractions (1 ml) collected from the cation exchange column measured using spectrophotometry (214 nm).

2.3.1.4 Reverse phase-high performance liquid chromatography

Electrospray mass spectrometry is adversely affected by the presence of salts leading to signal suppression. RP-HPLC using an ODS RP guard column was therefore investigated as an alternative to cation exchange for the clean up stage to remove the citrate phosphate buffer used for peptide elution. Flame photometric analyses showed that the buffer salts were still present at a high concentration, despite extensive washing. The retention of the peptides was also found to be poor in the presence of the citrate phosphate buffer. This resulted in low peptide recovery and blockage of the electrospray heated capillary and loss of sensitivity during the electrospray-mass spectrometric analysis.

The effect of introducing a cation exchange step prior to HPLC clean- up in order to remove the buffer anions was investigated using the cation exchange protocol described in section 2.2.3. Elution of the peptides with sodium hydroxide was possible with good recovery and after adjustment of the pH to 8, the peptides were efficiently pre-concentrated at the head of the HPLC column, whilst the column was washed with water. An analysis was performed to monitor the salt concentration in the eluted fractions using a flame photometer, the results for which are shown in Figure 2.8. It can be seen that if cation exchange is used prior to RP-HPLC, the sodium ion concentration falls to $< 10^{-5}$ M following a 20 min column elution with pH 8.5 water.

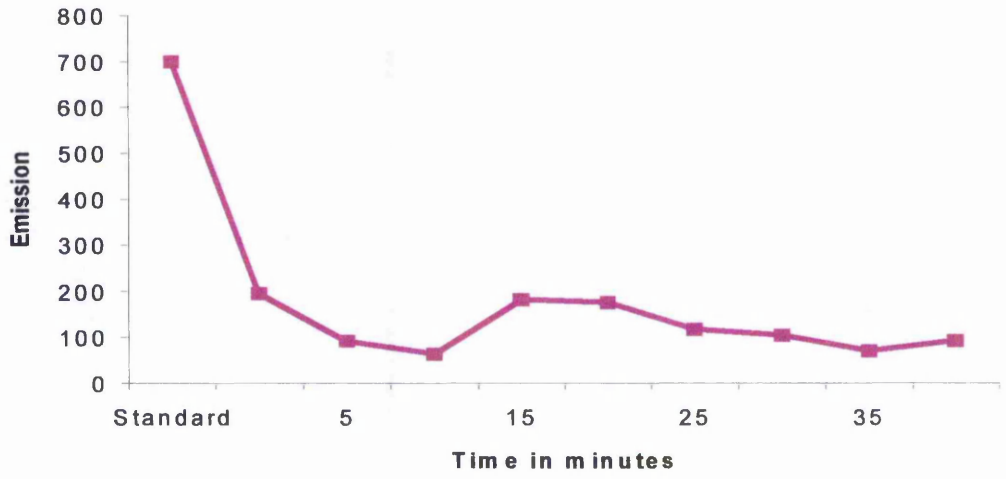


Figure 2.8: Flame photometric analysis measuring the sodium ion concentrations eluted after the citrate phosphate buffer- cation column eluate was loaded onto a RP ODS guard column during a 20 min wash with water pH 8.5.

2.3.1.5 Recovery of peptides through cation exchange and reverse phase - HPLC chromatography

Figure 2.9 shows the recovery of two model MHC class I synthetic peptides, A3 and β-gal after being subjected to cation exchange chromatography followed by HPLC on a reverse-phase guard and analytical column (4.6 x 150 mm). The overall recovery for the combined cation exchange/RP-HPLC clean-up was found to be 72% for the A3 peptide and 82% for the β-gal. This approach efficiently removes the salts that are detrimental to the electrospray, whilst reducing peptide losses to < 30%. Cation exchange chromatography was therefore employed to separate the peptides from the buffer salts, followed by separation of the peptide on the RP-

HPLC column. These two modes of chromatography allow a two dimensional mode of separation that is not achieved using multiple RP-HPLC steps alone. The use of cation exchange chromatography has not been previously applied to the biochemical clean-up of MHC class I or class II derived peptides, although other orthogonal chromatographic approaches have been employed. These include the use of hydrophobic interaction chromatography,[3] and anion exchange chromatography.[2] Herr *et al.* [11] reported the use of citrate phosphate buffer for the elution of cell surface MHC class I associated peptides derived from human blood infected with Epstein Barr virus. C_{18} cartridges were used as a de-salting step following the crude centrifugation of the eluates to remove remaining cell fragments from the supernatant. Although these are viable methods for the separation of MHC class I peptides from the buffer salts, in our experience, cation exchange chromatography gave the most consistent recoveries of the desalting methods investigated.

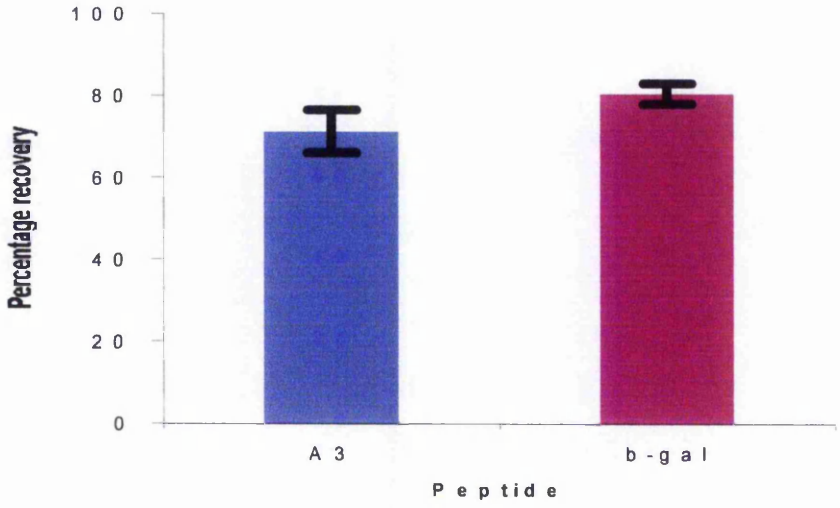


Figure 2.9: Percentage recovery of 2 model MHC class I peptides through cation exchange and RP-HPLC chromatography.

2.3.1.6. Concentration of cellular extracts

The next part of the protocol needed to be designed to concentrate the RP-HPLC peptide extracts to a sample of a few microlitres so as to enhance the sensitivity of the mass spectrometric analysis. Many different volume reduction techniques were investigated based on lyophilization of the RP-HPLC fractions. Lyophilization is commonly used to reduce volumes of biological extracts and advantages and disadvantages of this technique and the effect of the major parameters involved in the freeze-dry process, product, container, equipment and process have been reviewed by Franks[12]. Figure 2.10 shows the percentage recovery for a mixture of four different nonamers that were dried down from 2 ml to 60 µl in the freeze dryer under the best conditions achieved in this investigation. Freeze-drying was found to be a very inefficient method of sample volume reduction, and in many cases the recovery of some peptides was still less than 10%. L-lysine and poly-L-lysine were explored as carrier molecules, different ion-pairing reagents were employed to improve recovery of the peptide from the glass surfaces upon re-dissolving, and C_{18} Zip Tips (Millipore, Bedford, MA) were also investigated in an attempt to improve sample reduction. However, the percentage recovery could not be improved upon and the results shown in Figure 2.10 are typical of the optimum lyophilization/sample volume reduction methods employed.

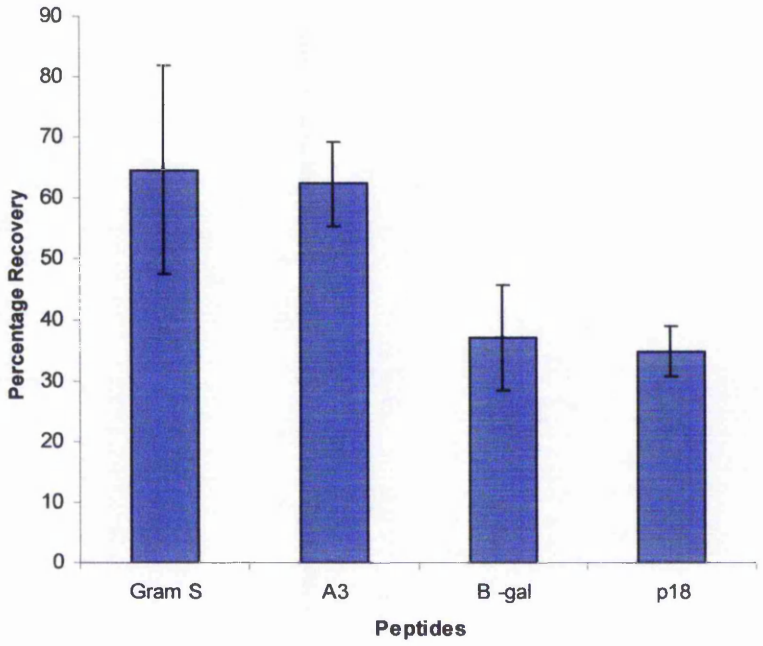


Figure 2.10: Recovery of Peptides (Mixture) drying 2 ml to 60 µl.

The difficulties experienced with the lyophilization of extracts led to the development of a microbore RP-HPLC procedure that combined chromatographic separation with concentration of the peptides in the cation exchange column fractions.

2.3.1.7 Introduction of microbore reverse phase chromatography employing a 6-port switching device

The LC-MS profile of the model MHC class I peptides p53 and gp70 (≈ 500 pmol) when subjected to the biochemical clean-up and chromatographic concentration and separation procedure outlined in the experimental section of this chapter are shown in Figure 2.11. The typical retention time for the MHC class II Hep B internal standard is also marked on the chromatogram. TFA was employed for use as the ion-pairing reagent during both loading and elution of peptides on the reverse-phase microbore column as it was found that retention times were more reproducible and recovery was higher. At pH values around 2-7, all basic peptidic residues and free amino termini will exist as cations and these ion-pairing reagents complex with the positively charged groups of the peptides. TFA in water can greatly increase the loading efficiency of the peptides onto a reverse-phase column and this ion-pairing reagent may be present in quite high concentrations (typically 0.1% v/v). For elution in LC-MS, however, the TFA concentrations should be at a level no higher than 0.02% v/v, as it is a major suppresser of ionisation if electrospray is employed.

TFA was added to the cation exchange eluant (2-3 ml) which was loaded onto the RP-HPLC guard column at 200 μl min^{-1}. This was loaded over 20 minutes while the buffer salts were washed to waste. Once the NaOH was separated from the peptides, the elution solvents were then applied and the flow redirected from pumps B and C (Figure 2.1) through both the pre column and analytical column. Reducing the flow rate of the microbore column to ≈ 20 μl min^{-1} during the elution allows peptides in 2-3 ml of cation exchange eluate to be concentrated to a few microlitres (2-5 μl). This protocol was performed using LC-ESI-MS to determine retention times for standard peptides, and for the analysis of MHC class I cell surface peptides, fractions were collected into glass vials for subsequent mass spectrometric analysis

using nano-electrospray ionisation. By collecting fractions every 20 seconds it is possible to collect the apex of the chromatographic peak and hence improve upon sensitivity further as this represents the most concentrated part of the eluting peptide.

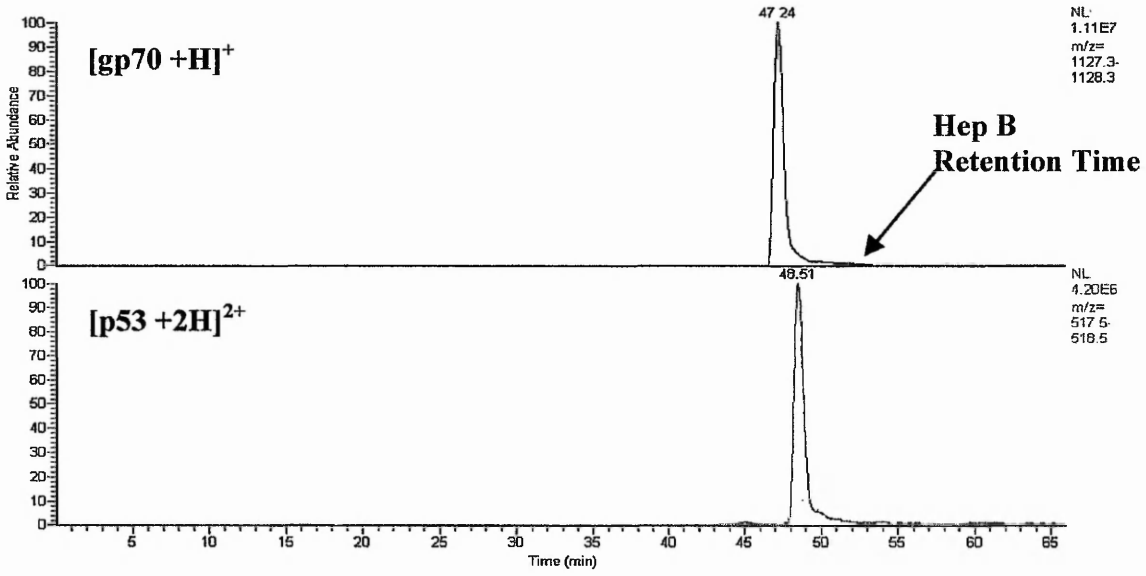


Figure 2.11: Extracted ion current traces for synthetic MHC class I peptides p53 and gp70 after subjection to TCA precipitation, cation and reverse-phase chromatography.

2.3.1.8 Recovery of model MHC peptides

The MHC class I peptide gp70 and the MHC class II peptide Hep B were subjected to the analytical procedure outlined above for the analysis of MHC class I peptides. The average recovery was found to be 20%. From this predicted 20% recovery it was estimated that, with a typical limit of detection being 2-10 fmol μl^{-1}, in the presence of a cellular matrix, that approximately 10^9 cells would be needed to

characterize cellular derived peptides. This figure is in accordance with the number of the cells typically used in the literature.

The Hep B peptide was chosen as the internal standard to monitor the recovery through the methodology employed for the analysis of cellular derived MHC class I eluted cell surface peptides. It not only acts as an internal standard for analyses, but also gives an indication of the chromatographic regions that are most likely to contain peptides because of the peptides late elution (see Figure 2.11). The Hep B peptide was also chosen, as it is a class II restricted antigen of viral origin, and would therefore not be expected to occur naturally in our cellular eluates since our target peptides are derived from tumour antigens. This methodology has been applied for the detection of MHC class I peptides from both transfected cell lines and clinical patient samples (Chapter 3).

2.3.2 Construction and optimisation of a nano-electrospray ionisation source.

2.3.2.1 Construction of a nano-electrospray ionization source

Figure 2.12 shows a schematic diagram of the in-house constructed nano-electrospray ionization source. Samples were introduced into the nano-electrospray ionisation source and were successfully ionized. Two different types of commercially available nanospray tips were employed (section 2.2.2.1). A reservoir consisting of a piece of teflon tubing was used for introducing solution into a New Objectives tip. As can be seen in Figure 2.3. the fused silica New Objective tips were precut with a defined spraying aperture diameter of 5±1μm, whereas the tips

from Protana (Figure 2.4) have a closed end due to the Au/Pd coating process and this has to be carefully broken in order for spraying to commence. This can be performed by gently breaking the tip against the heated capillary. The disadvantage of this process is that without a predefined I.D reproducibility of the flow rate for the tips from Protana will vary. The advantage of the Protana tips however is that they are much cheaper than those from New Objective.

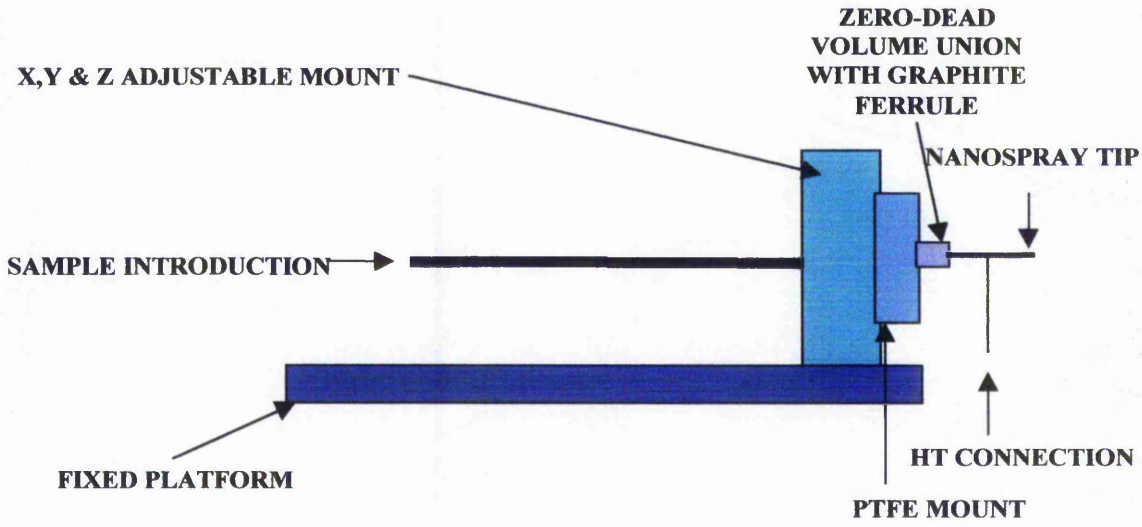


Figure 2.12: Schematic of the in-house constructed nano-electrospray ionization source.

As a variety of commercial nanospray tips are available offering a variety of apertures and coatings, an investigation was performed to see which tips would be the most efficient for the purpose of analysing MHC class I derived peptides. Table 2.1. shows the i.d. of the tip orifices as determined by SEM, the corresponding flow rates and droplet size produced. The radius of the droplets was calculated using equation 2.1[13]:

$$R = 1.73 \times 10^{-5} \quad 3\sqrt{(F/I)^2} \qquad \text{Equation 2.1.}$$

R= radius, F= flow rate in μl min^{-1}, I= ion current.

The Protana tip, which gave rise to a much higher flow rate had, as expected, a wider diameter (30 μm) than the tip, which gave rise to low flow rates (4 μm, 26 nl min^{-1}). Examination under the SEM, showed that the former tip had a very jagged break that extended much lower down the tip than the other tips. The low flow rate tip had been used for > 2 hours of peptide analysis and had a very flaky appearance under the SEM. This could have either been due to the gold/Pd coating flaking off due to the prolonged analysis time, or to the accumulation of sample residue at the tip. The New Objective tips proved difficult to load with sample solution as they are designed for on-line analysis with a microcapillary column. For off line analysis such as in this case, the system employed in these experiments required the use of a sample reservoir. It was therefore difficult to get an accurate flow rate as the volume of the reservoir was not clearly defined, however the flow rate was within the limits defined by Wilm and Mann.[15] The flow rate and spray current are determining factors in the diameter of the droplets produced. Typical figures reported by Wilm

and Mann are flow rates of 20-40 nl min^{-1} with droplet diameter < 200 nm[14-17], these are in accordance with the flow rates and droplet sizes shown in Table 2.1.

Table 2.1: Typical measured I.D, flow rate and calculated droplet size for commercial nanospray tips.

Tip	Tip i.d (μm)	Flow rate (nl min^{-1})	Calculated average droplet radius (nm).
New Objective	5	30	100
Protana unbroken	3.5	-	-
Protana (low flow rate).	4	26	92
Protana (high flow rate).	30	5000	3000

Two different spray voltages 1.1 kV and 1.3 kV were applied to the Protana tips. When applying a spray voltage of 1.3 kV, flow rates were measured at an average of 200 nl min^{-1}. This was repeated on several tips to ensure that analysis time and therefore the voltage and not the I.D of the tip were determining flow rate. When the experiment was repeated applying 1.1 kV, the average flow rate was 26 nl min^{-1} (range 24-27 nl min^{-1}) with a standard deviation of 1.5. From equation 2.1. the diameter of the droplets formed at 1.3 kV were 358 nm and those formed at 1.1 kV have a diameter of < 85.9 nm. By reducing the spray voltage, extended analysis times and greater sensitivity can be achieved. The droplet diameter for the New

Objective tips is approximately 100 nm, hence when spraying at the optimum spray voltage for each type of tip, the Protana tips show smaller droplet size.

2.3.2.2 Sensitivity and stability

The ion current observed for both types of nanospray tip was steady throughout the run with ion intensities being similar at the start and end of each run. However, the Protana tips gave slightly more stable TIC than the tips from New Objective. The sensitivity was similar for the two types of nanospray tip with limits of detection obtained by MS/MS analyses (LOD) in the range 6-50 fmol μl^{-1} (see Table 2.2). These detection limits were obtained before the optimisation of other mass spectrometric parameters and have since been improved by nearly ten fold. The MS/MS spectra obtained from both tips for a solution of 25 fmol/µl gp70 were compared (Table 2.3) and the ion counts were found to be comparable, however the signal to noise ratios (S:N) were higher in most cases for the peptides analysed using the Protana tips.

Table 2.2: Comparison of limits of detection for various synthetic peptides employing MS/MS analyses with commercial nanospray tips.

	Limit of detection fm μl^{-1} [a]	
Peptide	New Objective	Protana
A3	47	50
Gp70	25	10
Hep B	6	6

(a) The LOD was defined on the lowest concentration of peptide required to show at least three fragment ions with a S:N of greater than 3:1.

Table 2.3: Comparison of S:N ratio for product ions in the MS/MS spectrum of gp70 (25 fmol μl^{-1}).

Fragment ion	m/z	S:N Protana tips	S:N New Objective Tips
b8	962.2	16:1	8:1
y7	943.1	14:1	3: 1
b7	834.3	4:1	5:1
y3	431.0	3:1	1:1

2.3.2.3 Matrix Effect

Although stated that matrix effects are rarely seen when using nano-electrospray ionisation,[1] some matrix effects were being observed when using nanospray to analyze a complex mixture of peptides. A BSA tryptic digest was diluted by varying factors and spiked with known quantities of synthetic nonamers to investigate this occurrence and the signal to noise ratios of their fragment ions were then investigated for a suppressive effect. Figures 2.13-2.16 show the variation in the signal to noise ratio of the product ions produced by tandem mass spectrometry for four model peptides (A3, gp70, p53 and β-gal) in the presence of different concentrations of a BSA tryptic digest. As can be seen from Figures 2.13 and 2.14 a matrix effect seems to occur at in the presence of 50 pmol μl^{-1} of BSA, as the signal: noise ratio decreases significantly at this concentration. However this does not appear in Figures 2.15 and 2.16 as the signal to noise ratio remains constant at this dilution.

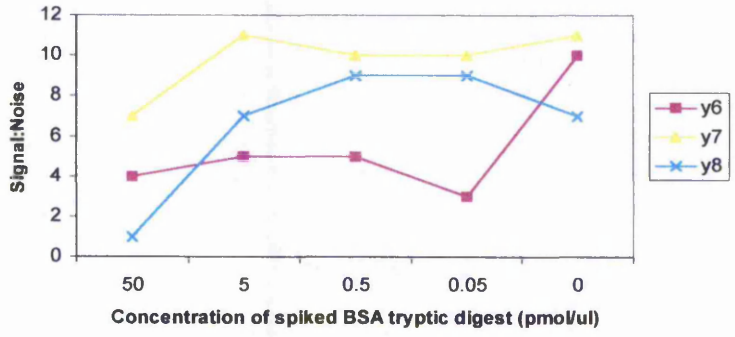


Figure 2.13: Matrix effect of BSA at various concentrations on the S:N for the y_6-y_8 MS/MS product ions of A3

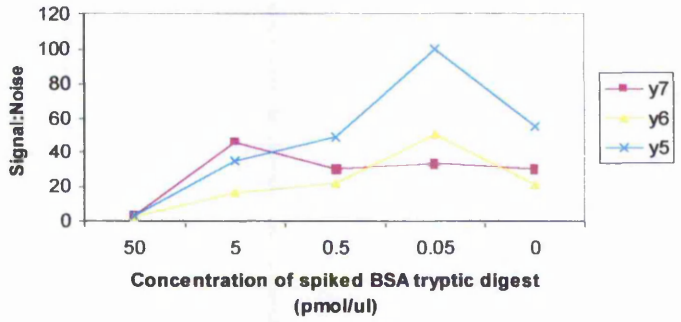


Figure 2.14: Matrix effect of BSA at various concentrations on the S:N y_5-y_7 MS/MS product ions of gp70.

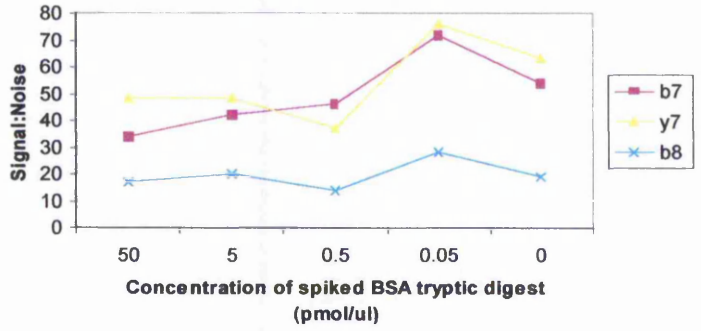


Figure 2.15: Matrix effect of BSA at various concentrations on the S:N of y_7, b_7 and b_8 MS/MS product ions of p53.

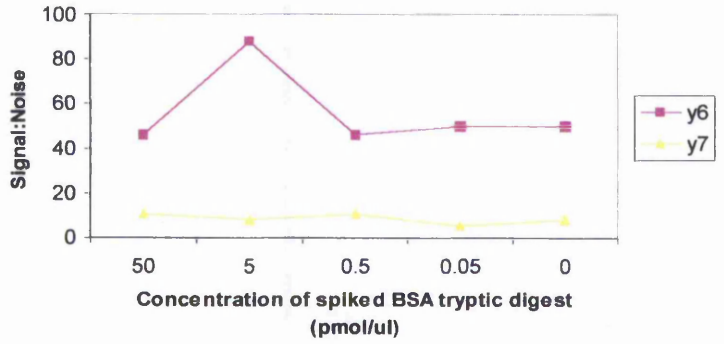


Figure 2.16: Matrix effect of BSA at various concentrations on the S:N for the y_6 and y_7 MS/MS product ions of β-gal.

The S:N ratios for the MS/MS product ions of the A3 (y_6-y_8) and gp70 (y_5-y_7) peptides, both at approximately 200 fmol μl^{-1} show significant suppression in the presence of a BSA concentration of 50 pmol μl^{-1}. The p53 and β-gal product ions were less affected at this concentration. This suggests that the presence of a matrix effect may dictated by the amino acids constituting the peptide, and may not be a general effect. However, for all of the peptides studied there is little suppression at BSA concentrations of less than 5 pmol μl^{-1}.

Overall the capabilities of the two different brands of nanospray tips are very similar. However, the tips from Protana give rise to a more stable flow and are also cheaper in cost. The tips from New Objective do however have an advantage in that they have a predefined accurate I.D. However, this investigation suggests that the breaking of the Protana tip can be performed reproducibly to ensure low flow rates, therefore producing droplet sizes comparable if not smaller than those produced by the New Objective tips. Decreasing the spray voltage to 1.1 kV can further extend these analysis times. Voltages lower than 1.1 kV were also investigated, however this affected ion intensities and the range of optimum intensity was found to be using spray voltages of between 1.1 kV and 1.4 kV. The Protana tips gave slower flow rates and hence smaller droplet diameters, both of which increase sensitivity to the ionization process.

2.3.3 Investigation into the effect of mass spectrometric parameters

The A3 peptide was chosen for an investigation into the effect of capillary temperature on charge state as singly, doubly and triply charged species are usually observed in the ESI mass spectrum of this peptide. Figure 2.17 shows that by

adjusting the temperature of the heated capillary, while the tube lens offset was set at –24 volts, significant differences were observed in the intensities of the charge status dominating the spectrum. In Figures 2.17 (b) and (c), the base peak ion count is ten fold less in intensity as for Figure 2.17 (a), where the heated capillary is set to 50°C and the $[M+3H]^{3+}$ is the most intense ion. The $[M+H]^{+}$ cannot be seen in this spectrum but, is at approximately the same absolute intensity when the temperature of the heated capillary was set to 150 and 300°C in Figures 2.17 (b) and (c) respectively. A heated capillary temperature of 50°C favours the production of the triply charged species, whilst the singly charged species becomes the dominant ion when the temperature is raised to 300°C. By varying the temperature of the heated capillary it is therefore possible to enhance the abundance of a particular peptide ion charge state.

The effect of changing the voltage of the tube lens offset was then investigated. The results showed that by increasing the tube lens offset voltage the singly charged species is favoured and the decreasing voltage favours the triply charged state. The highest ion count was observed when the temperature was set at 100 °C and the tube lens offset voltage set to 0 volts, with the $[M+3H]^{3+}$ (m/z 349.5) species being favoured. When the heated capillary temperature was set at 200°C, the highest ion count was observed at +20 volts, were the $[M+H]^{+}$ (m/z 1045.6) is dominant in the spectrum. Increasing the tube lens offset voltage to higher voltages results in the formation of fragments as a result of collisional activation of the protonated peptide ion, a process sometimes known as cone voltage fragmentation. These results were

also seen for other synthetic MHC class I peptides, although the majority of peptides investigated only showed singly or doubly protonated ions.

