The isolation and characterisation of MHC-presented peptides from CML-derived cell-lines, with a focus on post-translational modification.

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Declaration of intellectual rights

Abstract

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Phosphorylation is a key regulator of protein function and activity, and aberrant kinase activity is implicated in a wide range of malignancies, of which the bcr:abl fusion kinase found in chronic myeloid leukaemia is a classic example. As phosphopeptides are known to be presented by both the MHC class-I and class-II pathways, against which specific $CD4^+$ and $CD8^+$ T cell responses may be generated, study of MHC-presented phosphopeptides may reveal unique cancer antigens with direct links to the neoplastic state.

Mild acid cell-surface elution is a rapid and effective method for MHC class-I peptide capture, though complicated by contamination with non-MHC peptides and poor downstream compatibility, especially with IMAC, a popular method for phosphopeptide enrichment. As an alternative to the citrate-phosphate elution buffer, a TMA-formate elution buffer is proposed. This was developed for IMAC compatibility, and osmotically balanced and supplemented to minimise cell lysis, (assessed by several assays) and used with a pH 5.5 prewash to reduce non-MHC peptide contamination. MALDI-MS/MS of MHC class-I peptides from K562-A3 cells found a sequence with high homology to a known cancer antigen as the common peak for both citrate-phosphate and TMA-formate eluted cells.

Currently there are no published mechanisms for cell-surface elution of MHC class-II peptides (immunoprecipitation is widely used), though previous work at NTU led to the development of an IMAC compatible MHC class-II protocol. This was also subjected to supplementation and optimisation, reducing cell death to a level corresponding to that of the widely accepted citrate-phosphate class-I protocol.

Various chromatographic approaches were tested for phosphopeptide retention. Fe^{3+} IMAC remains optimal; methods were adjusted to increase peak fraction concentration (assessed by a modification the BCA protein assay improving suitability for peptides). Though further method development may be required to optimise mass spectrometry, a number of phosphopeptides were found in both MHC class-I and class-II eluates, many with known links to malignancy. It is hoped that these improved methods will be of use in the ongoing search for novel cancer antigens.

Thankyou.

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List of abbreviations

In addition to SI units, the following abbreviations have been used

1D	1-dimensional (in chromatography)
2D	2-dimensional (in chromatography)
ACN	Acetonitrile
ADP	Adenosine diphosphate
ADR	ATP detection reagent
AK	Adenylate kinase
AKDR	Adenylate kinase detection reagent
ALCL	Anaplastic large cell lymphoma
ALL	Acute lymphocytic leukaemia
AML	Acute myelogenous leukaemia
AMOL	Acute monocyclic leukaemia
APC	Antigen presenting cell
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLAST	Best linear alignment search tool
BSA	Bovine serum albumin
B_{reg}	B-regulatory cell
β2M	β-2 microglobulin
$CD4^+$	CD 4-positive (T cell)
$CD8^+$	CD 8-positive (T cell)
CHCA	α-cyano-4-hydroxycinnamic acid
CIITA	(MHC) class-II transactivator
CLIP	Class-II invariant chain peptide
CLL	Chronic lymphocytic leukaemia
CLP	Commited lymphoid progenitor (cell)
CMA	Chaperone-mediated autophagy
CM-Asp	Carboxymethylated aspartic acid
CML	Chronic myelogenous leukaemia
CMP	Commited myeloid progenitor (cell)
CREB	Cyclic AMP response element binding protein
CSC	Cancer stem cell
C-T	Cancer testis antigen
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell

ddH_2O	Double-distilled water (also ultrapure filtered)[1]
DEA	Diethanolamine
DNA	Dexoyribonucleic acid
DMSO	Dimethyl sulphoxide
ERK	Extracellular signal-regulated kinase(s)
ESI	Electrospray ionisation (mass spectrometry)
FAB	Fast atom bombardment (mass spectrometry)
FACS	Fluorescence-activated cell sorting (flow cytometry)
FIB	Fast ion bombardment (mass spectrometry)
FITC	Fluorescein isothiocyanate
EBV	Eppstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ESI	Electrosray ionisation
EtOH	Ethanol
FCS	Foetal calf serum
HLA	Human leukocyte antigen (see MHC)
GDP	Guanosine diphosphate
Grb2	Growth factor binding receptor 2
GTP	Guanosine triphosphate
GvHD	Graft vs host disease
HCl	Hydrochloric acid
HEA	Hexylamine
HEL	Hen Egg Lysozyme
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFBA	Heptafluorobutyric acid
HiLIC	Hydrophilic ligand interaction chromatography
HLPC	High performance(/pressure) liquid chromatography
HPS	Hydrophilic score
HSC	Haemopoietic stem cell
HSP	Heat shock protein
IDA	Iminodiacetic acid
IFN	Interferon
Ii	Invariant chain
IL	Interleukin (e.g. IL-2)
IMAC	Immobilised metal affinity chromatography
JAK2	Janus kinase 2

[1] To maximum electrical resistivity (=18.2 MΩ·cm)

LAMP	Lysosomal-associated membrane protein
LAT	Linker of activated T-cells
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization (mass spectrometry)
MAPK	Mitogen activated protein kinase
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MIIC	MHC class-II compartment
MOAC	Metal oxide affinity chromatography
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NK	Natural killer cell
NKR	Natural killer receptor
NKT	Natural killer T cell
NTA	Nitrilotriacetic acid
PA	Phosphoric acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phsophate buffered saline (pH 7.2)
PDGF	Platelet derived growth factor
PE	Phycoerythrin
pH	Per hydrogen
Ph	Philadelphia chromosome (typically +/-)
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PKC	Protein kinase C
PLC	Phospholipase C
PML	Pro-meylocytic Leukaemia
PPA	Phenylpropylamine
PTM	Post-translational modification
RB	Retinoblastoma rrotein
RCF	Relative centrifugal force (1 RCF = 1 x *g*)
RFX	Regulatory factor X
RNA	Ribonucleic acid
RP	Reversed phase
RPMI 1640	Roswell park memorial institute 1640 (growth media)

RT	Room temperature
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEREX	Serological analysis of recombinant cDNA expression libraries
SPE	Solid phase extraction
STAT	Signal transducer and activator of transcription
TAA	Tumour associated antigen
TAP	Transporter Associated with Antigen Processing (sic.)
TBP	Transactivator binding protein
TCA	Trichloroacetic acid
TCR	T-cell receptor
TED	Tris-carboxymethyl ethylene diamine
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
T_h	T-helper cell
T_h1	T-helper 1 cell
T_h2	T-helper 2 cell
TLR	Toll-like receptor
TMA	Trimethylamine
TNF	Tumour necrosis factor
TOF	Time of flight (mass spectrometry)
TSA	Tumour specific antigen
T_{reg}	T-regulatory cell
ULM	Ultra low weight marker
UV	Ultraviolet
v/v	Volume/ volume (0.1 ml / 1.0 ml = 10.0 %)
WHO	World Health Organisation
WHOSIS	World Health Organisation Statistical Information Service
w/v	Weight / volume (0.1 g / 1.0 ml = 10.0 %)
ZAP-70	Zeta-chain associated protein (70 kDa)

1.0 Introduction and Aims

1.1 Cancer

1.1.1 Definition and epidemiology

Cancer may be defined as the uncontrolled proliferation of cells from a single cell of origin. Cell division is a tightly controlled event responsible for tissue growth and repair/turnover, with the uncontrolled tissue growth exhibited in cancer typically having lethal consequences if left untreated. Cancers are diagnosed in over 10 million patients per year, and account for approximately 12% of deaths worldwide (WHO, 2005); though this figure rises to 27 % for the UK (CancerStats, 2009). While deaths from cancer have fallen by 19.2 % in men, and by 11.4 % in women in the US between 1990/1-2005 (Jemal *et al*., 2009), this is largely due to improvements in detection/therapy for small subsets of common malignancies, and the incidence of cancer is expected to rise along with the mean age of western populations (Pal *et al*., 2010).

1.1.2 Oncogenes and tumour suppressor genes

It is believed that cancers generally arise due to genetic insult, typically from the accumulation of errors and mutations within an organism's DNA over the course of its lifetime (Nordling, 1953; Lewin, 2000), though exposure to various agents such as carcinogens or ionising radiation may greatly increase the number of genetic errors over a short period of time (Westwood, 1999). As these errors accumulate they inactivate proteins or promoter regions, or (activate them via inactivation of their mediation pathways/proteins) until a point is reached at which the cell begins to replicate uncontrolled (Varmus and Weinberg, 1993). Hanahan and Weinburg (2000) laid out six characteristics that a cancer must acquire to develop into a tumour, namely: (1) independence from growth signal requirements; (2) insensitivity to antigrowth signals; (3) evasion of apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; and (6) tissue invasion/metastasis but there is no specific sequence for these events, and while the change in expression/functionality of a single gene may contribute to more than one of these steps, others may require alterations to multiple genes. It must be remembered that the potential causes for transformation of a cell from healthy to cancerous are legion, and far from exclusive (though some events are

common, such as loss of p53 function [Vousden and Lane, 2007]). Cancer stem-cell theory further complicates this picture, and the relationship(s) between tumour, normal stem cell and cancer stem cell is still far from elucidated (Clarke *et al*., 2006), though it is thought that at least in the leukaemias limitless self-renewal is restricted to a subpopulation of stem cells (Lane, *et al*., 2009).

At the root of carcinogenesis lie two classes of genes: oncogenes and tumour suppressors. The discovery in the early 20th century that some tumour causing agents were transmissible, followed by the identification of cancer-causing oncoviruses fifty years ago (Epps, 2005) eventually led to the Nobel prize-winning discovery that such genes were not originally viral in origin, but derived from mammalian genes that have become incorporated into a retroviral genome. The original genes, termed proto-oncogenes do not in the normal state of affairs lead to tumorigenisis, but are typically involved in cell division (Kwong *et al*., 2007). However mutations, translocations, or gene amplification may lead to permanent activation of these genes, which in turn may initiate the cell into a proliferative state, or enhance the progression of an existing malignancy. Proto-oncogenes may be divided into a range of functional groups including transcription factors (e.g. NOTCH1, mutated in the majority of T-ALL[1] patients); epigenetic remodelers (e.g. chromosomal translocations involving ALL1, common in acute leukaemias); growth factors or growth factor receptors (reviewed for melanoma by Kwong *et al*., 2007); signal transducers (particularly kinase signalling pathways) or inhibitors of apoptosis (BCL-2 being a classical example) (Alfano, 2006; Croce, 2008; Palomero and Ferando, 2010). Nevertheless, oncoviruses remains implicated in approximately 12 % of cancers globally (Schiller and Lowy, 2010) with the majority accounted for by human papillomavirus, Epstein-Barr virus (EBV), hepatitis B and C, and Kaposi's sarcoma–associated herpes virus. *Helicobacter pylori* infections are responsible for another 5.5 % of cancers (through activation of the Wnt/β-catenin signalling pathway) and the burden of infection-related (oncoviral or otherwise) malignancy is disproportionally borne by the developing world (Parkin, 2002).

However, a mutation that converts a proto-oncogene into their oncogenic counterpart will not necessarily cause an immediate transformation to malignancy. Tumour suppressors, such as RB and p53, regulate cell division, and according to the two-hit hypothesis of malignancy[2] the activity of such tumour

[1] T-cell acute lymphoblastic leukaemia.
[2] Not to be confused with the two hit hypothesis of immunity (Moore *et al*., 1993; Murphy *et al*., 2004)

suppressors must also be disrupted if a cell is to proliferate uncontrolled (Knudson, 1971; DeCaprio, 2009). This theory states that if disruption of a tumour suppressor is to occur at the genetic level both copies of the gene usually must experience deactivation (Chan *et al*., 2004); this contrasts with oncogenes which are typically dominant over their non-mutated counterparts (Viallet and Minna, 1990). Typically tumour suppressors operate as DNA-damage linked checkpoints in the cell cycle, either preventing mitosis or initiating apoptosis if DNA damage is unrepairable (Sherr, 2004). A related set of genes known as metastasis suppressors operate to prevent tumour cells down-regulating adhesion molecules (Vaidya and Welch, 2007).

1.1.3 The Leukaemias

1.1.3.1 Risk factors, classification and epidemiology

As the potential molecular bases for a cancer are legion, classification tends to be primarily based on the apparent tissue of origin. The leukaemias (from the Greek *leukoshaema* or 'white blood'), are cancers of the haematopoietic stem cells (HSC's), a non-uniform (yet $CD34^+$) pluripotent progenitor population contained in the bone marrow, with smaller numbers in the peripheral circulation (McKinney-Freeman and Goodell, 2004). These stem cells produce daughter cells which may be either committed myeloid or committed lymphoid progenitor (CMP/CLP) cells. While the former produce monocytes, neutrophils, basophils and eosinophils (as well as erythrocytes and platelets), the latter are the progenitors of B- and T-lymphocytes, and natural killer (NK) cells. Subsets of dendritic cells (DCs) may be produced by either progenitor class.

The theory of cancer stem cells (CSC's), that all the malignant cells within a tumour are derived from a single (un-differentiated or semi-differentiated) pluripotent cell with stem-cell like properties, is growing in acceptance, and in the case of CML and the other leukaemias is supported by a number of observations, including homogeneity of the bcr:abl (see 1.1.3.4) breakpoint amongst all leukaemic cells within a CML patient; the full or partial differentiation of these leukaemic cells into a range of hierarchical subtypes, all of which often carry the same chromosomal translocation (Haferlach *et al.*, 1997). Further evidence includes the findings by Lapidot *et al.* (1994) that only transplantation of $CD34^+$, $CD38^-$ acute myelogenous leukaemia (AML) cells led to the generation

of AML in SCID mice, where $CD34^-$ or $CD38^+$ cells could not. As in bone marrow the $CD34^+$, $CD38^-$ phenotype is expressed by immature haematopoietic progenitors, it is feasible that CSCs arise from mutations in normal stem or progenitor cells (which by definition are already in possession of the pluripotent capability) (Bannerjee *et al*., 2010).

Leukaemias may therefore be divided into lymphoid and myeloid (in practise primarily granulocytes) subsets, though the malignant cells typically fail to demonstrate full maturation, especially in later stages (Altucci, *et al*., 2005). The World Health Organisation (WHO) recently reported that in 2004 (the most recent year for which they carry full epidemiological statistics) leukaemias were collectively responsible for over 263,000 deaths (3.7% of total cancer deaths) (WHO, 2004) with the majority of leukaemias occurring primarily in adults (Westwood, 1999). Risk factors include ionising radiation [3] (Moloney, 1987; Dropkin, 2009; Pelissari *et al*., 2009); some chemical agents, including cyclophosphamide, benzene, and chloramphenicol (Adamson and Seiber, 1981); chromosomal translocations (e.g. Faber and Armstrong, 2007; Caudell and Aplan, 2008; Druker 2008; and Jamieson, 2008); as well as a variety of pre-existing conditions, including Down's syndrome (Malinge *et al*., 2009; Rabin and Whitlock, 2009), Fanconi's anaemia (Andrea, 2003), and hyper-eosinophilia (Owen and Scott, 1979). Viral infection of HSCs/progenitor cells is also a strong risk factor for many leukaemias, particularly for retroviruses, for example the Friends Leukaemia Virus, the Moloney Murine leukaemia virus and the Human T-cell lymphotropic virus type 1 (reviewed in Bannerjee *et al*., 2010), while the (non retroviral) herpes-virus EBV is linked with childhood acute lymphoblastic leukaemia (ALL) (Tedeschi *et al*., 2007) and there is some evidence that it contributes to the progression of chronic lymphoblastic leukaemia (CLL) (Dolcetti and Carbone, 2010). Interestingly EBV infection is known to activate the human endogenous retrovirus HERV-K18 present in the genome (Sutkowski *et al*., 2001), and it is possible that EBV may also employ this endogenous retrovirus as part of its life cycle, though how this may link with transformation is as yet unclear (Hsiao *et al*., 2006). However, the chronic myelogenous form of leukaemia (CML) which is the focus of this area of study has little in the way of recorded heritable components (Gunz, 1977; Lichtman, 1995).

[3]While a localized increase in CML incidences was a consequence of the 1945 atomic bomb detonations in Japan, a notably higher percentage of leukaemias in the Hiroshima survivors were diagnosed as CML than in Nagasaki (43.6 % *c.f.* 15 %) (Moloney, 1987).

Cancer	Diagnosed	Percentage of Total Cancers	Mortality	Percentage of Total Cancers
Breast	45972	15.427	12116	4.066
Lung	39473	13.246	35261	11.833
Colorectal	38608	12.956	16259	5.456
Prostate	36101	12.115	10168	3.412
Non-Hodgkin's Lymphoma	10917	3.664	4438	1.489
Malignant Melanoma	10672	3.581	2067	0.694
Bladder	10091	3.386	5002	1.679
Kidney	8228	2.761	3848	1.291
Oesophagus	7966	2.673	7606	2.552
Stomach	7784	2.612	5178	1.738
Pancreas	7684	2.579	7781	2.611
Uterus	7536	2.529	1741	0.584
Leukaemias (all)	7001	2.349	4367	1.465
Ovary	6719	2.255	4373	1.467
Oral	5410	1.815	1022	0.343
Brain with CNS	4676	1.569	3674	1.233
Multiple Myeloma	4040	1.356	2660	0.893
Liver	3407	1.143	3390	1.138
Mesothelioma	2401	0.806	2156	0.724
Other cancers	33305	11.177	22816	7.657
Total	*297,991*		*155,923*	

Table 1.1 Breakdown of cancer diagnosis and mortality in the UK (total population) in 2007. Note that diagnosis and death often occur months to years apart, and therefore changes in therapy or prevention may allow a greater number of fatalities from a cancer than are diagnosed within the same year, as evidenced by pancreatic cancer (derived from CancerStats, 2009). Cancer accounted for approximately 27.31 % of the registered deaths in the UK in 2007 (ONS, 2008).

Leukaemias	Diagnosed (2006)	Percentage of Total Leukaemias	Mortality (estimated 2010)	Percentage of Total Leukaemias
Chronic lymphocytic leukaemia	12,870	36.08074	4,390	20.10073
Acute monocytic leukaemia	10,140	28.42725	8,950	40.97985
Chronic monocytic leukaemia	4,650	13.03616	440	2.014652
Acute lymphocytic leukaemia	4,260	11.94281	1,420	6.501832
Other leukaemias	3,750	10.51304	6,640	30.40293
Total	*35,670*		*21,840*	

Table 1.2: Breakdown of diagnosis and mortality in the USA by leukaemia-subtypes. Diagnosis data for 2006 derived from Horner *et al.* (2006), estimated 2010 mortality figures derived from (LLS, 2010). Figures given are for total population, however the leukaemias account for a higher rate of mortality amongst males (WHO, 2004).

1.1.3.2 Chronic Myelogenous Leukaemia (CML)

As may be seen from table 1.2, lymphocytic and myelogenous leukaemias make up the vast majority of leukaemia fatalities in the USA. These may be divided into chronic and acute forms. The latter involves the rapid accumulation of immature white blood cells within the bone marrow, inhibiting haematopoietic stem cell activity. The former, first identified in 1845 (Virchow, 1845; Bennett, 1845; Cragie, 1845), typically involves the slower build up of (initially) normally maturing granulocytes in the bloodstream, though these cells showing increasingly poor chemotaxis as the disease progresses (Anklesaria *et al*., 1985). Unlike its acute counterpart, chronic myelogenous leukaemia (CML) may take years to progress (3-5 years to accelerated phase), and therefore immediate treatment is not always required. CML accounts for approximately 15% of adult leukaemias, affecting a global average of 10-20 individuals per million, per year, with the highest range between 40-60 years (the peak is 46-53 years), and like most leukaemias has a slightly higher prevalence in males[4] (Cartwright, 1992; Faderl *et al*., 1999). Symptoms relating to the high white cell count include fatigue, anorexia/weight loss, hyperviscosity of blood, headaches, tinnitus, blurred vision and retinal hemorrhage, splenomegaly, haemomegaly, hyperuricaemia, and resulting gout and bladder stones, priapis, confusion and stupor (Quintás-Cardama and Cortes, 2006).

Changes are usually observed in the white blood cells in the peripheral blood, with increases in basophils, eosinophils, myelocytes and neutrophils common, as well as potential increases in lymphocytes and platelets, the appearance of nucleated red blood cells may be observed, as may potential decreases in erythrocytes (Rodak *et al*., 2007). Within the bone marrow itself hypercellularity rapidly presents, along with depletion of fat and increasing fibrosis (Lorand-Metze et al., 1987; Buesche *et al*., 2004).

Chronic myelogenous leukaemia is bi- or tri-phasic, with the majority of patients diagnosed during the stable chronic phase, at which time ~40 % of patients are asymptomatic. This typically lasts for 3-5 years if untreated (Appleby *et al*., 2005) giving way to accelerated phase (defined as a proportion of > 20 %[5] leukaemic cells in peripheral blood), in which the cancer shows increasing

[4] The male-female patient ratio is 1.4:1 (Quintás-Cardama and Cortes, 2006)
[5] Formerly 30 %.

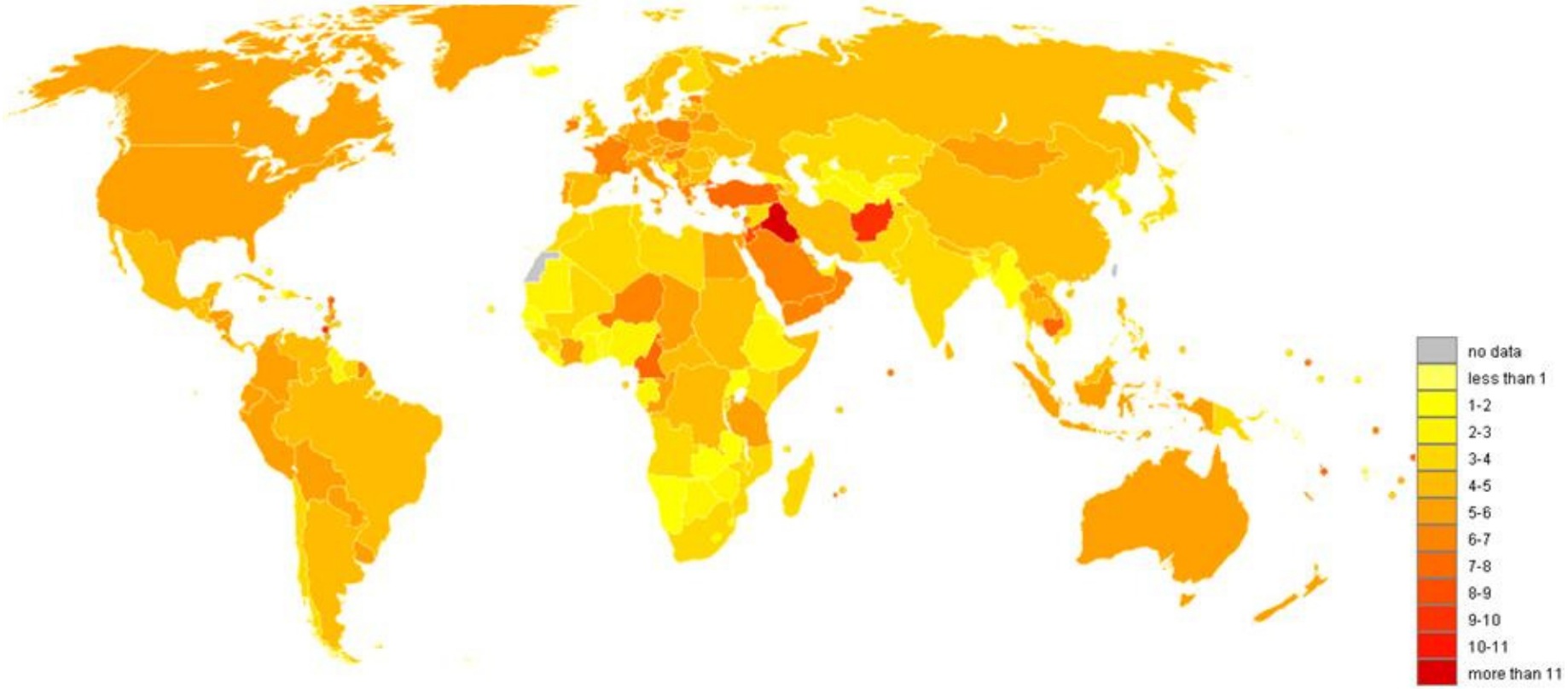


Figure 1.1 Global breakdown of mortalities from the leukaemias (per 100,000 inhabitants), standardised by age (WHOSIS, 2004) (image courtesy of Wikimedia Commons). The high rates of mortality in Iraq and Afghanistan are only partly due to the effect of the ongoing conflicts on the health service. In some regions of Iraq childhood leukaemia has more than doubled since 1995 (Hagiopan *et al*., 2010); while the cause of this has yet to be confirmed, public opinion largely ascribes this to the use of depleted uranium munitions in the 1990-1991 Gulf War.

genetic instability (Rivas *et al*., 2001) and the CML cell population shows reduced terminal differentiation (Mauro and Druker, 2001). As the disease progresses eosinophilia and basophilia are frequently observed, and Gaucher-like macrophage foam cells may be reported (Thiele *et al*., 1986). The activity of total leukocyte (or neutrophil) alkaline phosphatase is usually reduced, making this a common enzymatic assay (usually as a blood or bone marrow cell-count smear assayed under a microscope for a blue/violet colourimetric result) for indication of a potential leukaemic state (Ghanei and Vosoghi, 2002), prior to confirmation by cytogenetic or molecular approaches.

The advanced stage culminates in blast crisis, the terminal stage (the risk of progressing from the advanced phase to blast crisis rises by approximately 4 % per year (Sokal *et al.,* 1985)). This is symptomatically similar to acute myelogenous leukaemia (Faderl *et al*., 1999), and is defined as having greater than 20 % bone marrow composed of leukaemic blasts, or the presence of blast cells in peripheral blood, at which point median patient survival ranges from 6-9 months depending on sensitivity to therapy. Death is typically from infection or complications related to bleeding (Shah *et al*., 2002; Appleby *et al*., 2005; Radich, 2007).

As the condition progresses, 33% of patients in blast crisis exhibit cells with a lymphoid morphology expressing CD10 (a common acute lymphocytic leukaemia antigen also expressed in metastatic carcinomas [Velasquez *et al*., 2007]) CD19, and CD22. The remaining 67% exhibit a phenotype similar to acute myelogenous leukaemia (AML) expressing CD13, CD33, and CD117, with a small minority progressing to myelofibrosis, leading to bone marrow failure (Appleby *et al*., 2005; Quintás-Cardama and Cortes, 2006). The French-American-British (FAB) classification system divides myelogenous leukaemia into seven stages, primarily by cell morphology (Bennett *et al*., 1976; 1981; Lilleyman, 1991), though this system has been supplanted by the more prognostic/diagnostic WHO approach (reviewed in Heaney *et al*., 2000 and Vardiman *et al*., 2002) which includes blast classification and differentiation, key chromosomal translocations and cell morphology.

1.1.3.3 Chromosomal translocations and BCR:ABL

Abnormalities in chromosome structure, polyploidy and chromosome loss have been a commonly reported finding in cancer since the 1950's, however it was not until the advances in basic molecular biology at end of that decade that these were recognised as being linked to the cancerous state (Rowley, 2008), and the subsequent and ongoing elucidation of the roles of oncogenes and tumour suppressors in neoplasticity (Hanahan and Weinburg, 2000). Of particular interest are the translocations commonly found in the leukaemias (amongst many other cancers), indeed, chronic myelogenous leukaemia (CML) is often toted as "a paradigm of early cancer" (Clarkson *et al*., 2003), partly due to the discovery that the majority (>90 %) of patients exhibit a consistent chromosomal abnormality known as the Philadelphia (Ph) chromosome (Nowell and Hungerford, 1960) [6] . This is formed from a reciprocal chromosomal translocation (Rowley, 1973), fusing the gene for c-ABL[7] (Bartram *et al*., 1983), a proto-oncogene with non-receptor tyrosine kinase activity (from chromosome 9) (Lugo *at el*., 1990) with BCR[8] (Groffen *et al*., 1984), a hitherto unknown gene with serine/threonine kinase properties (from chromosome 22).

The cause of the translocation is unclear, though Goldman and Melo (2003) suggested that this may be facilitated by close proximity between the two genes in interphase, however the resulting fusion-gene product is a deregulated cytoplasmic tyrosine kinase (Mauro & Druker, 2001)[9]. This fusion protein lacks the N-terminal sequence of abl, and the loss of a myristoylated glycine coupled with the disruption of the adjascent SH3 domain by bcr together significantly upregulate the kinase activity (and thus transforming activity) of abl (Franz *et al*., 1989; Hanstschel *et al*., 2003). However Abl retains the actin-binding domain (enhanced by bcr coiled-coil and Grb-2-binding sequences which also enable dimerisation, and interaction with proteins such as Ras), nuclear localisation

[6] The remainder frequently exhibit mutations in the kinase Jak2, which is upstream of Ras, MAPK, Erk, PI3K, SHP-2 and STAT-5 (Tefferi and Gilliland, 2006; Kaushansky, 2007). Altered Jak2 activity is not specific to CML however, and Ph^+ CML also often demonstrates altered JAK2 phosphorylation, especially with regard to c-myc upregulation (Xie *et al*., 2002).

[7] c-ABL is the cellular homologue of the transforming protein v-ABL, from the Abelson murine leukaemia virus (Mauro and Druker, 2001), thought to be involved in cellular responses to oxidative stress, DNA damage, and integrin and (PDGF) signalling (Appleby *et al*., 2005).

[8] BCR is a serine kinase with DBL (guanine nucleotide exchanger) homology and GTPase activating homology domains. Neutrophils of BCR-negative mice exhibit increased oxidative burst (Appleby *et al*., 2005), though the cause for this is undetermined.

[9] Auto-tyrosine-phosphorylation down-regulates the kinase activity of the bcr region (Liu et al., 1996).

sequence and DNA-binding domain (Chung *et al*., 1996; He *et al*., 2002), further enhancing the activity of the fusion product.

The fusion most commonly occurs between bcr exon 13 (also known as b2) or 14 (or b3) and abl exon a2, producing a b2a2 or b3a2 fusion gene (Yaghmaie *et al*., 2007), which in turn is transcribed into a 210 kDa cytoplasmic protein; though one 190 kDa form is known to be commonly produced by alternative splicing (Heisterkamp *et al*.,1985; Grosveld *et al*. 1986; Bernards *et al*., 1987; Melo, 1996; ten Bosch et al. 1999; Mauro & Druker 2001). Alternative fusion points, such as b2a3 and b2a2 produce shorter (203 kDa) protein products (Yaghmaie *et al*., 2007), and similarly, additional splice variations have been found to produce shifts in reading frames and junction sequences which in turn may affect bcr:abl activity and produce differentially immunogenic sequences (Volpe *et al*., 2007). The presence of a specific breakpoint in Ph chromosomes of all CML cells within a patient has been regarded as one of the primary pieces of evidence for CML's clonal origin, and is supported by the activity of only one allele of glucose-6-phosphate dehydrogenase in heterozygous female patients (Rodak *et al*., 2007), the normal cells of whom express a heterogeneous phenotype.

However BCR:ABL fusion is not exclusive to CML, and is also found in chronic neutrophilic leukaemia patients (Pane *et al*., 1996), as well as a minority of acute lymphoblastic (Westbrook *et al*., 1992) and acute myelogenous leukaemia (Kurzrock *et al*,. 1987), lymphoma (Mitani *et al*., 1990; Fuji *et al*., 1990) and myeloma patients (Van den Berge *et al*., 1979), though these occurrences frequently involve alternative fusion points, such as a fusion between exon 1 of bcr and abl exon 2 (e1a2), common in Ph^+ ALL (and in CML in the later stages of blast crisis), or the a19a2 fusion common in chronic neutrophillic leukaemia (Yaghmaie *et al*., 2007).

Other chromosomal translocations are also common in the leukaemias, such as: t(15:17) PML-RARα found in >90% of acute promyelocytic leukaemia[10] (Altucci, *et al*., 2005); the t(8;21)(q22;q22)CBFT1-RUNX1 translocation (producing the AML1-ETO fusion protein) found in 10 % of AML (Schwieger *et al*., 2002). Also documented are the t(5:12)(q33:p13) TEL:PDGFRβ fusion commonly present in chronic myelomonocytic leukaemia (Golub *et al*., 1994); a TEL:AML1 t(12;21)(p13;q22) translocation found in 25% of juvenile ALL (Golub *et al*., 1995; McLean *et al*., 1996); the TEL:JAK2 t(9;12)(p24;p13) frequently occurring

[10] A subtype of AML.

in childhood T-cell leukaemia (Lacronique *et al*., 1997; Carron *et al*., 2000). Additional translocations include the t(5;10)(q33;q21)H4/D10S170:PDGFRβ translocation also found in (often bcr:abl negative) CML cases (Kulkarni *et al*., 2000; Schwaller *et al*., 2001; Garcia *et al*., 2003; Drechsler *et al*., 2007) or the t(8;13)(p11;q11-12)/t(6;8)(q27;p11) translocations that fuse the fibroblast growth factor receptor (FGFR1) with ZNF198 or with the oncogene FOP respectively (Xiao *et al*., 1998; Popovici *et al*., 1999). Additional chromosomal abnormalities such as trisonomy 8 and duplication of the Ph chromosome are routinely detected in accelerated-phase patients (Quintás-Cardama and Cortes, 2006).

Further complicating the issue however, bcr:abl positive cells have been identified in the white blood cells of non-leukaemic patients (Biernaux *et al.*, 1995; Bose *et al*., 1998; Bayraktar and Goodman, 2010); the genes for two fusion kinases associated with anaplastic large cell lymphoma (ALCL): NPM:ALK and ATIC:ALK are found in non-malignant tissue belonging to ALCL and Hodgkin's lymphoma patients (Maes *et al*., 2001), and the AML1/ETO fusion gene is present in non-leukaemic stem cells of AML patients (Miyamoto *et al*., 2000), and that such chromosomal translocations may occur prenatally (Mori *et al*., 2002). In these cases protein expression was not assessed however, and it is possible that the fusion proteins were either not properly expressed, that their activity might be inhibited by some unknown factor, that additional genetic events were required for malignancy, or that the occurrence of an (e.g.) t(9:22) chromosomal translocation in a non-stem cell cannot lead to self-renewing malignancy (Michor *et al*., 2006). Of these objections, the latter two are supported by the findings of Jaiswal *et al.* (2003) who found that the creation of an animal model which only expressed bcr:abl in non stem-cell myeloid cells led to myeloproliferative diseases in only a quarter of individuals, until the line was crossed with another strain lacking the pro-apoptosis gene BCL-2, whereupon 50 % were found to present with leukaemias transplantable to w/t counterparts.

The majority of these oncogenic fusion proteins are deregulated kinases like bcr:abl (as reviewed in Cross and Reiter, 2002), highlighting the role of phosphorylation within cancer (the remainder being typically transcription factors, or transcription regulatory factors). Though the human proteome is thought to contain over 10,000 phosphorylation sites on at least a third of the proteome, only around 2000 of these have been identified thus far (Zhang *et al*., 2002; Grimsrud *et al*., 2010), and altered expression or activity of kinases and

phosphatases has well documented links with cancer (Cantley *et al*., 1991; Capra *et al*., 2006) (see table 1.3 for some examples).

1.2 Protein phosphorylation

Protein phosphorylation may be broken down into *O*-linked (serine, threonine and tyrosine) (1800:200:1 respectively) (Hunter, 1998); *N*-linked (histidine, arginine and lysine); *S*-linked (cysteine); and acyl-linked (glutamic acid and aspartic acid) (Klumpp and Krieglstein, 2002; Barnouin *et al*., 2005; Han *et al*., 2008). To date, *O*-phosphorylation has received the lion's share of analysis, primarily due to abundance: while the most common *N*-linked phosphorylation, phosphohistidine is thought to be 10-times more common than phosphotyrosine in yeast, studies on the mammalian proteome have produced far fewer results (reviewed in Klumpp and Krieglstein, 2002). While histidine phosphatases have been documented in the mammalian proteome (Klumpp *et al*., 2002; Ek *et al*., 2002), the relative abundance of O-phosphorylation may however be a mirage produced by lower stability: surrounding amino acids have an immense impact on stability of (e.g.) histidine residue's phosphate group, to the extent that dephosphorylation may in some cases be spontaneous, e.g. as a potentially ephemeral modification in enzyme catalytic sites (Klumpp *et al*., 2002; Ek *et al*., 2002). Furthermore, the acid-lability of (e.g.) *N*-phosphorylation also renders analysis problematic by the mechanisms developed for *O*-phosphorylation (all non *O*-phosphorylations are acid labile, while phosphoarginine is unstable at either pH extreme), and currently no antibodies are available for detection of phosphorylated histidine residues (Klumpp and Krieglstein, 2002; 2005; Zu *et al*., 2006). Despite this, histidine phosphorylation has been linked to a number of proteins of interest to cancer research (reviewed in Steeg *et al*., 2003).

In all cases, phosphorylation may impact on protein activity, location and interaction (Zhang *et al*., 2002), and may result in the negatively charged phosphoryl group (which in the case of O-phosphorylation adds a mass of 80 Da to the amino acid in question [Mann & Jensen, 2003]) forming hydrogen bonds with positively charged amide groups (such as are found on asparagine, lysine or N-termini), or salt bridging to other residues (commonly arginine) (Petsko and Ringe, 2004). Such changes may then result in changes in protein conformation, bulk or charge; impacting either on function/activity, or creating recognition points for a second protein to bind (e.g. recognition of phosphotyrosine residues

by the highly conserved SH2 domain) (Campbell & Jackson, 2003). As the phosphate groups are removed by phosphatase enzymes these effects are often transient and reversible[11]. Phosphorylation, and its effects on protein structure and function is therefore a key factor in the activation or inactivation of enzymes, especially with regard to signalling cascades (Mann & Jensen, 2003) where it acts as a 'molecular switch' (Ishiai *et al*., 2003; Tournaviti *et al*., 2009). The kinase and phosphatase networks are therefore highly regulated (Mita *et al*., 2002) and the ramifications of dysregulation on the cell cycle are well documented, and of continuing interest to research (Sharrard and Maitland, 2007; Daub *et al*., 2008; Grimsrud *et al*., 2010).

Enzyme	Notes	Reference
RET/PTC3 (RP3)	Tyrosine kinase found in differentiated thyroid carcinomas, but also linked to inflammation	Russell *et al*., 2003
Protein Tyrosine Phosphatase γ	Tumour suppressor phosphatase linked to kidney and lung adenocarcinomas, lung neoplasms.	LaForgia *et al*., 1991 ; Zheng *et al*., 2000
PRL-3 Phosphatase	Found in colorectal and ovarian cancers and gastric carcinomas; has strong links to metastasis.	Polato *et al*., 2005, Miskad *et al*., 2004
Syk	Potential tumour suppressor kinase underexpressed in breast cancer. Widely expressed in haematopoietic cells.	Li & Sidell, 2005
Focal Adhesion Kinase (FAK)	Regulates cellular adhesion, and upstream of ERK; expression correlates with Pancreatic tumour size.	Sawai *et al*., 2005 Furuyama *et al*., 2006
Protein Phosphatase A2 (PPA2)	Broad specificity phosphatase with possible role as tumour suppressor. Inhibited by small t antigen of SV40 virus. Linked with various tumour types (*in vitro/ex vivo).*	Gallego and Virshup, 2005
Janus kinase 2 (Jak2)	Tyrosine kinase frequently deregulated in cancer, in particular the haemopoeitic malignancies, and Ph^- CML, but also breast, lung, pancreas, melanoma, and head and neck squamous cell carcinoma. Lies upstream from a number of key mitogenic signals, including MAPK, Ras, Erk and Pi3K.	Ferrand *et al.* (2005) Tefferi & Gilliland, (2006); Godeny & Sayeski (2007); Kaushansky (2007); Wagner and Rui, (2008); Pfeiffer *et al*., (2009)
Src	Non-receptor tyrosine-kinase linked to melanoma, sarcoma, and breast/colon cancers. Cellular homologue of v-src (transforming gene found in Rous sarcoma virus). Inhibition leads to mitotic arrest.	Moaser *et al*. (1999); Chong *et al*. (2005)
Epidermal Growth Factor Receptor (EGFR)	Tyrosine kinase over expressed in glioblastomas, oropharyngeal squamous cell carcinomas, bladder, breast, colorectal, lung prostate and ovarian cancers. Deletion of the extracellular domain can lead to constitutive activation (common mutation in some cancers, e.g. glioblastomas).	Lo & Hung (2006); Saloman *et al*. (1995); Nishikawa *et al*. (1994); Moscatello *et al*. (1995)

Table 1.3: Examples of kinases and phosphatases with links to malignancy.

[11] A notable exception would be phosphorylation-dependent protein degradation, for example the cyclin-dependent kinase inhibitor protein p27, responsible for regulation of the cell cycle, is degraded (via ubiquitination) only once phosphorylated (Nickeleit *et al*., 2007; Varedi *et al*., 2010)

1.3 The role of the immune system in combating malignancy

While the immune system is crucial for everyday defence against pathogens, it is also believed to play a vital role in destroying neoplasms at the oligocellular stage. The role of the immune system in tumour elimination was speculated at over a century ago (Erlich, 1908), though given the then immaturity of immunology and near non-existence of molecular biology it was largely overlooked until it was echoed by Burnet (1970), who hypothesised that:

> "…an important and possibly primary function of the immunological mechanisms is to eliminate cells which as a result of somatic mutation or some other inheritable change represent potential dangers to life[;] without immunological surveillance, cancer would be more frequent and occur at younger ages than it does, [and] immuno-suppressive agents (sic) [...] will increase the likelihood of neoplasia."
>
> Burnet (1970) in Schwartz (2000)

Research with a range of immunocompromised knockout mouse models has indeed shown consistent relationships between loss of immune function and increased vulnerability to carcinogen-induced or spontaneous cancers, and that these cancers are more immunogenic than those that arise in immunocompetent strains (Schreiber *et al*., 2004). The hypothesis is supported by the increased risk of cancer found in patients receiving immunosuppressive treatments to prevent transplant rejection, and the positive correlation between tumour infiltrating lymphocytes and survival rates (Hoover, 1977; Birkeland, 1995; Dunn *et al*., 2002).

This has in turn led to the modern theory that the immune system monitors the majority of tissues throughout the body for evidence of malignancy, with the exception of privileged areas such as (but almost certainly not limited to): the brain (Arshavsky2006); eye, ovaries and testis (Ferguson *et al.,* 2002); foetus and placenta (Guller and LaChapell, 1999); liver (Crispe *et al.,* 2006); and endothelial progenitor cell-derived endothelium (Ladhoff *et al.*, 2010)[12], and that as a malignancy comes to the attention of the immune system a form of natural

[12] Interesting, as there is conflicting data on the role of these cells in angiogenesis, which may feed through to another aspect of immune escape (Nolan *et* al., 2007; Purhonen *et al*., 2008; Ahn and Brown, 2009; Wickersheim, *et al*., 2009).

selection plays out ('immunological sculpturing') which either results in elimination of the cancer, or the generation of a non-immunogenic subpopulation which then develops into a tumour (Fassati and Mitchison, 2009). Therefore in addition to the six stages of cancer development laid out by Hanahan and Weinburg (2000), immune escape is also crucial to the development of any potentially harmful malignancy.

1.3.1 The nonspecific/innate immune system

The immune system is both complex and dynamic, and composed of cellular and humoral arms, each of which have innate and adaptive ('specific') components, with a high degree of cross-talk between them by way of cytokines. With the exception of natural killer (NK) cells, the innate cellular immune system has a limited role with regard to cancer immunosurveillance. While monocytes and macrophages are known to produce a range of tumouricidal responses (proinflammatory cytokines, and reactive oxygen species) when stimulated by $CD4^+$ and $CD8^+$ T cells (Bonnotte *et al*., 2001; Van Ginderachter *et al*., 2006), such environments may be pro-cancerous in the long term (de Visser *et al*., 2006). Natural Killer cells target cells that lack expression of MHC class-I (see 1.2.4.1), but may be evaded by expression of alternative MHC (or MHC-like) alleles in some tumours (Cretney *et al*., 1999; Godal *et al*., 2010) or possibly inactivated by regulatory T cells (T_{reg} cells) (Ralainirina *et al*., 2007). Nevertheless, NK infiltration correlates with improved prognosis in a number of cancers (Villegas *et al*., 2002; Kondo *et al*., 2003; Kim *et al*., 2007).

1.3.2 Tumour antigens

Cancer antigens (molecules which may be recognised by either the humoral or cellular aspects of the adaptive immune system) may be broken down into tumour-associated or tumour-specific antigens (TAAs and TSAs) (Neville *et al*., 1975). The former are expressed in a range of tissues, but with (often substantially) higher expression in malignancy (prostate specific antigen and HER2/neu are both classic examples), (Solvin, 2007; Hudis, 2007) and includes foetal and cancer-testis (C-T) antigens (such as HAGE), which are normally only expressed in immunoprotected tissues (Riley *et al*., 2009). Conversely tumour specific antigens are only found in malignant tissue, and include oncoviral

proteins (Khalili *et al*., 2008); frame-shifted sequences (Ishikawa *et al*., 2003); junction peptides from fusion proteins (Clarke *et al*,. 2001); and the idiotype for the T-cell or immunoglobulin receptors in T-cell or B-lymphocyte leukaemias (though of all the above these may be the least stable) (Davey *et al*,. 1986). While cancer antigens may be proteinaceous, carbohydrate or lipid; proteins tend to show higher immunogenicity (Westwood, 1999).

However, down-regulation of tumour antigens in response to immunological pressure is a major factor in immune sculpturing / immunoediting (Fassati and Mitchison, 2009). If a protracted, panoptic and lasting immune response is to be mounted against a cancer, the tumour antigen(s) in question must ideally be linked to the malignant state and therefore to at least one of the factors outlined by Hanahan and Weinburg (2000).

1.3.3 The humoral immune response

While there is extensive evidence that the humoral system is capable of recognising cancer related antigens in a variety of cancers, including p53 (though many of these antibodies do not discriminate between the w/t and mutant forms) (Soussi, 2000); and bcr and abl in CML patients (independently or fused, though not the fusion peptide) whether the production of endogenous antibodies against cancer-antigens can eliminate a tumour remains uncertain. Though monoclonal antibody therapy (e.g. trastuzumab/Herceptin®) may be highly successful (Hudis, 2007), many cancer antigens are not membrane bound (e.g. p53), and thus not accessible to circulating antibodies. When correlations between autoantibodies and prognosis exist, they tend to be negative (Soussi, 2000; Tan, 2001; Volkmanna *et al*., 2003), suggesting that despite their small size and potential for tumour penetration, they have limited potential to lead to full remission. The role of regulatory B cells (or 'B_{reg} cells') further complicates the issue (reviewed in Mizoguchi and Bahn, 2006).

1.3.4 The $CD4^+$ / $CD8^+$ immune system and the Major Histocompatibility Complex (MHC) system

The key to immune rejection of a tumour is therefore considered to be the $CD4^+/CD8^+$ immune response driven by $CD8^+$ cytotoxic T lymphocytes and the

$CD4^+$ T helper (T_h) cells (Li *et al*., 2005; Riley *et al*., 2009). Whilst this system is, like the humoral response, specific and adaptive, it is also capable of recognising the presence of intracellular proteins and is therefore not restricted to receptors and ion channels in the plasma membrane. Antigen is made visible to T-cells by way of the Major Histocompatibility Complex (MHC). While MHC class-I and class-II molecules share structural similarities, both being transmembrane proteins composed of two subunits (α and β) and a peptide (which is integral for the stability of the complex) (Miller and Sant, 1995; Koopman *et al*., 1997; Pieters, 1997) they differ crucially in terms of structure, expression, and function.

In simple terms, MHC class-I molecules are present on all healthy somatic cells, and present peptides derived from endogenously produced protein to $CD8^+$ CTLs, while MHC class-II molecules are only present on a subset of cells, known as antigen presenting cells (APCs), and present exogenous peptide (acquired by phagocytosis) to $CD4^+T_h$ cells, potentiating the specific immune response. Like B cells, T cells which recognise self-antigens are eliminated by negative selection in the thymus[13,14] (Starr, *et al*., 2003). However not all peptides have an equal chance of being presented by the MHC molecules, the potential presentome is restricted at three points within the system, namely: selective cleavage by proteolysis, peptide loading, and binding of the peptides to specific MHC allelotypes. The different aspects of the MHC class-I and class-II, and these aspects of their antigen processing mechanisms are dealt with briefly below.

1.3.4.1 Generation of MHC class-I peptides

Be it due to requirements to regulate enzyme activity or because of simple wear and tear, all cellular proteins have a limited lifespan, primarily controlled by the ubiquitin system and proteasomal degradation (Yewdell, 2005). Prior to destruction proteins are tagged with the small (8.5 kDa) ubiquitin protein by way of lysine-glutamine cross-linking in an ATP dependent manner. Ubiquitinylated proteins are typically diverted to the 26S proteasome, a ~2 mDa protease complex composed of two 19S activator complex 'gateway' assemblies either end of a 700 kDa 20Sbarrel-shaped destruction chamber; a multicatalytic

[13] With the exception of $CD25^+$ regulatory cells (Pacholczyk and Kern, 2008).
[14] APCs within the thymus express a wide range of antigens normally found in a diverse array of tissues, through expression of the Autoimmune Regulator (AIRE), a master transcriptional regulator that activates a wide range of normally tissue specific genes, allowing the expression of normal antigens to naïve $CD4^+$ and $CD8^+$ T cells (reviewed in Cohn, 2009; Gardner *et al*., 2009).

protease composed of two seven-membered rings of α and β subunits (Golberg and Rock, 1992; Groll, 1997).

The tagged proteins are then unfolded in an ATP-dependent process by the 19S activator complex (Liu *et al*., 2006), and fed into the 20S core, which breaks them down into short peptides by way of the proteolytic β subunits, each of which possess a different activity. Subunit β1 has a caspase-like activity, cleave primarily after acidic residues, subunit β2 cleaves after basic amino acids in a similar manner to trypsin; while subunit β5 has a chymotrypsin-like activity, cleaving after hydrophobic residues (Dick *et al*., 1998). Together these produce a range of peptides and polypeptides which may then be further metabolically degraded by peptidases (Yewdell *et al*., 2003).

A subset of the proteasome: the immunoproteasome differs in structure from the constitutive complex. Not only does it associated with an alternative activator gateway (the 11S activator) (Förster *et al*., 2005); it also may have some or all of any of the following specialised subunits:β1i; β2i and β5i (Scheffler *et al*., 2008; Guillaume *et al*., 2010), and β5t, (the latter of which is believed to be expressed solely in the thymus, and appears vital for $CD8^+$ T cell development) (Murata *et al*., 2008). These changes allow the immunoproteasome to produce a much greater proportion of 8-12 amino-acid peptides (Flutter and Gao, 2004) and the expression of the immunoproteasome can be up-regulated by cytokine exposure; e.g. interferon (IFN)-γ (produced by activated T- and NK cells) (Falk and Rötzschke, 2003), which also up-regulates leucine aminopeptidase, an enzyme which is thought to cleave N-terminal flanking residues from otherwise antigenic peptides (IFN-γ also down-regulates thimet oligopeptidase, a metalloproteinase known to destroy many antigenic sequences) (York *et al*., 1999).

Following proteolytic cleavage many peptides are further processed by other peptidases (Reits *et al*., 2004). In particular Tripeptidylpeptidase II (TPPII), a large (5-9 MDa) oligopeptidase complex may play a crucial role trimming larger peptides, as it possesses both endo- and exo-peptidase functions (Geier *et al*., 1999; Rockel *et al*., 2002; Reits *et al*., 2004). Indeed, the generation of some peptides may circumvent the immuno/proteasome entirely (Lankat-Buttgereit and Tampé, 2002), and that TPII may be able to target poly-ubuitinated proteins (Wang *et al*., 2000).

1.3.4.2 Loading of peptides into the MHC class-I complex

In either case thepeptides produced are trafficked across the membrane of the endoplasmic reticulum by the TAP 1 / TAP 2 / ATP Binding Cassette system (Saveanu *et al*., 2001), bringing them into proximity with the newly synthesised MHC class-I molecules. These class-I molecules are a heterocomplex, partly made up of a 44 kDa heavy α-chain (containing three extracellular domains - α1, α2 and α3 - and a cytoplasmic tail, linked by a transmembrane domain), and a conserved smaller 11.5 kDa aβ-chain (β2-microglobulin), both of which are synthesised in the ER (with folding aided by chaperones such as ERp57 and calnexin) (Carpenter, 2001; Lankat-Buttgereit and Tampé, 2002; Zhang *et al*., 2006), and glycosylated prior to encountering peptides. The α1 and α2 domains together form a structure of eight antiparallel β strands and two antiparallel α-helices, the groove between these domains is the peptide binding site (Bjorkman *et al*., 1987).

Each individual contains two copies of the MHC class-I haplotype (the three MHC class-I genes: A, B and C, found on the short arm of chromosome 6) inherited from each parent, and the expression of which are co-dominant (Choo, 2007). Unless homozygous, each individual therefore expresses six different class-I molecules (two MHC class-I A[15], two B, two C) on their cells, each gene showing a high degree of polymorphism, resulting in a great variety in affinity for different peptides, and a high degree of heterogeneity in MHC complement (and corresponding 'presentome') between individuals. However, some MHC alleles are common among certain populations: e.g. >5 % of Caucasians carry the alleles for MHC class-I A1 and B8; the class-I A2 allele is common in Northern Europe but becomes progressively rarer as more southern populations are sampled; and the Cw*14 allele is rare in Indo-European populations, but more common in southern India. This linkage disequilibrium may be exploited by population geneticists to trace migrations (Thomas *et al*., 2004; Valluri *et al*., 2005; Choo, 2007). Nevertheless, all MHC class-I molecule alleles share the common structure discussed above, with the majority of polymorphism being restricted to their peptide-binding sites (Bjorkman and Parham, 1990).

Within the ER, short peptides with high binding affinity are loaded into the groove of the MHC by the peptide loading complex (TAP 1/2 and tapasin), aided

[15] In the HLA nomenclature, these are described as e.g. HLA-A*0201, HLA-A*03, HLA-B*4501 etc.

by various chaperones including calreticulin (Turnquist *et al*., 2002; Flutter and Gao, 2004; Wearsch and Cresswell, 2008; Del Cid, 2009), which by this point has replaced calnexin (Lankat-Buttgereit and Tampé, 2002). The binding/release of peptides by the TAP involves a significant and partially ATP-dependent reorganisation of the peptide loading complex (Neumann, *et al*., 2002; Chen *et al*., 2003). The components of this complex - TAP in particular - may influence peptide selectivity as binding is primarily influenced in favour of peptides with hydrophobic and basic residues towards the COOH termini (which are produced in higher numbers by the immunoproteasome than by the standard 26S proteasome) (Rock and Goldberg, 1999; Lankat-Buttgereit and Tampé, 2002), though the first three N-terminal residues may also influence TAP binding. However, while TAP will not transport peptides below 7 amino acids in length, the optimum peptide range is 8-16 residues, (Lankat-Buttgereit and Tampé, 2002), and many peptides therefore require subsequent trimming by aminopeptidases before they may be loaded into the MHC peptide binding site (Saveanu *et al*., 2002; Yewdell *et al*., 2003). This may occur either in the ER, or following transport of the 'reject' peptides into the cytosol by the translocon (Koopman *et al*., 2000), after which they may be recycled back into the ER for a second (or possibly third) binding attempt (Roelse *et al*., 1994).

1.3.4.3 Alternate pathways: peptide editing and TAP-independent processing

Other peptides, while of correct length, may have poor binding kinetics, and these are exchanged in a serial fashion for stronger-binding peptides, a process described as peptide editing. The role of tapasin in peptide editing is currently unclear; the majority of reports have suggested that tapasin deficient cell lines either express MHC bound peptides with poorer binding stability than wild-type, with greater spontaneous MHC-peptide disassociation on the cell surface, or in lysates (Garbi *et al*., 2000; Momburg and Tan, 2002; Tan *et al.*, 2002; Williams *et al.*, 2002; Howarth *et al*., 2004). In contrast Zarling *et al*. (2003) and Everett and Edidin (2007) demonstrated that for the MHC class-I B8 allele, the peptide repertoire did not differ between tapasin$^{+/-}$cells (though these reports conflict with each other regarding whether greater spontaneous MHC-peptide disassociation occurs at the cell surface as a result of tapasin loss). However, Momburg and Tan (2002) note that the interactions between tapasin and the MHC heavy chain vary significantly depending on MHC gene and allele (Neisig *et*

al., 1996) and therefore it likely plays a greater role for some alloforms than others, while Wright *et al*. (2004) suggested that tapasin may be responsible for chaperoning peptide-binding, optimising binding kinetics while not significantly altering which peptides are expressed on the cell surface. A recent report by Praveen *et al*. (2010) using a intra-ER model suggests that tapasin allows the selective binding of low concentration high-affinity peptides in the presence of high (100-fold) concentrations of a lower-affinity peptide, by accelerating the disassociation of the low-affinity peptides when they are in a partially bound-intermediary stage. It should be noted that this used a murine (class-I H-2K^{b}) MHC molecule and human tapasin (from a Rajii cell line) and that the above caveats regarding allovariation may still apply. The mechanism by which tapasin may increase the lability of these low-binding peptides is as yet undetermined.

It is worth noting that peptide-MHC binding may also occur independent of TAP, (possibly via similar mechanisms that allow cross presentation between MHC class-I and class-II) (Jondal *et al*., 1996). The mechanisms by which this occurs are still under investigation: hydrophobic peptides containing or derived from signal sequences may enter the ER via the translocon, where they may be cleaved by ER peptidases, though this pathway cannot account for non-signal peptide presentation (Anderson *et al*,. 1991; Fromm *et al*., 2002), and expression of some signal sequences appears to be wholly TAP-dependent (Hombach *et al*., 1995). Song and Harding (1996) demonstrated that TAP-independent processing was proteasome-independent, and postulated that binding of peptide by empty MHC class-I may conceivably occur anywhere along the pathway including on the cell surface itself. However Sigal and Lock (2000) demonstrated a pathway for viral peptides that they believed occurred in endocytic vacuoles, this was supported by supported by Fromm *et al*. (2002), who demonstrated that MHC expression in TAP deficient cells was sensitive to changes in endosomal pH, and by Shen *et al*. (2004) who demonstrated that it depends on cathespin-S, a protein normally associated with MHC class-II peptide processing, indicating that the pathway may be similar to that involved in cross-presentation (see 1.3.4.6?). Furthermore there is evidence that TAP-independent processing may in some cases bear a relationship with the mechanisms of cell penetrating peptides (Brooks *et al*., 2010).

Like the classical pathway, expression by the TAP-independent pathway is up-regulated by IFN-γ, but in both cases this is largely a function of increased synthesis of MHC complex components rather than a selective effect (Fromm and

Erlich, 2001; Fromm *et al*., 2002). However while TAP-independent processing may be vital for presentation of some peptide vaccines (Sheikh *et al*., 2003), $TAP^{-/-}$ cells are less typically less efficient at presentation; they exhibit far fewer MHC molecules on their cell surface (possibly due to a higher ratio of MHC misfolding), and also have a higher proportion of empty surface MHC molecules (Jondal *et al*., 1996; Song and Harding, 1996; Sigal and Lock, 2000) underlining the crucial role for TAP in efficient antigen presentation. Accordingly, mutations in the TAP genes are one documented mechanism of achieving the MHC down-regulation common in tumours, and tend to correlate poorly with prognosis (Fowler and Frazer, 2003; Mclusky *et al*., 2004).

1.3.4.4 Transport of MHC class-I to the cell surface and interaction with $CD8^+$ CTLs

Once a strongly-binding peptide is loaded into the molecule, the heterotrimer is complete and is transported into the Golgi (Harter and Reinhard, 2000), where the complex is deglycosylated to allow TAP and calreticulin to disassociate (Turnquist *et al*., 2002), and following which it is trafficked to the cell surface. A simplified schematic of this pathway may be found in figure 1.3.A.

Once upon the cell surface, α/β T-cells (CTLs) interact with the class-I molecule via the (highly conserved) CD8 receptor and their (hypervariable) T-cell receptor (TCR). If the TCR recognises the peptide antigen, and co-stimulation is found by way of CD28/CD80 binding (or cytokine stimulation from $CD4^+$ T-helper cells), this leads to PI3K mediated activation of ERK to phosphorylate paxillin (Roberton *et al*., 2005), causing cytoskeletal reorganisation and polarisation of the CTL, and targeted release of granulysin, perforin, granzymes and tumour necrosis factor-α (TNF-α), as well as expression of the apoptosis-stimulating Fas-Ligand surface marker, followed by clonal expansion of the T cell population (Selleri *et al*., 2008; Hiroishi *et al*., 2010).

As stated above, MHC class-I expression is near ubiquitous amongst nucleated diploid cells (excluding some cell populations in developing trophoblasts, especially prior to implantation) (Shomer *et al*., 1998). Under normal conditions, lack of MHC class-I expression triggers NK cell-mediated killing (Aptsiauri *et al*., 2007), though this is not always the case; similarly the presence of MHC class-I may not result in CTL killing of a tumour cell for a number of reasons (Chang *et*

al.,2004). Nevertheless, infiltration of CD^{8+} T cells into tumours correlates with improved prognosis for many cancers (e.g. Schumacher *et al*., 2001; Zhang *et al*., 2003, Sato*et al*., 2005; Oble *et al.*, 2008), reinforcing the theory that they may be the key to tumour elimination.

1.3.4.5 MHC class-II synthesis and generation of peptides

As has already been stated MHC class-II is only expressed on a subclass of cells known as antigen presenting cells (APCs)[16], including B cells (Cheng *et al*., 1999), monocytes and macrophages (Ramachandra *et al*., 2009), and dendritic cells (DCs) (Leverkus *et al*., 2003), of which the latter are considered the most professional, and therefore potentially key to elimination of cancer by the immune system (Breckpot and Escors, 2009; Kalinski *et al*., 2009). The MHC class-II molecule is, like class-I, composed of an α- and β-chain; though in the case of MHC class-II these are of similar masses: 34 and 28 kDa respectively, and both contain transmembrane domains (Carpenter, 2001). Like class-I, each gene (HLA-DR, -DQ and –DP) is encoded on the long arm of chromosome 6; however for each class-II molecule both α- and β-chain show high variability, and as each chain is encoded individually, a wide range of combinations are possible across a population.

The genes responsible for MHC class-II (including Ii, HLA-DM, and the β-chain of HLA-DO) all lie under the control of a master transactivator CIITA (class-II transactivator) (Chen and Jensen, 2008). This co-operates with a number of other transcription factors (TBP, RFX, X2BP/CREB and NF-Y) and coactivators (e.g. p300 and PCAF) leading to nucleosome acetylation and gene transcription (Boss, 2003). The promoter for CIITA varies by cell-type, and CIITA is also affected by dimerization, posttranslational modification and GTP-binding[17] (Boss and Jensen, 2003).

Following transcription, both α- and β-chain are synthesised in the ER in conjunction with the stabilising invariant chain (Ii)[18] (Cresswell, 1994) which

[16] Endothelial cells and T-cells may also be induced to express MHC class-II in response to INF-γ (Chen and Jensen, 2008)

[17] The numerous modulators of activity may be crucial to differentiating CIITA's other functions, as it is also implicated in increased expression of 40 other (non-MHC related) genes (Boss, 2003).

[18] The invariant chain was originally believed to be a vital chaperone, without which class-II molecules would not fold properly. However this was later demonstrated to be allele-specific (Bikoff *et al*., 1995; Rajagopalan *et al*. , 2002).

dimerises with another MHC-class-II linked invariant chain forming a $\alpha\beta_3Ii_3$supercomplex. This is then trafficked to the MHC class-II compartment (MIIC), [19] (a multi-lamellar and/or multi-vesicular structure showing great regional homology in protein and lipid content) (Zwart *et al*., 2005) by way of the Golgi, where the invariant chain is cleaved sequentially by a number of peptidases (including Cathespin S), leaving individual MHC class-II molecules containing CLIP (class-II invariant chain peptide) in their binding groove (Chapman, 1998). The cleavage of CLIP disassociates the MHC from the cytoplasmic tail of Ii, which contains a targeting motif; without this the MHC is then free to be trafficked towards the cell surface.

Simultaneously, exogenous material (ranging from polysaccharides, proteins and peptides to bacteria and tumour cells) is internalised by phagocytosis in a receptor-binding mediated (usually TLR-binding mediated) fashion (Blander, 2008). Once internalised, this material moves from early endosomes through a series of increasingly acidic and proteolytic compartments, eventually being digested in lysosomes, which fuse with the MIIC (Chapman, 1998; Crotzer and Blum, 2009). Once here, peptides (12-30 amino acids in length) with strong binding affinity are then rapidly swapped for the CLIP peptide by way of the peptide editor[20] and dedicated chaperone, HLA-DM; which shows great homology with MHC class-II, except in that it is non-polymorphic and does not bind peptide directly (Denzin and Cresswell, 1995). While mediated by low-affinity hydrophobic association (Zwart *et al*., 2005), the interaction with HLA-DM is crucial to stabilising MHC class-II as new peptides bind, and while the disassociation of CLIP is optimal in a low-pH environment (Kropshofer *et al*., 1999), the chaperone function of HLA-DM is pH independent (Zwart *et al*., 2005).

Though not originally synthesised into sphingolipid and cholesterol-rich membranes, the class-II molecule (with Ii or CLIP) is incorporated into these lipid rafts within the vesicle's membranes during transport to the MIIC, and which will be anchored there by tetraspanins by the point at which the complex reaches the cell surface (Hitbold *et al*., 2003). Following binding of peptide antigen the complexes are trafficked with their raft to the cell surface in an IFN-γ stimulated manner (Poloso *et al*., 2004; Büning *et al*., 2005) (see figure 1.3.B.).

[19] The MIIC is primarily composed of late endosomes, but also fuses with early endosomes, phagosomes, and the ER (Robinson and Delvig, 2002).

[20] Though this primarily occurs in the MIIC, it is worth noting that in some cases MHC class-II-peptide complexing may also occur in the Golgi, phagosomes, or on the cell surface itself (Robinson and Delvig, 2002).

Once there it interacts with the TCR of CD4+ T_h or T_{reg} lymphocytes (again in a lipid raft-dependent manner, as CD4 is also carried within a raft structure) (Machy *et al*., 2002; Handin *et al*., 2002), and if TCR recognition is coupled with costimulation (e.g. by CD28 binding to CD80/CD86 on the target cell) (Zhang *et al*., 2004), this leads to serial activation of Lyk and Fyn, both (semi-redundant) Src-family kinases. These phosphorylate CD3 and the tyrosine kinase ZAP-70, which goes on to phosphorylate LAT (Linker of Activated T-cells); setting off a cascade of signals via PLC and PKC (Groves *et al*., 1996; Handin *et al*., 2002; Kyung Chan *et al*., 2011) and causing release of cytokines (which vary depending on whether the $CD4^+$cell is T_{h1}, T_{h2} or T_{reg} in nature), and which lead to either an immunostimulatory or immunoregulatory effect (Kaiko *et al*., 2008; Corthay, 2009).

Given the tissue of origin in leukaemia (and lymphoma), many are unique amongst cancers in expressing not just MHC class-I but also MHC class-II (Diaz, *et al*., 2009), with CML blasts often showing a DC progenitor cell-phenotype. While these show (normally) poor lymphocyte stimulation, their replacement with normal DCs appears to be crucial to successful Imatinib-dependent (see section 1.4) remission (Wang *et al*., 2004), and stimulation of AML blasts to form mature leukaemic-derived DCs has been presented as a possible route of immunotherapy (Tong *et al*., 2008; Kremser *et al*., 2010). Further investigation into the antigens these cells present may aid effective activation of the cellular immune system.

1.3.4.6 HLA-DM, HLA-DO and MHC class-II peptide editing

As well as stabilising the empty MHC molecule, HLA-DM is known to act as a peptide editor by altering the conformation of the class-II molecule and affecting conserved peptide bonds (in particular around the P1 binding pocket), thus allowing disassociation of bound peptide and reducing the inherent selectivity of the native MHC molecule, leading to a change in the peptide repertoire expressed (Busch *et al*., 2005; Narayan *et al*., 2009). Though there is evidence that HLA-DM may repeatedly bind to and disassociate from a class-II molecule, optimising the expression of high-binding peptides (Narayan *et al*., 2009), once a peptide with high binding kinetics has bound, the class-II molecule shows poor interaction with HLA-DM (Anders *et al*., 2011).

In some APCs (particularly mature B-cells and some DCs, depending on lineage) (Hornell *et al*., 2006) the activity of HLA-DM is modulated by a second chaperone, HLA-DO: another non-polymorphic class-II analogue[21], though one which (like empty MHC class-II) lacks stability in the absence of HLA-DM (Chen and Jensen, 2008). The function of HLA-DO is not yet clear, it is known to typically down-regulate HLA-DM's chaperone activity (in a pH-dependent manner) (Rocha and Neefjes, 2008), potentially resulting in lower cell surface MHC expression, a higher ratio of MHC-CLIP at the cell surface (common in mature B cells) (Chen *et al*., 2002) and a potential reduction in peptide editing. Indeed, the effect of HLA-DO is startlingly antigen-specific, with many experimental antigens showing cell-surface down-regulation, while others remain stable, or are even up-regulated (Fallas *et al*., 2004). Like HLA-DM, HLA-DO is only expressed intracellularly (though the murine homologue, H2O, can be trafficked to the cell surface) (Doueck and Altmann, 1997). Interestingly the expression of the HLA-DOα and β chains appears to be independently regulated, leading to the postulation of a second (as yet unknown) role for HLA-DOα (Hornell et al., 2006).

1.3.4.7 Cross-presentation

The classical pathway of antigen presentation, where MHC class-I molecules present endogenous peptide and class-II present exogenous peptide is complicated by cross-presentation of antigens. First reported by Bevan (1975), this may occur in either direction, and on the whole does not appear to be reliant on any special processes, rather operating through many of the mechanisms involved in the classical presentation pathway. Whilst initially considered a minor curiosity of the phagocytotic cells, it has become apparent that cross-priming (i.e. of $CD4^+$ cells against non-phagocytosed, or $CD8^+$ against exogenous peptide) plays an important role in many aspects of T-cell mediated immunity, including auto-immunity, graft-rejection and tumour immunology (Amigorena and Savina, 2010).