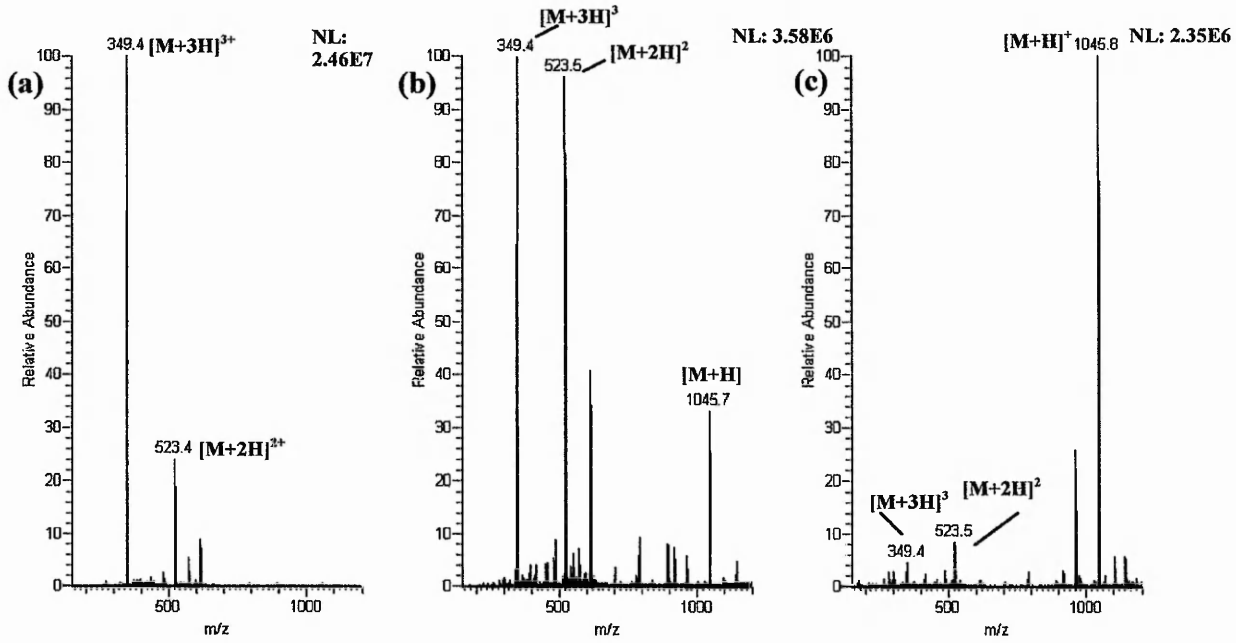


Figure 2.17: Nano-electrospray spectra of the A3 peptide with the tube lens offset voltage set at –24V and the heated capillary set at (a) 50°C, (b) 150°C and (c) 300°C.

Therefore, higher heated capillary temperatures and tube lens offset voltages favour the higher abundance of singly charged species, and lower temperatures and tube lens offset voltages favour the multiply charged species. Cationised peptide species such as samples contaminated with sodium or potassium ions were also seen to behave in this manner.

The general trend is that raising the tube lens offset voltage and temperature of the heated capillary favours $[M+H]^+$ but, peptides have different optimum values for the highest ion abundance. For example the highest ion intensities were observed at a tube lens offset of -10 volts for the Hep B peptide $[M+2H]^{2+}$ion, + 20 volts for the A3 peptide $[M+3H]^{3+}$ ion and + 40 volts for the β-gal peptide $[M+2H]^{2+}$ ion at a capillary temperature of 200 °C. Therefore in analysing a complex mixture of unknown peptides a compromise has to be made on the optimum operating conditions, as a peptide at a concentration near the limit of detection may not be seen under one set of parameters.

The investigation into the effect of tube lens offset voltage and temperature of the heated capillary of the LCQ were repeated using the conventional electrospray source with the same results. Hence the ionization process is not affecting the relative abundances of the different charge states of the peptide ions. There are several possible reasons why the tube lens offset voltage and heated capillary temperatures have such significant affects on ion intensities. The explanation may simply be the better transmission of the more highly charged species through the spectrometer interfaces, under certain parameters. Alternatively by raising the temperature of the heated capillary and the voltage of the tube lens offset,

fragmentation of higher clusters may be facilitated by interaction with ionic neutral species in the high-pressure interface region of the mass spectrometer. A charge stripping process could also be occurring where the peptide ion loses protons as a result of raising the voltage of the tube lens offset and the temperature of the heated capillary. However as nano-electrospray and conventional electrospray data behave in the same way this is unlikely. The reasoning behind the favouring of singly charged species being most abundant when the heated capillary is set at a high temperature could be due to a more efficient ion evaporation process during desolvation in the heated capillary.

A protein sample of cytochrome C (m/z 12360.0) was analysed by electrospray mass spectrometry to observe the effect of the temperature of the heated capillary and the voltage of the tube lens offset have on the higher charge states present in the protein spectrum. It was found that increasing the voltage of the tube lens offset (with the heated capillary temperature set at 100°C or 250°C) resulted in lower charge states being preferentially detected. Figure 2.18 shows the effect of an alteration in temperature and tube lens offset voltage on the charge states present in the cytochrome C spectrum. In Figure 2.18 (a) it can be seen that the charge states for cytochrome C range between +7 and +14 with the highest abundance for the $[M+10H]^{10+}$ ion when the tube lens offset was set at +40 volts the heated capillary at 250°C. In Figure 2.18(b), with the voltage set at –40 volts and heated capillary at 250°C, the charge state distribution has shifted to range from +9 to +16 with $[M+13H]^{13+}$ being the most abundant. By comparing Figures 2.18(c) and (d) where the heated capillary is set at 100°C and the tube lens voltages are +40V and –40V, it can be seen that again there is a shift in charge state distribution. It can be concluded

that lower heated capillary temperatures favour the formation of more highly charged species, and that the tube lens offset voltage can be used to manipulate the charge state of the ions in the spectrum.

Ions of a non-peptidic nature commonly observed in the LC-MS analysis of cell eluates were collected and subjected to nano-electrospray ionization to determine if these too were capable of undergoing an alteration in charge state. In all cases, transmission of a particular ion could be enhanced or decreased by changing the tube lens offset and capillary temperature, but the charge state could not be manipulated to become multiply charged.

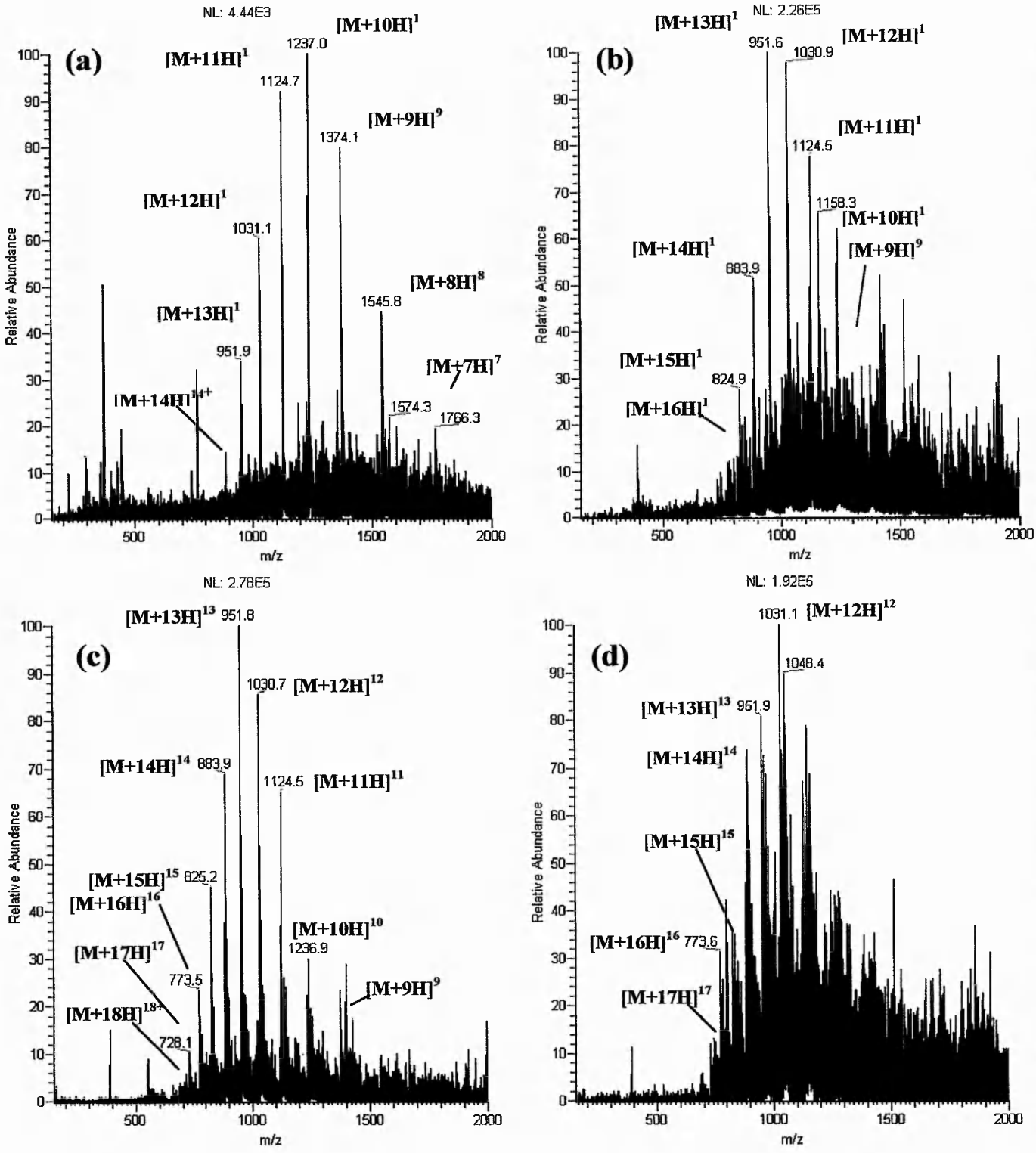


Figure 2.18: Electrospray mass spectra of Cytochrome C (a) heated capillary 250°C, tube lens offset +40 volts, (b) 250°C, tube lens offset –40volts, (c) 100°C, tube lens offset +40 volts and (d) 100 °C, tube lens offset –40 volts.

2.4 Conclusions

In conclusion, a method has been developed to isolate cell surface MHC class I peptides, to separate them orthogonaly by employing cation exchange and reverse-phase HPLC, and to characterise low fmol μl^{-1} concentrations of peptides using nano-electrospray ion trap tandem mass spectrometry. The techniques developed in this chapter are used in chapter 3 for the isolation and characterisation of MHC class I associated peptides from both transfected cell lines and patient material.

Decreasing the heated capillary temperature and the voltage of the tube lens offset enhances the formation of multiply charged peptide ions and reduces the number of fragment ions caused by tube lens offset induced fragmentation in the interface. It was observed that for peptides (9-13 aa) and large proteins that the more highly charged species are more abundant at lower heated capillary temperatures and tube lens offset voltages. Higher ion counts offering higher sensitivity and lower limits of detection were also observed under these conditions. By alternating between conditions favouring singly or multiply charged ions and performing a high resolution scan it is possible to distinguish peptidic material from synthetic polymeric material since these background ions investigated did not change charge states in the same way as the peptides. This manipulation can therefore provide a useful analytical tool when characterising peptides at low levels in a complex mixture where the most abundant peaks may be due to non-peptidic impurities that mask the spectrum. By utilizing heated capillary temperatures and tube lens offset voltages that favour the formation of multiply charge peptides, limits of detection can be increased by up to 20 fold and ease of peptide identification can be improved greatly.

Spectra obtained from nano-electrospray and conventional electrospray were shown to be identical, hence the ionization process is not affecting the relative abundances of the different charge states of the peptide ions. There are several possibilities why the tube lens offset voltage and heated capillary temperatures have such drastic effects on ion intensities. This observation may simply be due to better transmission of the low or high charged species, under certain interface conditions. Alternatively, by raising the temperature or the heated capillary and the voltage of the tube lens offset, fragmentation of higher clusters may be encouraged by interaction with ion neutral species in the high pressure region of the mass spectrometer, or is deprotonated in the interface. The reasoning behind the favouring of singly charged species being most abundant when the heated capillary is set at a high temperature could be due to a more efficient ion evaporation process during desolvation.

Although the heated capillary temperature and the tube lens offset voltage are the easiest of mass spectrometer interface parameters to manipulate to get an increase or decrease in charge state, other mass spectrometric conditions are of vital importance for the sensitive detection of low level biologics. After the initial introduction of ions into the mass spectrometer *via* the heated capillary, a voltage applied to this capillary can affect optimal transmission of ions and therefore sensitivity. When the "tune" function of the LCQ is applied to a specific ion, the mass spectrometer selects a voltage on the capillary that is ideal for the transmission of that ion. The voltages applied to the octapoles and the octopolar lens can also be manipulated to increase or decrease sensitivity and selectivity for high or low m/z ions. Again through the tune function, voltages can be selected for optimum transmission for a

particular ion of interest. The number of ions entering the ion trap must be very carefully balanced. Too few ions entering means the limit of detection can be reduced significantly; too many ions and a space charge effect may be observed. Therefore, by keeping the number of ions in the trap during full MS at a reasonable level to avoid space charging and by increasing the number of ions to gain the sensitivity that is needed during tandem mass spectrometric analysis, a competent set of data can be acquired. If the automatic gain control is set at a maximum value during MS^n and more sensitivity required, the AGC can be turned off and a function known as the injection wave form (IWF) can be switched on where by ions accumulate in the ion trap as a time function.

By constructing a nano-electrospray ionization source, extended analysis of low concentrations of cellular derived peptides has been made possible. Low femtomole μl^{-1} levels of peptides can be detected with only a few nanolitres being consumed during analysis. By acquiring in centroid mode, sensitivity can be enhanced by 10 fold and through manipulation of the voltages of the ion optics and through adjustment of the temperature of the heated capillary the charge state of the peptides can be altered and enhanced. Through a combination of these ionization and ion transmission techniques and with careful monitoring of the number of ions entering the trap, optimal performance of the ion trap for the detection of cellular levels of MHC class I associated peptides can be obtained.

The investigations reported in this chapter have led to the development of a method to isolate cell surface MHC class I peptides, to separate them by employing cation

exchange and microbore reverse-phase HPLC, and to characterise low fmol μl^{-1} concentrations of peptides using nano-electrospray ion trap tandem mass spectrometry. The techniques developed in this chapter are used in chapter 3 for the isolation and characterisation of MHC class I associated peptides from both transfected cell lines and patient material.

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CHAPTER THREE

CHARACTERISATION OF CELLULAR DERIVED MHC CLASS I ASSOCIATED TUMOUR ANTIGENS

3.1 Analysis of Chronic Myeloid Leukemia patient white blood cells.

3.1.1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the occurrence of the Philadelphia chromosome (Ph) which is expressed in about 95% of all CML patients (see section 1.1.4). This chromosome occurs due to a reciprocal translocation t(q:22)(q34:q11), resulting in the chimeric BCR-ABL gene, and is also present in other forms of leukemia including acute myeloid leukemia. The resulting BCR-ABL fusion protein is unique to this karyotype. Several class I HLA molecules have been reported to bind strongly to peptides spanning the BCR-ABL fusion region and several of these peptides were seen to elicit specific class I restricted CTL activity.[1-4] CML is treated currently with stem cell transplantation or interferon-γ therapy; both having a high degree of risk.[5] The alternative strategy of immunotherapy, especially the use of peptide vaccines, is becoming one of the most successful and risk free treatments for malignancies. Successful immunization is reliant upon the identification of T cell epitopes from antigens specific to the tumour/ tumour antigens. This section describes the application of the protocol developed in chapter 2 for the isolation, biochemical clean up and mass spectrometric characterisation of cell surface MHC class I peptides associated with the break-point region of the BCR-ABL protein[6] derived from CML patient white blood cells and transfected cell lines.

3.1.2 Experimental

CML cells were collected by leucophoresis and subjected to cell surface elution at the University of Liverpool, UK, using the method described in Chapter 2. The samples were transported to Nottingham and frozen at –80°C. Prior to biochemical clean-up, the cells were rapidly thawed in a water bath (37°C) and a Hepatitis B MHC class II restricted synthetic peptide (TPPAYRPPNAPIL$_{128\text{-}140}$; 900 fmol) was added as an internal recovery standard. The sample was subjected to TCA precipitation and the resulting supernatant was removed and loaded onto a cation exchange column (1 ml S catridge BioRad, UK) as described in Chapter 2. The eluant (2 ml) was collected and the ion pairing reagent triflouroacetic acid (TFA, 70 µl) was added. The acidified cation exchange eluate was then subjected to reverse phase HPLC using the chromatographic column switching configuration shown previously (Figure 2.1). The cation eluate was injected via an injection loop (3 ml) onto a pre-column (1 x 30mm, ODS, Jupiter, Phenomonex, UK) and washed with solvent A (0.1%TFA, 200 µl min^{-1}). The column effluent, containing buffer salts from the TCA precipitation and the IEC, was directed to waste. After 20 minutes the six port valve was switched to allow the flow of mobile phase from pumps B and C to pass through both the pre-column and the analytical column (1 x 150 mm, ODS, Jupiter, Phenomonex, UK). An elution gradient was applied consisting of 20% solvent B (acetonitrile + 5% acetic acid + 0.01% TFA): 80% solvent C (water + 5% acetic acid + 0.01% TFA) to 90:10 solvent B:C (45 min, 20 µl min^{-1}). After 20 minutes fractions were collected (1-4 µl) into silanized tapered glass vials (1.1 ml) and stored at 4°C until further analysis.

Prior to the analysis of cell eluate samples, method blanks consisting of the hepatitis B MHC class II spike (900 fmol) in TCA (2 ml) were subjected to biochemical clean-up, chromatographic separation and were analysed in the same manner as the eluates to ensure that no peptides relating to the BCR-ABL fusion protein were present. A solution consisting of HCl (0.1M, 2 ml) spiked with synthetic peptides gp70 ($SPSYVYHQF_{454-463}$ 500 pmol) and p53 ($LLGRNSFEV_{264-272}$ 500 pmol) was also prepared. This solution was subjected to cation exchange chromatography and RP-HPLC as described above to establish the correct chromatographic retention times for the collection of fractions.

Nano-electrospray analysis was performed with a quadrupole ion trap mass spectrometer (LCQ, Finnigan, UK) using the in-house constructed nano-electrospray source. Samples (1-2 μl) were introduced into a nanospray tip (Protana, Denmark) and analysed by applying a spray voltage (1.1 kV) to the tip. Spraying commenced once the tip had been gently broken against the heated capillary of the LCQ. Prior to the analysis, target masses corresponding to the $[M+H]^+$, $[M+2H]^{2+}$ and, where appropriate, the $[M+3H]^{3+}$ ion of predicted HLA class I alleles for the BCR-ABL fusion protein were calculated. Nano-electrospray ionization mass spectrometry was performed initially to search for the Hepatitis B class II synthetic spike, using the following mass spectrometric conditions; heated capillary 100 °C, capillary voltage – 46 V, tube lens offset voltage 20 V, octopole 1 –1.00 V, octopole 2 7.56 V and lens voltage – 46 V. Tandem mass spectrometry was performed by isolating the m/z 704.5, $[M+2H]^{2+}$ ion for the Hep B peptide with an isolation width of 3u and relative collision energy 30%.

Following confirmation of the presence of the Hep B class II recovery spike, analysis was performed in both MS (automatic gain control (AGC) 5 x 10^8-1 x 10^9, 3 microscans, m/z 300-1800) and MS/MS (ACG 5 x 10^9 or injection wave form (IWF) 1500 ms). Mass selected ions corresponding to the masses for singly, doubly and triply charged peptides predicted to be associated with specific HLA class I alleles were subjected to CAD and the product ion spectra were recorded. Other species observed in the full scan MS spectrum that had an isotopic distribution typical of a peptide were subjected to an alteration in charge state from +2 to +1 by changing typical mass spectrometric parameters from; heated capillary 100°C, capillary voltage 11 V, tube lens offset voltage 5 V, octopole 1 –1.25 V, octopole 2 –5.00 V and lens voltage –20 V, to heated capillary 150°C, heated capillary voltage 11, tube lens offset voltage 40 V. Zoom scans were performed before and after the change in the heated capillary and tube lens offset voltages to ensure correct assignment of the charge state. Where a charge shift occurred, the $[M+2H]^{2+}$ ion, or where available the $[M+3H]^{3+}$ ion, of the suspected peptides were subjected to MS/MS for sequencing with an isolation width 3u, and relative collision energy 25-35%.

For the analysis of HLA-A3 restricted BCR-ABL peptides from the bcr-abl protein fusion region, cells from patient A of HLA type -A2, -A3, -B51, -B60 (equivalent to 4 x 10^9cells) and patient B with a HLA-A3 positive haplotype (1 x 10^{12} cells) were eluted and subjected to the protocol as described above. Both of these patients expressed the b3a2 exon variant of the Philadelphia chromosome BCR-ABL gene (see section 1.1.4). Cells from patient C of HLA type -A3, -B37, -B62 (8 x 10^9 cells) who expressed the b2a2 exon variant of the Philadelphia chromosome BCR-

ABL gene and patient D, a HLA-A3 negative patient (10^{10} cells) expressing the exon variant b3a2 were analysed as the negative controls.

3.1.3 Results & Discussion

CML patient cells were extracted from HLA-A3 positive and HLA-A3 negative patients. These were subjected to biochemical clean-up and mass spectrometric analysis to characterize MHC associated and other eluted peptides from the cell surface, in particular the HLA-A3 restricted peptide derived from the BCR-ABL gene product.

3.1.3.1 Patient A: MHC class I type HLA-A2, -A3, -B51, -B60

Cells (4 x 10^9) from CML patient A were extracted by leucophoresis and subjected to mild acid elution, TCA protein precipitation, cation exchange chromatography and microbore RP-HPLC as outlined in the method section 3.1.2. The MHC class II Hepatitis B synthetic peptide was detected in the expected HPLC fraction and the MS/MS spectrum is shown in Figure 3.1.

The Hepatitis B spike served both as an internal standard to confirm recovery through the biochemical clean-up and as an indicator of the elution window for peptides from the RP-HPLC column. This peptide was previously observed to have a retention time slightly longer than that for a mixture of peptides derived from a BSA digest and other synthetic MHC restricted peptides. Fractions prior to this spike were therefore analysed for the presence of peptides from the predicted MHC class I restricted BCR-ABL fusion protein. In this patient's sample, the HLA-A3 restricted peptide KQSSKALQR (Figure 3.2(a)) was identified from the MS/MS

spectrum of the $[M+3H]^{3+}$ ion, m/z 349.5, which matched the product ion spectrum obtained for the synthetic peptide of the same sequence (Figure 3.2(b)). The product ion spectrum showed an almost complete set of the expected *y* fragment ions, although several of these are doubly charged which does make it more complicated to sequence *de novo*.

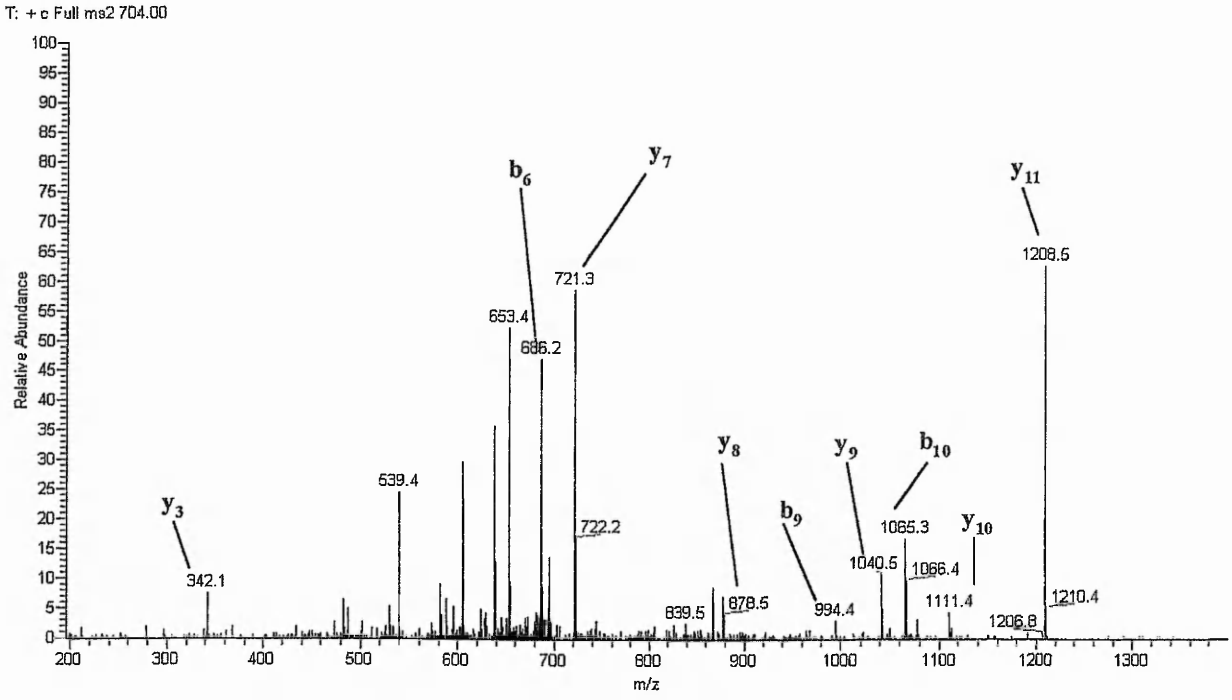

Figure 3.1. Tandem mass spectrum of the Hepatitis B MHC class II restricted synthetic peptide in CML patient A cell extract.

Chromatographic clean-up and separation was performed on two synthetic peptides, gp70, SPSVYVHQF (1 pmol) and p53, LLGRNSFEV (1 pmol), prior to the analysis of the cell eluate and the retention times of these two peptides were used to confirm the chromatographic fractionations corresponding to the elution window for 9-mer peptides. A method blank that consisted of the MHC class II Hep B peptide (900 fmol) in the citrate phosphate elution buffer was analysed prior to analysis of the cell eluate. There was no evidence indicating the presence of the HLA-A3 restricted peptide in the method blank.

In the MS/MS spectrum of m/z 349.5, $[M+3H]^{3+}$ ion of the cellular derived HLA-A3 restricted peptide KQSSKALQR, a strong ion was also present at m/z 129.1 (indicated in Figure 3.2) which corresponded to the mass-to-charge ratio for the b_1 ion. There has been much debate as to whether the b_1 ion can be formed by low energy CAD, as the mechanism involved in the formation of *b* ions is thought to involve a nucleophilic attack on the carbonyl carbon of the peptide bond between two neighboring amino acids.[7] Since the K residue of this peptide sequence has no "neighbour" it is more likely that the ion at m/z 129.1 is due to an arginine residue immonium ion (section 1.4). However, immonium ions are not typically seen in the ion trap and are usually more readily associated with high energy CAD. MS^3 was performed on the m/z 395.2 ion corresponding to the y_7^{2+} ion of a synthetic version of this peptide, and the ion at m/z 129.1 ion was still present, confirming that it is not a *b* type ion and is most likely derived from the C-terminus. Often when analysing peptides in the doubly and singly charged region by MS/MS, the low mass cut-off of the ion trap mass spectrometer means that low m/z ions, such as the immonium ions, are not detected, resulting in incomplete or ambiguous sequence

data. However due to the low m/z of the $[M+3H]^{3+}$ precursor ion in these MS/MS experiments, all the low mass species above m/z 102 were detected allowing complete sequence information to be obtained.

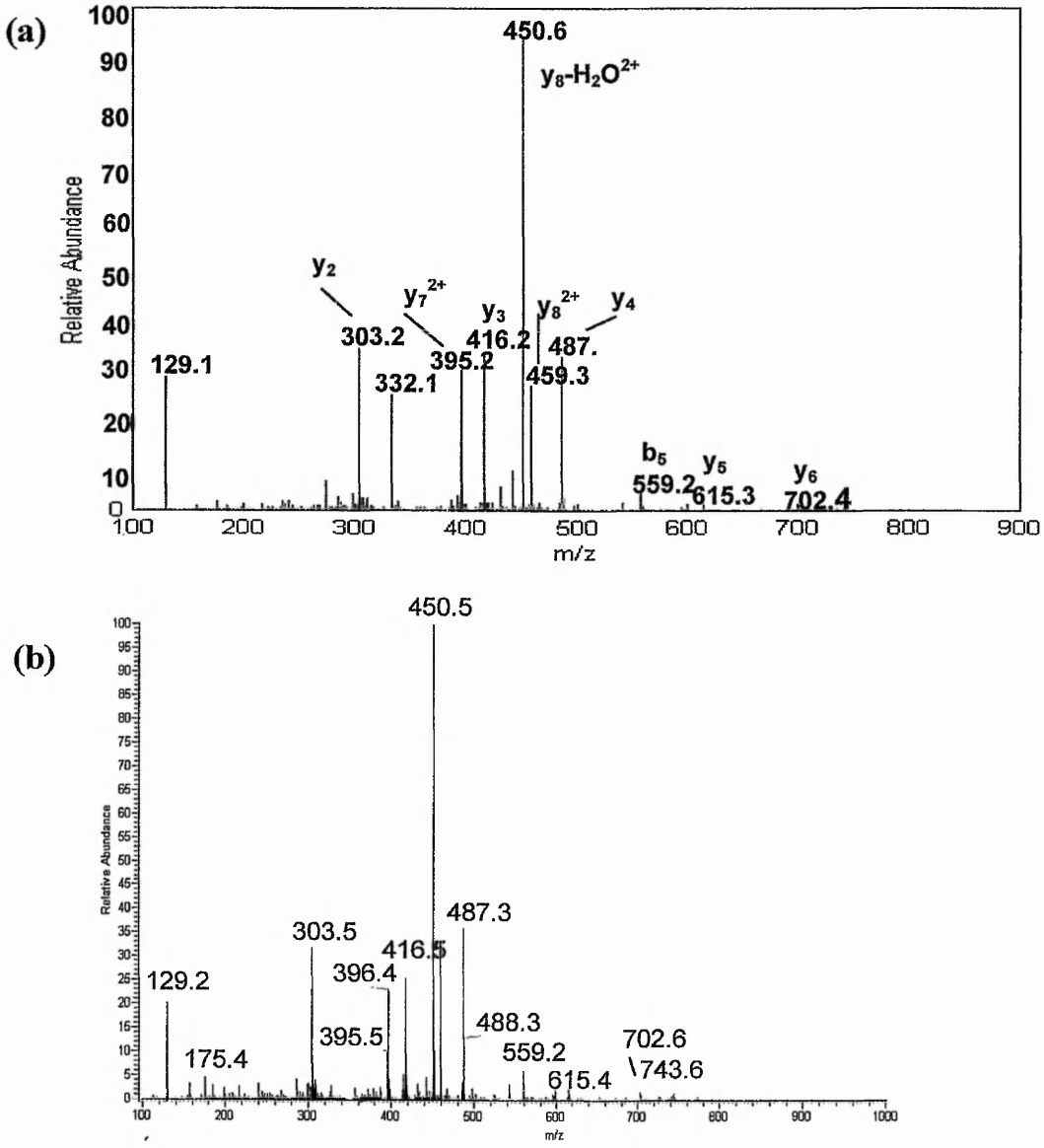

Figure 3.2: (a) MS/MS spectrum of m/z 349.5, $[M+3H]^{3+}$ ion corresponding to the HLA-A3 restricted BCR-ABL breakpoint fusion region peptide, KQSSKALQR from CML patient A white blood cells; (b) MS/MS spectrum of m/z 349.5, $[M+3H]^{3+}$ ion of synthetic peptide KQSSKALQR.

The MS/MS data obtained for the cell eluate for patient A corresponded with a positive identification of the sequence KQSSKALQR in several Internet peptide databases, e.g. MS-Tag (Figure 3.3). Figure 3.4 identifies where this peptide is derived from in the b3a2 exon arrangement of the BCR-ABL translocation product.

Result Summary

Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.6.17.2000 Accession #	Protein Name
(F)KQSSKALQR(P)	1045.6118	-0.6275	15814.5 / 5.50	HOMO SAPIENS	179385	(M25949) bcr/c-abl oncogene protein

Figure 3.3 Internet algorithm search result for the HLA-A3 restricted peptide KQSSKALQR derived from CML patient white blood cells.

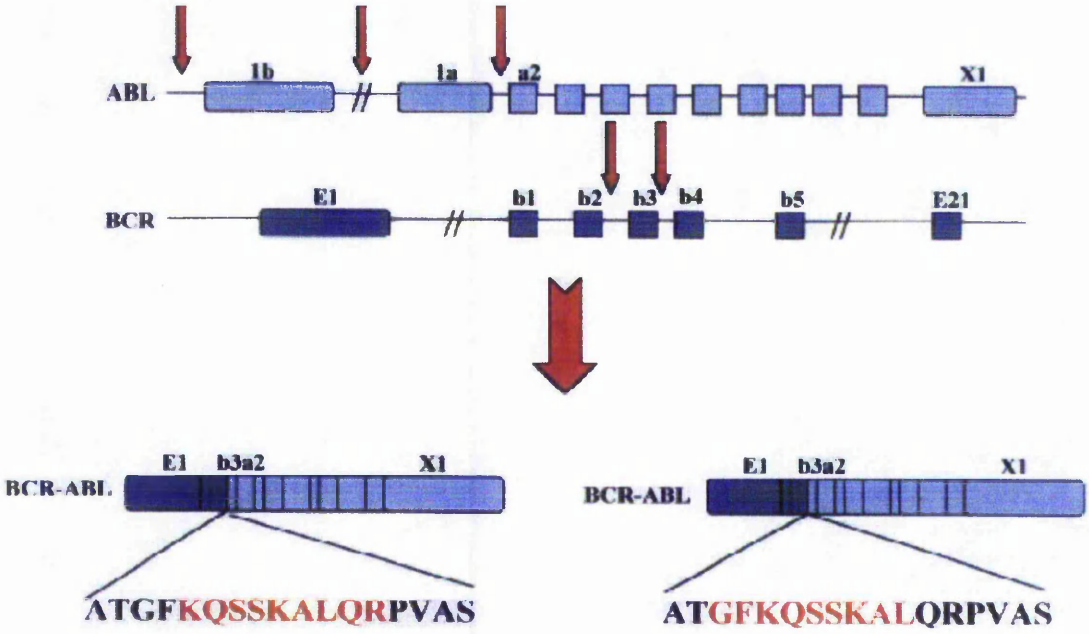

Figure 3.4: Predicted MHC class I restricted peptides derived from the BCR-ABL fusion protein.

The use of a fast gradient for the microbore RP-HPLC chromatography and the relatively high flow rates, resulted in the m/z 500 region of each full scan MS spectrum being extremely "noisy", with peptides and polymeric material present at a high abundance, making the spectra in this region difficult to analyse. However, the fact that the A3 restricted peptide KQSSKALQR can exist in a triply charged state and has relatively short RP-HPLC retention characteristics, due to the high abundance of basic amino acids residues, made it possible to detect and characterise this particular peptide with relative ease.

3.1.3.2 Patient B: MHC class I type, -A3.

Cells (1×10^{12}) from a CML patient expressing the HLA allele -A3, were collected by leucophoresis and subjected to the biochemical clean-up, separation protocol and mass spectrometric analysis as described above. A method blank was run prior to cellular analysis, which was free from contamination of the predicted MHC class I restricted BCR-ABL fusion region peptides. The Hepatitis B MHC class II spike was also detected at the correct retention time and at a detectable limit, indicating a satisfactory recovery through the biochemical clean-up. Ions at m/z 349.5 and m/z 523.5 corresponding to $[M+3H]^{3+}$ and $[M+2H]^{2+}$ of the HLA-A3 restricted BCR-ABL peptide KQSSKALQR were detected by full scan MS and the presence of this peptide in this sample was confirmed by tandem mass spectrometric analysis. Figure 3.4 below shows (a) the tandem mass spectrum of the $[M+3H]^{3+}$ ion m/z 349.5 and (b) the tandem mass spectrum of the $[M+2H]^{2+}$ ion, m/z 523.5.

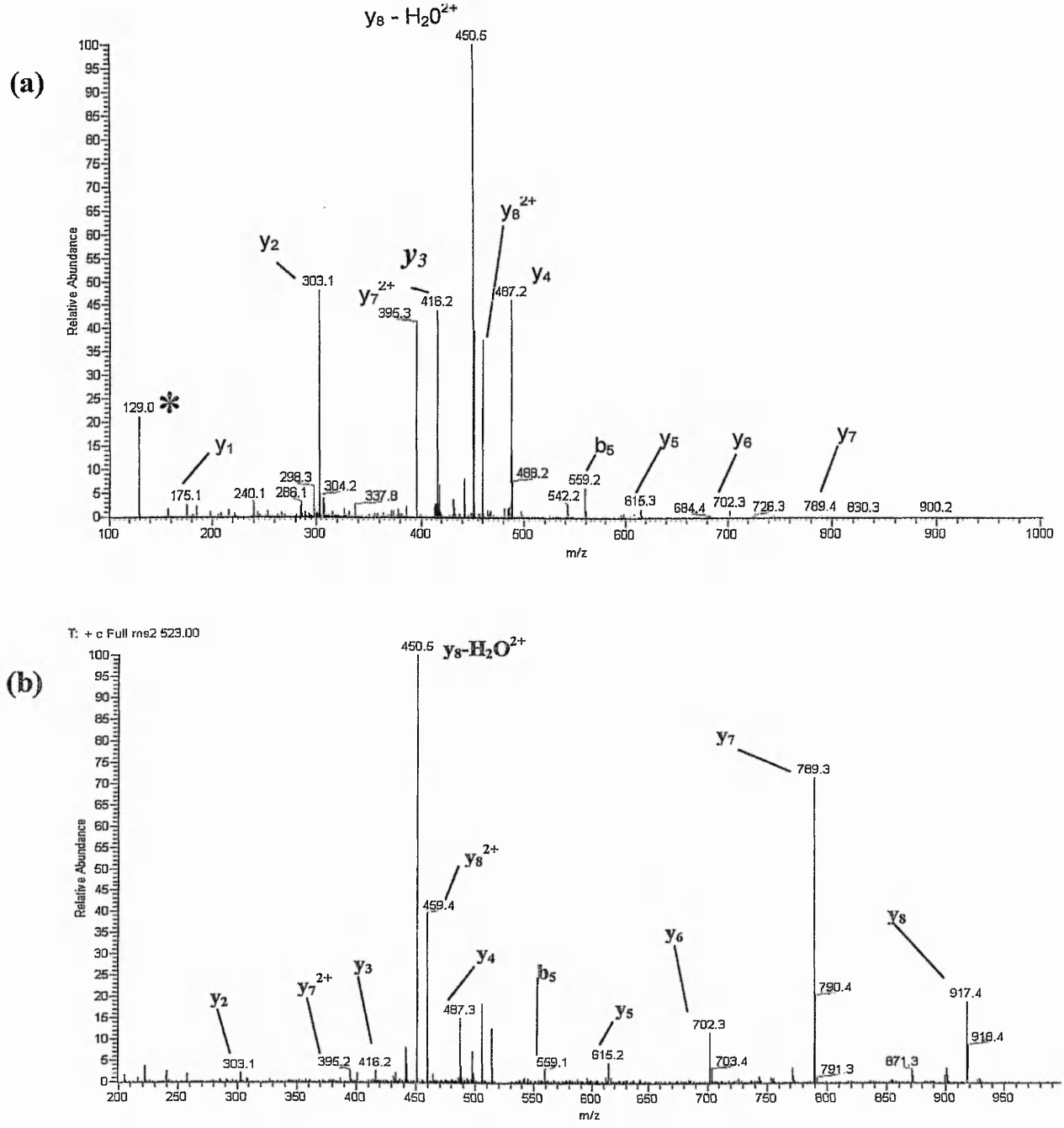

Figure 3.5: The tandem mass spectrum of (a) the $[M+3H]^{3+}$ ion, m/z 349.5 and (b) the tandem mass spectrum of the $[M+2H]^{2+}$ ion, m/z 523.5 from CML patient B white blood cells.

Each of the HPLC fractions analysed contained an abundance of peptides and in one of these an MS/MS spectrum was obtained which was tentatively assigned as the B8 restricted peptide GFKQSSKAL (Figure 3.6). This particular peptide is associated with the BCR-ABL breakpoint fusion region of the Philadelphia chromosome and was found in a single HPLC fraction at a very low concentration compared to the HLA-A3 peptide. The MS/MS data files were exported to Excel for background subtraction. Figure 3.6(a) shows the raw tandem mass spectrum before any subtractive analysis had been performed and 3.6(b) shows the data after the spectrum obtained from a neighbouring fraction was subtracted from the original mass spectrum. The Internet search algorithm results using MS-Tag are shown for this tandem mass spectrum in Figure 3.7.

The MS/MS spectrum for the B8 restricted BCR-ABL fusion region peptide, indicated the presence of a second peptide of the same precursor mass, which fragmented (Figure 3.8) to give completely different peptide sequence ions overlapping with the B8 restricted peptide. This peptide of $[M+H]^+$ m/z 965.5, was found to correspond to the sequence PTI/LTSGCRM, from a comparison of the fragment ions generated in the peptide fragmentation algorithm of the Ludwig ProteinProspector database with those ions observed upon fragmentation of the molecular ion at m/z 483.3. The ions labeled TITS-28 and ITSGC correspond to internal fragment ions. However, a positive identification for the protein from which this peptide is derived was not possible on any of the databases employed. The subsequences TSGCR and TITSG both gave a positive identification for a Myeloid/lymphoid or mixed lineage leukemia translocated to chromosomes 2 and 3 respectively. Although there is no clear indication that these two peptides are indeed

derived from a CML specific protein, it is possible that a novel translocation event may have occurred due to the instability of the karyotype during disease progression (see section 1.1.4) giving rise to a novel protein. This may have occurred as a patient specific event.

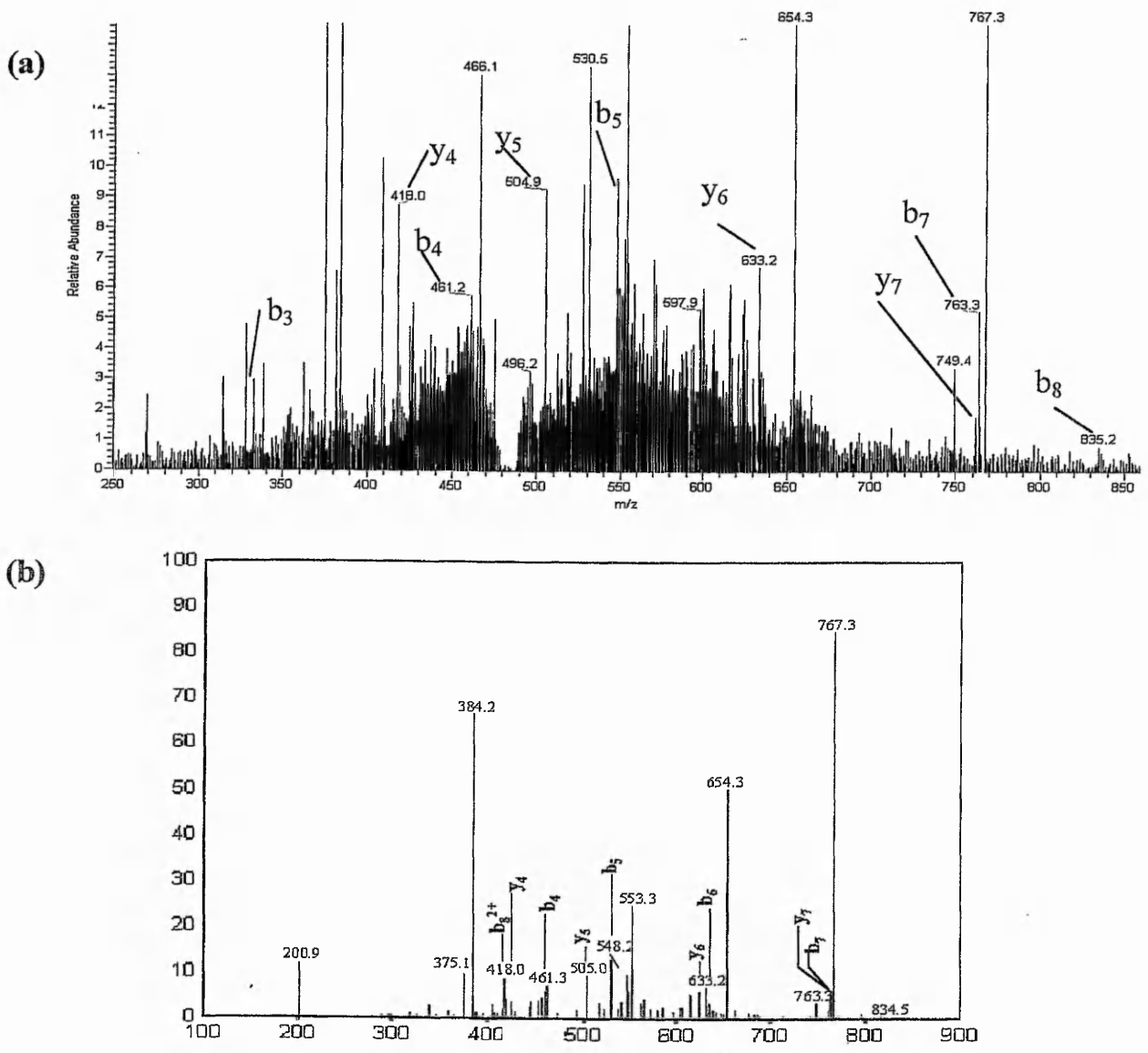

Figure 3.6: Tandem mass spectra of m/z 483.27 ion, $[M+2H]^{2+}$ of peptide GFKQSSKAL derived from CML patient B white blood cells (a) before subtractive analysis and (b) after subtractive analysis.

1	1/9	(T)GFKQSSKAL(Q)	965.5420	-0.0098	17118.0 / 5.47	HOMO SAPIENS	(M25946) bcr/c-abl oncogene protein

Figure 3.7 Internet algorithm search result for the HLA-B8 restricted peptide GFKQSSKAL derived from the white blood cells of CML patient B.

Peptides ions of between 5-6 amino acids were also identified in this cell eluate and Figure 3.9 shows a zoom scan of one such peptide. The tandem mass spectrum of a pentameric peptide assigned the sequence TSQYR is shown in Figure 3.10. In the NCBI and SwissProt databases this peptide was identified to be derived from a viral entity directly correlating to a sequence found in human herpesvirus (HSV), and human cytomegalovirus (CMV). This may be a reasonable assignment since CML patients can be severely immunocompromised allowing dormant viruses to re-activate. These smaller peptides may arise from the citrate phosphate cell surface elution process in which non-covalent cell surface interactions are disrupted during the elution. It has been reported in the literature that pentameric peptides have been found associated with MHC class I derived from Listeria monocytogenes, however these results were from murine subjects and not of human origin.[8] It may be therefore that these smaller peptides can be presented *via* the major histocompatibility complex for immune surveillance. It is also possible that these smaller peptides are derived from cleavage of larger peptides due to the elution process or during storage. However, none of the synthetic peptides investigated prior to these analysis showed signs of degradation and it seems unlikely that the elution, biochemical clean-up or storage has such a significant cleavage effect.

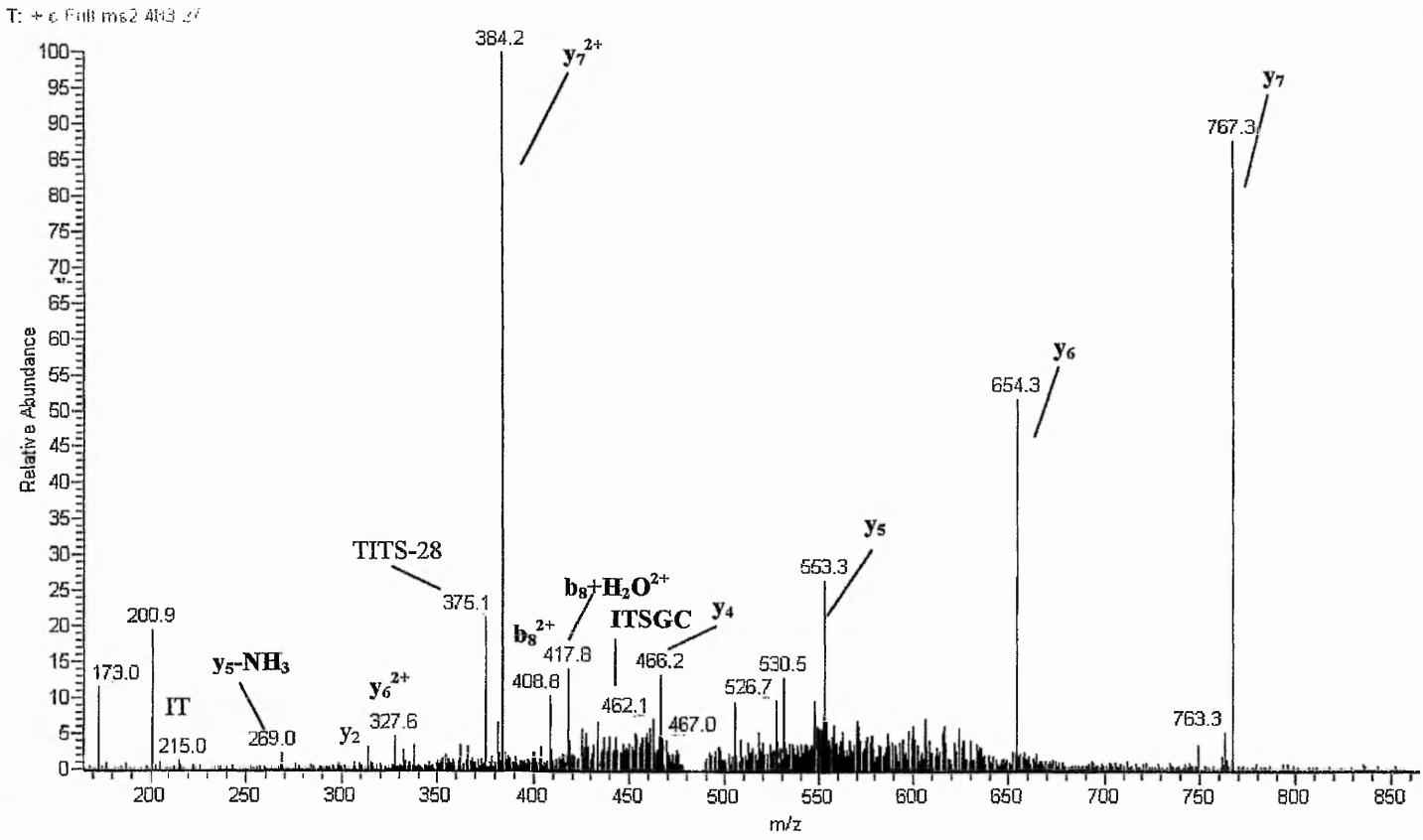

Figure 3.8: Tandem mass spectrum $[M+2H]^{2+}$ ion, m/z 483.27 of the peptide assigned as PTI/LTSGCRM from CML patient B white blood cells.

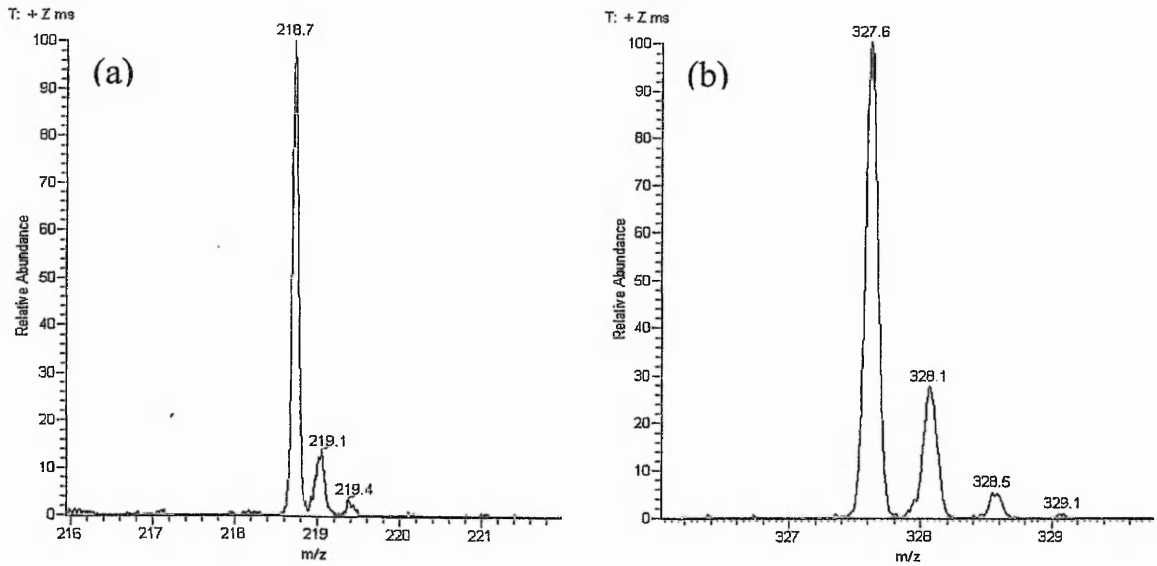

Figure 3.9: Zoom scans of the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ regions of pentapeptide TSQYR derived from CML patient B white blood cells.

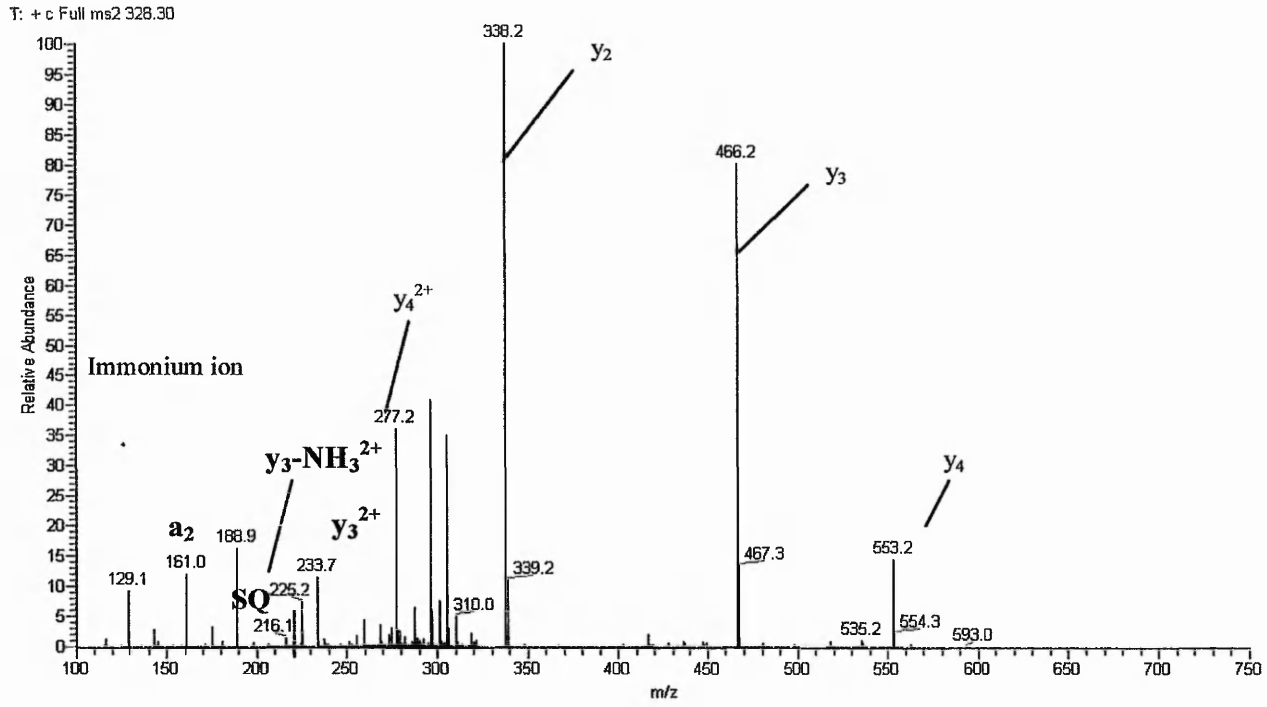

Figure 3.10: MS/MS of m/z 328.3, $[M+2H]^{2+}$ ion for pentapeptide TSQYR derived from CML patient B white blood cells.

The presence of two peptides of the same mass that fragment to give completely different sequences, as seen in the tandem mass spectrum for the B8 restricted peptide, is a common event and may be quite problematic in ascertaining correct characterisation of a peptide sequence. For example Figure 3.11 shows the zoom scan for ions in the region of m/z 726.0, $[M+H]^{+}$ and m/z 364.6, $[M+2H]^{2+}$ present in a single HPLC fraction. From these two zoom scans it might be supposed that a single peptide is present in both singly and doubly protonated states. However on performing tandem mass spectrometry on each of these ions, completely different tandem mass spectrometric data were obtained. Figure 3.12 shows the tandem mass spectrum of the m/z 726 ion, which gives rise to fragment ions corresponding to the amino acid sequence DKGAYT. This peptide was however, found to be associated

with a silanizing agent adduct which is discussed in more detail in section 3.3. This sequence matches by database search a transcriptional regulatory protein homologous to several types of bacteria. Fragmentation of the m/z 364.1 $[M+2H]^{2+}$ ion, yields product ions corresponding to the amino acid sequence CASK/QL/ICC (Figure 3.13). This sequence when entered into the databases did not correspond to any known protein, although, ASKLC is part of the core antigen from the hepatitis B virus. This data provides a good example of the power of nano-electrospray/tandem mass spectrometry to resolve and characterise overlapping peptides present in a single chromatographic fraction without the need for improved chromatographic separation. Table 3.1 lists peptides characterised from the cell eluate derived from CML patient B. Many of the peptides contain the isomers leucine/isoleucine (I/L) and glutamine/lysine (Q/K), therefore complicating characterisation. Sequences corresponding to virally derived peptides and also those derived from hemoglobin were found in several of the patient cell eluates analysed, regardless of HLA haplotype. This would suggest that these peptides are not MHC-related.

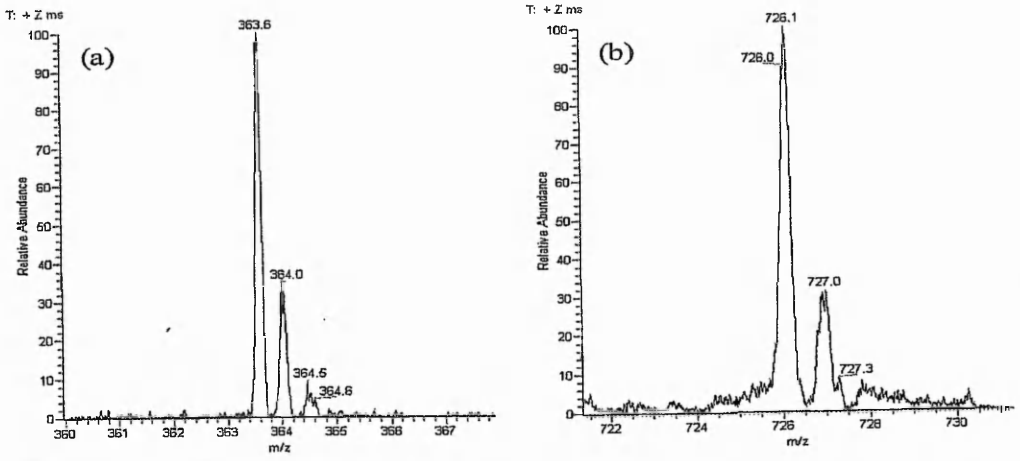

Figure 3.11: Zoom scans of overlapping peptides in HPLC fraction SJRL1176 (a) m/z 364.1 $[M+2H]^{2+}$ and (b) m/z 726.0 $[M+H]^{+}$ from CML patient B.