[21] Both HLA-DO and -DM show loss of conservation in the CD4+ binding region (Doueck and Altmann, 1997)

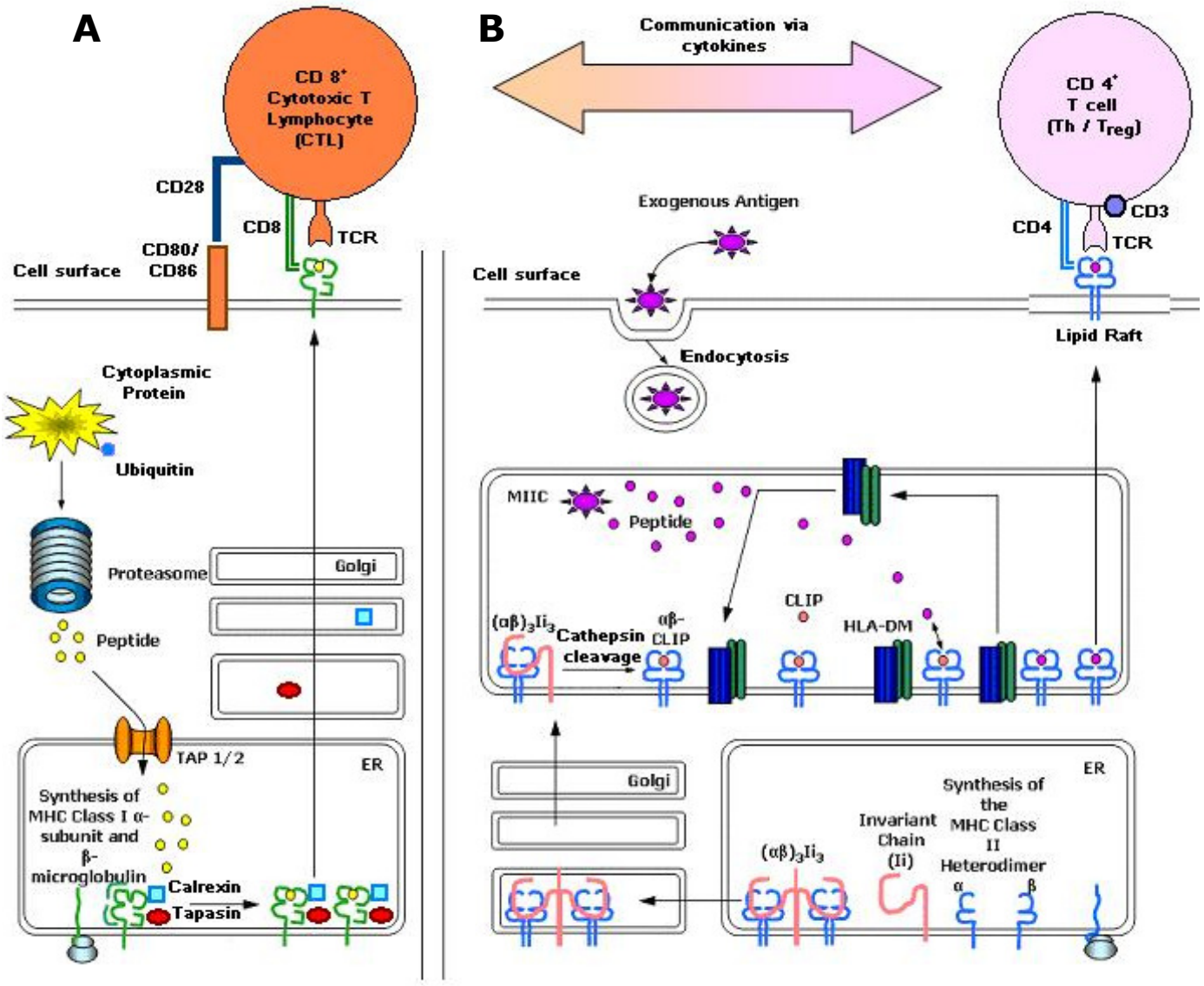


Figure 1.2: Simplified schematic of classical MHC class-I and class-II presentation. (**A**) Class I peptides are generated from endogenous/ intracellular protein and digested via the proteasome. Peptides are trafficked to the ER by TAP1/2 and loaded into the MHC class-I molecule with the aid of tapasin and calnexin, which then disassociate as the MHC class-I is then transported to the cell surface to interact with the TCR of $CD8^+$ CTLs. (**B**) The MHC class-II molecule is synthesised in conjunction with an invariant chain, which leads to dimerisation with another class-II molecule. Upon transport to the MIIC, the invariant chain is cleaved and the resulting CLIP peptide swapped for an exogenous peptide (acquired via endocytosis and digestion in endosomes) by HLA-DM, before being transported to the surface to interact with the TCR of $CD4^+$ T-helper/T_{reg} cells.

It is important to note that cross presentation of class-I peptides by the class-II pathway, and vice versa, does occur by mechanisms which are still being elucidated.

(Modified from Barry, 2006)

1.3.4.7.1 Presentation of exogenous antigen by MHC class-I

MHC class-I antigen presentation is not therefore solely restricted to endogenously produced peptides. The mechanisms by which exogenous material may lead to stimulation of CD8+ CTLs are varied, but can largely be broken down into antigens from a source with a unique ability to penetrate into the cytoplasm, e.g. bacterial disruption of membranes; antigens which may penetrate the membranes themselves such as cell penetrating peptides; antigens with high binding kinetics which displace and replace MHC-bound peptide on the cell surface, antigens passed between cells via gap junctions[22] or antigens internalised by phagocytosis (Rock, 1996; Neijssen *et al*., 2005). This latter category is of greatest interest, given that it does not appear to be restricted to a particular peptide population, because of it's probably involvement in vaccination (Groothuis and Neefjes, 2005), and because of the crucial role that phagocytotic APCs play in the immune system. Highlighting the importance of phagocytosis; cross presentation by class-I is considerably higher when antigen is bound to latex beads as compared to soluble antigen (Houde *et al*., 2003), and a far less common phenomenon in non-phagocytic APCs such as B cells than in macrophages and DCs (Rock, 1996); though of the two cross-presentation seems to be most crucial in DCs (Greer et al., 2009)[23].

In contrast with non-APC phagocytes such as neutrophils, and comparatively poor cross-presenting cells such as macrophages, both of which degrade phagocytosed material in an acid-dependent manner, the phagosomes of DCs may be maintained at a much higher pH for a prolonged period due to reactive-oxygen species production of NADPH oxidase 2 (NOX2) (Mantegazza *et al*., 2008)[24]. As NOX2 expression in myeloid cells is known to be modulated by a range of cytokines, including IL-6, TNF-α, or VEGF (Lechner *et al*., 2010), it is possible that the propensity towards cross-presentation may also be modulated

[22]These are inter-cytoplasmic connections between adjacent cells (often of different lineages) and especially commonly established by DCs and macrophages. They are established by connexins, and are capable of transferring molecules including peptides within the MHC class-I mass range. Tumour cells often close their gap junctions, potentially as another means of immune-evasion (Groothuis and Neefjes, 2005; Neijssen *et al*., 2005; Gafken and Lampe, 2006; Pang *et al*., 2009; Vyas *et al*., 2009).

[23] Ironic given that in the mid 1990s it was unclear at to whether DCs truly cross-presented antigen, with some ascribing experimental data to macrophage contamination (Rock, 1996).

[24] NOX2 and ROS levels only appear to affect cross presentation of antigens when peptide trimming is required; cross presentation of internalized shorter peptides is less affected by inhibition of NOX2 function, suggesting a specific neutral pH-dependent peptidase may be at work (Mantegazza *et al.*, 2008) . ER-associated peptidase 1 and 2 appear to be potential candidates, though they are primarily found in the ER, not phagosomes. A phagosomal homolog, insulin-regulated aminopeptidase may instead be responsible (Rock *et al*., 2010) especially given its optimum activity is within the neutral pH range (Mizutani *et al*., 1982).

in part cytokinetically in monocyte-derived DCs, though it is important to note that the larger the mass of the internalized material, the greater propensity towards phagosome acidification (Tran and Shen, 2008), presumably to ensure elimination of live pathogens or biologically active proteins.

Following internalisation and possibly enzyme-mediated hydrolysis, cross-presentation of antigen by MHC class-I may be either TAP dependent (Kovacsovics-Bankowski and Rock, 1995) or otherwise. TAP-dependent processing requires that the peptides are transported via the cytoplasm[25] (and possibly via the immunoproteasome, if not digested inside the phagosomes). The rate of transfer of peptides from phagosomes to cytoplasm is size dependent[26] (Tran and Shen, 2009), but given the distinct ion content of the ER and controlled pH commonly found in phagosomes, uncontrolled diffusion is unlikely. One mechanism responsible for this transfer is the bidirectional translocon Sec61 protein channel complex (Römisch, 1999), part of the ER antigen-degradation pathway, present in both the ER and phagosomes, and capable of trafficking peptides across the membrane of either (Inaba and Inaba, 2005; Osbourne *et al*., 2005; Rock, 2006). The complex is partly regulated by phosphorylation (Gruss *et al*., 1999) and is gated by the HSP chaperone BiP/Grp78, which requires ATP for activation along with numerous co-chaperones (Alder *et al*., 2005).

TAP-independent processing from the cytoplasm to the ER (or other bodies) is as much of a possibility for cross-presented peptides as it is for endogenous antigens. For example, the bidirectional translocon/Sec61 protein channel is responsible appears to be responsible for trafficking peptides from phagosomes into the cytoplasm, it may also provide a pathway for trafficking into the ER (Inaba and Inaba, 2005; Osbourne *et al*., 2005).

However a non-cytoplasmic cross-presentation route ('the vaculolar pathway') also exists, and for several years there has been debate on the exact route involved. While fusion of phagosomes (e.g. from autophagy – see 1.3.4.9) with the ER seems an obvious possibility, and is to some extent supported by experimental data (Ackerman *et al*., 2003), this theory is disputed by others (e.g. Touret *et al*., 2005, Groothuis and Neefjes, 2005); resolution of these conflicting viewpoints is problematic due to the difficulties of avoiding ER-contamination during phagosomes separation, as well as the potential for variation between cell

[25] TAP independent processing on the other hand may or may not be cytoplasmic.
[26] No data is available on concentration dependence.

types (Lin *et al*., 2008; Vyas *et al*., 2009). It is also further complicated by evidence that phagosomes may also contain many proteins involved in class-I antigen processing, including TAP, calnexin, calreticulin and ERp57 (Houde *et al*., 2003; Guermonprez *et al*., 2003), potentially giving phagosomes a role as 'self-sufficient cross-presenting organelles' (Houde *et al*., 2003; Ramirez and Sigal, 2004). Such ER-like bodies may go some way to explaining conflicting results on phagosomes-ER fusion (Ackerman et al., 2005), though again the existence of these bodies is also under debate. A third possible vacuolar route involves fusion of phagosomes with MHC containing endosomes (Greer *et al*., 2009) – an event which is relatively uncontroversial (Beron *et al*., 1995; Becken *et al*., 2010), though the extent to which this contributes to overall TAP-independent cross-presentation is unclear. However, like classical presentation the cytosolic/TAP-dependent route accounts for the overwhelming majority of cross-presented peptides (Shen *et al*., 2004; Rock and Shen, 2005).

DCs are particularly noted for cross presentation of exogenous antigen via the class-I pathway (Robson *et al*., 2010), possibly reflecting in part the junction role they play between the MHC class-I, -II and nonspecific immune system. It is important to remember that cross-priming is also capable of stimulating $CD4^+$ or $CD8^+$ regulatory T cells, leading to cross-tolerance rather than cross priming (Greer *et al*., 2009). Indeed, given that defects in NOX2 expression are linked with autoimmunity, cross-presentation may be a crucial junction within immunoregulation (Mantegazza *et al*., 2008).

1.3.4.7.2 Presentation of endogenous antigen by MHC class-II

MHC class-II has also long been known capable of presenting endogenous material as well as that taken in by phagocytosis, and though the former represents a minority of the peptides presented by APCs, some antigens such as peptides from hen egg lysozyme (HEL) are expressed on MHC class-II with vastly higher efficiencies when transfected into their targets rather than provided exogenously (though the exact rates vary between cell types and plasmid promoter). (Schnieder and Sercarz, 1997).

Again however, the exact mechanisms involved are yet to be fully elucidated. In some cases cross presentation appears to be TAP dependent (Lechler *et al*., 1996). Conversely however the addition of a four amino-acid ER-retention

sequence to HEL reduces the expression of five of the seven peptides cross-expressed in transfected B-lymphoma cell lines (when compared with the wild-type HEL plasmid) (Adorini *et al*., 1993).

One potential route for endogenous material to access the class-II pathway is by autophagy; the engulfing and degradation of intracellular protein complexes and organelles by phagosomes (macroautophagy) or lysosomes (microautophagy) (Yorimitsu and Klionsky, 2005; Strawbridge and Blum, 2007). This was long thought to be a random process, upregulated by nutritional deprivation, infection or other stresses, but has since been demonstrated to be specific and (at least in some instances) a chaperone-mediated (CMA) process, regulated by GTP (Bandyopadhyay *et al*., 2010), and also influenced by cytokines, including IFN-γ-mediated upregulation (Gutierrez *et al*., 2004), and TLRs (Vyas *et al*., 2008). Some forms of autophagy are organelle-specific, such as mitophagy, which degrades depolarised mitochondria in starvation conditions, or CMA, which largely targets proteins with a KFERQ motif (Li *et al*., 2008). The most-studied form: macroautophagy involves the formation of a double membrane which envelopes an area of the cytosol; while sometimes appearing random it is known to selectively target polyubiquitinylated protein aggregates in Parkinson's (Webb *et al*., 2003) and Huntingdon's (Shibata *et al*., 2006) diseases, while it is conceivable that the different forms of autophagy are more of a spectrum than discrete pathways, so each may have both selective and non-selective aspects (Li *et al*., 2008).

Paludan *et al*. (2005) demonstrated that expression of an endogenous EBV-related antigen was downregulated both by blocking lysosome acidification and by disrupting autophagy (with the type-1 PI3K inhibitor 3-methyladenine), with a reduction of between 30 and 70% expression on the cell surface, which along with the observation that autophagy is a constitutive function in APCs (Schmid *et al*., 2007), present as strong evidence for it's role in cross-presentation.

However, autophagy-deficient cell lines still cross-present peptides to the class-II pathway (Dani *et al*., 2004; Massey *et al*., 2006), suggesting that proteasomal (or immunoproteasomal) processing of protein may be another pathway to cross-presentation, following which the resulting peptides may be bound by class-II cross-presentation chaperones such as Hsp-40 and Hsp70 and transported to lysosomal-associated membrane protein (LAMP) 2a which traffics them into the lysosome; this in turn being bound for fusion with the MIIC (Zhou

et al., 2005; Strawbridge and Blum, 2007). It is believed that the degradation machinery may distinguish between long-lived (i.e. housekeeping) and short-lived proteins either prior to, or following autophagy, and that the former may be degraded while the latter are more likely to be cross-presented (Li *et al*., 2009b).

Thymic APC populations have a higher propensity towards cross presentation, highlighting its potential importance in immune-tolerance (Schnieder and Sercarz, 1997). A significant proportion of the peptides isolated from MHC class-II molecules are apparently derived from cytosolin/incracellular proteins (Dongre *et al*., 2001), and this proportion is upregulated when autophagy is induced (Dengjel *et al*., 2005b). The link between TLRs and autophagy is one factor that has encouraged investigation of TLRs as therapeutic agents for cancer (reviewed in So and Ouchi, 2010). Cross-presentation of endogenous material may also be induced in non-APCs (melanoma cells) following transfection with MHC class-II (Chen *et al*., 1994). Of course these cells lack the phagocytic properties of APCs, and the corresponding cellular machinery, but interestingly in these cases cross-presentation by MHC class-II is reduced if Ii is also expressed (Dissanayake *et al*., 2004). Crucially, the prime therapy for CML today, Imatinib mesylate (see 1.1.4) appears to upregulate autophagy as a survival mechanism (Bellodi *et al*., 2009), raising the possibility that cross-presentation may also be affected by the therapy.

1.3.5 The CD1 lipid-antigen presentation system

The CD1 processing system is a second antigen-presentation mechanism capable of stimulating a cellular immunity in an antigen-specific manner. There are five known CD1 isoforms, each with a structure similar to MHC class-I, being composed of a heavy chain with transmembrane domain, associated with β2-microglobulin molecule (Polakova *et* al., 1993). Of the five isoforms, CD1a, b and c present exogenous (primarily bacterial) lipid antigens[27] to α/β T cells, while CD1d presents endogenous lipid antigens to Natural Killer T cells (NKT cells: see 1.3.6) (Paul, 2008). CD1e is not trafficked to the cell surface; instead it is cleaved from its transmembrane domains and is retained in soluble form within the Golgi, where it plays a chaperone role similar to HLA-DM (Maître *et al*., 2008). Like the MHC system, the CD1d isoform is expressed by all cells, while

[27] Apolipoprotein E is implicated in uptake of lipid for CD1a-c presentation (van den Elzen *et al*., 2005)

CD1a-c and e are primarily expressed by DCs (Moody and Porcelli, 2003). Further similarities may be found in assembly of the CD1 complex, which is also calreticulin and calnexin dependent, though also relying on endoplamsic phosphatidylinositol, which plays a key role similar to Ii in MHC clas-II presentation. Trafficking of CD1 to the plasma membrane is reliant on microsomal triglyceride transfer protein (Sagiv *et al*., 2007). Furthermore, it is believed that CD1d will associate with Ii, suggesting that their distribution, or that of MHC class-II may alter in the presence of one another (Paul, 2008).

1.3.6 Alternative CD8+ T cell populations

In addition to the α/β TCR expressing CTLs, another population carrying the non-MHC γ/δ TCR also exists, making up 2-5 % of $CD3^+$ T cells in the blood, but a far higher proportion in certain areas such as the skin and gastro-intestinal tract. The role of these cells is still being explored, but it is known that they also possess strong cytotoxic effector function, and secrete a range of cytokines, including IFN-γ and TNF-α (Hayday, 2000). These cells do not express CD4/CD8, and may be further subdivided into $Vδ2^+$ (which make up 50-90 % of the total γ/δ T cell population) and $Vδ2^-$ ($Vδ1^+$ or $Vδ3^+$) populations. Of these, the latter do recognise MHC class-I chain-related molecules A and B (by way of the γ/δ TCR), and the UL16 binding proteins 1-3, all of which are up-regulated by heat shock or oxidative stress, leading to their constitutive expression in many epithelial tumours, as wells as and leukaemias and lymphomas (Groh *et al*., 1999; Poggi *et al*., 2004). $Vδ2^-$ γ/δ T cells on the other hand do not, but instead recognise a range of non-peptidic phosphorylated metabolic intermediates from the non-mevalonate pathway of isoprenoid biosynthesis, as well as mitochondrial F1-ATPase and delipidated apolipoprotein A-I, all of which are commonly expressed in tumours (Kabelitz *et al*., 2007).

The Vδ2+ population express MHC class-II and are capable of phagocytosis (Ichikawa *et al*., 1991; Wu *et al*., 2009), while the Vδ1+ population are capable of suppress CD8+ T cell responses and inhibit DC maturation, implying a regulatory function (Peng *et al*., 2007). Both populations are also capable of activation by NK receptors (which also recognise MHC class-I chain-related molecules A and B), tying them into the non-specific immune system (Rincon-Orozco *et al*., 2005; Kabelitz *et al*., 2007).

Natural killer T (NKT) cells is an umbrella term for a range of T-lymphocytes which express Natural Killer receptors (NKRs), subsets of which also express the variant TCR chains Vα24 and/or Jα18 (and which may be $CD8^{+/-}$). These TCRs recognise exogenous glycolipid antigens presented by the MHC-like CD1d molecule in a non hyper-variable manner (Peralbo *et al*., 2007), resulting in cytokine release and are capable of a memory-cell function. While these variant TCRs preclude recognition of peptide, the cells seem to play an important anti-tumour role, with reduced numbers being recorded in numerous epithelial cancers, and which may also be useful for immunotherapy (reviewed in Molling *et al*., 2008).

1.4 Therapies for cancer, and specifically CML

1.4.1 Current and historical therapies for CML

Though in general CML is largely chemotherapy-insensitive (Rivas *et al*., 2001) treatments for CML have historically relied on various chemotherapies such as Fowler's solution, busulfan, hydrea/hydroxyurea, (which, due to their different mechanisms of action, may be applied individually or in combination) or sometimes radiotherapy (e.g.: spleenic irradiation) (Deininger and Druker, 2001). The first chemotherapeutic agent: Fowler's solution (or black arsenic), is a weak potassium arsenite (arsenic trioxide) solution originally developed in the late 18th Century (though the use of arsenic as an anticancer agent dates back to the 1st Century AD) (Nicolis *et al*., 2009), but discontinued for therapeutic purposes (including at that time malaria and Hodgkin's lymphoma as well as the leukaemias) in the 1950's due to toxicity and carcinogenicity associated with long-term use. The use of arsenite has however seen a resurgence in later years following trials with late-stage promyelocytic leukaemia (PML) patients, and was re-approved for therapy by the FDA in 2001 at 0.15 mg / kg body wt (Jun *et al*., 2002; Nicolis *et al*., 2009).

Busulfan (1,4-butanediol dimethanesulfonate) was introduced as an anticancer agent in the 1950's. It is a DNA-alkylating and cross-linking agent known to affect stem cells, and typically able to control progression and symptoms of CML by reducing tumour burden for a minimum of three months, and extending patient lifespan to 3-4 years (Hehlmann *et al*., 1993; Iwamoto *et al*., 2004). It is usually prescribed at a daily dose of <0.1 mg/kg body weight, depending on

white cell count (Kolibab and Drucker, 2000) and may be used in combination with 'classic' chemotherapeutic agents such as cyclophosphamide (which also causes DNA-alkylation) (Kashyap *et al*., 2002) or fludarabine (which inhibits DNA synthesis) (de Lima *et al*., 2004). Side effects are severe however, including hepatic, pulmonary and cardiac fibrosis (potentially culminating in veno-occlusion), as well as myelosuppression, and not only is the drug typically incapable of inducing cytogenetic remission, but busulfan resistance has also been documented in some patients (Stone, 2004; Valdez *et al*., 2008). Furthermore busulfan also has some mutagenic properties which may actually impair long-term survival (Hehlmann *et al*., 1993).

Hydroxyurea, also known as hydroxycarbamide ($CH_4N_2O_2$) is a small molecule capable of inhibiting the reduction of ribonucleotides crucial for DNA synthesis, and is typically prescribed in the 1-4 g/day range, depending on white blood cell count (Kolibaba and Drucker, 2000). Comparisons with busulfan have shown it to be a similar or superior therapeutic agent, with a greater survival advantage a lower toxicity profile (Hehlmann *et al*., 1993). Again while it is capable of producing a haematological response (Bubnoff and Duyster, 2010), it is rarely able to cure the patient (Garcia-manero *et al*., 2002), instead it extends patient lifespan and aid control of their symptoms (Faderl *et al*., 1999), and side effects, while usually haematological (Kolibab and Drucker, 2000) may also include nausea, skin atrophy and myelosuppression in the short term, and ulceration, gangrene, cutaneous squamous cell carcinoma and potentially lethal pulmonary effects following chronic use (Stone, 2004). Unfortunately as stated, all chemotherapeutic approaches to CML lack specificity and generally fail to produce true remissions in the majority of patients (blast crisis in particular tends to be refractive to chemotherapy), but to some extent alleviates suffering and/or prolongs patient lifespan (Deininger and Druker, 2001; Quintás-Cardama and Cortes, 2006).

CML was also the first malignancy to be treated with (usually recombinant) IFN-α, a therapy which stimulates immune activity [28] through phosphorylation-dependent activation of STAT-1, STAT-3, STAT-4, STAT-5a and -5b which all lie downstream of the IFN-α/β receptor, as well as augmenting the effect of many other cytokines, including IL-2 and increasing INF-γ and IL-12 expression *in vivo* (Maitikainen *et al*., 1999). The net result is an upregulation of T-cell function

[28] INF-α and INF-β, and their shared receptor are essential for clearance of viral infections. (Müller *et al*., 1994).

(Fausel, 2007), CTL proliferation and survival (Tough *et al*., 1996) and a promotion of the T_h1 response (Roge *et al*., 1997). The therapy shows notably greater efficacy and survival rates than hydroxyurea (Chronic Myeloid Leukemia Trialists' Collaborative Group, 1997; Appleby, 2005); indeed, if deployed in the chronic phase INF-α is capable of producing haematological responses in up to 80 % of patients, and complete cytogenetic responses (i.e. patients which are negative for fusion product transcript by PCR) in between 5 and 15 % (Bubnoff and Duyster, 2010). IFN-α has been shown to be especially effective when combined with Ara-C [29] (Guilhot *et al*., 1997) or hydroxyurea (Hehlmann *et al*., 2003); unfortunately the key to IFN-α's success is also the root of its toxicity – the therapy is often poorly tolerated (Kolibaba and Druker, 2000; Stone, 2004), rendering therapeutic doses less applicable. Side effects may include fatigue, prolonged flu-like symptoms, and depression (Fausel, 2007), and potentially lethal renal dysfunction, though this is usually only following chronic use (Colovic *et al*., 2006).

Studies in the 1990's illustrated that allograft bone marrow (stem cell) transplants[30] (Apperley, 1998) and treatment with donor T lymphocytes can lead to remission and even complete cures of CML (Kolb *et* al., 1990; Collins *et al*., 1997; Falkenburg *et al*., 1999), especially when combined with chemotherapy (Riddell *et al*., 2002). While transplants were originally thought to be most efficacious if carried out within the first twelve months following diagnosis, some studies have indicated that transplants carried out within three years may have as high a success rate (Gratwohl *et al*., 1993). Prognosis of the benefit of transplant is largely assessed using the scoring system developed by Sokal *et al*. (1985), though the development of therapeutic kinase inhibitors (see below) has to some extent undermined this system (Quintás-Cardama and Cortes, 2006).

The primary hindrances to stem cell graft therapy are lack of suitable donors (for example only between one third – one half of patient siblings are suitable), and patient age, though obviously patient health / disease progression also factor heavily. The ideal conditions for transplant are otherwise healthy chronic-phase patients diagnosed less than one year ago and aged below 40, though even these have a post transplant mortality rate of up to 20 % (Peggs and Mackinnon, 2003; Quintás-Cardama and Cortes, 2006). Even with matched unrelated donors

[29] Also known as cytarabine: a chemotherapeutic agent similar in structure to nucleosides which target dividing cells.
[30] Studies which this author is proud to cite I.A. Dodi's involvement.

taken into account, transplants are not an option for over 40 % of patients, though this figure is down from 80 % in the late 1990s, mostly due to kinase inhibitor therapy (see below) (Sawyers, 1999).

Along with the risks typically implicit in surgery on cancer patients, one major complication of stem cell transplants is the risk of graft vs host disease (GvHD), where the immune cells which arise from the transplanted stem cells go on to reject the host's tissues (Crough *et al*., 2002; Kolb *et* al., 1990). The risks of this condition may be significantly reduced by careful matching of patient and donor MHC class-I A, B C, and class-II Dr alleles, mismatching of any one of which may be a significant risk factor (MHC class-I C being the least vital) (Morishima *et al*., 2002). When it does occur GvHD is usually controlled through corticosteroid therapy (Lee and Flowers, 2008), and has a strong negative correlation with CML relapse (Weiden *et al*., 1979; 1981), though this correlation is much weaker in AML and ALL (Horowitz *et al*., 1990).

CML therapy was revolutionised a decade ago with the first therapeutic kinase inhibitor. Imatinib mesylate (or STI571)[31], is a selective inhibitor for bcr:abl (Drucker and Lydon, 2000), and the first of a new family of small molecule inhibitors (reviewed in Zhang *et al*., 2009). Imatinib binds to the tyrosine kinase domain of bcr-abl in a competitive manner, stabilising the protein in an inactive conformation (Druker *et al*., 2001) and preventing ATP from accessing the active site (Quintás-Cardama and Cortes, 2006). It was designed based on the work of Yaish *et al*. (1988) in developing kinase-specific tyrphostins, and resulted from high throughput screening of the 2-phenylaminopyrimidine class kinase inhibitors. From these a number of compounds were selected and altered, until Imatinib was developed, initially as a platelet derived growth factor (PDGF) α/β inhibitor, but was also found to selectively inhibit *abl* kinases, and *c-kit* (Mauro and Druker, 2001).

Phase-I clinical trials (with patients unsuitable for IFN-α therapy) demonstrated a complete haematological response in 98 % of patients receiving >300 mg Imatinib/day, with over 50 % also demonstrating a cytogenetic response. While no significant toxicity was observed with doses three times higher (mild myelosupppression was documented), dropping the dose to below 250 mg/day significantly reduced response rates (Druker *et al.*, 2001). Similar results were observed in phase-II trials conducted on a largely late chronic-phase patient pool, dosed with 400 mg/day (now the standard dose, though subsequent trials have

[31] Developed by Novartis and marketed as Gleevec.

suggested benefits to raising this to 600 or 800 mg/day). As a result of this small molecule patient lifespans have been extended significantly, and when full cytogenetic responses occurred, they are maintained in 90% of these patients over three years later, though discontinuation of therapy often results in relapse (Quintás-Cardama and Cortes, 2006).

However, within the reservoir of CML cells, post-transformative mutations in bcr:abl (often within the kinase domain) are frequent[32] and may lead to Imatinib resistance (Corbin *et al*., 2003; Tauchi, *et al*., 2003). While newer small-molecule inhibitors such as Nilotinib (>20-fold greater potency than Imatinib) and Dasatinib (>100-fold greater potency than Imatinib and which also inhibits Fyn, Lck, and Src kinase activity) have been developed to circumvent this (Quintás-Cardama and Cortes, 2006; Deguchi *et al*., 2008), resistance to these newer inhibitors has also been recorded (Soverini *et al*., 2007; Mahom *et al*., 2008), especially amongst Ph^+ stem cells (Copland *et al*., 2006; Jiang *et al*., 2007; Konig *et al*., 2008) and the genetic instability typical of tumour cells may lead to the development of mutations which allow bcr:abl independent proliferation. As a result, Imatinib rarely succeeds in fully eradicating Ph^+ cells from the body and therefore is typically a lifelong treatment (Appleby *et al*., 2005). Indeed there are concerns that it may have immunosuppressive effects (Wolf *et al*., 2007) therefore additional therapeutic/preventative measures must be developed. As the overwhelming majority of cancers are destroyed by the immune system whilst still at the single cell-stage, successful malignancies must either escape from or be tolerated by the immune system (Khong and Restifo, 2002). Therefore breaking immune tolerance may be the key to curing cancers, with the added benefit that such an approach may be performed without the high-toxicity of radio/chemotherapy.

1.4.2 Cancer immunotherapy

Immunotherapy against bacterial and viral diseases, starting in the modern age with Jenner's cowpox-based vaccine for smallpox (then the cause of 10 % of mortality) has been a resounding success, and prophylactic vaccines are now readily available in Western society for a huge range of disease-causing agents, including: rabies; typhoid; cholera; plague; measles; varicella; mumps;

[32] Over 40 resistance-conferring mutations have been documented, each producing a different level of refractivity. Mutations in the glycine-rich P-loop, involved in ATP-binding are most common, and carry the poorest prognosis (Quintás-Cardama and Cortes, 2006).

poliomyelitis; hepatitis B and the tetanus and diphtheria toxins, many of which were previously fatal (Waldmann, 2003). However cancer immunotherapy is a far newer field, and is complicated by the immuno-sculpturing process that cancers undergo (typically long before diagnosis). The application of prophylactic vaccines is often prohibited by difficulty in identification of which tumours are likely to present in a currently disease-free patient; though one recent exception is the vaccine against human papillomavirus (HPV), some strains of which are sexually transmitted and responsible for 70 % of all cervical cancers (Lowy and Schiller, 2006). This vaccine is now given to adolescent females in numerous countries, with administration to males under consideration (Short *et al*., 2010; Yancey *et al*., 2010).

Unfortunately, as previously stated oncoviruses play an etiological role in a minority of human cancers (Schiller and Lowy, 2010), therefore vaccines against other malignancies must be therapeutic in nature; i.e. aiming to reactivate and restimulate the immune system against an existing tumour (Waldmann, 2003).

Unfortunately, as previously stated, oncoviruses play an etiological role in only a minority of human cancers (Schiller and Lowy, 2010), the remainder of which often lack a single major risk factor. As vaccines against other malignancies do not have such a direct link with an exogenous and immunogenic source, they must accordingly be therapeutic in nature; i.e. aiming to reactivate and restimulate the immune system against an existing tumour (Waldmann, 2003).

1.4.2.1 Determination of cancer antigens

A number of methods are available for determination of cancer antigens. While MHC elution and direct immunology is discussed in detail in section 4.1, a range of other options (reverse immunology) are available, each with specific strengths and weaknesses. One of the earliest approaches, autologous serological typing (e.g. Pfreundschuh *et al*., 1978; Ueda *et al*., 1979), and its successor, SEREX (serological analysis of recombinant cDNA expression libraries) (e.g. Chen 2004; Li *et al*., 2004) both rely on patient antibodies, which may represent a different set of antigens to the cellular immune response, while the T-cell equivalent (e.g. Kawakami *et al*., 1994) identifies T-cell epitopes, it may not take into account changes in reading frame or cryptic epitopes produced by translational modifications (Ho and Green, 2006), as well as requiring a known antigen to

begin with. Non-immune driven approaches tend to rely on differential expression of protein, as determined by (e.g.) protein microarrays (e.g. Gunawardana and Diamandis, 2007) or 2D-PAGE (though the latter is more commonly applied to serum reactivity) (Le Naour, 2001). These alone do not provide data on immunogenicity, and this data is usually acquired using peptides derived from *in silico* prediction of proteasomal digestion and MHC-binding for the protein of interest; though these algorithms are not perfect and may exclude peptides which are found *in vitro* (*Pers. Comm*., Dr Roger Horton, Nottingham Trent University), or include peptides which are not in fact naturally processed (Wang, 1997). Peptides which are predicted to be processed are then investigated using a variety of methods, including peptide binding assays, and the response (proliferation, cytokine release, and for class-I, the killing of target cells) of peptide-specific T cells[33] to (e.g.) a cell line known to express the protein of interest and relative MHC allele(s). However these algorithms also do not currently account for post-translational modifications which may alter peptide immunogenicity or processing (see 1.4.4).

1.4.2.2 MHC class-I antigens in leukaemia

A number of MHC class-I epitopes have been identified with potential relevance to the leukaemias. Peptides from the bcr:abl fusion region which bind MHC alleles have been identified by a number of studies, many of which also demonstrated a CTL response (Bocchia *et al*., 1995; 1996; Greco*et al*., 1996; Pinilla-Ibarz *et al*., 2000; Yotnda *et al*., 2000; and Clarke *et al*., 2001). Bocchia *et al*. (1995) demonstrated three peptides which bound well to MHC class-I A3 and A11 and/or B8, while Posthuma *et al.* (1999) demonstrated that individuals with these MHC alleles showed a decreased risk of developing clinically detectable CML. Buzyn *et al*., (1997) demonstrated processing and immunogenicity of a peptide from the fusion protein's counterpart abl:bcr. Vaccine trials with the bcr:abl peptides (either alone or in conjunction with Imatinib, cytokine therapy, or stem cell transplants) are ongoing, along with investigations into other antigens such as Wilms Tumour-1, Heat Shock Protein Peptide Complex 96; Proteinase 3 and Survivin, and have been recently reviewed in Dao and Scheinberg (2008) Guilhot *et al*. (2008), and Pinilla-Ibarz *et al*. (2009).

[33] Often generated by immunisation of a murine model with a peptide of interest and harvesting of ($CD4^+$ / $CD8^+$) T cells from the thymus a few days later.

1.4.2.3 Phosphopeptides as MHC antigens

As the genetic instability typical of neoplasms may result in aberrant kinase expression and/or other proteins not normally expressed in the tissue (Gisselsson, 2005), phosphorylation patterns not seen in normal tissue may provide novel tumour antigens. If the kinase and substrate are not normally co-expressed, such antigens may be tumour specific.

Andersen *et al*. (1999) demonstrated that the MHC class-I antigen processing system was capable of presenting phosphorylated peptides and that $CD8^+$ T cells could be generated that were specific for the phosphorylated form of the peptides, while Zarling *et al*. demonstrated that they occurred *in vivo* (2000) and that malignant cells present a different set of peptides from non-malignant (2006). However the phosphorylated peptides identified typically presented poor predicted binding scores in algorithms such as SYFPEITHI (mirrored by findings at NTU) and it was found that phosphorylation altered MHC binding as well as altering the antigenicity of the peptide (Mohammed *et al*., 2008; Petersen *et al*., 2009). More recently phosphorylated peptides have also been shown to be presented by the MHC class-II complex and that again, the phosphate group alters binding to the complex (Depontieu *et al.,* 2009; Li *et al.,* 2010).

Given the strong links between aberrant kinase activity and leukaemia, it is considered that phosphorylated peptides may present a novel class of tumour antigens when presented by both MHC class-I and class-II. As previous work at NTU has focused on the elution of MHC-presented peptides from class-I and class-II complexes direct from the cell surface, as well as the use of various chromatographic techniques to fractionation of MHC-presented peptides, the application of these approaches towards identification of MHC-presented phosphopeptides may generate novel candidate peptides for immunotherapy.

1.5 Aims and Objectives

The aims of this research focus on the peptidomic aspects of cancer immunology: namely isolation of MHC class-I and class-II peptides from the surface of leukaemic cell lines, and the use of chromatographic fractionation to isolate and

concentrate the phosphorylated subpopulation, prior to identification of sequences by mass spectrometry.

As will be discussed, this involved three main aspects of research:

1) Modification of the BCA assay commonly used for protein quantification so as to improve applicability for estimation of peptide content.

2) Alteration of the buffer components used for cell-surface elution of MHC peptides to minimise cell lysis. This involved utilisation of the BCA assay above as one determinant of cell death.

3) Investigation into various chromatographic methods (both selective and generic) for concentration of phosphopeptides (using both synthetic peptides and tryptic digests of bovine casein), again heavily relying on the modified BCA assay for quantification.

Following on from this, identification of MHC-presented phosphopeptides was performed by LC-ESI-MS/MS, though immunological significance is not determined at this stage.

2.0 Materials and Methods

2.1 List of Materials

2.1.1 List of plasticware, glassware and chromatographic stationary-phases

Reagent/material	Supplier	Notes
Bond-Elut 18 SPE Columns	Varian	
C_{18} SPE columns		*See 'Bond-Elut' and 'Strata C_{18}'*
Cell culture plasticware	Sarstedt	T-25, and T-75 tissue culture flasks (vented), 6-well plates, cryovials.
	Greiner Bio-one	T-175 tissue culture flasks (vented).
FACS Test Tubes	Tyco Healthcare	
HEA Hypercel Resin	Pall	
HiLIC	*See HEA Hypercel, PPA Hypercel and Strata NH_2.*	
Hi-Trap Chelating HP columns (IMAC)	GE Healthcare	Iminodiacetate resin.
IMAC columns		*See 'Hi TrapChelating HP'*
IMAC column dismantling tools	Constructed by Mr Robert Davy, with thanks.	
Microplates (opaque)	Fisher Scientific	96-wells, flat-bottomed.
Microplates (transparent)	Sarstedt	96-wells, flat-bottomed.
Micro-pippette-tips	Sarstedt	P 2 µl – P 10 ml
PPA HyperCel resin	Pall	
Screw-top tubes	Sarstedt	15 and 50ml, rounded bottom
	Sterilin	20 ml, flat bottomed.
Sealed boiling ('Eppendorf') tubes	Sarstedt	0.5, 1.5 and 2 ml
Strata C_8 SPE columns	Phenomenex	Strata C8 SPE columns
Strata C_{18} SPE columns	Phenomenex	Strata C18 SPE columns
Strata NH2 SPE columns	Phenomenex	Strata NH2 SPE columns
Strata X SPE columns	Phenomenex	Strata X SPE columns
Strata X-AW SPE columns	Phenomenex	Strata X-AW SPE columns
Tissue culture flasks		*See 'cell culture'*

2.1.2 List of reagents, solvents and media components

Reagent/material	Supplier	Notes
Acetic acid	Fisher Scientific	Reagent grade
Acetone	Sigma Aldrich	HPLC grade
Acetonitrile	Fisher Scientific	HPLC grade
Adenylate kinase (AK)	Sigma Aldrich (*kindly donated by*	From chicken muscle. Foreign (non AK)

	Dr.s John Dickenson and Laurice Fretwell.)	activity below 1 %.
Adenylate kinase detection reagent (AKDR)	Lonza Biosciences	includes luciferase and luciferin
ATP	Sigma Aldrich	Supplied at 90 % purity.
ATP detection reagent (ADR)	Lonza Biosciences	includes luciferase and luciferin
Acrylamide	Geneflow	30 (w/v) 29:1 acrylamide:bis acrylamide
Aldolase	GE Healthcare	From rabbit muscle.
Ammonium bicarbonate	Sigma Aldrich	
Ammonium persulphate	Sigma Aldrich	
Ammonium sulphate	Sigma Aldrich	
anti-β2 microglobulin antibody	Sigma Aldrich	Raised in mouse
Ascorbic acid	Sigma Aldrich	
Asparagine	Sigma Aldrich	
BCA assay kit	Fisher Scientific	1 l kit. Includes bicinchoninic acid reagent (BCA component A) and 4 % (w/v) CuSO4 (B).
BSA	Sigma Aldrich	
Casein	Sigma Aldrich	Bovine
Catalase	GE Healthcare	Bovine
Citric acid	Fisher Scientific	
Chloroform	Fisher Scientific	HPLC grade
Coomassie Brilliant-Blue	Sigma Aldrich	
Copper Sulphate ($CuSO_4$)	Sigma Aldrich	
Deoxycholate	Sigma Aldrich	
Diethanolamine (DEA)	Fisher Scientific	
Dimethylsulphoxide (DMSO)	Sigma-Aldrich	Cell culture grade
Ethanol (EtOH)	Fisher Scientific	
Ethylenediamine-tetraacetic acid (EDTA)	Sigma-Aldrich	
$FeCl_3$ (Iron III chloride)	Sigma-Aldrich	
Foetal Calf Serum	Hyclone	Cell culture grade
Formic acid	Fisher Scientific	
G-418	BD Biosciences	
Glucose (D-glucose)	Sigma-Aldrich	
Glutamine / L-glutamine	Lonza Biosciences	200 mM. Cell culture grade.
Glutaraldehyde	Melford Laboratories	
Glutathione (reduced)	Sigma-Aldrich	Hygroscopic.
Glycerol	Fisher Scientific	
Glycine	Melford Laboratories	
Guinea pig liver	Kindly supplied by Dr David Munoz	
HEPES	Melford Laboratories	
Hydrochloric acid (HCl)	Fisher Scientific	HPLC grade
Inositol	Sigma-Aldrich	Cell culture grade
Isoton	Beckman Coulter	
Laemelli sample buffer	Sigma-Aldrich	2x concentration

Methanol (MeOH)	Fisher Scientific	HPLC grade.
Anti-mouse (FITC conjugated)	Sigma-Aldrich	
Anti-mouse (PE conjugated)	Sigma-Aldrich	
NaCl	Sigma-Aldrich	
Phosphate buffered saline (PBS)	Lonza Biosciences	Cell culture grade.
Phosphoric acid (PA)	Fisher Scientific	HPLC grade
Penicillin/streptomycin solution	Lonza Biosciences	5000 [units/ml]/[mcg/ml]. Cell culture grade.
Peptides/phosphopeptides (synthetic)	4mer (LSRH): NTU synthesised by Dr S Patwardhan. 9-10mers: Pepceuticals 20-27mers: Alta Biosciences	All synthesised to > 80 % purity.
Potassium Chloride	Sigma-Aldrich	
Pro-Q Diamond Phosphostain	Invitrogen (Molecular Probes)	
Propidium idodide (PI)	Sigma-Aldrich	
RPMI 1640	Lonza Biosciences	Cell culture grade (without L-glutamine)
Serine	Sigma-Aldrich	
Sodium acetate	Sigma-Aldrich	
Sodium doecyl sulphate (SDS)	Melford Laboratories	
Sodium Hydroxide (NaOH)	Sigma-Aldrich	
Sodium formate	Sigma-Aldrich	
(di)Sodium Phosphate Na_2HPO_4	Sigma-Aldrich	
Sucrose	Sigma-Aldrich	
Tetramethyl Ethylenediamine (TEMED)		
Toxilight assay buffer	Lonza Biosciences	pH 7.5
Tricine	Fisher Scientific	
Trichloroacetic acid (TCA)	Sigma-Aldrich	
Trifluoroacetic acid (TFA)	Sigma-Aldrich	
Trimethylamine (TMA)	Fisher Scientific	37 % (v/v)
Tris	Melford Laboratories	
Trypan blue suspension	Sigma-Aldrich	0.4 % (w/v) in PBS
Trypsin	Sigma-Aldrich	(Porcine)
Tween 20	Fisher Scientific	
Ultra low weight marker (ULM)	Sigma-Aldrich	Peptide PAGE ladder (26.6 – 1.06 kDa MW)[1]
W6/32 Antibody	Kindly purified in-house by Mr Stephen Reeder.	

[1] Composed of: Rabbit Triosephosphate Isomerase (26.6 kDa); Horse Cardiac Myoglobin (17 kDa); Bovine Aprotinin (6.5 kDa); bovine Insulin Chain B (3.5 kDa) and Bradykinin (1.06 kDa).

2.1.3 List of suppliers

Supplier	Address
Alta Bioscience	University of Birmingham, Edgbaston, Birmingham, B15 2TT.
BD Biosciences	21 Between Towns Rd, Cowley, Oxon, OX4 3LY UK.
Beckman Coulter UK, Ltd.	Oakley Ct, Kingsmead Business Park, High Wycombe, Bucks, HP11 1JU.
GE Healthcare.	Pollards Wood, Nightingales Ln, Chalfont St Giles, Bucks, HP8 4SP.
Fisher Scientific UK, Ltd.	Bishop Meadow Rd, Loughborough, Leics, LE11 5RG.
Geneflow, Ltd.	Fradley Business Centre, Wood End Ln, Lichfield, Staffs, WS13 8NF.
Greiner Bio-one, Ltd.	Brunel Way, Stroudwater Business Park, Stonehouse, Gloucs, GL10 3SX.
Hyclone UK, Ltd.	Unit 9 Atley Way, North Nelson Industrial Estate, Cramlington, Northumb., NE23 1WA
Invitrogen, Ltd.	3 Fountain Dr, Inchinnan Business Park, Renfrew, Renfrewshire, PA4 9RF
Lonza Biosciences (Lonza Group Ltd.)	Lonza Group Ltd. 228 Bath Rd, Slough, Berks, SL1 4DX
Melford Laboratories Ltd.	Bildeston Rd, Chelsworth, Ipswich, Suffolk, IP7 7LE
Pall Life Sciences	Europa House, Havant St., Portsmouth, Hamps, PO1 3TD
Pepceuticals Ltd.	Nottingham Biocity, Pennyfoot Street, Nottingham, NG1 1GF
Phenomenex Ltd.	Melville House, Queens Av, Macclesfield, Cheshire SK10 2BN
Sarstedt Ltd.	68 Boston Rd, Beaumont Leys, Leicester, Leics, LE4 1AW
Sigma Aldrich	Sigma-Aldrich Company Ltd., The Old Brickyard, New Rd, Gillingham, Dorset, SP8 4XT.
Sterilin, Ltd	Angel Ln, Aberbargoed, Bargoed, Caerphilly, CF81 9FW.

2.2 Cell Culture

Stock Solutions

A: RPMI 1640 (as supplied)

B: FCS (as supplied)

C: L-glutamine solution: 200 mM (as supplied)

D: Penicillin/streptomycin solution: 5000 [units/ml]/[mcg/ml] (as supplied)

E: Precept solution: 2 tablets dissolved in 500 ml dH_2O, plus the addition of 1 % (v/v) Tween (normal grade).

F: DMSO.

G: PBS: cell culture grade (as supplied)

H: 0.1 % (w/v) Trypan blue: prepared by diluting 0.4 % (w/v) Trypan blue suspension fourfold with PBS.

N.B.: All tissue culture was carried out using aseptic techniques in a Containment Level 2 laboratory. Solutions were all tissue culture grade unless stated otherwise.

2.2.1 Preparation of cell culture media

Culture media was prepared by the addition of the following per 500 ml bottle of RPMI 1640. Before addition to the media, foetal calf serum (FCS) was heat inactivated at 59 °C for 60 minutes.

Component	Stock concentration	Volume added	Final concentration	Role
Heat-inactivated Foetal calf serum (FCS)	100 % (v/v)	50 ml	10 % (v/v)	Provides necessary growth factors and hormones
L-glutamine solution	200 mM	5 ml	2 mM	Essential amino acid and secondary energy source
Pen/strep solution	5000 units/ml potassium penicillin and 5000 mcg/ml streptomycin sulphate	5 ml	50 units/ml potassium penicillin and 50 mcg/ml streptomycin sulphate	Penicillin: gram-positive bactericidal β-lactam antibiotic, Streptomycin: gram-negative bacteri-cidal aminoglycoside antibiotic.[2]

Table 2.1: Components added to RPMI 1640 cell culture media (500 ml), referred to hereafter as "complete RPMI 1640"

[2] Penicillin and streptomycin work synergistically, with penicillin enhancing the penetration of streptomycin into the bacterial cell wall (Winstanley & Hastings, 1989).

When necessary the antibiotic G-418 (prepared as a 500 mg/ml stock in PBS) was also added to a final concentration of 1 mg/ml for the culture of the transfected cell lines K562-A2, K562-A3 and T2.Dr4. G-418 is an aminoglycoside antibiotic toxic to mammalian cells lacking the gene APH 3` II (encoded as the reporter gene on the HLA-A2, HLA-A3 and HLA-Dr.4 plasmids), selectively killing those cells which have lost the gene of interest.

2.2.2 Culture of frozen cells

10^6 cells stored in a 1 ml aliquot of culture media (as above) plus 10 % v/v dimethylsulphoxide (DMSO) and frozen in liquid nitrogen were briefly thawed at room temperature and washed in 20ml RPMI 1640. The cells were then pelleted by centrifugation at 400 r.c.f. for 3 minutes using a refrigerated centrifuge (Eppendorf model 5702 R) and the supernatant discarded. Cell pellets were resuspended in 20 ml complete RMPI 1640 and seeded in a T-25 cell culture flask which was then cultured at 37 °C in a Heto Holten cellhouse 170 incubator supplied with a 5 % (v/v) CO_2 atmosphere. Size and number of flasks were increased as required by the growth of the cells in question. Aside from incubation and centrifugation, all work took place within a Walker Safety Cabinet laminar flow hood, model 1290 CLII.

2.2.3 Maintaining cell cultures

Suspension cells lines JY, K562-A3, LAMA-84, T2.Dr4, & THP-1, were used for the course of experimentation. Cells were cultured in close contact and were typically passaged every 3-4 days, with a minimum period of 24 hours (for high-confluency cultures) and a maximum period of no more than 9 days, due to the instability of glutamine, which has a half life of between 6.7-18 days (Ozturk & Palsson, 1990) and degrades into pyroglutamate and ammonium, which may be toxic to the culture depending on concentration (Schneider *et al*., 1996). The necessity to passage the cells was determined by the colour of the phenol red pH indicator present within the RPMI 1640.

Culture media was replaced by one of two methods. Firstly, the cell suspension could be transferred to a 20 ml universal or a 50 ml falcon tube, and centrifuged at 400 r.c.f. for 3 minutes, using either an Eppendorf model 5702 R refrigerated

centrifuge or a MSE Falcon model 6/300 refrigerated centrifuge, following which the supernatant was discarded and the pellet resuspended in a fresh volume of media prior to being returned to the culture flask. Whilst this allows nearly 100 % of the media to be changed per passage, it becomes a more demanding strategy in terms of time and disposable plasticware as culture volumes increase.

Alternatively, and more conveniently for large numbers of cells, the flask could be placed in a vertical position and the cells allowed to settle by gravity over the course of 2-3 hours, following which the top 75-90 % (v/v) of the culture media was aspirated off (taking care not to disturb the cell sediment), and replaced with fresh complete RPMI 1640.

Cells were sub-cultured 1:2 or 1:4 to maintain log phase growth (typically every 8-12 days), either by centrifugation and division of the cell resuspension, or by transfer of 25 - 50 % v/v of the cell suspension from one flask to another, each of which was then brought to maximum culture volume by the addition of fresh media. Aside from incubation and centrifugation, all work took place within a laminar flow hood (Walker Safety Cabinet model 1290 CLII) and sealed flasks were cultured at 37 °C in a Heto Holten cellhouse 170 incubator supplied with a 5 % (v/v) CO_2 atmosphere. All waste was inactivated by incubating at a ratio of no greater than 1:4 with a Precept:H_2O solution for > 1 hr at RT.

2.2.4 Long-term storage of cells in liquid nitrogen

When necessary cells were frozen in liquid nitrogen according to the following method:

2.2.4.1 Preparation of freezing media (RPMI 1640)

Freezing media was prepared by the addition of 2 ml 10 % (v/v) sterile DMSO (sterile filtered[3], hybridoma tested) to a complete RPMI 1640, typically to a final volume of 20-50 ml depending on the number of cells required. It should be noted however that while this was suitable for those cell lines frozen during the course of this research, other cell lines (or fragile cultures) may benefit from a

[3] DMSO dissolves the plastic in most disposable filters; therefore DMSO pre-filtered by the manufacturer is required, and must be kept sterile for obvious reasons.

higher concentration of FCS in the freezing medium (90 % (v/v) FCS, 10 % (v/v) DMSO, 0 % (v/v) RPMI is not unheard of).

2.2.4.2 Freezing cells

Before freezing cells were counted by trypan exclusion (see section 2.4.4.1), washed once in RPMI 1640, and resuspended in freezing media at a concentration of 10^6 per ml. This was then transferred to 1.5 ml cryovials which were then frozen first at -80 °C for 3 hours, then transferred to liquid nitrogen storage (monitored and replenished as needed) until they were next required.

2.3 Development of the BCA Peptide Assay

2.3.1 BCA protein assay

Stock Solutions

A: Bicinchoninic acid (BCA) component A (as supplied)

B: 4 % (w/v) $CuSO_4$ (as supplied)

C: 10 mg/ml BSA solution for standard curve preparation (as supplied)

2.3.1.1 Unmodified BCA protein assay protocol

A protein assay was performed by transferring 25 µl of each vortex-mixed sample to a clear, flat-bottomed 96-well microplate. When necessary the sample was diluted 1/5, 1/10, 1/20 or 1/50 in water or buffer (dilutions may be assayed as an alternative to, or in addition to the undiluted original) to compensate for high protein content, interfering substances, or a low pH.

A standard curve was prepared using the ampoule of 10 mg/ml BSA provided with the BCA kit. Dilutions are made ranging from 0.1-1 mg/ml in a matrix appropriate for the samples to be assayed (along with blanks), and transferred in 25 µl volumes to the 96 well microplate. For all quantitative assays, replicates of no less than 3 were carried out for both the sample(s) and standard curve.

BCA standard working reagent was then prepared by mixing bicinchoninic acid reagent (BCA component A) with 4 % (w/v) $CuSO_4$ (component B) in a ratio of 50:1. A 200 µl volume of this coloured reagent was then added in a randomised pattern to each well that contained a standard or sample, before the microplate was covered with sealing film, and incubated at 37 °C for 30 minutes, following which the microplate was unsealed, and the absorbance read at 570 nm in a Bio Rad microplate reader (model 680).

Where a time course was performed the plate was incubated unsealed within the microplate-reader (built-in incubator pre-warmed to 37 °C), and absorbances read every 5 minutes over a 120 minute period.

2.3.2 Investigation into the applicability of the BCA assay for the quantification of peptides

Stock Solutions:

A: Bicinchoninic acid (BCA) component A (as supplied)

B: 4 % (w/v) $CuSO_4$ (as supplied)

C: Synthetic peptide solutions (all to >80 % purity), dissolved in 100 % DMSO to 10 mg/ml. Sequences as per Table 2.3.1

D: Protein solutions (aldolase, BSA casein and catalase) for tryptic digestion, dissolved in 10 mM Tris-acetate pH 8 to 25 mg/ml.

E: Trypsin solution, dissolved in 10 mM Tris-acetate pH 8 to 10 mg/ml

2.3.2.1 Preparation of synthetic peptide standards for BCA assay

Synthetic peptide solutions in DMSO were diluted in appropriate reagents as required to produce working concentrations and standard curves from 0.015 – 1 mg/ml. Samples were then assayed as per 2.3.1.1. See chapter 3, table 3.2 for details of peptide sequences and physical properties.

2.3.2.2 Tryptic digestion of standard proteins

2.3.2.2.1 Preparation of tryptic digests of standard proteins for BCA peptide assay

Rabbit muscle aldolase, bovine serum albumin, bovine casein and bovine liver catalase were dissolved at 25 mg/ml in 10 mM Tris-acetate pH 8 and tryptically digested by the addition of 1 % (w/w) porcine trypsin. Samples were incubated at 37 °C for 72 hours to achieve maximal digestion, following which standard curves ranging from 0.05 – 0.5 mg/ml were prepared from these digests by diluting in water, or other reagents as required. Samples were then assayed as per 2.3.1.1, with or without denaturing in SDS-NaOH (see 2.3.4).

2.3.2.2.2 Preparation of low-concentration standard curves from tryptic digests of BSA to determine BCA peptide assay sensitivity

Bovine serum albumin was digested as per 2.3.2.2.1 and standard curves ranging from 0.005 – 0.05 mg/ml were prepared from these digests by diluting in water, or other reagents as required. Samples were then assayed with or without denaturing in SDS-NaOH (see 2.3.4), as per 2.3.1, or with the 25:1 bicinchoninic acid reagent (BCA component A):$CuSO_4$ ratio as per 2.3.5.1.

2.3.2.2.3 Preparation of weighted standards from tryptically digested casein

Bovine casein was digested as per 2.3.2.2.1 and serial dilutions prepared. These were then denatured and assayed according to 2.3.4. The tryptic digests were then quantified against standard curves prepared from the 9-10mer peptides, and assigned the average concentrations from the pooled peptide standards. The weighted digests were then dispensed in to aliquots and frozen at -20 °C.

In cases when a peptide subpopulation was expected, the weights were adjusted to reflect the relevant (e.g. phosphorylated) subpopulation.

2.3.3 Comparative solubilisation strategies for hydrophilic and hydrophobic peptides prior to BCA assay

Two synthetic peptides, SQKGQESEY (hydrophilic score 0.9) and YISPLKSPY (hydrophilic score -0.5) were utilised to investigate potential BCA-compatible solubilisation methods prior to the assay, with the aim of reducing the variations in inter-peptide reactivity.

Stock Solutions:

A: Bicinchoninic acid (BCA) component A (as supplied)

B: 4 % (w/v) $CuSO_4$ (as supplied)

C: Synthetic peptide solutions (all to >80 % purity), dissolved in 100 % DMSO to 10 mg/ml. Sequences as per Table 2.3.1

D: 100 % (v/v) DMSO

E: 100 % (v/v) methanol

F: 0.18 % (w/v) deoxycholic acid solution

G: 10 % (w/v) SDS solution

H: 200 mM disodium phosphate solution

G: 1 M sodium hydoxide solution

2.3.3.1 Peptide Solubilisation

Peptides were assayed at a final sample concentration of 0.2 mg/ml under the following conditions: **(A)** H_2O; **(B)** 0.15 % deoxycholic acid*; **(C)** 1 % SDS; **(D)** 5 % SDS*; **(E)** 1 % SDS + 10 % MeOH; **(F)** 5 % SDS + 10 % MeOH; **(G)** 50 mM disodium phosphate*; **(H)** 100 mM disodium phosphate*; **(I)** 1 % SDS + 0.1 M NaOH*; **(J)** 3 % SDS + 0.1 M NaOH*; **(K)** 5 % SDS + 0.1 M NaOH*; **(L)** 1 % SDS + 10 % MeOH; **(M)** 5 % SDS + 10 % MeOH; **(N)** 1 % SDS + 50 mM Disodium phosphate + 10 % MeOH; **(O)** 5 % SDS + 50 mM Disodium phosphate + 10 % MeOH; **(P)** 5 % SDS + 0.1 M NaOH + 10 % MeOH; **(Q)** 5 % SDS + 0.1 M NaOH + 10 % DMSO; and **(R)** 5 % SDS + 0.1 M NaOH + 10 % MeOH + 10 % DMSO.

The percentage concentration figures given represent (w/v) for SDS and deoxycholic acid, and (v/v) for DMSO and MeOH. ***** indicates treatment both at room temperature, and heat-denaturing for 5 minutes in a Biometric Uno Thermoblock set to 95 °C. Samples were assayed as per 2.3.1.

2.3.3.2 Data Analysis and Statistical Evaluation

In order to achieve cross-plate comparability, the absorbance for both the SQKGQESEY and YISPLKSPY peptide following each solubilisation regimen standardised against a third peptide (VHSATGFKQSSKALQRPVA-SD) at 0.3 mg/ml in water.

The adjusted absorbance values were then pooled and the standard deviation across both peptides calculated, under the hypothesis that a reduction in spread between the SQK and YIS would lead to a lower cross-peptide standard deviation. Once the final regime was decided upon (see 2.3.4), the comparative standard deviations of the pooled peptides with and without denaturing was compared using the student T-test to generate a statistically reliable result (n = 21).

2.3.4 BCA peptide assay (final method)

The following is the standard method adopted following the solubilisation experiments.

Stock Solutions:

A: Bicinchoninic acid (BCA) component A (as supplied)

B: 4 % (w/v) $CuSO_4$ (as supplied)

C: 10 M NaOH

D: 10 mg/ml synthetic peptide solutions (SQKGQESEY and YISPLKSPY peptides in DMSO)

2.3.4.1 Preparation of SDS-NaOH for BCA peptide assay

To prepare 20 ml of 2 % (w/v) SDS in 0.2 M NaOH (2x SDS-NaOH), 0.2 g SDS was dissolved in 15 ml pre-warmed dH_2O, and 200 µl of 10 M NaOH stock added. The solution was then brought to 20 ml with warm dH_2O, and filter-sterilised prior to storing in a sealed 20 ml sealed sample tube. The SDS-NaOH was pre-warmed in a 37 °C water bath for 30 minutes and gently agitated, to ensure that SDS was fully dissolved prior to each use, following which samples were diluted 1:1 with the 2x reagent and heat-denatured prior to analysis.

When a 1x solution was required this was prepared by diluting the X2 solution 1:1 with ddH_2O. This 1x solution was only used to dilute samples in a ratio of >20:1 SDS-NaOH : sample.

2.3.4.2 Heat-denaturation of peptide samples in SDS-NaOH

Sealed 500 µl microfuge tubes containing peptide samples diluted 1:1 in 2x SDS-NaOH were vortex-mixed, pulse-centrifuged at 20,800 r.c.f. and heated to 95 °C for five minutes in a Biometric Uno Thermoblock. Samples were then vortex mixed before being assayed as per 2.3.1.

2.3.5 Investigation of additional variations on the standard protocol

2.3.5.1 BCA assay with increased-copper standard working-reagent

In order to investigate the effect of higher $CuSO_4$ concentrations on the peptide BCA assay, BCA standard working reagents was also prepared in a ratio of 25:1 bicinchoninic acid reagent (BCA component A) to 4 % (w/v) $CuSO_4$ (component B). Samples were then assayed as per 2.3.1.1.

2.3.5.2 Analysis of samples over an incubation time course

Where a 120 minute time course was pursued, samples were prepared in H_2O or denatured in SDS-NaOH, and 25 µl transferred to a 96-well microplate in triplicate as previously described. However once BCA standard working reagent was added, the 96-well microplate was transferred directly to the Bio Rad microplate reader (model 680), where the built in incubator was pre-warmed to 37 °C. Readings were then taken every 5 minutes over a two hour period.

2.3.5.3 Microwave incubation

The method of Atkins and Tuan (1992) utilising microwave incubation as an alternative to the 37 °C incubation period was adapted to investigate its applicability to peptide quantification, and whether the period of microwave-incubation could also simultaneously replace the heat-denaturing step. A 600 w Hinari '*lifestyle*' model equipped with a rotating tray was utilised.

The effect of empty wells on microwave incubation was determined by adding 200 µl of H_2O to 24, 46 or all 96 wells within a microplate, which was then visually observed when heated for up to 60 seconds on full power. The effect of microplate location on the rotating microwave tray was also investigated, with plates filled with 200 µl of H_2O in all 96 wells placed in the centre or on the edge of the rotating tray, and again visually observed.

The final method established for even heating of the microplate without boiling required all unused wells to be filled with 200 µl of H_2O, following which the microplate was covered with sealing film and placed on the near edge of the microwave tray. The microwave was set to heat the microplate for 15 seconds, during which time the microwave tray would rotate though 180 °. The plate was then moved across the tray to its original position, with the result that the wells previously on the outer-edge of the rotating tray were now on the inner side, and subjected to a second 15 second incubation. Following this the plate was then allowed to cool to room temperature over the course of 2 minutes, then the seal removed[4] and the absorbance read at 570 nm in a Bio Rad microplate reader (model 680).

Using this method a tryptic digest of BSA, as well as the two synthetic peptides SQKGQESEY (hydrophilic score 0.9) and YISPLKSPY (hydrophilic score -0.5) were then assayed across a short standard curve of 0.1 - 0.5 mg/ml. Samples were assayed in water, or in SDS-NaOH (+/- heat denaturing), and the data analysed in a similar manner to 2.3.4.

[4] Care must be taken not to allow the microplate to boil, or the contents of the wells to expand to the point where capillary action draws liquid up out of the wells to mix on the underside of the seal, as this may alter both well volume, and lead to sample mixing, skewing results. Alternatively the microwave incubation may be attempted without the seal, however the effects of evaporation have not been evaluated.

2.3.5.4 Heat denaturation in BCA Reagent A + SDS

Stock Solutions

A: Bicinchoninic acid (BCA) reagent A (as supplied)

B: 10 % (w/v) SDS solution

C: 1 % (w/v) SDS solution

D: 10 mg/ml synthetic peptide solutions (SQKGQESEY and YISPLKSPY in DMSO)

E: 4 % (w/v) $CuSO_4$ (as supplied)

G: 2x SDS-NaOH (prepared as per 2.3.4)

Two synthetic peptides were dissolved to 0.066 mg/ml (final assay concentration) in reagent A with increasing concentrations of SDS as per table 2.3.2. Samples were aliquoted into three replicates of 250 µl, vortex-mixed, pulse-centrifuged at 20,800 r.c.f. and heated to 95 °C for five minutes in a Biometric Uno Thermoblock.

Both peptides (along with appropriate blanks) were also prepared to the same (final assay) concentration in water, and in SDS-NaOH and assayed with the other samples described above, as per 2.3.1 and 2.3.4 respectively.

Final SDS concentration	Synthetic Peptide Stock/ DMSO only	10 % (w/v) SDS solution	1 % (w/v) SDS solution	H20	BCA reagent A	Final Volume
0 % (w/v)	5 µl	0 µl	25.7 µl	92.5 µl	660 µl	757.5 µl
0.05 % (w/v)	5 µl	0 µl	51.8 µl	66.8 µl	660 µl	757.5 µl
0.01 % (w/v)	5 µl	20.6 µl	0 µl	40.7 µl	660 µl	757.5 µl
0.25 % (w/v)	5 µl	41.3 µl	0 µl	71.9 µl	660 µl	757.5 µl
0.50 % (w/v)	5 µl	82.5 µl	0 µl	51.3 µl	660 µl	757.5 µl
1.00 % (w/v)	5 µl	82.5 µl	0 µl	10 µl	660 µl	757.5 µl

Table 2.2: Preparation of synthetic peptides (and DMSO-only blanks) in BCA reagent A, with standard curve of increasing SDS concentration. Both SQKGQESEY and YISPLKSPY peptides were prepared.

$CuSO_4$ (4.5 µl of 4 % w/v) was added to each well of a transparent 96-well flat-bottomed microplate (excepting those wells required for BCA, which were plated out as per 2.3.1 or 2.3.4). To each of these wells 225 µl sample/blank was added, and the plate sealed and incubated at 37 °C for 30 minutes prior to reading absorbance at 570 nm as described in 2.3.1.

2.3.6 Peptide BCA of biologically-derived material

Stock solutions

A: 1 M Tris stock solution
B: 20 mM Tris-acetate pH 8.0
C: Guinea pig liver homogenate: (20 % w/v) in 20 mM Tris-acetate pH 8.
D: 10 mM TMA-HCl pH 8
E: Dubecco's phosphate buffered saline (isotonic and sterile as supplied)
F: 100 % Chloroform (as supplied)
G: 100 % Methanol (as supplied)
H: 37 % Hydrochloric acid (as supplied)
I: Acidified chloroform:methanol (as per Jackowski and Rock (1989)
J: 100 % Acetone (as supplied)
K: 72 % (w/v) Trichloroacetic acid (TCA)
L: Bicinchoninic acid (BCA) component A (as supplied)
M: 4 % (w/v) $CuSO_4$ (as supplied)
N: SDS-NaOH (2x).

2.3.6.1 Precipitation of protein by ammonium sulphate

10 ml of a guinea-pig liver homogenate was transferred to a sterile 50 ml beaker and 8 g of ammonium sulphate crystals were added (final concentration 80 % w/v) and allowed to dissolve at 4 °C overnight. Precipitated material was collected by transferring this material to sealed 2 ml microfuge tubes and centrifuging at 10,000 r.c.f. at 4 °C for 45 minutes, after which the supernatant was discarded, the pellets transferred to sterile dialysis tubing and dialysed against a volatile digestion buffer (10 mM TMA-HCl pH 8) for 24 hours at 4 °C, during which time the buffer was changed regularly.

2.3.6.2 Cell lysis and Precipitation of protein by acidified chloroform:methanol

T2 and JY cell lines were cultured as per section 2.2 to populations of approximately 5 x 10^8. Cells were transferred to sealed 50 ml solvent-proof tubes and washed twice with PBS to remove serum proteins. Cellular protein was

then extracted by the method developed by Jackowski and Rock (1989); 25 ml acidified chloroform:methanol was added to the cell pellet and thoroughly vortex-mixed. Following this the lysate began to separate into two phases, a clear bottom-phase, and a debris-rich top-phase. Cell lysates were then centrifuged at 10,000 r.c.f. for 60 minutes at 4 °C to allow the formation of a protein plug between the two phases. The nucleotide-, peptide- and carbohydrate-rich methanolic top-phase was aspirated and frozen at -80 °C, and the plug removed with tweezers and washed twice in 20 ml ethyl acetate before being dried under inert gas (N_2). The lipid-rich chloroform bottom-phase was discarded.

Cell pellets were then transferred to sterile dialysis tubing and dialysed against a volatile digestion buffer (10 mM TMA-HCl pH 8.0) for 24 hours at 4 °C, during which time the buffer was changed regularly.

2.3.6.3 Tryptic digestion of biologically derived material

Once dialysis was complete the contents of the tubing was transferred to sealed 20 ml tubes and the protein concentration estimated by protein BCA (see 2.3.1). Following this, 1 % (w/w) porcine trypsin was added and the samples incubated at 37 °C for 72 hours (see 2.3.2.2).

2.3.6.4 Acetone precipitation of undigested material

Following trypsinisation, undigested protein was precipitated by the addition of 9 volumes of ice-cold acetone to the sample, which was incubated at -20 °C overnight, and then centrifuged at 10,000 r.c.f. for 20 minutes at 4 °C, following which the supernatant was dried under inert gas (N_2), and the peptides weighed out and redissolved at 10 mg/ml in DMSO, before being stored at -80 °C pending dilution in water or SDS-NaOH for assaying by BCA. Following the absorbance being read at 570 nm, the data was analysed in a similar manner to 2.3.4, though T-tests were not performed.

2.3.6.5 Trichloroacetic acid-precipitation of polypeptides and proteins

When necessary proteins and large polypeptides were removed from samples prior to assaying by the addition of 1 volume of 72 % (w/v) TCA stock : 9 volumes of sample, bringing the final concentration to 7.2 % (w/v) TCA. Precipitated material was then removed by centrifugation at 10,000 r.c.f. for 10 minutes at 4 °C, and the supernatant diluted 1:5 in ddH_2O before being mixed 1:1 with 2x SDS-NaOH, and assayed against a standard curve also containing 0.72 % (w/v) TCA, diluted 1:1 in SDS-NaOH.

2.4 Cell Surface Elution Methodology

2.4.1 Preparation of MHC elution buffers

N.B.: While dissolution of powdered reagents often took place either at RT or using ddH_2O pre-warmed to higher temperatures, all solutions involved in MHC elution were brought to pH at 4 °C, and used at this temperature, on the principle that lower temperatures minimises membrane trafficking of receptors, and thus prevents or reduces MHC-internalisation during elution.

2.4.1.1 Preparation of isotonic citrate-phosphate buffers ('Storkus buffer') for elution of MHC class-I or pH 5.5 prewash

Stock Solutions:

A: 0.131 M disodium phosphate (isotonic solution)

B: 0.066 M citric acid (isotonic solution)

Once both solutions were cooled to 4 °C, the disodium phosphate was brought to pH (typically pH 3.3) with citric acid, producing an isotonic buffer. This was then sterile filtered, and used within 4 hours of preparation.

2.4.1.1.1 Supplemented citrate-phosphate

A version of the isotonic citrate-phosphate pH 3.3 ("Storkus buffer") and pH 5.5 ("prewash buffer") were also prepared containing those supplements added to

the TMA formate and sodium-formate buffers (excluding the sucrose and KCl). This was prepared as per 3.1.1, except with the addition of 10 mM D-glucose, 2 mM *myo*-inositol and 1 mM reduced glutathione in each of the buffers.