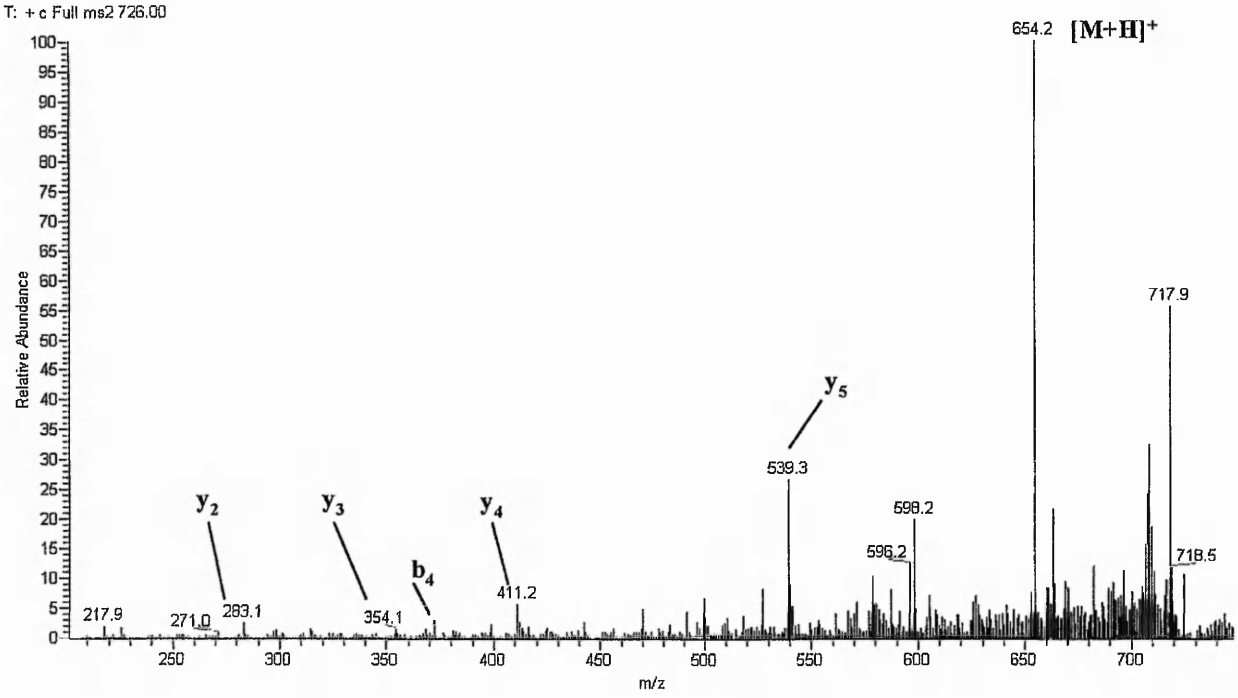

Figure 3.12: MS/MS spectrum of m/z 726.0

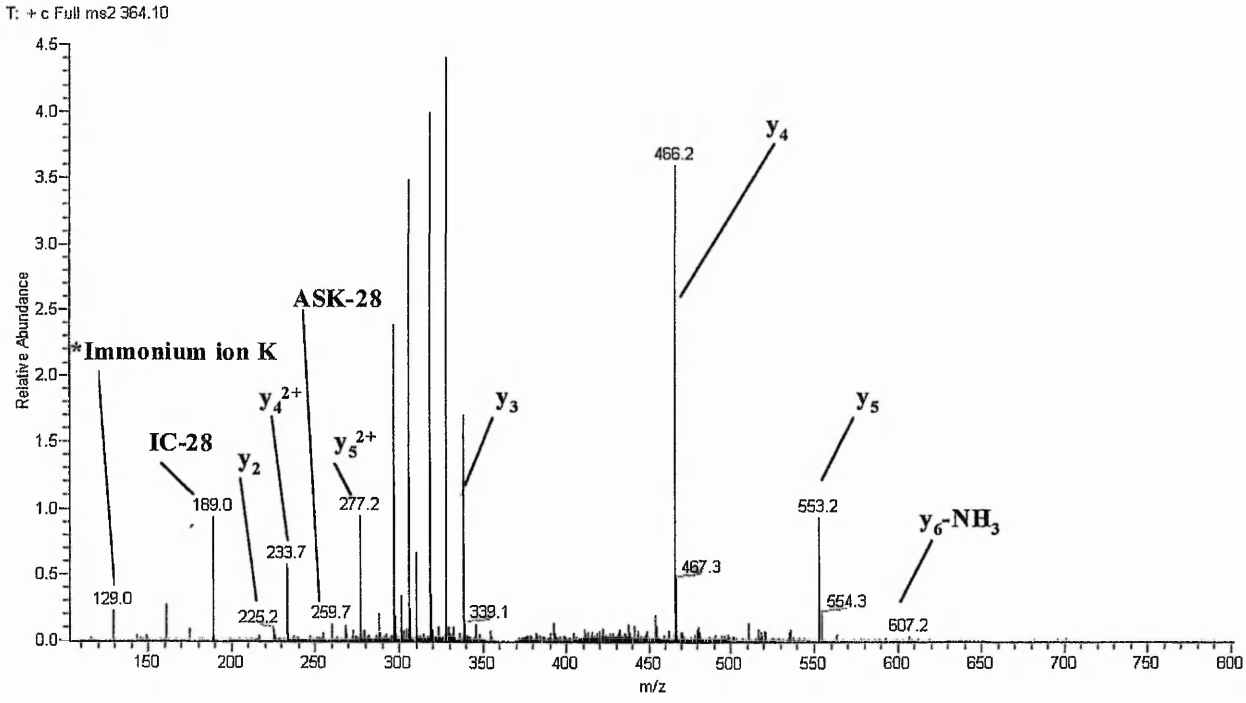

Figure 3.13: MS/MS spectrum of m/z 364.1

Table 3.1 Peptides characterised from the cell surface of HLA-A3 positive CML patient B cell sample.

MS/MS precursor (m/z)	$[M+H]^+$	Assigned sequence	Protein of origin
577.3	1153.6	VSTVLTSK/QYR	α chain hemoglobin
453.3	905.6	HMVCVTCI/L	Unknown
429.7	859.4	(N)FK/QGPNGK	Mitochondrial
431.8	862.6	K/QI/LATPLYG	Unknown
483.3	956.6	K/QNRSMR	Translation initiation factor/ homologue to vitamin B12 receptor
400.1	799.2	SPCQAMY	Human herpes virus U53 protease assembly protein
466.7	932.4	VPMTTA	Unknown
328.3	655.6	TSQ/KYR	HSV or HCM protein
654.2	654.2	ASK/QLC	Hepatitis B core antigen
371.3	1111.9	K/QYI/LI/LAPRG	Unknown

3.1.3.3 Cellular negative controls: Patients C and D

Cells (4×10^9) from CML patient C of HLA type –A3, -B37 and -B62 expressing the b2a2 exon arrangement of the Philadelphia chromosome BCR-ABL gene product was used as a cellular blank to see if the HLA-A3 restricted peptide KQSSKALQR was being processed and presented by MHC class I. No HLA-A3 restricted peptide KQSSKALQR was detected from these cells above the limit of detection of the LCQ, after subjection to the biochemical and mass spectrometric protocol described in 3.1.2. This was as expected, as the b2a2 exon variant of the Philadelphia chromosome expresses a different set of amino acids and hence peptide sequence to the b3a2 exon. A further cellular blank was analyzed consisting of HLA-A3 negative cells (10^{10}) derived from CML patient D, expressing the b3a2

exon arrangement of the Philadelphia chromosome BCR-ABL gene product. Again, no peptides corresponding to the HLA-A3 restricted peptide KQSSKALQR were observed above the instrumental limit of detection. This data provides evidence that cells need to express both the b3a2 exon arrangement of the BCR-ABL protein and the MHC class I haplotype HLA-A3 in order to process and present the HLA-A3 restricted peptide KQSSKALQR.

3.2 Analysis of peptides derived from the HLA-A3 transfected cell line K562

3.2.1 Introduction

The lymphoblastoid cell line K562 is a mutated cell line that does not express any HLA molecules on its surface. Due to its lack of MHC expression it is susceptible to natural killer (NK) cell attack,[8] but once the genes for the cell surface MHC class I molecules have been transfected these cells become less prone to NK killing. The K562 cell line naturally expresses the Philadelphia chromosome and so this cell line, transfected with an expression construct for HLA-A3, was investigated to see whether this too presents any peptides derived from the BCR-ABL protein fusion region at the cell surface.

3.2.2 Method

Transfected K562 cells (2×10^9) (donated by A. Dodi and A. Madrigal from the Anthony Nolan Bone Marrow Trust, London) were harvested and subjected to mild acid elution, biochemical clean-up and mass spectrometric analysis as described for the CML patient samples (section 3.1.2). Method blanks (HCl, 0.1M containing the Hep B spikes (900 fmol) and cellular blanks (5×10^8) were also analysed, the

cellular blanks being non-transfected K562 cells which were subjected to the same protocol as the transfected cellular samples. Synthetic Hepatitis B MHC class II restricted peptide (900 fmol) was added to the samples as the internal recovery standard and was detected by nano-electrospray tandem mass spectrometry at the expected retention time in both the method blank and the K562 cell extract cellular blank.

3.2.3 Results and Discussion

The A3 peptide KQSSKALQR, was specifically sought in the cleaned-up extract from the transfected K562 cell line to see if the antigen processing and presenting machinery of the transfected cell lines behave in the same manner as the clinical samples. No molecular ion corresponding to the peptide could be identified when the extract was analysed in full scan MS. However a positive identification was made through the MS/MS spectrum obtained from m/z 349.5 ($[M+3H]^{3+}$) ion for the A3 peptide that contained fragment ions with masses matching the peptide sequence KQSSKALQR (Figure 3.14(a)). The method and cellular blanks were found to contain the Hep B internal recovery standard and were free from this BCR-ABL fusion protein MHC class I restricted related peptide above the limit of detection. In order to gain an estimate of how much material was present in this sample an HPLC fraction that was negative for the HLA-A3 restricted peptide was spiked with a synthetic version of the peptide KQSSKALQR to a final concentration of 2.5 fmol μl^{-1} (Figure 3.14(b)).

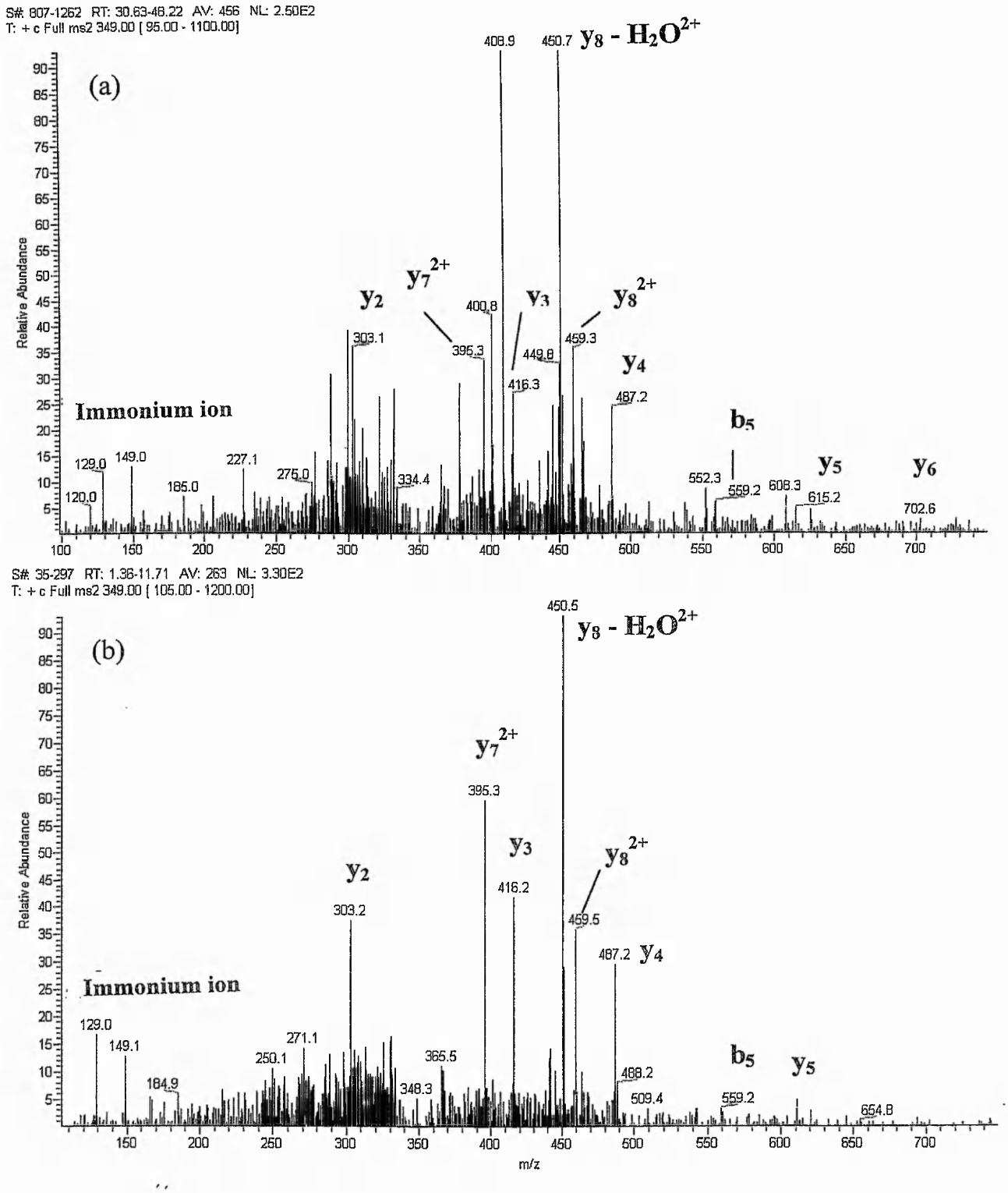

Figure 3.14: (a) MS/MS spectrum of the m/z 349.5, $[M+3H]^{3+}$ ion from HLA-A3 transfected K562 cells ($2x10^9$) and (b) MS/MS spectrum of the m/z 349.5, $[M+3H]^{3+}$ ion of peptide KQSSKALQR (2.5 fmol μl^{-1}) spiked into a K562 cellular HPLC fraction.

The product ion spectra in Figures 3.14 supports the assignment of the A3 peptide sequence and suggests that the amount of peptide KQSSKALQR in the K562 cellular fraction present was in the low femtomole μl^{-1} range. By spiking the cellular HPLC fraction with a standard addition of the A3 peptide, the matrix effects that may be occurring due to the cellular and other extracted components will have a similar ion suppression effect on the MHC presented and synthetic peptide, therefore allowing a clearer estimation of the concentration of peptide present. It must be pointed out, however, that quantitative analyses of complex biological samples by nano-electrospray ionization presents problems because of matrix and interference effects, and therefore any estimation of concentration must be viewed with caution. No other peptides were found in the triply charged region (m/z 300-400) of the K562 cellular HPLC fractions containing the targeted A3 peptide. The background mass spectrum "noise" was quite high around the m/z 500 region, and therefore it was much easier to sequence a triply charged peptide from the m/z 300-400 region where interferences were significantly lower.

This data is the first direct mass spectrometric proof that a haematological malignancy can express leukemia-specific peptides on the cell surface, in association with class I HLA. These results are in accordance with the observations made by our collaborators at the University of Liverpool, whereby patients are shown to mount a CTL response *in vitro* against BCR-ABL peptides expressed by their CML cells.[6] The demonstration that BCR-ABL peptides can be eluted directly from CML cells is also consistent with observations that appropriate synthetic 9-mer b3a2 peptides can bind to HLA-A3 and B8 molecules.[1,4] Eluates obtained from b3a2 positive HLA-A3 negative patients lacked the KQSSKALQR peptide,

providing further proof that this peptide was derived from endogenously processed BCR-ABL protein presented in the HLA peptide binding groove in HLA-A3 positive cells. This provides evidence that CML cells express surface BCR-ABL junctional peptides in association with HLA class I.[6]

3.3 Adduct formation of cellular derived peptides in the presence of a silylating reagent.

3.3.1 Introduction

Silylation *via* substitution of an active hydrogen by a trimethylsilyl group is a widely used method for the derivatisation of non-volatile biological analytes to aid their detection by gas chromatography (GC),[9,10] or GC-MS[11] and to improve their distillation properties. These silylating techniques have been applied to the derivatisation of flavonoids,[12] biles,[13] sugars[14] and amino acids[15,16] employing a variety of silylating agents including hexamethyldisalazane[17] (HMDS), trimethylchlorosilane[17] (TMCS) and trimethylsilyldiethylamine[18] (TMSDEA). Polar solvents such as acetonitrile[19] or pyridine[19] are traditionally employed along with high temperatures to achieve efficient silylation. Carboxyl groups are silylated more readily than hydroxyl groups which are, in turn, more easily silylated than amino groups.[19] Due to the requirement for the silylated products to be sufficiently volatile for GC analysis, this area of derivatisation has focused on simple amino acids and has not been generally employed for the analysis of peptides or proteins. These derivatisation methods have now generally been superceded by the introduction of biologically compatible liquid chromatography-mass spectrometric techniques that allow these analytes to be analysed without prior derivatisation.

Although silylation of peptides is rarely employed in the modern laboratory, one research team purposefully employed this technique in protein analysis. Goldblum[20] *et al.* used silylated bovine serum albumin (BSA) as a control in their enzyme linked immunosorbant assays (ELISA), which was performed to show that patients who have undergone silicone elastomer implants may undergo an immune response

against these prostheses. Harsh conditions and two different silylating agents had to be employed for silylation. In this section, preliminary evidence is presented for the formation of peptide adducts when MHC associated peptides come into contact with silylating reagents commonly used to deactivate laboratory glassware.

3.3.2 Method

3.3.2.1 Silylation of synthetic MHC class I associated peptides

The following peptides were synthesized at the Queens Medical Centre, Nottingham, UK; gp70, SPSVYVHQF; p53(2), RMPEAAPPV; and A3, KQSSKALQR. Mass spectrometric analysis was performed using a quadrupole ion trap mass spectrometer (LCQ, Finnigan, UK).

An aliquot of each of the above peptides (≈ 500 pmol in 5 μl 50:50 acetonitrile: water + 5% v/v acetic acid) was transfered to both a non-silanised 1.1 ml tapered glass vial and also to a vial that had been pre-treated with the silanising agent, Sigmacote (Sigma, UK) by adding 2 μl of the reagent to the vial and immediately removing the excess reagent using a glass syringe (25 μl, Hamilton, SGE, UK). The peptides were left in contact with the silylating agent for 1 hour, before the solution was withdrawn from the vial and loaded into a nanospray tip (NanoES, Protana, Denmark) and subjected to nano-electrospray ion trap mass spectrometry using the in-house constructed nano-electrospray ionization source. MS was performed on the sample to identify ions corresponding to adducts in the singly, doubly and, where present, triply charged mass regions. Tandem mass spectrometry was performed with an isolation width of 3u and a relative collision energy of 30-45%. Mass

spectrometric conditions employed included a heated capillary temperature of 100°C (expect where stated), a spray voltage of 1.1 kV and the automatic gain control set at 1x 10^8.

The effect of temperature was investigated by storing the A3 peptide solution in freshly silylated vials at 4°C, room temperature and 60°C for 1 hour prior to analysis. The solutions were then analysed using nano-electrospray MS and tandem mass spectrometry as described above. The effect of the temperature of the heated capillary on adduct formation was also investigated by varying the temperature by 50°C increments from 50-300°C.

3.3.3 Results and Discussion

3.3.3.1 Silylation of naturally derived cellular peptides

During the nano-electrospray mass spectrometric analysis of cellular derived MHC class I peptides, unidentified ions showing isotopic abundances characteristic of peptides were fragmented to identify the peptide sequence. An example of such an ion is the peak at m/z 373.7 observed in the full scan MS spectrum of an RP-HPLC fraction from patient B (Figure 3.15). Inset is the zoom scan of this triply charged ion present in the cell eluate. The ion at m/z 373.7 was subjected to CAD and the MS/MS spectrum is shown in Figure 3.16.

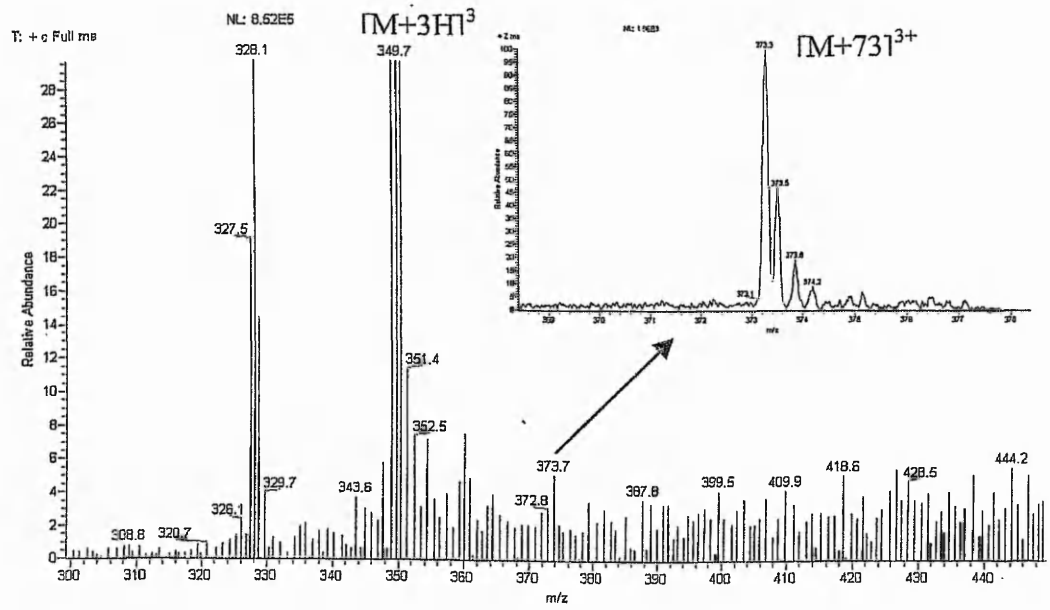

Figure 3.15: A full scan MS spectrum of an RP-HPLC fraction from CML patient B in the m/z 300-450 region. Inset, a zoom scan of the triply charged ion, m/z 373.7.

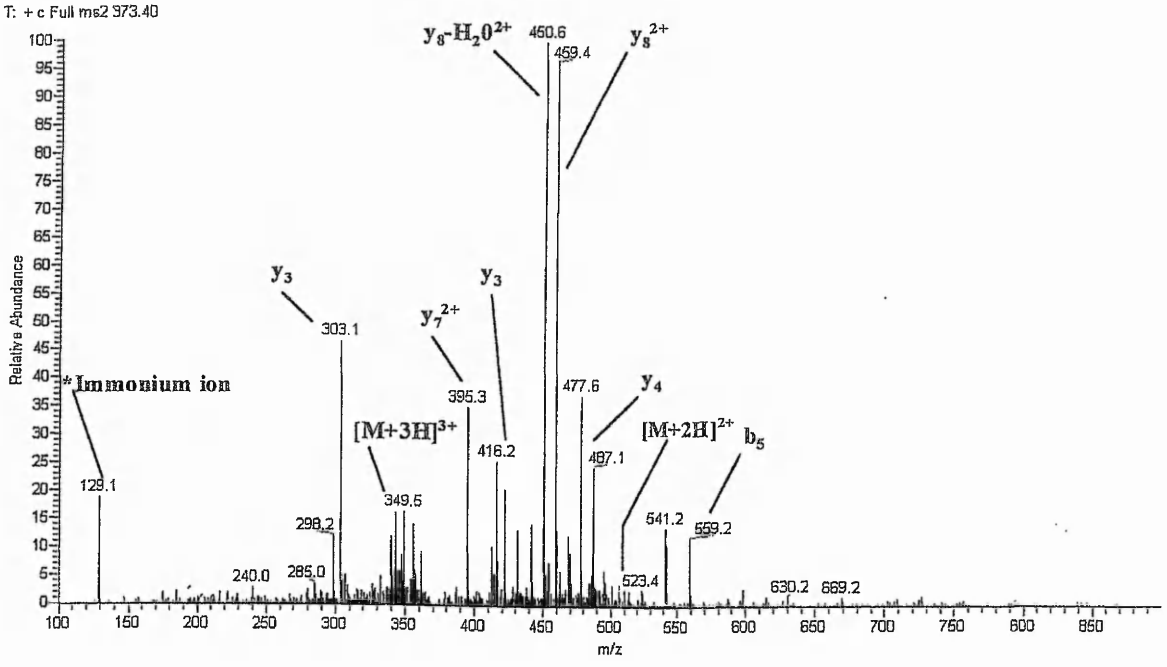

Figure 3.16 MS/MS spectrum of ion m/z 373.7 derived from an HLA-A3 positive CML patient sample.

The tandem mass spectrum shows a full complement of product ions corresponding to the HLA-A3 restricted peptide KQSSKALQR, derived from the BCR-ABL fusion region of the Philadelphia chromosome, despite the fact that the m/z 373.7 ion corresponds to a molecular weight 72 Da higher than that for the protonated A3 peptide. The same phenomenon was observed for several other peptides derived from the cell eluate, including a cell surface hexameric peptide sequence DKGAYT arising from the tandem mass spectrometric fragmentation of an ion at m/z 726.0 (Figure 3.17), although the sequence information corresponded to a peptide with a molecular weight of 653.2 Da. These observations suggest the formation of an adduct that is readily lost under CAD to reveal the sequence of the original peptide. The mass difference of 72 Da does not, however, corresponded to any commonly observed adduct such as a post-translational modification or a cationised species. One common process leading to an increase in molecular weight of 72 Da, however, is the substitution of a hydrogen by a trimethylsilyl group. The only source of a trimethylsilyl group in this experimental protocol was the Sigmacote reagent used to treat the glassware used for the storage and clean-up of cell extracts. Further investigations were therefore carried out on synthetic MHC class I associated peptides to investigate this hypothesis.

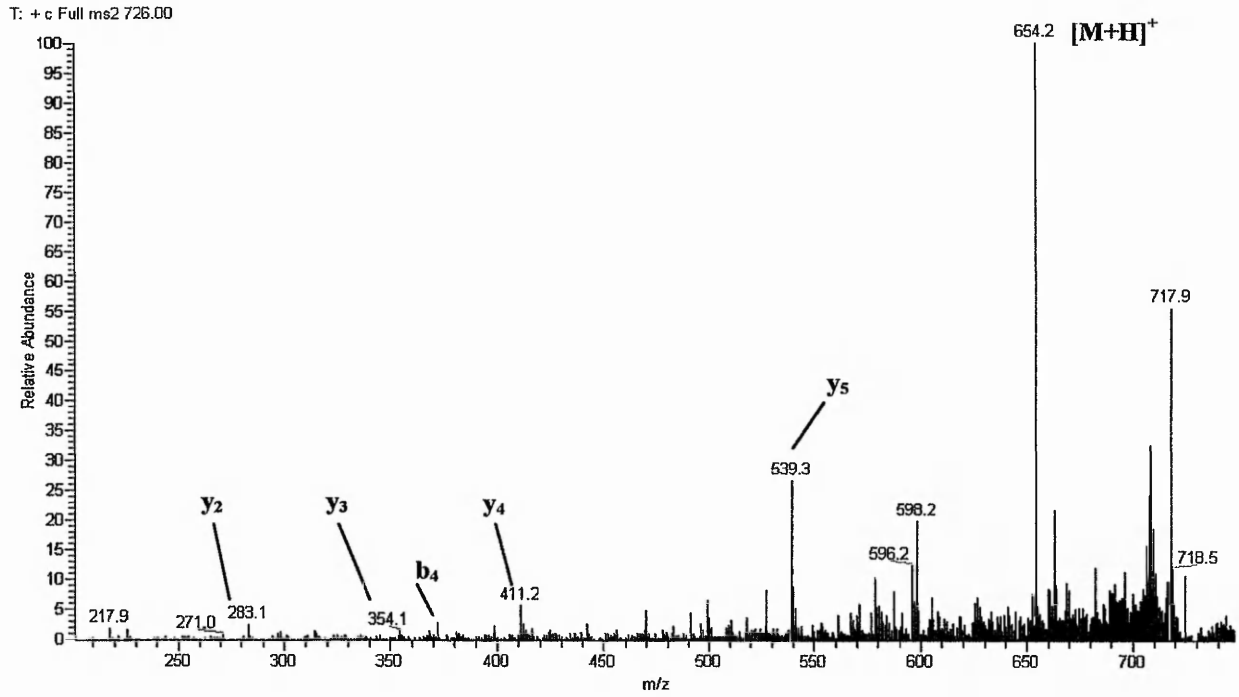

Figure 3.17: MS/MS spectrum of m/z 726.0, a silylated cellular derived peptide of sequence DKGAYT.

3.3.3.2 Silylation of synthetic MHC class I associated peptides

The synthetic peptide KQSSKALQR was exposed to Sigmacote and analysed mass spectrometrically. In addition to the expected $[M+3H]^{3+}$ ion at m/z 349.5, the resulting spectrum contained an ion at m/z 373.7, $[M+3H]^{3+}$ which was not observed when the peptide was analysed without exposure to Sigmacote. Tandem mass spectrometry was performed on this ion and the resulting spectrum is shown in Figure 3.18. Several ions in the spectrum correspond to product ions of the pure triply protonated A3 peptide when the m/z 349.5 is selected and dissociated. It is known that carboxyl groups are the most readily silylated group[19] belonging to peptidic species, and this is therefore the most likely site for the substitution of a

hydrogen by a trimethylsilyl group to occur. This is supported by the observation that the serine analogue *N-tert*-butoxycarbonyl serine methyl ester ($H_2NCH(CH_2OH)COOCH_3$) does not form an adduct corresponding to $[M+73]^+$ in the presence of Sigmacote, hence suggesting that the TMS group is not associated with the amino terminus of the amino acid/peptide. Further evidence is that the A3 peptide only formed a mono-silylated adduct, which may be due to this peptide containing only one COOH group, located at the carboxyl terminus.

In order to investigate the idea that this TMS group is forming an adduct with the carboxyl residue further, a peptide of sequence RMPEAAPPV, p53(2), was chosen, which contains an additional carboxyl group at the fourth amino acid residue from the N-terminus, glutamic acid (E). Mass spectrometric analysis of this peptide prior to exposure to Sigmacote, resulted in an ion being observed at m/z 967.6, corresponding to the $[M+H]^+$ ion and no ions were observed corresponding to any possible TMS adducts. After exposure to the silylating reagent however, two peaks corresponding to the mono and disubsitituted TMS derivatives $[M+73]^+$, m/z 1039.6, and $[M+145]^+$, m/z 1112.6 were observed. Figure 3.19(a) shows the zoom scan of the m/z 1039.6 mass range before exposure to Sigmacote and 3.19(b) after exposure. This data provides strong evidence that the TMS group was indeed forming an adduct with the carboxyl group of these peptides.

An analysis was performed on a further MHC class I restricted synthetic peptide, gp70, SPSYVYHQF. Once more, before exposure to Sigmacote, only a strong peak corresponding to the $[M+H]^+$ ion at m/z 1127.5 was observed. However. analysis of the peptide solution after exposure to the silylating reagent, showed peaks at m/z

1199.5 and m/z 1271.5 corresponding to $[M+73]^+$ and $[M+145]^+$. This was not expected as this peptide contains only one COOH group located at the carboxyl terminus. However, gp70 does contain two serine residues and two tyrosine residues and is therefore rich in OH side chain amino acids. It may be due to this high number of hydroxyl residues that this peptide is able to form the disubstituted TMS adduct. Two of these OH containing serine residues are located either side of a proline residue, which may bend the peptide promoting exposure of the serine residues and therefore encouraging formation of TMS adducts with these side chain groups. Tandem mass spectrometry was performed on the ion at m/z 1199.50, $[M+Si(CH_3)_3]^+$ in the gp70 samples which had not been exposed to Sigmacote and also to a sample of equal concentration and volume after exposure to the silylating reagents. Figure 3.20 shows (a) the MS/MS spectrum before exposure to Sigmacote and (b) after exposure.

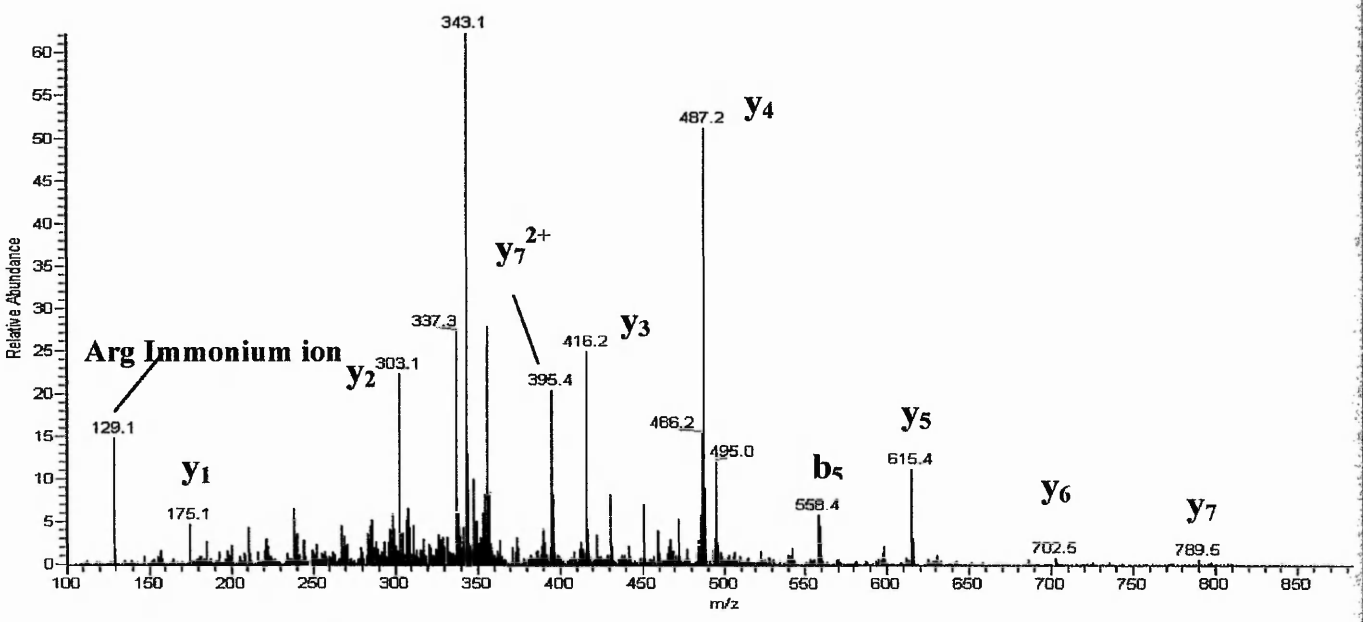

Figure 3.18: Tandem mass spectrum of the ion m/z 373.7, $[M+3H]^{3+}$ in a sample containing the synthetic peptide KQSSKALQR in the presence of Sigmacote.

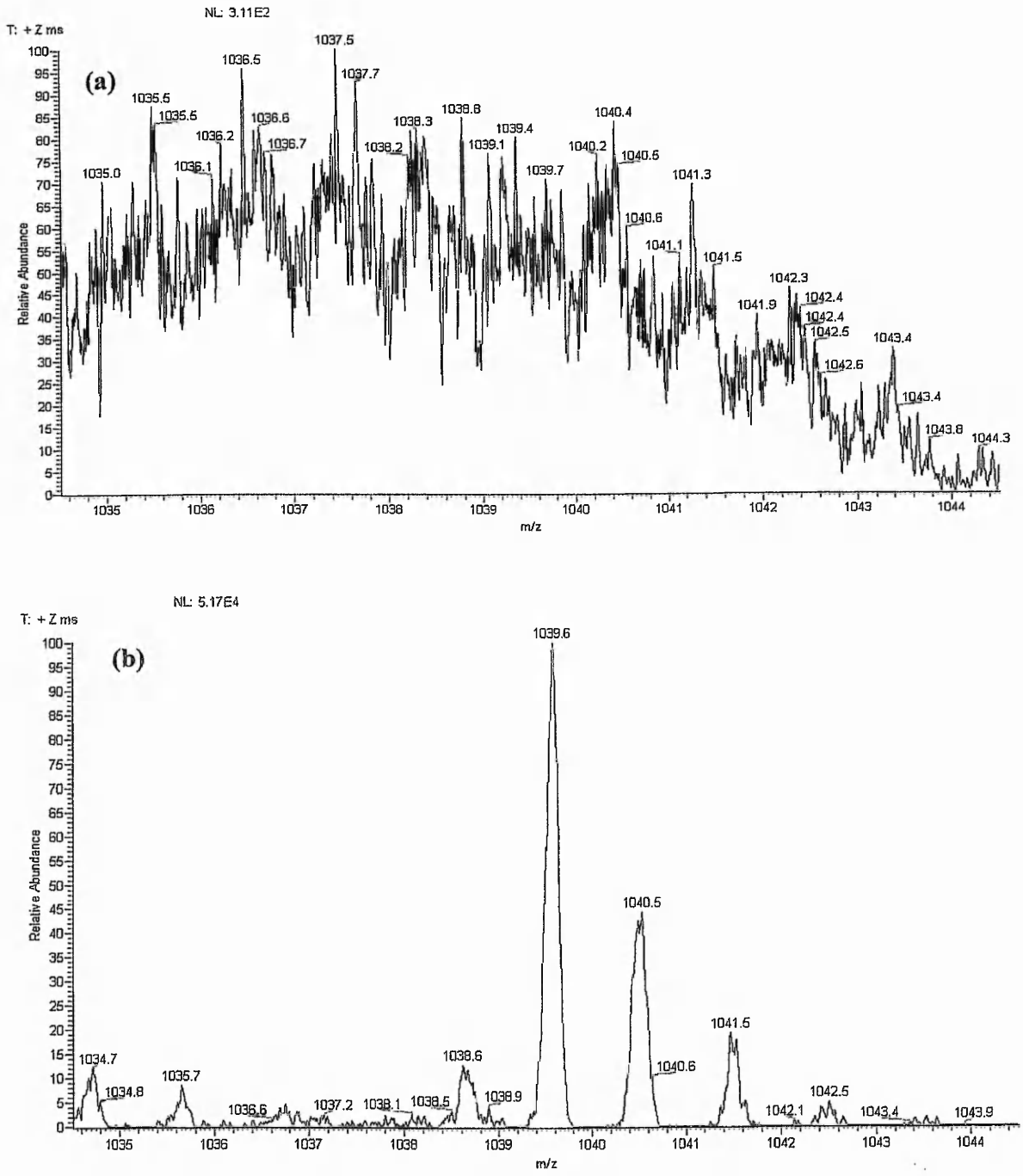

Figure 3.19 (a) Zoom san of m/z 1039.6 mass range before exposure of the MHC class I peptide RMPEAAPPV to Sigmacote and (b) after exposure.

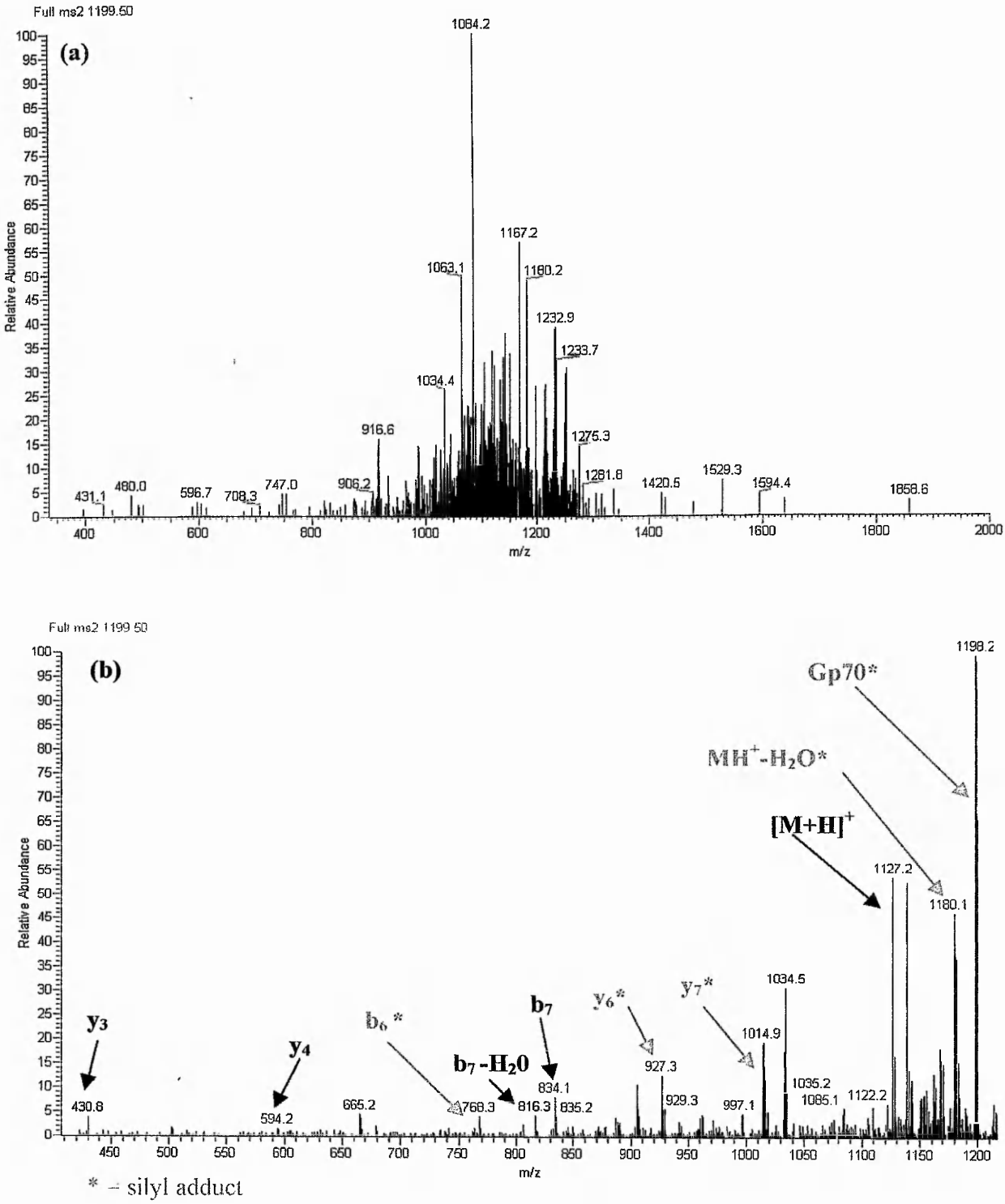

Figure 3.20 (a): MS/MS of the ion m/z 1199.5 in a synthetic MHC class I peptide gp70 not exposed to Sigmacote and (b) after exposure to Sigmacote.

Figure 3.20(a) reveals only background noise, whereas in Figure 3.20(b), fragmentation of m/z 1199.5, $[gp70+Si(CH_3)_3]^+$ ion, results in both the $[M+H]^+$ and a series of both protonated and silylated fragment ions. MS^3 was performed on the suspected $[M+H]^+$ ion, m/z 1127.5 and sequence information confirmed that this ion does indeed correspond to the singly protonated gp70 peptide.

It has been reported in the literature that high temperature plays an important role in producing these trimethylsilyl adducts.[19] It was therefore decided to see if the high temperature of the heated capillary was in some way enhancing the production of these adducts, an experiment was conducted in which the heated capillary temperature was set at 50°C, 100°C, 150°C, 200°C, 250°C and 300°C during the nano-electrospray mass spectrometric analysis. It was found that the ratio of protonated to silylated peptide did not alter with changing the heated capillary temperature and remained constant at approximately 300:1 $[M+H]^+$: $[M+Si(CH_3)_3]^+$. A further investigation into the role of heat in producing these silyl adducts was performed on the synthetic A3 peptide, in which the peptide was stored in a freshly silanised vial for 1 hour at either 4°C, room temperature or 60°C. Both of these temperatures resulted in the same degree of adduct formation, and hence it would seem that temperature does not play a significant role in the formation of these silyl adducts. This is surprising for, as stated above, the literature reports on the need for high temperatures for the encouragement of these TMS adducts to form with biological compounds.[16] However, the ratio of the protonated to silylated biological products observed in these investigations suggests that small amounts of the TMS adducts may be formed even under mild conditions. Further work is required to clarify the mechanism and site of silylation.

3.8 Conclusion

The HLA-A3 restricted peptide sequence KQSSKALQR and the B8 restricted sequence GSKQSSKAL have been identified in cell eluates derived from CML patient white blood cell samples. This is the first evidence of a direct analysis of cell surface MHC class I peptides derived from an *in vivo* human malignant sample. It is also the first evidence that peptides derived from the break-point region of the Philadelphia chromosome are being processed (by the proteasome) and presented by different MHC class I alleles to the cell surface. The analysis of peptides eluted from the transfected K562 cell line shows that when cells deficient in cell surface MHC are transfected with the gene for HLA-A3, the processing and presentation of these fusion region peptides occurs in the same manner as cells naturally expressing this HLA allele.

The absence of the HLA-A3 restricted peptide in the HLA-A3 negative patient sample suggests that this peptide was being eluted from the cell surface and not derived through total cell lysis. Killing *via* cytotoxic T lymphocytes has been demonstrated for the HLA-A3 restricted peptide proving the immunogenicity of this break-point fusion protein MHC class I epitope. Tetrameric MHC class I complexes have also been made specifically for the HLA-A3 allele and are proving to induce a high percentage of killing. These HLA-A3 and B8 restricted BCR-ABL fusion protein derived peptides show potential as an immunotherapeutic agent for the treatment of this malignancy.

Other peptides have been identified in patient white blood cell extracts, which correspond to house keeping proteins that are thought to be non-immunologically

relevant. Pentameric peptides have also been found in the CML patient eluates that represent viral epitopes, specifically from cytomegalovirus and herpes simplex virus. Since these patients are in an immunocompromised state, particularly after transplantation, it is highly likely that the "natural" load of these peptides is increased.

Preliminary evidence is presented that trimethylsilyl adducts of peptides may occur when exposed to silylating reagents. Mass spectrometric analysis of synthetic peptides suggests that trimethylsilylation may be associated with the terminal carboxylic acid or hydroxyl side chain amino acid residues of the peptides. These TMS adducts may cause ambiguity when analysing cell surface eluted MHC class I peptides.

It can be concluded that cell surface MHC class I bound tumour antigens may be extracted and identified using mild acid elution, TCA precipitation, cation exchange chromatography and microbore RP-HPLC with nano-electrospray tandem ion trap mass spectrometry. The MHC class I peptides derived from the break-point region of the Philadelphia chromosome are molecularly unique and are therefore strong immunological candidates for immunotherapeutics for the treatment of the leukemias expressing this translocation product.

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CHAPTER FOUR

NANO-ELECTROSPRAY AND MICROBORE LIQUID CHROMATOGRAPHY ION TRAP MASS SPECTROMETRY STUDIES OF TRANSITION METAL COMPLEXATION WITH MHC RESTRICTED PEPTIDES

4.1 Introduction

Interactions of peptides with alkali metal,[1,2] alkali earth metal [3,4] and transition metal ions [5-9] have been demonstrated using ESI-MS and MALDI-MS,[10] and molecular modeling [8, 11] has played an important part in predicting metalation sites. Copper ion/peptide complexation has also been explored for model peptides using ESI-MS [7, 12-16] because of the important role of copper in biochemical systems. [17] These metal/peptide interactions have been exploited for the selective separation of peptides from complex mixtures via metal chelate affinity chromatography [9, 11, 18] and iron complexation has been employed for the selection and isolation of phosphorylated peptides and proteins from complex mixtures. Silver is well known as a stain on SDS polyacrylamide gels for the detection of peptides and proteinaceous material. [19] Tandem mass spectrometric studies have indicated that histidine containing peptides have a particularly strong affinity for transition metals, although copper can bind to other amino acids through association with the C-terminus of the peptide. [11,20] Mass spectrometric analysis of metal/peptide complexation has however not been extended to naturally occurring peptides in the range of 8-22 amino acids. This chapter describes an investigation into the formation of transition metal-peptide complex ions of MHC class I and class II restricted peptides with copper, nickel and silver salts using nano-electrospray ionization and micro-bore LC/MS with post-column complexation.

4.2 Experimental

All experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, Hemel Hempstead, UK) under the following conditions: heated capillary 200°C, AGC $1x10^8$, maximum ion injection time 400 ms, 3 microscans/scan, tube lens offset 40 V and capillary voltage 38 V. Spectra were acquired in full scan mode in the range of m/z 300-1700 or in high resolution scan mode.

4.2.1 Nano-electrospray

The standard electrospray source was removed and replaced with the in-house constructed nano-electrospray source (Section 2.1.1). An Au/Pd coated nanospray tip (Nano-ES tip, Protana, Denmark) supported in a PTFE holder was attached to an adjustable platform allowing movement along all three axes for the precise positioning of the tip near the heated capillary inlet. The tip was gently broken against the heated capillary to initiate spraying. The nano-electrospray tip was maintained at voltages in the range of 0.9-1.1 kV by connection to the instrumental high voltage power supply.

The following human or murine derived MHC associated peptides were supplied by the Queens Medical Centre, Nottingham: HLA-A3 restricted *bcr-abl* fusion peptide, $KQSSKALQR_{33-41}$ (A3); Tumour antigen p53, $LLGRNSFEV_{264-272}$ (P53); H2-kd restricted murine glycoprotein, $SPSYVYHQF_{454-463}$ (gp70); H2-kd restricted murine β-galactosidase, $TPHPARIGL_{377-386}$ (β-gal) and the MHC class II restricted Hepatitis B HBVc, $TPPAYRPPNAPIL_{128-140,}$ (Hep B).

4.2.2 Copper/Peptide complexation

An aliquot (5µl) of the murine MHC class I restricted peptide gp70 (500pmol μl^{-1}) or a mixture of human and murine MHC class I and class II restricted peptides (gp70, A3, Hepatitis B, and p53 each at ~300 pmol/µl) in 50:50 acetonitrile (BDH, UK):water + 5% glacial acetic acid (Fisher Scientific, Loughborough, UK) was introduced into a nanospray tip. A molar excess of copper (II) acetate solution in deionised water (5µl of a 5mM or 32 mM solution adjusted in the range pH 3.5 - 11.5 with ammonium hydroxide) was added to the tip and the two solutions were allowed to mix during the nano-electrospray analyses. Data were acquired in full scan MS mode with a spray voltage of 0.9 kV applied to the nanospray tip.

4.2.3 Copper complexation with a peptide derived from a clinical cell eluate

Chronic myeloid leukemia cells (10^{12}) were eluted, subjected to biochemical clean-up and analysed using the methodology described in Chapter 3. After positive identification of the HLA-A3 restricted peptide $KQSSKALQR_{33-41}$ by nano-electrospray ionization/tandem ion trap mass spectrometry, the scan mode was returned to full MS and an aliquot (2µl) of copper acetate solution (20 molar excess, pH 8.8) was introduced into the back of the nanospray tip. Data was acquired while the peptide and copper solutions mixed and a spray voltage of 1.1 kV was applied to the tip during analysis.

4.2.4 Nickel/Peptide complexation

An aliquot (5 µl) of an equimolar solution of MHC class I restricted peptides p53 and gp70 and the MHC class II restricted peptide Hep B (1nmol μl^{-1}) in 50:50

acetonitrile: water + 1% acetic acid was mixed with an aliquot (5 μl) of nickel acetate (20 molar excess, pH 8.8) and analysed in full MS scan mode with a spray voltage of 0.9 kV applied to the tip.

4.2.5 Copper/Nickel mixed metal complexation

An aliquot (2 μl) of the histidine containing peptide gp70 (1.2 nmol μl^{-1}) in 50:50 acetonitrile:water + 5% acetic acid was mixed with an aliquot (2μl) of an equimolar solution of nickel acetate:copper (II) acetate (20 x molar excess, adjusted in pH range 2.7 – 9.6 with ammonium hydroxide or acetic acid). The solutions were allowed to mix prior to analysis and data was acquired in full scan MS mode with a spray voltage of 1.1 kV applied to the tip.

4.2.6 Silver/Peptide complexation

An aliquot (2μl) of the non-histidine containing peptide p53 (2.6 nmol μl^{-1}) in 50:50 acetonitrile: water + 1% acetic acid was mixed with a molar excess of silver sulphate solution in deionized water (2 μl, 20 x molar excess, pH 10). The solutions were allowed to mix prior to analysis and data was acquired in full MS scan mode with a spray voltage of 1.1 kV applied to the nano-electrospray tip. Tandem mass spectrometry was performed on the $[p53+^{107}Ag(I)]^+$, m/z 1140.5 ion, and the $[p53+^{109}Ag\ (I)]^+$, m/z 1142.5 ion respectively (isolation width 1-2 u, relative collision energy 40%).

The procedure was repeated with the histidine containing MHC peptide gp70 (1.2 nmol μl^{-1}). An aliquot (2μl) in 50:50 acetonitrile: water + 1% acetic acid was mixed

with an aliquot (2 μl) of silver sulphate (20 x molar excess, pH 10). Data was acquired in both full MS and MS/MS mode on the $[gp70+^{107}Ag(I)]^+$, m/z 1233.5 ion and the $[gp70+^{109}Ag(I)]^+$, m/z 1235.5 ion under the same mass spectrometric conditions as above.

4.2.7 Copper/ Silver mixed metal complexation

An aliquot (2μl) of the histidine containing peptide gp70 (1.2 nmol μl^{-1}) or the non-histidine containing peptide p53 (2.6 nmol μl^{-1}) in 50:50 acetonitrile:water + 1% acetic acid was mixed with an equimolar solution of silver sulphate:copper acetate (7nmol μl^{-1}, pH 8.8) prior to analysis. Data was acquired in full scan MS mode with a spray voltage of 1.1 kV applied to the tip. Tandem mass spectrometry was performed on the $[M-2H+Cu(II)+Ag(I)]^+$ ion for both gp70 (m/z 1269) and p53 (m/z 1201). An isolation width of 3u was applied with 35-40% relative collision energy.

ESI-MS (5μl min^{-1}) of p53 and gp70 copper/silver complexes was also performed on an FT-ICR-MS instrument (IonSpec, UK, courtesy of the Rademacher Group, London, UK). The peptide gp70 (4 nmol μl^{-1}) was mixed with an equimolar solution of silver sulphate/ copper acetate at 20 molar excess (pH 9). The peptide p53 (2.58 nmol/μl) was mixed with the silver/copper solution again to 20 molar excess (pH 9). Data was recorded in full-MS with a mass range of ± 10 Da for the peptide/metal complexes.

4.2.8 Microbore LC/MS with post-column metal complexation

The instrumental arrangements for the HPLC/ESI-MS analysis of MHC associated peptides are shown schematically in Figure 4.1. Post column addition of copper was carried out using either a T-piece located between the column and the ESI source (Fig. 4.1a) or *via* the ESI source sheath liquid inlet (Fig. 4.1(b)). The LCQ syringe pump was used in both configurations to introduce the copper (II) acetate solution (32 mM adjusted to pH 10.3 with ammonium hydroxide) into the HPLC eluant at a flow rate of 5 $\mu l\ min^{-1}$. MHC class I and class II peptides (A3, gp70, Hepatitis B and p53 each at ~ 700 pmol μl^{-1}) were separated by microbore reverse phase-HPLC using an ODS column (1x150mm, Jupiter, Phenomonex, UK). Samples were introduced using an injection valve (Rheodyne 7010) fitted with a 5 µl injection loop. The eluant was delivered to the column by two HPLC pumps (Milton Roy, Constametric 300) connected to a microflow splitter (SGE, UK) fitted with a fused silica restrictor (50µm I.D x 20cm) which reduced the flow to 40-60 $\mu l\ min^{-1}$. Peptides were eluted using a gradient consisting of water (pH 8.6) for 5 min followed by 20-70% acetonitrile + 5% (v/v) glacial acetic acid + 0.01% (v/v) TFA: water + 5% (v/v) glacial acetic acid + 0.01% TFA over 60 min. Three different electrospray conditions were employed for the formation of copper adducts; (i) spray voltage 2.5 kV, nebulizing gas 140 (arb units); (ii) spray voltage 4.2kV, nebulizing gas 100 (arb units); (iii) spray voltage 7.0 kV, nebulizing gas 20 (arb units).

Tandem mass spectrometry was carried out following microbore HPLC separation and post-column addition of copper *via* the T-piece. A multiple scan monitoring routine [25] was used to isolate the $[M-H+Cu(II)]^+$ ion (where M= peptide) of each

peptide in turn for collision activated dissociation (CAD). Electrospray ionization conditions were; spray voltage 7 kV and nebulizing gas, 20 (arb) units. CAD was performed with a 40% relative collision energy and an isolation width of 3 u.

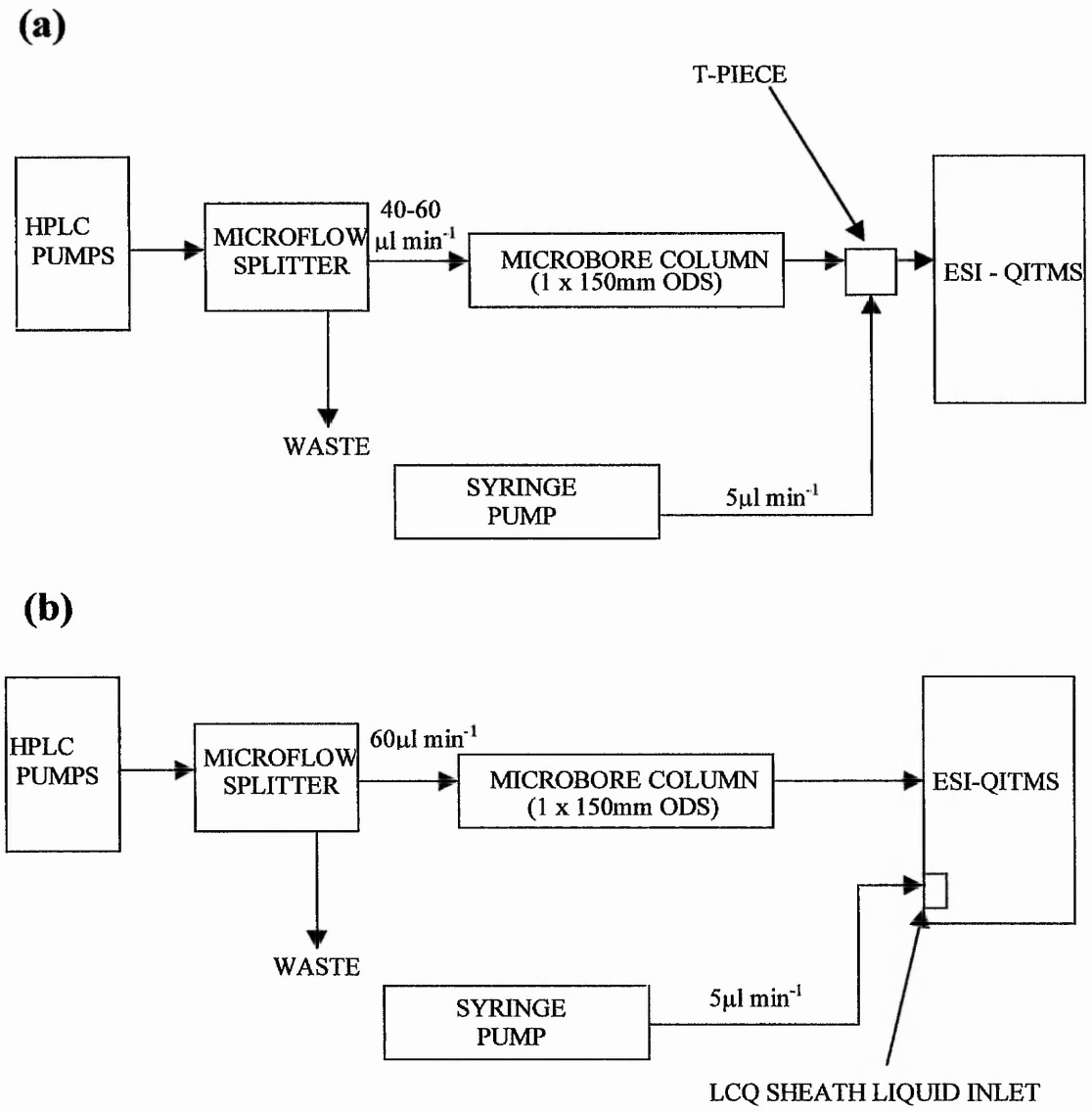


Figure 4.1: Schematic diagram of the experimental configuration for microbore LC/MS with of the post-column addition of copper (a) *via* a T-piece situated between the HPLC column and the ESI source and (b) *via* the ESI source sheath liquid inlet.

4.3 Results and Discussion

4.3.1 Nano-electrospray ionisation studies of copper/peptide complexation

The formation of copper/peptide complex ions was investigated initially for the murine MHC class I peptide gp70 using the nano-electrospray source. In the absence of copper a strong $[M+H]^+$ ion is observed under nano-electrospray conditions. Addition of a molar excess of copper (II) acetate solution (100 fold excess, pH 10.3) to the peptide solution in the nano-electrospray tip resulted in a decline in the intensity of the protonated peptide ion and a corresponding increase in the intensities of copper adduct ions over a period of approximately fifteen minutes as the solutions mixed (Figure 4.2). The principal copper adduct ions correspond to $[gp70\text{-}H+Cu(II)]^+$ (m/z 1188.5 and 1190.5) and $[gp70+Cu(II)]^{2+}$ (m/z 594.7 and 595.6) for the ^{63}Cu and ^{65}Cu isotopes (Figure 4.3). $[gp70+Cu(I)]^+$ and $[gp70+Cu(I)+H]^{2+}$ ions are also present at m/z 1189.5/1191.5 and m/z 595.1/596.0 as a result of reduction of copper (II) to copper (I) by electron capture during the nano-electrospray process. Reduction of Cu(II) to Cu(I) has also been reported by Lavanant *et al.* [10] in an ESI source and forms the basis of the biuret reaction for peptides and proteins.[18,21] The characteristic copper isotope pattern can be seen in detail in the high resolution scan (Figure 4.3 inset). The other peaks in the spectrum are assigned to alkali metal/peptide adduct ions.