2.4.1.2 Preparation of TMA-formate MHC class-I elution buffer

A 250 ml volume of elution buffer was prepared by mixing 0.998 ml (37 %) TMA (final concentration 25 mM); 25.67 g sucrose (final concentration 0.3 M); 8.39 g KCl (final concentration 0.45 M); 0.9 g D-glucose (final concentration 10 mM); 0.18 g myo-inositol (final concentration 2 mM); and 0.08 g glutathione (final concentration of 1 mM) in 225 ml ddH_2O. This solution was cooled to 4 °C, and the pH adjusted to 3.3 with formic acid before being brought to 250 ml with ddH_2O. The buffered solution was then sterile filtered prior to use, which took place within 4 hours of preparation.

2.4.1.2.1 TMA-formate MHC class-I elution buffer variants

In developing the final minimal-lysis MHC class-I buffer, variations on this buffer composition were investigated, including various concentrations of the supplements as illustrated in table 2.3 below.

Supplement	Concentrations tested
Sucrose	0, 0.075, 0.15, 0.3, 0.45 and 0.6 M
KCl	0, 0.075, 0.15, 0.3, 0.45, 0.6, and 0.75 M
Glucose	0, 0.5, 2, 5, 10, 20, 30 and 50 mM
Inositol	0, 0.5, 1, 2, 4 and 5 mM
Glutathione	0, 0.2, 0.5, 1, 2, 4 and 5 mM
Ascorbic acid	0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 mM

Table 2.3: Range of supplement concentrations investigated for MHC class-I elution buffer.

Apart from the differing concentrations of the supplements, these were prepared identically to the TMA-formate buffer described in 2.4.1.3.

2.4.1.3 Preparation of sodium-formate MHC class-II elution buffer

A 250 ml volume of class-II elution buffer was prepared by dissolving: 0.85 g sodium-formate (final concentration 50 mM); 25.67 g sucrose (final concentration 0.3 M); 8.39 g KCl (final concentration 0.45 M); 0.9 g D-glucose (final concentration 10 mM); 0.18 g myo-inositol (final concentration 2 mM); and 0.08 g glutathione (final concentration of 1 mM) in 225 ml ddH_2O. This solution was cooled to 4 °C, and the pH adjusted to 2 with formic acid before being brought to 250 ml with ddH_2O. The buffered solution was then sterile filtered prior to use, which took place within 4 hours of preparation.

2.4.1.3.1 Sodium-formate MHC class-II elution buffer variants

In developing the final minimal-lysis MHC class-II buffer, variations on this buffer composition were investigated, including various concentrations of the supplements as illustrated in table 2.4.2.

Apart from the differing concentrations of the components, these were prepared identically to the sodium-formate buffer described in 2.4.1.3.

Supplement	Concentrations tested
Sodium formate	50, 100 and 200 mM
Sucrose	0, 0.15, 0.3, and 0.45 M
KCl	0, 0.3, 0.45, and 0.6 M
Glucose	0, 5, 10, and 20
Inositol	0, 1, 2, and 4 mM
Glutathione	0, 0.5, 1, and 2 mM

Table 2.4: Range of supplement concentrations investigated for MHC class-II elution buffer

2.4.2 Cell surface elution protocol

In order to obtain enough material to identify peptide sequences by mass spectrometry, large numbers of cells are required. While this figure is ideally in excess of 1×10^9 (Bonner *et al*., 2002) smaller populations may be used if there is confidence that the peptide in question is highly expressed. Here all cultures were grown to $> 1.8 \times 10^9$, in duplicate. In developing the minimum lysis buffers,

smaller figures of 1-10 x 10^6 cells were used in triplicate or greater, and the buffer volumes scaled down to the 1–2 ml range, but the eluate was not carried through to mass spectrometry.

2.4.2.1 Elution of MHC class-I by isotonic citrate-phosphate pH 3.3 ('the Storkus method')

Stock Solutions:

A: Citrate-phosphate pH 3.3

B: Cell culture-grade PBS.

Following a cell count (see 2.4.4), the cell population is pooled by centrifugation at 500 RCF for three minutes into the minimum feasible number of sealed 50ml sample tubes and washed twice with sterile PBS. These washes are discarded and the citrate-phosphate buffer added to the cells and gently mixed by hand for one minute, before being centrifuged again. The supernatant is retained and stored at -80 °C.

2.4.2.2 Elution of MHC class-I by TMA-formate minimal-lysis buffer

Stock solutions

A: Citrate-phosphate pH 3.3

B: RPMI 1640 (as supplied by Lonza Biosciences)

C: Isotonic saline solution (0.9 % v/v NaCl)

D: 25 mM TMA-formate pH 3.3 MHC class-I elution buffer (prepared as per 2.4.1.2, unless stated otherwise).

Following a cell count, the cell population was pooled by centrifugation at 500 RCF for three minutes into the minimum feasible number of sealed 50 ml sample tubes and washed with 25 ml isotonic citrate-phosphate pH 5.5 to lyse fragile cells and deplete potential contaminants. The cell suspension was then brought back to physiological pH by two washes with 50 ml RPMI 1640, and then de-buffered by washing twice with isotonic saline. Following this, 15 ml MHC class-I elution buffer is added to each 50 ml tube, and the suspension gently mixed by hand for one minute, before being centrifuged again. The cells were not exposed to the elution buffers for a period of less than four, or greater than five minutes.

The supernatant was retained and transferred to 2 ml sealed boiling tubes before being clarified by centrifuging a second time at 20,800 RCF for fifteen minutes to remove any cellular debris or large protein complexes which may have carried over. The clarified supernatant was then repooled and stored at -80 °C until required.

2.4.2.2.1 Elution of MHC class-I from adherent ALC cells by TMA-formate minimal-lysis buffer.

These were eluted as per 2.4.2.2.1 with the following modifications. 2 x 10^8 cells[5] provided in T-75 tissue culture flasks were washed with 2x RPMI 1640, and de-buffered with 2x washes of isotonic saline. No prewash was performed. 30 ml supplemented 25mM TMA-formate pH 3.3 was added to the first flask, the cells gently washed with the buffer for 60 seconds, and then transferred to the second flask, and so on in a serial manner. This eluate was then centrifuged at 500 RCF for three minutes, and the supernatant then transferred to 2 ml sealed boiling tubes. These were then clarified by centrifuging a second time at 20,800 RCF for fifteen minutes to remove any cellular debris or large protein complexes which may have carried over. The clarified supernatant was then repooled and stored at -80 °C until required.

2.4.2.3 Elution of MHC class-II by sodium-formate minimal-lysis buffer

Stock solutions:

A: Isotonic citrate-phosphate pH 5.5

B: RPMI 1640

C: Isotonic saline solution (0.9 % v/v NaCl)

D: 50 mM sodium-formate pH 2 MHC class-II elution buffer

E: 25 mM TMA-formate pH 3.3 MHC class-I elution buffer

OR Isotonic citrate-phosphate pH 3.3

Following MHC class-I elution, above, 25 ml isotonic citrate-phosphate pH 5.5 was added to each cell pellet, which is then disrupted by gentle agitation. The cell suspension was then brought to 50 ml with RPMI 1640, pelleted by centrifugation, and washed once more with 50 ml RPMI 1640. This process was

[5] Kindly donated by Dr Selman Ali and Ms Anisha Bannerjee (Nottingham Trent University)

repeated to ensure full pellet disruption, and the cell suspension was then de-buffered by washing twice with isotonic saline. Following this, the MHC class-II peptides were eluted with 15 ml (per tube) sodium-formate and centrifuged to pellet the cell population (again the cells were not exposed to the elution buffer for a period of less than four, or greater than five minutes). The supernatant was retained and transferred to 2 ml sealed boiling tubes before being clarified by centrifuging a second time at 20,800 RCF for fifteen minutes to remove any cellular debris or proteins which may have carried over. The clarified supernatant was then repooled and stored at -80 °C until required.

If class-I peptides are not required for analysis, isotonic citrate-phosphate pH 3.3 may be utilised to elute them from the cell surface prior to class-II elution, instead of TMA-formate. This also eliminates the need for the pH 5.5 prewash steps.

2.4.3 Flow cytometry analysis to confirm cell surface elution

In order to determine whether the TMA-formate buffer leads to disruption of the MHC class-I complex, flow cytometry was utilised using the monoclonal α-MHC class-I antibody W6/32 (produced from hybridoma in-house, and kindly purified by Mr Steve Reeder), and a mouse anti-β2 microglobulin antibody.

2.4.3.1 Cell surface elution and staining of cells for flow cytometry

Stock Solutions:

A: FACS Buffer

B: W6/32 Primary monoclonal anti-MHC class-I antibody

C: Anti β2M Antibody

D: FITC-conjugated anti mouse secondary antibody

E: MHC class-I elution buffers. Prepared as per 2.4.1 with the following modifications: The TMA-formate and isotonic citrate-phosphate buffers were prepared as per 2.4.1, except in 20 ml volumes along a pH gradient with the following pH values: 7.5; 7; 6.5; 6; 5.5; 5; 4.8; 4.6; 4.4; 4.2; 4.0; 3.8; 3.6; 3.5; 3.4; 3.3; 3.2; 3.1; 3.

F: Isotonic saline solution (0.9 % v/v NaCl).

The following elution protocols were investigated:

1) An isotonic citrate-phosphate pH gradient with the K562-A3 cell line.
2) A TMA-formate pH gradient with both the K562-A3 and JY cell lines.
3) An untreated control for both K562-A3 and JY cell lines (washed with PBS only)

For each buffer pH, 4 x 10^6 cells were eluted as per the relevant sections of 2.4.2 (though the pH 5.5 prewash step was not utilised in this instance), and washed twice by centrifuging at 500 RCF for three minutes, then resuspending in PBS. Each cell suspension was then divided in two for staining against either MHC class-I expression or β2 microglobulin, and transferred to FACS tubes before being then washed twice in FACS buffer.

The cell suspension was gently mixed and resuspended with 100 µl FACS buffer before 5 µl primary antibody was added, and the cells incubated on ice for 30 minutes.

Following this, the cells were washed 2x with FACS buffer, and then 20 µl of secondary antibody, a fluorescein isothiocyanate (FITC) conjugated (rabbit) anti-mouse polyconal, was added and the cells again incubated on ice for 30 minutes, and protected from light. Once incubation was complete, the cells were again washed 2x with FACS buffer and resuspended in 1 ml isoton solution before being subjected to analysis by flow cytometry.

2.4.3.2 Flow cytometry analysis of cells following elution

Following staining the distribution of relative fluorescence within the cell resuspensions were analysed using a Beckman Coulter flow cytometer.

2.4.4 Determination of cell viability following elution protocols

In developing the minimum lysis MHC class-I and –II elution buffers, various methods of measuring cell death were employed.

2.4.4.1 Post-elution determination of cell viability by trypan blue exclusion

In order to optimise the TMA-formate MHC class-I elution buffer for minimal cell death, trypan blue staining was utilised.

TMA-formate buffers containing the range of supplement concentrations detailed in Table 2.4.1 were prepared as per 2.4.1.2.1. Two million cells were then eluted in replicates for each buffer as per the relevant sections of 2.4.2, washed twice with RPMI 1640 by centrifuging at 500 RCF for three minutes, then resuspended in 2 ml RPMI 1640.

Following this, 20 µl of each cell suspension was assayed for viability by trypan blue exclusion, as per 2.4.4.1. The percentage dead cells within the haemocytometer was then calculated by the following formula:

(Mean number of stained cells counted / total number of cells counted) x 100

Again, each sample was eluted, washed, prepared and counted prior to preparation of the next, in order to minimise variations in Trypan exposure.

Once the buffer supplements were optimised, the addition of these into the sodium-formate pH 2 MHC class-II elution buffer was also investigated, along with a reduction in buffer molarity from 200 mM to 50 mM, as per 2.4.1.3.1.

2.4.4.2 Post-elution determination of cell viability by propidium iodide exclusion

2.4.4.2.1 Preparation of a phycoerythrin-conjugated colour control

In order to calibrate the flow cytometer to the excitation and emission spectra for PI, an appropriate colour control was prepared using a secondary antibody conjugated to the fluorochrome phycoerythrin (PE), which has a similar emission spectra to PI (575 nm, compared with 550 nm for PI). Cells were labelled with the W6/32 α-MHC class-I monoclonal antibody as detailed in 2.4.3.1 (no MHC elution steps were performed on this sample), with a PE-conjugated secondary antibody taking the place of the FITC-conjugated antibody described.

2.4.4.2.2 Analysis of cell viability by propidium iodide

TMA formate and sodium-formate buffers (supplemented or unsupplemented) were prepared, along with isotonic citrate-phosphate buffers, pH 3.3 and 5.5, PBS and isotonic saline (see section 2.4.1). Cells were eluted as per the protocols detailed in 2.4.2, after which they were washed twice with FACS buffer, before being resuspended in 0.2 ml Isoton. Controls took the form of an untreated cell population and a heat-killed population heated to 95 °C for 20 minutes, then cooled to room temperature.

Twenty µl of a 100 µg/ml PI solution was added to each sample, which were then incubated on ice for 2 minutes, protected from light, after which the volume was brought to 1 ml with Isoton, the cells pelleted by centrifugation at 500 RCF for three minutes, the supernatant discarded, and the cell pellet resuspended in 1 ml isoton solution (Beckman Coulter, UK) (again protected from light), before being analysed by flow cytometry using an excitation and emission wavelength of 488 and >550 nm respectively.

2.4.4.3 Determination of peptide/protein loss during elution as a marker for cell damage

Stock solutions

A: Isotonic citrate-phosphate pH 3.3

B: Isotonic citrate-phosphate pH 5.5

C-D: 25 mM TMA-formate pH 3.3: unsupplemented (without osmotic balancing)/osmotically balanced (without glucose, inositol or glutathione).

E-F: 50 mM sodium-formate pH 2: unsupplemented (without osmotic balancing)/osmotically balanced (without glucose, inositol or glutathione).

G: Isotonic saline solution (0.9 % v/v NaCl)

H: Phosphate buffered saline

2.4.4.3.1 Elution of MHC class-I and II

Replicates of 10 x 10^6 cells were prepared in RPMI 1640, washed twice in PBS and eluted by the protocols illustrated in 2.4.2, with the following modifications:

PBS was utilised instead of RPMI 1640. The elution took place using a 600 µl volume, of which 400 µl was aspirated following cell centrifugation, and taken for analysis by peptide-BCA assay (see 2.4.4.3.2).

2.4.4.3.2 Analysis by modified BCA

For each replicate, 10 µl of the MHC elution supernatant was diluted in 40 µl ddH_2O and this then mixed with 0.2M NaOH and 2 % (w/v) SDS in a 1:1 ratio. This 100 µl mixture was then heat-denatured and assayed as per Kapoor *et al*. (2009) (method detailed in section 2.3.4), with appropriate blanks and a weighted casein digest standard curve.

2.4.4.4 Investigation into the applicability of the ToxiLight cell viability assay to determine cell death during MHC elution

2.4.4.4.1 Denaturing and renaturing of adenylate kinase

Stock solutions:

A, B, C:	Isotonic citrate-phosphate pH 7.4, 5.5, and 3.3
D, E, F:	25mM TMA formate (unsupplemented) pH 7.4, 5.5, and 3.3
G:	RPMI 1640 cell culture medium (as provided; free of serum, L-glutamine and penicillin/streptomycin.
H:	2 mM HEPES pH 7.4
I:	ToxiLight assay buffer (ass supplied)

200 units of chicken muscle AK (Sigma Aldrich, UK) was dissolved in 100 µl of 2 mM HEPES pH 7.4. Five microlitres of this solution was then vortex-mixed with 20 microlitres of buffer (TMA-formate / citrate-phosphate or RPMI 1640 media), incubated at 4 °C for five minutes, and then brought back to pH 7.4 by diluting 1:10 with ToxiLight assay buffer (optimal ratio predetermined using pH paper). This was then compared with a sample prepared in ToxiLight buffer alone.

2.4.4.4.2 Determination of renatured adenylate kinase activity

Stock solutions

A: Adenylate kinase detection reagent (AKDR)

25 µl of the rebuffered AK solution was then tranferred to a white opaque-bottomed 96-well plate in triplicate. To this, 100 µl of AKDR was added, and the plate incubated for five minutes at 37 °C before being transferred to a Fluostar OPTIMA luminometer / fluorimeter, the sum luminescence was read over 10 cycles (11.4 minutes) at 22 °C.

2.4.4.5 Determination of ATP loss during MHC elution as a marker for cell damage

Stock solutions

A: Isotonic citrate-phosphate pH 3.3
B: Isotonic citrate-phosphate pH 5.5
C: Unsupplemented 25 mM TMA-formate pH 3.3
D: Osmotically balanced 25 mM TMA-formate pH 3.3
E: Fully supplemented 25 mM TMA-formate pH 3.3
F: Unsupplemented 50 mM sodium-formate pH 2
G: Osmotically balanced 50 mM sodium-formate pH 2
H: Fully supplemented 50 mM sodium-formate pH 2
H: Isotonic saline solution (0.9 % v/v NaCl)
I: Cell-culture grade PBS (as supplied)
J: 5 mM ATP stock
K: 5 mM Tris acetate pH 7.8
L: ATP detection reagent (ADR): stock solution
M: ATP detection reagent (ADR): working solution

2.4.4.5.1 Determination of relative intracellular ATP content

In order to establish the intracellular ATP content of the four cell lines under investigation, 5 x 10^6 cells were washed twice with 2 ml 0.9 % (w/v) saline, before being resuspended in 2 ml ddH_2O. These suspensions were then heat-killed by immediately subjecting to 95 °C for ten minutes, then vortex-mixed for

one minute before being subjected to a second 95 °C incubation for another ten minutes, in a method similar to one of those employed by Bagnara and Finch (1972). Following this, the suspensions were clarified by centrifuging at 20,800 RCF for thirty minutes, after which the top 1.5 ml of supernatant was aspirated and immediately transferred to -80 °C until the was required for analysis.

Analysis was carried out using opaque 96-well microplates. Replicates of 5 µl were transferred to each well and mixed with 20 µl of 5 mM Tris-acetate pH 7.8. To this 175 µl of ADR working solution was added, and the plate gently agitated for 10 seconds before being loaded into a Fluostar OPTIMA set to incubate plate at 25 °C[6], and the cumulative luminometry measured over 15 240-second cycles.

2.4.4.5.2 MHC Elution for ATP assay

The elution protocol was carried out as per 2.4.4.3.1, with the following modifications: 5 x 10^6 cells were utilised for each elution, in a 0.6 ml elution volume, and once the supernatant was aspirated it was clarified by centrifuging at 20,800 RCF for twenty minutes, following which the top 200 µl of supernatant was aspirated to a fresh 500 µl sealed boiling tube and immediately transferred to -80 °C.

2.4.4.5.3 Choice of assay buffer

Stock solutions:

A - F: 0.02, 0.05, 0.1, 0.2, 0.5 and 1 M Tris-acetate pH 7.8

G-L: 0.02, 0.05, 0.1, 0.2, 0.5 and 1 M HEPES-HCl pH 7.8

M: 25 µM ATP

N: ATP detection reagent (ADR): working solution

Standard molarity curves of both Tris and HEPES buffers were assessed for compatibility with the Luciferase assay.

5 µl of 25 µM ATP was transferred to each well of a white opaque 96-well flat-bottomed microplate. This was followed by 120 µl of buffer (in triplicate), and 75 µl of ADR working solution. The plate was then gently agitated for 10 seconds

[6] Optimum temperature for firefly luciferase (Gális & Jirásková, 1992)

before being loaded into a Fluostar OPTIMA set to incubate plate at 25 °C, and cumulative luminometry was measured over twenty 180-second cycles.

2.4.4.5.4 Determination of optimum buffer : eluate ratios

Stock solutions:

A: 0.2 M HEPES-HCl pH 7.8

B: 1 M NaOH

C: Elution buffers (as 2.4.4.5)

Volumes of unsupplemented elution buffers were titrated against 0.2 M HEPES, with or without the addition of different volumes of NaOH to increase pH above the pKA of the elution buffer. The resulting pH was then assessed first using universal indicator pH paper and then confirmed using a pH meter. Once the optimum ratios to provide a pH of 7.8 were determined, the ability to rebuffer the osmotically balanced and fully supplemented elution buffers were also then confirmed. To ensure full rebuffering the ratio of HEPES to buffer was then doubled before use with the assay.

2.4.4.5.5 Compatibility of elution buffers with the luciferase-determined ATP assay

Stock solutions:

A: 0.2 M HEPES-HCl pH 7.8

B: 25 µM ATP

C: ATP detection reagent (ADR): working solution (2)

D: Elution Buffers (as 2.4.4.5)

MHC class-I TMA-formate buffers were diluted 1:4 in 0.2 M HEPES, while citrate-phosphate buffers and MHC class-II sodium-formate buffers were diluted 1:9. In each of these buffer mixes standard concentration curves (0.1 – 0.5 µM) of ATP were prepared, along with an ATP-negative blank.

25 µl of sample was transferred in triplicate to a white opaque 96-well flat-bottomed microplate. Once all samples were laid out within the plate, they were followed by 175 µl ADR working solution. The plate was then gently agitated for

10 seconds before being loaded into a Fluostar OPTIMA set to incubate plate at 25 °C, and cumulative luminometry was measured over twenty-five 180-second cycles.

2.4.4.5.6 Luciferase-determined assay of MHC eluate ATP content.

Stock solutions:

A: 0.2 M HEPES-HCl pH 7.8

B: 25 µM ATP

C: ATP detection reagent (ADR): working solution (2)

D: Elution Buffers (as 2.4.4.5)

E: MHC class-I and class–II eluates (as 2.4.4.5.1)

MHC eluates were diluted with 0.2 M HEPES as per 2.4.4.5.5. Again standard concentration curves (0.1 – 0.5 µM) of ATP were prepared in matching buffer mixes, along with ATP-negative blanks, and the opaque 96-well plates assayed for ATP as per 2.4.4.5.5.

2.4.5 Potential to re-culture cells post elution

Following on from determining cell viability, the possibility of re-culturing a cell population after elution of MHC class-I and/or class-II was investigated, with a view to the potential for a single population to provide multiple elutions.

2.4.5.1 Reculture potential determined by media turnover

Following on from determining cell viability, the possibility of re-culturing a cell population after elution of MHC class-I and/or class-II was investigated, with a view to the potential for a single population to provide multiple elutions.

Stock solutions:

A: Isotonic citrate-phosphate pH 3.3

B: Isotonic citrate-phosphate pH 5.5

C-E: 25 mM TMA-formate pH 3.3 (unsupplemented / osmotically balanced /fully supplemented)

F-H 50 mM sodium-formate pH 2 (unsupplemented / osmotically balanced / fully supplemented)

I: Isotonic saline solution (0.9 % v/v NaCl)

J: Cell-culture grade RPMI 1640

K: Complete RPMI 1640.

Two x 10^6 cells were eluted in triplicate as per 2.4.2, producing cell populations which had been eluted for class-I by citrate phosphate or TMA-formate buffers ($^+/_-$ supplements) and for class-II by first eluting MHC class-I in citrate-phosphate pH 3.3, then eluting class-II in sodium-formate ($^+/_-$ supplements).

Once eluted, cells were washed once with citrate-phosphate pH 5.5, then twice with RPMI 1640, before being recultured in complete RPMI 1640 in covered six-well culture plates incubated at 37 °C in a Heto Holten cellhouse 170 incubator supplied with a 5 % (v/v) CO_2 atmosphere, and the plates monitored for a change in media colour over the next 7 days.

2.4.5.2 Post-elution cell proliferation determined by [3H]-thymidine incorporation

Stock solutions:

A: MHC elution buffers (excluding the sodium-formate buffers)

B: Cell-culture grade PBS

C: 'Complete' RPMI 1640

D: Tritiated thymidine solution

E: Scintillation fluid

One million cells were treated with MHC class-I elution protocols using citrate phosphate, or TMA-formate ($^+/_-$ supplements) buffers in triplicate. Elutions took place as per 2.4.2, though in 1 ml volumes. These cells were then washed twice in RPMI 1640 and recultured in complete RPMI 1640 for 48 hours in 6 well plates, as per 2.4.5.1.

Following this forty-eight hour period, cells were counted and resuspended in fresh complete RPMI 1640. From these resuspensions 1 x 10^3 cells from each treatment were plated out in triplicate into a 96 well microplate. Each well was

then treated with tritiated thymidine to a final concentration of 37 MBq/µl and incubated for 18 hours prior to harvesting.

Following incubation cells were harvested onto a 96-well filter plate and 40 µl scintillation fluid added to each filter well, following which a Top-Count Scintillation counter was utilised to perform counts over 1 minute for each well.

2.5 Optimisation of chromatography

2.5.1 Immobilised Metal Affinity Chromatography (IMAC)

2.5.1.1 Fe^{3+} IMAC

Stock Solutions:

A: 15 mM $FeCl_3$ in ddH_2O.
B: Wash buffer (1): 25 mM TMA-formate, pH 3.3 (unsupplemented).
C: Tryptic digests of casein (prepared as per 2.3.2.2) and brought to 25 mM TMA-formate, pH 3.3.
D: Elution buffer (1): 100 mM DEA-HCl, pH 8.4.
E: 50 mM EDTA.
F: ddH_2O
G: Storage solution: 20 % (v/v) ethanol in ddH_2O
H: Wash buffer (2): 5 mM TMA-formate, pH 3.3 (unsupplemented).
I: Elution buffer (2): 100 mM DEA-HCl, pH 9.0.
J: Elution buffer (3): 50 mM DEA-HCl, pH 9.0.
K: Elution buffer (4): 100 mM DEA, pH 10.45.

N.B: Unless stated otherwise, each wash of change in solution took place using >5 ml (five column volumes) to ensure full alteration of intra-column conditions. With the exception of the sample each solution was sterile filtered prior to use. Flow rates were maintained at 0.5 ml/min.

2.5.1.1.1 Standard method

Hi-Trap-chelating HP IMAC columns were connected to a Gilson MiniPulse eVolution peristaltic pump and the flow rate adjusted to 0.5 ml/min. The

columns were washed with ddH_2O and charged with 1 ml 15 mM $FeCl_3$. Unbound metal ions were washed away with ddH_2O and the column equilibrated with wash buffer (1) before the sample was applied. Unbound material was washed away with wash buffer and bound material eluted using elution buffer (1), and collected in 1 ml fractions which were then quantified by peptide BCA (see 2.3.4).

Buffer was then washed away using ddH_2O and metal ions removed using 50 mM EDTA, which was in turn removed using ddH_2O. Following this the column was either subjected to a second wash and re-charged with metal ions for a second sample, or washed with 20 % (v/v) EtOH and stored at 4 °C.

2.5.1.1.2 Increased elution pH

Performed as per 2.5.1.1.1, with the exception of elution, which took place using elution buffer (2) (100 mM DEA-HCl pH 9.0). This was then followed with 10 column volumes of elution buffer (4) (100 mM DEA pH 10.45), and again 1 ml fractions collected.

Performed as per 2.5.1.1.1, with the exception of elution, which took place using elution buffer (2) (100 mM DEA-HCl pH 9.0).

2.5.1.1.3 Addition of a low-molarity wash stage.

Performed as per 2.5.1.1.2, with a second 5 mM wash stage (wash buffer 2) after the wash with 25 mM TMA-formate pH 3.3 (wash buffer 1).

2.5.1.1.4 Reduction in elution buffer strength

Performed as per 2.5.1.1.3, with the exception of elution which took place using elution buffer (3) (50 mM DEA-HCl pH 9.0).

2.5.1.1.5 Addition of a second column

A second IMAC column was attached in line with the first and chromatography was performed as per 2.5.1.1.4, except that the volume of each solution applied to the column was increased by a factor of 2.5.

2.5.1.2 Cu^{2+} IMAC

Stock Solutions:

A: 15 mM $CuSO_4$ in ddH_2O.
B: Wash buffer (1): 100 mM HEPES-NaOH, pH 7.2.
C: Tryptic digests of casein (prepared as per 2.3.2.2) and brought to 25 mM TMA-formate, pH 3.3.
D: Elution buffer (1): 0.1 % (v/v) TFA in ddH_2O.
E: 50 mM EDTA.
F: ddH_2O
G: Storage solution: 20 % (v/v) ethanol in ddH_2O
H: Wash buffer (2): 5 mM HEPES-NaOH, pH 7.2.
I: Elution buffer (3): 50 mM DEA-HCl, pH 9.0.

N.B: Unless stated otherwise, each wash of change in solution took place using >5 ml (five column volumes) as per 4.4.1.1 and with the exception of the sample each solution was sterile filtered prior to use.

2.5.1.2.1 Standard method

Hi-Trap-chelating HP IMAC columns were connected to Gilson MiniPulse eVolution peristaltic pump and the flow rate adjusted to 0.5 ml/min. The columns were washed with ddH_2O and charged with 1 ml 15 mM $CuSO_4$. Unbound metal ions were washed away with ddH_2O and the column equilibrated with wash buffer (1) before the sample was applied. Unbound material was washed away with wash buffer and bound material eluted using elution buffer (1), and collected in 1 ml fractions which were then quantified by peptide BCA (see 2.3.4).

Buffer was then washed away using ddH_2O and metal ions removed using EDTA, which was in turn removed using ddH_2O. Following this the column was either

subjected to a second wash and re-charged with metal ions for a second sample, or washed with storage solution and stored at 4 °C.

2.5.1.1.2 Increased elution pH

Performed as per 2.5.1.1.1, with the exception of elution, which took place using elution buffer 2 (0.5 % (v/v) TFA in ddH_2O).

2.5.1.1.3 Addition of a low-molarity wash stage.

Performed as per 2.5.1.1.2, with a second 5 mM wash stage after the 100 mM HEPES-NaOH pH 7.2

2.5.2 Reversed-phase (RP) chromatography

N.B: Unless stated otherwise, each wash of change in solution took place using >1 ml (five column volumes) to ensure full alteration of intra-column conditions. With the exception of the sample each solution was sterile filtered prior to use. Application was performed by hand using a 1 ml syringe, at a flow rate of 1 ml/min (visually assessed).

2.5.2.1 Choice of resin

Stock Solutions:

A: Phosphopeptide solutions: 0.2 mg/ml in 0.1 % (v/v) TFA.

B: Conditioning/wash solution: 0.1 % (v/v) TFA.

C: Acetonitrile 80 % (v/v) in ddH_2O.

D: Acetonitrile 100 % (v/v).

E: Non-chromatographic standards: 0.2 mg in 82.5 % (v/v) ACN.

2.5.2.1.1 Chromatography

Varian Bond-Elut C_{18}, and Strata C_8 and C_{18} 50 mg SPE columns were activated with 2 ml of 80 % (v/v) ACN and conditioned using 1 ml 0.1 % (v/v) TFA. Samples (0.2 mg in 1 ml) were loaded onto the column and unbound material removed by washing with 1 ml 0.1 % (v/v) TFA. Bound peptides were then eluted using 1 ml 80 % (v/v) ACN, followed by 0.5 ml 100 % ACN. Eluted fractions were all collected in 1.5 ml volumes stored in 2 ml sealable boiling tubes and spiked with 20 µl DMSO.

Non chromatographic standards were prepared by the addition of 20 µl synthetic peptide stock solution (in DMSO) to 1.5 ml 82.5 (v/v) ACN.

2.5.2.1.2 Rotary evaporation

Eluates and standards were then placed in a Juan Rotary Evaporator and dried to below 50 µl (the presence of DMSO prevents full evaporation), following which they were brought to 200 µl with ddH_2O and the peptide content assayed by peptide BCA (as per section 3.1.4). Recovery of phosphopeptides following RP-chromatography was then calculated as a percentage of the non-chromatographic standards.

2.5.2.2 Alteration of sample acidification

Stock Solutions:

A-I: Acidified ALR(p) phosphopeptide solutions: 0.2 mg/ml in TFA, HCl or phosphoric acid (PA) at 0.1, 0.5, 1 % (v/v).

JR-: Conditioning/wash solutions: TFA, HCl or PA as above (without peptide).

C: Acetonitrile 80 % (v/v) in ddH_2O.

D: Acetonitrile 100 % (v/v).

E: Non-chromatographic standard: 0.2 mg ALR(p) in 82.5 % (v/v) ACN.

Varian Bond-Elut C_{18} 50 mg SPE columns were activated with 2 ml of 80 % (v/v) ACN and conditioned using 1 ml conditioning solution. Samples (0.2 mg ALR(p) acidified as above in 1 ml) were loaded onto the column and unbound material removed by washing with 1 ml of the corresponding wash solution. Bound

peptides were then eluted using 1 ml 80 % (v/v) ACN, followed by 0.5 ml 100 % ACN. Eluted fractions were all collected in 1.5 ml volumes stored in 2 ml sealable boiling tubes and spiked with 20 µl DMSO.

Non chromatographic standards were prepared by the addition of 20 µl synthetic peptide stock solution (in DMSO) to 1.5 ml 82.5 (v/v) ACN. Samples were then dried by rotary evaporation, brought to volume and assayed by peptide BCA as per 2.5.2.1.2.

2.5.2.3 Inclusion of salt in loading conditions

Stock Solutions:

A-E: Tryptic digests of casein (0.3 mg/ml) in 0.2 mM Tris-acetate pH 8 (15 mg/ml digest diluted 1/50), acidified with 0.5 % (v/v) TFA containing: 0, 0.1, 0.2, or 0.5 M KCl or 20 µl DMSO.

F-I: Wash solution (1) 0.5 % (v/v) TFA containing: 0, 0.1, 0.2, or 0.5 M KCl.

I: Conditioning/wash solution: 0.5 % (v/v) TFA.

J: Acetonitrile 80 % (v/v) in ddH_2O.

K: Acetonitrile 100 %.

Varian Bond-Elut C_{18} 50 mg SPE columns were activated with 2 ml of 80 % (v/v) ACN and conditioned using 1 ml 0.5 % (v/v) TFA ($^{+}/_{-}$ KCl). Samples (0.3 mg casein in 1 ml 0.5 % (v/v) TFA $^{+}/_{-}$ KCl) were loaded onto the column and unbound material removed by washing with 1 ml of wash solution. Bound peptides were then eluted using 1 ml 80 % (v/v) and 0.5 ml 100 % ACN. These were spiked with 20 µl DMSO and subjected to rotary evaporation and assayed by peptide BCA as per 2.5.2.1.2 against a non-chromatographic standard.

2.5.2.4 Final method

Stock Solutions:

A: 80 % Acetonitrile.

B: Conditioning/wash solution 0.5 % (v/v) TFA.

Varian Bond-Elut C_{18} SPE columns (500 g) were activated with 8 ml of 80 % (v/v) ACN and conditioned using 8 ml 0.5 % (v/v) TFA. Samples (in MHC elution

buffers – see 2.4) were diluted 1:1 in ddH_2O, acidified by the addition of 0.5 % (final volume) TFA and loaded onto the column[7]. Unbound material was removed by washing with 8 ml of wash solution and bound peptides eluted using 8 ml 80 % (v/v) and 3 ml 100 % ACN. These were assayed by peptide BCA as per 2.3.1.

2.5.3 Mixed mode and ion exchange resins

Again, these were investigated using the hydrophilic SQK(p) and the hydrophobic YIS(p) phosphopeptides.

N.B: Unless stated otherwise, each wash of change in solution took place using >1 ml (five column volumes) to ensure full alteration of intra-column conditions. With the exception of the sample each solution was sterile filtered prior to use. Application was performed by hand using a 1 ml syringe, at a flow rate of 0.5 drops per second (visually assessed).

2.5.3.1 Strata X

Stock Solutions:

A: Phosphopeptide solutions: 0.2 mg/ml SQK(p)/YIS(p) in 2 % (v/v) phosphoric acid.
B: 2 % (v/v) phosphoric acid.
C: 25 % (v/v) MeOH in ddH_2O.
D: 50 % (v/v) MeOH in ddH_2O.
E: 50 % (v/v) MeOH in acetonitrile.
F: 100 % acetonitrile.
G: ddH_2O
E: Non-chromatographic standards: 0.2 mg in 82.5 % (v/v) ACN.

Strata X 50 mg SPE columns were activated with 2 ml of 100 % ACN and conditioned using 1 ml ddH_2O and 1 ml 2 % (v/v) phosphoric acid. Samples (0.2 mg in 1 ml) were loaded onto the column and washed with 1 ml 2 % (v/v) phosphoric acid; followed by 1 ml 25 % (v/v) MeOH in ddH_2O and 1 ml 50 %

[7] In cases where two-rounds of C_{18} SPE was used, the peak fraction(s) from the first round were diluted 1:19 with 0.5 % (v/v) TFA and applied to a fresh column.

(v/v) MeOH in ddH_2O. All three wash/elution stages were retained for analysis. Bound material was then eluted with 1 ml 50 % (v/v) MeOH in ACN and 1 ml 100 % ACN. Solvent washes and eluates were collected in 1 ml volumes stored in 2 ml sealable boiling tubes and spiked with 20 µl DMSO.
Non chromatographic standards were prepared by the addition of 20 µl synthetic peptide stock solution (in DMSO) to 1 ml 50 (v/v) MeOH in ACN. These and the methanolic fractions were then subjected to rotary evaporation and assayed by peptide BCA as per 2.5.2.1.2 against the standards.

2.5.3.2 Strata X-AW

Stock Solutions:

A: Phosphopeptide solutions: 0.2 mg/ml SQK(p)/YIS(p) in 2 % (v/v) phosphoric acid.
B: 2 % (v/v) phosphoric acid.
C: 20 mM sodium acetate, pH 5.0.
D: 50 % (v/v) MeOH in ddH_2O.
E: 50 % (v/v) MeOH in acetonitrile.
F: 100 % methanol.
G: 2 % ammonium bicarbonate, 0.5 % TFA in MeOH.
E: Non-chromatographic standards: 0.2 mg in 82.5 % (v/v) ACN.

Strata X-AW 50 mg SPE columns were activated with 2 ml of 100 % ACN and conditioned using 1 ml ddH_2O and 1 ml 2 % (v/v) phosphoric acid. Samples, either SQK(p) or YIS(p) (0.2 mg in 1 ml) were loaded onto the column and washed with 1 ml 20 mM sodium acetate pH 5 and then with methanol, followed with 1 ml 2 % (w/v) ammonium bicarbonate, 0.5 % (v/v) TFA in MeOH. Both the sodium acetate and methanolic fractions were collected in 1 ml volumes stored in 2 ml sealable boiling tubes and spiked with 20 µl DMSO.

Non chromatographic standards were prepared by the addition of 20 µl synthetic peptide stock solution (in DMSO) to 1.5 ml 50 (v/v) MeOH in ACN. These and the methanolic fractions were then subjected to rotary evaporation and assayed by peptide BCA as per 2.5.2.1.2 against the standards.

2.5.4 Hydrophilic Ligand Interaction Chromatography (HiLIC)

A form of normal-phase chromatography, Hydrophilic Ligand Interaction Chromatography (HiLIC) may be considered the opposite of reversed-phase chromatography: samples are loaded in a non-polar solvent, and bind to a resin with hydrophilic components, before they are eluted in a polar (typically aqueous) medium.

N.B: Unless stated otherwise, each wash of change in solution took place using >1 ml (five column volumes) to ensure full alteration of intra-column conditions. With the exception of the sample each solution was sterile filtered prior to use. Unlike the previous chromatographic approaches, the work with HiLIC involved both small-volume (< 200 µl) SPE cartridges loaded by syringe (at a flow rate of 1 ml/min), and 1 ml HPLC-compatible columns, loaded by peristaltic pump (at a flow rate of 0.5 ml/min). With the exception of the Strata NH_2, these were all packed in-house.

Stock Solutions:

A: Synthetic peptides/phosphopeptides: 10 mg/ml in DMSO.

B: Casein digest: 25 mg/ml in 50 mM DEA pH 9.0.

C: Post-IMAC phosphopeptide enriched casein digest: typically > 4 mg/ml in 50 mM DEA-HCl pH 9.0.

D: 20 % (v/v) ethanol (storage solution).

2.5.4.1 Comparison of resins

Stock Solutions:

A: ALR(pS)NFERI: 0.2 mg in 2 ml 90 % ACN (+ 1 % v/v DMSO[8]).

B-F: 90.0; 75.0; 50.0; 25.0; 0 % (v/v) ACN

2.5.4.1.1 Preparation of columns

Phenomenex Strata-NH_2 columns were used as supplied. HEA (hexylamine) and PPA (phenylpropylamine) HyerCel columns (Pall, UK) were constructed from used Varian Bond-Elut C_{18} cartridges. The C_{18} resin was removed from these

[8] A result of the peptides being stored at 10mg/ml in 100 % DMSO.

columns by a combination of back pressure applied through the nozzle with a steel rod, and use of a slender spatula to fish out the filter-frits and dry resin. Filters and columns were cleaned with detergent and hot water, and then by sonication in 1 % (v/v) SDS in 0.1 M NaOH, before final rinses in 100 % ACN and 100 % ddH_2O. End frits were then placed back into the columns, and HEA or PPA HyperCel resin suspensions (in 20 % v/v EtOH) added in 0.3 ml volumes until the bed volume appeared similar to the Strata-NH_2 columns, at which point the top-frits were replaced atop the resin and the column washed with water and then with 20 % EtOH as a storage solution. Columns were used for a maximum of 10 analytical runs before the resin was removed and repacked.

2.5.4.1.2 Application of material

Columns were activated with ddH_2O and equilibriated with 90 % (v/v) ACN. The synthetic phosphopeptide ALR(p) was loaded onto each column in 2 ml (~ 5 column volumes) and the column eluted with a stepwise elution from 90.0, 75.0, 50.0, 25 and 0 % (v/v) ACN, with fractions of ~250 µl collected for analysis by peptide BCA. These were diluted 1:5 in ddH_2O before being mixed 1:1 with 2x SDS-NaOH, heat denatured (see 2.3.4.2) and analysed by the peptide BCA assay (Kapoor *et al*., 2009), as per 2.3.1.

2.5.4.2 Effect of DMSO

Stock Solutions:

A: ALR(pS)NFERI: 0.2 mg in 2 ml 90 % ACN (1 % v/v DMSO).

B-E: 90.0; 75.0; 50.0; and 25 % (v/v) ACN (+/- 1 % v/v DMSO)

F: ddH_2O

HEA columns were prepared as per 2.5.4.1.1. Casein digests were clarified by centrifugation at 20,800 r.c.f. for 15 minutes and the supernatants loaded onto the column. Unbound material was washed away with 90 % (v/v) ACN, and a stepwise elution gradient applied from 75 to 25 % (v/v) ACN (+/- 1 % v/v DMSO). Finally each column was washed with ddH_2O. Fractions of ~0.33 µl were collected throughout and were analysed by peptide BCA in a similar manner to 2.5.4.1.2.

2.5.4.3 HPLC setup

2.5.4.3.1 Preparation of column

A 1 ml volume HEA HyperCel column was prepared from a used Hi-Trap-chelating HP IMAC cartridge. End caps were removed using tools built in-house[9] and filter/frits and resin removed and the filters and cartridge cleaned in a similar manner to 2.5.4.1.1. The column was packed with HEA HyperCel under a flow rate of 0.2 ml/min ddH_2O using a peristaltic pump connected as per 2.5.1.1.1, and the top-frit replaced. The column was then washed with 10 ml ddH_2O and then with 5 ml 20 % EtOH as a storage solution. Columns were used for a maximum of 10 analytical runs before the resin was removed and repacked.

2.5.4.3.2 HPLC methodology

The HEA HyperCel columns were connected to an ÄKTA FPLC platform (GE Healthcare) and subjected to a flow rate of 0.1-0.5 ml/min. A maximum pressure of 40 psi was set, and a UV absorbance of 210 nm. Analyte-free runs were performed using a gradient from 90 – 0 % (v/v) ACN in the presence or absence of 1 % (v/v) DMSO though increases in pressure beyond the maximum tolerance of the resin were experienced and method development further optimised using quantification via peptide BCA (see 2.3.4).

Columns were subsequently connected to a PerkinElmer series 200 HPLC under similar conditions to the above and phosphopeptide enriched digests of casein loaded at 75 % ACN. Issues with TFA absorbance of UV led to a second return to BCA-determined elution, and investigation into maintenance of a continuous TFA concentration (2.5.4.7), or the use of TFA/alternative acid gradients to fractionate bound material (2.5.4.8).

2.5.4.4 Selectivity for casein or BSA

Stock Solutions:

A-C: 75.0; 50.0; and 25 % (v/v) ACN.

[9] Tools designed and created by Mr. Robert Davey, with thanks.

D-I: BSA or casein dissolved in 75.0; 50.0; and 25 % (v/v) ACN to a concentration of 5 mg/ml.

J: ddH_2O

K: 0.1 % (v/v) TFA.

In order to determine the applicability to isolate phosphorylated protein, BSA and casein were both prepared in varying amounts of ACN: 75.0; 50 and 25 (v/v)[10] and 2 ml applied to 1 ml volume HEA HyperCel columns using a Gilson MiniPulse eVolution peristaltic pump at 0.5 mg/ml. Again, a stepped gradient to ddH_2O was employed, followed by a 0.1 % (v/v) TFA wash. Fractions of 1 ml were collected and peptide concentration determined by BCA as 2.5.4.1.2.

2.5.4.5 Selectivity for casein IMAC eluates

Stock Solutions:

A-C: 75.0; 50.0; and 25 % (v/v) ACN.

D: Post-IMAC phosphopeptide enriched casein digest, typically > 1 mg/ml in 75 % (v/v) ACN, 12.5 mM DEA-HCl pH 9.0.

E: ddH_2O

F: 0.1 % (v/v) TFA.

Following on from the casein and casein digest experiments, a phosphopeptide enriched fraction from a Fe^{3+}-IMAC of trypsinised casein was employed to determine the potential for HEA and PPA HyperCel to fractionate a heterogeneous phosphopeptide population. Peptides were loaded at 75 % (v/v) ACN and the columns subjected to a stepwise gradient to ddH_2O followed by a 0.1 % (v/v) TFA wash. Again 1 ml fractions were collected and analysed by peptide BCA as 2.5.4.1.2.

2.5.4.6 Inclusion of TFA in mobile phase

Stock Solutions:

A-F: 75.0; 50.0; and 25 % (v/v) ACN, +/- 0.1 % (v/v) TFA.

G: Post-IMAC phosphopeptide enriched casein digest, typically > 0.5 mg/ml in 75 % ACN (v/v), 12.5 mM DEA-HCl pH 9.0.

[10] Problems with solubility were experienced at 90 % ACN.

H: ddH_2O

I: 0.1 % (v/v) TFA.

To determine if the inclusion of TFA might allow peptide fractionation along a solvent gradient the above experiment (2.5.4.6) was repeated with HEA HyperCel only, with or without the inclusion of 0.1 % TFA in the mobile phase. Peptides were loaded at 75 % (v/v) ACN and the columns subjected to a stepwise gradient to ddH_2O following which a 0.1 % (v/v) TFA wash was applied to both columns. Again 1 ml fractions were collected and analysed by the peptide BCA assay as per 2.5.4.1.2.

2.5.4.7 Elution with acid-gradients and use of alternative acids

Stock Solutions:

A: 75 % (v/v) ACN.

B: ddH_2O

C-D: 0.001 % (v/v) TFA; HCl.

E-H: 0.01 % (v/v) TFA; HCl; formic acid; phosphoric acid.

I-L: 0.1 % (v/v) TFA; HCl; formic acid; phosphoric acid.

M-P: 1 % (v/v) TFA; HCl; formic acid; phosphoric acid.

Q: Post-IMAC phosphopeptide enriched casein digest, typically > 0.5 mg/ml in 75 % ACN, 12.5 mM DEA-HCl pH 9.0.

To determine if an acid gradient with TFA or alternative acids might facilitate fractionation of a heterogeneous phosphopeptide population (2.5.4.6) was repeated with HEA HyperCel only, and a range of concentrations of four commonly utilised acids. Peptides were loaded at 75 % (v/v) ACN and the columns washed with ddH_2O following which a logarithmic acid gradient was applied (0.001 – 1 % v/v), following which remaining peptide was eluted using 1 % (v/v) TFA followed by 90 % (v/v) ACN. Again 1 ml fractions were collected and analysed by the peptide BCA assay as 2.5.4.1.2.

2.5.4.8 Evaluation of binding potential for synthetic (phospho/)peptides

Stock Solutions:

A-B: 75.0; 50 % (v/v) ACN.

C: ddH_2O

D: 0.5 % (v/v) TFA.

E: 0.01 % (v/v) TFA; HCl; formic acid; phosphoric acid.

F: Phosphopeptide mix: 1 ml containing 0.2 mg of each of the following: ALR(p); SQK(p); TVE(p); YIS(p) in 75 % (v/v) ACN.

G: Non-phosphopeptide mix: 1 ml containing 0.2 mg of each of the following: ALR(x); SQK(x); TVE(x); YIS(x) in 75 % (v/v) ACN.

To determine whether the HiLIC eluates contain phosphorylated, unphosphorylated, or mixed peptides, a mixture of peptides (phosphorylated or not) were applied to a 1 ml HEA column at 75 % (v/v) ACN, and the column subjected to a stepwise elution protocol to 0.5 % (v/v) TFA. Again, 1 ml fractions were collected and analysed by the peptide BCA assay as 2.5.4.1.2.

2.5.5 Peptide PAGE

The peptide-PAGE methods below are based on Yim *et al*. (2002)'s modification of the method developed by Schagger & von Jagow (1987).

2.5.5.1 Preparation of 18 % polyacrylamide tricine gel buffer

Stock Solutions:

A: Acrylamide: 30 % (w/v) solution (29:1 acrylamide:bis-acrylamide).

B: 5x Buffer/electrolyte solution (0.6 M tris-HCl; 500 mM tricine; 2.5 % (w/v) glycerol and 0.5 M each of serine, glycine, and asparagine.

C: 10 M NaOH.

To prepare 500ml 18% acrylamide peptide gel buffer 100 ml of (room temperature) solution B was mixed with 300 ml 30 % (w/v) acrylamide. The pH was adjusted to 7.4 with 10 M NaOH and the volume brought to 500 ml with ddH_2O.

2.5.5.2 Gel polymerisation

Stock Solutions:

A: 18 % (w/v) acrylamide gel buffer (see 2.5.5.1).

B: 1 % (w/v) Ammonium persulphate (APS)

C: Tetramethylethylenediamine (TEMED)

D: Running buffer: 3.27 g Trizma Base, 15.014 g glycine, and 1.00 g SDS in 100 ml ddH_2O, pH 8.3 with HCL.

To 20 ml of acrylamide gel buffer, 150 µl of APS and 15 µl of TEMED were added, swiftly mixed and transferred to the gel plates along with the well-combs. Once polymerisation was complete the gel kits were rinsed with ddH_2O to remove any unpolymerised acrylamide, and transferred to the gel tanks. Running buffer was then added to both inner and outer chamber.

2.5.5.3 Sample preparation and electrophoresis

Stock Solutions:

A: 2x Laemlli sample buffer.

B: Ultra-low weight marker/ladder (ULM) in 2x Laemlli sample buffer.

Following quantification by the peptide BCA assay, sample volumes were adjusted with ddH_2O to ensure equal loading and mixed 1:1 with 2x Laemlli sample buffer and denatured by heating to 95 ºC before each well was loaded with no more than 100 µg total peptide. One well was loaded with 10 µg ULM to show molecular weights. Electrophoresis was performed at 45 v/gel for approximately 3 hours, following which gels were removed from the gel plates and placed in shallow ~ 40 ml volume covered trays and briefly rinsed twice with ddH_2O to remove excess tris/SDS.

2.5.5.4 Pro-Q Diamond Staining

Stock Solutions:

A: 5 % (v/v) Glutaraldehyde

B: Pro-Q Diamond phosphostain.

C: Pro-Q Diamond destain: 20 % (v/v) ACN , 50 mM Na-acetate, pH 4.0.

Gels were incubated on a gel shaker with 10 ml 5 % (v/v) glutaraldehyde for one hour to fix peptides within the gels, following which the gel was washed repeatedly in ddH_2O over 36 hours to dialyse out any remaining glutaraldehyde. The gel tray was then wrapped in foil before the addition of 20 ml Pro-Q Diamond and incubated on a gel shaker for 150 mins under protection from light. This was then poured off and the unbound stain removed by incubating with 20 ml destain solution for 3 x 30 min periods. Following two washes in ddH_2O the gels were then imaged using a Fujifilm, FLA-5100 gel scanner armed with a cye-5 laser.

2.5.5.5 Coomassie blue staining

Stock Solutions:

A: Coomassie blue gel stain: 1 mg/ml Coomassie Brilliant-Blue powder in 45 % MeOH and 10 % acetic acid (both v/v) in ddH_2O.

B: Gel destain: 45 % MeOH and 7 % acetic acid (both v/v) in ddH_2O.

Gels were incubated on a gel shaker for one hour with 20 ml 5 % (v/v) coomassie blue stain, following which the gel was rinsed twice in ddH_2O and incubated overnight on a gel shaker with destain solution and a small twisted piece of tissue paper to bind coomassie stain as it disassociates from the gel. Following this a digital photograph of the gel was taken in a UVLtec gel imager equipped with a light-box and visible-range bulb.

Mass Spectrometry

2.6.1 MALDI-TOF Mass Spectrometry

Stock Solutions:

A: α-Cyano-4-hydroxycinnamic acid solution (CHCA): 5 mg/ml in 50 % (v/v) ACN, 0.05 % (v/v) TFA.

B: Peptide standard calibrant II [Bruker pt no 222570] (700-4000 Da) mg/ml in 33.3 % (v/v) ACN, 0.066 % (v/v) TFA.

C: MHC class-I peptide eluates: ~1 mg/ml in 80 % (v/v) ACN (HPLC grade) (following C_{18} RP SPE).

N.B: Unless stated otherwise, all reagents are mass-spectrometric grade.

MALDI mass spectrometry was performed in a similar manner to Matharoo-Ball *et al.* (2007) and Vafadar-Isfahani *et al*. (2010) Following desalting using C_{18} RP SPE columns (as per 2.5.2.4) 1 µl of sample was mixed with 1 µl of CHCA solution on-plate. Standard calibrants were prepared in the same method, excluding the RP stage, and at a ratio of 1 calibration spot : 4 sample spots. These were air dried at RT and the plates loaded into the Bruker Ultraflex III TOF/TOF MALDI mass spectrometer. Peptide-CHCA complexes were then fired upon, and data from 2000 pulses per spot collected in positive reflectron mode, and the mass spectra from 500 – 4000 Da analysed in Flex Analysis software (Bruker).

2.6.2 LTQ-ESI-MS/MS

Stock Solutions:

A: 5 % (v/v) ACN, 0.1 % (v/v) formic acid, 0.005 % (v/v) heptafluorobutyric acid (HFBA) in H_2O.

B: 0.1 % (v/v) formic acid, 0.005 % (v/v) heptafluorobutyric acid (HFBA) in 95 % (v/v) ACN.

C: Heptatitis B standard peptide ($TPPAYRPPNAPIL_{128-140}$)

D: Sample: diluted to 1 fmol/µl in 0.1 % (v/v) formic acid in H_2O.

N.B: Unless stated otherwise, all reagents are mass-spectrometric grade.

2.6.2.1 HPLC fractionation

HPLC and ESI-MS/MS were performed in a similar method to Clarke *et al*. (2001). Samples were chromatographically analysed on a Dionex U3000 nanoflow LC system equipped with a Dionex C_{18} 20 mm 300 µ trap column to desalt analytes, and a Dionex Pepmap (15 cm length; 15 µ internal diameter; 3 µ particle size) analytical column for sample fractionation.

Twenty µl of each sample/standard at 1 fmol/µl was loaded by injection loop onto the trap column at a 30 µl/min flow rate. After desalting the flow was redicrected by means of a 10-port switching valve and the flow-through from the trap column directed onto the analytical column at 300 µl/min. The columns were subjected to an A:B gradient of 100:0 – 30:70 A:B (40 mins) and from 30:70 – 0:100 A:B (2 mins) at 300 µl/min. This was then held for 5 mins before a reverse gradient to 100:0 A:B (1 min) was applied. Columns were then reequilibriated in (A) for 32 mins (total run time of 80 mins).

2.6.2.2 Sample ionisation and fragmentation

Upon elution from the column samples were ionised across a unconditioned glass capilliary 10 µ ESI tip with a 1.8 kV charge held at 200 °C (typical injection time 400 ms, total microscans 3) and introduced to the Finnegan linear quadruple ion trap (LTQ) mass spectrometer (Fisher, Thermo Scientific), running in positive mode. The signal was tuned with LTQ autotune and the synthetic Heptatitis B MHC class-II restricted peptide TPPAYRPPNAPIL standard, and fragmentation carried out with relative collision energy of 35 % (automatic gain control was used and an isolation width of 3 Da set). Data-dependent acquisition was carried out on the top 5 tandem mass spectra throughout the run.

2.6.3 Identification

Tandem mass spectra RAW files were either analysed with Bioworks Browser (Thermo) or later with Proteome Discoverer (Thermo) using the MASCOT search algorithm to search the SwissProt protein database against the (unless indicated) *Homo sapiens* (hereafter: Human) Proteome catalogue. Post-translational modifications included were phosphorylation on serine, threonine, and tyrosine, or for Cu^{2+} IMAC samples, oxidation of methionine.

2.6.4 Database design

A simple non-relational database of peptide sequences was created using Microsoft Access (version 2003). Fields were created using Table design view, primarily using either text boxes (maximum field size 255 chars) or Boolean

checkboxes, with the SwissProt accession numbers of the suggested protein sources used as a key field. A mononumeric "potential significance" field allows users to score the peptide from 0-9, while Boolean checkboxes allow sorting by protein function(s), links with different cancers, and possible roles within cancer. Data is entered manually at this time.

3.0: Modification of the BCA assay for quantification of peptide content

3.1 Introduction

The accurate measurement of peptide in solution is vital to developing appropriate protocols for sample fractionation, isolation, and analysis. As reproducible analytical chromatography and mass spectrometry both require accurate loading of sample prior to fractionation/analysis, knowledge of sample concentration is crucial. While the majority of peptide analyses involve tryptic digests of (often precipitated, weighed and reconstituted) proteins, estimation of peptide concentration may usually be circumvented. However as the samples obtained by mild acid elution of MHC complexes contain peptides rather than proteins, measurement of peptide concentration is highly desirable.

With the rise in peptidomics over the last few years, it is curious that though there are a wide range of methods for estimating protein concentration, far fewer approaches are available for quantifying the peptide content of a sample. Whilst UV absorbance at 280 or 200 nm is often recommended for synthetic peptides, it is far from ideal for biochemical samples since at 280 nm absorbance is strongly influenced by aromatic rings (e.g. those found in the side chains of Trp and Tyr, as well as purines and pyrimidines), whilst absorbance at 200 nm may be complicated by many other compounds, especially those with carbon-carbon or carbon-oxygen bonds (Stoshcheck, 1990). Fujinari *et al*. (1994) increased the accuracy of this approach by combining UV-absorbance with chemiluminescent detection of nitrogen in HPCL eluates, though this is a difficult approach to apply to crude biological samples. Conversely amino acid analysis, whilst highly accurate, usually requires significant and lengthy sample cleanup prior to analysis, and full hydrolysis of amino acids from peptide may take anywhere from a few hours to several days (Kaspar *et al*., 2009).

Alternatively a number of fluorescent approaches to protein and peptide quantification have been put forward, for example, Fluorescamine (4'-phenyl-spiro [furan-2(3H),1'-phthalan]-3,3'-dione) (Udenfriend *et al*., 1972), o-phthaldialdehyde (OPA) (Goodno *et al*., 1981) and 3-(4-Carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) (Liu *et al*., 1991; You, *et al*. 1997) are all nonfluorescent compounds that become fluorescent when conjugated to primary

amines (such as N-termini and lysine residues), rendering them suitable for sensitive detection of peptide and protein. However, these assays may be strongly influenced by changes in frequencies of different amino acids between samples (e.g. lysine), and changes in the order of some residues (especially those with terminal prolines) (Schiltz *et al*., 1977). They are also susceptible to false positives from other primary amines; e.g. glycine, and to an extent Tris buffers, or amino sugars such as sialic acid (Goodno *et* al., 1981; You, *et al*. 1997). The fluorescent agents are often light sensitive, and some, such as fluorescamine, are rapidly degraded in aqueous solutions or hydroxylic solvents (e.g. methanol, ethanol, propanol) (Böhlen *et al*., 1973; De Bernado *et al*., 1974). The assays' tolerance for other compounds also varies considerably, and though most are quite compatible with lipids, the secondary structure of proteins and peptides can affect sensitivity, and limited compatibility with detergents and thiols means that they cannot be easily denatured (an exception being CBQCA, where the addition of Triton-X may actually enhance sensitivity) (Haughland, 1996; You, *et al*. 1997; Molecular Probes, 2001a, C6667).

Similarly, epicocconone, the basis for the LavaPrep™ assay (Veal, 2007) and Deep Purple™ protein stain, is also applicable to peptide quantification, but is highly selective for histidine, arginine and lysine residues (Bell *et al*., 2003; Coghlan *et al*., 2005), leading to problems with interpeptide variability, as well as being complicated by free amino acids. It also exhibits very limited tolerance for detergents and acids (Veal, 2007; Interchim, 2008).

Two alternative fluorescent assays, Nano-Orange and the Quant-iT protein assay, bind through hydrophobic-interactions with the sample protein (Molecular Probes, 2001b; 2007). While this reduces specific amino acid-based variations in fluorescence proteins, it may present a greater issue with regard to peptides, especially for the Nano-Orange assay, given that there is a notable difference in the reactivity between proteins of different sizes (Dr. P. Roach, University of Nottingham, pers. comm. 2009). Additionally given that these assays operate by hydrophobic binding, they present limited compatibility with detergents and lipids, and therefore sample cleanup may be required prior to use. A third assay, the EZQ Protein Quantitation Kit (Molecular Probes, 2005), is performed on a dot-blot after washing to remove contaminants, and may therefore be problematic for small molecules such as heterogenous peptide populations, which may bind poorly to the membrane. Alternatively relative/semi-quantitation by way of mass spectrometry is also an option, though this has its own obstacles

in terms of time, cost and convenience, and is far more applicable to specific peptides (ideally sequences known prior to analysis) within a sample rather than total peptide content. It also requires the inclusion of an appropriate internal standard, and may be complicated by chromatographic losses (Zhu and Desiderio, 1996).

However, there are no standard colourimetric approaches to peptide quantification. With regard to colourimetric protein assays, both modern forms of the biuret assay: (a) the Lowry (Lowry *et al*., 1951) and (b) the bicinchoninic acid (BCA) (Smith *et al*., 1985) assays are occasionally utilised to quantify peptide (Hua & Scheller, 2001; Faber *et al*., 2003; Saleem *et al*., 2005), though neither is recommended for such. The other 'classic' colourimetric protein assay, the Bradford method, is sequence specific (Stoscheck, 1990), and not applicable to peptides below 3 kDa (Pierce, 2005).

The BCA assay, which has largely replaced the older Lowry method due to its convenience, compatibility, linearity and comparatively lower inter-protein variability (Stoshcheck, 1990) is based on two reactions. Firstly the co-ordination and reduction of a Cu^{2+} ion by 4-6 peptide bonds to form a complex, which allows the reduction of the copper to Cu^{1+} (the biuret reaction). The reduced copper is then chelated by two BCA molecules, producing a shift in absorbance to 562 nm (in practice 540-570 nm may be utilised) (Olsen and Markwell, 2007). There is also a sequence-specific reduction of copper involving tryptophan, tyrosine, cysteine and cystine residues, but as this is temperature independent (unlike the peptide-backbone reaction), sequence specific effects may be reduced by carrying the experiment out at higher temperatures (Wiechelman *et al*., 1988). Given that biuret-based assays react primarily with the peptide bonds in anything larger than a tripeptide, rather than the NH^{+} terminus or a reaction specific to functional groups, the BCA assay is theoretically applicable to reproducible estimation of peptide content, in a range consistent with the requirements for prep chromatography and peptide PAGE; however, despite the examples of this cited above, there is little published data on its practical applicability to peptide quantification.

This chapter details evaluation of the BCA assay for analysis of total peptide concentration within a sample, and a modification to the standard BCA protocol to improve applicability to estimation of peptide content. Synthetic peptides and tryptic digests were both evaluated, as well as the effect of phosphorylation as a

relevant post-translational modification. The most significant results were published in Kapoor *et al*., 2009.

3.2 Suitability of the BCA assay for estimating the peptide content of a sample

Previous work has suggested that the BCA assay, normally restricted to the quantification of protein, would also be applicable to quantifying peptides. This work was based around a single peptide from BCR-ABL, VHSATGFKQSSKALQRPVASD (hereafter: 'VHS'), and a reproduction of this assay demonstrated that a calibration curve could be generated using standards ranging from 16.25 to 250 µg/ml (see Figure 3.1). The peptide was prepared and assayed by BCA as per section 2.3.1, producing a repeatable and linear standard curve implying potential viability for assaying peptide concentration. However, inter-sample variability remained uninvestigated.

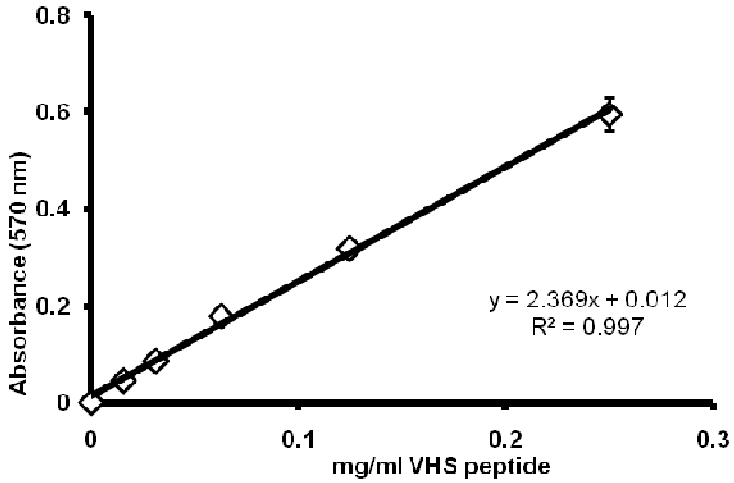


Figure 3.1: A typical BCA assay calibration curve generated using VHSATGFKQSSKAL-QRPVASD peptide standards from 0.0163 – 0.25 mg/ml. Peptide dilutions were prepared in water and 25 µl transferred to a 96 well plate in triplicate. 200 µl of BCA working reagent was then added to each well, and the plate incubated for 15 minutes at 37 ℃ before the absorbance was read at 570 nm. $n = 4$, error bars indicate SD.

3.3 Inter-sample variability for peptide BCA

Tryptic digests of BSA and casein were prepared in 10 mM Tris pH 8 (as per 2.3.2.2), and a standard curve of dilutions prepared, and then assayed as per the BCA protein assay protocol (2.3.1) (see Figure 3.2).

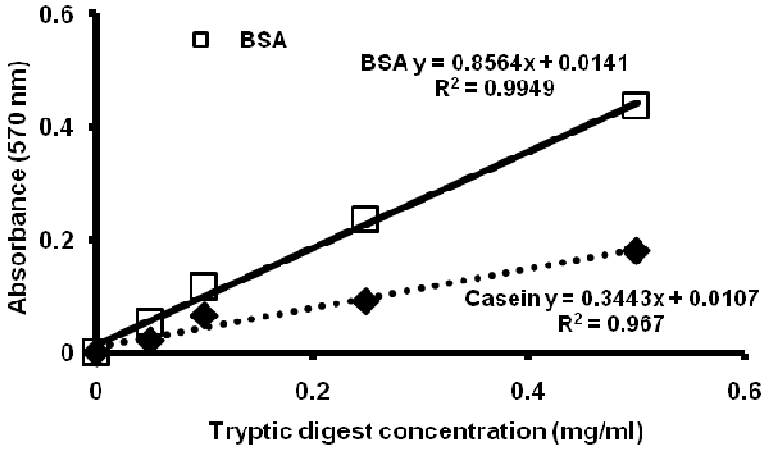


Figure 3.1.2: Representative BCA assay calibration curves generated using tryptic digests of BSA and mixed caseins from 0.05 – 0.5 mg/ml. Digests were diluted in water and 25 µl transferred to a 96 well plate in triplicate. 200 µl of BCA working reagent was then added to each well, and the plate incubated for 30 minutes at 37 ℃ before absorbance was read at 570 nm. Similar observations were found with multiple digests. *n* = 4, error bars indicate SD.

This illustrated a notable difference in BCA reactivity between BSA and casein peptides. Whilst according to their sequence data, the two should both render peptides of broadly similar lengths when digested (with marginally larger peptides produced from the caseins), peptide gel electrophoresis indicates that the BSA is not readily trypsinised, but consistently produces fragments of a larger length than the caseins (see Figure 3.3), potentially reflecting the function of casein as a performed on undigested BSA and casein, a similar pattern is seen to the digests (see figure 3.4).

As the BCA assay is known to be influenced by sequence-specific effects (Wiechelman *et al*., 1988), it is conceivable that the difference between BSA and casein reactivity may be due to the higher incidence of cysteine, tyrosine and tryptophan in BSA compared with the caseins (see table 3.1), but given the temperature independent nature of these reactions and the large difference in

reactivity between the two proteins it was believed that other factors were involved.

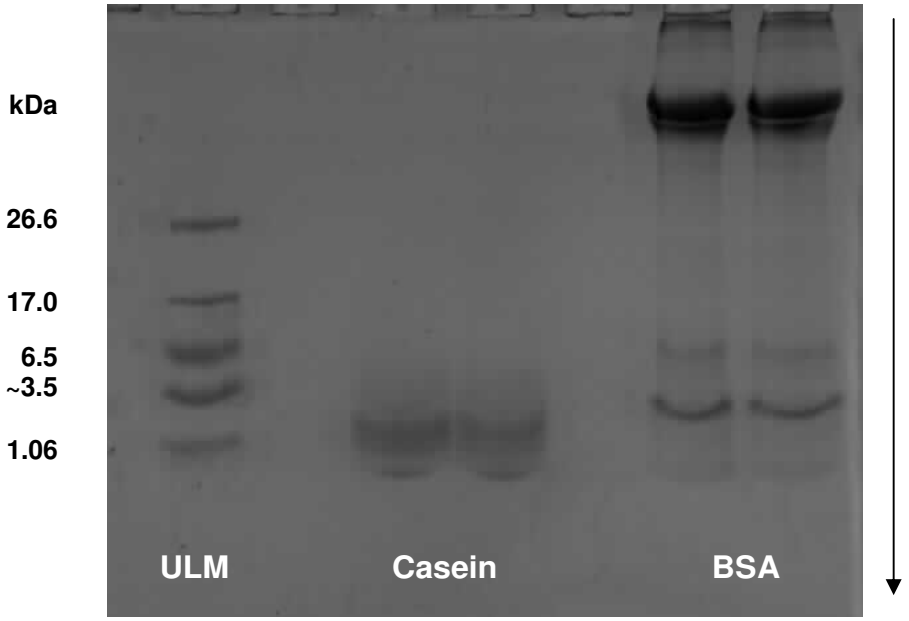


Figure 3.3: Coomassie-blue stained 18 % (v/v) acrylamide peptide PAGE (prepared as per Yim *et al*., 2002, see: 2.5.5) containing tryptic digests of casein and BSA (prepared as per 2.3.2.2) along with an ultra low-weight marker (ULM), illustrating differential digestion between the two proteins. Similar staining patterns were observed for numerous digests.