The nano-electrospray spectra of a mixture of MHC class I and class II restricted peptides in the singly charged region (m/z 1000-1500) obtained before and after mixing of the peptides with a molar excess of copper (II) acetate solution, (100 fold, pH 10.3) is shown in Figure 4.4. The spectrum acquired at the start of the analysis, immediately after the peptide and copper solutions were loaded into the nano-

electrospray tip, shows $[M+H]^+$ ions for each of the peptides (Fig. 4.4(a)). A weak copper/gp70 adduction cluster is also present in this spectrum, which is formed as soon as copper ions come into contact with this peptide. Copper adduct ions are observed for all the peptides after complete mixing of the solutions in the nano-electrospray tip (Fig. 4.4 (b)) and the intensities of the singly charged copper adduct ions are significantly more intense than the corresponding protonated ions for the gp70, A3 and p53 peptides. Reducing the concentration of copper acetate solution from a 100 to a 20 fold molar excess at the same pH, still led to the formation of copper/peptide complex ions. However, under these conditions, the protonated/peptide ions dominate the spectrum with the copper adducts being relatively less intense.

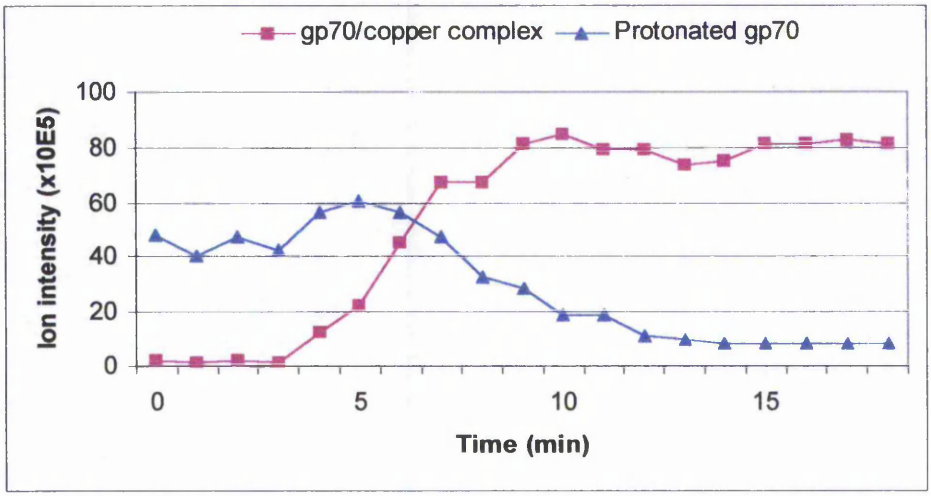


Figure 4.2: Variation of ion intensities with time for the protonated and copper adduct ions of gp70 peptide using nano-electrospray ionization.

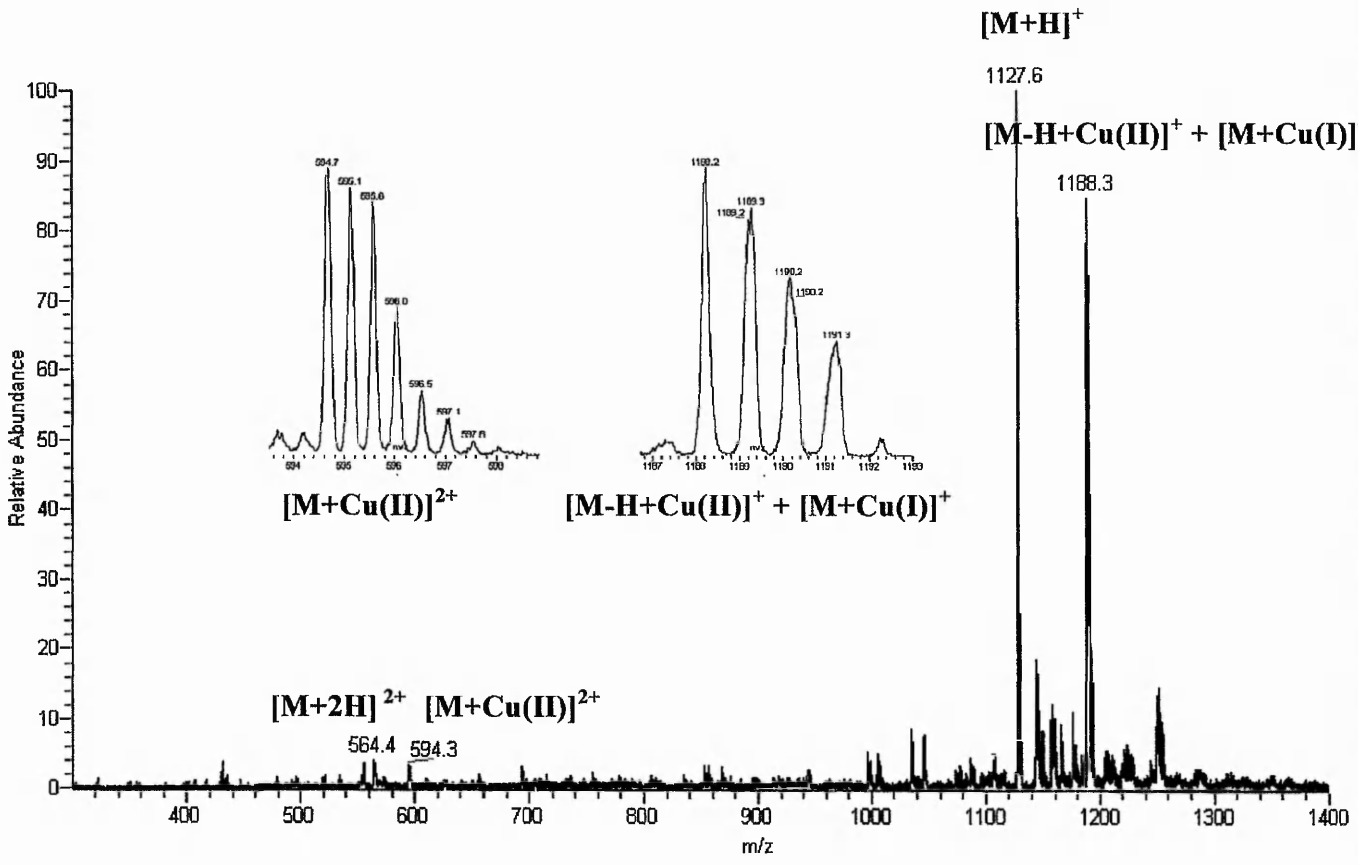


Figure 4:3 Nano-electrospray spectrum of the copper adducts of the murine MHC class I gp70 peptide.

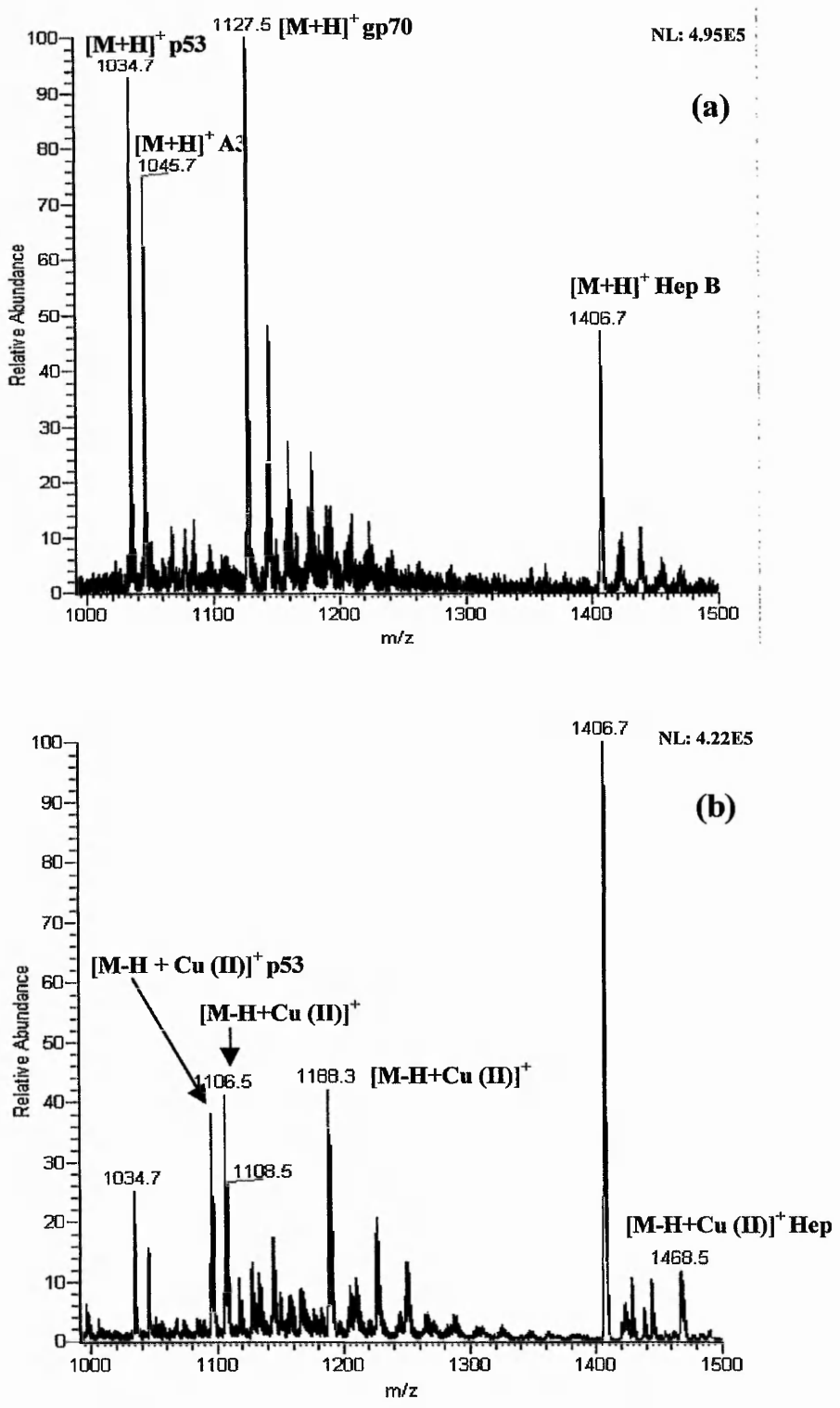


Figure 4.4: Nano-electrsopray mass spectra of an MHC class I and II peptide mixture (a) immediately following addition of copper acetate solution (pH 10.3, 100 fold molar excess) (b) after mixing of peptide and copper acetate solutions.

If the relative copper adduct/protonated peptide ion intensity ratios for the MHC peptides are taken as an indication of the copper ion affinity, gp70 appears to have the highest copper affinity followed by the A3 and p53 peptides. Gp70 peptide contains a histidine residue which is known to promote transition metal complexation,[20] whilst the complexation of the A3 and p53 peptides may be promoted by the significant number of basic residues present in these peptides. The MHC class II peptide Hep B was observed to undergo complexation, but to a much lesser extent than the other peptides. The poor association of the Hep B peptide with copper may be due to the unusually high number of proline residues present in this sequence, since peptides containing proline near the amine terminus are known to have a lower affinity for copper ions.[18] This is supported by the observation that the β-gal derived MHC class I peptide (TPHPARIGL), which contains a histidine residue located between two proline residues at the N-terminus, also shows weak copper ion affinity.

The effect of pH on the formation of copper/peptide complex ions was investigated by adjusting pH of the copper (II) acetate solution (20 fold molar excess) in the range pH 3.4 to 11.5. The spectra obtained at pH < 11 showed similar protonated ion/copper complex ion ratios and the most abundant copper/peptide ion intensities were observed on addition of copper acetate solution at pH 11.5. Under these conditions deprotonation of the C-terminus is facilitated and the copper peptide ion intensities were greater than the protonated species for all the peptides except Hep B, which reached a maximum relative intensity of 75%. Increasing the molar excess of copper (II) acetate solution at this pH resulted in suppression of all ion responses. Optimized conditions for the analysis of copper/peptide complexes were therefore

observed in the presence of a 20 fold molar excess of copper (II) acetate solution at pH 11.5.

Immobilized metal affinity chromatography was also previously investigated and the same results were seen on the column as in the gas phase. These columns may therefore be suitable for separating out histidine or sulphur containing proteins and polypeptides, it is not however, specific enough for peptides of smaller amino acid chain lengths such as those used in this study.

4.3.2 Copper complexation with a clinical cell eluate derived peptide

The ability to detect trace levels (< 1 nmol) of MHC peptides in microlitre sample volumes by nano-electrospray and to obtain ion shifts associated with copper complexation in the same experiment, without interrupting the run or switching off the HT, suggests that this technique has analytical potential for peptide characterization. This was investigated for a native peptide from a clinical cell eluate. Figure 4.5 shows the mass spectra obtained from the nano-electrospray analysis of a HPLC fraction containing the HLA-A3 restricted peptide $KQSSKALQR_{33-41}$ $[M+3H]^{3+}$, isolated from CML patient white blood cells (10^{12} cells) (a) before addition of copper acetate solution and (b) after addition of copper acetate. In Figure 4.5(b) an additional ion cluster is observed at m/z 390.7, corresponding to $[M+2Cu(II)-H]^{3+}$. These spectra show that copper complexation can be used on a cellular derived peptide even in the presence of a cellular matrix to help confirm the assignment of a peptide peak in the mass spectrum.

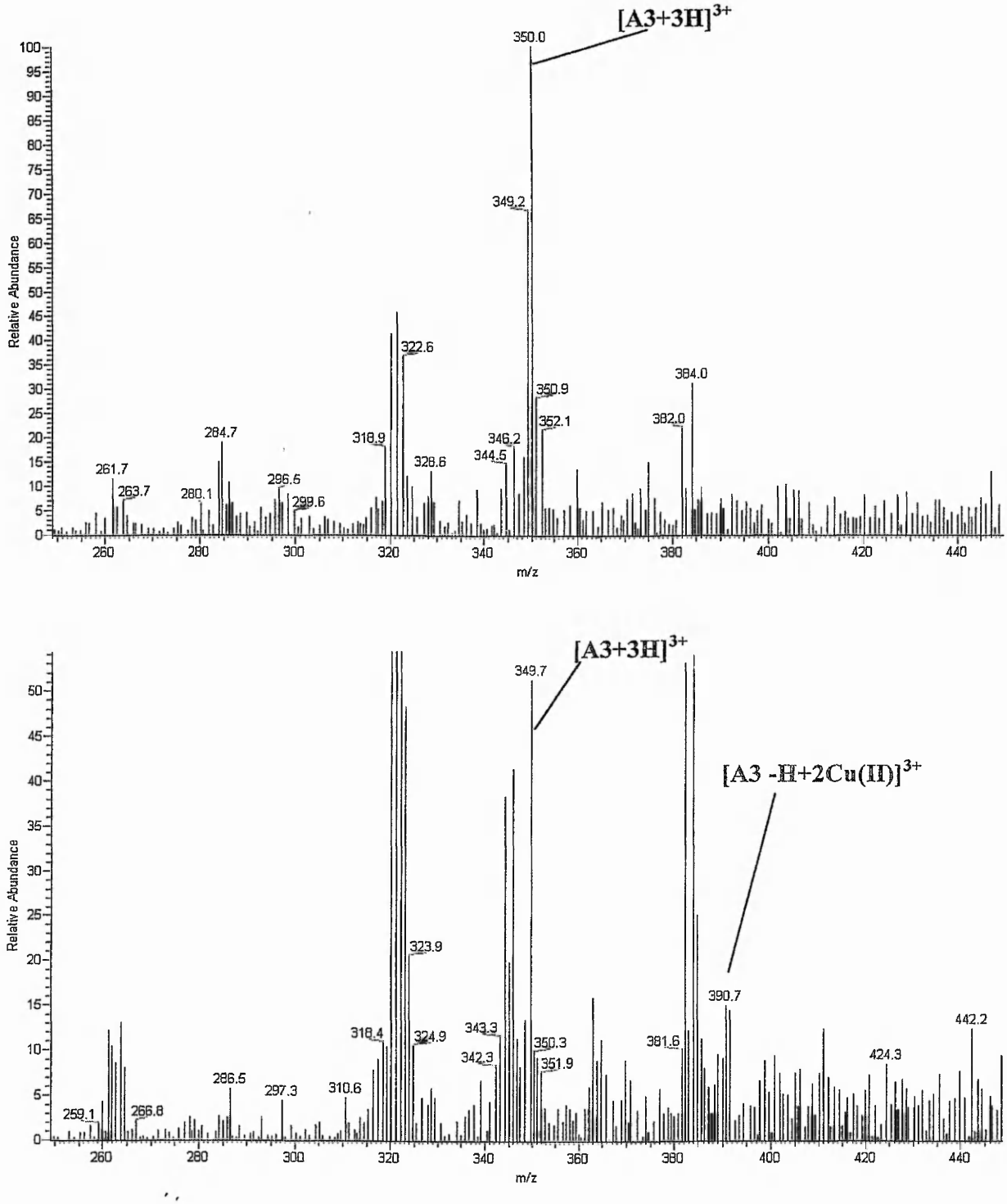


Figure 4.5: Mass spectra for the HLA-A3 restricted peptide KQSSKALQR$_{33-41}$ $[M+3H]^{3+}$ isolated from CML patient white blood cells (a) before the addition of copper and (b) after complexation with copper.

4.3.3 Nickel/Peptide complexation

Figure 4.6 shows the mass spectrum in the singly charged region for the Hep B, p53, and gp70 peptides in the presence of a 20 fold molar excess of nickel acetate. A peak is observed at m/z 1183.4, corresponding to the $[gp70\text{-}H+Ni(II)]^+$ ion, but the presence of weak peaks at m/z 1090.5, $[p53\text{-}H+Ni(II)]^+$, and m/z 1463.5, $[HepB\text{-}H+Ni(II)]^+$, ions, suggests that nickel does not bind strongly to these two peptides. Nickel is capable of forming a chelation complex with histidine containing peptides such as gp70 and this is supported by the presence of the stronger $[gp70\ –H+Ni(II)]^+$ ion, although the intensity of this ion is still weaker than the $[M+H]^+$ ion. Figure 4.7(a) shows a zoom scan of the $[gp70\text{-}H+Ni(II)]^+$ ion region confirming the presence of ^{58}Ni and ^{60}Ni isotopes in the singly charged region. Only Ni (II) is present in these spectra, the isotopic distribution of the peptide/nickel complex therefore differs from that observed for the copper complexes where both copper (I) and (II) isotopes were observed.

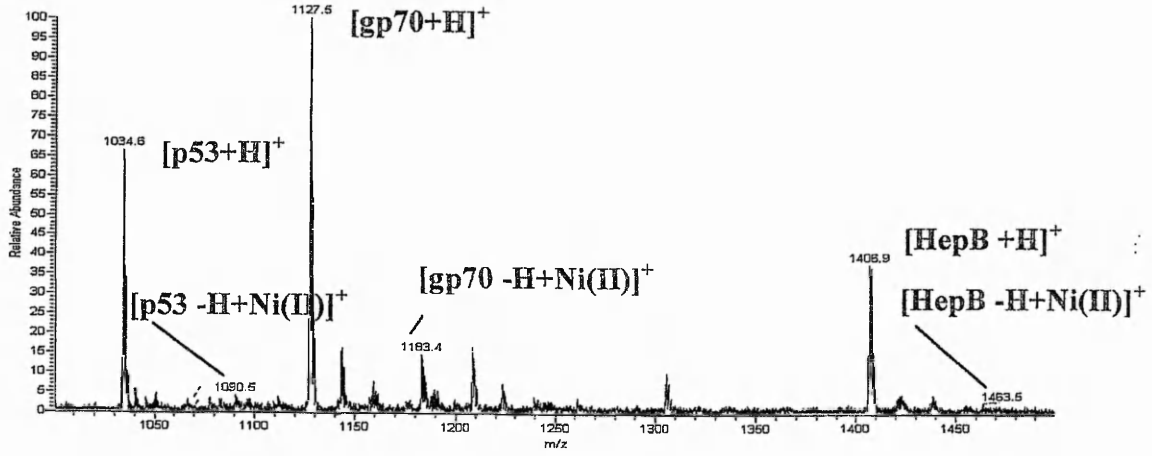
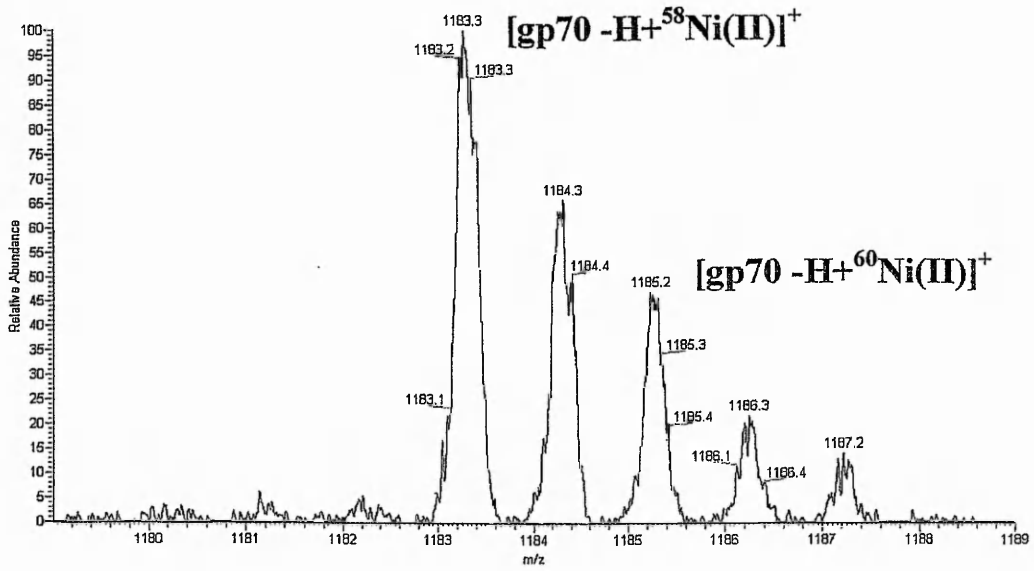


Figure 4.6: Mass spectrum of a mixture of MHC class I and class II peptides in the presence of a 20 x molar excess of nickel acetate.

(a)



(b)

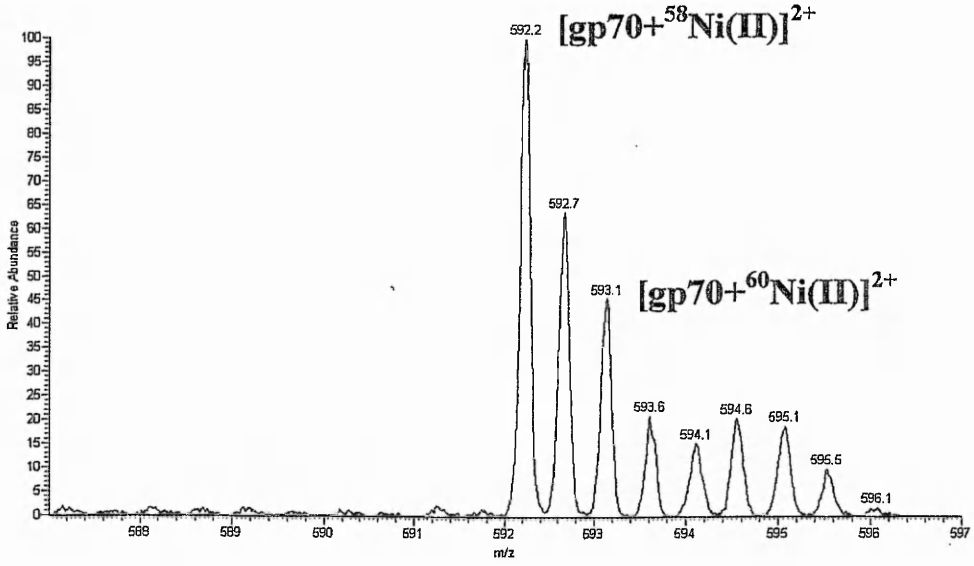


Figure 4.7: High resolution scan of $[gp70 -H+Ni(II)]^+$ (a) singly charged region and (b) doubly charged region.

4.3.4 Mixed metal complexation studies-copper/nickel.

The complexation of copper and nickel with the peptide gp70 was further studied by mixing equimolar amounts of nickel and copper (20 molar excess) with gp70 at different pH in the range 2.7-9.6. Figure 4.8 shows the mass spectrum resulting from conducting this experiment at pH 5.2. The $[gp70\text{-}H+Cu(II)]^{+}$ (m/z 1188.2) dominates the spectrum, and is representative of the results obtained throughout the pH range investigated. The corresponding nickel complexed ion is not detected above the background. Similarly, in the doubly charged region, $[gp70+Cu(II)]^{2+}$, (m/z 594.7) is formed, but not the nickel complex. It can therefore be concluded that the relative affinity of nickel is weaker than that of copper for this histidine containing peptide. In Figure 4.8, ions corresponding to $[gp70+Cu(II) +Ni(I)]^{2+}$, (m/z 622.8) and $[gp70\text{-}2H+Cu(II)+ Ni(II)]^{+}$, (m/z 1244.3) can also be seen indicating that it is possible for mixed copper/nickel complexes to be formed with the gp70 peptide. For this to occur, the nickel or copper is likely to be binding to the histidine residue as it has been shown that nickel in particular, forms only a weak complex with the non-histidine containing peptides. The higher affinity of the copper compared to nickel suggests that copper may bind preferentially to the histidine of the gp70 in these mixed metal complexes in order for both these complexation events to be occurring.

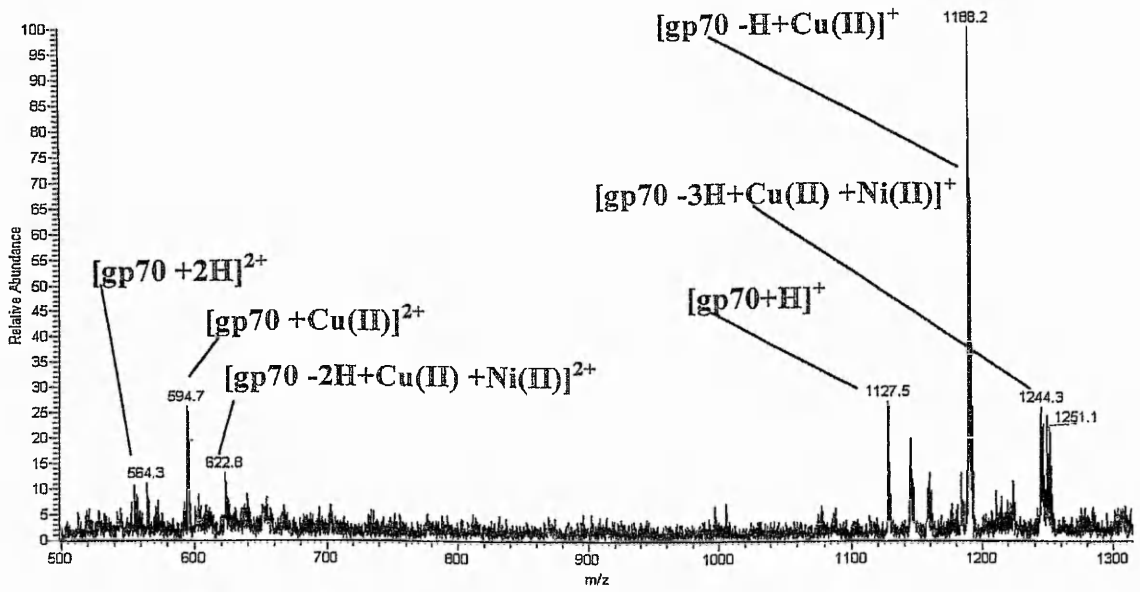


Figure 4.8: Complexation competition between gp70 in the presence of a 20 x molar equimolar solution of nickel acetate and copper acetate.

4.3.5 Silver/Peptide complexes

Silver is used commonly to stain polyacrylamide gels for the detection of peptides and proteins and the investigation was expanded to the nano-electrospray analysis of MHC peptides with silver salts. Silver(I):peptide complexes in the gas phase have previously been explored for methionine and non-methionine containing peptides (2-11 aa) and proteins using electrospray ionization.[23] Tandem mass spectrometric analyses have also been performed on these silver (I) peptide complexes yielding silver associated product ions.

Silver is known to form a strong bond with the sulphur containing amino acid methionine. However, none of the peptides used in this investigation contained this residue and so specific binding to the side chains of sulphur containing amino acids

was not expected with these particular MHC peptides. The results for the copper and nickel complexation experiments reported in this work showed that pH > 9 facilitated deprotonation at the C-terminal carboxylate group and N-terminal amine group of the peptide, increasing the affinity of metals for this terminus. Experiments were therefore carried out at higher pH values to ensure binding of the silver to the peptide. Figure 4.9(a) shows the spectrum of the p53 peptide complexed with silver (I) at pH 10. Adducts corresponding to $[p53+^{107/109}Ag]^+$, (m/z 1140.5/1142.5), $[p53-H+2^{107/109}Ag]^+$, (m/z 1246.3/1248.3) $[p53-2H+3^{107/109}Ag]^+$ and (m/z 1354.1/1356.1) were observed in the spectrum following addition of silver ions. Ions corresponding to the doubly charged adducts $[p53+H+Ag(I)]^{2+}$ and $[p53 +2Ag(I)]^{2+}$ were also present, but at low abundance. Figure 4.9(b) shows a high resolution scan of the $[p53+Ag(I)]^+$ region around m/z 1142.5. The isotopic peaks for the silver 107 and 109 are clearly detected. Figure 4.10 shows the full MS spectrum of the histidine containing peptide gp70 complexed with silver at pH 10. Ions corresponding to $[gp70+Ag(I)+H]^{2+}$, (m/z 616.3/618.3) ion, $[gp70+2Ag(I)]^{2+}$, (m/z 669.3/671.3) ion, $[gp70+Ag(I)]^+$, (m/z 1233.4/1235.4) ion and $[gp70-H+2Ag(I)]^+$, (m/z 1339.3/1341.3) for the ^{107}Ag and ^{109}Ag isotopes are observed. Comparing Figures 4.9 and 4.10 it can be seen that the $[M+Ag]^+$ ion intensity is similar to the $[M+H]^+$ intensity for gp70, whereas the sum of the intensities corresponding to all of the metallated ions for p53 is much weaker than the protonated ion. This suggests that silver binds more strongly to the gp70 peptide than p53 peptide, which may be due to the presence of the histidine residue in gp70.

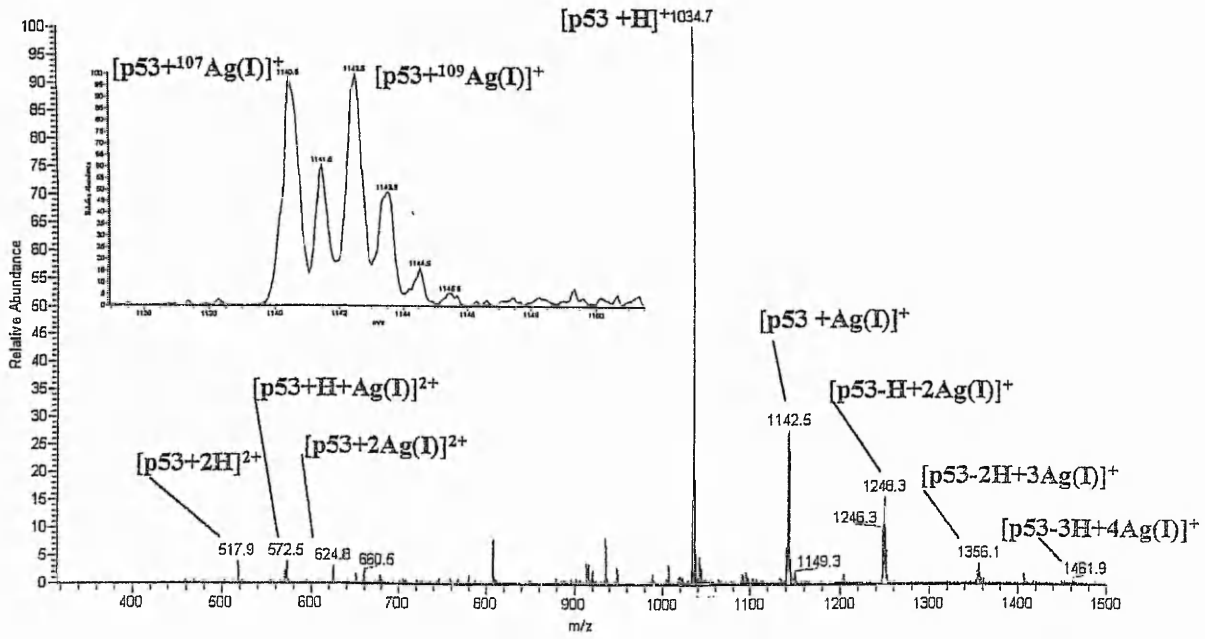


Figure 4.9: (a) Mass spectrum of the non-histidine containing peptide p53 complexed with silver and (b) a high resolution scan of the $[M+Ag(I)]^+$, m/z 1142.5.

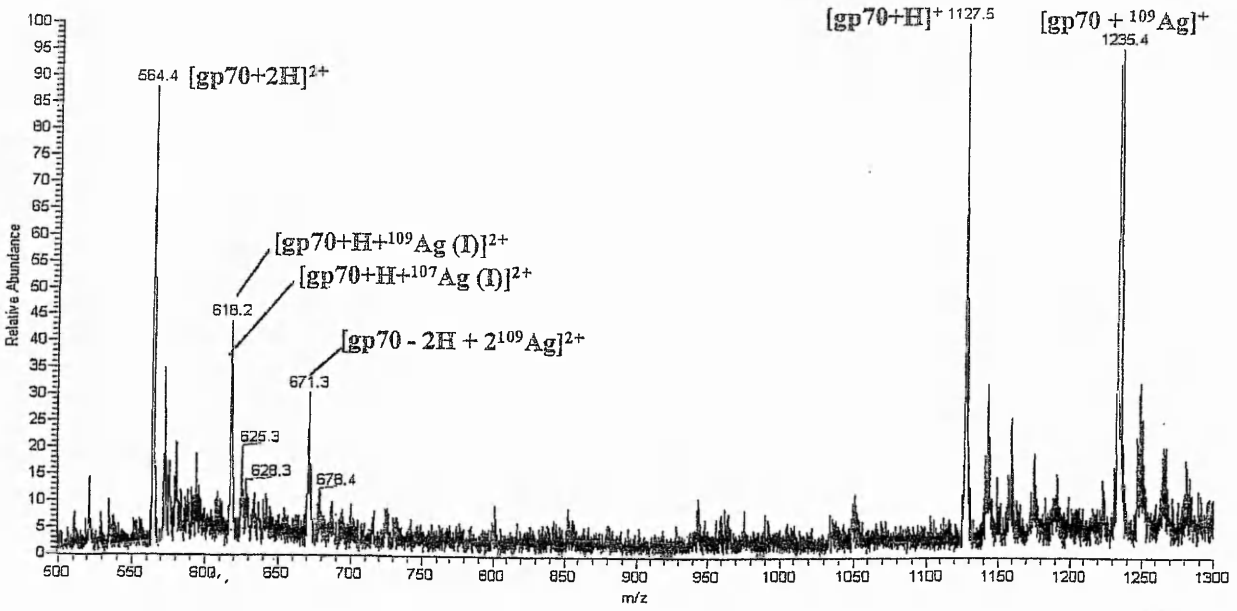


Figure 4.10: Mass spectrum of the histidine containing peptides gp70 complexed with silver.

Figure 4.11 shows the tandem mass spectrum for [p53 + ^{107}Ag(I)]$^+$, m/z 1140.5. A sequence of [b-H+ Ag(I)]$^+$ ions associated with the N-terminus of the peptide dominate the spectra. This suggests that the silver is binding predominately at the N-terminus of the p53 peptide.

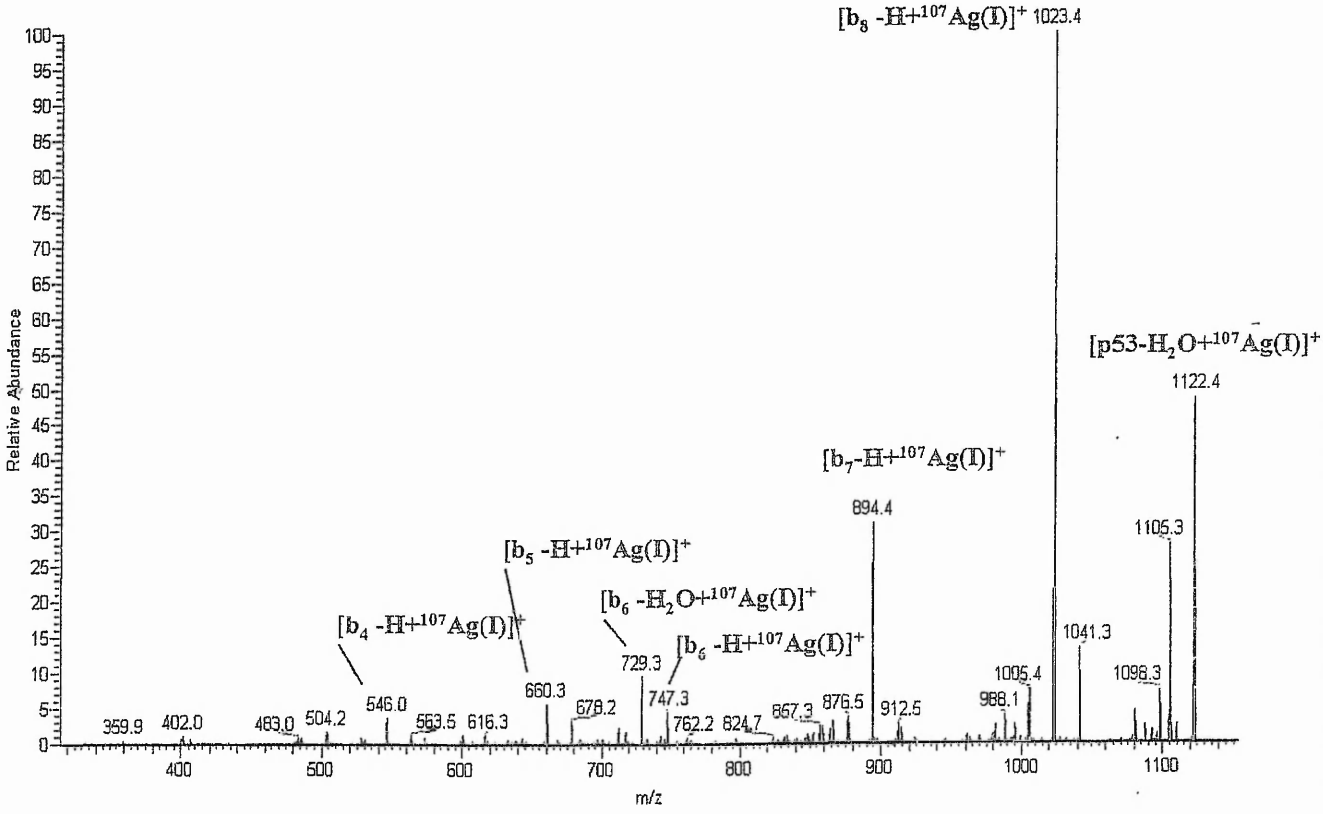


Figure 4.11: Tandem mass spectra for [p53 + ^{107}Ag]$^+$, (m/z 1140.5)

Figure 4.12 shows the tandem mass spectra for [gp70 + Ag(I)]$^+$, m/z 1233.5 ion. In contrast to the p53 peptide, silver containing product ions corresponding to both *b* and *y* fragment ions, ([b_n-H +Ag(I)]$^+$ and [y_n-H+Ag(I)]$^+$) are observed in the tandem mass spectrum. Two *a* type fragment ions were also present at m/z 775.2 and 912.3 corresponding to [a_6-H+Ag(I)]$^+$ and [a_7-H+Ag(I)]$^+$, which were not expected as these are not formed when CAD is performed on the [M+H]$^+$ of this peptide.

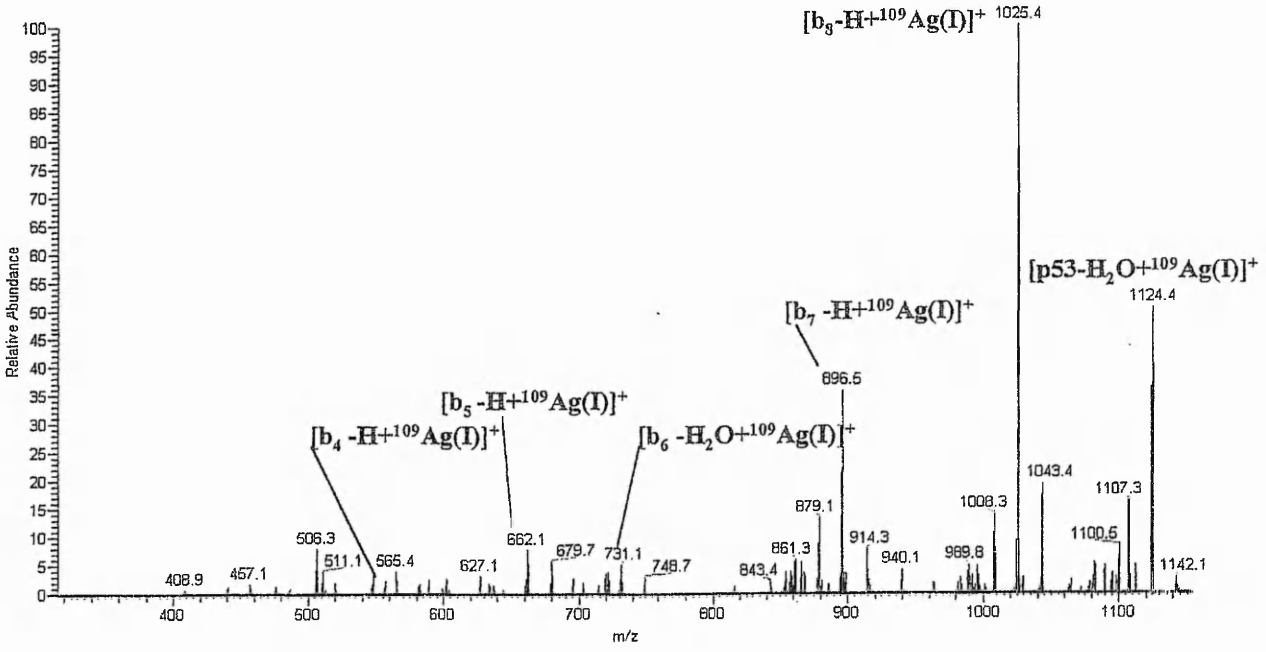


Figure 4.12: Tandem mass spectrum for [gp70 + ^{107}Ag]$^+$, (m/z 1233.5).

Figure 4.13 shows zoom scans for the b_7 –H+Ag(I) and y_6-H+Ag(I) fragment ions isolated during MS/MS of both $[gp70+Ag(I)]^+$ isotopes with an isolation width of 3u.

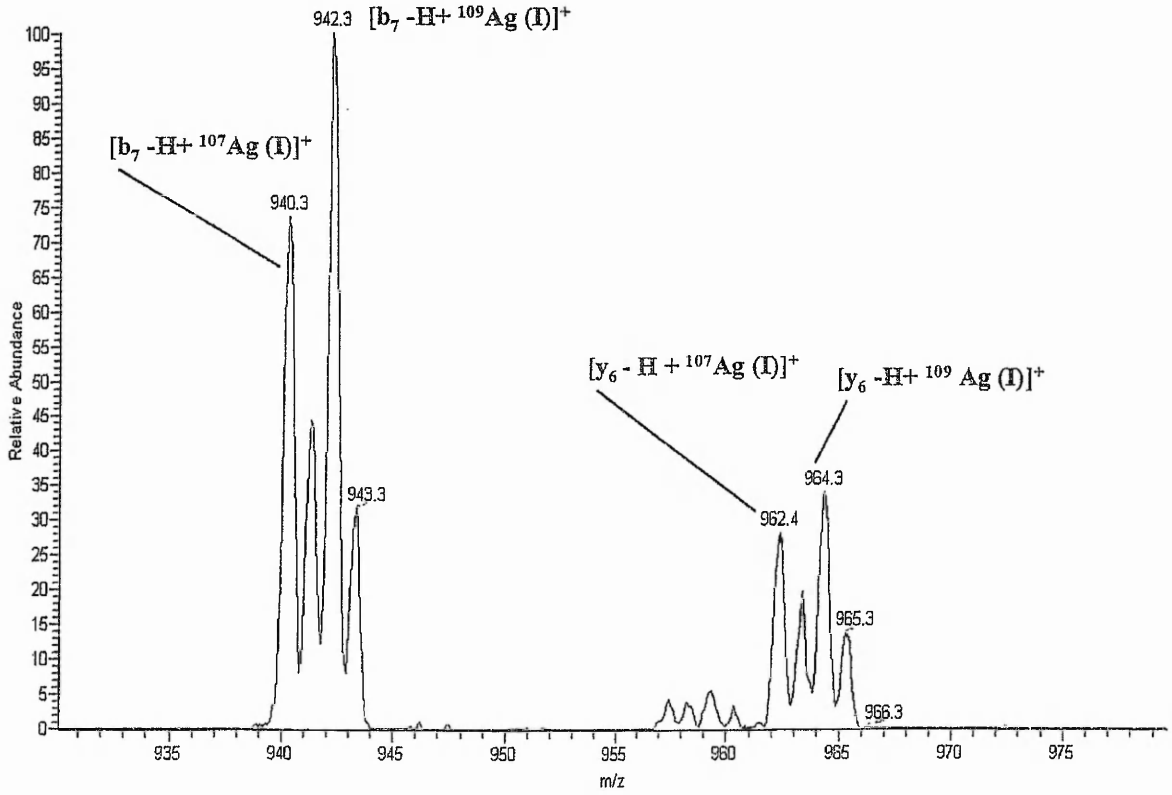


Figure 4.13: Zoom scans for the b_7-H+Ag(I) and y_6-H+Ag(I) ions isolated from MS/MS of both $[gp70 +Ag(I)]^+$ isotopes.

The tandem mass spectrometry data for the silver complexes of p53 and gp70 clearly demonstrates that the complexation influences the fragments observed. The tandem mass spectrometric results for silver complexation with the p53 peptide shows the tandem mass spectra is composed solely of *b* ion silver adducts, suggesting N-terminal binding. This is supported by cesium-ion bombardment studies of solid peptide samples deposited on silver suggesting that silver was likely to form a chelated bond with the N-terminal nitrogen and the carbonyl oxygen of the

second amino acid residue. [24] However, the tandem mass spectrum of the silver/gp70 peptide complexes shows metallated ions corresponding to both *b* and *y* series fragments, which suggests that metalation is not exclusive to the N-terminus. This may be due to binding with histidine or with the N and O atoms of the peptide backbone C-terminus, which is a strong binding site for nickel and copper as proposed by Li *et al.* [25]

4.3.6 Mixed metal complexation studies- copper/silver

An experiment was carried out to investigate the relative binding affinities of copper and silver to the MHC class I peptide, gp70. Equimolar concentrations of the two metal solutions (silver (I) sulphate and copper (II) acetate) were mixed in a 20 fold molar excess with the peptide gp70 and the mixed solution was loaded into a nanospray tip and analysed in full MS scan mode. Figure 4.14 shows the resulting MS spectrum in the singly charged region. The $[gp70–H+Cu(II)]^+$ ion, (m/z 1188.5) and $[gp70+Cu(I)]^+$ ion, (m/z 1189.3) dominate the spectrum, whereas only a very small adduct can be seen corresponding to $[gp70 + Ag(I)]^+$, (m/z 1235.3). This suggests that copper has a stronger affinity than silver for this histidine containing peptide, if electrospray ionization, ion introduction and detection efficiencies are the same for both metals. This is probably due to its ability to form a strong chelating complex with the histidine residue at amino acid position 7 of this peptide. A cluster of ions centered at m/z 1296.1 corresponding to $[gp70–2H+Cu(II) +Ag(I)]^+$ was also detected, demonstrating that it is possible for both Cu(II/I) and Ag(I) to bind to the same peptide.

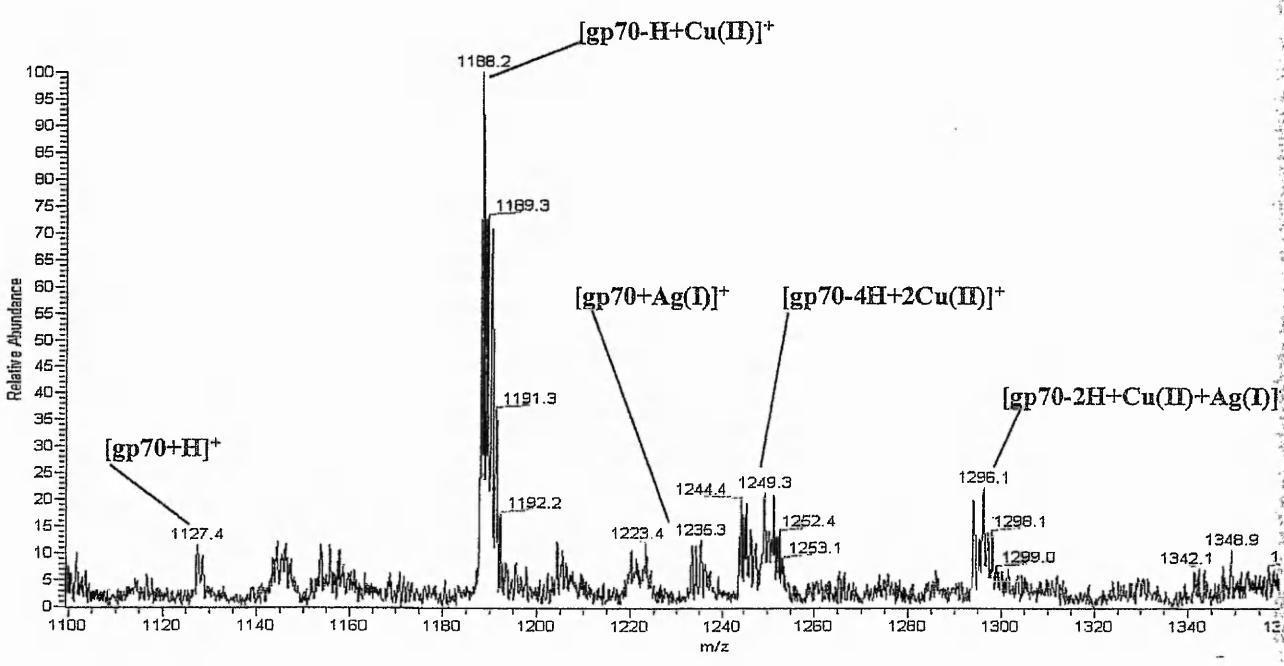


Figure 4.14: Full scan MS spectrum of gp70 in the presence of a 20 x molar excess solution of silver sulphate and copper acetate.

For the non-histidine containing peptide p53, silver and copper, when present in equimolar concentrations, are shown to bind to the peptide with similar affinity. Figure 4.15 shows the full scan spectrum in the singly charged region containing ions corresponding to $[p53 - H+Cu(II)]^+$, (m/z 1095.3), [p53+Ag(I)], (m/z 1142.5), $[p53 - 2H+Cu(II) + Ag(I)]^+$, (m/z 1203.2) and also $[p53-H+2Ag(I)]^+$, (m/z 1248.3). In the absence of a histidine residue, the interaction of both copper and silver with the p53 peptide must be non-specific, with silver showing a strong affinity for the

N-terminus group of this peptide. The ion at m/z 1203.2, which corresponds to [p53 –2H+Cu (II) +Ag(I)]$^+$, indicates that both silver and copper can also bind to this peptide at the same time at different binding regions. These two sets of results indicate that copper has a higher binding affinity for histidine than silver when this residue is present in the peptide. However, without the presence of this amino acid, the non-specific interactions appear to be of approximately equal affinity.

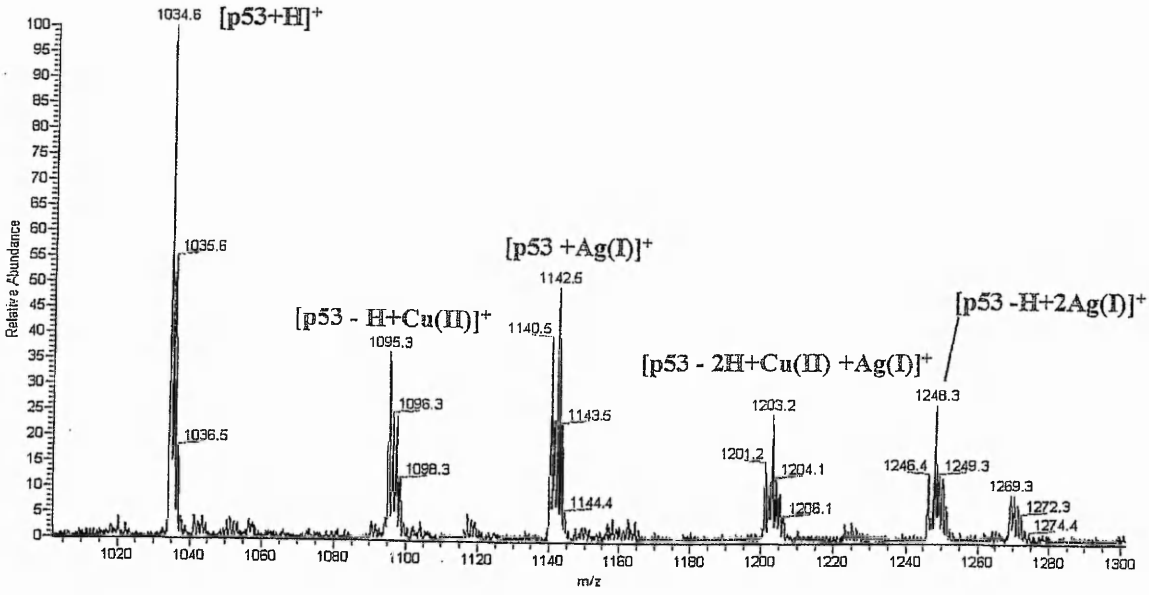


Figure 4.15: MS spectrum of p53 in the presence of a 20 x molar excess of silver sulphate: copper acetate.

These copper/silver p53 peptide complexes were also analysed using FT-ICR mass spectrometry at higher resolution (18,000). Figure 4.16 shows the FT-ICR high resolution mass spectrum for $[p53 -2H +Ag(I) + Cu(II)]^{2+}$ in the region m/z 1200-1206. The isotopic distribution was clearly resolved at this resolution. The same isotopic distribution for this metallated p53 peptide observed in full scan MS mode on the LCQ are observed in Figure 4.17. The predicted isotopic distributions (http://www.sisweb.com/cgi-bin/masses) correspond to those observed in our mass spectral zoom scans obtained on the ion trap mass spectrometer and the high resolution data obtained on the FT-ICR (Figures 4.18 and 4.19).

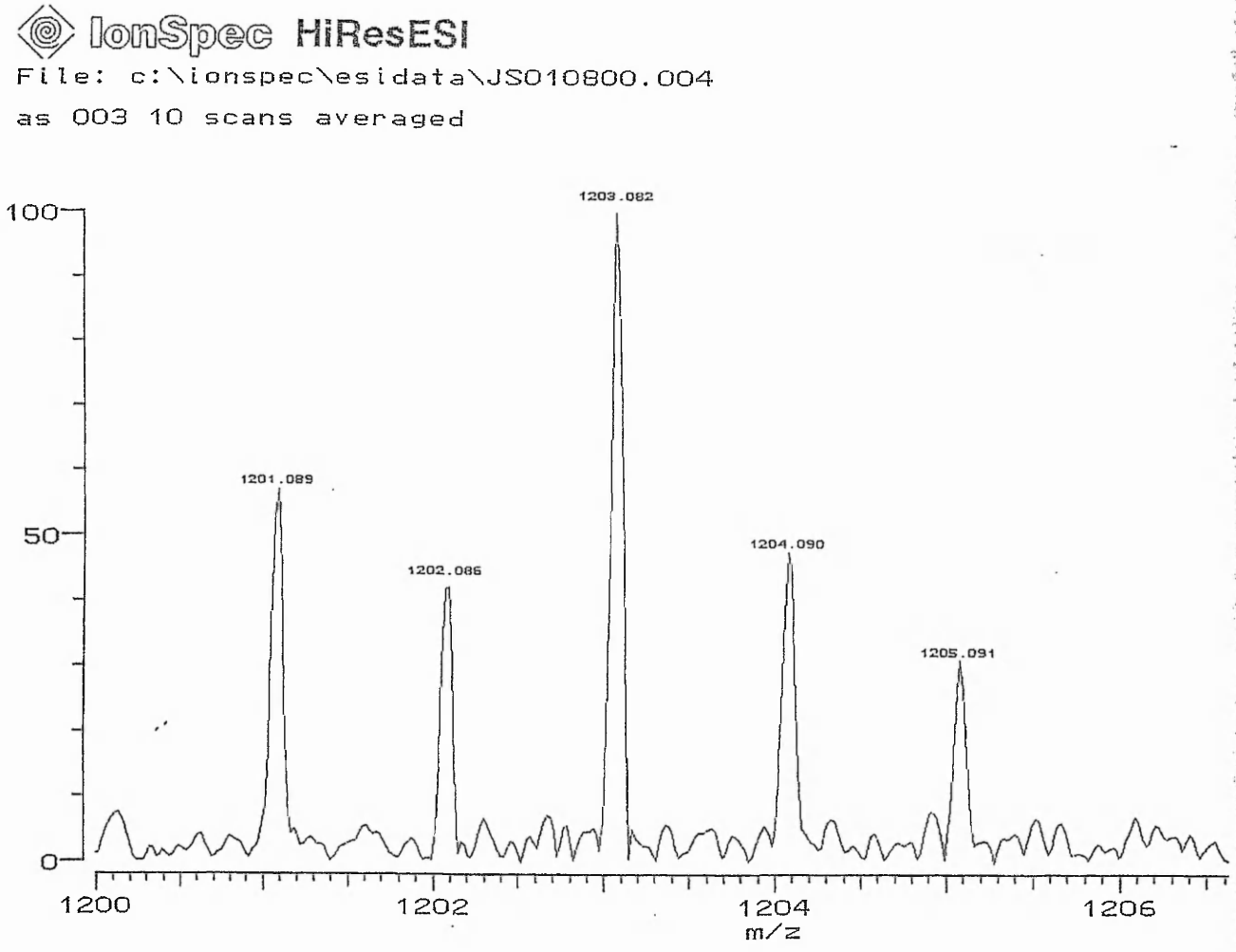


Figure 4.16: FT-ICR-MS spectrum for $[p53 -2H +Cu(II) + Ag(I)]^{+}$

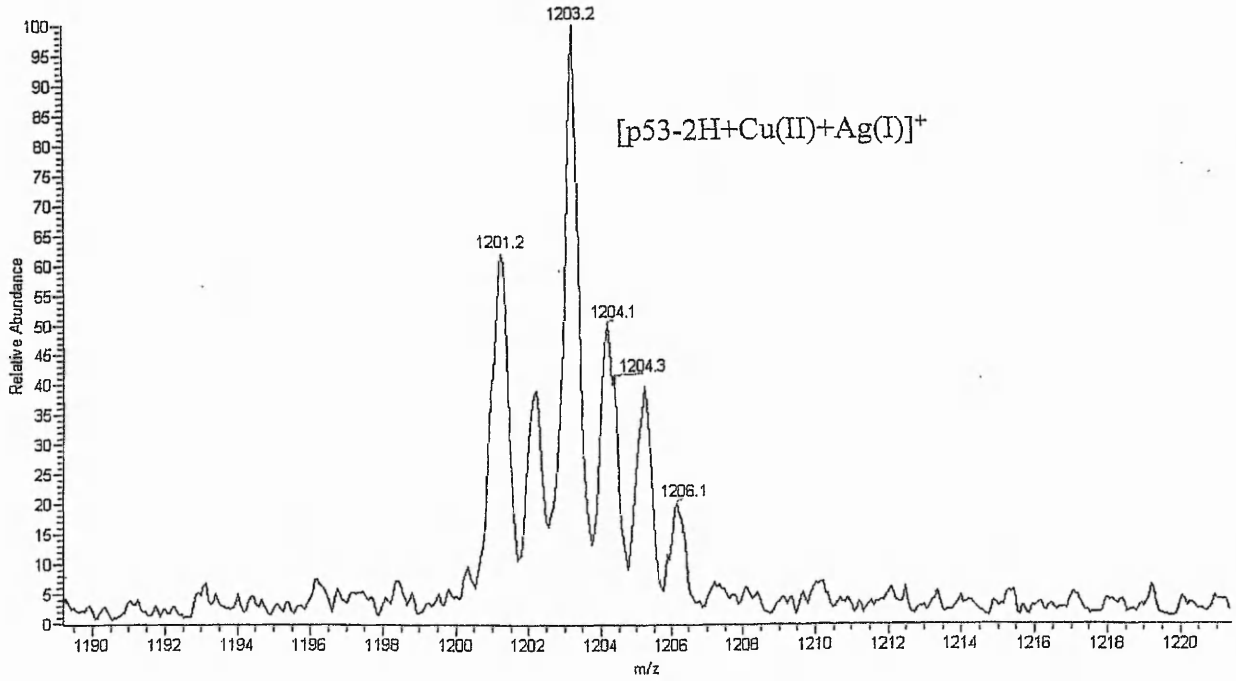


Figure 17: Full scan MS spectrum isotopic distribution of [p53 –2H +Cu(II) + Ag(I)]$^{+}$ using the LCQ ion trap mass spectrometer.