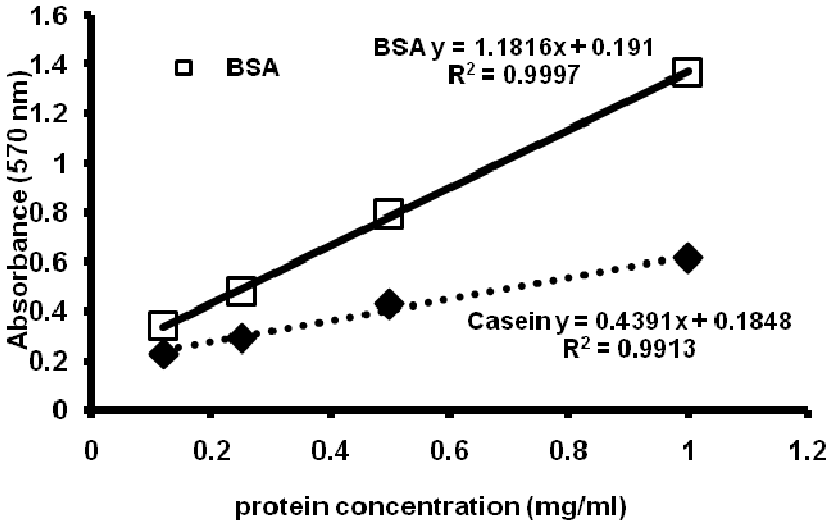


Figure 3.4: Typical BCA assay calibration curves generated from undigested BSA and mixed caseins from 0.12 – 1 mg/ml. Protein solutions were diluted in water and 25 µl transferred to a 96 well plate in triplicate. 200 µl of BCA working reagent was then added to each well, and the plate incubated for 30 minutes at 37 ℃ before absorbance was read at 570 nm. $n = 4$, error bars indicate SD.

Protein	No of residues per protein molecule		
	Cysteine	**Tryptophan**	**Tyrosine**
BSA	35	3	21
Casein α S1	1	2	10
Casein α S2	3	2	12
Casein β	1	1	4

Table 3.1: Relative incidence of BCA-reactive amino acids (as defined by Weichelman *et al*., 1988) in BSA and bovine caseins. Sequence data derived from the ExPASy proteomics server (www.expasy.ch).

In order to further investigate this aspect of the assay, 13 synthetic peptides (including 4 phosphopeptides and their non-phosphorylated counterparts, all with high predicted binding scores to MHC class-I A2 or A3 alleles, as determined by the SYFPEITHI algorithms) were utilised (see table 3.2 for sequences and physical characteristics), all from proteins with known links to leukaemia or malignancy.

Peptide sequence	**Size**		**pI**	**HPS**	**Sequence Source**	**Short name**
	a.a.	**Da.**				
LSRH	4	511.6	11	0.3	TP53BP1	LSRH
ALRSNFERI	9	1105.3	10.9	0.3	bcr	ALRx
ALR(pS)NFERI	9	1185.2	10.9	0.3	bcr	ALRp
SQKGQESEY	9	1055.1	4.3	0.9	SHP-1	SQKx
SQKGQESE(pY)	9	1135	4.3	1.1	SHP-1	SQKp
YISPLKSPY	9	1067.3	9.5	-0.5	Rb protein	YISx
YI(pS)PLKSPY	9	1147.2	9.5	-0.5	Rb protein	YISp
TVEAVAYAPK	10	1048.2	6.9	-0.1	hRad51	TVEx
TVEAVA(pY)APK	10	1128.2	6.9	0.1	hRad51	TVEp
LYGFNLVIVHSATGFKQSSK	20	2196.5	10.2	-0.5	bcr:abl	LYG
QAGILARNLUPMVATVQGQN	20	2080.4	11	-0.5	GP64	QAG
VHSATGFKQSSKALQRPVASD	21	2214.5	10.6	0.2	bcr:abl	VHS
VIVHSATGFKQSSKALQRPVASDFEPQ	27	2928.3	9.9	0	bcr:abl	VIV

Table 3.2: Synthetic peptide sequences utlised to determine the suitability of the BCA assay for peptide quantification and in development of the modified assay. Physical properties derived from the online peptide property calculator at http://www.innovagen.se/. All are from human protein sequences, with the exception of QAG (derived from human herpes virus 5). Key: a.a., amino acids; Da, molecular weight in Daltons; pI, isoelectric point; HPS, hydrophillic score. All 9-10 amino acid peptides have high predicted MHC class-I A*201 and A*301 binding scores.

Peptide concentration curves from 0.125 – 1 mg/ml were prepared (as Figure 3.5) and samples assayed as per the method outlined in section 2.3.2.1. The divergent standard curves produced illustrate the diverse ability of assorted peptides to react with the BCA standard working reagent. A time course of BCA reactivity over 120 minutes indicate that this difference is not primarily based around differential rates of reaction, but a near-absolute difference in reactivity. While the inter-peptide variation was found to decrease over time, a linear regression curve extrapolated from the final 30 minutes of the time course indicated that inter-peptide variation would require an incubation of over eight hours to drop below 10 %, and therefore merely extending the incubation period was not a realistic solution (data not shown).

However when the effect of the peptides' physical properties on BCA reactivity was investigated, a non-parametric correlation co-efficent of 0.788 became apparent between peptides' hydrophilic scores (HPS) and BCA reactivity, rising to 0.83 if phosphorylated peptides are excluded, and 0.907 if the sample is restricted to non-phosphorylated peptides of 9-10 amino acids in length, though neither phosphorylation status nor peptide length correlated well with BCA reactivity (even if peptides were divided into subgroups according to HPS). Furthermore, larger peptides appeared to show a greater sensitivity of BCA reactivity to changes in hydrophilicity (see Figure 3.6), though this may be an artefact of the small population studied.

3.4 Reducing interpeptide variability in BCA reactivity through peptide solubilisation

As the prime determinant of BCA reactivity appeared to be hydrophilicity, attempts were made to solubilise peptides by a variety of regimens, including addition of NaOH, SDS, deoxycholate, disodium phosphate, DMSO and methanol, alone or in various combinations (see 2.3.3). Two peptides with widely divergent hydrophobicities were utilised: YISPLKSPY (YISx, HPS -0.5) and SQKGQESEY (SQKx, HPS 1.1) and following solubilisation the peptides were assayed by the standard BCA protocol (see section 2.3.1) at 0.5 mg/ml. The absorbance of both peptides was pooled and standard deviation calculated to determine spread between the two samples, compared against assaying both in water (see Figure 3.7). A comparison of these solubilisation methods indicated that while many approaches led a to decrease in spread between the two peptides, boiling the

sample in 1 % (w/v) SDS in 100 mM NaOH provided the greatest reduction in interpeptide variability.

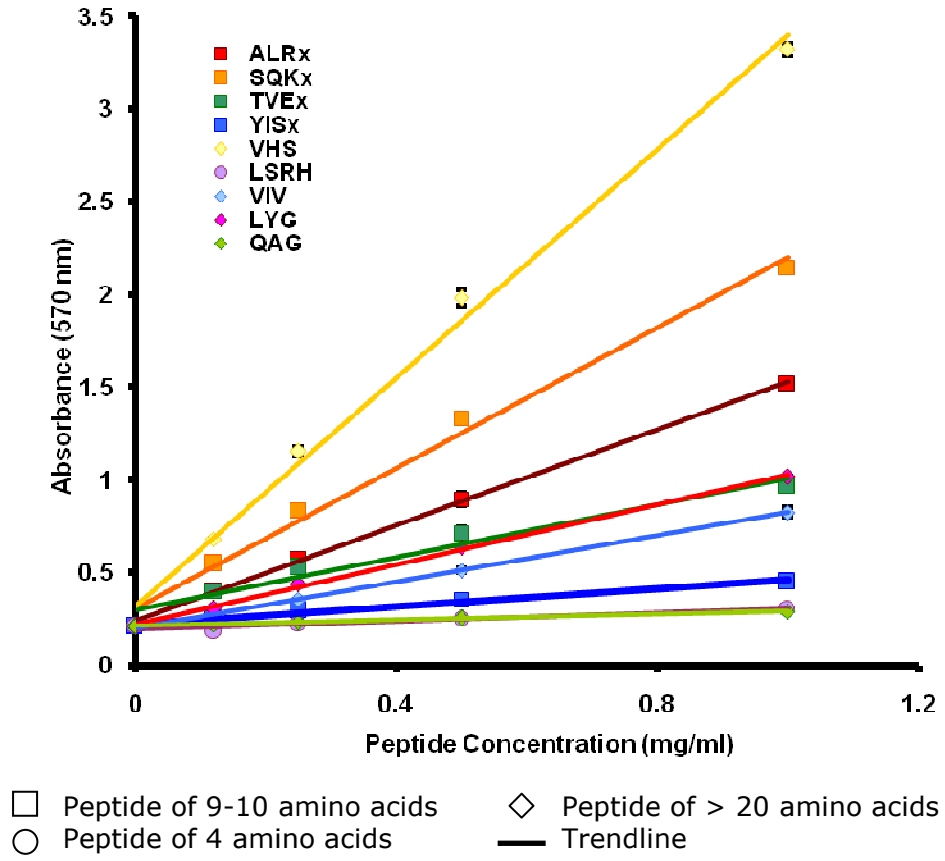


Figure 3.5: BCA assay calibration curves generated from 9 synthetic peptides (all non-phosphorylated) from 0.12 – 1 mg/ml. Peptide dilutions were prepared in water and 25 µl transferred to a 96 well plate in triplicate. 200 µl of BCA working reagent was then added to each well, and the plate incubated for 15 minutes at 37 ℃ before the absorbance was read at 570 nm. $n = 4$, error bars indicate SD.

Peptide	Trendline Equation	R^2
ALRSNFERI	Y = 1.2922x + 0.2393	0.9985
SQEGQESEY	y = 1.8948x + 0.3055	0.9905
TVEAVAYAPK	y = 0.7149x + 0.2958	0.9719
YISPLKSPY	y = 0.2366x + 0.2221	0.9839
VHSATGFKQSSKALQRPVASD	y = 3.0076x + 0.3236	0.9931
LSRH	y = 0.1146x + 0.1929	0.9326
VIVHSATGFKQSSKALQRPVASDFEPQ	y = 0.6263x + 0.1978	0.9983
LYGFNLVIVHSATGFKQSSK	y = 0.8056x + 0.2198	0.9983
QAGILARNLVPMVATVQGQN	y = 0.0813x + 0.2104	0.9977

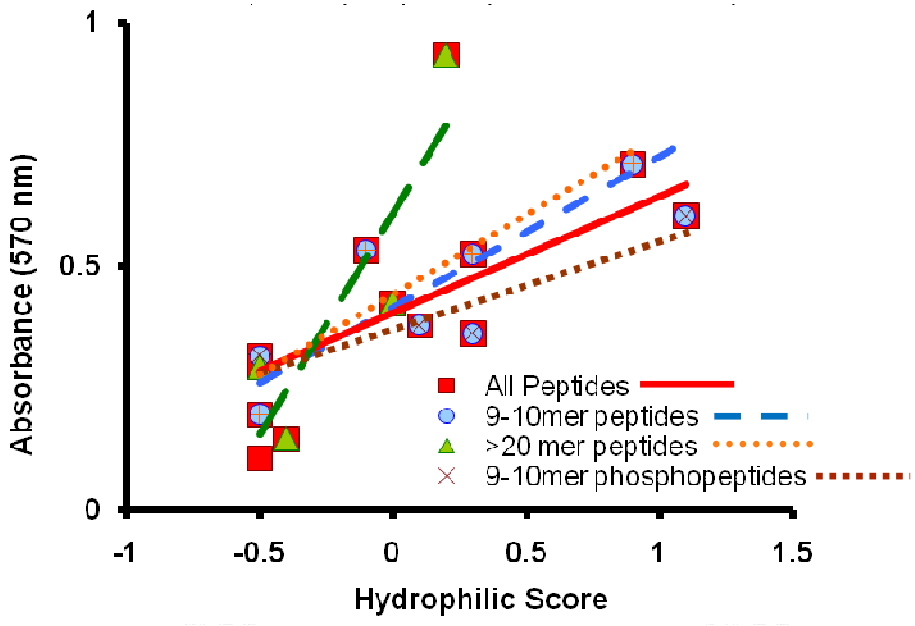


Figure 3.6: Scatterplots of peptide hydrophilic score (HPS) and BCA reactivity for 13 synthetic peptides (*n* = 4) when assayed by the standard BCA protocol at 0.5 mg/ml, including trendlines for total peptide population and subpopulations divided by peptide length and phosphorylation status (hence symbol overlap). Peptides were prepared in H_2O and 25 µl transferred to a 96 well plate in triplicate. 200 µl of BCA working reagent was then added to each well, and the plate incubated for 30 minutes at 37 ℃, following which the absorbance was read at 570 nm. HPS derived from the peptide property calculator at http://www.innovagen.se.

Peptide Population	Population Size	Mean Absorbance	Mean HPS	Spearmans' Rho (*P* value)
All peptides	**13**	**0.42**	**0.03**	**0.788 (0.00)****
9-10mer subpopulation	**8**	**0.45**	**0.2**	**0.762 (0.00)****
9-10mer Phosphorylated subpopulation	**4**	**0.4**	**0.25**	**0.734 (0.007)****
9-10mer Non-phosphorylated subpopulation	**4**	**0.49**	**0.15**	**0.907 (0.00)****
> 20mer subpopulation	**4**	**0.45**	**-0.18**	**0.777 (0.03)***
All non-phosphorylated peptides	**9**	**0.43**	**-0.1**	**0.83 (0.00)****

Spearmans *Rho* correlation co-efficients for the relationship between synthetic peptide BCA reactivity and hydrophillic score (HPS).

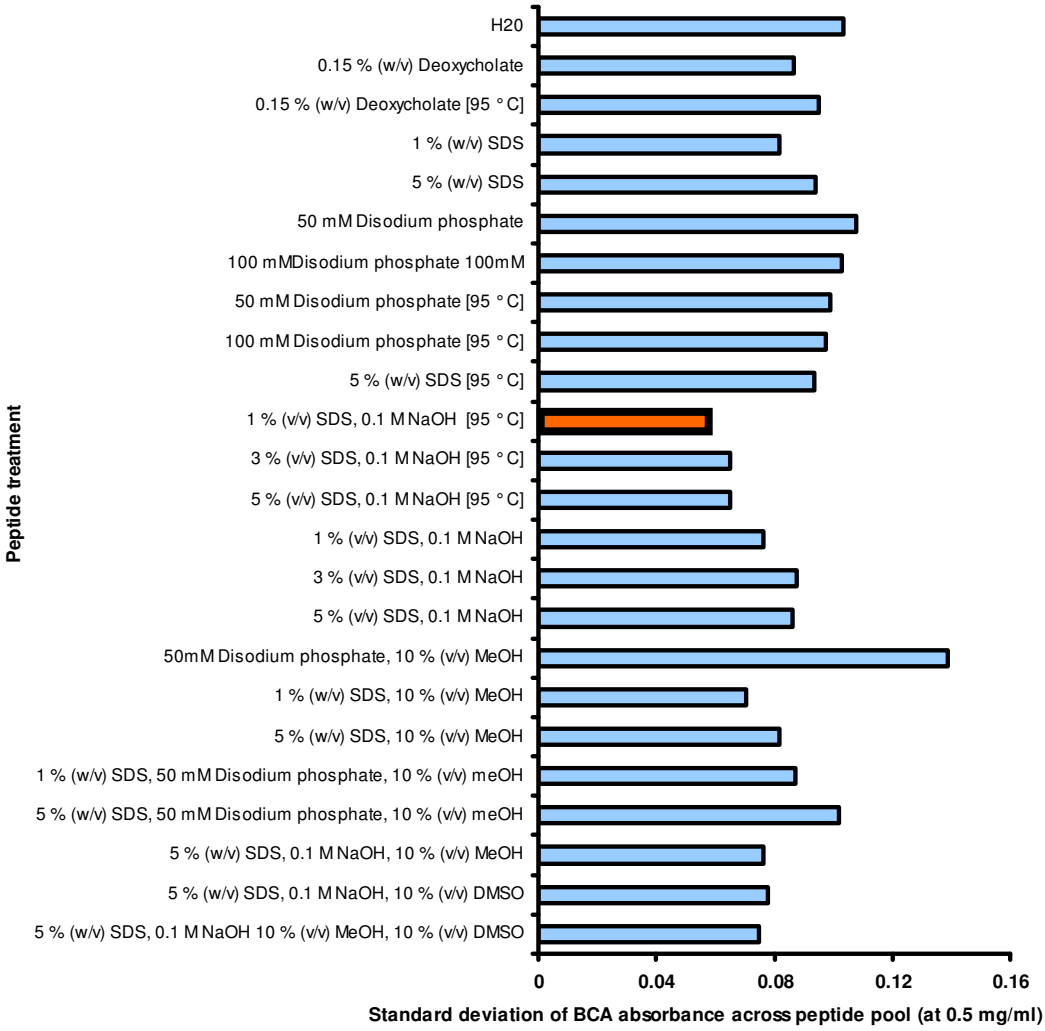


Figure 3.7: Cross-peptide standard deviation following various solubilisation regimens. Attempts were made to increase the solubility of two peptides YISPLKSPY (HPS -0.5) and SQKGQESEY (HPS 1.1) by various means (n = 3), following which 25 µl of each sample was transferred to a 96 well plate and 200 µl of BCA working reagent added to each well before the plate was incubated at 37 °C for 30 minutes, and the absorbance read at 570 nm. Following this absorbances (n = 3 or greater) were normalised against a water control and the standard deviation calculated across all absorbances for each solubilisation regimen. This indicated that whilst many approaches led to a reduction in spread between the two peptides when compare with the non-solubilised control, heating the samples for five minutes at 95 °C in the presence of 1 % (w/v) SDS in 0.1 M NaOH prior to the addition of BCA working reagent gave the greatest reduction in interpeptide variation.

This solubilisation method was then reapplied to the wider synthetic peptide population and a reduction in spread of 32.12 % was found, rising to 52.46 % when the assay was restricted to nonamers and decamers. When the trypsinised samples were analysed was found to only reduced the spread of standard protein digests (rabbit aldolase; BSA, bovine casein and catalase) by 11.5 %; while digests of protein isolated from guinea pig liver, and two suspension cell lines (the EBV-immortalised JY line and the T2 hybridoma line) showed a greater reduction in intrersample variation of 34.63 % (full graphical and numerical information is available in figures 3.8 – 3.10). Reproducible linear standard curves were achievable in all cases. Surprisingly however the reduction in spread was not achieved solely by increasing the reactivity of hydrophobic peptides, but also byreducing the absorbance of formerly highly-reactive peptides (especially notable in the case of the VHS peptide, though also for SQKx, and to some extent for ALRx and VIV), leading to a notable reduction in the mean absorbance for each peptide pool, though the median absorbance was not dramatically altered, indicating that peptides of medium to low-reactivity were not similarly affected.

3.5 Investigation into addition of SDS directly to BCA reagent A

In was hypothesised that the number of reagents required could be streamlined by the addition of SDS and the peptide sample directly into BCA reagent A, which itself contains 100 mM NaOH. This mixture could then be heat-denatured prior to mixing with the $CuSO_4$ required for the BCA reaction. Once again the hydrophobic YISPLKSPY (YISx, HPS -0.5) peptide and the hydrophilic SQKGQESEY (SQKx, HPS 1.1) peptide were utilised to test the feasibility of this approach. These were included at a fixed concentration (0.5 mg/ml) in the preparation of two standard curves (one for each peptide) of SDS concentration in BCA reagent A (0.05 – 1 % w/v SDS). Both standard curve preparations were then heat-denatured and added to a 96-well microplate containing 4 µl of $CuSO_4$ in each well, following which the plate was incubated for 30 minutes at 37 °C and absorbance measured as previously. The absorbance of both peptides was pooled and the cross-peptide standard deviation calculated to determine spread between the two peptides, compared against assaying both in water, or by denaturing in the previous SDS-NaOH protocol (see Figure 3.11).

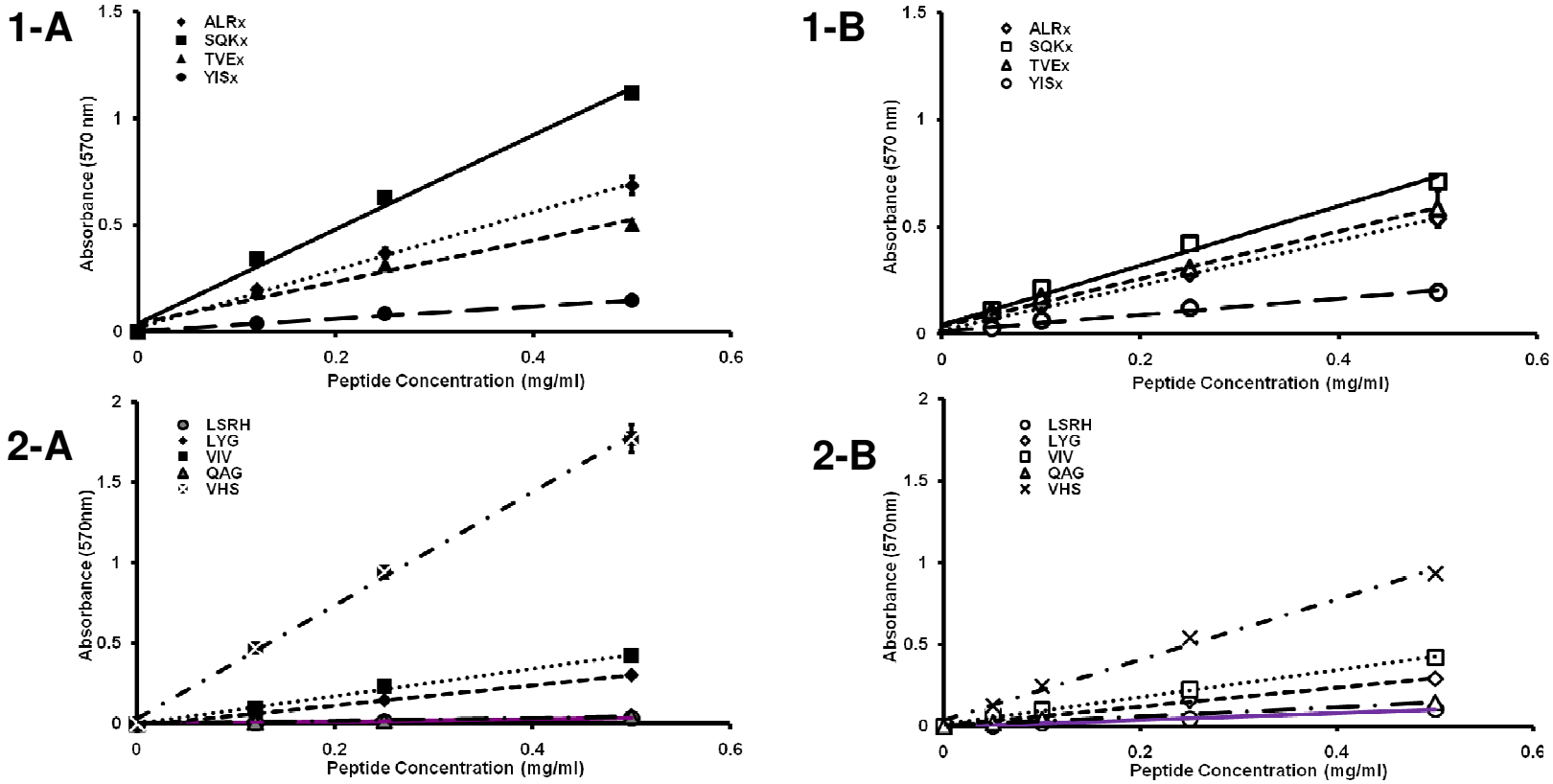


Figure 3.8: Typical standard calibration curves for synthetic **(1)** unphosphorylated 9-10mer and **(2)** 4 and >20mer peptides (in each case *n* = 4). Peptides were assayed by BCA in **(A)** water or **(B)** following heat-denaturing in 1 % (w/v) SDS in 0.1 M NaOH. Standard curves of peptide concentration were prepared and 25 µl assayed in a 96 well plate in triplicate by addition of 200 µl of BCA working reagentand incubated for 30 minutes at 37 ℃ prior to reading absorbance at 570 nm. Linear standard curves were achieved for each peptide, with heating in SDS-NaOH reducing inter-peptide variation by >32 %, by both reduction in reactivity of formerly re peptides, and increases in reactivity of hydrophobic peptides. Error bars indicate SD.

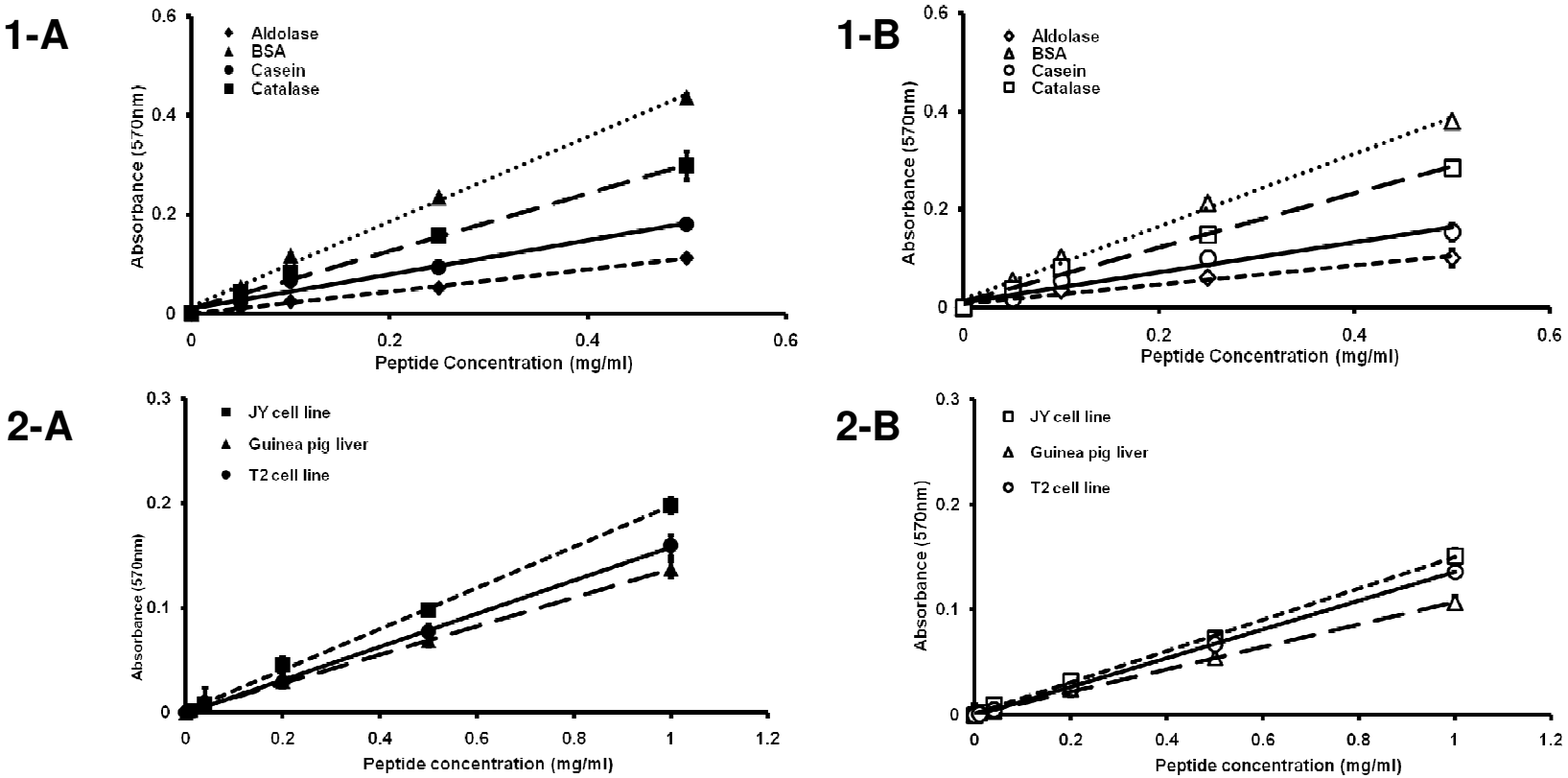


Figure 3.9: Typical standard calibration curves for tryptic digests of **(1)** standard proteins (aldolase, BSA, casein and catalase) and **(2)** biologically derived material (protein from guinea pig liver, and the JY and T2 suspension cell lines) (in each case n = >3). Digests were assayed by BCA in **(A)** water or **(B)** following heat-denaturing in 1 % (w/v) SDS in 0.1 M NaOH. Standard concentration curves were prepared and 25 µl assayed in a 96 well plate in triplicate by addition of 200 µl BCA working reagent and incubated for 30 minutes at 37 ℃ prior to reading absorbance at 570 nm. Linear curves were achieved for each peptide, with heating in SDS-NaOH reducing inter-peptide variation by 11.5 % for the standard proteins, and 34.63 % for the biologically derived material. Error bars indicate S.D.

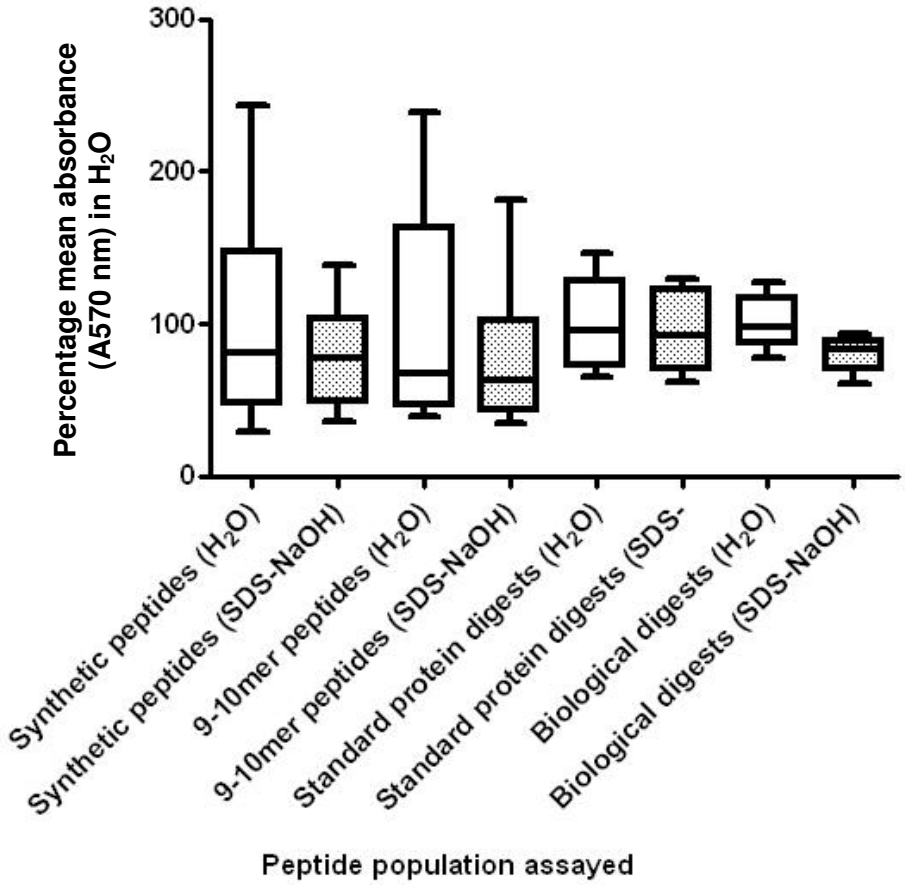


Figure 3.10: Box and whisker plot indicating total data spread and interquartile range of synthetic and tryptically-derived peptide populations assayed by the standard BCA (Smith *et al*., 1985), or by denaturing in SDS-NaOH prior to assay. Standard proteins: aldolase, BSA, catalase and casein. Biological material: protein extracts from guinea pig liver, and JY and T2 suspension cell lines. Data is expressed as a percentage of mean absorbance for that population when assayed by the standard BCA method. In each case $n = 3$.

Sample	Assayed in H_2O		Assayed in SDS-NaOH		Percentage reduction SD
	median	mean (±SD)	median	mean (±SD)	
Synthetic peptides (n=13)	0.6685	0.8656 (± 0.5811)	0.647	0.671 (± 0.3944)	32.12%
Synthetic 9-10mer peptides (n=8).	0.0582	0.0829 (± 0.0539)	0.0544	0.0651 (± 0.0256)	52.46%
Standard protein digests (n=4)	0.417	0.4368 (± 0.1289)	0.4025	0.41 (± 0.1141)	11.5%
Biological material digests (n=4)	0.0795	0.0812 (± 0.0137)	0.0677	0.065 (± 0.009)	34.63%

Median and mean A_{570} nm (± standard deviations) for synthetic and tryptically-derived peptide populations assayed by the standard BCA or by heat-denaturing in SDS-NaOH prior to assay. Standard proteins: aldolase, BSA, catalase, and casein. Biological material: protein extracts from guinea pig liver, and JY & T2 suspension cell lines. Synthetic peptides as previously detailed.

It was found that this method resulted in inter-peptide variation comparable to the standard BCA approach, with the absorbances for SQKx being similar across the standard curve, often without any statistically significant difference in absorbance. However a slight and statistically significant increase in absorbance for the YISx peptide was observed across the standard curve. Though this was highest for reagent A containing 0.1 % (w/v) SDS, it was statistically indistinguishable from that achieved using reagent A in the absence of SDS, implying that heat denaturing YISx within reagent A may be the determining factor (similarly SQKx also showed small but a statistically significant increase in absorbance when heated in reagent A in the absence of SDS).

Overall however there is little reduction in inter-peptide spread when compared with the standard BCA approach, while heat-denaturing the peptides in 1 % (w/v) SDS in 0.1 M NaOH prior to mixing with working reagent was again shown to reduce inter-peptide variation by over 50 % (Kapoor *et al*., 2009).

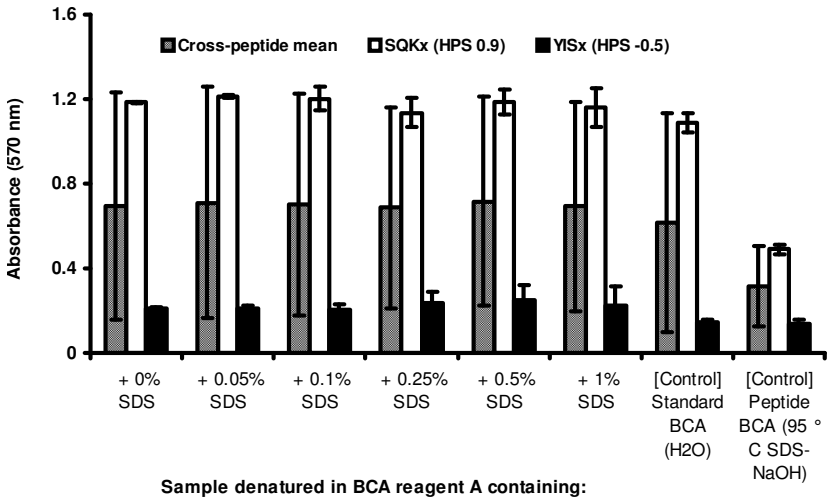


Figure 3.11: Assessment of SDS addition into Reagent A. Absorbance of two peptides (and cross-peptide mean) (n = 5) at 0.5 mg/ml following heat-denaturing in BCA reagent A in the presence of increasing concentrations of SDS, following which samples were mixed with $CuSO_4$ (at a 50:1 ratio of reagent A:$CuSO_4$), incubated at 37 ºC for 30 minutes, and the absorbance read at 570 nm; compared with peptides assayed in H_2O, or following heat-denaturing in 1 % (w/v) SDS in 0.1 M NaOH. As can be seen SQK and YIS absorbances are largely constant across the increasing SDS concentrations, while heat denaturing in 1 % (w/v) SDS in 0.1 M NaOH dramatically reduces interpeptide spread, as indicated by the error bars (S.D.).

3.6 Determination of assay sensitivity following modification

In order to determine to what extent the modified assay conditions may have impacted upon sensitivity, standard curves from 5 – 50 µg/ml were prepared from tryptic digests of BSA as per 2.3.2.2, and assayed as per 2.3.1 and 2.3.4, with both the 50 : 1 BCA reagent A : $CuSO_4$ ratio described in 2.3.1 and the 25 : 1 ratio described in 2.3.5.1. It was found that at concentrations of 10 µg/ml and above, linear standard curves were achievable, but below this absorbances were statistically indistinguishable from the blank for both the unmodified and modified BCA at the 50:1 ratio of BCA working reagent (see Figure 3.12). As expected, the 25:1 ratio of BCA working reagent gave a minor increase in absorbance to the standard BCA assay, though without increasing the sensitivity of the assay, while this ratio appeared to impart a reduced sensitivity to the modified assay, rendering the 10 µg/ml BCA peptide standard statistically indistinguishable from the blank, and barely distinguishable from 20 µg/ml. Regression co-efficient (R^2) values of 0.98 or greater were achievable in all cases apart from the modified assay when carried out with the 25:1 ratio of BCA working reagent (R^2 = 0.67).

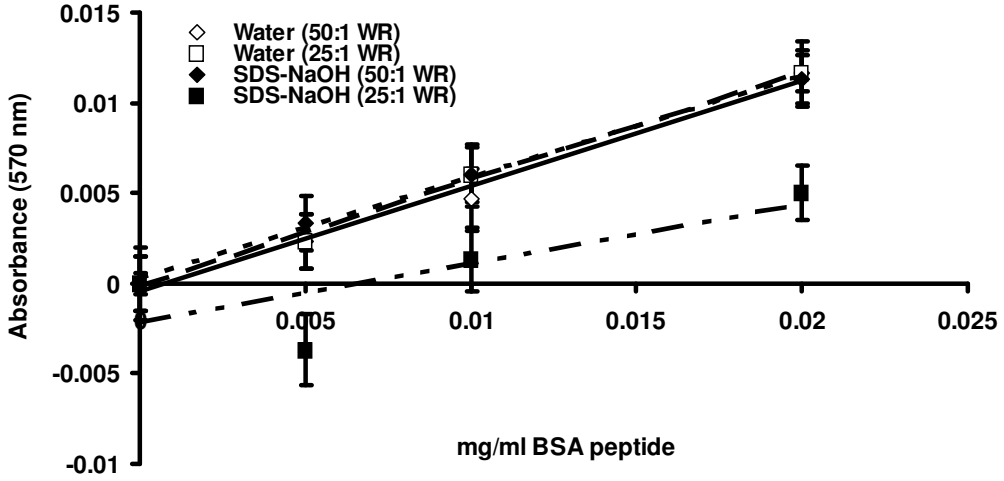


Figure 3.12: Typical standard BCA assay calibration curves for BSA digests (n = 4) over 5 – 20 µg/ml to determine the limits of assay sensitivity. BSA tryptic digests were assayed in a standard calibration curve: either in H_2O, or following heat-denaturing in 1 % (w/v) SDS in 0.1 M NaOH. 25 µl of each sample was transferred to a 96 well plate in triplicate and 200 µl of BCA working reagent (prepared at either 50 : 1 or a 25 : 1 ratio of reagent A to 4 % (w/v) $CuSO_4$) was added to each well. The plate was then incubated for 30 minutes at 37 ℃, and the absorbance was read at 570 nm. The higher concentration of $CuSO_4$ in the 25:1 working reagent produces a decrease in sample reactivity when peptides were were heat denatured in SDS-NaOH prior to the assay. Error bars indicate S.D.

3.7 Applicability of microwave incubation to replace either the heat-denaturing step or the 37 °C incubation step

In order to determine whether the microwave-incubation proposed by Akins and Tuan (1992) was transferable to replace the 30 minute 37 °C incubation required for the modified assay, and furthermore, whether it might also be utilised to simultaneously replace the heat-denaturing step at 95 °C, the two peptides previously utilised for solubilisation studies: YISPLKSPY (YISx, HPS -0.5) and SQKGQESEY (SQKx, HPS 1.1) were exploited. Short standard curves over the 0.1 – 0.5 mg/ml range were prepared from each peptide, either in H_2O, or in 1 % (w/v) SDS in 0.1 m NaOH (+/- heat denaturing) as per 2.3.4.

Before the incubation could take place, control microplates containing only water were microwave-incubated in a number of positions on the revolving tray. These were then heated to boiling and visually observed. It was found that placing the microplate in the centre of the revolving tray lead to rapid boiling, but this only occurred in the centre of the microplate. Offsetting the microplate lead to those wells closest to the centre heating to the greatest degree, while the most even

incubation was achieved by moving the microplate to the edge of the revolving tray, allowing it to complete a 180° revolution over 15 seconds, then pausing the incubation and moving it to the opposite edge of the tray (thereby alternating which wells are on the inside of the revolving tray) before continuing the incubation for a further fifteen seconds (see 2.3.5.3). It was also observed that the fewer wells of the 96-well microplate that contained aqueous sample, the more rapidly those wells that were utilised would reach boiling point, therefore for purposes of standardisation, 200 µl of dH_20 was added to any unutilised wells.

Once this was optimised, incubation of a 96-well microplate containing the peptide standard curves and BCA working reagent were subjected to the microwave incubation and the reduction in data spread calculated as previously described (see Figure 3.13).

While standard curves could be produced using the microwave method, it was found that inter-well variability was higher when plates were incubated by this method, and the R^2 values lower than usually observed.

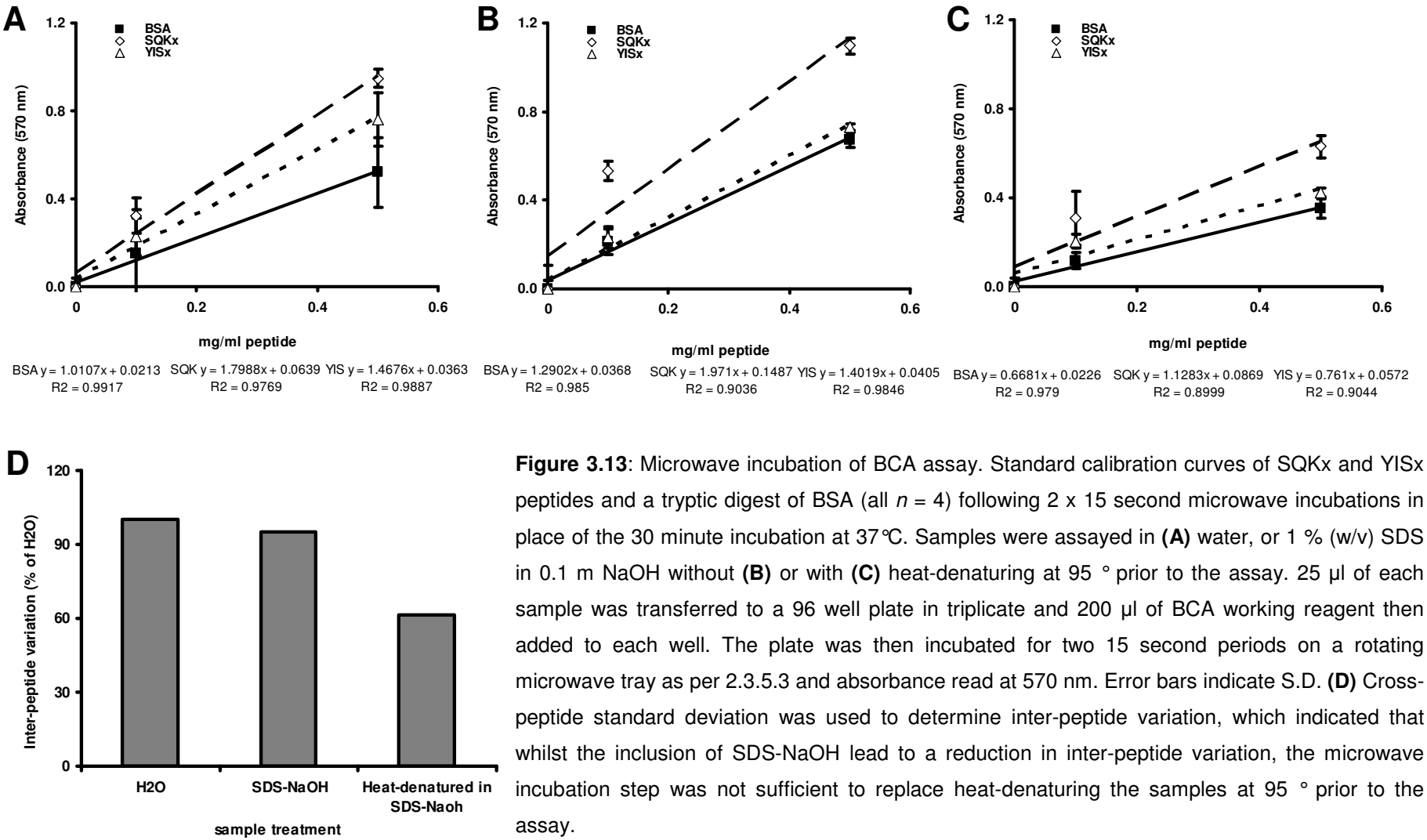


Figure 3.13: Microwave incubation of BCA assay. Standard calibration curves of SQKx and YISx peptides and a tryptic digest of BSA (all *n* = 4) following 2 x 15 second microwave incubations in place of the 30 minute incubation at 37°C. Samples were assayed in **(A)** water, or 1 % (w/v) SDS in 0.1 m NaOH without **(B)** or with **(C)** heat-denaturing at 95 ° prior to the assay. 25 µl of each sample was transferred to a 96 well plate in triplicate and 200 µl of BCA working reagent then added to each well. The plate was then incubated for two 15 second periods on a rotating microwave tray as per 2.3.5.3 and absorbance read at 570 nm. Error bars indicate S.D. **(D)** Cross-peptide standard deviation was used to determine inter-peptide variation, which indicated that whilst the inclusion of SDS-NaOH lead to a reduction in inter-peptide variation, the microwave incubation step was not sufficient to replace heat-denaturing the samples at 95 ° prior to the assay.

The inclusion of 1 % SDS in 0.1 M NaOH within the peptide matrix was found to reduce inter-peptide variation by approximately 5 % when compared with the peptides assayed in ddH_2O. Curiously this was less than the reduction found in unheated samples incubated at 37 °C, where it was reduced by 26 % (see figure 3.7). This may be a function of the higher inter-well variability which is believed to be a consequence of non-uniform heating of each well (despite the repositioning of the microplate within the microwave), or possibly a longer 37 °C incubation in SDS-NaOH provides greater solubilisation and linearisation than the brief microwave incubation allows. In either case, it is clear that microwave-incubation is not sufficient to replace heat denaturing prior to incubation, which produced a reduction in inter-peptide variation of 38.6 % (though this is a smaller improvement than the 52.46 % reduction achieved by a 30 minute incubation at 37 °C).

3.8 Investigation into the effect of peptide phosphorylation on BCA reactivity

Finally, given the overall aims of selectively, the effect of phosphorylation on the BCA peptide-assay was briefly investigated using the four 9-10mer phosphopeptides and their non-phosphorylated counterparts. When the reactivity of the phosphorylated and non-phosphorylated forms of these peptides are compared a clear discrepancy is observed (see figure 3.14), in that the phosphate group appears to produce a reduction in BCA reactivity (significant to $P < 0.05$) in three of the four peptides (ALR, SQK and TVE), though to differing degrees, irrespective of whether the peptides are heated in 1 % (w.v) SDS in 0.1 M NaOH or not. A very minor increase in BCA reactivity is apparent in the final peptide (YIS), though this was not statistically significant if heat-denatured in SDS-NaOH.

It is notable that in the case of the ALR peptide, phosphorylation only reduces BCA reactivity by approximately 12 % (when assayed in SDS-NaOH), while in the case of SQK and TVE, this rises to 45% and 35% respectively. Conversely, the hydrophobic YIS peptide shows a minor statistically insignificant increase in reactivity when phosphorylated. As the SQK and TVE peptides are tyrosine phosphorylated, it is conceivable that this may be linked to the sequence-specific temperature independent secondary reaction noted by Wiechelman *et al*. (1988). Though within the peptide pool studied tyrosine-rich peptides were not found to

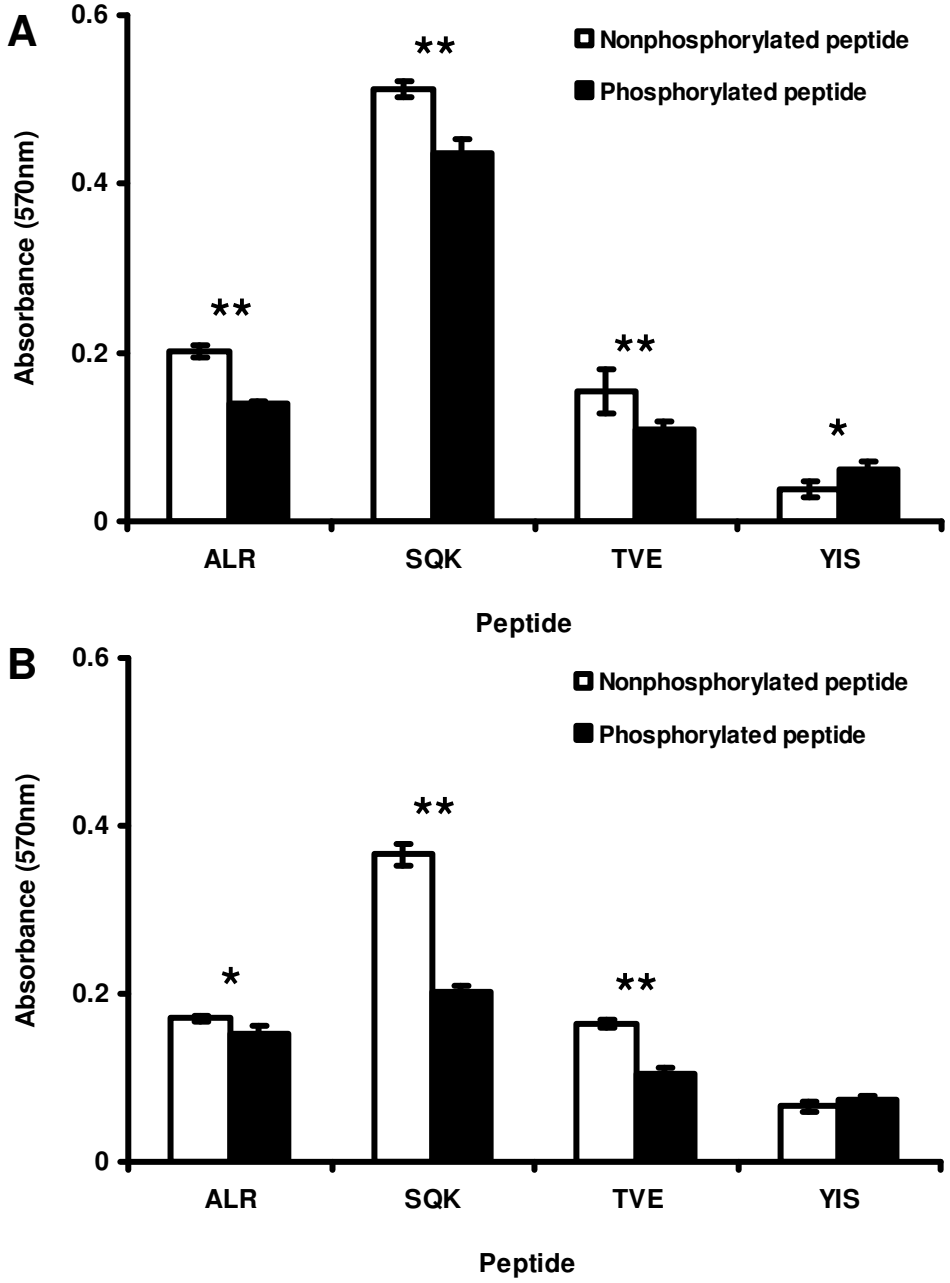


Figure 3.14: Differential BCA reactivity of four peptides with and without phosphorylation. Peptides were assayed at 0.125 mg/ml **(A)** in water or **(B)** following heat denaturing in 1 % (w/v) SDS in 0.1 m NaOH. 25 µl of each sample was transferred to a 96 well plate in triplicate and 200 µl of BCA working reagent then added to each well. The plate was then incubated for 30 minutes at 37 ℃, following which the absorbance was read at 570 nm. See table Y.2 for peptide sequences. For all but the most hydrophobic peptide, phosphorylation appears to produce a statistically significant reduction in BCA reactivity, for both denatured and native peptides (in each case $n = 4$). * = significant to a P value of <0.05, ** = significant to a P value of <0.01.

have a statistically greater reactivity than those without this residue, the range of peptide lengths and hydrophilicities, combined with a small overall sample pool may have hidden such sequence specific effects. As another post-translational modification, sulphation, may also occur to tyrosine residues (Huttner, 1982), it is conceivable that this may have similar effects, as may modification of tryptophan or cysteine, which are targets for glycoslation (Schnaebel *et al*., 2004), and prenylation/palmitoylation respectively (Marshall, 1993; Deitrich and Ungermann, 2004).

Conversely, ALR and YIS are both serine phosphorylated, and while a slight reduction in BCA reactivity is noted in the phosphorylated form of ALR compared with the unphosphorylated control, there is no known role for serine in the secondary BCA reaction. In this case it is conceivable that a phosphoserine may simply interfere sterically with the co-ordination and reduction of the Cu^{2+} to Cu^{1+}, though in the case of the highly hydrophobic YIS peptide, this may be balanced against a possible increase in solubility imparted by the phosphate group.

When peptides are assayed by the standard BCA protocol (i.e. without SDS-NaOH) the differences in absorbance between phosphorylated and nonphosphorylated peptides is greater for the serine-phosphorylated peptides (ALR, YIS), but smaller for the tyrosine phosphorylated peptides (SQK, TVE). The reasons behind this are unclear; while the purpose of the SDS-NaOH is to linearise and solubilise the peptides prior to the assay, unphosphorylated tyrosine containing peptides showed relative reduction in reactivity compared to their phosphaorylated counterparts. Therefore it is not due to a resulting increase in the accessibility of the naked tyrosine residues and concurrent increase of the secondary (sequence specific) BCA reaction compared with the native controls. It is possible that the same solubilisation and linearization may reduce the steric hindrance of the phosphoserine residues on Cu^{2+} reduction or co-ordination, leading to a concomitant ablation of the differences in BCA absorbance. Analysis of the interpeptide variation reveals that spread between the phosphorylated peptides is less than 50 % of that found between their non-phosphorylated counterparts. This naturally leads to the hypothesis that the modified BCA assay may potentially show even greater accuracy when the content of a phosphopeptide enriched sample is analysed (assuming an appropriate phosphorylated standard is utilised). However given the limited phosphopeptide population utilised these hypotheses are difficult to verify, and

will require further evaluation before their accuracy and applicability may be fully determined.

3.9 Discussion

While in theory a wide number of assays are available for the measurement of peptide and protein concentration, in practice many of these require extensive sample clean-up, lengthy assay time, specialised equipment, or are heavily influenced by sequence-specific effects. The versatility and robustness of the BCA protein assay, along with its rapidity and convenience has lead to and maintained its widespread use as one of the leading protein assays for several decades (Stoscheck, 1990). While not designed for the determination of peptide concentration, these qualities - along with the fact that it does not primarily rely on a sequence-specific reaction - have lent it to adoption in the assay of peptides and polypeptides (Hua & Scheller, 2001; Faber *et al*., 2003; Saleem *et al*., 2005).

However, it is evident that there is a wide variation in inter-peptide BCA reactivity; though some variations in reactivity between samples is not unusual and generally accepted in protein assays (see: Gornall *et al*., 1949; Smith *et al*., 1985); this is usually minimised by choice of appropriate standards. In this case it is apparent that the variations were primarily due to differences in peptide hydrophilicity (which was implicated in 90 % of the variation in nonphosphorylated peptides of the same length) and, it is presumed, solubility. Therefore using two peptides of differing hydrophilicities, attempts were made to solubilise the peptides by a variety of means, many of which reduced this variation to some extent, with boiling the sample in 1 % (w/v) SDS in 0.1 M NaOH prior to mixing with working reagent showing the greatest reduction. When this protocol was applied to a variety of peptide populations: both synthetic and tryptic digests from purified proteins (aldolase, BSA, casein and catalase) or biological samples (lysates of guinea pig liver and the JY and T2 cell lines), a reduction in data spread was achieved in each case, ranging from 11.5% - 52.5%, with a mean of 32.7%; a variation well within that accepted for the original protein BCA assay. This modification did not appear to significantly impact negatively on the assay's linearity, optimum incubation period, applicability of microwave-incubation, or the lower limits of the overall assay sensitivity, though some reduction in hydrophilic peptide reactivity was observed.

When investigating solubilisation strategies it was apparent that combination treatments were, for the most part, more effective than any single reagent. It is therefore conceivable that the inter-peptide variation could be further reduced by also including methanol, DMSO, or other peptide solubilising reagents. However, it is assumed that each addition to the assay will likely reduce the tolerance of the assay for complicating reagents.

The effect of post-translational modification – specifically phosphorylation, while normally of minor interest with regard to the protein assay, was also investigated. Our data suggests that phosphorylation notably reduces BCA reactivity, and that this effect is more pronounced in the case of tyrosine phosphorylation, possibly due to the sequence-specific secondary BCA reaction. Furthermore the effect of tyrosine phosphorylation was increased when peptides were heat-denatured in SDS-NaOH, highlighting the need for appropriate standards when samples are assayed. The effect of other post-translation modifications (e.g. glycosylation or prenylation) are at this point unknown, however it is considered likely that some effect would be observed, if only due to steric hindrance.

Of particular note is an effect which might be called the hydrophilic elasticity of reactivity by length: changes in overall peptide hydrophilicity had a greater impact on reactivity in longer peptides than in shorter ones. While this may seem a semi-obvious observation, for a 20–amino acid peptide to increase in hydrophilicity to the same extent as 10-amino acid one, the effect on the amino acid(s) itself must be greater. Similarly, one would therefore expect a single phosphorylation point to have a lesser effect on reactivity in longer peptides than shorter, though this hypothesis is as yet untested.

In conclusion, a simple modification to the BCA assay protocol markedly improves its applicability to peptide quantification. Despite this, some inter-peptide variation remains, and appropriate standards (including any expected post-translational modifications) must be used to generate an accurate result.

4.0: MHC cell-surface elution buffers (development of minimal-lysis IMAC-compatible MHC elution buffers)

4.1 Introduction

As previously discussed (see 1.3.4), the $CD8^+$ and $CD4^+$ T-cell responses are the cornerstone of the specific immune system, and rely on the expression of peptides by surface MHC molecules (which in humans are often termed HLA). MHC class-I expression is near universal in healthy nucleated cells, and involves the expression of short peptides primarily derived from intracellular protein (Clarke, et al., 2001), which are monitored by a circulating antigen-specific $CD8^+$ CTL population. MHC class-II is normally found only on typically phagocytotic antigen presenting cells (APCs), and displays longer peptides usually derived from exogenous protein, and monitored by the antigen-specific $CD4^+$ T-cells, which regulate other elements of both the specific and non-specific immune system (Assudani *et al*., 2006). Numerous groups have attempted to develop immunotherapy strategies against a range of cancers (using peptides/peptide constructs, whole proteins, plasmids, or attenuated cells) and whilst there has been a significant degree of success *in vitro* and using animal (typically murine) models, most human trials have shown little success (Parish, 2003).

There are numerous theories for why this is the case, including but not limited to immunoregulatory functions, and our limited understanding and control of the patient immune system, the late stage of many patients, the need to striate patient groups/move towards a more personalised approach, and a requirement for improvements in the vaccines themselves (the target, the delivery mechanism, and the adjuvant), (Copier *et al*,. 2009). This last category may also be paired with the need to investigate combination therapies (including chemical inhibitors, and when applicable, allogenic stem cell therapies for leukaemia patients) (Barret & Rezvani, 2007; Dao and Scheinberg, 2008) hopefully leading to improvements in the success rate of T-cell mediated tumour immunotherapy. Identification of putative vaccines is therefore an important aspect of both general cancer immunotherapy, and the personalised approach which may become more dominant in future (Castelli *et al*., 2004).

The methods used when searching for vaccines may be divided into two primary categories: namely direct or reverse immunology. Reverse immunology (discussed in 1.4.3) involves the development of a peptide-based vaccine typically from a gene or protein known to be commonly expressed/over-expressed in the tumour of interest (e.g. tumour associated antigens, fusion proteins or cancer-testis antigens). This was initially achieved using overlapping peptides from the protein of interest, but was later aided by algorithms such as Pcleavage and SYFPEITHI, which predict likely points of cleavage by the immune-proteasome, and strength of binding to different MHC-alleles (Rammensee, *et al*., 1999; Bhasin and Raghava, 2005); and confirmed using MHC binding assays. Peptides likely to be presented are then assessed by CTL-mediated killing assays of the target cells (Osanto, 1997). Currently however the limited data regarding the effects of phosphorylation on peptide processing and the strength of binding to MHC molecules makes a predictive approach difficult to achieve.

Direct immunology involves identification of naturally occurring MHC-associated peptide epitopes direct from the cell surface, typically though not exclusively from malignant or virus-infected cells/cell lines. The method stems originally from the work of Sugawara *et al*. (1987) who determined that treatment of either primary or cultured cells with isotonic citrate-phosphate pH 3.3 supplemented with 1 % (w/v) BSA led to elimination of MHC class-I mediated antigenicity in a pH dependent manner, without affecting either MHC class-II or non MHC antigens, and with minimum cytoloysis. This approach was later taken up by Storkus *et al*. (1993a) to elute MHC-associated peptides from a range of cell lines, and following fractionation of an eluate from an influenza-A infected Mel-624 cell line (MHC class-I A2-positive) by reverse-phase HPLC on a C_{18} column, they were able to confirm the presence in the eluate of a previously identified immunogenic peptide (Hogan *et al*,. 1988) by a CTL-mediated specific cytolysis assay. This approach was then used to identify immunogenic HPLC fractions from the surface of a number of melanoma cell lines (Storkus *et al*., 1993b), and six months later the first of these peptides was characterised using tandem mass-spectrometry (Cox *et al*., 1994), a pairing of methods which would become the accepted paradigm for direct immunology, and would be utilised by Jimenez *et al*. (1996); Ostankovitch *et al*., 1998 ; Herr, *et al*. (1999) ; Nakatsuka *et al*. (1999); Fan, *et al*., (2000); Santin *et al*. (2000); Clark *et al*. (2001); Ishii *et al*. (2003); Chromik *et al*. (2005); Gannage, *et al*., 2005; Gebreselassie *et al*. (2006); Pend *et al*. (2007) and Antwi *et al*. (2009).

However the pH 3.3 isotonic citrate-phosphate buffer is known to cause between 5 - 10 % cell death as determined by trypan-blue staining (Storkus *et al*., 1993a and 1993b; Fan *et al*., 2000; Clark *et al*., 2001), which may contaminate eluates with intracellular peptides not associated with MHC molecules (Bonner *et al*., 2002). As the chelating properties of the citric acid render it incompatible with on-line mass spectrometry, samples eluted from cells must be desalted and concentrated by (e.g.) C_{18} SPE or cation-exchange chromatography prior to subfractionation and analysis (Clark *et al*., 2001), following which candidate peptides may be chosen, and assessed for their immunogenicity (routinely using synthesised counterparts). While the direct immunology method requires a higher degree of sample handling and data analysis than reverse immunology, it does not restrict the search to peptides from a (usually single) known target (Greten and Jaffee, 1999), and ensures that the peptide is not only present on the cell surface, but is processed and presented efficiently; a potential weakness of the reverse immunological approach (Dao and Scheinberg, 2008). Furthermore direct elution of MHC peptides also prevents the products of unexpected variations in proteasomic cleavage from being disregarded, and may also be used to determine the presence of post-translationally modified peptides within the MHC presentome.

The most common alternative, papain cleavage of MHC molecules or membrane solubilisation and subsequent capture of MHC class-I molecules onto an immunoaffinity column is both expensive, and in the case of papain digestion risks cleavage of peptides and hydrolysis of esters and amides within the peptides' functional groups (Kato *et al*., 1987; Meos *et al*,. 1995)[1].

Direct elution of class-II peptides has long been thought to be problematic. Whilst it has previously been shown that the MHC class-II molecule will destabilise at pH 2.2 (Schneider and Secarz, 1997), high cell lysis has been observed at this pH (Bonner *et al*., 2002; Kessler *et al*., 2003). Therefore the primary approaches for acquisition of class-II restricted peptides again tend to involve either papain cleavage (Gorga *et al*., 1991; Nag *et al*., 1994) or

[1] Though the deamidation function of papain has an optimum pH of 10.0, at pH 7 it has only fallen to 25 % and the protein shows optimum deamidation activity at 20 °C (c.f. 50 °C for the protolytic activity). The esterfication activity has a optimum of pH 6-7. While C-terminal hydrophobic residues are important for MHC-binding (MHC consortium, 1999), Meos *et al*. (1995), also report that the presence of terminal hydrophobic residues are important for papain activity.

membrane solubilisation, followed by immunoprecipitation and elution (e.g. Tomlinson *et al*. 1996a 1996b and 1997). Again however, papain cleavage risks damage to peptides prior to their analysis, and furthermore unlike the class-I molecule which may be cleaved from the cell surface within 20 minutes, approximately 90% of class-II molecules remain uncleaved after a 3 hour incubation, even with a 1:3 ratio of papain:membrane protein (Kaufman and Strominger, 1979)[2]. Membrane solubilisation also presents problems in that a proportion (ranging up to 50 %) of MHC class-II molecules remain lipid insoluble (Poloso *et al*., 2004), impacting on immunoprecipitation and the subsequent elution Poloso *et al*., 2004. However this population may include the majority of molecules from the MIIC and so poorly bound peptides which would not normally be trafficked to the cell surface may be depleted from the eluate as a result (Poloso *et al*., 2004), it may also be enriched in those peptides which stimulate a T_h1 response (Buatois *et al.*, 2003), an effect which may be beneficial to the search for T_h2 stimulating epitopes, and also contain the majority of cell-surface class-II molecules presenting CLIP (Goebel, 2002). An alternative method, using noble-metal complexes to displace class-II antigens has been described by De Wall *et al*. (2006), though no subsequent information has become available, and compatibility with protein cleanup/fractionation techniques has yet to be assessed.

Recent work within NTU involved research into the elution of MHC class-II peptides direct from the cell surface using a pH 2 buffer compatible with a broad range of chromatographic methods, including IMAC and ESI-MS/MS (Barry, 2006). This was performed using the TAP and HLA-DM deficient T2 cell line transfected with MHC class-II DRB1*0401. As a result of HLA-DM deficiency, the class-II molecules present on the cell surface largely present the CLIP peptide (LPKPPKPVSKMRMAATPLLMQA). Flow cytometric staining against class-II and CLIP revealed disassociation of the peptide from the MHC at pH 2 (see figure 4.1.A), and this was confirmed by ESI LC MS/MS (figure 4.1.B).

As the importance of $CD4^+$ T_{h1} in potentiating the $CD8^+$ CTL response is well documented (Kaiko *et al*., 2008), it is conceivable that this may allow an alternate route for development of class-II peptide vaccines by direct immunology. Alternatively and as discussed previously (1.2.4.2) unlike the

[2] As determined by radio immuno-assay.

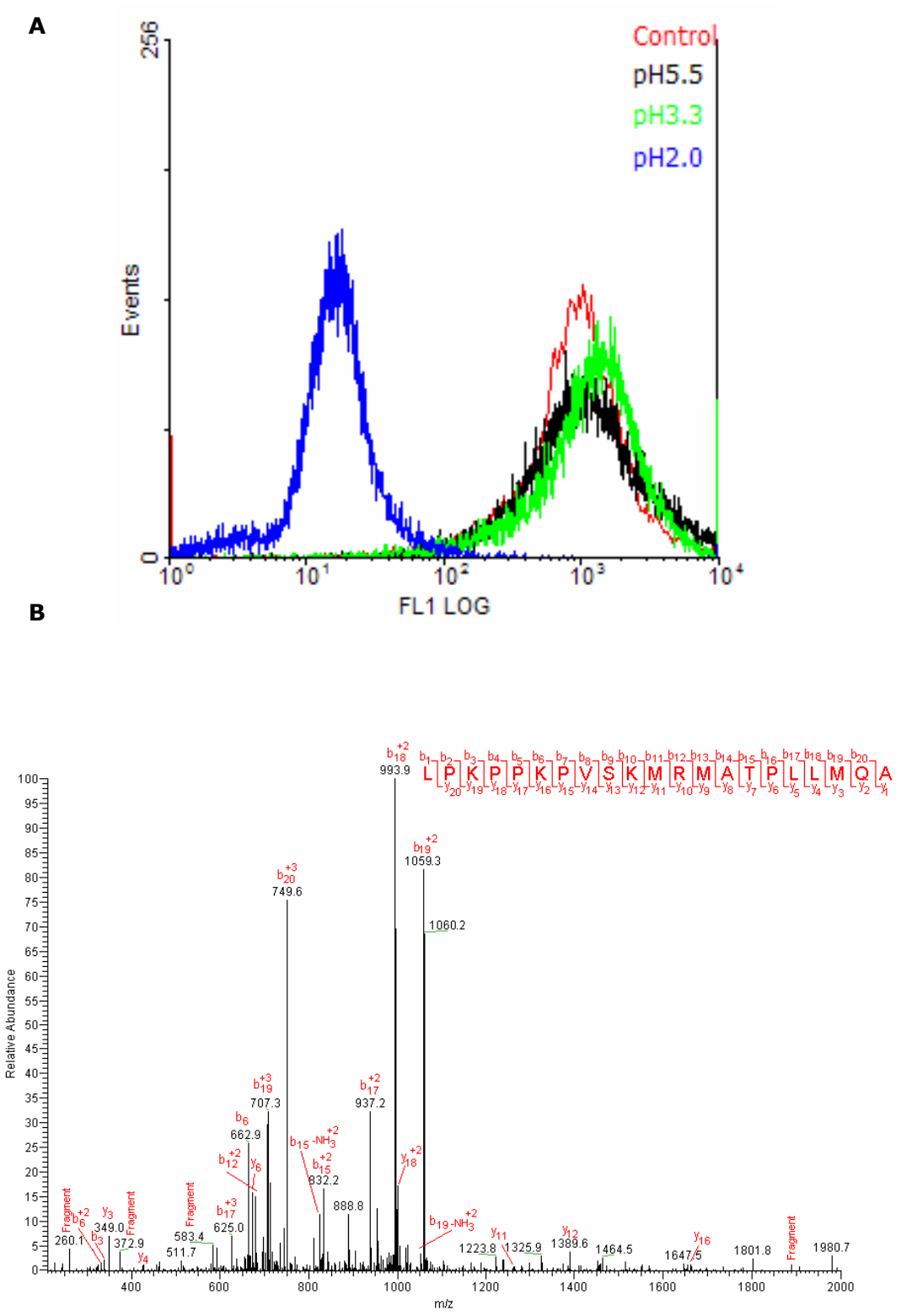


Figure 4.1: Confirmation of CLIP elution from MHC class-II of T2.Dr4 cells determined by **(A)** flow cytometry and **(B)** tandem mass spectrometry for the CLIP peptide (from Barry, 2006).

majority of tumours, MHC class-II expression is reasonably common in the leukaemias (Guy *et al*., 1986; Drexler *et al*., 1988, Yunis, *et al*., 1989) it may be coupled with the use of MHC class-II expression by leukaemic cells themselves as the basis for a cell-based vaccines, similar to the method of transfecting tumour cells or cell lines with class-II as a means of stimulating activation of T_h1 $CD4^+$ cells to cause an anti-tumour $CD8^+$ response leading to rejection of a tumour or metastases (Ostrand-Rosenberg *et al*., 1990; Baskar *et al*., 1995; Armstrong *et al.,* 1997; Pulaski and Ostrand-Rosenberg, 1998; Dolan *et al*., 2004).

In a similar vein, previous work carried out at NTU indicates that a 25 mM TMA-formate buffer pH 3.3 may be an IMAC compatible substitute for the isotonic citrate-phosphate Storkus' buffer, and the potential for a pre-elution wash with a low pH buffer to remove membrane-associated serum peptides prior to MHC elution (see table 4.1) (Barry, 2006). This chapter describes the development and optimisation of these novel buffers for the elution of class-I and class-II peptides direct from the surface of numerous cell lines with minimal cell lysis, reducing the time required for sample processing and purification.