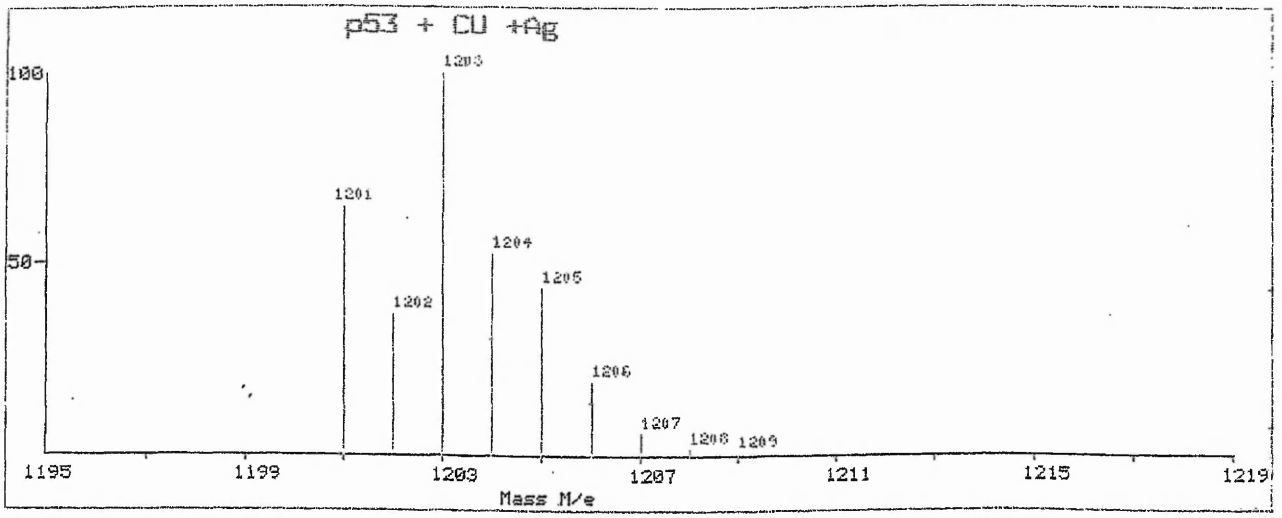


Figure 4.18: Predicted isotopic distribution of [p53 –2H +Cu(II) + Ag(I)]$^{+}$ using the Internet based program.

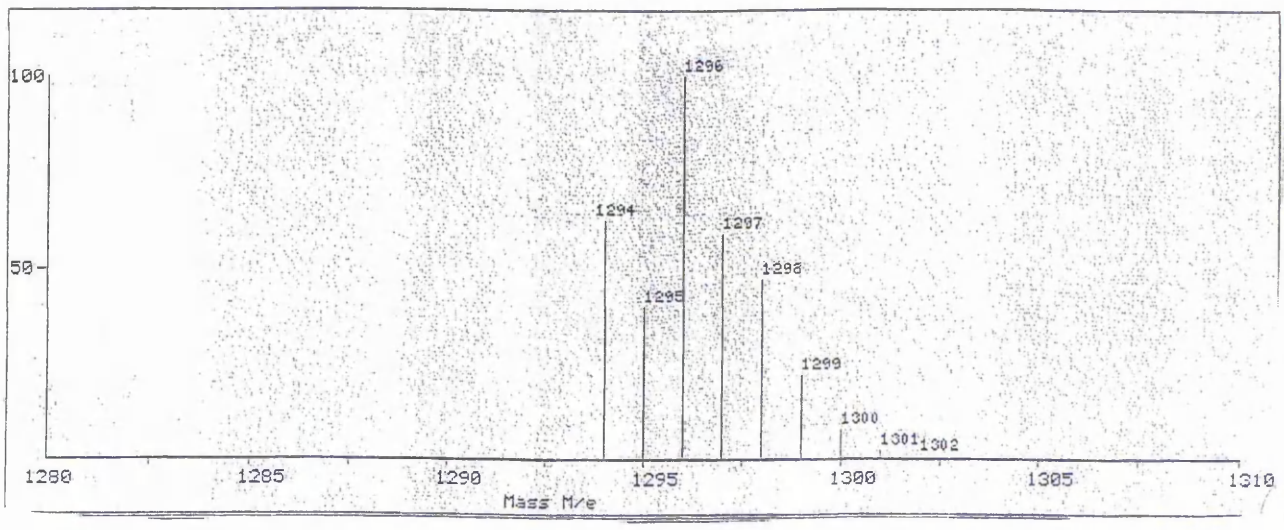


Figure 4.19: Predicted isotopic distribution of [gp70 –2H +Cu(II) + Ag(I)]$^{+}$ using the Internet based program.

4.3.7 Microbore LC/MS with post-column metal complexation

On-line copper complexation in microbore LC/MS was explored using post-column addition of copper acetate. The configuration shown in Figure 4.1(a) employed a zero-dead volume T-piece located between the microbore column and the ESI source. In the alternative configuration (Figure 4.1(b)) post-column addition of copper was achieved by introduction of the copper solution *via* the ESI source sheath liquid inlet. The single ion chromatograms obtained for the LC/MS analysis of the MHC class I and II restricted peptides with post-column addition of copper acetate solution (70 fold molar excess, pH 9 post-mixing) using the T-piece configuration are shown in Fig. 4.20.

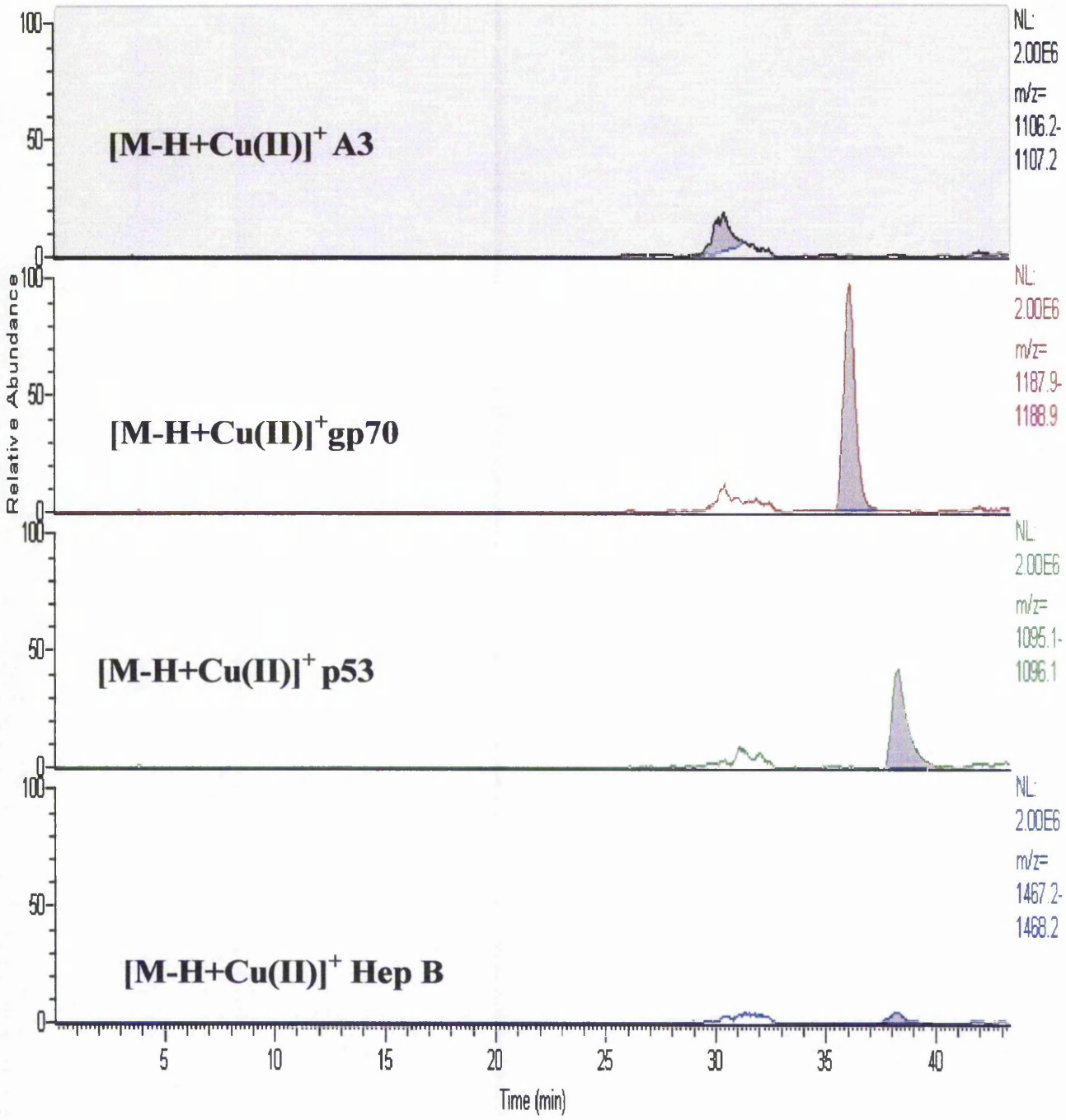


Figure 4.20: Single ion chromatograms of MHC class I and II peptides separated by microbore LC/MS with post column addition of copper acetate *via* a T-piece, (70 fold molar excess, pH 9).

The LC/MS results correlate well with those obtained using nano-electrospray, with copper adducts being observed for all the MHC associated peptides. As with the nano-electrospray data, the relative ion intensities indicate that gp70 peptide has the highest copper ion affinity and a much weaker response is observed for the Hep B peptide/copper complex. The copper (II) acetate solution concentration used in the LC-MS experiments (32mM) was the highest that could be used for post-column addition, since higher concentrations resulted in blockage of the heated capillary. The relative intensities of the protonated versus the copper adduct ions for the gp70 peptide (copper was added via the T-piece) under different ESI source conditions are shown in Figure 4.21. For post-column addition of copper *via* the T-piece, an increase in spray voltage and a decrease in nebulization gas flow promoted the formation of copper adducts (Figure 4.21 (a)).

Copper/peptide ions were also observed for the gp70, A3 and p53 derived peptides when copper solution was added *via* the sheath liquid inlet, but complexation was not observed for the Hep B peptide under these conditions. Figure 4.22 shows the spectra obtained under various ESI source conditions for the gp70 peptide introduced *via* the sheath liquid inlet. In this configuration, the formation of copper containing ions requires efficient mixing of the HPLC eluant and the liquid sheath flow. The nebulization gas flow is a critical parameter in this mixing process and metal/peptide complex ion formation is favoured by a high nebulization gas flow and low spray voltage (Figure. 4.22(a)). This is in contrast to the case for the post-column addition of copper *via* the T-piece, where the copper solution has an extended mixing time with the HPLC eluant and nebulization gases are therefore not required to promote the mixing process, but high electrospray voltages favour the

overall ionization event. In the extreme case, when a copper/peptide solution was introduced into the ESI source through the sheath liquid inlet in the absence of an applied spray voltage, complex ions could still be detected if a sufficiently high gas flow was applied in combination with a low sample flow rate (<10 μl min^{-1}). The detection of copper/peptide ions in the absence of an ESI voltage, but in the presence of a nebulizing gas, has also been reported by Bayer *et al.* [26] However, at the higher flow rates used for LC-MS with post-column addition used in this work, it was not possible to detect copper adduct ions without applying a minimum voltage of 2.5 kV and nebulizing gas was necessary for electrospray ion formation even at the higher needle voltages.

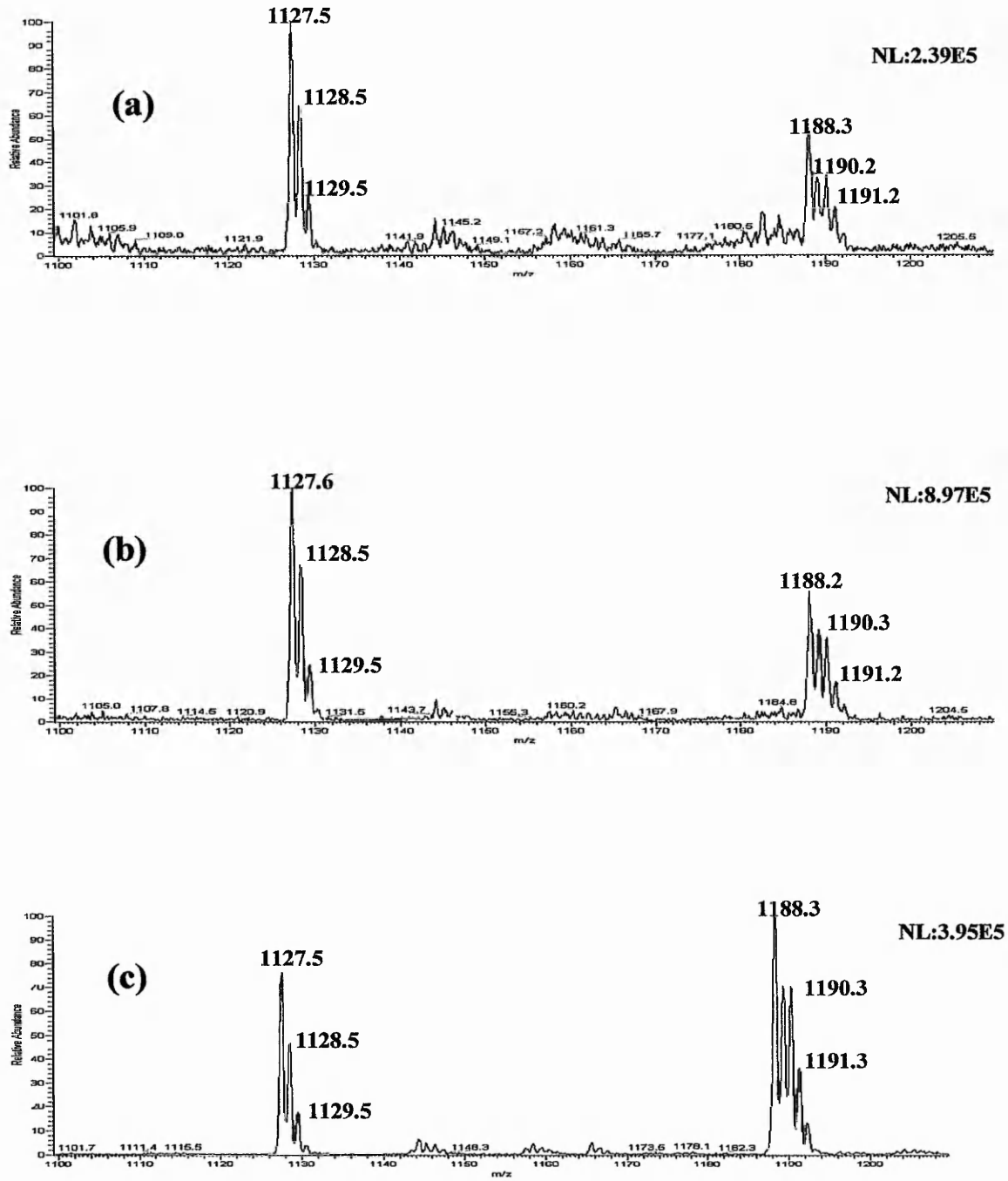


Figure 4.21: LC/MS spectra of gp70 peptide with post addition of copper acetate *via* a T-piece; (a) spray voltage 2.5 kV, sheath gas 80 (arb) and auxiliary gas 60 (arb), (b) spray voltage 4.2 kV, sheath gas 80 (arb) and auxiliary gas 20 (arb) and (c) spray voltage 7 kV, sheath gas 20 (arb) and auxiliary gas 0 (arb).

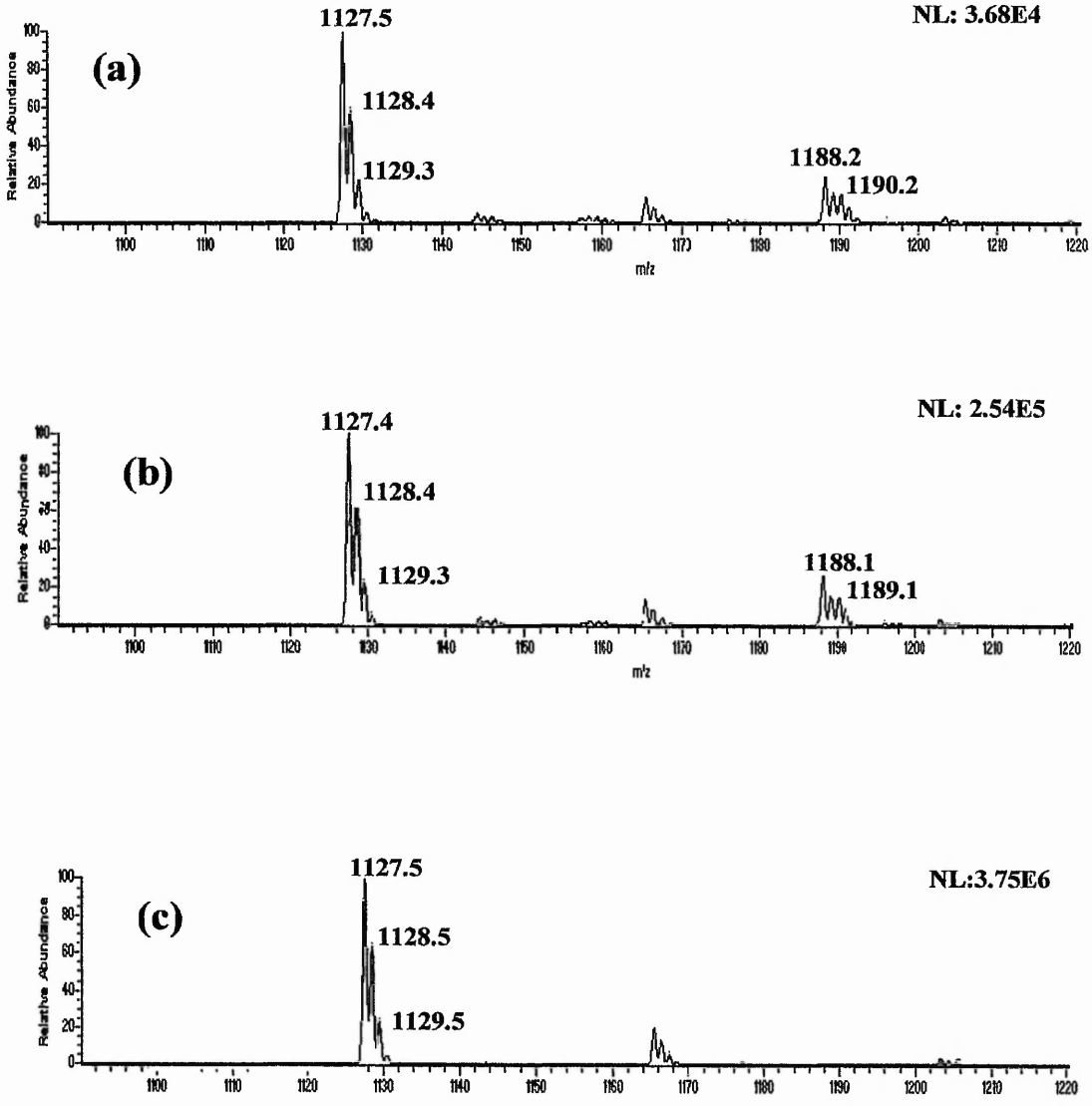


Figure 4.22: LC/MS spectra of the gp70 peptide with post addition of copper acetate *via* the LCQ sheath liquid inlet; (a) spray voltage 2.5 kV, sheath gas 80 (arb) and auxiliary gas 60 (arb), (b) spray voltage 4.2 kV, sheath gas 80 (arb) and auxiliary gas 20 (arb) and (c) spray voltage 7 kV, sheath gas 20 (arb) and auxiliary gas 0 (arb).

Tandem mass spectrometric analysis of copper/peptide complexes yields evidence about the location of copper complexation on the peptide.[7, 11-14] The use of tandem mass spectrometry to generate peptide sequence information for the copper/peptide complex ions was therefore investigated in combination with microbore LC/ESI-MS of the MHC associated peptides using post-column complexation. Figure 4.23 shows the tandem mass spectrometric data for the gp70/copper ion $[M-H+Cu\ (II)]^+$ at m/z 1188.5 (isolation width 3u). The product ion spectrum is dominated by copper adducts of the characteristic *a, b* and *y* peptide fragment ions, suggesting that the copper ion is associated predominately with the histidine residue of gp70 rather than to the C-terminus. If binding were only due to interactions at the carboxy-terminus, the tandem mass spectrum would be expected to consist of predominately copper adducts of *y* fragment ions. The product ion of spectra for the three non-histidine containing peptides supports this observation since these showed mainly copper adducts of *y* fragment ions, together with some non-complexed *b* and *y* fragment ions, suggesting C-terminal binding.

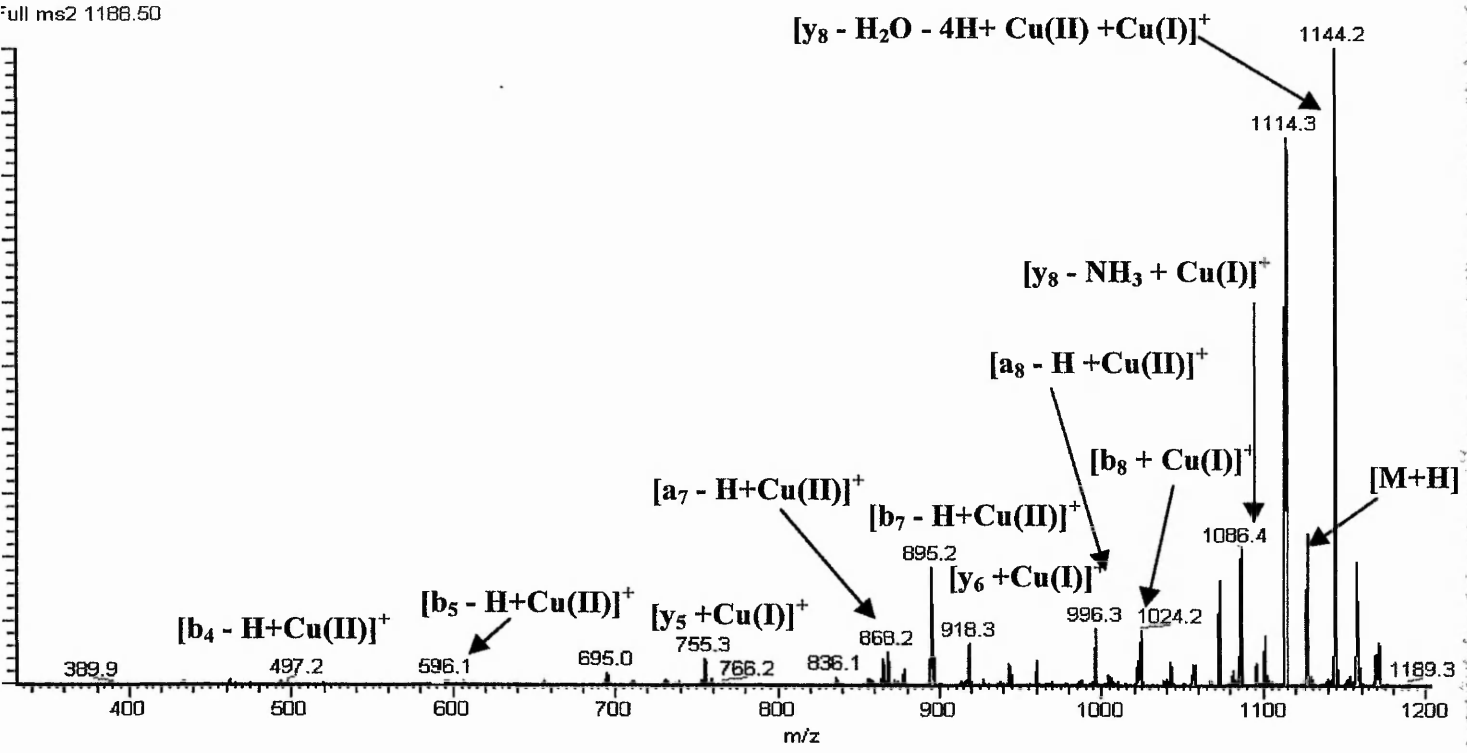


Figure 4.23: LC/MS/MS product ion spectrum of the $[M-H+Cu(II)]^+$ ion of gp70 peptide (m/z 1188.5) with post-column addition of copper acetate (spray voltage 7kV, sheath gas 20 (arb), no auxiliary gas).

4.3 Conclusion

Copper complexation with MHC class I and class II associated peptides has been demonstrated using nano-electrospray ionization and microbore LC/electrospray mass spectrometry with post-column addition of copper. Post-column mixing using a T-piece was found to be much less susceptible to changes in ESI source conditions than mixing *via* the sheath liquid inlet of the ESI source. Tandem mass spectrometry of Cu/peptide ions indicates that binding occurs preferentially at histidine residues or non-selectively at the C-terminus in the absence of a histidine residue. Post-column addition of copper in LC/MS and the use of nano-electrospray ionization in the mass spectrometric analysis of peptides suggests that copper complexation may be a useful analytical tool for the characterization and sequencing of peptides particularly those containing a histidine residue. Nickel has also been shown to bind to peptides, but with a much weaker affinity than for that for the copper: peptide complexes. Silver has been used in the identification of proteinatious material on polyacrylamide gels for many years and complexation was also observed using nano-electrospray ionisation. Tandem mass spectrometry was used to determine the sites of metallation.

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PUBLICATIONS, PRESENTATIONS & AWARDS

- Characterisation of MHC class I bound tumour antigen peptides using RP-HPLC and electrospray ionisation quadrupole ion trap mass spectrometry. C S Creaser, J R Lill, J W Stygall, R C Rees, S C Hill & P L R Bonner. Presented at the 35th Royal Society of Chemistry Annual congress 1998.

- Identification of MHC class I bound tumour peptides using HPLC and a quadrupole ion trap mass spectrometer: a comparison of nanospray (NSI) and electrospray (ESI). C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Proceedings of the 23rd Annual meting of the British Mass Spectrometry Society, 1998.

- Procedures for the characterisation of cell surface MHC class I bound tumour antigens. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Presented at the 36th Royal Society of Chemistry Annual congress 1999.

- Characterisation of cell surface MHC class I bound tumour antigens. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Presented at SAC 1999.

- Metal complexation studies of MHC associated peptides by electrospray ion trap mass spectrometry. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Proceedings of the 24th Annual meeting of the British Mass Spectrometry Society, 1999.

  Awarded the Michael Barber prize for best presentation at the 24th Annual meeting of the British Mass Spectrometry Society.

- Nano-electrospray and microbore liquid chromatography ion trap mass spectrometry studies of copper complexation with MHC restricted peptides. C S Creaser, **J R Lill,** P L R Bonner, S C Hill & R C Rees. ***Analyst,* 125, 599-604 (2000).**

- Copper complexation with MHC restricted peptides using microbore liquid chromatography/ion trap mass spectrometry. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Presented at the 37th Royal Society of Chemistry Annual congress 2000.

- Identification of MHC class I derived peptides from *in vivo* malignant material and transfected cell lines using nano-electrospray quadrupole ion trap mass spectrometry. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Proceedings of the 48th American Society of Mass Spectrometry Annual Conference, 2000.

  Awarded the ProteoMetrics Student Travel Award at the 48th American Society for Mass Spectrometry Annual Conference 2000.

- Characterisation of MHC class I restricted peptides from transfected cell lines and malignant cells using nano-electrospray ion trap mass spectrometry. C S Creaser, J R Lill, P L R Bonner, S C Hill & R C Rees. Presented at the International Mass Spectrometry Conference, Barcelona, 2000.

- Direct evidence that leukaemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. Richard E Clark[1*], I Anthony Dodi[2*], Seran C Hill[3*], **Jennie R Lill[4*]**, Geraldine Aubert[2], Andrew R Macintyre[1], Jose Rojas[3], Audrey Bourdon[2], Philip LR Bonner[3], Lihui Wang[1], Stephen Christmas[5], Paul J Travers[2], Colin S Creaser[4], Robert C Rees[3], J Alejandro Madrigal[2]. Accepted for publication *Blood* (2001).