No prewash.		**Following prewash.**	
Peptide sequence	Proposed Source	Peptide sequence	Proposed Source
TLAAHLPAEFTPAVHASLDKF	Haemoglobin	LSGITGARNLAVS QVVHKVVS	Alpha-1 antichymotrypsin pre-cursor
YQKVVAGVANALAHKYH	Haemoglobin	QNLKLFRH	Protein kinase AMP-activated alpha 2 catalytic subunit
LSFPTTKTYFPHF	Haemoglobin	FVSNHAY	Fructose bisphosphate aldolase
SPADKTNVKAAWGKVGAHAG EYG	Haemoglobin	HDSFLKAVPSQKR T	S100 calcium binding protein – calgizzarin
DAHKSEVAHRFKDLGEENFK	Human Serum Albumin	FGPKGFGRGGEA SHTFK	Cysteine Rich Protein 1
PGHLQEGFGCVVTNRFDQL	PAI-1 mRNA binding protein	MAHMASKE	Glyceraldehyde 3-phosphate dehydrogenase
TLAAHLPAEFTPAVHASLDKF	Haemoglobin	ECQSHKLTVE	Proteosome Subunit

Table 4.1: Highest confidence peptides eluted from LCL-BM cell line MHC class-II with unsupplemented 50 mM sodium formate pH 2 with or without first washing the cells with a pH 5.5 prewash, and isolated by Cu^{2+} IMAC prior to ESI MS/MS (from Barry, 2006).

4.2 Confirmation of MHC class-I elution by citrate-phosphate or TMA formate elution buffers

Previous work at NTU suggested that a TMA-formate buffer at pH 3.3 would be capable of causing disassociation of peptide from the MHC class-I complex in a pH dependent manner similar to the citrate-phosphate elution protocol devised by Storkus *et al*. (1993), while remaining compatible with a wide variety of downstream chromatographic approaches such as IMAC. In order to confirm this, populations of 4 x 10^6 JY and K562-A3 cells were treated with TMA-formate and citrate-phosphate elution buffers as per section 2.4.2, washed and rebuffered in PBS, and treated with the W6/32 anti-MHC class-I monoclonal antibody or an anti-β2 microglobulin monoclonal antibody and then treated with the FITC conjugated secondary antibody prior to analysis by flow cytometry, as described in section 2.4.3.

This illustrated comparable disassociation of β2 microglobulin from the MHC class-I complex with TMA-formate or citrate-phosphate (see Figure 4.2), and that despite research which suggests different MHC alleles may present tighter or looser global association with their presentomes (Gebreselassie *et al*., 2006), at pH 3.3 the effect was not limited to a single MHC allele. While the disassociation at pH 3.4 and above seemed to be greater for the citrate-phosphate buffer over the TMA-formate, this is probably due to the greater capacity and broader buffering range of the citrate-phosphate buffer. The pH 5.5 citrate-phosphate buffer appears to have no appreciable effect on MHC expression or β2 microglobulin retention, and is thus a suitable prewash medium.

Though this data does not document MHC expression levels following buffer treatment, any internalisation of the MHC complex is expected to take place after the disassociation of the peptide, especially given that the buffers used were pre-cooled to 4 °C, and the rate of MHC internalisation is known to be temperature dependent (Tse and Pernis, 1984).

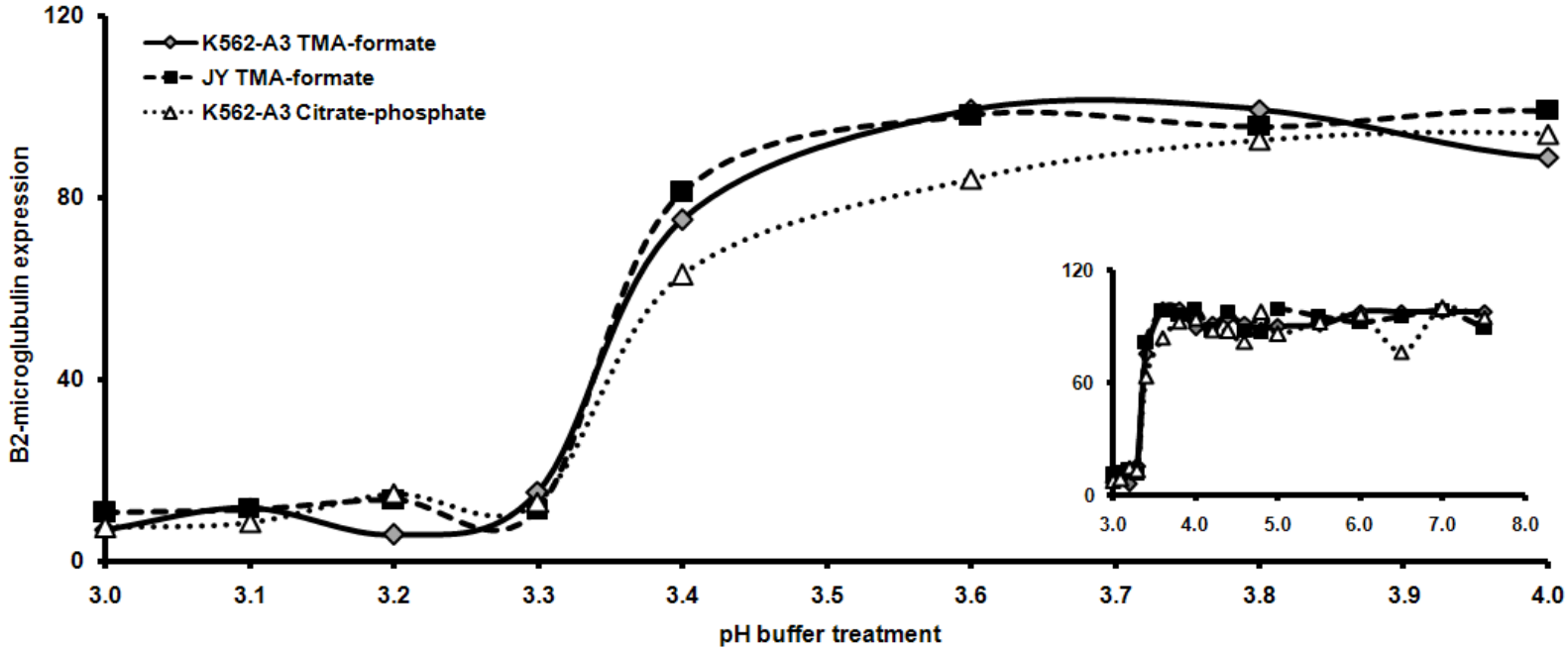


Figure 4.2: Confirmation of pH–dependent β2-microglobulin elution by FACS (expressed as a percentage of the range between stained / unstained untreated controls). FITC stained K562-A3 and JY cell populations analysed by flow-cytometry following treatment with citrate-phosphate or TMA-formate buffers prepared across a pH range (displayed as % expression on untreated cells). 4 x 10^6 cells were subjected to TMA-formate or citrate-phosphate elution buffers across a pH range from 3 to 7.5. Following treatment, cells were stained with a monoclonal murine antibody against β2-microglobulin, washed, and stained with an FITC-conjugated α-mouse secondary before being analysed by flow cytometry, as per 2.4.3. The data indicates β2-microglobulin disassociation occurs between pH 3.4 and pH 3.3 with both TMA-formate and citrate-phosphate buffers, illustrating a pH-dependent mechanism for eluting bound peptide from the MHC class-I complex. No significant differences appear to exist between the disassociation pH of the MHC class-I A*0201 presenting JY cell line, and the K562 cell line transfected with MHC class-I A*0301. Finally, washing with pH 5.5 citrate-phosphate has no apparent effect on β2-microglobulin integrity. Main graph illustrates the pH scale from 3 to 4.0, while the full pH scale is visible in the inset (bottom-right).

4.3 Optimisation of minimal-lysis elution buffers using trypan-blue staining as an indicator of cell mortality

The trypan blue dye was introduced by Nobel laureate Paul Ehrlich in 1904 (Ruis and Muir, 2005), and is routinely used within the context of cell culture to differentiate between viable and non-viable mammalian cells, as non-viable cells lack the membrane integrity required to exclude the dye and thus prevent cell staining. It was first applied to leukocytes by Evans and Schulemann (1914) and lymphocytes by Pappenheimer (1917), where it was demonstrated to rapidly stain damaged or traumatised cells. Its advantages are the speed and convenience with which it may be used, cells require little preparation prior to staining, and no specialised equipment beyond a microscope with (x)10 or (x)40 objective lens and a haemocytometer, as well as its reproducibility, low cost, and low acute toxicity (Pappenheimer, 1917).

4.3.1 Osmotic balancing and supplementation of TMA-formate elution buffer

While the pH gradient experiments illustrate that MHC class-I elution is pH dependent, the unsupplemented TMA-formate buffer was found to cause high cell death, raising concerns with regard to sample contamination by intracellular peptides, proteases, kinases and phosphatases, which may alter or mask those peptides which are derived from the MHC complex. It is suggested that this was primarily due to the high water potential of the 25 mM buffer leading to osmotically determined cell lysis, osmotic balancing of the buffer with sucrose and KCl was attempted. Cell lysis was determined by trypan blue exclusion as per 2.4.4, and an optimum concentration of 0.3 M sucrose, and 0.45 M KCl found to produce 8.4 % (± 2.72) trypan-staining staining for JY cells (see figure 4.3).

This was then confirmed with the K562-A3 and THP-1 cell lines using a limited sucrose/KCl matrix, producing a similar pattern of trypan staining, with a minimum value of 8.69 (± 1.68) % cell death at 0.3 M sucrose, 0.45 M KCl (see Table 4.2).

The osmotic balancing brought trypan-determined cell lysis within the ~ 10 % range described by Storkus *et al*. (1993) and others (Clark *et al*., 2001; Bonner, *et al*., 2002), and a Mann-Whitney U test failed to find a statistical difference in

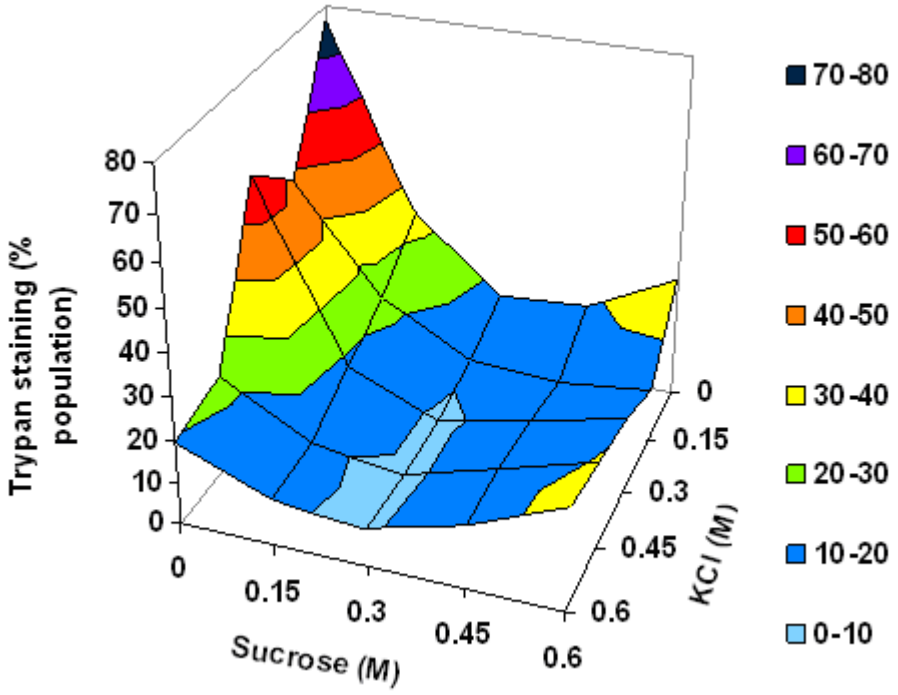


Figure 4.3: Trypan staining of the JY cell line (*n* = 6) following treatment with pH 3.3 25mM TMA-formate elution buffers with varying concentrations of sucrose and potassium chloride included as osmotic agents. 2 x 10^6 cells were washed in PBS, treated with elution buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage cell death, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. An optimum concentration of 0.3 M sucrose and 0.45 M KCl was determined to reduce the proportion of cell-staining to 8.4 (± 2.72) %. Numerical data is detailed below, and reproduced for other cell lines in table 3.2.2 overleaf.

Percentage post-elution trypan staining of JY cell population when treated with 25 mM TMA-formate (pH 3.3) elution buffers containing various concentrations of sucrose and KCl as osmotic agents (± values indicate standard deviation).

Sucrose (M) \ KCl (M)	0	0.15	0.3	0.45	0.6
0	76.71 (± 5.07)	48.05 (± 8.54)	58.53 (± 8.2)	22.86 (± 9.02)	19.6 (± 2.05)
0.15	33.8 (± 16.01)	23.02 (± 10.10)	17.69 (± 1.65)	11.19 (± 2.49)	10.79 (± 1.99)
0.3	16.8 (± 4.56)	11.57 (± 1.76)	8.53 (± 1.72)	8.40 (± 2.72)	8.96 (± 3.93)
0.45	16.06 (± 3.32)	10.39 (± 1.68)	13.39 (± 5.39)	14.64 (± 3.56)	15.09 (± 2.98)
0.6	28.63 (± 4.89)	12.97 (± 2.57)	18.47 (± 5.72)	20.75 (± 4.67)	23.99 (± 3.87)

Sucrose (M)	KCl (M) 0.3			0.45			0.6		
	JY	**K562-A3**	**THP-1**	**JY**	**K562-A3**	**THP-1**	**JY**	**K562-A3**	**THP-1**
0.15	17.69 (± 1.65)	19.7 (± 2.59)	17.46 (± 1.27)	11.19 (± 2.49)	12.23 (± 1.26)	8.47 (± 1.61)	10.79 (± 1.99)	11.27 (± 2.26)	10.45 (± 1.1)
0.3	8.53 (± 1.72)	9.03 (± 1.22)	8.85 (± 1.09)	8.4 (± 2.72)	8.69 (± 1.68)	7.66 (± 1.41)	8.96 (± 3.93)	8.85 (± 1.64)	7.96 (± 1.59)
0.45	13.39 (± 5.39)	14.05 (± 3.14)	13.21 (± 1.32)	14.63 (± 3.56)	14.85 (± 1.81)	13.66 (± 1.13)	15.09 (± 2.98)	16.7 (± 0.99)	15.7 (± 1.29)

Table 4.2: Percentage post-elution trypan staining of the JY, K562-A3 and THP-1 cell lines following treatment with pH 3.3 25mM TMA-formate elution buffers, with varying concentrations of sucrose and potassium chloride as osmotic agents across a narrow molar range. 2 x 10^6 cells were washed in PBS, treated with elution buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage cell death, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. An optimum concentration of 0.3 M sucrose and 0.45 M KCl was found in each case. ± values indicate standard deviation, and in all cases $n = 6$.

trypan staining between osmotically balanced TMA-formate and isotonic citrate-phosphate for either the JY or K562-A3 cell lines (see figure 4.4).

It was also noted that trypan-blue staining was marginally lower for both K562-A3 and JY cell lines when they were re-buffered in RPMI 1640 *c.f.* PBS following elution (see figure 4.5), echoing the results of Pappenheimer (1917), who noted that lymphocytes produced lower staining counts when the trypan blue was prepared in Locke's solution (an isotonic buffer containing NaCl, KCl, $CaCl_2$, and a sodium bicarbonate buffer pH 7.4) rather than isotonic saline.

It was therefore determined to investigate whether the components of the RPMI 1640 culture media might improve survival under elution conditions, as the media was itself designed primarily for the culture of lymphocytes, albeit within a physiological pH range. Amongst other components, the RPMI 1640 culture media contains: 11.1 mM D-glucose; 194 µM *myo*-inositol and 3.25 µM reduced glutathione (Moore *et al*., 1967), each of which was tested at a range of concentrations to determine the effect on immediate post-elution cell viability. All proved to provide some benefit to cell survival (see figure 4.6.A), with optimum concentrations determined to be 10 mM D-glucose, 2 mM *myo*-inositol, and 1 mM reduced glutathione, each of which reduced post elution trypan staining of the JY cell line from 8.44 % (± 2.77) to between 3.79 – 5.36 % (with

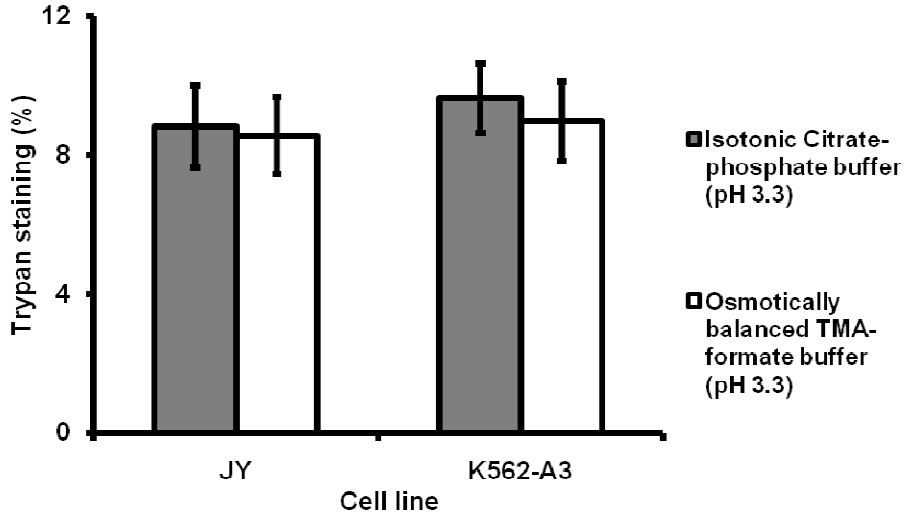


Figure 4.4 Trypan staining of the JY and K562-A3 cell lines (n = 12) following treatment with pH 3.3 isotonic citrate-phosphate or 25mM TMA-formate (osmotically balanced with 0.3 M sucrose and 0.45 M KCl). 2 x 10^6 cells were washed in PBS, treated with elution buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. Both student T-tests and Mann-Whitney U tests indicated no statistical difference apparent between the isotonic citrate-phosphate and osmotically balanced TMA-formate treated populations for either cell line (to $P < 0.05$). Error bars indicate S.D.

standard deviations of between 1.49 and 2.4). The anti-oxidant ascorbic acid was also tested, and though this too offered a minor benefit with regard to cell survival (reducing trypan staining of the JY cell line to 6.47 %), it proved incompatible with Fe^{3+} IMAC and was therefore not carried forward.

The benefits provided by the D-glucose, inositol and reduced glutathione proved at least semi-cumulative, and their addition in combination reduced post-elution trypan-determined cell death to 1.17 (± 0.35) %, 2.31 (± 1.06) % and 2.12 (± 1.06) % for the JY, K562-A3 and THP-1 cell lines respectively (see figure 4.6.B). Not only were these staining levels significant statistically (P value = <0.01) when compared by Mann-Whitney U test with an unsupplemented osmotically balanced buffer, but it brought staining within each cell line to a point statistically indistinct from the untreated populations.

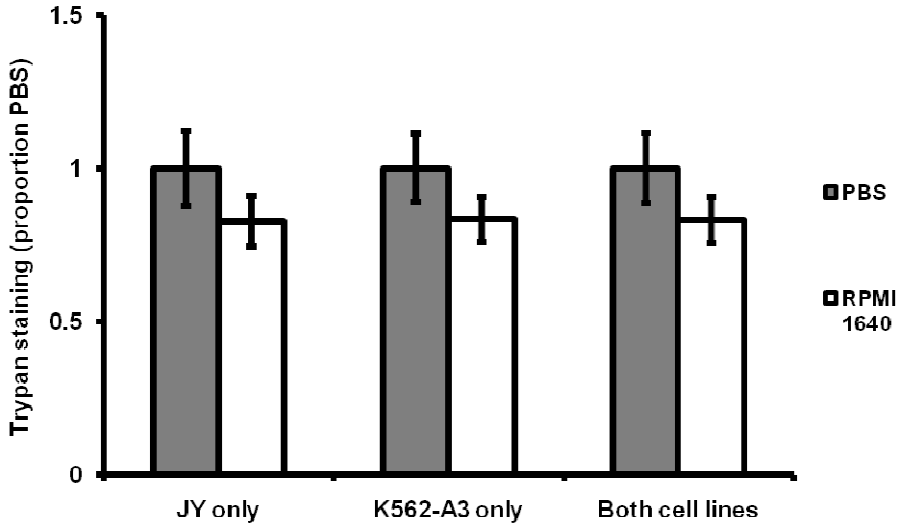


Figure 4.5: Post-elution trypan staining of the JY and K562-A3 cell lines following rebuffering to pH 7.5 with either PBS or RPMI 1640. 2 x 10^6 cells were washed in PBS, treated with elution buffers, and centrifuged to pellet the cells, following which cells were rebuffered in either PBS or RPMI 1640. Percentage viability was determined by mixing 20 µl of each cell suspension with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. In each case a Mann Whitney U test found statistical differences between PBS and RPMI 1640 rebuffered populations, to $P < 0.05$ (signified by *). Error bars indicate S.D.

Though the approach of Sugawara *et al*. (1987) included 1 % BSA in the citrate-phosphate buffer to stabilise cell viability, this was excluded from the methods of Storkus *et al*. (1987) and our own procedures for the simple reason that the contamination of bovine serum peptides would complicate sequencing of MHC-derived peptides. Similarly, the amino acids and phosphate ions found in RPMI 1640 were not considered as potential supplements as it was feared they would complicate isolation, quantification and identification, however the addition of these to the elution buffer may reduce cell death further, as may the inclusion of metal ions such as calcium or magnesium.

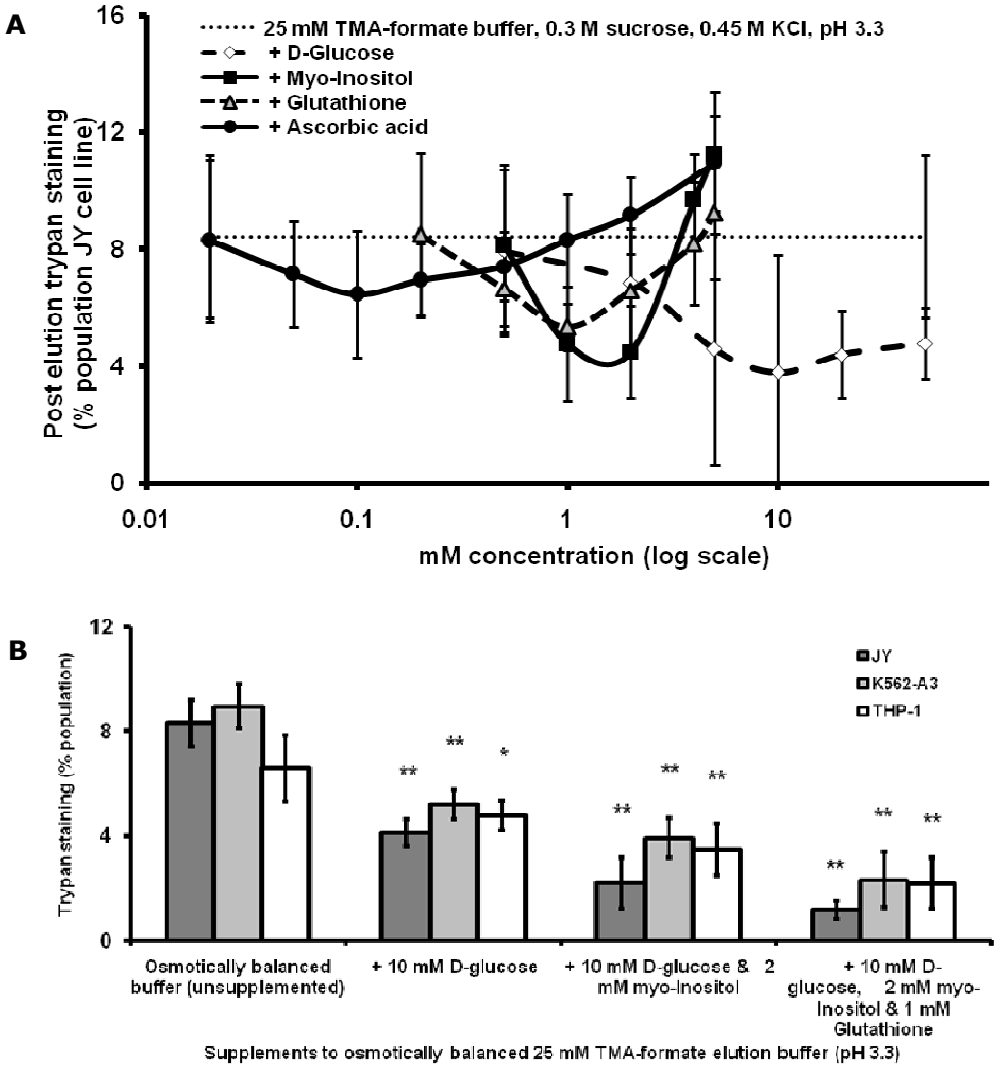


Figure 4.6: Trypan determined post-elution cell death of **(A)** JY cell line eluted with osmotically balanced 25mM TMA-formate buffers (pH 3.3) containing a range of concentrations of either D-glucose, myo-inositol, reduced glutathione or ascorbic acid. The level of cell staining observed in the unsupplemented buffer is denoted by the dotted level line. **(B)** JY, K562-A3 and THP-1 cell lines eluted with osmotically balanced 25mM TMA-formate buffers containing serial supplementation with 10 mM glucose, 2 mM inositol and 1 mM glutathione (each of which proved statistically distinct from the unsupplemented osmotically-balanced buffer). In each case 2 x 10^6 cells were washed in PBS, treated with elution buffers containing the various supplements/concentrations, before the cells were washed twice and re-suspended in PBS. Percentage viability was determined by mixing 20 µl of each suspension with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. An elution buffer containing all three supplements reduced post-elution trypan-determined cell death to 1.17 (± 0.35) %, 2.31 (± 1.06) % and 2.12 (± 1.06) % for the JY, K562-A3 and THP-1 cell lines (significant to $P < 0.01$ when compared with the unsupplemented buffer, $n = 5$).

4.3.2 Effect of osmotic balancing, adjustment of molar concentration, and further supplementation of sodium-formate MHC class-II elution buffer

It was noted that the MHC class-II elution buffer originally designed at NTU provided an extremely high buffering capacity, however when cells were rebuffered in RPMI 1640, the phenol red did not present the standard reddish colouration indicative of pH 7.4 until at least the third or fourth wash leading to concerns that the high cell staining - 30.9. (± 0.55) % for the JY cell line, 35.7 (± 0.75) % for THP-1, might be a result of prolonged exposure to a low pH. When investigated, reductions in the concentration of the buffer were found to reduce trypan staining to 22.4 (± 0.81) % for the JY cell line and 24.2 (± 0.84) % for THP-1 at 50 mM sodium formate (see figure 4.7), each to a statistically significant degree. Furthermore, a Spearman's rho of -0.945 (to a *P* value of <0.01) was found between buffer strength and trypan staining.

While cell staining - and by implication, cell lysis - remained high with the 50 mM buffer, the 200 mM buffer required a much higher concentration of formic acid to bring to pH 2.0. Though following clarification eluates are stored at – 80 °C, high formic acid concentrations may result in formylation of peptides, or degradation of aromatic amino acids, especially if samples are to be stored for extended periods (Beavis and Chait, 1990; Morrison *et al*., 1990).

Although initial research on the 200 mM sodium-formate buffer had indicated a similar optimal range of KCl and sucrose to the TMA-formate buffer, this was confirmed using the MHC class-II positive JY and T2.DR4 cell lines, treated with 50 mM sodium-formate buffers across a limited sucrose/KCl gradient. This produced a similar pattern of trypan staining to the TMA-formate buffer, with a minimum value of 11.97 (± 1.0) % cell staining for the JY cell line, 13.63 (± 1.16) % for the T2.Dr4 hybridoma line, and 14.58 (± 1.18) % for the THP-1 cell line at 0.3 M sucrose, 0.45 M KCl (see Table 4.3).

The inclusion of short concentration ranges of glucose, inositol or glutathione in the pH 2 buffer were assayed by trypan-blue staining, and demonstrated a similar pattern to Figure 4.6.A. This was followed by the serial addition of 10 mM glucose, 2 mM inositol and 1 mM glutathione, which again provided a cumulative benefit to post-elution trypan-exclusion, reduced cell staining to 1.17 (± 0.35) % and 2.31 (± 1.06) % for the JY and K562-A3 cell lines respectively (see figure

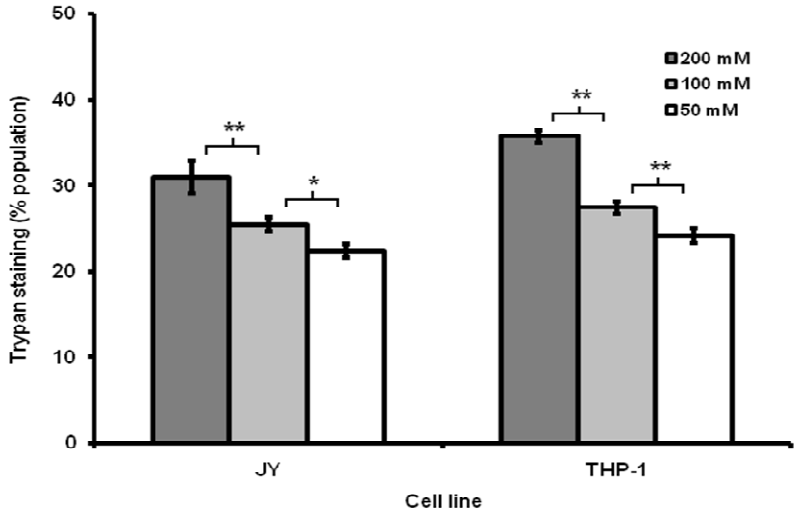


Figure 4.7: Trypan determined post-elution cell death of JY and THP-1 cell lines eluted with unsupplemented sodium formate pH 2 at decreasing buffer molarity. 2 x 10^6 cells were washed in PBS, treated with elution buffers containing the various supplements/concentrations, and centrifuged to pellet the cells, following which cells were washed five times and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. Each reduction in buffer strength led to a statistically significant drop in trypan-blue staining for both the JY and THP-1 cell lines (in each case with a *P* value of 0.05 or greater) , and an inverse Spearman's rank correlation of -0.945 (to a *P* value of 0.01) was found for both cell lines between cell death and buffer strength. At 50 mM staining of JY and THP-1 cell line was reduced to 22.4 (± 0.81) % and 24.2 (± 0.84) % respectively (*n* = 6).

		KCl (M)								
		0.3			0.45			0.6		
		JY	K562-A3	THP-1	JY	K562-A3	THP-1	JY	K562-A3	THP-1
Sucrose (M)	0.15	18.18 (± 1.81)	19.81 (± 0.87)	21.62 (± 1.03)	16.73 (± 1.07)	17.94 (± 0.92)	19.44 (± 0.97)	15.61 (± 0.97)	16.49 (± 1.26)	18.78 (± 1.12)
	0.3	13.66 (± 1.15)	15.28 (± 1.06)	15.28 (± 1.37)	11.97 (± 1.0)	13.63 (± 1.16)	13.56 (± 1.43)	12.96 (± 1.43)	14.58 (± 1.18)	14.51 (± 1.59)
	0.45	14.96 (± 0.88)	15.31 (± 1.06)	15.53 (± 0.99)	15.76 (± 0.92)	16.55 (± 0.91)	16.87 (± 1.84)	16.48 (± 1.21)	18.21 (± 1.07)	18.7 (± 1.29)

Table 4.3: Percentage post-elution trypan staining of the JY, T2.Dr4 and THP-1 cell lines following treatment with pH 2 50 mM sodium-formate elution buffers, with varying concentrations of sucrose and potassium chloride as osmotic agents across a narrow molar range. 2 x 10^6 cells were washed in PBS, treated with elution buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage cell death, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. An optimum concentration of 0.3 M sucrose and 0.45 M KCl was found in each case. ± values indicate standard deviation, and in all cases *n* = 6.

4.8). Again, these staining levels were statistically significant when compared by Mann Whitney U test with an unsupplemented osmotically balanced buffer, though in the case of THP-1 cell line treated with the class-II elution buffer containing 10 mM glucose, this significance was only apparent at a *P* value of <0.05.

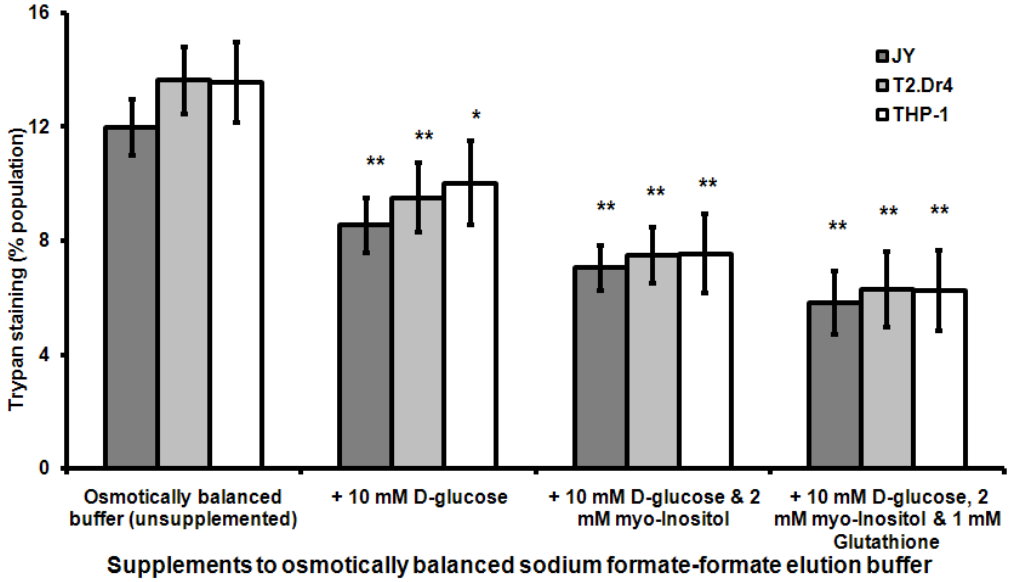


Figure 4.8: Trypan determined post-elution cell death of JY, T2.Dr4 and THP-1 cell lines eluted with osmotically balanced 50 mM TMA-formate buffers (pH 2.0) containing serial supplementation with 10 mM glucose, 2 mM inositol and 1 mM glutathione (each of which was statistically distinct by ANOVA from the unsupplemented osmotically-balanced buffer for every cell line). In each case 2 x 10^6 cells were washed in PBS, treated with elution buffers containing the various supplements/concentrations, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. Treatment with an elution buffer containing all three supplements reduced post-elution trypan-determined cell death to 5.82 (± 0.35) %, 6.29 (± 1.06) % and 6.25 (± 1.06) % for the JY, T2.Dr4 and THP-1 cell lines respectively, and the addition of each supplement was statistically significant (to a *P* value of at least <0.05) against the unsupplemented but osmotically balanced buffer treatment.

4.3.3 Supplementation of isotonic citrate-phosphate with non-osmotic supplements

Once ideal concentrations of the additional supplements were confirmed, the effect of their addition into the isotonic citrate-phosphate buffers at pH 5.5 and 3.3 was tested; while no significant effect was apparent at pH 5.5, surprisingly a minor increase in mean cell staining for each of the cell lines was observed at pH 3.3 (see figure 4.9), though this was only statistically significant (to a *P* value of <0.05) for the K562-A3 line. As these buffers are already isotonic with regard to the contents of the suspension cell lines tested, this may be due to osmotic effects, though Rous and Turner (1916), noted that the survival of erythrocytes in media containing agents such as glucose was modulated by citric acid on a concentration dependent scale. Furthermore, as one of the aims of this research was to replace the citrate-phosphate elution buffer with one directly compatible with the IMAC approach for selection of phosphorylated or histidine-containing peptides, supplementation of the citrate-phosphate buffer would do little to achieve these goals, as the same sample clean-up steps would be required.

4.3.4 Comparative post-elution trypan staining following treatment with MHC class-I and class-II buffers

Finally, comparative trypan staining of cells was performed following MHC class-I (figure 4.10) and MHC class-II (figure 4.11) elution with the buffers developed above. In the case of the MHC class-I buffers, elution was performed with and without the pH 5.5 prewash step, while for MHC class-II elution was performed with and without elution of MHC class-I peptides, either by isotonic citrate-phosphate pH 3.3, or by prewashing with isotonic citrate-phosphate pH 5.5 then eluting MHC class-I with the complementary (osmotically balanced or fully supplemented) TMA-formate buffers prior to class-II elution. Results were compared with MHC elution by the isotonic citrate-phosphate approach utilised by Storkus *et al*. (1993a/b). This confirmed a reduction in cell lysis with progressing supplementation of the pH 3.3 TMA-formate and pH 2 sodium-formate buffers.

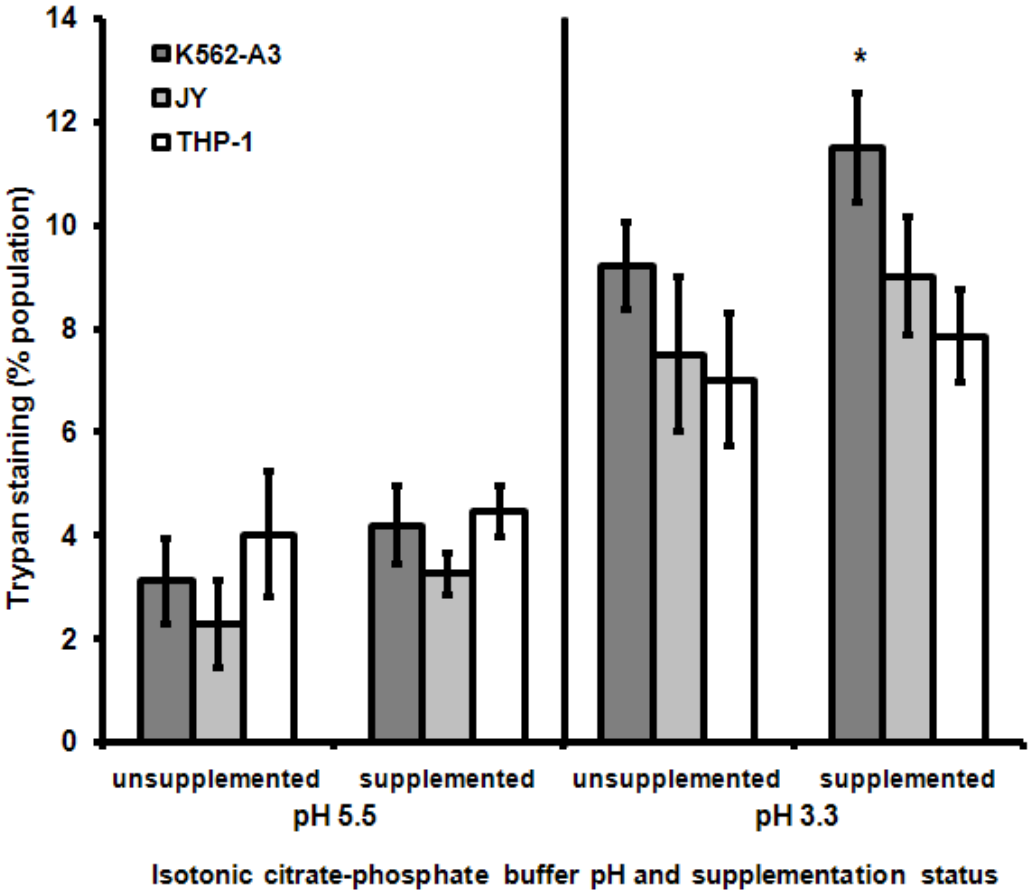


Figure 4.9: Trypan determined cell death of K562-A3, JY and THP-1 cell lines following treatment with isotonic citrate phosphate buffers with ('supplemented') or without ('unsupplemented') the addition of 10 mM glucose, 2 mM myo-inositol and 1 mM glutathione. 2 x 10^6 cells were washed in PBS, treated with buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. While little difference was observed between the pH 5.5 buffers, at pH 3.3 the mean cell staining increased for all three cell lines when supplements were added to the isotonic citrate-phosphate, and the K562-A3 cell line exhibited a statistically significant difference in trypan staining between supplemented and unsupplemented pH 3.3 buffers. Also interesting to note is the comparative staining of the three cell lines, particularly THP-1 which seems to show increased resistance to the citrate-phosphate buffer at pH 3.3 relative to K562-A3 and JY, but comparative or higher cell staining at pH 5.5 when compared with the other cell lines.

It also indicated that prewashing the cells with isotonic citrate-phosphate at pH 5.5 prior to MHC class-I elution did not significantly increase the levels of cell staining except in the case of the osmotically balanced and fully supplemented TMA-formate buffer, where trypan staining increased, but became statistically indistinguishable from the prewashed-only controls. Indeed, when trypan staining levels at the preceding stages are taken into account, it appears that prewashing with citrate phosphate leads to a reduction in cell lysis at the MHC class-I elution stage, potentially by lysing the more fragile elements of the subpopulation prior to the pH 3.3 stage. As a result the resultant trypan staining for each prewashed cell line following exposure to the optimised TMA-formate buffer is calculated to be under 1 % (see figure 4.10).

For MHC class-II elution (figure 4.11) the situation appears slightly more complex. When MHC class-I elution by TMA-formate (with a pH 5.5 prewash) took place prior to MHC class-II elution, mean staining increased, but remained statistically insignificant from populations subjected to class-II elution only. When class-I peptides were removed by citrate-phosphate pH 3.3 (without a prewash), mean cell staining increased even further and became statistically significant (P <0.05) when compared with cells where MHC class-I peptides were not removed, though this result remained statistically insignificant when compared with those which had undergone a pH 5.5 prewash and TMA-formate elution prior to MHC class-II elution. Again, when the trypan staining levels of the populations which had undergone either the prewash followed by TMA-formate or the citrate-phosphate elution were accounted for it was found that both led to a reduction in cell staining after the pH 2 treatment, with citrate-phosphate pH 3.3 producing a greater reduction in staining than the prewash followed by TMA formate buffer.

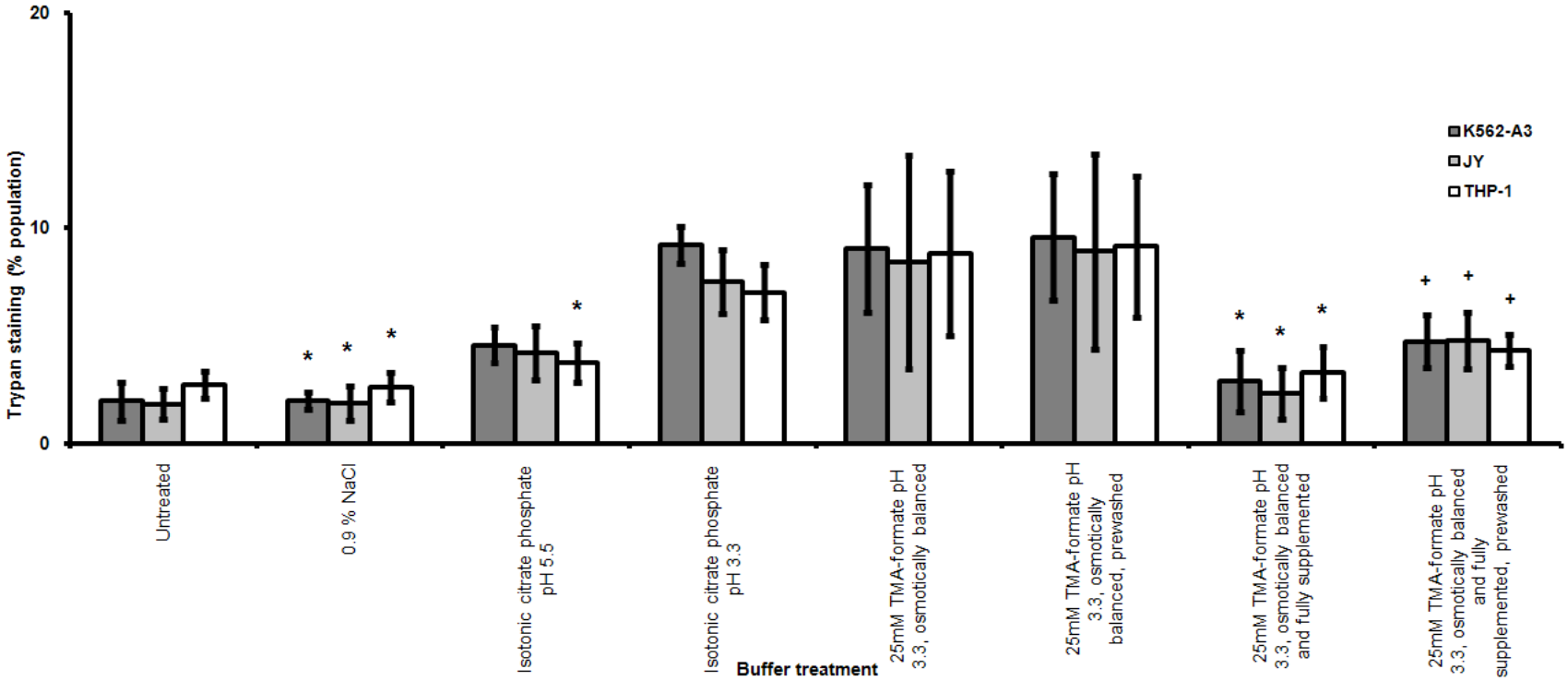


Figure 4.10: Trypan-determined cell death of K562-A3, JY and THP-1 cell lines (*n* = 6) following treatment with the buffers used in MHC-class-I elution, and variants thereupon. 2 x 10^6 cells were washed in PBS, treated with buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and percentage staining determined with a haemocytometer. The osmotically balanced TMA-formate buffer produces trypan staining levels statistically indistinguishable from citrate-phosphate at pH 3.3, while the fully supplemented buffer is indistinguishable from untreated cells (P <0.05). Prewashing at pH 5.5 led to a slight rise in mean trypan staining, though this was only statistically significant to (P <0.05) for the osmotically balanced and fully supplemented buffer. * indicates no statistical difference to untreated controls (to a P value of <0.05); $^+$ indicates no statistical difference to pH 5.5 prewash-only controls (to P <0.05).

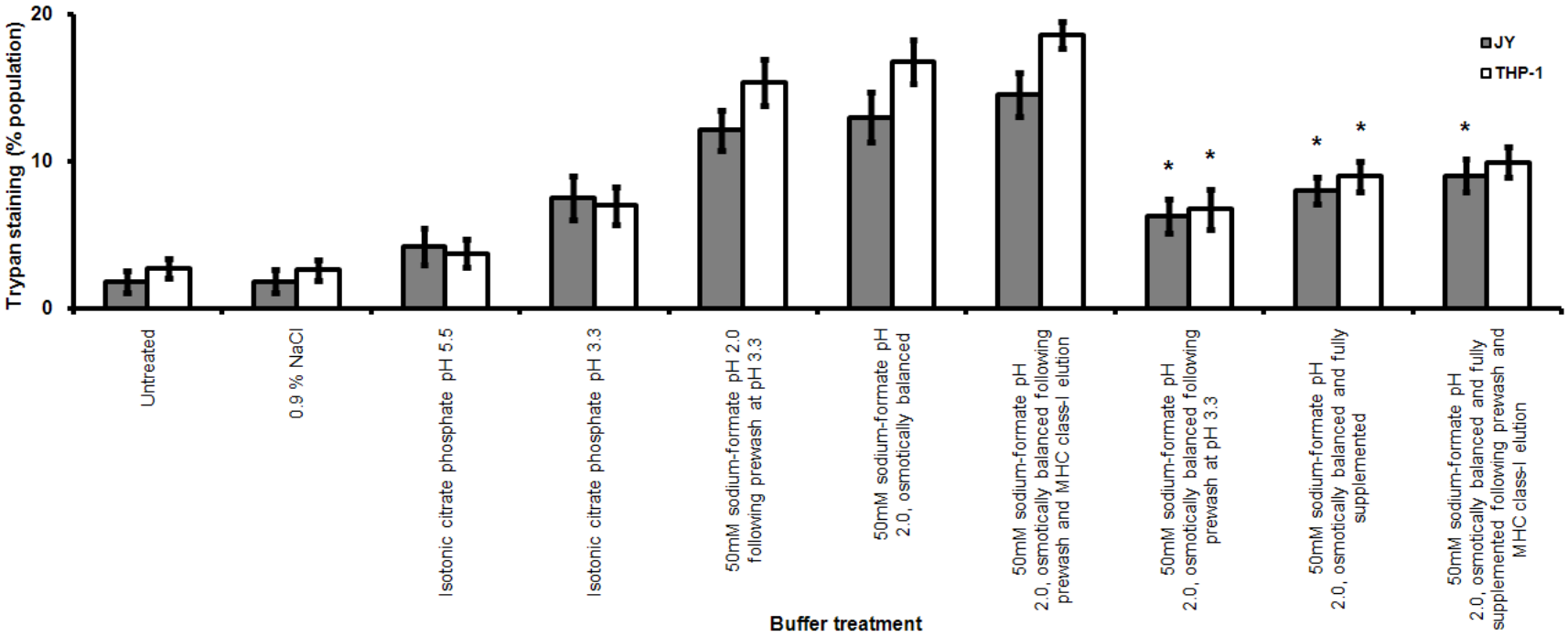


Figure 4.11: Trypan determined cell death of JY and THP-1 cell lines following treatment with the buffers used in MHC class-II elution, and variants thereupon. 2 x 10^6 cells were washed in PBS, treated with buffers, and centrifuged to pellet the cells, following which cells were washed twice and re-suspended in PBS. To determine percentage viability, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the percentage staining determined with a haemocytometer. As with the TMA-formate this confirms the decreasing cell staining observed with osmotically balancing and supplementing the sodium-formate buffers (significant to P <0.05), and that the osmotically balanced and fully supplemented class-II elution buffer produces trypan staining levels statistically indistinguishable from isotonic citrate-phosphate at pH 3.3. Additionally treatment of cells with isotonic (0.9 % w/v) saline produced no increase in cell staining compared with untreated controls. * indicates no statistical difference to Storkus *et al.* (1993) pH 3.3 citrate-phosphate buffer for elution of MHC class-I (to P <0.05).

4.4 Measurement of cell death by propidium iodide exclusion and flow cytometry

Following on from trypan exclusion, a second cell stain, propidium iodide (PI) was employed to confirm minimal cell death and comparative staining between buffers. Like trypan blue, this fluorescent dye is excluded by live cells with intact membranes, but unlike trypan, the fluorescence of PI increases dramatically when intercalated between the guanine-cytosine base pairs of DNA or RNA (Suzuki *et al*., 1997, Le Goff, *et al*., 1992), and the non-visual assessment offered by flow cytometry reduces subjectivity. It was first utilised in conjunction with flow cytometry by Nicoletti *et al*. (1991) to determine apoptosis, though subsequent work indicated that it does not fully differentiate between apoptosis and necrosis, it is less sensitive to necrotic cell death due to the occurrence of random DNA fragmentation (Lecoeur, 2002).

The viability of the three cell lines: K562-A3, JY and THP-1 were assessed by propidium iodide exclusion and flow cytometry following elution of MHC class-I class-II peptides by isotonic citrate-phosphate, and the TMA-formate and sodium-formate buffers (unsupplemented, osmotically balanced, or fully supplemented) (see figure 4.12). The resulting data largely supported that gathered by trypan exclusion, in that progressive supplementation of the TMA-formate and sodium-formate buffers reduced the mean level of cell staining, though the levels of cell staining for the unsupplemented TMA-formate and sodium-formate buffers were significantly lower and higher than that found by trypan exclusion, while that observed in the untreated controls was significantly higher. Despite this, osmotic balancing of the TMA-formate buffer resulted in a level of mean cell staining comparable to that caused by isotonic citrate-phosphate pH 3.3, while full supplementation brought mean cell staining close to that of the untreated controls, and below that caused by citrate-phosphate elution. Full supplementation of the sodium formate buffer also brought cell staining to a level statistically indistinguishable from the level caused by MHC class-I elution with citrate-phosphate pH 3.3.

Whilst this data appears to support that gathered from the earlier trypan blue exclusion experiments, both assays rely on penetration and retention of a dye introduced after MHC-elution and rebuffering. It is conceivable that any significant damage to membrane integrity during MHC elution may be repaired

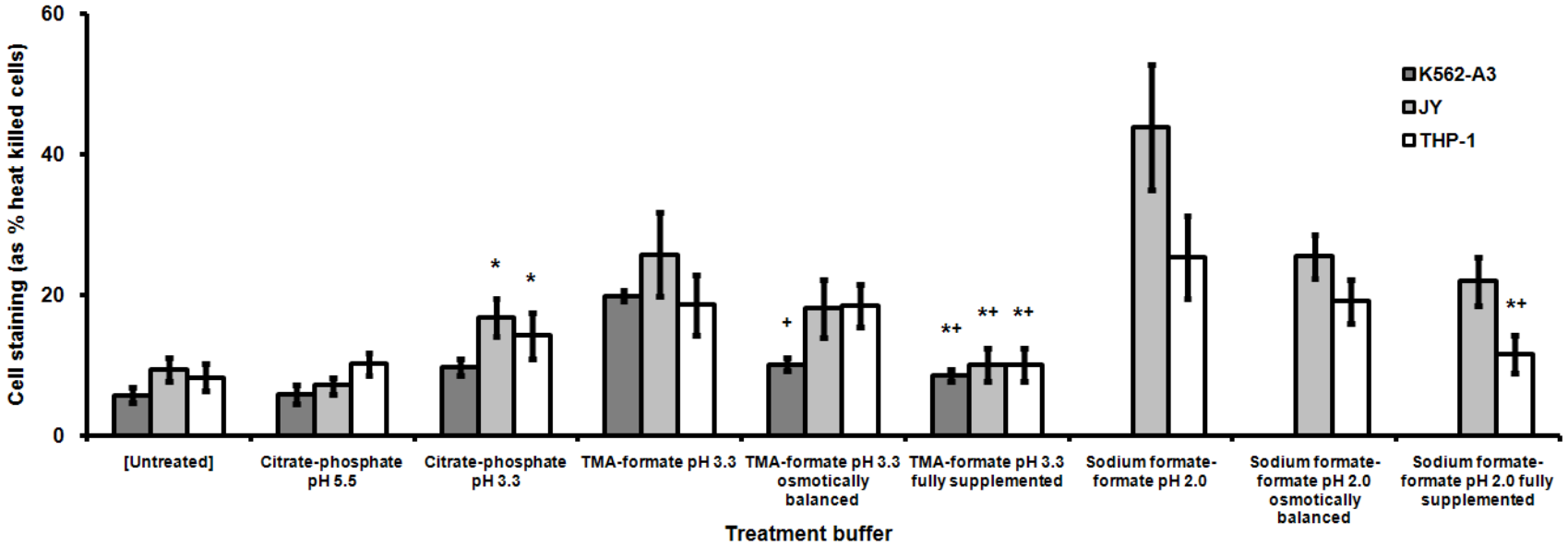


Figure 4.12: Post-elution staining of K562-A3, JY and THP-1 cell lines with propidium iodide as determined by flow cytometry. 4 x 10^6 cells were washed in PBS, treated with elution buffers (JY and THP-1 were treated with both MCH class-I and class-II elution protocols, K562-A3 with class-I only), and pelleted by centrifugation, following which cells were washed twice in FACS buffer and re-suspended in 0.2 ml Isoton. 20 µl of 100 µg/ml propidium iodide was added to each cell suspension and incubated at room temperature (protected from light) for 2 minutes, prior to being washed by centrifugation and resuspension in Isoton prior to analysis by flow cytometry (calibrated with cells stained with W6/32 (MHC class-I) and a phycoerythrin-labelled secondary antibody as a positive colour control). Supplementation of buffers reduced cell staining to 8.6 – 10.1 % for the MHC class-I TMA formate buffer (significant to $P < 0.05$ when compared with unsupplemented or osmotically balanced buffers for each cell line), and 11.6 – 21.9 % for the MHC class-II elution buffer (significant to $P < 0.05$ when compared with unsupplemented buffers for both cell lines, and in the case of THP-1 when compared with the osmotically balanced buffer). This may be compared with the citrate-phosphate buffer (pH 3.3) which produced 9.8 – 16.8 % cell staining. * indicates no statistical difference to untreated controls (to a *P* value of <0.05); $^+$ indicates no statistical difference to pH 5.5 prewash-only controls (to a *P* value of <0.05).

by this point, or that the stress of a low pH environment may leave a subpopulation of cells in a fragile or proapoptotic state. Either could lead to a misreporting of cell integrity, and therefore an erroneous estimation of intracellular peptide contamination.

4.5 Determination of relative protein and peptide loss as a marker for cell viability

In order to further and more accurately determine relative cell integrity during MHC elution, and the benefits conferred by buffer supplementation, the modified BCA assay for estimation of peptide concentration (as detailed in chapter 3) was utilised to determine the relative proteinaceous content of MHC eluates from four cell lines (see figure 4.13).

As stated in 3.1, the BCA assay largely relies on the co-ordination and reduction of Cu^{2+} to Cu^{1+} by the peptide bonds of a protein or peptide, leading to a concentration-dependent shift in absorbance at 562 nm. Therefore any agents which alter the rate of $CuSO_4$ reduction will cause a variation in the final results. Unfortunately, the inclusion of glucose (a reducing sugar) and glutathione (a tripeptide) in the fully supplemented buffer lead to high peptide-independent levels of copper reduction that proved unfeasible to blank out, and therefore only the unsupplemented and osmotically balanced IMAC compatible buffers were compared with the isotonic citrate-phosphate elution buffer. The results largely corresponded with previous data, insofar as the osmotic balancing appeared to reduce the mean levels of peptide/protein loss (to a *P* value of <0.05) for both the TMA-formate and the sodium-formate buffers (except in the case of MHC class-II elution of the THP-1 cell line, following a prewash). Furthermore, the use of a pH 5.5 prewash step also reduced the levels of protein/peptide loss (to *P* <0.05) for all samples, with the exception of the K562-A3 cell line which exhibited an increase from 96.7 (± 2.6) µg/ml to 132.7 (± 2.5) µg/ml.

Unfortunately as stated earlier, exact quantification of protein/peptide loss is difficult. This is partly due to inter-protein/peptide variation in BCA reactivity: as figure 3.9 in chapter 3.5 shows, tryptic digests of protein derived from the JY and T2.Dr4 cell lines show a minor difference, even after heat-denaturing in SDS-NaOH; and is further complicated by the lack of a 100 % accurate standard.

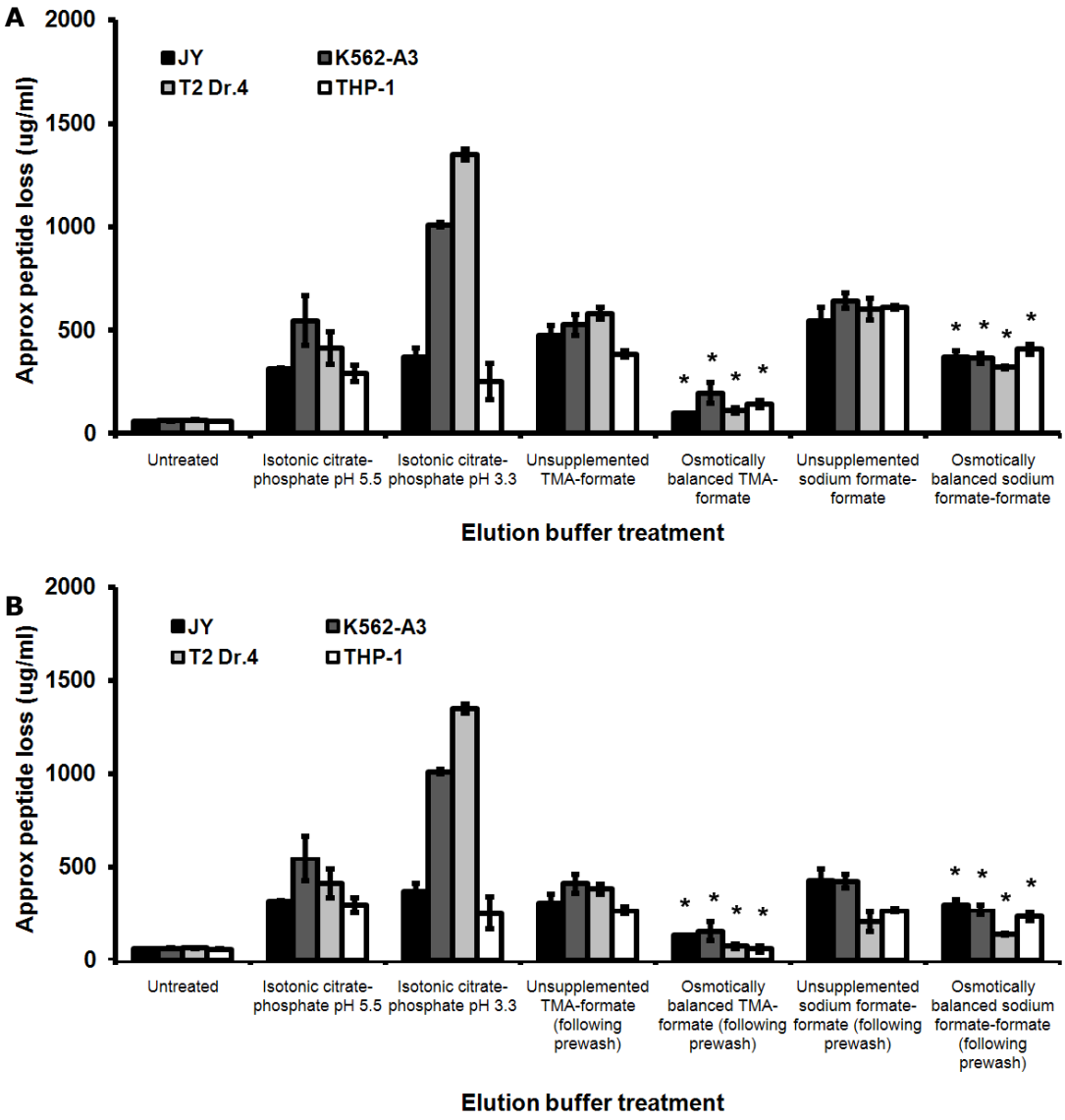


Figure 4.13: Peptide and protein loss from JY, K562-A3, T2.Dr4 and THP-1 cell lines following treatment with MHC elution buffers, including isotonic citrate-phosphate pH 3.3 and the IMAC compatible TMA-formate pH 3.3 and sodium-formate pH 2 buffers (unsupplemented or osmotically balanced) either without **(A)** or with **(B)** first treating the cells with a pH 5.5 isotonic citrate-phosphate prewash. 10 x 10^6 cells were washed in PBS, treated with 0.6 ml of the elution buffers, and following centrifugation to pellet the cells the top 0.4 ml was aspirated and assessed for proteinaceous content by diluting 1:4 in water and then mixing 1:1 with 2 % (w/v) SDS in 0.2 M NaOH, prior to heat-denaturing and analysis by peptide BCA (see chapter 3) against standards prepared in appropriate matrices. In each case osmotic balancing of class-I and class-II buffers significantly reduced intracellular peptide-loss from each cell line (to $P < 0.05$, $n = 3$), and the osmotically balanced buffers exhibited significantly lower peptide loss than treatment with the isotonic citrate-phosphate pH 3.3 (excepting MHC class-II elution of THP-1). * indicates statistical difference to unsupplemented results ($P < 0.05$).

Additionally, the susceptibility of the BCA assay to reducing agents also raises the problem that loss of any intracellular reducing agents, including glucose and many intermediate products of the respiratory pathway, glutathione, and some ions such as magnesium (Lucarini and Kilikian, 1999), may lead to erroneous quantification of the proteinaceous content of each sample. Such variations are however characteristic of most protein/peptide assays and while the figures given for peptide-loss are therefore approximate, internal comparability is still valid.

As citric acid is a chelating agent, known to interact with Cu^{2+} ions (Molinari *et al*., 2004) it is conceivable that this could also impair the assay results, however the linearity of the standard curves obtained (R^2 values were obtained of 0.99 for the isotonic pH 5.5 buffer and 0.984 for the pH 3.3 buffer) imply that accurate measurement is possible. Furthermore, if the chelating properties of citric acid were to play a role, one would expect a drop in reactivity and a subsequent underestimation of peptide loss. In fact the comparative standard curves for pH 3.3 and pH 5.5 indicate that the pH 5.5 buffer actually showed a lower BCA reactivity than the pH 3.3, producing an A_{570nm} of 0.245 (SD ± 0.016) c.f. 0.284 (SD ± 0.005), despite the lower ratio of citric acid : sodium phosphate[3]. This may be due to either a buffering effect between the sodium phosphate and the components of the BCA assay (in order of concentration: sodium carbonate; bicinichoninic acid; sodium-bicarbonate; NaOH; and sodium tartrate) or possibly due to an ionic interaction between the negatively charged phosphate groups and the positively charged sodium tartrate, which may in turn impair the solubilisation of the Cu^{1+} by the sodium tartrate, leading to a marginally lower colour formation. Though in either case the absorbance of the standard curves prepared in the other buffers were in a similar range, demonstrating that the citric acid is unlikely to be a major source of error. However, the unfortunate inability of the assay to compare the osmotically balanced buffers with the fully supplemented versions required further experimentation to confirm the trypan and PI staining data.

[3] As demonstrated in 3.1.3, the presence of sodium phosphate produces a higher inter-peptide variation between the SQKx and YISx peptides (see figure 3.2.7), which may also go some way to explaining the difference observed between these two buffers.

4.6 Applicability of the ToxiLight Assay to determine cell permeability during MHC elution

In order to further determine relative cell integrity during MHC elution, the ToxiLight non-destructive cell toxicity assay kit (Lonza Biosciences) was assessed for its applicability to measure cell-permeability during MHC elution. The assay operates on the principle of ATP production from ADP by adenylate kinase (AK) derived from cell culture supernatants, and subsequent measurement of ATP concentration using firefly luciferase and luciferin (Lonza, 2007). Therefore the difference in luminescence between samples is - *cēterīs paribus* - dependent on differential AK concentrations between samples and therefore differences in cell lysis or permeability.

Human AK has an optimal pH of 7.4 – 7.5 (Hamada *et al*., 1982; Sheng, 1999), however, though most AKs have a reasonably broad pH range (Dinbergs and Lindmark, 1989; Ulschmid *et al*., 2004) it was uncertain whether the enzyme released into the elution buffer medium (pH 5.5, 3.3 and 2.0) would show compromised activity, and whether it would fully renature when brought back to pH 7.4. To test this, a solution of 200 units chicken muscle AK was mixed with isotonic citrate-phosphate and (unsupplemented) 25 mM TMA-formate buffers at pH 7.4, 5.5, and 3.3[4], and incubated at 4 °C for five minutes before being rebuffered to pH 7.4 with ToxiLight assay buffer (RT).

Following exposure to the isotonic citrate-phosphate and 25 mM TMA-formate buffers at pH 3.3 a notable drop in AK activity was observed (see Figure 4.14). This was more pronounced in the case of the isotonic citrate-phosphate buffer, which dropped to 5.73 (SD ± 5.2) %, while the pH 3.3 TMA-formate buffer only dropped to 23.07 (SD ± 7.9) %. At pH 5.5 the difference between the two was similarly pronounced, with exposure to the citrate-phosphate buffer reducing AK activity to 60.57 (SD ± 15.5) % when rebuffered to pH 7.4, while the TMA-formate was 82.78 (SD ± 9.5) %. Despite the broad pH range it appears that the AK fails to re-nature following exposure to low-pH buffers, making it less than ideal for the purposes of this assay, hence the supplemented and class-II elution buffers were not investigated.

[4] Note that pH 7.4, and 5.5 lie more than the 1 pH point outside the pKa of the TMA formate buffer, and thus a relatively poor buffering effect might be expected at these pHs.

The difference between the TMA-formate and isotonic citrate-phosphate buffers was investigated further by comparing the enzyme treated with pH 7.4 buffers (i.e. without a pH denaturing effect) with those treated with RPMI 1640 or the ToxiLight buffer alone. At pH 7.4 the 25 mM TMA-formate buffer demonstrated no statistically significant difference to the RPMI 1640 and ToxiLight buffer only controls, however the isotonic citrate-phosphate buffer displayed only 72.63 (SD ± 5.4) % activity (to P <0.01). This, and the relatively lower activity displayed by all the isotonic citrate-phosphate buffers, might be due to both the relative strength of the isotonic citrate-phosphate buffer compared to the 25 mM TMA-formate, and the broader pKa range of the citrate-phosphate buffers (the phosphate has multiple acidic groups and thus three dissociation constants). The isotonic citrate phosphate is therefore a much greater buffer at pH 7.4 and pH 5.5, and has a higher concentration (and thus buffering capacity) throughout the pH range examined, and so it may be argued that this allowed it to fully denature the AK, while the TMA-formate failed to do so.

However, the isotonic citrate-phosphate buffer also possesses chelating properties which, given that the luciferase assay is magnesium-dependent, may be ablating the assay luminescence, and would explain why the assay failed to show full AK activity following exposure to the citrate-phosphate buffer even when the buffer pH matched the optimum activity. As the pH of the buffer drops, the citric acid concentration increases, producing a greater impact on the luciferase assay as well as denaturing the AK enzyme, and reducing its activity. This double-effect complicates adoption of the assay for the purpose of determining cell permeability during MHC elution, and it was therefore decided not to pursue the ToxiLight assay any further. Furthermore there were concerns that measurement of AK-loss may not be comparable between different cell lines without first quantifying and identifying the activity and distribution of the different isoforms within each cell line studied (which may change as a result of stress, for example as a response to alteration of the cell membrane potential, e.g. when subjected to mild acid elution) (Abanese, *et al*., 2009), and that use of AK as a marker for cell permeability may lead to underestimation of intracellular peptide contamination, given the relative size of the adenylate kinases (194 – 562 amino acids depending on the isoform) (von Zabern *et* al., 1976; Van Rompen *et al*., 1999) compared with MHC class-I and class-II peptides.

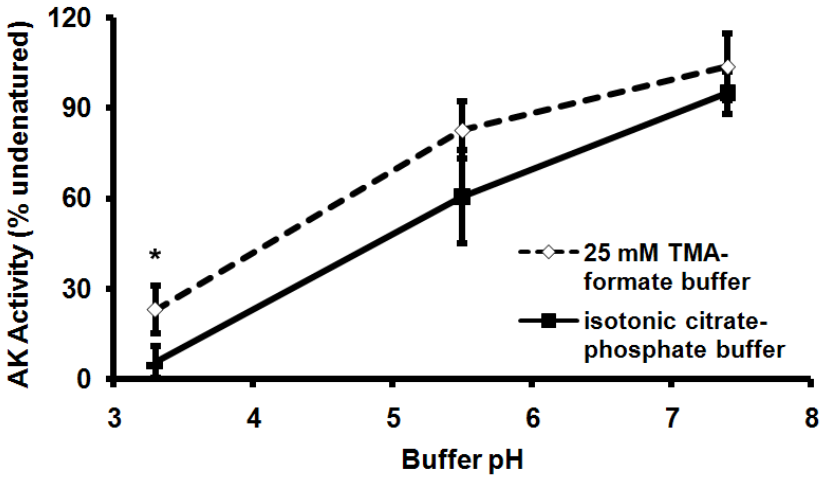


Figure 4.14: Luciferase-determined post-elution adenylate kinase (AK) activity assayed by the ToxiLight cell viability assay. 0.455 units of AK (n = 4) was denatured in either TMA-formate or citrate-phosphate buffers across a pH range, and brought back to pH 7.4 by diluting in ToxiLight assay buffer. This was then mixed with AK detection reagent, incubated for five minutes at 37 ℃, and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 3 over 10 cycles (11.4 minutes) at 22 ℃. Data is expressed as a percentage activity of that treated with pH 7.4 buffers. Error bars indicates SD, * indicates statistical significance at $P < 0.05$) as determined by Mann-Whitney U-test.

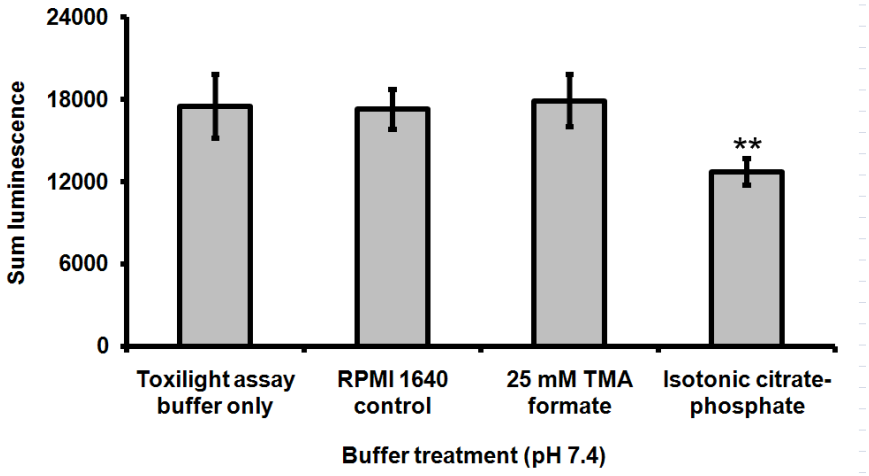


Figure 4.15: Luciferase-determined adenylate kinase (AK) activity assayed by the ToxiLight cell viability assay following exposure to three buffers at pH 7.4. 0.455 units of AK (n = 6) was exposed to RPMI 1640, 25 mM TMA-formate or isotonic citrate-phosphate buffers all at pH 7.4, and then diluted in ToxiLight assay buffer (also pH 7.4). This was then mixed with AK detection reagent, incubated for five minutes at 37 ℃, and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 3 over 10 cycles (11.4 minutes) at 22 ℃. Error bars indicates SD, ** indicates statistical significance at P <0.01) as determined by ANOVA.

4.7 Use of ATP leakage as a marker for cell permeability during MHC-elution

It was therefore decided to measure ATP-loss directly using the ATP-monitoring reagent found in the ViaLight cell cytotoxicity assay (Lonza Biosciences). While this assay is normally applied to lysates to determine total cellular ATP, it was considered that any permeability of the plasma membrane large enough to allow intracellular peptides to contaminate a MHC eluate would also be large enough to allow the relatively much smaller ATP molecules to migrate from the cytoplasm to the supernatant. Firefly luciferase bioluminescence is considered a fast and highly sensitive ATP assay with high reproducibility (Karl, 1980; Zhao and Haslam, 2005).

4.7.1 Optimisation of buffer compatibility and buffer : sample ratios

Given the possibility for the buffer pH to denature the luciferase, the post-elution supernatant would require full rebuffering prior to the addition of the ADR. Two buffers: Tris and HEPES were assessed for their compatibility with the assay at high molarities (0.2 - 1 M) each containing 5 µl 25 µM ATP. As can be seen in figure 4.16, increasing the strength of either Tris or HEPES buffers leads to a corresponding decrease in assay sensitivity, though this decrease is greater in the case of Tris, which has previously been reported to inhibit other enzymatic processes (Oda *et al*., 1988; Desmarais *et al*., 2002; Ghalanbor *et al*., 2008). Both Tris and HEPES are known to inhibit carbamoyl-phosphate synthase activity (Lund and Wiggins, 1987), again with Tris leading to greater inhibitor than HEPES, though in these cases neither was tested at the high molar concentrations required here. It is also worth noting that at low buffer concentrations, i.e. below 10 mM HEPES-HCl or 5 mM Tris-acetate, sum luminescence was statistically indistinguishable from 5 µM ATP in water.

0.2 M HEPES was decided upon, as this gave a high buffering capacity with a comparatively modest trade-off in sensitivity: ATP assayed in 0.2 M HEPES produced luciferase bioluminescence equivalent to 80.45 (SD = ± 0.9) % of an 'unbuffered' control (the unbuffered ATP was assayed with ATP detection reagent working solution containing 0.625 mM Tris-acetate pH 7.8), while reducing the concentration of HEPES to 0.05 M only increased the bioluminescence to 87.75 (SD = ± 2.44) % (see figure 4.16).

The elution buffers were then titrated against 0.2 M HEPES pH 7.8 to determine the optimum ratios of elution buffer : HEPES which would produce a pH of 7.8. A ratio of approximately 1 : 2 was found for the TMA-formate buffers, while the isotonic citrate-phosphate and sodium-formate buffers required approximately 1 : 4 and 1 : 5 respectively, which was confirmed by pH probe.

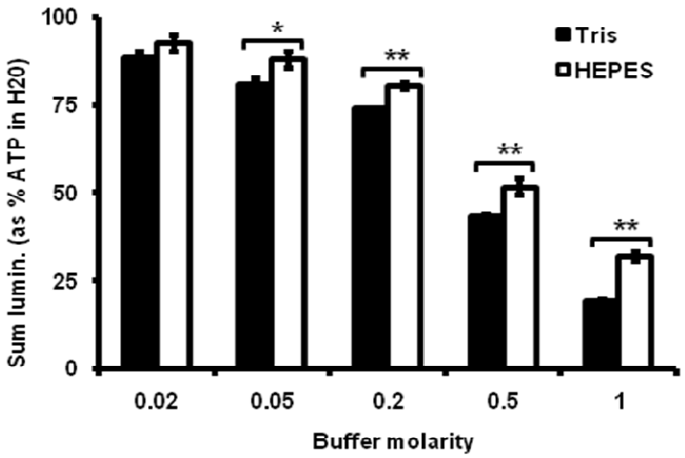


Figure 4.16: Sum luminescence produced by firefly luciferase in the presence of ATP buffered in increasing molarities of Tris or HEPES pH 7.8. Five µl of 25 µM ATP was mixed with 75 µl of either buffer across a standard curve of molarity from 0.02 - 1 M Tris/HEPES at pH 7.8. To these 120 µl of ATP detection reagent was added and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 20 180-second cycles (60 minutes) at 22 ℃. *n* = 5, error bars indicate SD, */** denote statistical significance to $P < 0.05$ / < 0.01 respectively, as measured by T-test.

To ensure full rebuffering these were increased to 1:4 for the TMA-formate buffers and 1 : 9 for the citrate-phosphate and sodium-formate buffers. Short standard curves of ATP were assayed in the elution buffers following rebuffering with HEPES for linearity and sensitivity, and whilst the elution buffers did appear to effect assay sensitivity, linear standard curves were produced in all cases, all with R^2 values greater were than 0.99 (see figure 4.17). The effect of osmotic balancing agents and supplements on the assay appeared to be negligible.

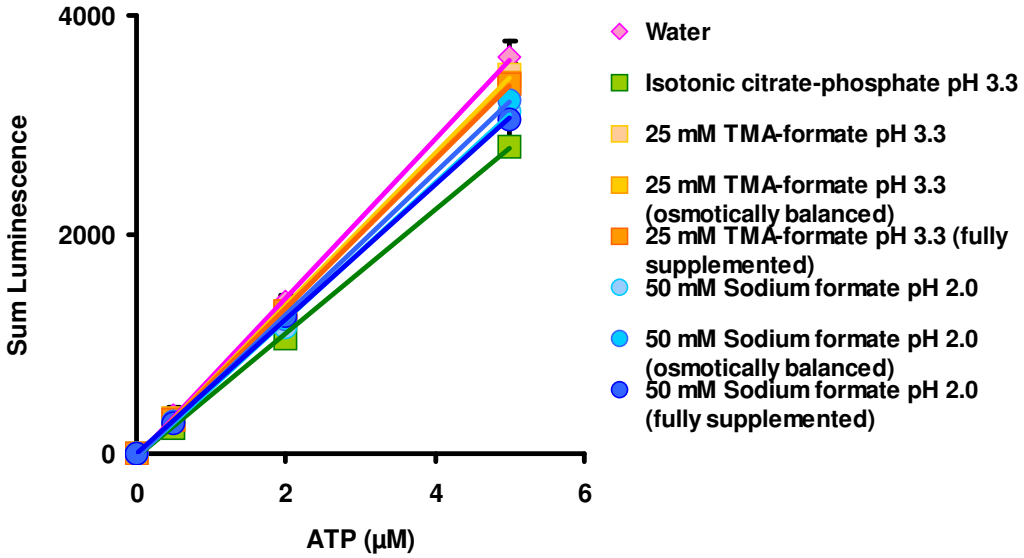


Figure 4.17: Typical standard curves of ATP concentration against sum luminescence in the various MHC class-I and class-II elution buffers. Various concentrations of ATP (*n* = 4) were prepared in ddH_2O, isotonic citrate-phosphate pH 3.3, 25 mM TMA formate pH 3.3 (+/- osmotic agents and supplements) or 50 mM sodium-formate pH 2 (+/- osmotic agents and supplements), then rebuffered with 0.2 M HEPES pH 7.8 as described in 2.3.4.5.5 producing ATP standard curves from 0.5 – 5 µM final concentration, and assayed in triplicate by the addition of 175 µl of ATP detection working reagent and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 20 x 120-second cycles (40 minutes) at 25 ℃. Error bars indicate SD.

4.7.2 Determination of relative intracellular ATP content

Before loss of ATP during MHC elution could be studied, the intracellular ATP content of the four cell lines studies was investigated. Cells were heat-killed in 5 % (v/v) acidified methanol, and supernatants clarified by centrifugation prior to being buffered and assayed as described in 2.3.4.5.1.

A noticeable difference in ATP content was observed between the cell lines. The immortalised JY cell line and the hybridoma T2.Dr4 both exhibited intracellular ATP levels of 3.867 (± 0.13) and 3.779 (± 0.17) fmol/cell, whist the leaukaemic lines K562-A3 and THP-1 both contained a much higher concentration of ATP – 17.189 (± 0.29) and 14.9 (± 0.35) fmol/cell (see figure 4.18).

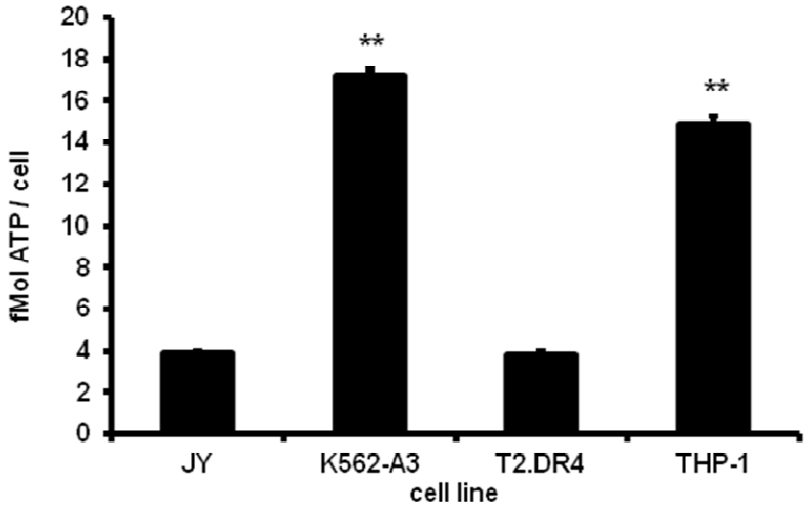


Figure 4.18: Intracellular ATP content of four suspension cell lines. 5 x 10^6 cells were lysed by for 2 x ten minute incubations at 95 °C and the supernatant clarified by centrifugation at 20,800 r.c.f. for 30 mins. 5 µl supernatant was buffered by the addition of 20 µl 5 mM Tris-acetate pH 7.8. To these 120 µl of ADWR was added and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 15 x 240-second cycles (60 minutes) at 25 °C. n = 3, error bars indicate SD, ** denotes statistical significance to $P < 0.01$, as measured by ANOVA.

While somatic cells are commonly described as containing approximately 2 fmol ATP per cell (Wettermark *et al*., 1975; Harber, 1982; Lundin *et al*., 1986; Stanley *et al*., 1976) the actual value can vary greatly. Mouse embryo fibroblast cells contain 6.6 fmol ATP per cell (Bershadsky and Gelfand, 1981), while chick embryo spinal astrocytes and dorsal root neurons are known to contain approximately 7.9 and 11.9 fmol per cell (Schousboe *et al.,* 1970). With regard to myeloid and lymphoid cells/cell lines, Tarnok and Tarnok (1987) report 1.22 (S.D. ± 0.18) fmol ATP per human neutrophil, while murine perinoteal macrophages appear to contain only 0.5 fmol[5] (Loike *et al*., 1979). Little data is available for suspension cell lines, however with regard to adherent lines when in log phase growth the HeLa cell line is known to contain 0.79 (± 0.09) fmol ATP per cell (Karu *et al*., 1995); the human breast-cancer cell line MDA-231 contains 6 (± 0.8) fmol ATP (Geier *et al*., 1994); the human lung adenocarcinoma line A549 displays as much as 100 fmol ATP/cell (Chi and Pizzo, 2006); while the ATP content of the murine Erlich ascites tumour cell line ranges from ~ 7 to >30 fmol/cell as the cell goes through the mitotic cycle (Skog *et al*., 1987). A Friend-

[5] Though macrophages are thought to contain a second high-energy phosphate pool in the form of creatine phosphate from which the cellular ATP stores are rapidly replenished (Loike *et al*., 1970).

virus induced erythroleukaemic murine cell line on the other hand has been recorded as containing 0.0357 fmol ATP per cell[6] (Hobbs *et al*., 1995), while the Raji lymphoma[7] cell line is recorded as containing only 0.0085 fmol ATP/cell[8] (Chignola *et al*., 2007).

It is easy to conclude from this data that the levels of intracellular ATP may vary considerably between cell types, and likely across the cell-cycle, though some of the variations between cell types are undoubtedly a consequence of different approaches to cell lysis, as there is a trade-off to be made between maximum ATP release (Bagnara and Finch, 1972) and the effect of interefering substances (e.g. solvents, surfactants, detergents, buffers or antioxidants) (Betz, 2009) on the assay. Taking this into consideration it is still notable that the two leukaemic cell lines studied exhibit much higher ATP content than the EBV immortalised JY cell line and T2.Dr4 hybridoma.

4.7.3 Effect of prewashing and buffer supplementation on ATP loss during MHC-elution

Prior to analysis of the four cell lines, the effect of an isotonic citrate-phosphate prewash on MHC class-I elution of the K562-A3 cell line using either osmotically balanced or fully supplemented 25 mM TMA-formate pH 3.3. As previously observed, full supplementation of the elution buffer led to a statistically significant reduction (*P* value = < 0.05) in cellular ATP loss, as did prewashing the cells with isotonic citrate-phosphate pH 5.5. Either approach was found to reduce the level of ATP loss, while prewashing followed by elution with the fully supplemented TMA-formate buffer reduced ATP loss to 13.3 % of the osmotically balanced buffer alone (see figure 4.19).

[6] Based on the figure of 0.181 ng ATP (m.w. 507.18) per 10^4 cells.
[7] Derived from a human B lymphoblastoid Burkitt's lymphoma (Karpova *et al*., 2005)
[8] Based on the figure of 4.32 fg ATP per cell.

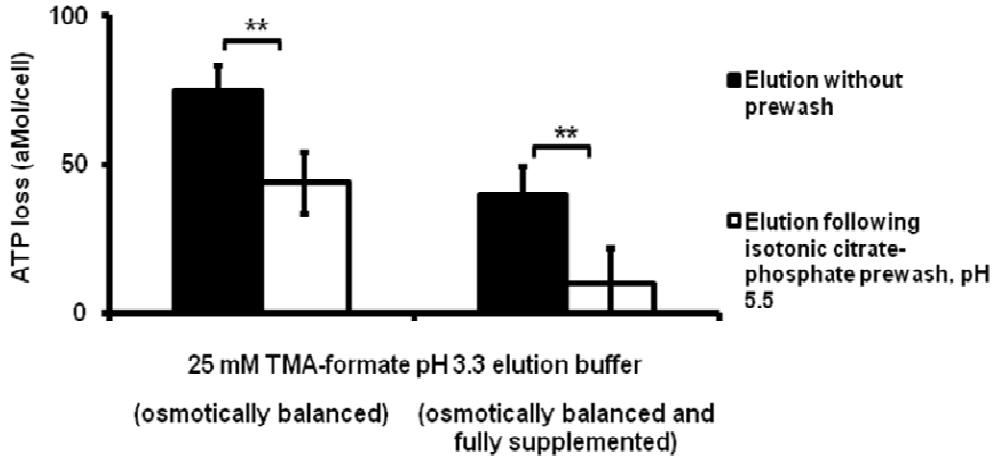


Figure 4.19: Intracellular ATP loss from the CML cell line K562-A3 when eluted with either osmotically balanced or fully supplemented 25 mM TMA formate buffers at pH 3.3, with or without subjecting the cells to an isotonic citrate-phosphate pH 5.5 prewash prior to MHC elution. Data is expressed as amol ATP loss/cell. 5 x 10^6 cells were subjected to MHC elution as described in 2.4.4.5, the eluates rebuffered with HEPES and analysed in replicates of 25 µl, to which 175 µl of ADWR was added and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 15 x 240-second cycles (60 minutes) at 25 ℃. ATP concentration was calculated using a standard curve of ATP prepared in appropriate matrices. n = 5, error bars indicate SD, ** denotes statistical significance to $P < 0.01$, as measured by T-test.

4.7.4 Determination of comparative intracellular ATP loss during MHC elution

After optimisation, ATP loss from the four cell lines during MHC elution with unsupplemented or fully supplemented buffers, or the isotonic citrate-phosphate buffer utilised by Storkus *et al*. (1993) was analysed. 5 x 10^6 cells were subjected to MHC elution, the eluates clarified by centrifugation and rebuffered with 0.2 M HEPES (in a ratio of 1:4 for TMA buffers and 1:9 for citrate-phosphate and sodium-formate buffers) as described in 2.4.4.5.4 before being assayed in replicates of 25 µl by the addition of 175 µl ADR working solution. ATP loss (amol per cell) was then calculated using a standard curve prepared in the same matrix (see figure 4.20) and expressed as a percentage of the total cellular ATP previously determined (see figure 4.21).

Again, the supplementation of the 25 mM TMA-formate buffer appeared to have a large and statistically significant (P value = < 0.05) impact on cell permeability,

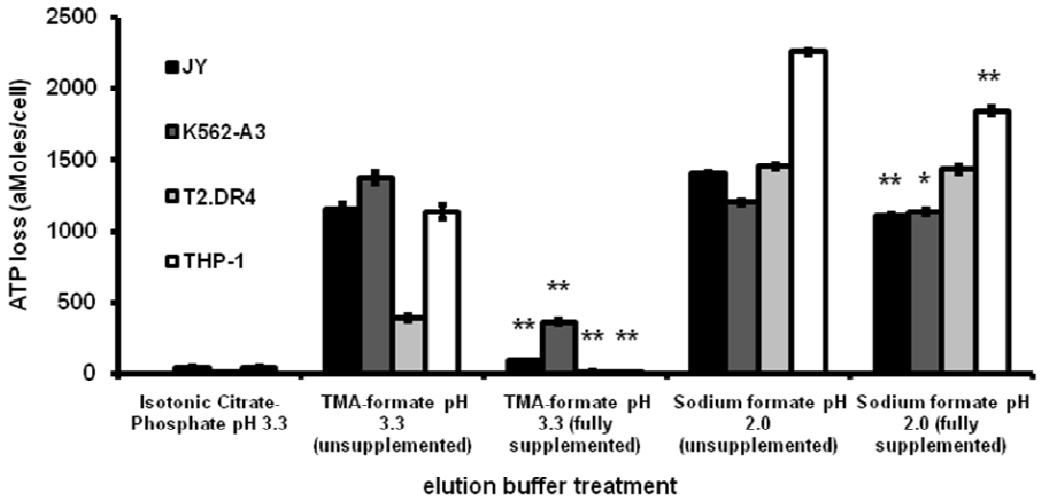


Figure 4.20: Absolute ATP loss from four cell lines when subjected to different MHC elution protocols. 5 x 10^6 cells were subjected to MHC elution as described in 2.4.4.5, the eluates rebuffered with HEPES and analysed in replicates of 25 µl, to which 175 µl of ADWR was added and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 15 x 240-second cycles (60 minutes) at 25 ℃. ATP loss per cell was determined using a standard curve prepared in identical matrices, and revealed a consistent reduction in ATP-leakage (to $P < 0.01$) from following supplementation of the TMA-formate buffer, while for the class-II elution, supplementation produced a drop in ATP loss (to $P < 0.05$) for all but THP-1. $n = 4$, error bars indicate SD, */** denotes statistical significance between supplemented and unsupplemented buffers to $P < 0.05$ / < 0.01, as measured by T-test.

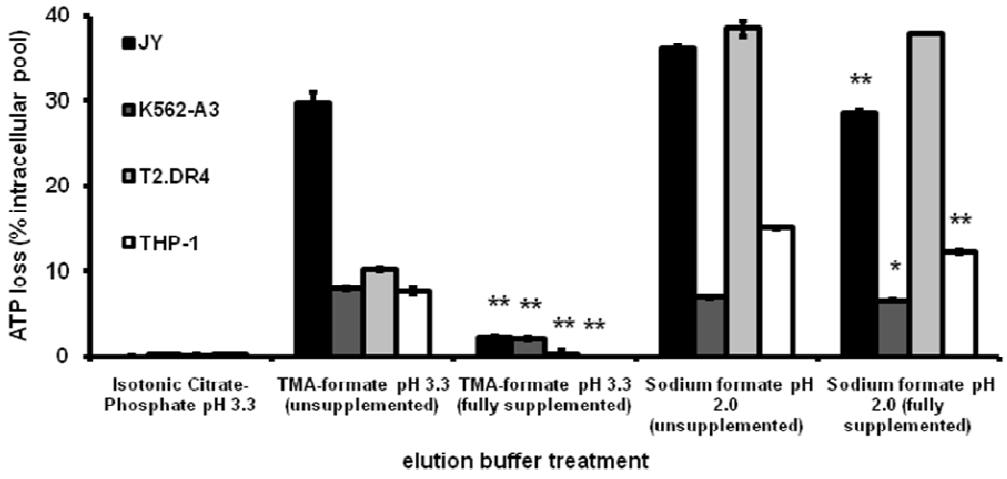


Figure 4.21: Percentage cellular ATP loss from four cell lines when subjected to different MHC elution protocols. Data displayed in Figure 4.20 expressed as a percentage of the cellular ATP content of the four cell lines (as determined in Figure 4.18). $n = 4$, error bars indicate SD, */** denotes statistical significance between supplemented and unsupplemented buffers to $P < 0.05$ / < 0.01, as measured by T-test.

cutting ATP-loss to an average of one tenth that of the unsupplemented buffer. However as may be seen from figure 4.20, this effect varied significantly from cell line to cell line, with the permeability of the THP-1 cell line showing the greatest elasticity to supplementation (ATP loss fell to 0.93 % that produced by the unsupplemented TMA-formate buffer) and the K562-A3 cell line exhibiting the lowest elasticity (26.37 %); while the ATP loss from the JY and T2.Dr4 cell lines exhibited a reduction to 7.46 % and 3.11 % when compared to the unsupplemented buffer.

Supplementation of the sodium-formate buffer on the other hand did not produce large shift in cell permeability; the JY and THP-1 cell lines showed the greatest reduction, with ATP falling to 79.07 and 81.42 % respectively (both to $P < 0.01$). However the K562 A3 line only showed a 5.37 % drop in ATP loss ($P < 0.05$), while the ATP release from the T2.Dr4 hybridoma line remained statistically indistinct from treatment with the unsupplemented buffer.

When the fully supplemented TMA-formate buffer is compared with the isotonic citrate-phosphate elution buffer (both pH 3.3), the results vary considerably, and mostly in the favour of the Storkus' method. Whilst treating the T2.Dr4 and THP-1 cell lines with the fully supplemented TMA-formate buffer leads to 128.6 % and 23.5 % ATP loss compared with the citrate-phosphate buffer, treatment of the K562-A3 cell line with fully supplemented TMA-formate results in 8.1 times the level of ATP loss than that caused by the pH 3.3 citrate-phosphate approach, while the ATP loss found in the JY cell line is four orders of magnitude higher than that caused by treatment with the isotonic citrate-phosphate buffer. The ATP loss caused by treatment with the fully supplemented pH 2 sodium-formate buffers ranges from a minimum of 25 x that caused by the pH 3.3 citrate-phosphate buffer (K562-A3), up to a maximum of 29,000 x the ATP loss (JY), a somewhat suspect figure.

When the luminometry data was examined however, it is notable that the lowest readings were in the low nanomolar range, possibly outside of the optimum range for the assay, and certainly outside of the determined linearity of the assay. To test this a second set of standard ATP curves were prepared in 0.9 % (w/v) saline, isotonic citrate-phosphate pH 3.3, and 25 mM TMA-formate pH 3.3, with or without supplementation. These were then diluted in 0.2 M HEPES producing a final concentration of ATP from 20 to 100 nM (see figure 4.22). It is notable that all of the standard curves retain linearity (R^2 = > 0.99) with the

exception of the isotonic citrate-phosphate (R^2 = > 0.96), which on inspection reveals that at 20 nM the sum luminescence is indistinguishable from 0 nM ATP.

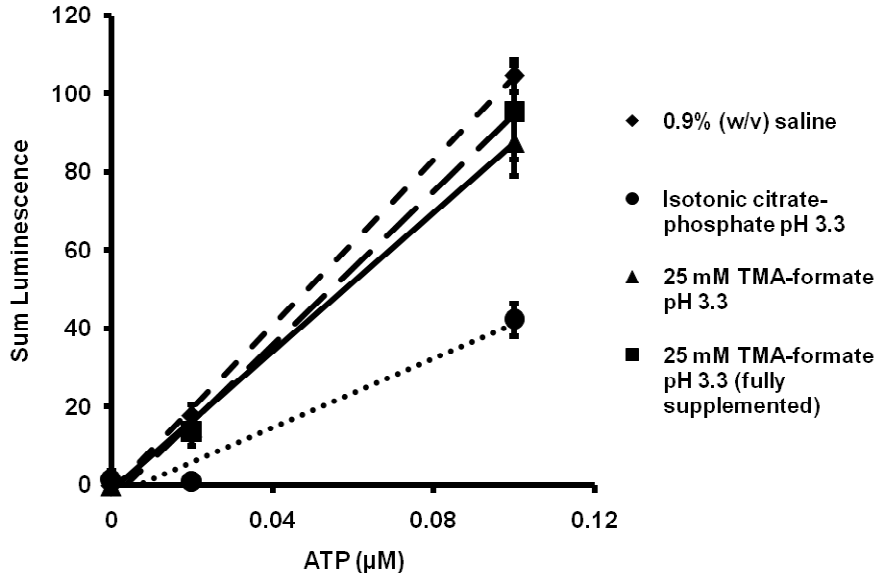


Figure 4.22: Standard curves of ATP concentration against sum luminescence in MHC class-I elution buffers (*n* = 5). Various concentrations of ATP were prepared in 0.9 % (w/v) NaCl, 25 mM TMA formate pH 3.3 (+/- osmotic agents and supplements) or isotonic citrate-phosphate pH 3.3. Each standard was rebuffered with 0.2 M HEPES pH 7.8 (as described in 2.4.4.5.4) producing ATP standard curves from 0.02 – 0.1 µM final concentration, and assayed in triplicate by the addition of 175 µl of ATP detection working reagent and the sum luminescence read using a FluoStar OPTIMA fluorimeter / luminometer set to gain 6 over 20 x 120-second cycles (40 minutes) at 25 ℃.

This lack of linearity and apparent ablation of the assay at low ATP concentrations may well be a consequence of the chelating properties of the citric acid, and calls into question the values of ATP loss recorded during treatment with the isotonic citrate-phosphate elution buffer, implying that cell permeability during MHC elution by the Storkus approach may be higher than the figures suggested by this experiment. However, the TMA-formate buffers appear to retain their linearity at these concentrations, suggesting that the low ATP losses recorded with the bioluminescent assay reflect an actual reduction in cell permeability when cells are treated with the fully supplemented TMA-formate MHC class-I buffer. In addition, the determination of ATP loss as a percent of the cellular pool depend on the lysis method producing close to 100 % ATP release, and whilst the cellular ATP content appears consistent between replicates (though different lysis methods were not compared), there remains the possibility that some lines may show greater sensitivity to heat-lysis than others.

4.8 Post MHC-elution population growth and potential for serial elutions

Storkus *et al*., (1993) describe that following MHC class-I elution with isotonic citrate-phosphate, cells briefly downregulate MHC expression, within 10-18 hours antigen presentation is fully restored, following which a second MHC class-I elution may take place.

In order to test the potential for serial elutions, it was required that post-elution population viability (c.f. death of membrane permeability) first be established. Cells were subjected to elution protocols as detailed in section 2.3.2, washed twice in PBS and twice in RPMI 1640 before being recultured in a small volume of complete RPMI 1640, under normal cell culture conditions. This was observed visually on a daily basis to record any colour change to the phenol red pH indicator present within the RPMI 1640 which would indicate a drop in pH due to media depletion/build up of waste products. In each case the PBS-treated cultures showed a change in media colour within 48 hours, while those treated with MHC class-I elution buffers (with or without supplementation) also demonstrated a pH change within the seven-day period (see table 4.4). However those treated with the MHC class-II elution buffers invariably failed to demonstrate any change in media colour, and upon inspection under the microscope at 100x magnification, appeared highly granulated with a significant degree of debris, an appearance consistent with non-viable populations.

In order to further quantify the growth rates of cell populations following MHC class-I elution, the tritiated thymidine incorporation assay was employed. Following MHC class-I elution (as above) populations of JY, K562-A3 and THP-1 were recultured in complete RPMI 1640 for 48 hours to allow a sufficient recovery window. They were then incubated for a further 18 hours in the presence of 37 MBq/µl [3H]-thymidine[9], following which scintillation counts were taken. During mitosis the thymidine is incorporated into the DNA of the daughter strands (as unlike the other nucleotides, thymidine does not occur in RNA) and once the background is eliminated, the resulting radioactive signal from the cultured population correlates well with cell division (Hughes *et al*., 1958), it may also be influcenced though other factors such as mitochondrial replication (Parsons, 1965).

[9] Performed with the aid of Dr. Morgan Mathieu (NTU) and Dr. Stephanie Laversin (NTU).

In each case the results obtained indicated a massive decrease in cell proliferation following MHC class-I elution (Figure Table 4.5). While the isotonic citrate-phosphate buffer outperforms the TMA-formate buffers by far in this regard (with the exception of the K562-A3 cell line), the post-elution proliferation rate for each cell line is still below 6 % (S.D. = ± < 1.26) that of PBS-treated cells. Supplementation and osmotic balancing of the TMA-formate buffer appeared to have no significant impact on post-elution cell proliferation, with all three lines demonstrating less than 0.5 % (S.D. = ± < 0.09) of the proliferative capacity of their untreated counterparts (see Table 4.5).

	Cell line			
	JY	**K562-A3**	**T2.Dr4**	**THP-1**
PBS-treated control	+	+	+	+
Heat-killed control	-	-	-	-
Isotonic citrate-phosphate pH 5.5	+	+	+	+
Isotonic citrate-phosphate pH 3.3	+	+	+	+
25 mM TMA-formate pH 3.3	+	+	+	+
25 mM TMA-formate pH 3.3 (osmotically balanced)	+	+	+	+
25 mM TMA-formate pH 3.3 (fully supplemented)	+	+	+	+
50 mM sodium-formate pH 2.0	-	-	-	-
50 mM sodium-formate pH 2 (osmotically balanced)	-	-	-	-
50 mM sodium-formate pH 2.0 (fully supplemented)	-	-	-	-

Table 4.4: Colour change observed in cultures of JY, K562-A3, T2.Dr4 and THP-1 cell lines when recultured in complete RPMI 1640 following treatment with MHC elution protocols (n = 3). 2 x 10^6 cells were subjected to MHC elution as per 2.4.5. Isotonic citrate-phosphate pH 5.5 was used as a prewash before eluting MHC class-I with TMA-formate buffers, while citrate-phosphate pH 3.3 was used prior to eluting MHC class-II with sodium-formate buffers. Following elution cells were washed twice in PBS and twice in RPMI 1640 before being resuspended in 5 ml complete RPMI 1640 media and recultured in 6-well plates under standard conditions. Media colour was observed visually for a period of 7 days.

Trypan-blue staining of cells 48-hours after MHC class-I elution demonstrated high population fragility in the unsupplemented TMA-formate treated cultures: 73.49 – 83.88 % (S.D. = ± < 3.31) of each population failed to exclude the dye. However, in these cases osmotic balancing and further supplementation did appear to affect cell staining, and in each case led to a drop in trypan staining of 23.98 – 29.18 % (± < 3.15) and of 35.87 – 37.45 % (± < 2.49) respectively (as a percentage of trypan blue stained cells derived from treatment with the

unsupplemented buffer). The isotonic citrate-phosphate on the other hand led to a much lower trypan staining of 15.09 – 19.42 % (± < 1.57) (see figure 4.23).

Scintillation counts for each cell line following treatment (± S.D).			
Treatment	**JY**	**K562-A3**	**THP-1**
PBS-treated cells	18737.33 (±2538.97)	68357 (±13378.71)	39532 (±5172.10)
Isotonic citrate-phosphate pH 3.3	703 (±85.02)	145 (±62.22)	2273 (±499.22
25 mM TMA-formate pH 3.3	68.33** (±17.47)	61.33* (±10.41)	72** (±3.61)
25 mM TMA-formate pH 3.3 (osmotically balanced)	43** (±7.81)	64.33* (±11.93)	61.33** (±1.53)
25 mM TMA-formate pH 3.3 (fully supplemented)	81.67** (±15.63)	75 (±21.00)	66.33** (±20.55)

Table 4.5: Scintillation counts of JY, K562-A3 and THP-1 cell line populations 48 hours after treatment with MHC class-I elution buffers, and following 18 hours culture in tritated thymidine-containing media. 1 x 10^6 cells were subjected to MHC elution as per 2.3.2. Isotonic citrate-phosphate pH 5.5 was used as a prewash before eluting MHC class-I with TMA-formate buffers. Following elution cells were washed twice in PBS and twice in RPMI 1640 before being resuspended in 10 ml complete RPMI 1640 media and recultured in 6-well plates under standard conditions for 48 hours. After this incubation cells were plated out in triplicate into a 96-well microplate, and treated with [3H]-thymidine to 37 MBq/µl and incubated for 18 hours prior to harvesting onto 96-well filter plate; to which 40 µl scintillation fluid was added per well, following which a Top-Count Scintillation counter was utilised to perform counts over 1 minute/well. n = 3, error bars indicate SD, */** denotes statistical significance between supplemented and unsupplemented buffers to $P < 0.05$ / < 0.01, as measured by T-test.

An inverse Spearman's correlation coefficient of -0.774 (to a P value of <0.01) was found between trypan staining and proliferation rate for the cell populations assayed, dropping to a weaker coefficient of -0.562 if the untreated controls were excluded. As would be expected from the [3H]-thymidine incorporation assay results, no correlation was found between trypan staining and proliferation rate when the data for cells treated with the TMA-formate buffers only were analysed.

This lies somewhat at odds with the data reported by Storkus *et al*., (1993) which suggested that following elution with isotonic citrate-phosphate, full antigen presentation is restored within 10 – 18 hours, allowing serial elutions.

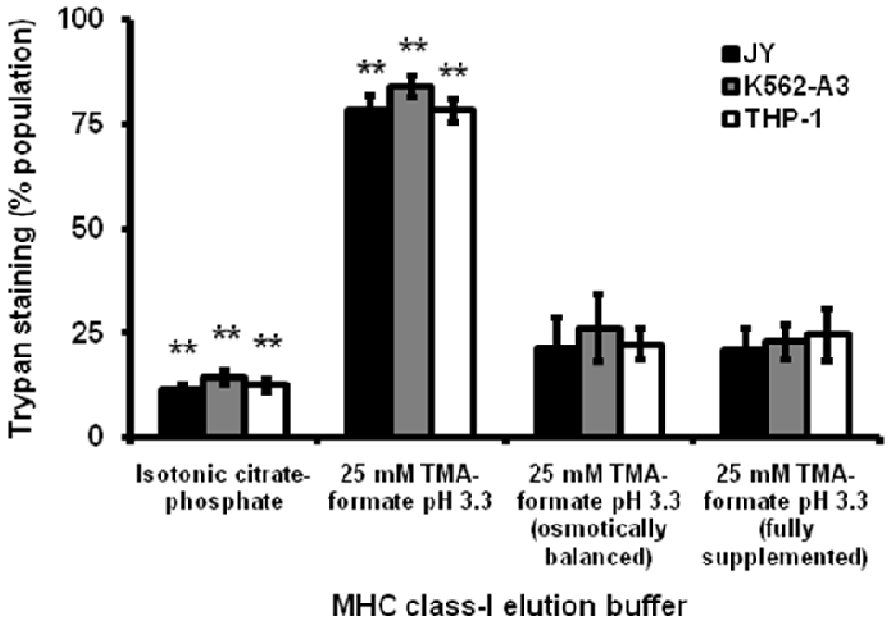


Figure 4.23: Trypan-blue staining of JY, K562-A3 and THP-1 cell lines 48 hours after treatment with MHC class-I elution buffers. 1 x 10^6 cells were subjected to MHC elution as per 2.3.2. Isotonic citrate-phosphate pH 5.5 was used as a prewash before eluting MHC class-I with TMA-formate buffers. Following elution cells were washed twice in PBS and twice in RPMI 1640 before being resuspended in 10 ml complete RPMI 1640 media and recultured in 6-well plates under standard conditions for 48 hours, following which cells were pelleted and resuspended in PBS. To determine percentage cell death, 20 µl of each cell suspension was then mixed with 180 µl of 0.1 % (w/v) trypan blue, and the stained and total number of cells counted using a haemocytometer. n = 3, error bars indicate SD, ** denotes statistical significance when compared with osmotically balanced or fully supplemented TMA-formate buffers to $P < 0.01$, as measured by ANOVA.

This data suggests that even with the isotonic citrate-phosphate buffer, the proliferative capacity of eluted cells is severely impeded, and therefore any subsequent MHC-elutions may characterise an altered presentome. Furthermore it is conceivable that multiple elutions may lead to higher levels of cell lysis and contamination with intracellular peptides, and even should a return to log-phase growth be achievable through a prolonged return to culture, there remains the issue of potential selective pressure on the cultured population. The comparatively much lower proliferation rate of cells treated with the TMA-formate MHC class-I elution buffers also therefore precludes any possibility of multiple elutions when these buffers are employed.

It was therefore decided to avoid serial MHC elutions, especially given that in add7ition to the above factors, reculturing is not possible for populations which have also been subjected to elution of MHC class-II peptides with the sodium-formate buffers.

4.9 Discussion

Though the pH-dependent disassociation of MHC-restricted peptides from the class-I complex has been utilised for almost 20 years (Storkus *et al*., 1993), little in the way of method development has occurred, and the technique presents largely the same problems for downstream analysis today as it did in the early nineties, namely limited compatibility with chromatographic methods, and the potential for acid-induced cell lysis or disassociation of membrane associated non-MHC peptides to lead to sample contamination.

As this chapter demonstrates, a TMA-formate buffer may be used as an alternative to isotonic citrate-phosphate for elution of MHC class-I peptides, though as illustrated, the high water potential of the buffer leads to high cell lysis, though this may be abrogated to some extent by the inclusion of 0.3 M sucrose and 0.45 M KCl as osmoticants, which appear to reduce overall cell lysis (though to differing extents depending on the assay by which cell death/permeability is measured). Sucrose may confer a secondary benefit in the form of cushioning during centrifugation (a feature which often lends it to organelle preparations by ultracentrifugation) (Dr Alan Hargreaves and Dr Shakthi Dookie, NTU, *Pers. Comm*., 2008). Following on from this, the inclusion of 10 mM D-glucose, 2 mM *myo*-inositol and 1 mM reduced glutathione also allowed a reduction in cell staining/permeability to the point where by all measurements bar ATP-loss it led to less cell death/permeability than the isotonic citrate-phosphate elution buffer, and in several cases became statistically indistinguishable from the untreated (or prewashed-only) controls.

The role that D-glucose plays in reducing cell permeability/lysis suggests that surviving low pH stress is an energy-dependent process. Locke's solution, an early media for cell culture, often (though not always) contained glucose at varying concentrations (Rous and Turner, 1916), though sadly Pappenheimer (1917) did not state the components of his Locke's solution used for culture of

lymphocytes. However the addition of sucrose and glucose to Locke's solution is known to improve the long-term viability of erythrocytes, and dextrose is also known to confer a survival benefit, especially in samples with heavy handling (and which may also be of benefit here).

In the case of erythrocytes, the inclusion of citric acid in Locke's solution seems to confer a survival benefit, though this benefit is not consistent between species and may also heavily modulate the benefit or otherwise of other additives such as glucose or dextrose (Rous and Turner, 1916), possibly explaining why the addition of the non-osmotic supplements to the citrate-phosphate Storkus buffer or prewash buffer did not appear to reduce cell staining. Hendry (1951) noted that suspension of erythrocyes in solutions of 12 mM of glucose delayed haemolysis, especially when coupled with cooling below room temperature, and it is possible that that the inclusion of glucose merely delays a significant percentage of the cell lysis until after elution has taken place (a theory somewhat supported by the relatively high trypan staining of the fully supplemented buffer 48 hours post elution (see figure 4.23). It is possible that titrating the concentrations of the various supplements against cell staining might lead to reductions in isotonic citrate-phosphate elution mediated cell death, though given the downstream chromatographic requirements this might not facilitate the further aims.

The role of inositol in minimising post-elution lysis is less clear. The best known role of the molecule is as the basis for the intracellular secondary messenger compounds of inositol phosphates (e.g. IP_3), which have a number of functions, including modulation of membrane potential (Berridge, 1988) and intracellular Ca^{2+} levels (Gerasimenko *et al*., 2006). Silver *et al*. (2002) report that under hypertonic conditions the addition of *myo*-inositol to rat brains leads to a concomitant increase in intracellular *myo*-inositol, reducing osmotic stress. Each of these functions may be a potential source of the reduction in cell fragility during MHC elution when *myo*-inositol is added to the TMA-formate and sodium-formate elution buffers. Additionally *myo*-inositol has been shown to reverse D-glucose-induced reductions in cell proliferation (Ziyadeh *et al*., 1991); though in this case of this research cells underwent a far shorter exposure period (< 5 minutes), it is possible that it may also contribute to a reduction in any glucose-mediated cell-shock that may occur.

Like inositol, glutathione also has numerous functions, though it is known first and foremost as an antioxidant (Larbi *et al*., 2010), it is also capable of inactivating many toxins and carcinogens, both organic and inorganic, and acting as a cofactor for numerous peroxidases. It is one of the most abundant antioxidants, reaching cellular levels in the milimolar range in humans, though never higher than ~ 10 mM (in the liver) (Meister, 1995). Related to it's antioxidant activity, it is a potent scavenger of free radicals, and donator of electrons and sulphydryls, and is also involved in glutamate synthesis and the conversion of ribonucleotides into deoxyribonucleotides (Kidd, 1997). As with glucose however, it is suggested that the most common role: the antioxidant activity, may provide the beneficial effect (though it may also be ameliorate the theoretical release of waste products as a response to pH stress). Glutathione has already proven its protective effects towards tumour cells (which are often glutathione rich) (Arrick *et al*., 1982), though it is interesting that the optimum concentration of this supplement was so low, given the far higher cellular concentrations discussed above (Kidd, 1997), and that the low pH of the MHC elution buffers would (at RT) be a pro-oxidative environment. Indeed, the yeast *Candida utils* up-regulates glutathione synthesis when exposed to low pH environments (Nie *et al*., 2005), though obviously this is a different biological system.

Despite the efforts to minimise cell death/leakage it undoubtedly has not been eliminated entirely given the pH stress that it necessitates, and it is probable that alterations to the methodology may further drive down cell lysis (as well as potentially counteracting growth inhibition). For example inclusion of metal ions such as Ca^{2+} in low-pH buffers has been shown to reduce (PI determined) sensitivity to pH stress in *Arabidopisis thaliana* (Koyama *et al*., 2001). It is conceivable that similar effect might be achievable here, though complications to downstream chromatography must be avoided. Moore and Hood (1993) recommended the addition of Hetastarch (esterified amylopectin-containing starch) to their modified low-pH RPMI 1640 medium, and therefore the addition of it or other non-proteinaceous colloids such as dextrose to the elution buffers may further reduce cell death, especially given the extensive handling protocols detailed in the methodology.

The use of the prewash step, while increasing the overall handling and experimental time involved in peptide elution also appears to reduce lysis/leakage during the peptide elution stage, further reducing the likelihood of

contamination by intracellular peptides. This coupled with the previously determined pH-dependent disassociation of membrane-associated (often serum) peptides (Barry, 2006) also aids decomplexing irrelevant peptides from the eluate. This is of particular note when the findings of Antwi, *et al* (2009) are taken into account – a mild acid elution of the PANC-1 pancreatic tumour cell line, produced 148 peptides, of which only 32 % were in the appropriate 7-13 amino acid mass range for MHC class-I presentation. Similarly Torabi-Pour *et al*., (2002) note that treatment of both MHC- class-I positive and negative Fen cells with a mild acid elution buffer will produce peptides in both cases. Depletion of these peptides by prewashing significantly reduces the complication they may present to antigen discovery.

Indeed, there may be benefits to reducing the prewash pH closer to the β2-microglobulin disassociation pH, as not only would this hypothetically decrease the remaining membrane associated peptides prior to MHC-elution, it would also aid removal of MHC class-II invariant chain peptides which might otherwise elute during the pH 3.3 stage (Urban *et al*., 1994). However, use of the prewash buffer on the murine CT26 cell line led to cellular disassociation from the polystyrene culture surface. Given that EDTA is also used to remove adherent cells, the higher citric acid concentration found in the isotonic Storkus buffer or a lower pH prewash buffer may lead to even greater disassociation of cells – a problem which the non-chelating TMA-formate buffers may avoid.

The validity of prewashing cells with isotonic citrate-phosphate pH 5.5 to reduce intracellular 'leakage' during MHC class-I elution was validated by measuremement of ATP loss. However when an adherent CT26 cell line was treated with the elution protocol, the chelating properties of the buffer were found to partially disrupt cell adhesion, leading to disassociation of some, but not all of the cell population into the prewash buffer. It is possible therefore that an alternative buffer composition may be required for prewashing of adherent cell lines. With this said, the chelating properties of the citric acid may also provide the optimal disassociation of membrane-associated non-MHC peptides, and a change in prewash buffer may lead to a greater proportion of contaminating peptides in the final pH 3.3 MHC eluate. At this time it is unknown whether the cells which disassociated (or those that did not) represent any meaningful subpopulation of the CT26 cells (for example a certain point in the cell cycle), though their numbers (~ 20 % by visual mass) probably preclude the effect being limited to a stem cell subpopulation.

An alternative solution to this problem might be supplementation of the prewash with additional chelating agents (e.g. EDTA) to disassociate all cells, and then elute them in the same manner as non-adherent cells. As yet however the effect that this might have on MHC expression is also currently unknown.

If however a serious commitment to further buffer optimisation were to be made, the first point of call might be the buffer itself, which was originally chosen for volatility and compatibility with M/S. Though the supplements added to reduce cell death compromise volatility, the loss of mass-spectrometric compatibility is a minor drawback given the complexity and likely impurities to be found in a MHC-eluate (lipids being of particular concern to analytical column degredation). Of the two, TMA is an intermediate product in numerous metabolic pathways and should be readily broken down (Rehman, 1999), while formic acid is not tolerated as well, and is the 'active' agent in methanol intoxication (Seme *et al*., 1999), inhibiting cytochrome oxidase (Eells *et al*., 2004) and degrading to carbon monoxide (Araña *et al*., 2001), all of which may damage mitochondria; as well as facilitating Ca^{2+} entry into cells and altered ATP production (Liesivuori, and Savolainen, 1991).

Formic acid may therefore play a role in the growth retardation/arrest seen as a result of the MHC class-I / class-II elution protocols (though given that citrate-phosphate elution also significantly ablates growth, it is certainly not the sole factor). Replacement of the acid with another, such as HCl, or further reduction in buffer concentration may both pay dividends in reduction in cell leakage/death. This mitochondrial damage may be caused/exacerbated by ATP depletion (Dorman *et al*., 1993; Wallace *et al*., 1997) and there may be a benefit to supplementing post- or pr-elution buffers with ATP, though if it were to be included in the elution buffers themselves it might increase kinase or protease activity, altering results.

Interestingly Eells *et al*. (2004) found that red to near infra-red light (already known to speed wound healing) appears to stimulate cytochrome C function, and upregulation of gene pathways related to mitochondrial energy and antioxidant function. It is conceivable that such 'light-therapy' might improve post elution recovery, or if applied prior to MHC elution might even pre-condition the cells against damage, though doing so would further distance the cell line from a true *in vivo* blast crisis and may undermine the research. In any case the reasons for

not pursuing multiple MHC elutions from a single CML cell line population remain relevant.

With regard to ATP, the relative differences in content between the cell lines warrants further examination. Loo *et al*., 2006 noted a positive correlation between ATP content and aggressiveness in invasive breast tumours. Similarly, a Spearman's *rho* of 0.84 (to a *P* value of <0.01) was found between ATP content of the four cell lines, and their proliferation-assay determined growth rates (the data for the untreated controls), further highlighting the potential for ATP to be used as a marker for tumour prognosis/staging. Martin *et al*. (2000) reviews findings that depletion of ATP sensitised cancers to chemotherapy, while Fine *et al*. (2009) found that ATP uncoupling agents alone produced a tightly linked drop in both growth and ATP content.

Despite optimisation, the MHC class-II elution buffer shows higher levels of cell death than the TMA-formate. This may be due to both the additional stress of a lower pH, and the toxic (relatively pH independent) effects of the comparatively much higher formic acid content. Again, this may be avoided by use of an alternative buffer, though Barry (2006) records that a glcyine-HCl composition was not suitable for cell surface elution. Nevertheless while the levels of cell death have been minimised to a point comparable (by most means of measurement) with that found in the widely accepted isotonic citrate-phosphate approach for class-I elution, it is likely that further optimisation is possible.

Despite the advances in mild acid elution detailed here, the cell-surface elution method retains a number of complications. Firstly, a mild-acid eluate from cells naturally expressing MHC class-I will contain peptides derived from A, B and C alleles. As these may be found in many different combinations (though the expression of some alleles correlates to some extent with ethnicity), single peptides derived from a cell eluate cannot be considered viable therapeutic agents until the MHC-allele they bind is characterised. This may be considered an argument for using MHC-bound antibody columns as opposed to cell surface elution, or using homoallelic cell lines such as K562-A3. These too have their own drawbacks however: the former will include newly synthesised MHC molecules not expressed on the cell surface, potentially containing peptides with poor processing efficiency (Bonner *et al*., 2002); while the latter may be restrictive, and of potentially limited relevance to an *in vivo* malignancy). In addition given that the W6/32 antibody often used for MHC antibody columns

binds to an epitope including both the α-heavy chain and β2-microglobulin of the MHC class-I molecule, it is conceivable that this may in some instances stabilise the complex in acidic conditions, and that not all peptides may elute properly.

Indeed, Gebreselassie *et al.* (2006) found that the mild acid elution and MHC-immunoaffinity produce only mildly overlapping peptide populations, with the former skewed towards poor binding peptides, and the latter strong binders. It is conceivable that poor binding peptides failed to be retained during the chromatographic stage, and given that elution of peptides from immuno-precipitated MHC molecules is usually performed by boiling the samples in 10 % (v/v) acetic acid for 10 minutes, a strong-binding subpopulation may indeed fail to elute at pH 3.3 (or pH 3 in the case of Gebreselassie *et al.,* 2006). Though the data in figure 4.2 suggests that mild acid treatment is sufficient to elute the vast majority of peptides from MHC class-I-A2 and A3, Ciccone *et al*. (1995) report that a mild-acid elution of pH 2.2 is required to achieve 100 % disassociation of peptide from MHC class-I $Cw3^+$ and $Cw4^+$ CR-1 and 81.22 cell lines. Whilst this tightly bound peptide subpopulation was not characterised, nor were the pH required to achieve complete disassociation of the peptide from other MHC alleles investigated (Ermanno Ciccone, Université de Genève, *Pers. Comm*., 2010) together these data suggest mild acid elution at pH 3.3 is not be sufficient to analyse the total presentome of a culture, and that cell-surface elution and elution from immunoprecipitated MHC may be complementary methods rather than exclusive ones. Whether similar patterns may be seen for MHC class-II elution when compared to eluates from immunoprecipitates is currently uninvestigated. Yet it also implies that some MHC class-I peptides may contaminate class-II cell-surface eluates, complicating any cell-surface elution based research into cross presentation.

The remaining drawbacks to mild-acid elution are primarily methodological: larger than ideal sample volumes and potential contamination with prewash-resistant serum peptides may both be at least partially overcome by additional chromatographic methods; including rebinding peptides to immobilised MHC molecules (e.g. class-I or class-II tetramers) before re-elution. If the non-MHC contaminating peptides are characterised, determination of common characteristics may allow alternative/specific methods of depletion.

Increasing reliance on serum-free media will also reduce the presence of serum peptides (this would also reduce intra-culture variation and the ethical issues

surrounding serum collection) (FoA, 2005) though in all probability at least a proportion of membrane-associated peptides are produced by the culture rather than derived from the FCS.

It appears that despite the weaknesses of the Storkus *et al*. (1993a;b) approach to mild-acid elution it remains a part of the immunology toolkit, and that immunoprecipitation of MHC molecules may in fact be a complementary rather than a competing technique. With this in mind it is hoped that the modifications to the elution reagents and protocol suggested here may improve the efficiency of the approach, and though in of itself the method does not discriminate between immunogenic and nonimmunogenic peptides, that the use of these methods may aid in the search for novel T-cell epitopes in cancer immunotherapy.

5.0: Optimisation of chromatography.

5.1 Introduction

Once a heterogeneous population of MHC-bound peptides have been eluted from the cell surface as putative vaccines their sequences require identification. While a variety of chromatographic approaches have been applied to MHC-eluted peptides prior to mass spectrometry (MS), decomplexing or fractionation of samples along a first dimension (1-D) prior to analysis on a second dimension (which may be LC or TOF) and identification by MS is a common feature of many latter direct immunology publications (Clark *et al*. 2001; Gebreselassie *et al*., 2006; Fortier *et al*., 2008), for the simple reason that complex samples such as MHC-eluates require simplification/fractionation to reduce the high background signal caused by multiple co-eluting peptides; while low abundance peptide populations (e.g. phosphopeptides) must be concentrated and isolated from the non-phosphorylated majority if they are to be confidently identified.

Various forms of chromatography have been used for over a century to separate components of a heterogeneous solution by passing them in solvent through a stationary phase (Tswett 1906). The most basic method, paper chromatography was rapidly supplanted by thin layer chromatography (later found to be capable of isolating serine-phosphorylated (pSer) peptides from threonine- or tyrosine-phosphorylated (pThr/pTyr) peptides at low pH) (Martensen, 1984), which in turn fell out of favour with the arrival of techniques such as poly-acrylamide gel electrophoresis (PAGE) (in both 1-D and 2-D) and HPLC, both of which show improved resolution and reproducibility (Martensen, 1984; Görög, 2004).

Along with column fractionation, PAGE is one of the most common proteomic methods of analysis (Minden, 2007), with staining either done in-gel, or by western blotting following transfer to nitrocellulose/PVDF, or alternatively spots may be excised (and usually trypsinised) for identification by mass-spectrometry. Due to the limitations of resolution and identification methods, 1-D PAGE is rarely utilised for complex unknown samples except in conjunction with western blotting (Ahmad *et al*., 2005), and despite its limitations in resolution has major advantages over its 2-D counterpart in terms of reproducibility, throughput, and the timescale involved (Ahmad *et al*., 2005; Minden, 2007). Nevertheless, 2-D PAGE (typically using immobilised pH gradient strips) in conjunction with MS is now the gold-standard for the majority of proteomic investigations (Gorg *et al*.,

1988; Kubota *et al*., 2005), and is capable of determining the net acidic/basic weighting of a protein, as well as differentiating between isoforms. However, despite advances in solubilisation methods, large (>100 kDa) or membrane-bound proteins remain difficult to run (Ong & Pandey, 2001) and proteins and peptides below 10 kDa remain outside the range of the technique (Kubota *et al*., 2005). There have however been some efforts to utilise iso-electric focusing followed by 2-D PAGE for peptides (Gatti and Traugh, 1999), though high-acrylamide concentrations (15-50 % w/v) are vital for any meaningful separation of peptides, which may differ from each other by only a few kDa. A wide variation in methods exists for 1-D PAGE for peptides, e.g.: Andersen *et al*. (1983); West *et al*. (1983); Fling and Gregerson (1986); Schagger & von Jagow (1987); Ahn *et al*. (2001); and Yim *et al*. (2002) and the use of peptide PAGE for analytical purposes is constrained by the limited facility for western-blotting of peptides onto PVDF (Xu *et al*., 2004)[1], and the competition of MS (especially MALDI-TOF) to rapidly determine peptide masses with high throughput.

As previously discussed (see 1.2) protein phosphorylation is a relatively common post-translational modification, however, as proteins are typically hundreds of amino acids in length, often with only one or two phosphorylation sites, and that the phosphorylated form of a protein is often (though not always) of low abundance (Wagner *et al*., 2007), phosphopeptides are expected to make up the minority of the presentome. Isolation/enrichment of phosphopeptides is therefore an important requirement for analysis, without which a 'drowning out' of the modified peptides by higher abundance non-phosphorylated peptides may be observed, leading to very poor signal strength (by both gel-based analyses or mass-spectrometry) (Steen *et al*., 2006; D'Ambrosio *et al*., 2006).

Enrichment of phosphorylated proteins and peptides is achievable by a variety of methods, but the majority of approaches use one of the following three: antibody-affinity; immobilised metal affinity chromatography (IMAC) or more recently metal oxide (typically titanium dioxide/TiO_2) affinity (MOAC). Antibody-based methods were highly limited as though global anti-pTyr antibodies were available, global anti-pSer/anti-pThr antibodies were not until the last decade. Antibodies against Ser- or Thr-phosphopeptides had to be raised using synthetic peptides or purified proteins, and were usually specific for the peptides in

[1] Kuhar and Yoho (1999) found a lower limit of 4 kDa for blotting of CART peptides (involved in the neuroendocrine control system), while Xu *et al*. (2004) and Ghosh *et al*. (2004) both found it necessary to ligate small (10-20 amino acid) peptides to carrier proteins prior to western blotting.

question (Patton, 2002), rendering them unusable for purification of heterogeneous phosphopeptides from MHC eluates and the vast majority are also not compatible with immunoprecipitation (Rush *et al*., 2005). Furthermore as Tyr is by far the least common site of *O*-phosphorylation in mammals (a ratio of 1:200:1800 pTyr : pThr : pSer is often cited) (Hunter, 1998) the use of anti-pTry antibodies alone excluded a large proportion of phosphorylated peptides, only remedied by the advent of global anti-pSer and anti-pThr antibodies in the early 2000's (Grønbørg *et al*., 2002; Stannard *et al.,* 2003; D'Ambrosio *et al*., 2006), though even these are usually of limited applicability to immunoprecipitation, constraining their use (Han *et al*., 2008a). Furthermore, disassociation of the peptide from the antibody often requires harsh temperatures and/or incubation with detergents and reducing agents, producing a suspension far from compatible with electrospray mass spectrometry (Patton, 2002), as well as risking peptide oxidation which may complicate the analysis.

Immobilized metal ion affinity chromatography (IMAC) (reviewed extensively in Chaga, 2001) was first described for protein chromatography by Porath *et al*. (1975) who employed zinc and copper ions to isolate proteins from plasma. It has since proven to be highly selective, and operates on the basis of differential affinity arising between the metal ions and the functional groups on the amino acids; allowing a protein to be isolated from a sample and eluted under non-denaturing conditions. Whilst not as stringently selective as immunoaffinity chromatography, IMAC allows great flexibility with a low number of columns, as well as high recovery, milder elution conditions and lower cost, and may be applied independently to; or on-line with electrospray mass spectrometry (D'Ambrosio *et al*., 2006; Kaur-Atwal *et al*., 2007).

The metal ions employed vary depending on the proteins to be isolated, and are typically classified by Pearson's (1963) three-tier "hard and soft acids and bases" system (HSAB); determined by reactivity with nucleophiles. At a low pH, "hard" ions such as Fe^{3+}, Ga^{3+}, and Al^{3+} co-ordinate unprotonated oxygen ions (Sulkowski, 1985), and depending on running conditions may be used to target carboxylic amino acids, Tyr residues or, crucially, phosphorylated amino acids (Andersson and Porath, 1986). The ability of IMAC to isolate and enrich phosphorylated proteins/peptides using hard ions such as Fe^{3+} and the more selective Ga^{3+} (Posewits & Tempst, 1999) has become increasingly important in proteomic research, though there is on goinging research into the utilisation of intermediate ions for investigation of other PTMs (Chaga, 2001). Once optimised

however, Fe^{3+} IMAC may be utilised to strongly enrich phosphopeptide fractions from heterogeneous samples, though the approach has a higher affinity for multiply phosphorylated peptides (Scanff *et al*. 1991). Additionally, Zn^{2+} ions (normally be thought of as "intermediate" under the Pearson system) may also be used to isolate phosphoproteins (Ueda *et al*., 2003; D'Ambrosio *et al*., 2006). However, the resins commonly used for IMAC may also inadvertently select out peptides containing acidic residues (aspartic acid [Asp], glutamic acid [Glu]) or electron donors (e.g. histidine (His))[2].

The intermediate metal ions (e.g. Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+}) co-ordinate nitrogen and sulphur ions as well as oxygen, and have an optimum adsorption in neutral pH. Cu^{2+} and Ni^{2+} may also be used to target His and cysteine (Cys) residues, though Cys residues are typically rarer (Hansen *et al*., 1996). Finally the soft ions (Cu^{+}, Hg^{2+}, and Ag^{2+}) preferentially retain sulphur – and may be used to isolate methionine, Cys and sulphated Tyr. As the hard and soft ions interact with different side chains, they may be used in tandem to improve separation/isolation. Indeed IMAC techniques have also been used in conjunction with other separation methods such as HPLC to achieve impressive levels of isolation (Chaga 2001). Over 50 % of recombinant proteins are now routinely isolated through the use of IMAC, aided by either the addition of terminal poly-His tags to improve isolation (Hochuli *et al*., 1987 and 1988) using Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} ions (in increasing order of His affinity) (Sulkowski, 1985), or via the generation of novel binding sites through point mutation (Smith, 1991; Yilnaz *et al*., 1995).

The choice of metal ion may strongly impact the efficiacy of any column. For example, Cu^{2+} columns will retain approximately 50 % of proteins from any biological sample, while Ni^{2+}, Co^{2+} or Zn^{2+} will retain a far smaller proportion, due to a higher stringency for binding (Chaga, 2001) (Cu^{2+} binds exposed His, which makes up approximately 1 % of the average protein [Klapper, 1977]). Conversely, Fe^{3+} will adsorb over 30 % of proteins from any biological mixture at below pH 5.0, though a proportion of this may be non-specific interactions between proteins and the resin rather than the metal ion (Thingolm and Jensen, 2009). The choice of resin may also therefore be expected to impact on purification efficiency, though currently only a small variety of adsorbent resins are commonly used in IMAC: IDA (iminodiacetic acid) [e.g. as produced by GE Healthcare]; NTA (nitrilotriacetic acid) [e.g. Quiagen] and CM-Asp

[2] This is obviously less of an issue when Cu^{2+} IMAC is used to enrich His-containing peptides.

(carboxymethylated aspartic acid) [e.g. Clontech]; or TED (tris-carboxymethyl ethylene diamine) [e.g.: Inovata], offering the formation of tri-, tetra- and penta-dentate metal-ion complexes respectively (as most metal ions can co-ordinate between four to six ligands, the choice of resin-metal complex may have large effects on sample binding) (Kaur-Atwal *et al*,. 2008). While other adsorbant resins have been synthesised, these are rarely used due to lack of commercial availability (Chaga, 2001).

Any IMAC protocol consists of 6 key steps: (1) charging the column with metal ion; (2) removing the excess metal; (3) equilibration of the adsorbant; (4) loading of the sample; (5) washing away unbound protein and; (6) eluting the enriched material. However no standard methods exist for the majority of these steps. As stated the metal chosen varies depending on the target in question, but may be loaded in a small concentrated volume, or a more dilute one, and unbound metal may be washed away using glycine, ultrapure water, weak acids or imidazole. Similarly, equilibration (bringing the column to the required loading pH) may be performed with a wide range of buffers (e.g. Na_2HPO_4 and MOPS for intermediate metal ions, and sodium acetate, MES, PIPES for hard ions). Kinoshita-Kikula *et al*. (2006) were even able to both selectively bind and elute phosphoprotein at a constant pH of 7.5 from a zinc(II)-based resin (the *Phos-Tag* system), disassociating the bound material with Na_2HPO_4 and NaCl. At the same time reductions in pH have also been shown to allow at least partial elution of phosphopeptides (Thingolm and Jensen, 2009) and Imanishi *et al*. (2007) demonstrated elution in an acetonitrile (ACN)/phosphoric acid mobile phase, while Hart *et al*. (2002) were able to elute Fe^{3+} IMAC using 2', 5'-dihydroxybenzoic acid (DHB), which is now commonly utilised in conjunction with TiO_2 approaches, but which is of limited LC-MS/MS compatibility (Imanishi *et al*. 2007). Though Tris is commonly utilised as a mobile phase it should be avoided (despite many manufacturers recommendations), as weak co-ordination of the ions by the buffer results in decreased adsorbancy and even weak metal leakage from the column[3], though in the case of high affinity ligands low concentrations of Tris may reduce non-selective binding (Proteus, 2004; GE Healthcare, 2005). Similarly, chelators such as EDTA should be avoided for all stages except removal of metal ions from the column. For the isolation of phosphorylated proteins and peptides, pH extremes should also be avoided, as *O*-phosphorylations (especially pSer and pThr) are succeptible to β-elimination in basic conditions (pTyr residues show higher stability), while *N*- phosphorylations

[3] Though this is a greater issue with intermediate metal ions.

are labile at a low pH (as determined by ^{32}Pi loss) (Mecham and Olcott, 1949; Martensen, 1984; D'Ambrosio *et al*., 2006).

When samples are loaded onto the column, there are three possible outcomes: (1) the peptide of interest binds to and is retained by metal ion; (2) the peptide of interest is not retained by the metal ion; or (3) the peptide of interest co-ordinates and removes the metal ion from the resin (metal ion transfer). Obviously leakage of metal ions during column operation will seriously affect both column efficiency and capacity, and for this reason columns are typically underloaded with metal ion (often by ~20 %) or a second uncharged column is placed in-line with the first, leaving room for disassociated ions to rebind (Kaur-Atwal *et al*., 2008). Certain buffers may exacerbate metal ion leakage, especially at high concentrations, therefore Yruela *et al*. (2003) and Pingoud *et al*. (2005) recommend a maxiumum concentration of 50 mM MES in conjunction with IMAC. This said, IMAC adsorption matrices have excellent regenerative properties, and old ions are easily removed with strong chelators (e.g. EDTA), following which the column may be rapidly washed and recharged with fresh metal ions.

Previous work at NTU entailed the comparative use of Cu^{2+} IMAC as a prefractionation step before HPLC, where it allowed identification of low-abundance peptide peaks and the isolation and identification of His-containing peptides from tryptic digests of K562 cell line protein (Barry, 2006); as well as the use of on-line Cu^{2+} IMAC for decomplexing of samples and simplification of mass spectra during ESI-MS/MS. Preliminary investigations were also carried out into the use of Cu^{2+} IMAC for fractionation of MHC class-I presented peptides, using either the isotonic citrate-phosphate MHC elution buffer developed by Storkus *et al*. (1993) with IMAC following sample cleanup by C_{18}, or by Ga^{3+} IMAC following elution with the unsupplemented 25 mM TMA-formate pH 3.3 buffer.

A related technology; metal oxide affinity chromatography (MOAC) (Han *et al*., 2008a) is advocated by some as cheaper, more readily optimisable and with greater selectivity than IMAC (D'Ambrosio *et al*., 2006). The most common form utilises TiO_2 (occasionally referred to as 'titania') which had previously been utilised for binding of organic phosphates (Matsuda *et al*., 2000) and phospholipids (Ikeguchi and Nakamura, 2000), and like IMAC (often) involves binding at low pH and eluting with a intermediate-high pH buffer (e.g. Sano and Nakamura, 2004). This similarity between the two methods is all the more striking when it is considered that as well as zirconium, oxides of gallium and

aluminium have all also been utilised at some stage (Dunn *et al*., 2010), and that both appear to involve interaction between the metal ion and the oxygen or hydroxyls of the phosphate group (Leitner, 2010), along with electrostatic repulsion at the higher elution pH. Unlike IMAC however little data is available on comparable specificity between different ions (Leitner, 2010) and there are conflicting reports on the applicability of iron(III)-oxide for phosphopeptide enrichment (Han *et al*., 2007; Shan *et al*., 2008; Lee *et al.,* 2008). Again, non-specific binding is primarily due to interactions with acidic residues (Glu/Asp) though unlike IMAC, these can be reduced by inclusion of 2,5-dihydroxybenzoic acid (a potent chelator), or reducing the loading pH beyond that typically employed for IMAC (Pinkse *et al*., 2004), and the methodology is far more tolerant of detergents, higher buffer strengths, and salts (etc).

The majority of proteins contain a number of hydrophobic patches on their exterior (Hochuli, 1992), a feature which is exploited by reversed-phase (RP) approaches[4], the simplicity, broad binding capacity and strong reproducibility (Hengström and Irgum, 2006) of which have for several decades made them the most popular method for peptide and protein fractionation, either for sample cleanup/concentration or for analytical purposes along an increasing solvent gradient. Adjustment of loading, washing and elution conditions can allow selective isolation and/or elution of particular fractions, e.g. for removal of abundant serum proteins such as albumin (Freeman *et al*., 2006; Matharoo-Ball *et al*., 2007). These resins contain silica beads to which long hydrophobic alkyl hydrocarbon chains are bound, the resin name being dependent on the number of carbon molecules per chain: C_8 corresponding to an octyl-carbon chain, C_{12} to a duodecyl-carbon chain, etc. While C_4, C_{12} and C_{18} are the most commonly used resins, largely due to commercial availability, resins of up to C_{60} have been developed (Vallant *et al*., 2007).

Samples are typically loaded at an acidic pH (2 or below) to protonate the side chains, minimising overall charge, and bind by primarily hydrophobic interations with the resin. The column is then washed, and subjected to increasing solvent concentration(s), allowing either fractionation across a hydrophobic gradient (though charge and size also play a role), or concentration into a small volume by a rapid increase in solvent. Unlike hydrophobic interaction chromatography (an earlier method), the presence of salt is not required to encourage peptides to

[4] Derived from hydrophic interaction chromatography (HIC) methods first described by Porath *et al*. (1973) and Hjerten *et al*. (1974)

bind to the column; hence C_{18} is frequently used to desalt samples prior to MS. Furthermore, as identification of MHC peptides by LC-MS/MS is almost always performed using C_{18} columns they are also ideal for sample concentration and clean-up, as peptides which bind poorly to the SPE resins will typically also be poorly retained by the analytical column, leading to inferior spectra.

Though well investigated, the effect of phosphorylation on reversed-phase binding is complex; though in general the addition of an anionic phosphate group is believed to increase the polarity and the hydrophilicity of a peptide, data is at times contradictory (Steen *et al*., 2006; Kim *et al*., 2007). Ohguro and Palczewski (1995) found that when a variety of phosphopeptides were studied by RP chromatography, each eluted prior to, or with the nonphosphorylated forms, with differences of up to 20 minutes found between elution times (though this corresponded to an increase in solvent concentrations of only 4.22 % v/v), with the ion pairing agent heptafluorobutyric acid (HFB) found to provide the greatest difference in elution times. Matsumoto *et al*. (1997) on the other hand found no difference between phosphorylated and nonphosphorylated forms using either a C_4 or a POROS 20R2 reversed-phase resin (analogous to a C_8 silica resin) when acidified with either TFA or HFB, but found that on the 20R2 system increasing the pH to 11.5 (silica resins are not alkaline stable) phosphopeptides were resolvable from their naked counterparts and again were found to elute prior to the nonphosphorylated form (though the low alkaline stability of O-phosphorylation required samples to be neutralised with TFA as soon as they eluted from the column). However, Steen *et al*. (2006) found that each of the fifteen monophosphorylated peptides (varying from 7-17 amino acids in length and with a range of physical characteristics) tested on a C_{18} resin (acidified with formic acid) eluted after the nonphosphorylated form, and that two of the three doubly phosphorylated peptides eluted after both the singly or non-phosphorylated peptides (the third peptide eluted before either the non- or the mono-phosphorylated form), and postulated that peptides with basic amino acids might experience net reduction in charge from phosphorylation, increasing retention.

Matsumoto *et al*. (1997) also noted that as monophosphorylated peptides increase in size, the difference in retention times decreases; presumably due to a fall in the net effect of a single phosphate group. This was mirrored by Kim *et al*. (2007), but the opposite was found by Steen *et al*. (2006), who found that the smallest of their peptides demonstrated the greatest overlap between peaks.

Kim *et al*. (2007) studied 33 peptides from 4-35 amino acids in length with single, double and in one case quadruple phosphorylation, and found mixed results. In general phosphorylation was found to decrease retention on the RP column, however there were several exceptions, for which they implicated hydrogen bonding and stearic hindrances from adjascent residues. They also found that phosphorylation of Tyr lead to the greatest reduction in peptide retention time, which may be of special importance given the relatively low abundance of Tyr phosphorylation (Hunter, 1998).

The use of a porous graphitic carbon column to both desalt and separate hydrophilic phosphopeptides from their nonphosphorylated counterparts along an increasing solvent gradient as developed by Chin and Papac (1999) may be thought of as a loose analogue of reversed-phase approaches, though it likely also involved additional interactions, as peptides which normally bind poorly to C_{18} may be retained. Vallant *et al*. (2007) compare their C_{60} reversed-phase resin favourably with the porous graphite approach, though there are concerns about column reusability (Larsen *et al*., 2002). The method has also been used (with MALDI-MS) by Vascratis *et al*. (2002) and Larsen *et al*., (2002; 2004).

Hydrophilic Interaction Liquid Chromatography (HiLIC) (sometimes termed Hydrophilic Interaction Chromatography), is a normal-phase approach often considered as an orthoganal counterpart to the reversed-phase resins such as C_{18}. Unlike reversed-phase, HiLIC requires samples be loaded onto a polar stationary phase in non-polar conditions, and eluted along an increasingly polar gradient; and as would be expected from such an approach, much greater retention and fractionation of polar molecules are possible (Bai and Wang, 2009). Though methods employing a polar stationary phase, including HiLIC have been in use since the 1970's, these were primarily applied to small molecules, metabolites and carbohydrates (Yoshida, 2004); at the time normal phase approaches to protein biochemistry were limited by poor reproducibility when compared to reversed-phase (Hengström and Irgum, 2006) - HiLIC as a method for peptide fractionation was therefore not introduced until 1990 by Alpert (1990) who was able to fractionate proteins, peptides, amino acids, oligonucleotides, and carbohydrates.

As well as direct hydrogen bonding and ionic interactions between the analyte and the resin (Bai and Wang, 2009), the mechanism depends on partitioning of sample into the stationary aqueous layer associated with the hydrophilic

(typically silanol) groups present within the resin (Yoshida, 2004) (as suspected by Alpert, 1990), and must therefore not be confused with other normal phase approaches, which may allow samples to be loaded in the absence of H_2O.[5] As well as resin and sample composition, binding efficiency is known to be affected by salt and solvent concentrations, and by pH and ionicity. The potential for HiLIC for the study of phosphorylation was noted by Alpert (1990), and Linder *et al*. (1997) were able to isolate phosphorylated histones from the Raji cell line. The use of HiLIC for phosphopeptides did not gather pace until 2008, when Albuquerque *et al*. (2008) and McNulty and Annan (2008) both used it as part of a multidimensional phosphopeptide enrichment strategy. The latter later found that prefractionation of samples with HiLIC (as opposed to the post IMAC methodology they previously employed) improved phosphopeptide selectivity of IMAC to >95 % (McNulty and Annan, 2009).

Alternate technologies for phosphopeptide enrichment often involve chemical derivatization (Patton, 2002): examples include binding phosphopeptides to thiol-containing compounds (subsequent to amino-group tert-butyloxycarbonyl modification and amidation of the carboxylate-groups), which were then covalently bound to glass beads and released through an acid wash (Zhou *et al*., 2001); or β-elimination of phosphate groups under alkali conditions, followed by the Michael addition of ethanedithiol and subsequent tagging of the thiol group with biotin (via the resulting sulphydryl group), which may then be isolated via a captavidin column (Oda *et al*., 2001, as described in Araces *et al*., 2004). Knight *et al*. (2003) were able to convert pSer/pThr to lysine analogues, and then identify these sites by differential proteolytic cleavage (though this method is far less applicable to MHC eluates). These approaches also circumvent the issue of Ser/Thr phosphate-loss during mass-spectrometric fragmentation (Wolshin & Weckwerth, 2005), however, they are also limited to phosphorylation of these residues, and therefore overlook pTyr events (Bai and Wang, 2009).

Similarly, chemical modification of samples prior to IMAC or TiO_2 chromatography can be used to reduce non-specific binding (typically by Asp and Glu), and such methods typically improve the quality of the resultant mass-spectra (He *et al*., 2004; Simon *et al*., 2008). However, they can also significantly complicate peptide sequencing and render unbound fractions unusable, as incomplete/partial sample modification, combined with secondary or non-specific reactions (e.g.: esterification of arginine; β-elimination of O-

[5] Depending on sample solubility in nonaqueous conditions.

linked glycosylations producing intermediates with similar molecular weights to a phosphate group) can produce numerous molecular weights from a single peptide, greatly increasing mass spectrometric analysis time, and potentially reducing concentrations of many modified peptide isoforms below the levels required for confident identification (Ficarro *et al*., 2002; McLachlin and Chait, 2003; Karty and Reilly, 2005; D'Ambrosio *et al*., 2006). Furthermore the chromatography may be complicated by increases in sample volume or the presence of low-compatibility reagents during modification. A third alternative, ^{32}P-labelling followed by Edman-sequencing (De Carvalho *et al*., 1996), while highly accurate carries both the complications associated with beta-emitter isotope work (e.g. inapplicability to patient samples) and long analysis times/low throughput (Bai and Wang, 2009). Additional chromatographic methods for isolation of phosphopeptides include thin-layer chromatography as already mentioned (Martensen, 1984), and more recently the use of soluble nanopolymers which may be used in conjunction with or distinct from TiO_2 (Iliuk and Tao, 2009)

A single pSer peptide was found by Hogan *et al*. (1998) in an eluate from immunoaffinity-purified MHC class-I molecules from the VBT2 carcinoma cell line, following fractionation on HPLC (without any phosphopeptide-enrichment stage), noting at the time the potential phosphorylation may have to influence the $CD8^+$ T cell response. Since then investigations into MHC-presented phosphopeptides have primarily been carried out by Zarling *et al*., (2000; 2006; Mohammed *et al*., 2008; Depontieu *et al*., 2009) using Fe^{3+} IMAC of peptides eluted from immunopurified MHC molecules. The IMAC protocol utilised however varies notably, with the initial approach using a 100 mM NaCl in 25 % ACN, 1 % (v/v) acetic acid wash to minimise non-specific binding, and the column eluted with Na_2HPO_4 (pH 9.0); and later using Ficarro *et al.* (2003)'s methylation protocol (Zarling *et al*., 2006; Mohammed *et al*., 2008). In both cases they use POROS 20MC resin and peptides were sequenced using LC-ESI-MS/MS. Meyer *et al*. (2009) on the other hand used a similar MHC class-I and -II isolation procedure, with the exception that phosphopeptide enrichment took place using TiO_2, and peptides were characterised by LC-ESI Q-TOF.

The MHC class-I / class-II peptides acquired by mild acid elution are contained in a semi-volatile low-pH matrix (TMA-formate or sodium-formate, plus salts, sugars and other supplements) that is not directly compatible with mass spectrometry or quantification of peptide content by peptide BCA. Furthermore

the peptides are highly heterogeneous, with the phosphorylated component expected to make up the minority of the sample. This chapter primarily deals with improving the use of IMAC and reversed-phase approaches to retain and concentrate model phosphopeptides, and to elute them in a matrix-compatible with quantification, secondary chromatographic fractionation, and identification by mass spectrometry. There is also some investigation into the use of HiLIC for phosphopeptide selection/retention, and ion exchange chromatography as an alternative to reversed-phase. A descriptive flowchart (Figure 5.1) below illustrates the main concerns at each stage.

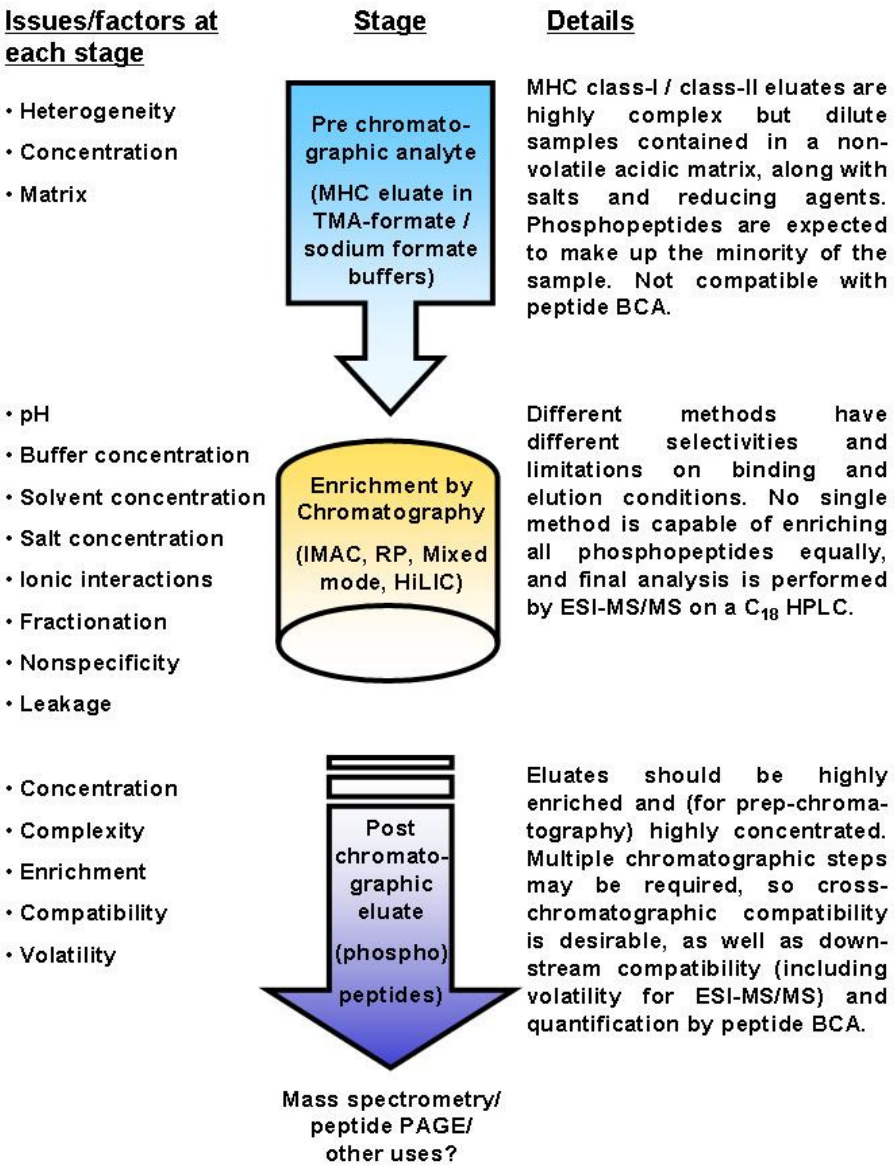


Figure 5.1: Major concerns at each stage of the chromatographic process. Low concentration and high complexity of the MHC peptides and poor compatibility may be rectified by chromatographic concentration and enrichment, though this may be affected by a range of factors. Eluates should be quantifiable and compatible with all downstream approaches.

5.2 Use of IMAC for fractionation/selection of peptide subpopulations

By nature the peptide populations derived from MHC eluates are highly heterogenous, and unlike protein digests, rarely contain two or more abundant peptides from the same protein. In order to reduce background noise and improve identification of peptides, decomplexing of samples by chromatographic fractionation is highly desirable. While the use of LC-MS/MS includes separation of peptides along a hydrophobic gradient, prefractionation by a second dimension typically reduce sample complexity dramatically and may lead to identification of lower abundance peaks than the original unfractionated sample might allow. As deregulation of kinase activity has a strong link with malignancy (Capra et al., 2006) and MHC-presented phosphopeptides are known to modulate the immune response (Zarling, 2006), identification of MHC phosphopeptides may be key to novel immunotherapeutic vaccines. It was therefore sought to improve the use of IMAC as a preparative-chromatographic stage for MHC phosphopeptides prior to MS analysis.

5.2.1 Isolation of phosphopeptides using Fe^{3+} IMAC – proof of principle

To confirm the potential for IMAC to isolate phosphorylated peptides from a heterogeneous mix, a casein digest was subjected to the Fe^{3+} IMAC protocol previously utilised at NTU[6]. The peak unbound and eluted fractions were then quantified by peptide BCA (Kapoor e*t al*., 2009) as per chapter 3, analysed by peptide-PAGE along with unfractionated casein and BSA digests, and stained with the Pro-Q diamond phosphoprotein stain (see figure 5.2). This confirmed that IMAC was capable of producing a heavily phosphopeptide-enriched fraction, and that the unbound material was depleted of casein phosphopeptides to a level below the detection limits of the stain). This enrichment was significant when compared with an un-enriched casein digest which was loaded at double the concentration of the IMAC material, while as expected a BSA digest failed to show any staining. Compatibility of the DEA-HCl buffer with peptide PAGE was also demonstrated.

[6] Loaded in 25 mM TMA-formate pH 3.3, washed with five column volumes, and eluted with five column volumes 0.1 M DEA-HCl pH 8.4.

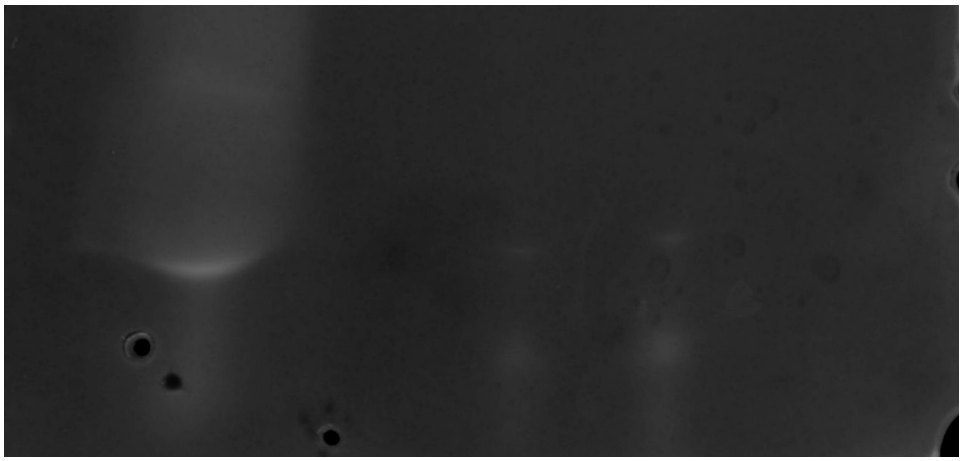


Figure 5.2: Peptide PAGE of BSA and casein digests (+/- IMAC) stained with Pro-Q Diamond phosphoprotein stain. Lanes are as follows: **A** – casein digest subjected to Fe^{3+} IMAC, peak fraction from eluate (~ 6 µg); **B** - casein digest subjected to Fe^{3+} IMAC, peak unbound fraction (~ 6 µg); **C** – casein digest (~ 6 µg); **D** – casein digest (~ 12 µg); **E** – BSA digest (~ 12 µg). Digestion was performed as per 2.3.2.2, IMAC as per 2.5.1.1, peptide quantification as per 2.3.4, and peptide PAGE and staining as per 2.5.5.

When the peak IMAC eluate fraction was subjected to LC-MS/MS (as per 2.6.2) analysis by MASCOT indicated that 9 of the 12 casein peptides confidently identified were phosphorylated (data not shown).

5.2.2 Modification of Fe^{3+} IMAC protocol to increase peak fraction concentration

While analytical chromatography usually depends on fractionation of a sample across a steady gradient, the generation of numerous samples from each chromatographic step complicates analysis by RP-mass spectrometry, dramatically increasing analysis time. Therefore, using IMAC as a prep-chromatograpic method, the bound material should be concentrated into as small a sample volume as feasible. This also allows increased dilution of the sample prior to analysis, reducing background noise and any buffer compatibility issues.

An increase in elution buffer pH from 8.4 to 9 was investigated with the aim of increasing the pH gradient across the five column volumes used to elute bound material (in this case a tryptic digest of casein) from the column. This led to an increase in peak fraction concentration from ~ 43.1 (S.D ± 2.61) % to 59.89 %

(S.D ± 3.46) of total eluted material, as well as altering the shape of the elution profile, bringing the peak forward to the second fraction (see figure 5.3).

The effect of reducing the wash buffer molarity was also tested, under the hypothesis that a lower molarity would present lower buffering capacity when confronted with the pH 9 elution stage. The addition of a second wash stage of 5 column volumes 5 mM TMA-formate pH 3.3 (following the 25 mM wash) was found to increase peak fraction content even further, to 74.36 % (S.D ± 3.17) total eluted material (see figure 5.3), without any sign of protein or metal ion leakage during the additional stage (data not shown).

Following these alterations to the protocol, the effect of dropping the elution buffer molarity from 100 mM to 50 mM DEA-HCl (pH 9.0) was also tested, theoretically doubling the compatibility of the buffer and halving any dilutions required for downstream analysis. This was achievable with a mild-trade-off in peak fraction concentration, which dropped to 67.85 % (S.D ± 1.6) total eluted material.

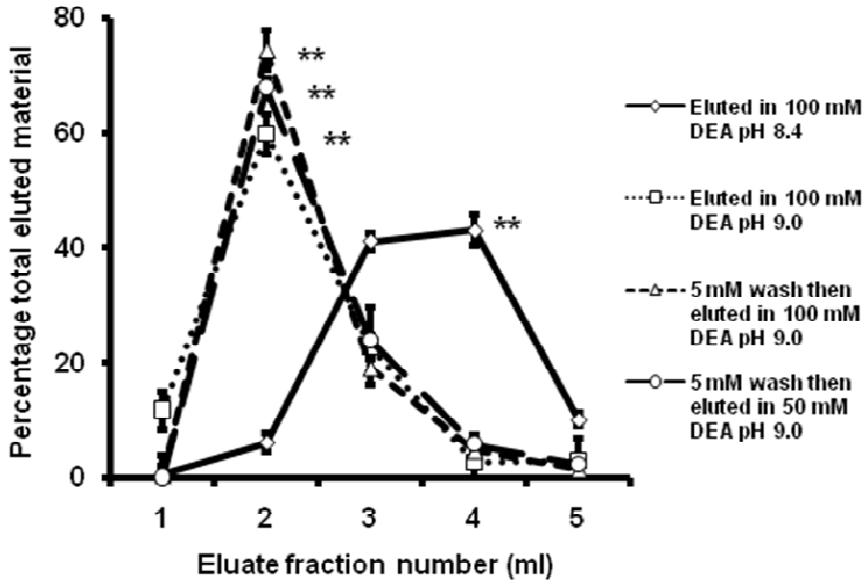


Figure 5.3: Typical peptide BCA-determined elution profiles (1 m / fraction) for casein digests subjected to Fe^{3+} IMAC. Samples were loaded in 25 mM TMA-formate pH 3.3, washed with at least 5 column volumes of TMA buffer (+/- low molarity 5 mM TMA wash stage), and eluted with DEA 50/100 mM pH 8.4/ 9.0. Each alteration to the method led to a change in peak fraction concentration (significant to a *P* value < 0.01, *n* = 4). Error bars indicate SD.

The use of a second charged Fe^{3+} IMAC column, in line with the first was also investigated. By providing an increase in bed volume, a greater charged surface is available to interact with the phosphopeptides, increasing the total eluted material from 7.18 mg (S.D ± 0.22) to 10.97 mg (S.D ± 0.528) (see figure 5.4.A). However, the peak fractions only rose from 4.54 mg/ml (S.D ± 0.2) to 5.0143 mg/ml (S.D ± 0.16), which represented a drop in percentage bound material per peak fraction from 66.79 % (S.D ± 2.92) to 45.73 % (S.D ± 1.48) (see figure 5.4.B), which may reduce the potential for a single fraction to be representative of the total bound material, a consequence of the broader peaks produced by two columns in tandem. This may also be in part an artefact from the points at which fractions were collected, the peak fraction may be divided between fractions 5 and 6, and may possibly be remedied by staggering fraction collection by 0.5 ml. In either case however the use of a second column required slower flow rates (from 0.5 to 0.2 ml/min) to mitigate for increased back-pressure and leakage of mobile phase, leading to longer run-times, and on occasion appeared to exhibit greater metal ion loss.

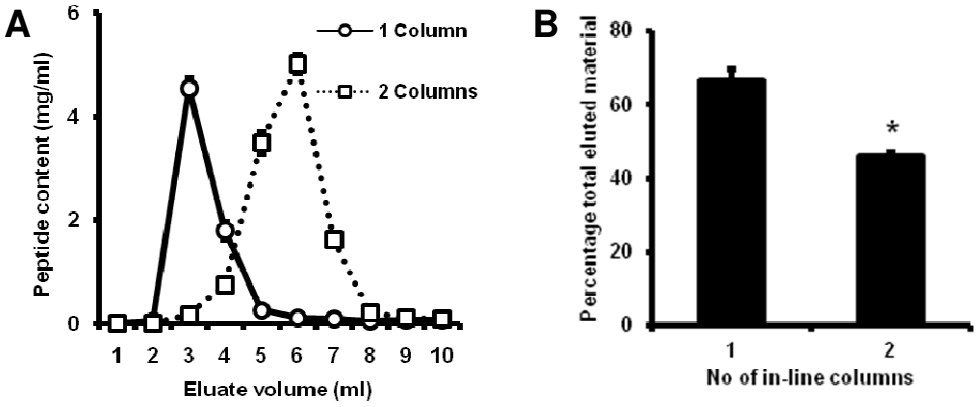


Figure 5.4: (**A**) Peptide BCA-determined elution profiles for casein digests subjected to Fe^{3+} IMAC on a single or dual column set-up. Samples were loaded in 25 mM TMA-formate pH 3.3, washed with 5x 2-column volumes of 25 mM then 5 mM TMA-formate pH 3.3, then eluted with 10 ml 50mM DEA pH 9.0. While the dual column approach nearly doubled the amount material bound and eluted, peak fraction concentration only increased by ~ 10 % (significant to a *P* value of < 0.05), which when expressed as a percentage total eluted material (**B**) indicated a drop from 66.79 % (S.D ± 2.92) to 45.73 % (S.D ± 1.48), significant to $P < 0.05$, $n = 4$. Error bars indicate SD.

5.2.3 Modification of Cu^{2+} IMAC methodology

The applicability of these modifications to the Cu^{2+} IMAC protocol were also investigated using a BSA digest, with the addition of a 5 mM HEPES wash stage found to increase peak fraction concentration from 37.12 % (S.D ± 1.82) to 59.11 % (S.D ± 1.18). In this case however increasing the pH gradient (normally 0.1 % v/v TFA) was incompatible with reducing concentration of the solution; however increasing TFA to 0.5 % (v/v) led to an increase in peak fraction concentration to 72.64 % (S.D ± 5.4) of the total eluted material (see figure 5.5).

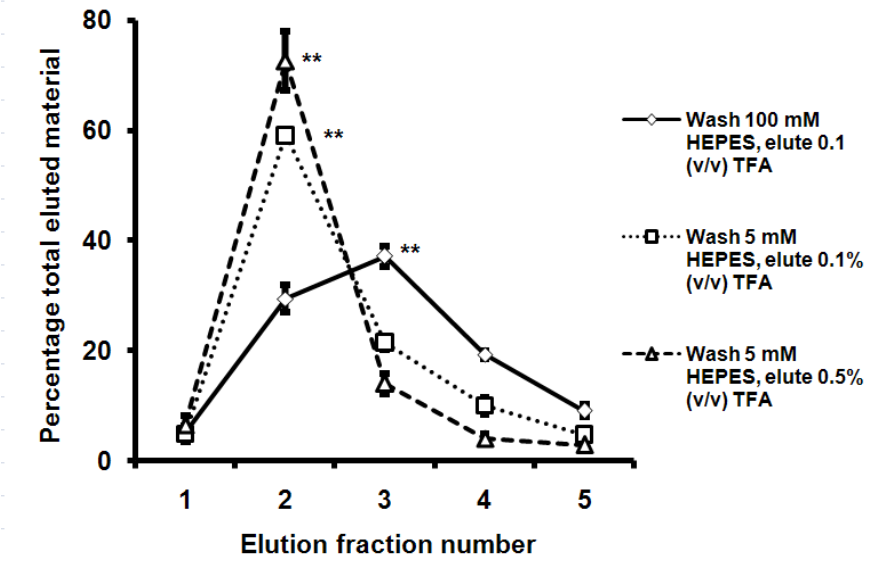


Figure 5.5: Peptide BCA-determined elution profiles for BSA digests subjected to Cu^{2+} IMAC by the standardised method, or by modifications similar to those previously utilised for Fe^{3+} IMAC. Samples were loaded and washed with 100 mM HEPES-NaOH pH 7.2, with or without a second low-molarity wash stage (5 mM HEPES-NaOH), prior to bound material being eluted with 0.1 or 0.5 % (v/v) TFA in 1 ml fractions. Again each alteration to the method produced a statistically significant (to P <0.01 , n = 4) increase in peak fraction concentration. Error bars indicate SD.

5.3 Alternative chromatographic methods

The use of alternative SPE-chromatographic resins for sample cleanup and/or selective phosphopeptide capture was also investigated. Again, as these were to be used primarily for sample clean-up/concentration, the emphasis was on peak sharpness and recovery of sample in as small a volume as feasible.

5.3.1 Reversed-phase SPE resins

Whilst the fractionation of phosphopeptides from their unphosphorylated counterparts has been extensively documented using reversed-phased columns such as C_{18}, there is little data available regarding increasing phosphopeptide binding to reversed-phase resins. The choice of resin and modification of loading conditions was therefore investigated to maximise phosphopeptide recovery, with the aim of coupling this method with IMAC.

5.3.1.1 Choice of optimum reversed-phase resin

Three different reversed-phase resins: Bond-elut C_{18} (Varian), Strata C_{18} and Strata C_8 (Phenomenex) were tested for their potential to capture and retain two phosphorylated peptides with differing hydrophilic characteristics, SQK(p), HPS = 1.1; and YIS(p), HPS = -0.5. Peptides (0.2 mg) were loaded onto a column containing 30 mg resin in 0.1 % (v/v) TFA, unbound material washed away, and the peptides eluted in 1.5 ml volumes. Eluates were then spiked with 20 µl DMSO[7] and concentrated by rotary evaporation. Once significantly reduced in volume they were brought back to 200 µl with ddH_2O and the peptide content assessed by peptide BCA as per (as per chapter 3) quantified against standards subjected to the same rotary evaporative process (without the addition of DMSO) (see figure 5.6). While recovery of YIS(p) was consistent at ~ 22 % (S.D ± <1.21) between the resins, SQK(p) recovery dropped from ~25 (S.D ± <1.26) % binding to the C_{18} resins to 16.6 % (S.D ± <0.75) binding to Strata C_8. It was therefore decided to utilise a C_{18} SPE resin for any sample cleanup, and the Varian Bond-elut C_{18} cartridges were chosen for reasons of availability.

[7] Chosen for it's low volatility and preference as a solvent for dissolving peptides, as well as it's presence in the non-chromatographic standards.

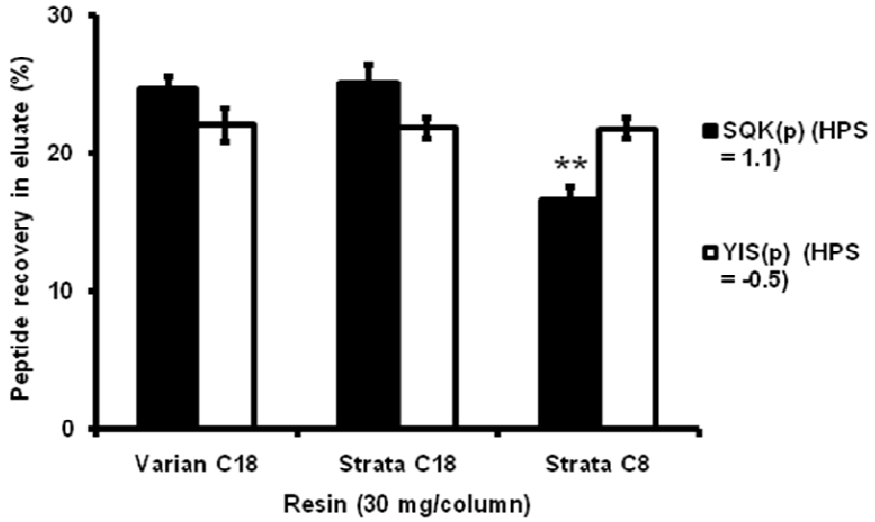


Figure 5.6: BCA-determined recovery of two peptides in eluates from three reversed-phase resins following concentration by rotary evaporation. 0.2 mg of each peptide was loaded in 0.1 % (v/v) TFA and eluted in 1 ml 80 % (v/v) ACN before being spiked with DMSO and further concentrated by rotary evaporation. While binding of the YIS(p) peptide remained constant, the C_8 showed poor recovery of the hydrophilic SQK(p) peptide. ** Indicates statistical significance to $P < 0.01$ when compared with other resins as determined by ANOVA ($n = 5$). Error bars indicate SD.

Given the hydrophilic score of SQK(p), the higher recovery when compared with YIS(p) was surprising. While the lower BCA reactivity of the YIS(p) peptide may play a role in this result, or potentially higher volatility/adsorption of the hydrophobic peptide, the use of peptide standards not subjected to reversed-phase chromatography makes this unlikely. As stated, the presence of a phosphate group is generally believed to alter the interaction between the peptide and the resin. However, in this case there does not seem to be a distinction between serine or tyrosine phosphorylation, as the 25.2 % of the ALR(p) peptide (HPS = 0.3) was recovered from an eluate of a Varian Bond-Elut SPE column when subjected to the same procedure (see Figure 5.7, column 1). If one is to follow the data of Steen *et al*. (2006), it is conceivable that the phosphate group aids retention of a hydrophilic peptide, and that the effects balance out in the loss in binding that would normally be seen by such a hydrophilic peptide, but further data would be required before this could be proven. As each peptide was supplied at > 80 % purity, it also is possible that retention and quantification of mis-synthesised sequences may also have had an effect. These would be expected to be shorter than the sequences stated, and might be expected to present lower BCA reactivity unless they also possessed fewer hydrophobic residues.

5.3.1.2 Sample acidification with TFA, HCl or Phosphoric acid

In order to determine the optimum acidification protocol for maximum retention of phosphopeptides on the Varian Bond-Elut C_{18} columns, three different acids were tested at different concentrations (see figure 5.7). It was found that neither HCl nor phosphoric acid were a suitable alternative for TFA. However increasing the TFA concentration from 0.1 to 0.5 % (v/v) lead to an increase in peak fraction concentration to approximately 33 %, statistically higher (to P <0.01) than any other protocol investigated (with the exception of 1 % (v/v) TFA, which was statistically indistinct from both 0.5 and 0.1 % TFA).

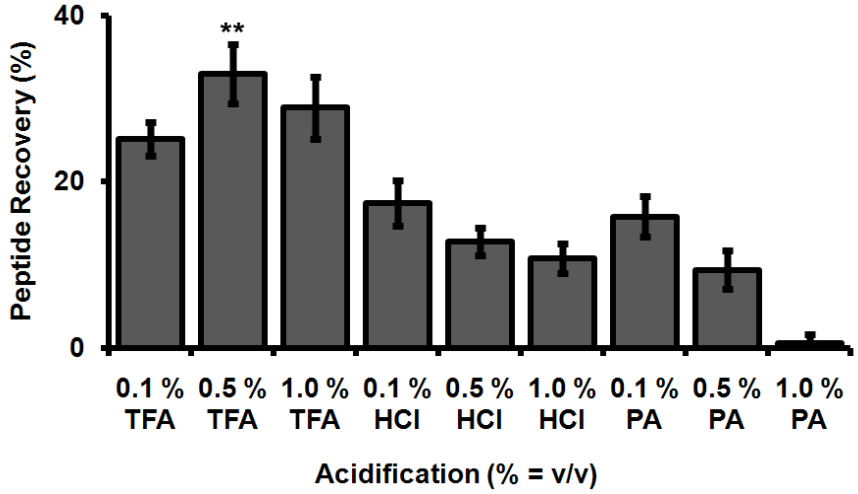


Figure 5.7: The effect of three different acids at different concentrations on BCA-determined recovery of synthetic peptide ALR(p) in eluates from Varian Bond-Elut reversed-phase SPE columns. 0.2 mg peptide was loaded in trifluoroacetic (TFA), hydrochloric (HCl) or phosphoric acid (PA) (all % v/v), and washed with the same before elution in 1 ml 80 % (v/v) ACN and 0.5 ml 100 % (v/v) ACN, before being spiked with DMSO and concentrated by rotary evaporation. Acidifying with 0.5 % TFA allowed the greatest recovery, and was statistically distinct (to $P < 0.01$, $n = 5$) from all other protocols bar 1 % (v/v) TFA when assessed by ANOVA. Error bars indicate SD.

5.3.1.3 Inclusion of KCl

In order to determine whether the inclusion of neutral salts might affect recovery from the C_{18} SPE columns, KCl was tested at various concentrations, on the basis that it was the primary supplement in the minimal-lysis MHC class-I and class-II

elution buffers. Tryptic digests of casein were loaded in 0.5 % (v/v) TFA containing 0.0, 0.1, 0.2, or 0.5 M KCl. The columns were then washed with the same, and then again with 0.5 % (v/v) TFA (no salt), before being eluted in 1 ml 80 % (v/v), and then 0.5 ml 100 % ACN. Fractions were then assayed by peptide BCA (as per chapter 3) with appropriate standards. While the inclusion of 0.1 and 0.2 M KCl failed to produce a statistically significant change in peptides recovered, 0.5 M lead to a decrease in peptide recovery from 42.1 (S.D ± <2.8) to 36.4 (S.D ± <2.4). In no case was bound material found to elute in the second (salt-free) wash (data not shown).

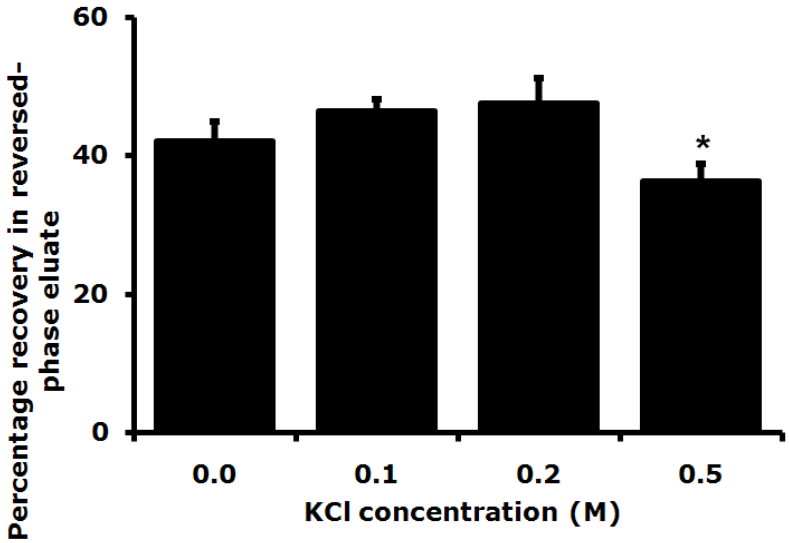


Figure 5.8: The effect of the inclusion of salts in the loading phase on BCA-determined recovery of a casein digest in eluates from Varian Bond-Elut C_{18} SPE columns. 0.3 mg of digest was loaded in 0.5 % (v/v) TFA containing varying concentrations of KCl and, washed with 1 ml 0.5 % TFA (no KCl) and eluted in 1 ml 80 % and 0.5 ml 100 (v/v) ACN. Eluates were then spiked with 0.2 ml DMSO and subjected to rotary evaporation prior to quantification by peptide BCA. With the exception of 0.5 M, the inclusion of KCl was found to have no statistically significant effect (to a *P* value of <0.05, *n* = 4). Error bars indicate SD.

5.3.2 Mixed mode SPE resins

Two recently-developed SPE resins from Phenomenex were also tested for their ability to bind phosphopeptides. Strata X, a mixed mode resin with both hydrophilic and hydrophobic binding properties; and Strata X-AW, a weak anion-exchange resin with reversed-phase properties (methodologies based on personal communication between P.L.R. Bonner and Phenomenex). Again the

phosphopeptides SQK(p) and YIS(p) were utilised to assess the resins' abilities to retain dissimilar phosphopeptides. The results (see figure 5.9) indicate that the two resins show divergent selectivity - the Strata X showed a high selectivity for the YIS(p) peptide, 30.33 % recovery (S.D ± <4.9) (total recovery 31.769 %); while the peak fraction recovery of the SQK(p) peptide was below 10.29 % (S.D ± <0.6) (total recovery 18.84 %).

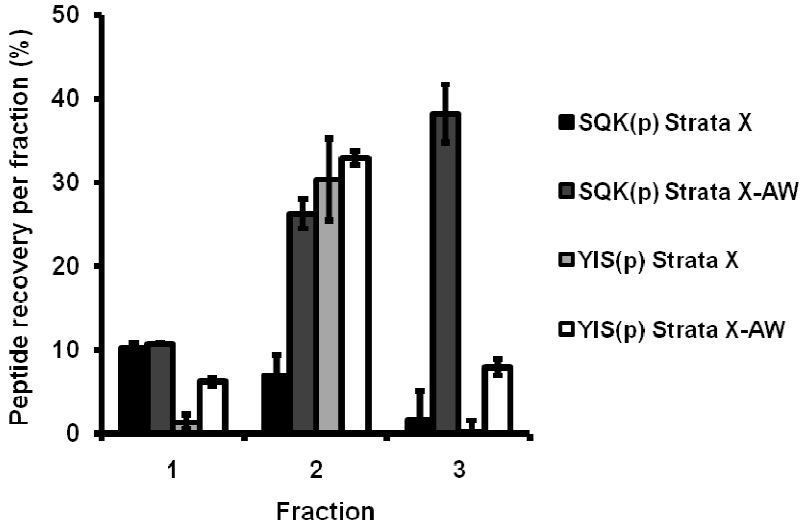


Figure 5.9: Recovery of two synthetic peptides in the three wash and elution stages of Strata X and X-AW. While the Strata X resin appeared to have little affinity for the hydrophilic SQK(p) phosphopeptide, YIS(p) was retained and selectively eluted in the second fraction (50 % (v/v) MeOH in ddH_2O). Strata X-AW showed a more even selectivity for both peptides.

Strata X: samples were loaded in 2 % (v/v) phosphoric acid and washed with 1 ml of the same (fraction 1) before being eluted with 1 ml 25 % (fraction 2) and 50 % (fraction 3) (v/v) MeOH in ddH_2O. Strata X-AW: samples were also loaded in 2 % (v/v) phosphoric acid. They were then washed with 1 ml 20 mM Na acetate pH 5 (fraction 1), followed with 1 ml MeOH (fraction 2), and finally with 1 ml 2 % (w/v) ammonium bicarbonate, 0.5 % (v/v) TFA in MeOH (fraction 3).

The Strata X-AW resin on the other hand showed some selectivity for both peptides, with greater amounts of the SQK(p) peptide eluting in each fraction, peaking at 38.25 (S.D ± <1.8) in the third (ammonium bicarbonate and TFA in MeOH) wash (total recovery 75.25 %), while the YIS(p) peptide eluted primarily in the second wash (MeOH only) at 32.9 % (S.D ± <0.9). This resulted in a higher total recovery (46.97 %), and though the multiple elution points are

unexpected for a single peptide, it should be noted that SQK(p) also eluted in several fractions when applied to the Strata X resin, and 6.13 and 7.93 % of YIS(p) was found in the first and third elution conditions. As this chromatography was performed by hand, it is conceivable that these variations might stem to some extent from changes in flow-rate or pressure, but it was decided not to pursue this approach.

5.3.3 Hydrophilic Ligand Interaction Chromatography (HiLIC) resins

Though in the simplest terms HiLIC resins may be considered normal-phase counterparts to the reversed-phase resins such as C_{18}, their mechanisms of action are still somewhat unresolved, and along with hydrophilic partitioning of sample from the mobile to the stationary phase, the approach is also known to overlap to some extent with reversed-phase and ion-exchange chromatography, with interaction between resin and sample usually being a combination of hydrogen bonding, dipolar interactions and electrostatic effects, as most resins exhibit a charge within some regions of the pH scale (Sequant, 2009). The use of HiLIC to isolate phosphopeptides, either alone or in conjunction with IMAC/TiO_2 is a recently developing field, and no work into it's applicability to MHC peptide fractionation exists in the literature.

Two resins from Pall Biosciences: HEA (hexylamine) HyperCel and PPA (phenylpropylamine) HyperCel, were assessed, each is normally utilised for industrial feed stock preparation. Both are mixed-mode resins with a combination of both HiLIC and hydrophobic-interaction chromatographic (HIC) properties, though lacking the latter's need for high salt concentrations. The Strata-NH_2 HiLIC resin (Phenomenex) was also investigated.

5.3.3.1 Assesment of various HiLIC resins for retention of a standard phosphopeptide

In order to determine which of the three HiLIC resins (HEA HyperCel, PPA HyperCel and Strata-NH_2) tested demonstrated the best potential for phosphopeptide retention, the ALR(p) peptide (HPS = 0.3) was employed as a standard; 0.2 mg peptide was loaded in 2 ml 90 % (v/v) ACN and the HiLIC columns (HyperCel columns constructed in-house using spent Varian Bond-Elut C_{18} cartridges, Strata-NH_2 cartridge as supplied) washed/eluted with a stepwise gradient from 90 to 75, 50, 25 and 0 % ACN. While neither the PPA-HyperCel nor the Strata-NH_2 resins retained a high proportion of the peptide, the HEA resin bound and eluted up to 53 % of the peptide in the 90 % ACN wash phase (see Figure 5.10). As the loading phase contained 0.1 mg/ml peptide which was derived from a 10 mg/ml stock in DMSO, it was speculated that the loss of DMSO from the mobile phase may have led to elution of the peptide.

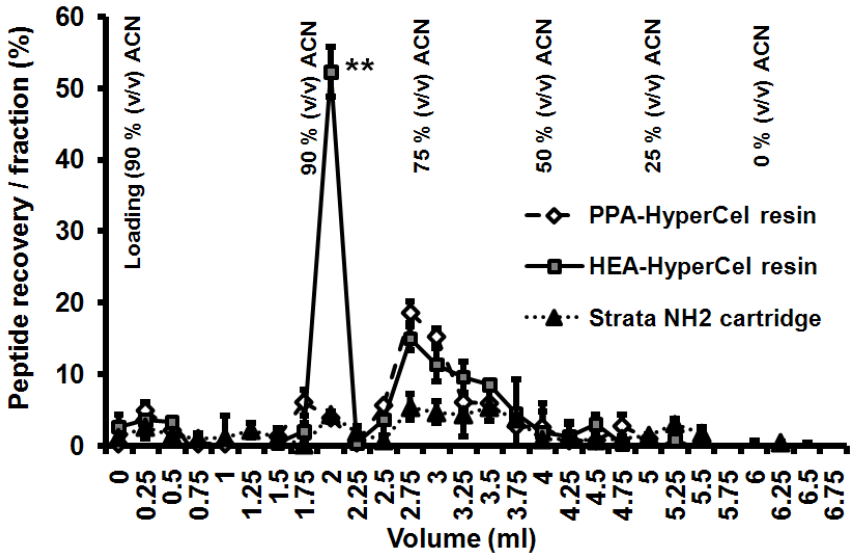


Figure 5.10: Typical peptide BCA-determined elution profiles of synthetic phosphopeptide ALR(pS)NFERI in fractions obtained from three HiLIC resins: HEA-HyperCel and PPA-HyperCel (both Pal Biosciences), and Strata NH2 (Phenomenex) in SPE column setups. 0.2 mg peptide was loaded in a 2 ml volume of 90 % (v/v) ACN, and the columns washed and eluted with a stepwise gradient of 90; 75; 50; 25; and 0 % (v/v) ACN, with fractions of 6-drops collected (= ~0.3 - 0.4 ml). Fractions were vortexed mixed, diluted 1:5 with ddH_2O and the diluted samples assayed by peptide BCA (as per section 3.1.4). The HEA appeared to retain and elute up to 53 % (to $P < 0.01$, $n = 3$) of the phosphopeptide during the 90 % ACN wash (which unlike the loading phase lacked 1 % (v/v) DMSO), while both HyperCel resins showed smaller comparable peaks at 75 % (v/v) ACN. Error bars indicate SD.

5.3.3.2 Effect of DMSO on retention of peptides

In order to determine the effect DMSO might have on ligand-column interaction, as well as determine whether other peptides would also elute at the same point on the solvent gradient, a tryptic digest of casein was prepared in 90 % (v/v) ACN. Precipitated material was removed by centrifugation at 20,800 r.c.f. for fifteen minutes and the supernatant applied to a HEA-HyperCel SPE cartridge and washed/eluted along the same stepwise gradient in the presence or absence of 1 % (v/v) DMSO in the mobile phase (see figure 5.11). The presence of DMSO was found to exert an increase in peak area, and at the lower end of the ACN:H_2O gradient to produce peaks not found within the DMSO-free chromatographic runs. As casein is primarily serine phosphorylated (as is ALR(p)) phosphorylation of different amino acids (threonine/tyrosine) is unlikely to be responsible for the multiple elution peaks, though it could be hypothesised that the multiple phosphorylation sites within casein (which upon tryptic digestion usually lead to multiply-phosphorylated peptides) might be responsible for the multiple elution peaks. However, the binding of casein phosphopeptides to the column remained unproven.

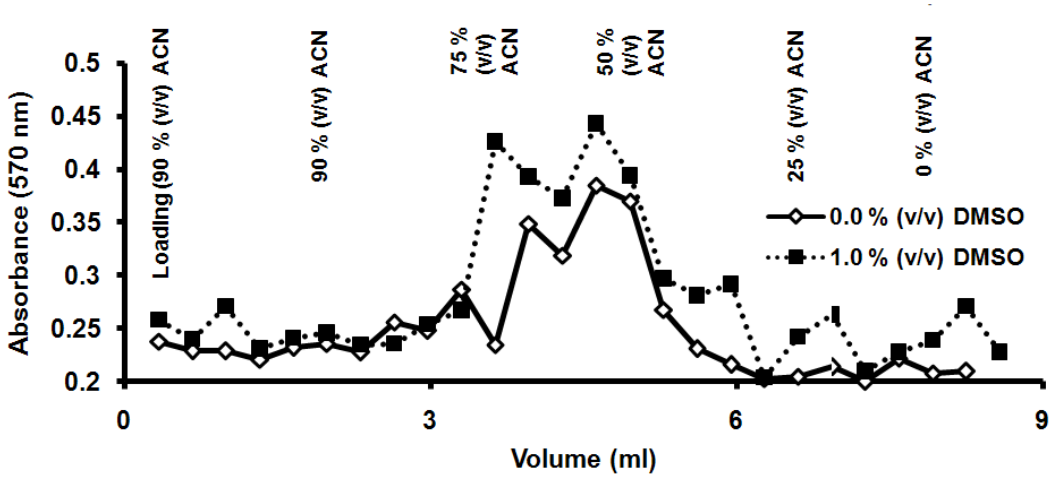


Figure 5.11: Typical peptide BCA-determined elution profiles of casein digests following SPE-fractionation with the HEA-HyperCel HiLIC resin with or without the inclusion of DMSO in the mobile phase (excluding H_2O). 5 mg tryptic digest was prepared in 2 ml 90 % (v/v) ACN, and (following removal of precipitate by centrifugation) the supernatant applied to the column. The columns were washed and eluted with a stepwise gradient and the 330 µl fractions collected and assayed by peptide BCA (see figure 3.3.9). Multiple peaks were observed, and the inclusion of DMSO was found to increase the amount of bound material and create additional peaks in the 25 % (v/v) ACN (1 % DMSO) and H_2O-only (DMSO-free) final phase.

Following on from this a 1 ml column was packed with HEA Hypercel resin and attempts made to replicate this data using a HPLC setup, with the aim of confirming the presence of phosphorylated material by peptide-PAGE and Pro-Q Diamond staining. However issues with pressure fluctuation (possibly linked to the precipitation observed at 90 % (v/v) ACN and highly exacerbated in the presence of DMSO) proved impossible to resolve (data not shown). Given this, and the relative incompatibility of DMSO with ESI-MS/MS, it was also decided to shift focus away from using DMSO to alter retention on the column.

5.3.3.3 Binding studies of undigested casein and BSA protein to HEA HyperCel HiLIC.

In order to simplify the analysis and compare phosphorylated with unphosphorylated material, undigested BSA and casein were prepared in 70 %; 50 % and 25 % (v/v) ACN[8] and applied to a 1 ml HEA HyperCel column with a similar stepwise gradient as per 5.3.1 with the addition of a 0.1 % (v/v) TFA wash following 0 % ACN (see Figure 5.12). It is notable that as the concentration of solvent in the loading conditions decreases, selectivity for casein also falls, while selectivity for BSA rises. At 50 % (v/v) ACN a double peak is observed for BSA which may correspond to (e.g.) the monomeric and dimeric isomers of the protein or differential sulphydryl content (Janatova *et al*., 1968). However the most significant result is that without the TFA wash the majority of sample does not disassociate from the column, though it is unclear whether this effect is due to the acidic conditions or whether the ion-pairing effect TFA is commonly employed for may be facilitating disassociation.

5.3.3.4 Potential to retain a Post-IMAC phosphopeptide-enriched fraction

In order to determine the potential for the two HyperCel resins to retain and fractionate a heterogeneous phosphopeptide population, a pre-enriched IMAC eluate from a casein digest was applied to each resin. Unlike the single synthetic ALR(p) peptide, with the exception of a minor peak at 50 % (v/v) ACN, elution of bound material did not occur until the column wash flushed with 0.1 % (v/v) TFA. Unlike the ALR(p) peptide, similar binding was found between both resins, though a sharper elution peak was obverved for HEA HyperCel (see figure 5.13).

[8] Casein would not dissolve in 90 % (v/v) ACN.

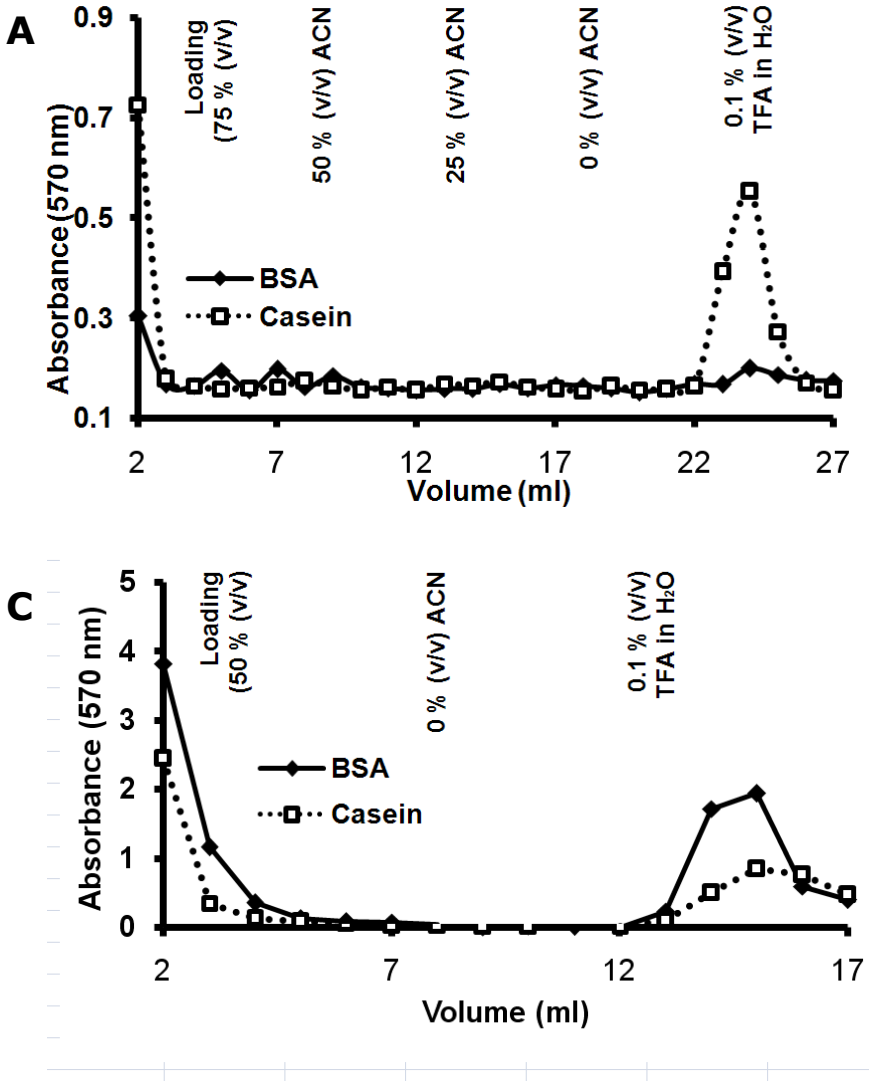


Figure 5.12: Typical peptide BCA-determined elution profiles of undigested casein and BSA loaded in (A) 70 %; (B) 50 %; (C) 25 % (v/v) ACN, and eluted along a stepwise gradient, followed by a final elution with 0.1 % (v/v) TFA. As the solvent concentration of the loading stage dropped, selectivity for casein followed, while BSA selectivity increased. It is notable that the 0.1 % TFA wash stage is crucial for full elution of bound material from the column, though the cause of this is unknown.

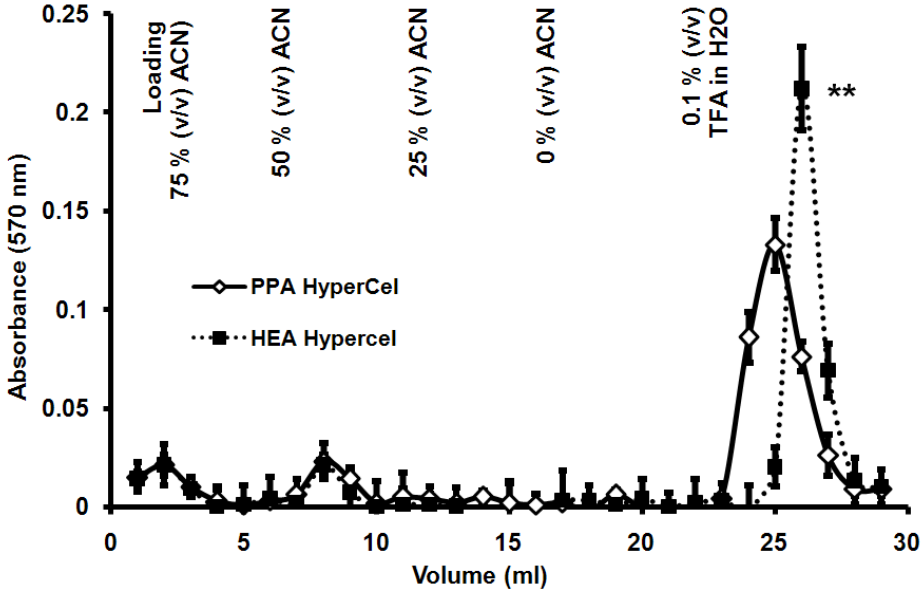


Figure 5.13: Typical peptide BCA-determined elution profiles (1 ml/fraction) of HEA and PPA HyperCel resins (1 ml columns packed in-house) when loaded with phosphopeptide-enriched casein IMAC eluates at 75 % (v/v) ACN and eluted by a stepwise gradient followed by a 0.1 % (v/v) TFA wash. Fractions were assayed by peptide BCA (as previously described). HEA HyperCel was found to elute in a tighter peak than PPA, but peak areas did not appear to differ between resins. ** denotes statistical significance to $P < 0.01$ ($n = 3$). Error bars indicate SD.

5.3.3.5 Inclusion of TFA in mobile phase

Given the effect of 0.1 % (v/v) TFA on bound material, the inclusion of 0.1 % (v/v) TFA in the mobile phase on casein protein retention was investigated. Casein was loaded at 75 % (v/v) ACN and the stepwise elution carried out with the presence or absence of 0.1 % (v/v) TFA, following which five column volumes of 1 % (v/v) TFA was applied to elute any remaining bound material (see figure 5.14). However, no binding/elution was observed, and therefore inclusion of a steady TFA concentration along a solvent:H_2O gradient would require a lower TFA concentration, if at all.

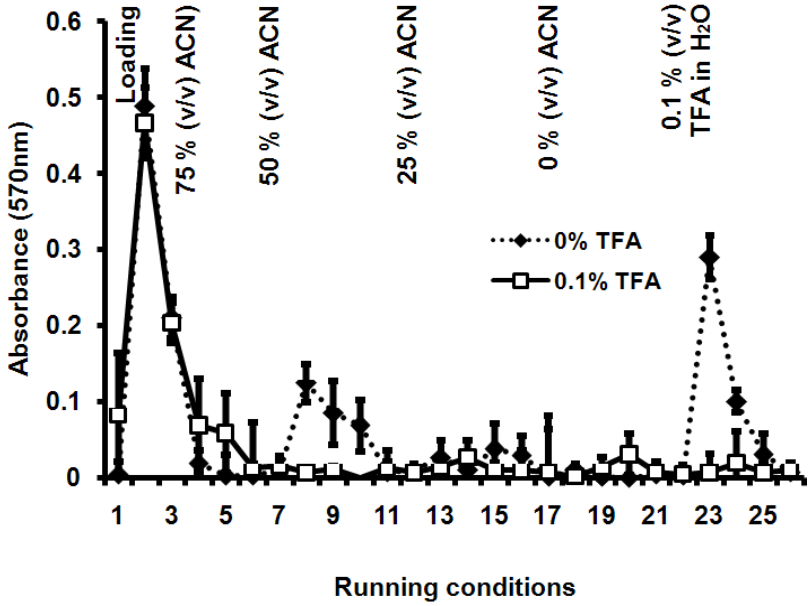


Figure 5.14: Typical peptide BCA-determined elution profiles (1 ml/fraction) of phosphopeptide-enriched casein IMAC eluates loaded onto HEA HyperCel resin at 75 % (v/v) ACN and eluted by a stepwise gradient in the presence or absence of 0.1 % (v/v) TFA, followed by a 1 % (v/v) TFA wash. Fractions were vortexed mixed, diluted 1:5 with ddH_2O and the diluted samples assayed by peptide BCA (as per chapter 3). Presence of 0.1 % (v/v) TFA in the mobile phase was found to prevent peptide binding. Error bars indicate SD.

5.3.3.6 Elution along an acid gradient / elution with alternative acids

As elution seemed dependent on acidification (known to reduce hydrophilic interactions [Hjertén, 1973]), yet TFA seemed less than ideal for downstream purposes (given that it absorbs strongly in the UV at 210 nm precluding use of UV peak identification, and as an ion pairing agent it may suppress LC-MS/MS ionisation (Apffel, *et al*., 1995; Kuhlmann *et al*., 1995) the potential for alternative acids/concentrations was investigated by way of a stepwise gradient applied to a post-IMAC casein digest collected in 1 ml fractions and assayed by peptide BCA. HCl, formic, and phosphoric acid were all assayed alongside TFA to determine if they were suitable substitutions. Each column was finally flushed with 1 % TFA to determine if any material remained bound (see figure 5.15).

Unfortunately only HCl was able to provide full elution of bound material, but in this case signs of resin damage rapidly became evident upon re-dehydration to 70 % ACN, limiting re-use if these elution conditions were required in future.

Fortunately formic acid only produced a minor peak at 1 % (v/v), raising the potential for unbound R-P C_{18} or Fe^{3+} IMAC fractions to be retained by HEA HyperCel as a secondary chromatographic pathway (though in the case of IMAC these may be depleted of phosphopeptides). Phosphoric acid produced peaks at 0.1 % and 1 % (v/v) but did not fully elute at this concentration. It is possible that higher levels of phosphoric acid may allow full disassociation of bound material, but as a non-volatile acid, high concentrations must be avoided in conjunction with LC-MS/MS. Interestingly the column subjected to a TFA gradient did not fully elute at 0.1 % (v/v) TFA, suggesting that if TFA is used for fractionation a gradient will be required (i.e. a steady concentration of TFA along a solvent:H_2O gradient may not allow full elution).

5.3.7 Assessment of phosphopeptide selectivity

Once elution conditions were optimised, the preference of the resin for phosphopeptides was reassessed. A mixture of four synthetic phosphopeptides or their non-phosphorylated counterparts were loaded at 0.2 mg/peptide onto 1 ml HEA HyperCel columns in 75 % (v/v) ACN, and a short stepwise gradient to 50 % (v/v) ACN, H_2O, and then 0.5 % (v/v) TFA applied. Again, eluates were collected in 1 ml fractions and assayed by peptide BCA as previously described (see figure 5.16). No significant difference in binding was observed between the phosphorylated and non-phosphorylated, both of which appeard to elute between 50 – 0 % (v/v) ACN. Despite the variation in peptide's physical characteristic multiple peaks were not observed, and the introduction of 0.5 % (v/v) TFA to the column similarly produced no effect. It is unknown however if all four peptides bound to the column, or whether a gradient between 50 – 0 % might resolve the unmodified peptides from the phosphorylated, however, these data suggests that any difference in binding may well be a matter of relative retention rather than true selectivity under the current conditions.

The nature of the strongly bound post-IMAC casein peptides remains unresolved, however if they are phosphorylated they may be a distinct subpopulation of phosphopeptides. Given the failure of the synthetic peptides (each of which's non- phosphorylated form was chosen for a high MHC class-I binding score) to bind and elute at this point, they may not be representative of MHC eluted nonphosphorylated peptides, however as the effects of phosphorylation on MHC binding are only currently being elucidated (Mohammed *et* al., 2008), the

overlap between MHC eluted phosphopeptides and HEA HyperCel-binding peptides may only be determined by direct experimentation.

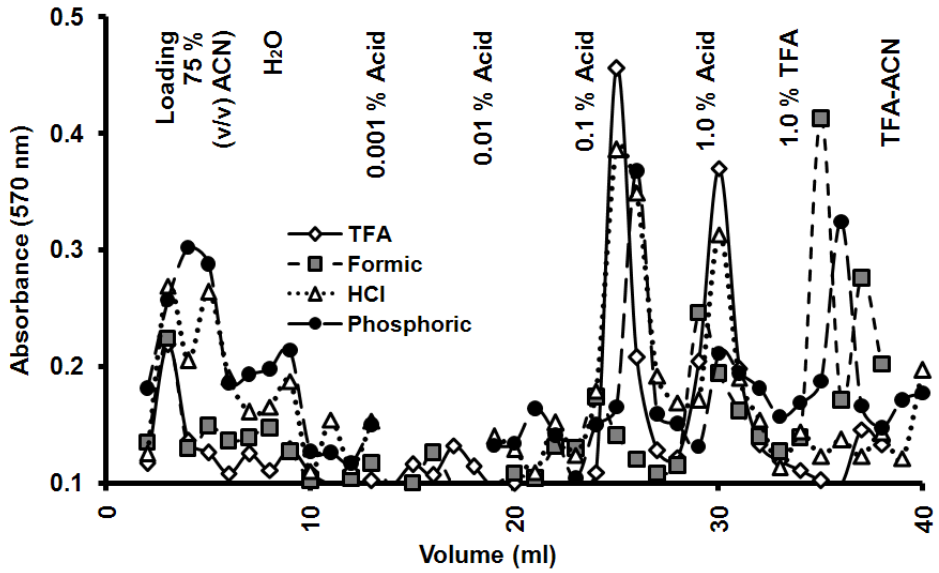


Figure 5.15: Typical peptide BCA-determined elution profiles (1 ml/fraction) of phosphopeptide-enriched casein IMAC eluates loaded onto HEA HyperCel resin at 75 % (v/v) ACN and eluted by a stepwise gradient of acid (all v/v) followed by a 1 % (v/v) TFA wash and a short TFA-ACN gradient. Fractions were vortexed mixed, diluted 1:5 with ddH_2O and the diluted samples assayed by peptide BCA (as per chapter 3). TFA remains the optimum acid for sample disassociation.

5.4 Discussion

Phosphopeptide isolation, enrichment and fractionation is crucial for proper characterisation of this typically sub-stochiometric peptide population, and while a number of methods are available, immunoaffinity, IMAC and TiO_2 are by far the most commonly utilised (Bai and Wang, 2009). As with all chromatographic approaches, IMAC may be utilised as either an analytical or a preparative chromatography approach, the former involving long run times, slow gradients and high separation into a large number of (dilute) fractions; while the latter involves rapid gradient changes with the aim of producing a single concentrated bound fraction which may then be taken on for downstream analysis. While the established IMAC protocol previously used at NTU has been shown to isolate phosphorylated peptides from a heterogenous mixture; by including a low-molarity wash stage and increasing the elution pH to 9 a significant increase in

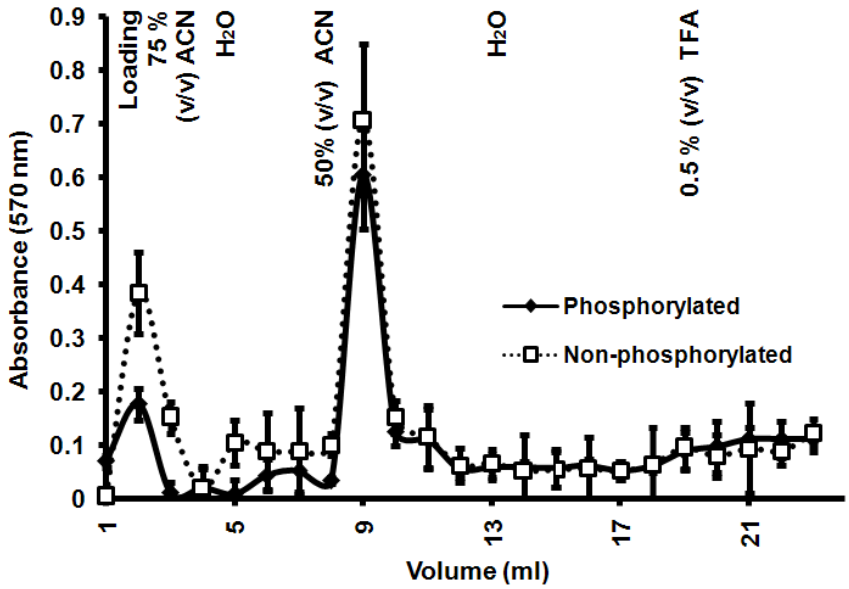


Figure 5.16: Typical peptide BCA-determined elution profiles (1 ml/fraction) of phosphorylated or non- phosphorylated peptide mixtures loaded onto HEA HyperCel resin at 75 % (v/v) ACN and eluted by a stepwise gradient to 0.5 % (v/v) TFA. Fractions were vortex-mixed, diluted 1:5 with ddH_2O and the diluted samples assayed by peptide BCA (as per section 3.1.4). No significant distinction in binding was observed between the phosphorylated peptides or their nonphosphorylated counterparts. Error bars indicate SD.

peak fraction concentration may be achieved, and the molarity of the elution buffer halved (thus doubling downstream compatibility) without a punitive trade-off in peak height. These developments are also applicable to other IMAC procedures, as may be seen from the improved peak fraction concentration for Cu^{2+} IMAC (Fig 5.5).

The analysis of phosphopeptides in the mass range of MHC eluates are much less restricted by the steric hindrances that complicate IMAC work with phosphorylated proteins and polypeptides (Han *et al*., 2008a), and while the technique carries a reasonable selectivity for phosphorylated peptides, IMAC is however not considered as robust as MOAC/TiO_2 chromatography, which has seen a boom in use over recent years (D'Ambrosio *et al*., 2006). Like IMAC, acidic residues are the primary cause of non-specific sample binding, however this may be reduced by the inclusion of low DHB concentrations, or by reducing the loading pH (Pinkse *et al*., 2004; Larsen *et al*., 2005; Thingholm and Larsen, 2009), as at pH 2 and below carboxyl groups such as the side chains of acidic amino acids are largely uncharged (Bai and Wang, 2009). While reductions in

the IMAC loading pH are capped at pH 1.7 or above, below which multiply-phosphorylated peptides begin to disassociate from the column (Thingolm and Jensen, 2009)[9], lowering the loading pH from 3.3 to 2 may reduce non-specific binding. Alternatively including wash stages of varying pH or solvent concentration may also aid disassociation of nonphosphorylated peptides, though this may require scaling up of the chromatographic setup to guarantee detection by peptide BCA[10]. Under-loading of sample onto the columns should also be avoided as this may lead to non-specific interactions which would normally be outcompeted by the phosphate groups (Dunn *et al.,* 2009). The effect of salts or solvent on selectivity (as opposed to gross binding) is also worth investigating, as either may further reduce non-specific interactions or possibly even metal ion leakage. However care must be taken as it is unknown whether these improvements in selectivity, or indeed the higher stringency some have found TiO_2 to have over IMAC (Larsen *et al*., 2005) may result in a decrease in the population of phosphopeptides which are retained on the column. Methods may be further optimised by the inclusion of low concentrations of Tris to reduce binding (Proteus, 2004; GE Healthcare, 2005). While our own data suggests ascorbic acid can cause metal ion loss from the column, very low concentrations, or another antioxidant may be included to minimise metal-catalysed oxidation on the column [11] (Krishnamurthy, *et al*., 1995) which would complicate mass spectrometric analysis [12], though these in turn must be compatible with downstream analysis. Loss of metal ions may be adjusted by using higher-affinity resins such as the tetradentate NTA (Hochuli *et al*., 1987; Holmes and Schiller, 1997), though as already mentioned this may affect column capacity and peptide binding/selectivity (Kaur-Atwal *et al*,. 2008). A new variation on IMAC using GMA-EDMA polymer beads as a resin, which operates by the coordination of free phosphonate to in turn immobilise metal ions was found to produce a higher specificity for phosphopeptides than TiO_2 or ZrO_2 (reviewed in Han *et al*., 2008a).

[9] Though inter-resin variations may also play a role here, as they found full elution of all phosphorylated peptides did not occur until pH 10-11, when multiply phosphorylated peptides also disassociated from the column.

[10] An alternative approach would be to concentrate unbound fractions by freeze-drying (if their matrix is volatile) or by another chromatographic method, however this may have implications for accuracy of quantification.

[11] It is conceivable that the 1 mM glutathione content of the MHC elution buffers may do just this, but this has not been assessed at the time of writing.

[12] Araña *et al*., (2001) note that formic acid may be degraded by Fe 2+/3+, or by TiO2. If this leads to carbon monoxide production a formic acid based buffer may promote metal ion loss or sample oxidation.

As previously mentioned, another method to reduce non-specific binding involves chemically modification of samples prior to IMAC. Methylation is the most common approach, and in 2002 Ficarro *et al*., (2002; Salomon, 2003) developed a novel method for methylation of acidic residues involving incubation with 2N HCl in methanol. An alternative which seems to have been mostly overlooked with regard to IMAC is carbodiimide-catalysed methyl-esterification of carboxylic groups[13]. Using the improved protocol published by Staros *et al*. (1986) the addition of N-hydroxysuccinimide may be used to increase the efficiency of reaction with the methyl-ester. However, as with all modifications, conditions must be carefully chosen to avoid both incomplete modification (which can significantly complicate mass spectra, reducing the clarity of results and inhibiting analysis) and cross reactivity with other residues, or functional groups (D'Ambrosio *et al*., 2006; Han *et al*., 2008a)[14]. Preliminary work in this area carried out at NTU appeared not to have any negative effects on the phosphate groups, but was found to significantly dilute samples. However pre-IMAC sample concentration, either by HiLIC (McNulty and Annan, 2009) or by reversed-phase may compensate for these changes in volume, be they prior to or following modification. Indeed, it has been suggested that the reaction might be carried out in an organic solvent such as ACN (Dr Ray Wallace, Nottingham Trent University, Pers. Comm., 2007), similar to the methylation protocol developed by Ficarro *et al*. (2002), and this would also leave the samples compatible with the high-solvent loading/washing buffer used by Ndassa *et al*. (2006).

However the largest stumbling block to proper method development remains the limited understanding of both TiO_2 and IMAC's mode of action (Thingolm *et al*., 2008), there is evidence that IMAC may yet prove as robust as TiO2 (Liang *et al*., 2007). Furthermore, both IMAC and TiO_2 chromatography are still undergoing continuous and highly fractured method development, and while the lack of standard methods allows flexibility and tailoring of methods to specific samples and analytes, it also produces a highly heterogeneous body of literature, which renders comparisons between publications challenging to say the least. A standardisation effort between multiple research groups using a large panel of phosphorylated peptides (+/- nonphosphorylated peptides with acidic amino acids) with a range of different physical properties (not to mention the

[13] Note that this modification may render the aspartic acid residues trypsin-cleavable (Wang & Young, 1978), and that a secondary reaction may also (irreversibly) modify the sulphydryl group of cysteine (Carraway and Koshland, 1968 in Timkovich, 1977) at approximately the same rate as the carboxyl groups and the hydroxyl group of tyrosine (Hoare & Koshland, 1966) (though this is a slower reaction and is reversible by addition of hydroxylamine) (Carraway & Koshland, 1972).
[14] For examples of carboxymethylation of alternative residues see Gurd (1967).

underexplored world of *N*-, *S*- and *acyl* linked phophorylation) would go a long way towards determining the relative advantages of each methodology, as well as possibly further elucidating the mechanisms by which both methods operate, and why neither will bind the full range of phosphopeptides.

Reversed–phase resins have been the most commonly utilised peptide concentration/fractionation method for over twenty years, and (unlike IMAC and TiO_2) have a simple methodology which is relatively homogenous between research groups [15] . However despite the recognised importance of phosphorylation in signalling pathways, the binding kinetics of phosphopeptides to reversed-phase columns are not fully understood. While we did not compare binding between phosphorylated and nonphosphorylated peptides, the hydrophilic (C-terminal) tyrosine-phosphorylated SQK(p) peptide and the hydrophobic (mid-peptide) serine-phosphorylated YIS(p) both showed comparable binding to C_{18} reversed-phase SPE cartridges despite their divergent physical properties, but recovery for both peptides was not high. While this could be increased by raising the TFA concentration, the increased ion-pairing effect may dampen the resulting M/S signal, although acids which did not ion-pair to the same extent did not provide the same effect. However these approaches may still be fruitful for HPLC fractionation or use of RP as a first dimension before applying to IMAC, peptide-PAGE or simply drying under vacuum (e.g. prior to MALDI-TOF MS/MS).

Through the mixed mode work indicated a variation in selectivity for the two peptides tested, the Strata X-AW anion exchange resin appeared to be capable of binding both peptides (though a greater amount of SQK(p) was recovered than YIS(p), and was split between two elution conditions. Nevertheless, Dai *et al*. (2007) note that cation exchange resins struggle to isolate phosphopeptides with a pI lower than 4.5, while anion exchange resins are capable of capturing these peptides, which is to some extent borne out by our data: SQK(p) has a pI of 4.3, while YIS(p) is much higher at 9.5. Han *et al*. (2008b) report that the use of strong anion exchange chromatography (SAX) to isolate and fractionate phosphopeptides may be as effective as Fe^{3+} IMAC, though the extent to which their selectivities overlap is as yet unknown.

[15] Acidification and loading solvent concentration (typically 0 – 10 %) are the factors most likely to vary, the majority of HPLC takes place using either MeOH or ACN.

HiLIC show some promise as a method to concentrate phosphorylated peptides either prior to or following IMAC. While the preliminary data in figure 5.16 suggests that HEA HyperCel does not appear to have any true selectivity for phosphopeptides under the current conditions, this is similar to the results found by Albuquerque *et al*., 2008; and McNulty and Annan, 2009; both of whom state that HiLIC alone does not appear to be suitable for phosphopeptide purification. Ideally utilisation of HiLIC with a HPLC setup would have provided rapid and reproducible results for method development in this area. Unfortunately such a marriage of technologies was not achieved during this research period, issues with pressure were especially prevalent, and perhaps expected once the primary use of the resins (industrial feedstock seperation) are taken into account. It was however possible through careful packing and low flow rates to generate a HPLC setup within the pressure tolerance of the resins in the absence of analyte or DMSO. The inclusion of either, especially injection of a tryptic casein digest led to pressure increases, possibly due to micellular formations and low solubility even at 75 % ACN. Furthermore, even if one were willing to accept the damage to the resin from exceeding pressure tolerance, the research to date suggested TFA was required to fully elute a heterogeneous digest from the resin, however TFA absorbs strongly in the UV at 210 nm, complicating peak identification, hence research was restricted to the more laborious BCA assay approach, as it has greater accuracy for biological samples. Crucially the effect of loading pH and salt concentration were not investigated fully, samples were merely loaded in differing solvent concentrations, however it is possible that the higher pH of the tryptic digests (pH 8.0) and IMAC eluates (pH 9.0) may explain the difference in peak elution points between these and the synthetic peptides, which were loaded without buffering. Whilst there is little literature available for either HEA or PPA Hypercel, Pezzini *et al*. (2009) and Brenac Brochier *et al*. (2009) both noted a benefit to eluting along a pH gradient when utilising HEA Hypercel for purification of recombinant protein.

Nevertheless it is hoped that the mixed-mode aspects of HEA (or even PPA) HyperCel might allow better selectivity for phosphopeptides with further method development. Even utilising the methods as they are in combination with RP and Fe^{3+} IMAC should allow significant decomplexing of resultant mass spectra, though again, ion pairing agents such as TFA are widely regarded as problematic for ESI-MS/MS due to the resulting neutralisation of cation charge, masking analytes from positive-mode mass spectrometers (Apffel, *et al*., 1995; Kuhlmann *et al*., 1995), and it may be beneficial to employ vacuum drying or application

onto a second chromatographic dimension (such as IMAC) prior to LC-MS/MS. Furthermore the current methodology for Cu^{2+} IMAC may not be downstream compatible with HEA/PPA HyperCel HiLIC, though figure 5.15 indicates that formic acid would be a suitable loading matrix in place of TFA (especially following sample dilution in ACN), this may require some optimisation as a Cu^{2+} IMAC elution buffer. With this exception, care has been taken to maintain inter-method compatibility as far as is possible, therefore an eluate from any method should be capable of fractionation by a subsequent approach, or by LC-ESI-MS/MS, if room for sample dilution prior to loading is allowed, and peptides are decomplexed and concentrated to the point that supression effects of remaining ion pairing agents (e.g. TFA) are tolerated.

It must be remembered however that each chromatographic step reduces the material left for the next stage. At the $2 * 10^9$ suspension cell number used for MHC class-I elution, stochiometric concentrations of at least 10 copies of each peptide are expected per cell, resulting in at least fmolar concentrations of each peptide following MHC elution (Bonner, 2002), though how these figures relate to phosphorylated peptides (and/or class-II phospho/peptides) is much less clear. Chromatographic losses must be weighed up against poor signal/noise ratios, or effective underloading of the final analytical approach due to low interaction between peptide fractions and the method in question. Furthermore comparisons of chromatographic methods, especially with regard to IMAC are plagued by the lack of standardisation within the field, and the complementary (and only partly overlapping) binding profiles between the main phosphopeptide isolation methods also complicate inter-chromatographic comparisons (Bodenmiller *et al*., 2007). Nevertheless the above data indicates that IMAC, already well regarded as a LC-MS/MS compatible approach for phosphopeptide enrichment can be further tailored as a prep-chromatographic stage, and that HiLIC appears capable of binding a subpopulation of phosphorylated peptides following IMAC enrichment.

6.0 Mass spectrometry

6.1 Introduction

Following sample fractionation and phosphopeptides enrichment, peptide sequences require identification by mass spectrometry, followed by a BLAST to determine their likely protein of origin.

It is something or an irony that mass spectrometry (MS), based on approaches developed by Thomson (1913) and Aston (1933), has existed since the mid 1950's, as long as the Edman sequencing method, yet while the former is far more common for determining peptide sequencing today, it did not achieve this status until the late 1970's, and far more recently. The reasons behind this are primarily technological; early MS methods used a gas chromatographic mass spectrometer (Biemann, 1995), and while small molecules were 'easily' ionisable using electron-impact, polar/thermally labile molecules such as peptides required extensive and laborious chemical modification to render them volatile and readily ionisable. These methods also required relatively abundant samples, and struggled to sequence peptides of greater than 8 amino acids accurately (Griffiths *et al*., 2001); while the Edman method was rapidly automated, and capable of dealing with larger polypeptides, and much less concentrated samples. However, pioneering research across the globe (including both sides of the Iron Curtain) gradually eroded these problems; the 1980's saw the advent of soft ionisation approaches, such as fast atom/ion bombardment (FAB-/FIB-MS), electrospray ionisation (ESI) and matrix-assisted laser desorption-ionisation (MALDI) (usually with a time-of-flight [TOF] detector), rapidly increasing ionisation rates. These, along with improvements in mass detector sensitivity allowed the sample requirements to fall to the pico and femtomolar concentrations commonly used today. Indeed, the miniaturisation of LC-ESI-MS/MS was to some extent driven by the requirements for analysis of MHC peptides, which typically display high heterogeneity and sample scarcity (Biemann, 1995).

Though mass spectrometers may be divided into numerous subtypes (e.g.: ESI, MALDI, ion mobility mass spectrometry), all involve the ionisation of a sample ("source"), the fractionation/isolation of analyte within that sample by mass/charge (*m*/*z*) by a mass analyser, and the determination of ion abundance

values (mass detector). While this gives limited information, combining it with a tandem mass spectrometric approach (MS/MS), where the precursor ion is further fragmented (usually by collision-induced disassociation) (Wells and McLucky, 2005), allows peptide sequences to be determined from the daughter ions (Griffiths *et al*., 2001).

The majority of mass spectrometric based peptidomic approaches rely on either MALDI (e.g. MALDI-TOF-MS or MS/MS[16]) or ESI (frequently LC-ESI-MS/MS[1]) as their ion source. MALDI involves the complexing of volatile sample (often tryptically digested and desalted by RP) with a matrix such as α-Cyano-4-hydroxycinnamic acid (α-CHCA) or sinapinic acid on a MALDI plate. This mixture is allowed to co-crystallise, and the resultant spot targeted with a laser pulse. The matrix absorbs the energy of the laser, transferring it to the sample, which is ionised and launched towards the detector (which in modern models almost always utilises an ion trap). Smaller, strongly ionised peptides travel faster, and the time-of-flight may then be used to determine the peptide *m/z*.

Electrospray ionisation (ESI) is commonly used with a liquid chromatography (LC) setup, typically an HPLC with a RP column. Unlike MALDI it is a semi-continuous approach, samples are concentrated onto an analytical column and eluted along an acidified aqueous to solvent (ACN) gradient. As these elute they are pumped through a charge which is subjected to a high voltage, imparting a (usually positive) charge to the sample which exits the needle into a vacuum. The positive charge of the ionised analyte molecules repel one another, which combined with the low pressure forms an ionised gas ("Coulomb explosion"); this enters the MS analyser (typically an ion trap) as a beam of ions, which may then be fragmented by collision with electrons or an inert gas to determine peptide sequence (Cole, 1997; Griffiths *et al*., 2001).

Whilst MALDI allows high throughput and efficient ionisation of peptides, the fractionation approach of LC-ESI MS/MS (along both hydrophobicity and *m/z*) provides a greater resolution for peptides of similar masses (e.g. MHC-eluates). Quadropole setups such as the Thermo Fisher Finnegan LTQ (Linear Trap Quadropole) are among the most common mass analysers used in conjunction with ESI (El Aneed, 2009), especially with regard to MHC-associated peptides (Lemmel and Stevanovi, 2003; Bonner *et al*., Zarling *et al*., 2006; 2002; Milo *et*

[16] These are common combinations, though in theory any MS source should be combinable with any mass analyser.

al., 2007) though MALDI is used by some groups (Butterfield *et al*., 2001; Ramos *et al*., 2004).

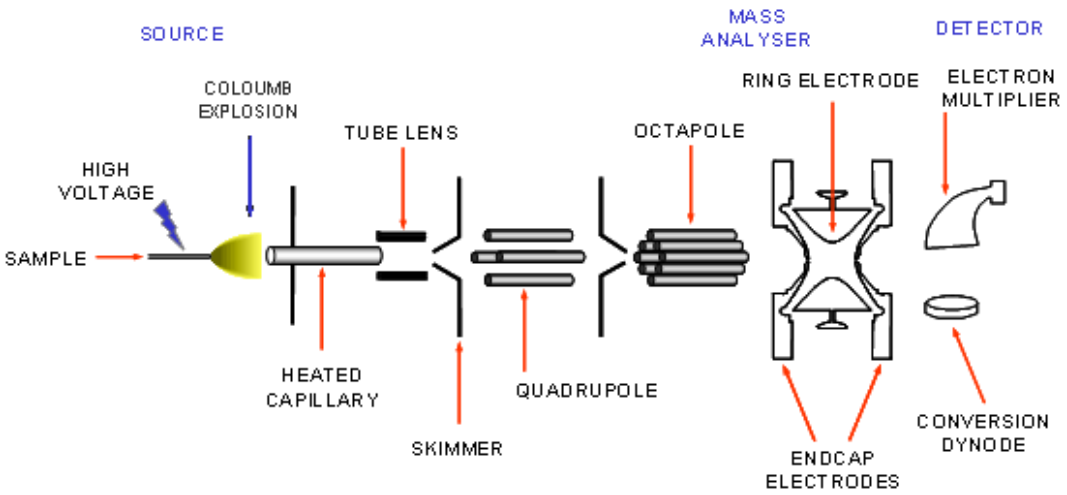


Figure 6.1: Schematic design of a Fisher Thermo LTQ ESI MS/MS (adapted from Fisher online).

6.2 Comparison of citrate-phosphate and TMA-formate MHC class-I eluates by MALDI-TOF/TOF mass spectrometry

Cultures of 2 x 10^9 K562-A3 and LAMA-84 cells were subjected to MHC class-I elution using either the isotonic citrate-phosphate (K562-A3 only) or supplemented TMA-formate (K562-A3, LAMA-84) elution buffers (both pH 3.3) by the methods outlined in 2.4.2. 10 % of each eluate was subjected to SPE sample cleanup by two rounds of chromatography by C_{18} (as per 2.5.2.4) and 1 µl of eluate assayed by MALDI-TOF (CHCA matrix) using methods optimised by Matharoo-Ball *et al*. (2007) and Vafadar-Isfahani *et al*. (2010) in a mass range from 600 to 3500 Da (see figure 6.2). When analysed these indicated similar spectra for both citrate-phosphate and TMA-formate eluted K562-A3 samples. The spectra obtained from the LAMA-84 cell line was distinct from all four from K562-A3, indicating that the peaks identified are linked to the cell line in question (i.e. not a product of the culture conditions, which were identical for all five samples).

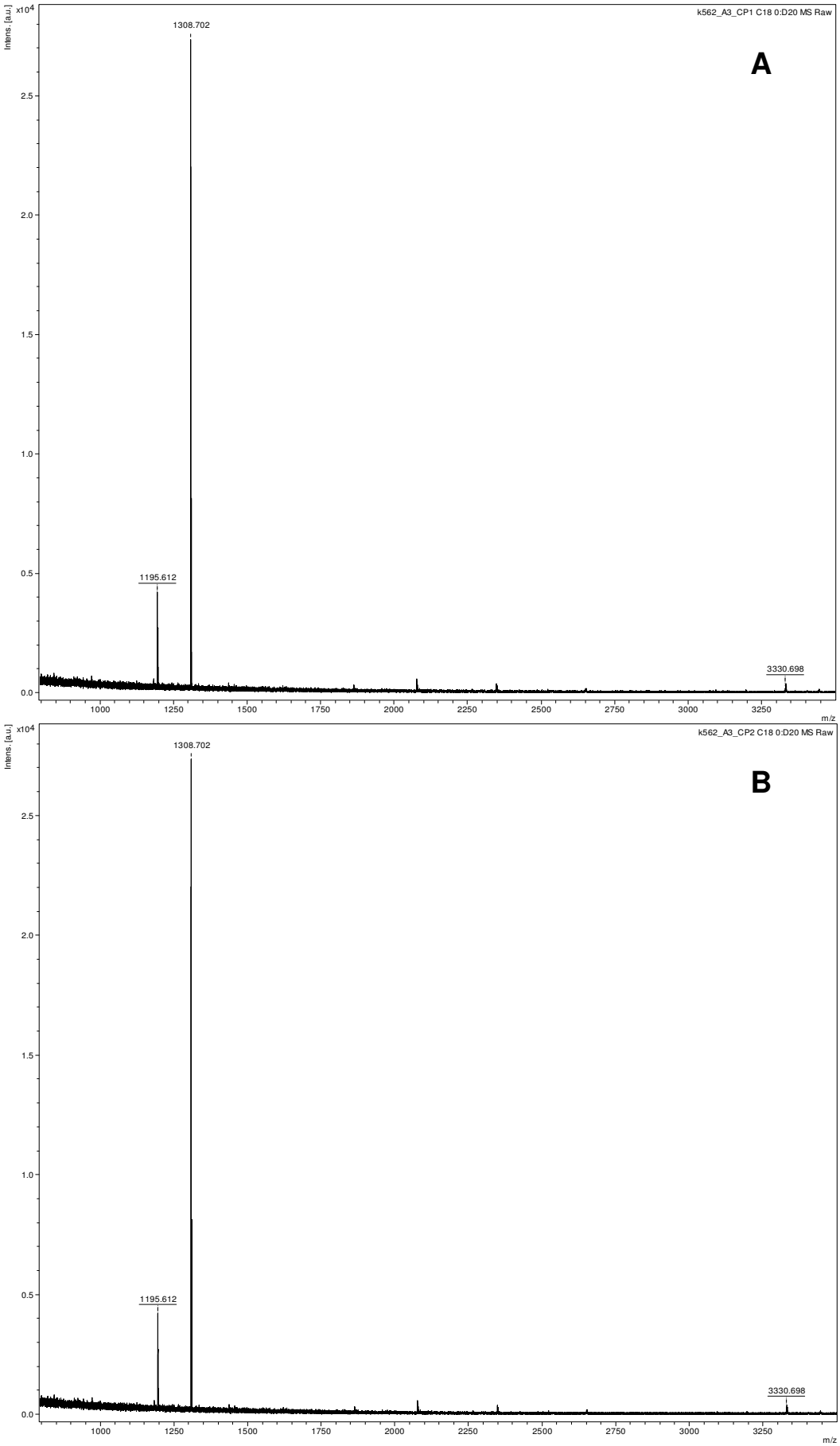
The major peak in the K562-A3 samples (1308.702 Da) was sequenced by tandem mass spectrometry (see figure 6.3) and the sequence compared to the

human proteome (SwissProt database). It was subsequently found to be a ten amino acid peptide (LVVYPWTQRF), which lies in a region of the haemoglobin sequence common to the β, γ-1; γ-2; and ε subunits. Though sequence data alone cannot rule out the potential for contamination with serum peptides, again the presence of this peptide in all five K562-A3 eluates (and as they major peak in all but one) but apparent lack within the LAMA-84 eluate implies that it is restricted to the K562-A3 cell line, which is known for haemoglobin synthesis (Zuhrie *et al*., 1988).

Of note is the relative signal strength between the peptides. A signal intensity of 2.5 x 10^4 a.u. was observed for both K562-A3 samples eluted in isotonic citrate phosphate. However for the TMA-formate eluted K562-A3 this ranged from 2.5 - 5 x 10^4 a.u., while the LAMA-84 cell eluate was only 1 x 10^4 a.u.. It is important to consider that while mass spectrometry is a concentration-dependent technique, the apparent abundance of this peptide may not be reflected in the original samples, but may instead be a product of ionisation or chromatographic retention. It is possible that the LVVYPWTQRF peptide may have been preferentially retained on the C_{18} columns to a greater degree than other peptides, and that some enrichment may have taken place, a possibility which is supported by the peptides predicted hydrophilicity (-1.0)[17].

In terms of the peptide's origin, the SYFPEITHI MHC-binding prediction algorithm (Rammensee *et al*., 1999) assigned a MHC class-I A3 binding score of 14 to the LVVYPWTQRF peptide; below that normally considered for reverse immunology, while the stringency of the algorithm may incorrectly exclude peptides which do bind (*Pers. Comm*., Dr Roger Horton, 2006, Nottingham Trent University). Lin *et al*., (2008) found the algorithm to have a success rate of 80-90 %, and therefore again, a serum/membrane-associated origin for the peptide cannot be ruled out. However a near-identical peptide LVVYPWTQRY (HBB_{33-42}) was recently under investigation (in a murine sarcoma model) by Komita *et al*. (2008), lending weight to the possibility of presentation by the MHC[18].

[17] Determined as per 2.3.2.1.
[18] Tyrosine and phenylalanine share similar structures, and each are coded for by two codons. An A/T SNP can lead to the conversion of one to in respective codon pair (Tan *et al*., 2005; Schneider *et al*,. 2008).


k562_A3_CP1 C18 0:D20 MS Raw
A
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1195.612
3330.698
m/z
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B
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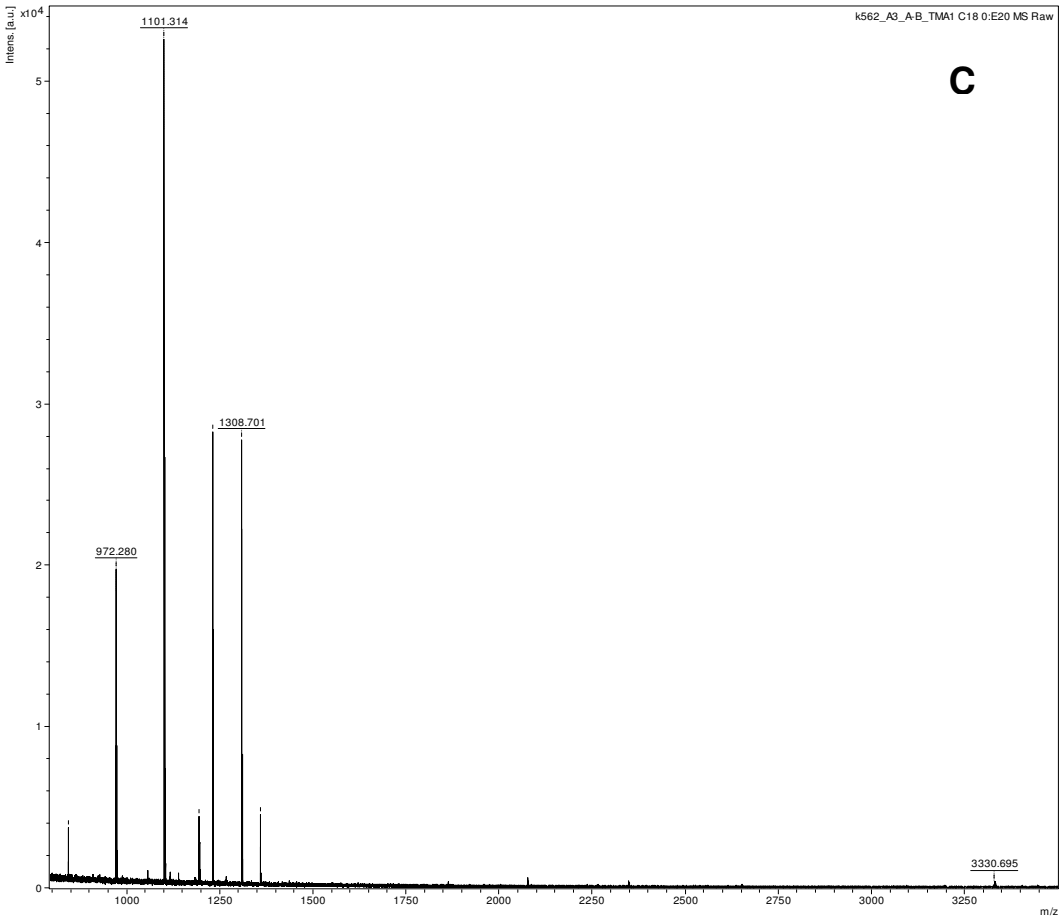
m/z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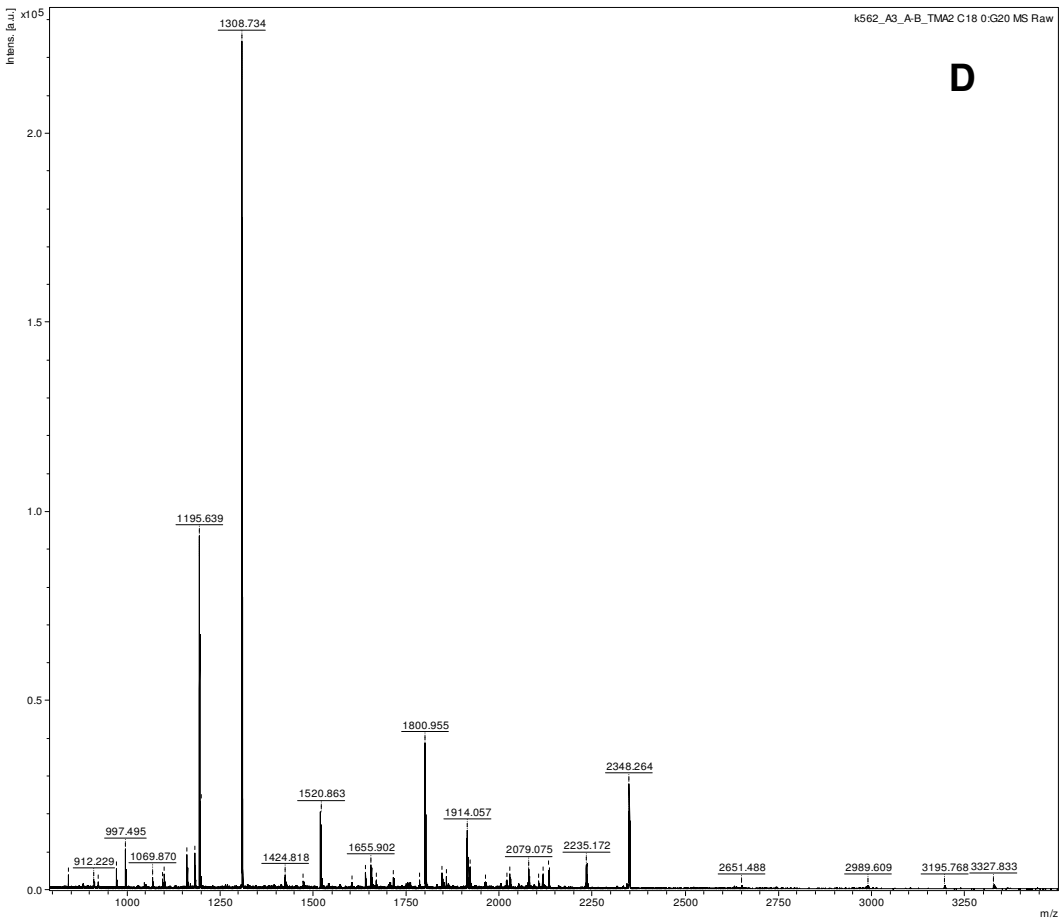
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m/z

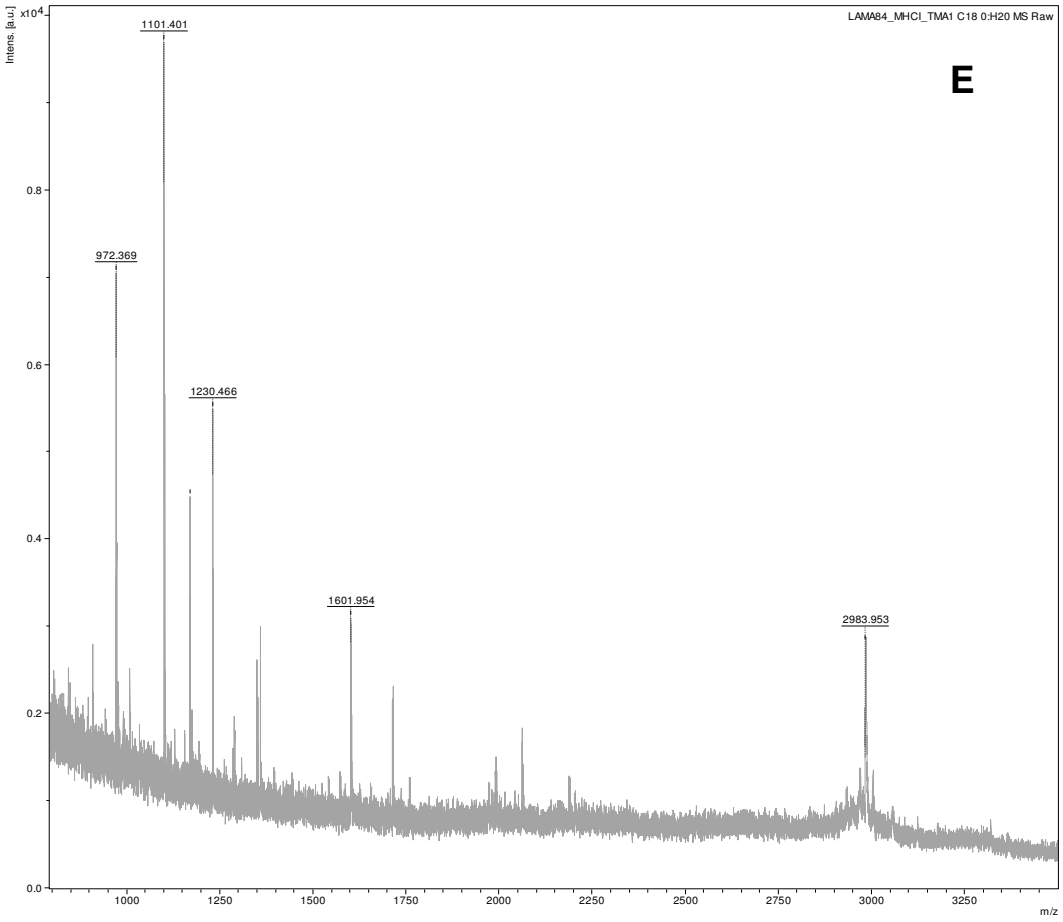

Figure 6.2: MALDI TOF-TOF mass spectra (600 – 3500 Da) for MHC class-I eluates from ~ 2 * 10^9 K562-A3 (**A-D**) and LAMA-84 (**E**) cell lines eluted with isotonic citrate-phosphate (**A, B**) or supplemented TMA-formate (**C-E**) as per 2.4.2. Of particular note were the peaks 1195, 1308 and 3330 present in all K562-A3 samples, but absent in LAMA-84 (see Figure 6.2 for MS/MS analysis of 1308 Da peak).

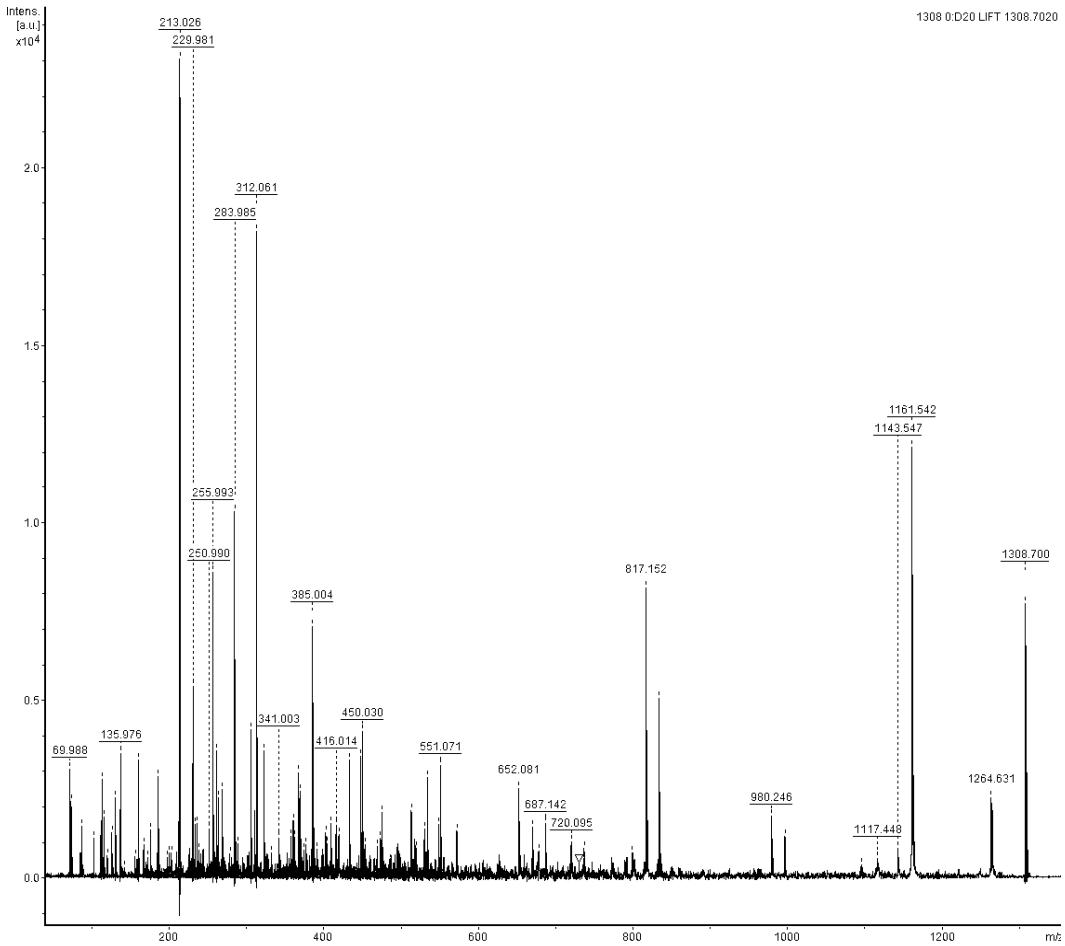

Figure 6.3: Tandem mass spectra (MALDI-TOF/MS) for major peak (1308 Da) from K562-A3 MHC class-I eluate (this peak was common to all K562-A3 samples, this spectra was generated from sample D in Figure 6.1). A MASCOT search against the SwissProt Human database revealed it to be LVVYPWTQRF, with a proposed origin in the Haemoglobin sequence (β, γ-1; γ-2; or ε subunit), closely matching the HBB_{33-42} peptide (LVVYPWTQRY) presented by the CMS-4 sarcoma cell line, and against which a $CD8^+$ CTL response may be generated (Komita *et al.*, 2008).

6.3 LC-ESI MS/MS mass spectrometry of phosphopeptides from MHC class-I eluates

MHC class-I peptides were eluted from 2 x 10^9 cells of the cell lines JY, K562-A3, and THP-1 (cultured as per section 2.2) using the supplemented 25 mM TMA-formate buffer (pH 3.3) as detailed in 2.4.2. The phosphopeptide subpopulation was enriched by Fe^{3+} IMAC as per 2.5.1.1.4 and peak fraction subjected to ESI-MS/MS following RP (C_{18}) fractionation in the second dimension by HPLC (as per 2.6.2).

Some typical mass spectra are presented in figure 6.3, in this case supplemented TMA-formate eluted MHC class-I peptides from the K562-A3 cell line. Similar spectra (though different peptides) were observed in MHC eluates from the EBV immortalised JY cell line and the AML cell line THP-1. Some notable examples are listed in Table 6.1.

6.3.1 Identification of MHC class-I restricted peptides from a transfected cell line

MHC class-I peptides were eluted (as per 2.4.2.2.1) from cultures of 2 x 10^8 murine CT26 cells transfected with the immunogenic GP63 protein[4] (expressed by way of a CMV promoter) from *L. mexicana*, a strain of parasite responsible for the Leishmaniasis (Ali *et al*., 2009) and subjected to two rounds of SPE sample cleanup using a C_{18} RP column as per 2.6.2.

Three GP63 peptides in the 7-12 mass range were putatively identified: AGSAGSH; CTAEDILTDEK and TDEKRDTLVKHL, though only one (CTAEDILTDEK) with a confident score (for spectra see figure 6.4). No evidence of hydrophobic peptide enrichment was seen (peptide hydrophilic scores are 0.1, 0.8 and 1 respectively), though sample decomplexing was observed as an attempt to subject the sample to ESI-MS/MS following a single round of C_{18} SPE showed far higher background signal (data not shown). However, as no prewash step was used higher contamination of non-MHC peptides would be expected, and the presence of these peptides must therefore be validated, and following on from this their immunogenicity assessed. While the 7- and 12-amino acid peptides are outside of the mass range found for most MHC class-I peptides (typically 8-11

[4] Kindly donated by Dr Selman Ali and Ms Anisha Bannerjee (Nottingham Trent University)

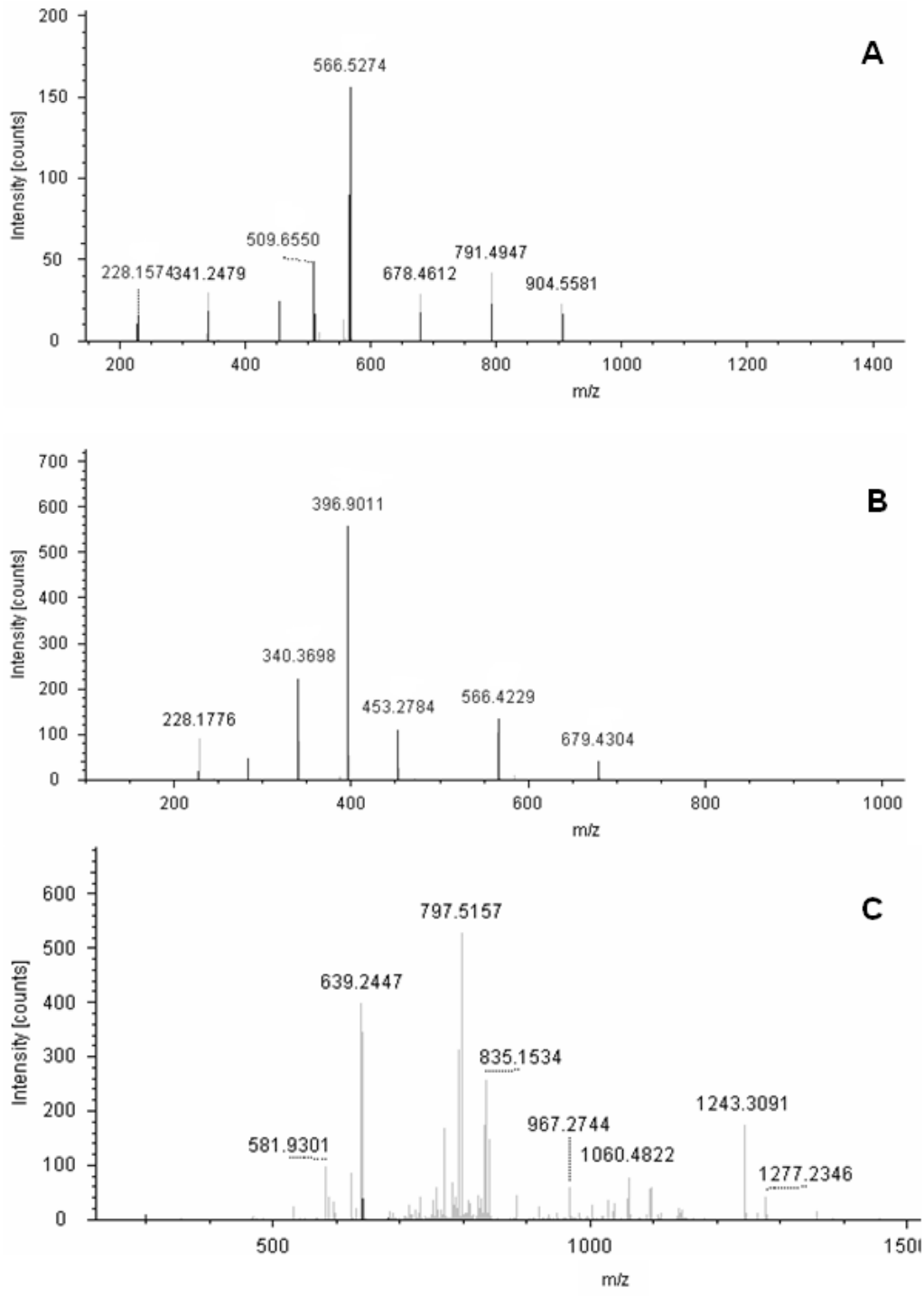

Figure 6.4: Typical tandem mass spectra (ESI-MS/MS) for phosphopeptides from MHC class-I eluates of the K562-A3 CML cell line. Sequences determined by ESI-MS/MS following C_{18} fractionation of a Fe^{3+} IMAC eluate. (**A**) [Q13467] (pS)APQLLLLLL from Frizzled 5; (**B**) [Q9UI38] LLLLLLLLR(pS) from Testis-specific protease-like protein 50; (**C**) [Q16825] SNP(pS)ITGS from Tyrosine-protein phosphatase non-receptor type 21.

Peptide(s)	Protein Name and Accession No	Details
FIQQRL(pS)Q(pT)EP	(Q8WZ42) Elastic titin	Involved in chromosomal condensation.
(pT)AAKPRQKALP	(Q12934) Filensin,	Cytoskeletal protein.
(pY)REV(pS)RAFHLN	(P55160) Membrane-associated protein HEM1/ NCKAP1L	Hematopoietic protein
LLLLLLLLR(pS)	(Q9UI38) Testis-specific protease-like protein 50 (TP50) (Cancer/testis antigen 20)	Expression formerly considered testis-specific, but now known in kidney, liver and pancreas (Scanlan *et al.*, 2004). Expressed in breast cancer and regulated by p53 (Xu *et al.*, 2007).
LIAMK(pT)EKEE	(Q15596) Nuclear receptor coactivator 2	Fuses with MOX in AML and results in P53 inhibition.
KKKKEKVLA(pT)	(Q02878) Ribosomal protein HL6, cytosolic	Associated with human T-cell leukemia virus
LEFHEAILRL(pS)	(Q99871) UCHL5-interacting protein	Part of the 26S proteasome
(pS)GGGGGLLL	(P78426) Homeobox protein Nkx-6.1, NKX6A, NKX6-1	Involved in β cell development. Gene methylated/downregulated in number of cancers.
LLLLLPLS(pS)(pS)	(Q16270) Insulin-like growth factor-binding protein 7	Proto-oncogene differentially expressed in many cancers. Known expression in K562 cell line (Jin *et al.*, 2007).
KPNLDV(pT)(pS)(pT)	(P12821) Angiotensin converting enzyme	Overexpressed in AML, and in K562 cell line. Linked to tumour progression. Inhibition may have anti-leukaemic effect (*De la Iglesia Iñigo et al., 2009*)
(pS)APQLLLLLL	(Q13467) from Frizzled 5 (FZD5)	Linked to Wnt / β-catenin signalling (both proto oncogenes) (He *et al.*, 1997; Lai *et al., 2009*)
KVRFQA(pS)IHL	(P56499) Mitochondrial uncoupling protein 3	Involved in thermostasis.
LVQLP(pS)GQ(pT)I	(Q03060) Indiucible cAMP-early repressor (ICER/CREM)	Often downregulated in cancer, often by phosphorylation in leukaemia, leading to proteasomic degredation (Pigazzi et al*., 2008)*
LLLLLLL(pY)K(pY)K	(P07333) Macrophage colony stimulating factor 1 receptor (M-CSF-1R) (aka cFMS proto-oncogene)	Membrane Y-kinase involved in myeloid-development. Implicated in many cancers and sensitive to Imatinib (Dewar *et al.*, 2005).
SNP(pS)ITGS	(Q16825) Tyrosine-protein phosphatase non-receptor type 21.	Regulator of Tec kinase family. Binds Src (Jui *et al.*, 2004). Frameshift mutations common in colorectal cancer (Korff *et al.*, 2008)

Figure 6.1: MHC class-I phosphopeptides eluted from the CML cell line K562-A3 and isolated by Fe^{3+} IMAC. Where references are not indicated, details are adapted from UniProtKB (http://www.uniprot.org/uniprot/), OMIM (http://www.ncbi.nlm.nih.gov/omim/) and iHOP (http://www.ihop-net.org/UniPub/iHOP/) databases.

Peptide(s)	Protein Name and Accession No	Details
(pS)EGA(pS)SD	(P26442) Autocrine motility factor (AMF) receptor	Involved in tumour metastasis, AMF release is induced by EBV infection. (Baumforth *et al*., 2005)
IINLG(pS)LVIED	(O15164) Transcription intermediary factor 1-alpha (TIF1-alpha)	Transcription factor that interacts with estrogen receptors. Fusion with RET produces the PTC6 oncogene.
IPIQINVG(pT)(pT)	(P46379) Large proline-rich protein BAT3 (Protein G3)	Involved in DNA damage stimulated P53 acetylation (Sasaki *et al*., 2007). Transcription induced by EBV protein LMP2A (capable of circumventing p53) in lymphoma (Bieging *et al*., 2009.

Figure 6.2: MHC class-I phosphopeptides eluted from the EBV-immortalised JY cell line and isolated by Fe^{3+} IMAC. Where references are not indicated, details are adapted from UniProtKB (http://www.uniprot.org/uniprot/), OMIM (http://www.ncbi.nlm.nih.gov/omim/) and iHOP (http://www.ihop-net.org/UniPub/iHOP/) databases.

Peptide(s)	Protein Name and Accession No	Details
FSPCAEK(pS)P	(P35916) Vascular endothelial growth factor receptor 3 (VEGFR-3)	Receptor Y-kinase implicated in leukaemia proliferation/resistance to therapy and MAPK activation (reviewed in Su *et al.,* 2007; 2008)
(pS)SSTPSSLPQSF *(borderline score)*	(O60732) Melaloma-associated antigen (MAGE) C-1	C-T antigen with unknown function, expressed in multiple myeloma (Jungbluth *et al*., 2005).

Figure 6.3: MHC class-I phosphopeptides eluted from the AML cell line THP-1 and isolated by Fe^{3+} IMAC. Where references are not indicated, details are adapted from UniProtKB (http://www.uniprot.org/uniprot/), OMIM (http://www.ncbi.nlm.nih.gov/omim/) and iHOP (http://www.ihop-net.org/UniPub/iHOP/) databases.

amino acids in length[5]), immunogenic septomeric and duodecomeric peptides are known (e.g. Fu *et al*., 1994; Chen *et al*., 1992) and so they remain potential candidates for further investigation. However given that the cell population from which this eluate was sourced was an order of magnitude below that normally used for direct immunology, the immunogenic peptide within GP63 may be none of the above, and a larger sample may be required.

While no 3000 / 5000 w.m. cutoff filters have been applied to the MHC class-I elutates prior to chromatographic fractionation, it is possible that doing so (or including a size-exclusion chromatographic stage) may be worthwhile, especially in this case. Though for post Fe^{3+} IMAC analysis of phosphopeptides, the non-phosphorylated β2-microglobulin molecule is not expected to be retained, in this instance no phosphopeptide enrichment stage was performed, and it is possible

[5] Though MHC class-I H2-Kd (one allele expressed by the CT26 cell line) shows a high preference for nonamers, with only 16% of peptides found by Suri *et al*. (2006) exceeding this length.

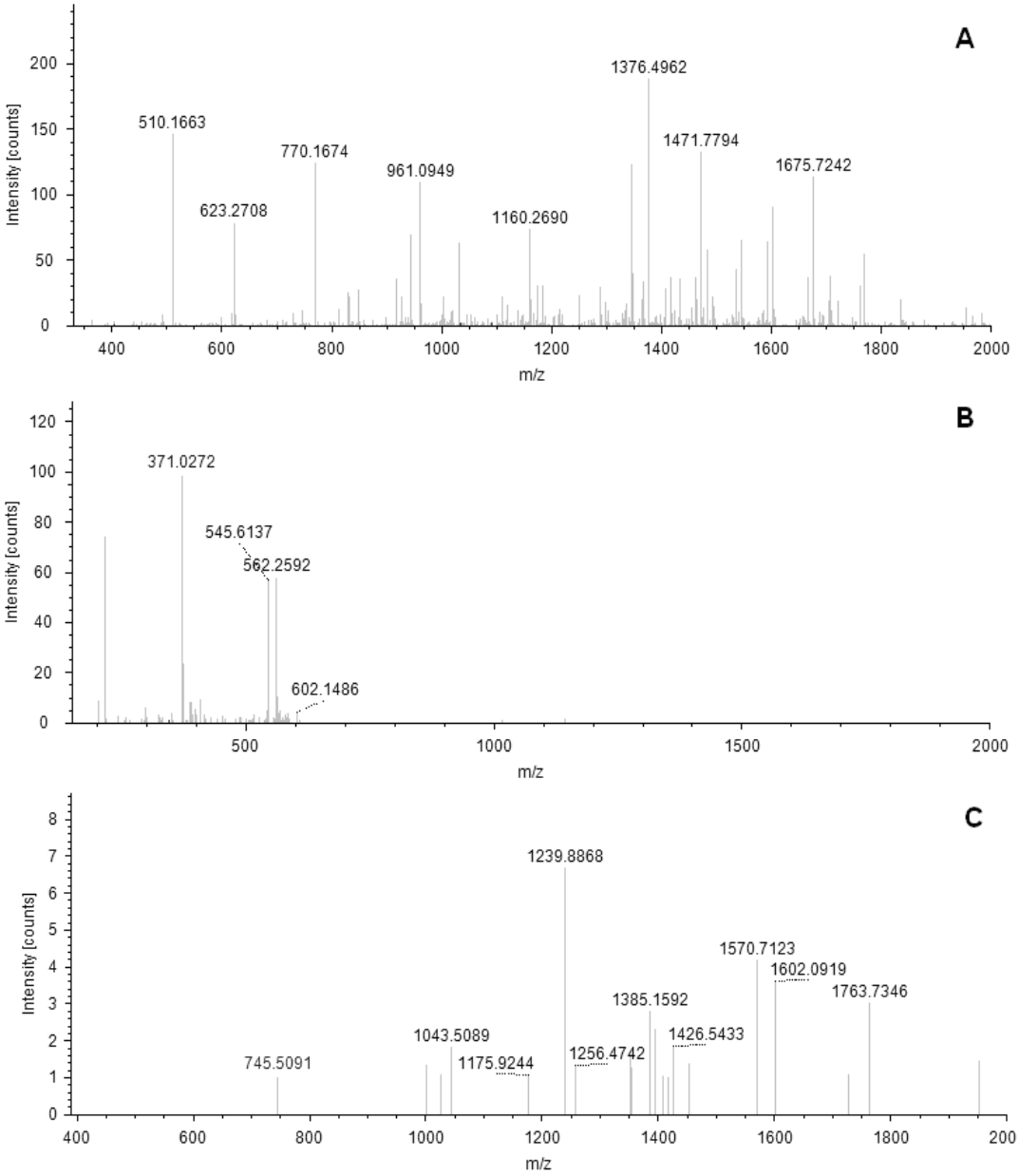

Figure 6.5: Tandem mass spectra (ESI-MS/MS) for three peptides from MHC class-I eluates of the GP63 transfected murine ALC cell line. Peptide sequences: (**A**) CTAEDILTDEK; (**B**) AGSAGSH; and (**C**) TDEKRDTLVKHL. Determined by ESI-MS/MS following serial C_{18} fractionation (2x C_{18} SPE, 1x HPLC).

that the 12 kDa β2M molecules (which should be in a 1:1 ratio with MHC class-I peptides) have drowned out the MHC peptides, both in terms of BCA quantification (*ceteris paribus*, β2M may lead to a 10-fold overestimation of peptide content) and in terms of chromatographic retention.[6]

6.4 LC-ESI MS/MS mass spectrometry of phosphopeptides from MHC class-II eluates

MHC class-II peptides were eluted from 2 x 10^9 cells of the THP-1 (known to present low levels of MHC class-II DRB0101, DRB1501, DQB05, DQB06, DQA0101, and DQA0102) (Berges *et al*., 2005) and JY ($DR4^+$, $DR6^+$) (Kaufman and Strominger, 1979) cell lines using the supplemented MHC class-II elution buffer (as per 24.2.3); and subjected to Fe^{3+} IMAC, from which the peak fractions were further concentrated and desalted by C_{18} RP SPE, following which samples were subjected to LC ESI-MS as per 6.3.

In this case however poor spectra were found in almost all cases. LC-ESI-MS/MS of the IMAC eluates prior to C_{18} concentration were indistinguishable from background in every case (peak ion intensities of as low as 17 counts were observed). When peak fractions from IMAC eluates were subjected to concentration by C_{18} RP SPE, the majority of all samples failed to bind to the SPE column, but the signal strength rose by one order of magnitude. Despite this no confident spectra were found in MHC class-II eluates from the JY cell line, while a number of borderline spectra were found in the THP-1 cell eluate.

Of these, three represented peptides within the 14-26 amino acid MHC class-II range (see figure 6.5), however only two (Protocadherin Fat 1 and Nuclear receptor corepressor 1) could be identified in the *Homo sapiens* proteome. The third was found to correspond to cell division protein zipA homolog in the bacterial *Pseudomonas putida* proteome.

A MCH class-II eluate of the CML MHC class-II negative LAMA 84 cell line processed in the same fasion failed to present any confident spectra, and the top

[6] Though the BCA figure may be an overestimate given the low (-0.2) HPS of the β2M polpeptide, this low hydrophilicity may lend itself to C_{18} binding, reducing the capacity for MHC peptide retention except by way of any secondary interactions with the β2M itself.

10 peptides listed were all over 27 amino acids in length, with a mean peptide length of 37.33 amino acids. This supports the hypothesis that the above peptides from the THP-1 cell line have a MHC class-II origin, though whether they originated in extracellular material or from the intracellular proteome by way of cross-presentation is unclear, and their immunogenicity is currently uninvestigated.

6.5 MHC peptide database design

The mass spectrometry of MHC eluates rapidly led to a build up in lists of peptide sequences. In order to maintain a high level of order a database was required to contain sequences, protein source data and cell line data. It was decided to utilise Microsoft Access (ver 2003) to generate and manage the database for a number of reasons primarily related to speed and convenience. Chief amongst these were prior experience in database design using this facility, flexibility of the software to accept changes in the structure of an existing database (allowing the addition of new fields, or deletion of pre-existing ones) and the availability of the software, being present on all machines within NTU.

To prevent duplication of data, the SwissProt accession number of each protein was chosen as the key field (/unique record identifier). This allowed peptide sequences to be stored by parent protein, along with details on the protein's function, distribution and potential links to malignancy. In order to facilitate searches, a range of Boolean check-boxes were utilised, allowing exclusions of extraneous records as well as sorting by alphabetic or numeric fields (e.g. accession number or common titles). Secondary research was carried out regarding each protein, and a brief literature review of relevant information composed (the check-boxes conveying additional information). Depending on the protein's function and the information found in the literature review, a 'potential significance' score was assigned manually from 0-9. A screen-capture of the database can be found in figure 6.6.

While data entry into the database is an ongoing effort (currently it contains approximately 200 records), the end result is a tool which allows a user to search for peptides from proteins by their sequence, the protein's role (e.g. receptors, transcription factors, kinases), or links to cancer in general, or specific

forms of malignancy. The search may also be restricted to include or exclude posttranslationally modified peptides, or peptides from a single cell line. It also

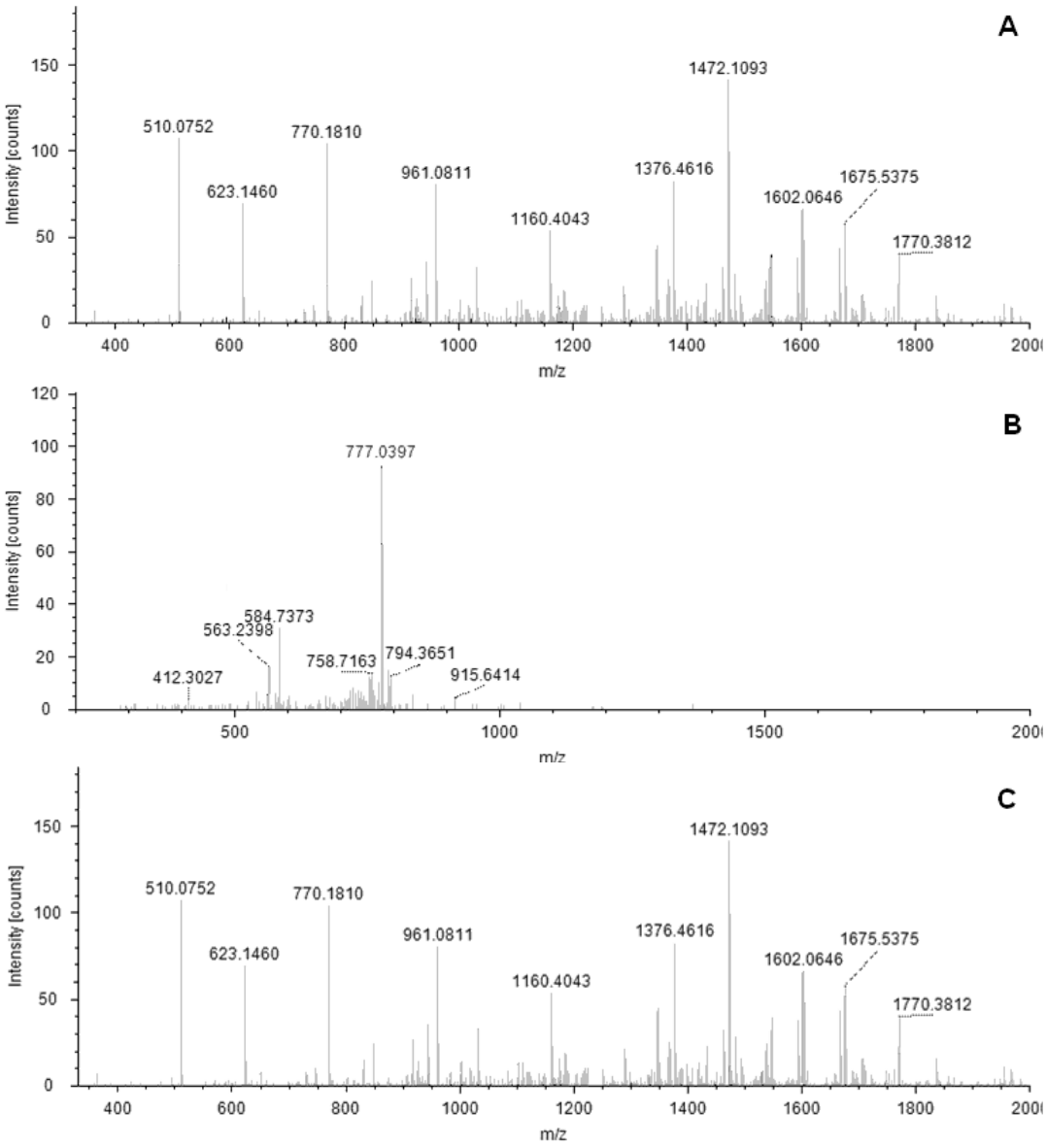

Figure 6.6: Tandem mass spectra (LC-ESI-MS/MS) for three peptides in the MHC class-II mass range from eluates of the AML cell line THP-1 following Fe3+ IMAC and C_{18} RP SPE cleanup. Sequences: (**A**) (Q14517) GKLVYAVSGGNED(pS)CFMIDMET from Protocadherin Fat 1; (**B**) (O75376) K(pS)LITGPSKLSRGM from Nuclear receptor corepressor 1; and (**C**) (ZIPA_PSEP1) (pS)DDDFAADNNRSSGAAPASSSVKE from Cell division protein zipA homolog (from *P. putida*).

allows novel fields (text or check-boxes) to be applied as required, as well as their content to be automatically applied retroactively to all previous records (though if this content is non-uniform, it must be entered/amended manually).

However if this database is considered as a prototype, further versions may require additional features. Currently the primary limitation is the manual nature of data entry. For maximum efficiency, future versions should be compatible with Proteome Discoverer, and allow importing of mass-spectrometric data directly into the database. The ability for keyword-searching of online protein/gene databases such as Expasy and OMIM (and possibly iHOP) would also allow autopopulation of the checkboxes and an automatic generation of significance-score, avoiding any user bias, or complications from multiple user scoring.

6.5 Discussion

While it has been demonstrated that it is possible to elute MHC restricted peptides from the surface of suspension and adherent cell lines using the minimal lysis buffers, and following fractionation and sample concentration/cleanup by chromatography to identify sequences related to malignancy and EBV immortalisation, the results are often restricted to a small pool of high quality spectra, especially in the case of phosphopeptides and MHC class-II peptides.

There are numerous reasons which may lie behind this observation. First and foremost the sample size may not be adequate: the figure of $2x10^9$ cells used for these elution (Bonner 2002) may not be sufficient for a sub-stochiometric population such as phosphorylated peptides, especially given the transient nature of this modification (Ishiai *et al*., 2003), and that peptide phosphorylation alters MHC binding (Mohammed, 2008). Zarling *et al*. (e.g.: 2000; 2006; Depontieu; 2009) typically employed up to 5×10^9 cells for both MHC class-I and class-II analysis, indicating that higher numbers may be required. This may be especially true for MHC class-II phosphopeptides, which given their greater length may allow greater sequence variation and therefore even lower stochiometric concentrations of each peptide. Higher confidence spectra may also be found by the increased sensitivity of the latest ESI and MALDI mass

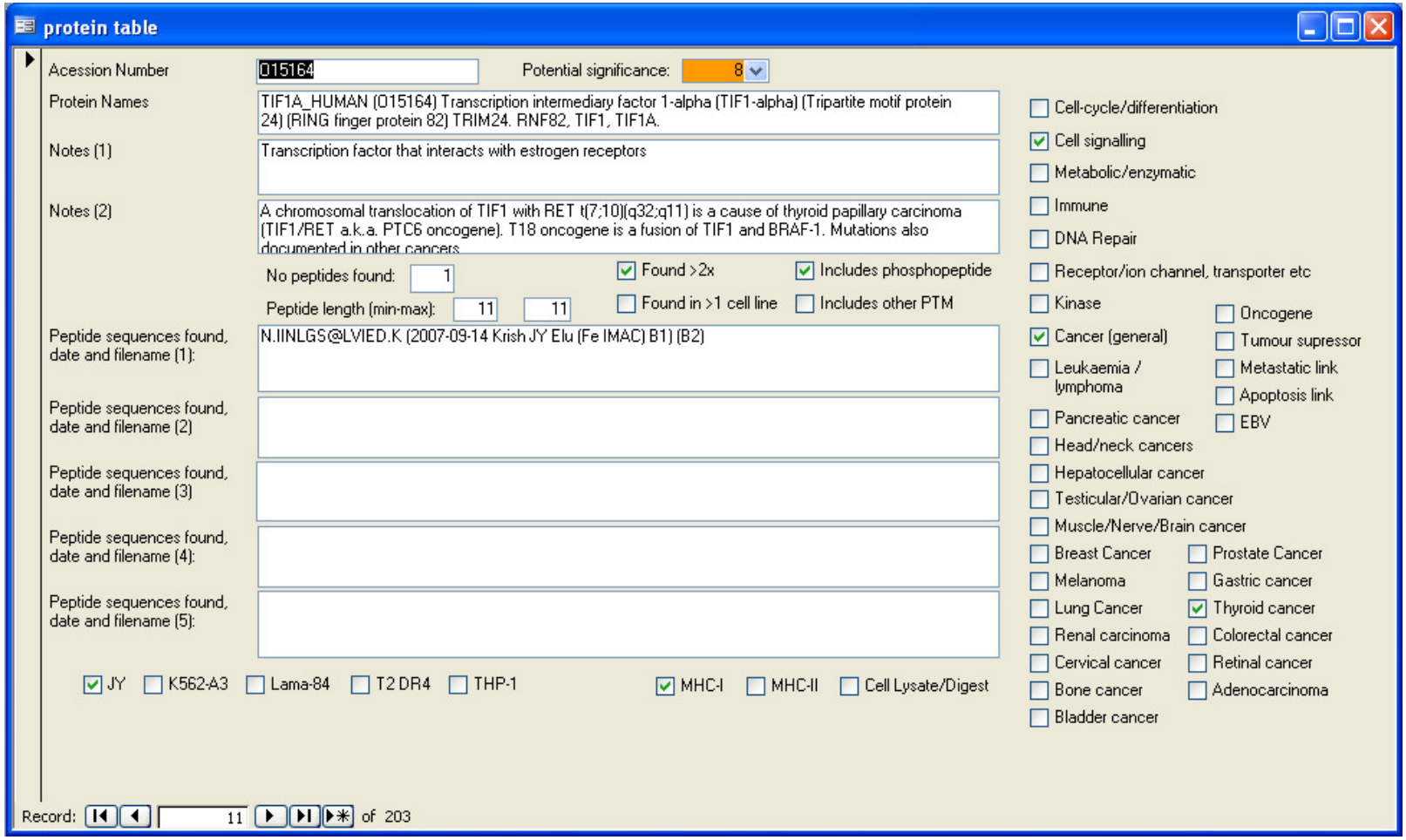

Figure 6.67: In-house MHC peptide database (form view) with the record for TIF1-alpha$_{417\text{-}427}$ ('@' denotes phosphorylation). While this protein is known to undergo multiple serine phosphorylation events in response to DNA-damage, the point in question (pSer422) has not yet been documented. This phosphopeptide was found in duplicate cultures of the same cell line (JY), but not in subsequent elutions.

spectrometry technology, which are > 2 orders of magnitude more sensitive than the Thermo LTQ ESI model (*Pers. Comm*. Dr. David Boocock, Nottingham Trent University, 2010).

As has been shown no one method is capable of isolating the full range of phosphopeptides within the proteome (Bodnar *et al*., 2007); in all probability therefore, Fe^{3+} IMAC therefore only enriches a proportion of MHC-presented phosphopeptides, and whether this proportion differs from tryptic peptides is not known at this time. As when MHC class-II peptide Fe^{3+} IMAC eluates were subjected to C_{18} SPE cleanup the total sample material was found to drop significantly, it is believed that there is a poor overlap between C_{18} and Fe^{3+} IMAC binding which may significantly hinder a concentration dependent method such as mass-spectrometry, and may have lead to an effective underloading of the RP HPLC set-up. As MHC-eluates show high scarcity and long lead-times, method optimisation has largely been performed using tryptic digests of single standard proteins; it is possible that greater optimisation may be achievable using proteolytic digests of cell material.

While it might be argued that there is therefore a strong argument for performing C_{18} SPE concentration prior to Fe^{3+} IMAC, reducing the sample to those which will interact with a C_{18} resin prior to enriching the phosphorylated subcomponent, doing so may remove a high proportion of Fe^{3+} IMAC binding peptides from the sample, and encourage non-specific binding during the IMAC stage. It is believed that merely increasing the concentration of the material loaded onto the LC-ESI-MS/MS will produce improvements in spectra quality, though the use of RP SPE following Fe^{3+} IMAC may allow the selection of phosphopeptides with stronger C_{18} binding properties, and exclude the unbound population, the corresponding chromatographic losses may prove prohibitive.

A simple, though crude method of estimating the increase in concentration required might be to determine the proportion of peptides which bind to a C_{18} SPE column, and multiply the sample loading concentration by this amount (with perhaps a 10-20 % safety margin), though this assumes good inter-resin compatibility and inter-sample variation might lead to overloading of the column.

A preferable option would be to perform the LC stage prior to ESI-MS/MS using a nanoflow Fe^{3+} IMAC HPLC system in a manner similar to Kaur-Atwal *et al*. (2007), though this would require a dedicated HPLC-ESI-MS/MS set-up which was not

available during the current research period, and would of course require its own optimisation.

It is worth noting that many peptides that appeared in earlier MS/MS analyses (analysed with Bioworks Browser/SEQUEST) were not found when those same files were reanalysed by MASCOT, and for the purposes of clarity these are not currently included in the database (Wan *et al*. [2008] noted that MASCOT is superior to SEQUEST in assigning phosphorylation sites). Furthermore, numerous peptides present multiple candidate proteins as parents. For some this is due to identical or near-identical sequences (or masses, leucine and isoleucine are particularly difficult to distinguish from one another by mass). Others have missing ions, requiring the MASCOT algorithm to predict missing amino acids from the net remaining mass (total peptide mass minus the mass of the ions identified). With single absent ions this is easily resolved, as few amino acids will exactly match the net remaining mass. However, two or more missing ions may lead to misidentification as these amino acids may produce different proteome matches when exchanged. The lack of these ions may be due to poor ionisation or fragmentation, or PTMs that were not included in the MASCOT search, yet a greater number of modifications allow a larger number of mass permutations that must be eliminated prior to a putative sequence and protein source, and thus each modification adds an exponential increase to the demands in terms of computing power/time.

The mass spectrometric analysis of phosphopeptides has long been considered more challenging than that of unmodified peptides (Steen *et al*., 2005). While the effect of phosphorylation on binding to reversed-phase columns has already been discussed (see 5.3.1), it is often also stated that the negatively charged phosphate groups hinder positive ionisation of the peptide for mass spectrometry (Barnouin *et al*. (005) (termed lower ionisation efficiency), though work by Steen *et al*. (2005) indicates that this may be less true than is thought, and is a highly variable effect dependent on running conditions, especially for multiply-charged ions (Steen *et al*., 2005). Barnouin *et al*. (2005) and Edelson-Averbukh *et al*. (2006) recommend the use of negative ion mode rather than the classic positive ion approach for improved ionisation efficiency, though this may not be suitable for all phosphopeptides, and may result in reduced efficiency for nonphosphorylated peptides (Gunawardena *et al*., 2006).

Under the collision induced disassociation (CID)[7] approach to ion-fragmentation, phosphate groups on serine and threonine show higher lability, and may be lost from the parent mass as phosphoric acid (-98 Da) prior to full fragmentation (Boersema *et al*., 2009). This neutral loss event (determined by the division of the 90 Da mass between doubly- or triply- charged ions) (Wagner *et al*., 2006; 2007) can be measured to identify phosphorylation sites that would otherwise be missed (Schroeder *et al*., 2004; Syka *et al*., 2004; Wolshin & Weckwerth, 2005). Though this approach currently requires semi-manual data analysis, recently improvements in spectral analysis software (often combining data from multiple mass spectra), used in conjunction with phospho-site prediction algorithms have shown a significant improvements in phosphopeptide identification accuracy (reviewed in Han *et al*., 2008).

Many however have found it simpler to chemically modify the phosphate groups to one with greater durability/convenience for mass spectrometry, often by β-elimination and Michael addition[8] (e.g. Steen and Mann, 2002; Wolshin and Weckwerth, 2005; Xu *et al*., 2007b), though as always reaction efficiencies may play an important role. It has also been suggested that samples processed using reverse-phase set-ups (for either MALDI and ESI-based MS/MS) may be acidified using phosphoric acid (0.1-1%) to reduce hydrophilicity and minimise resultant sample loss (Kim *et al*., 2004; Liu *et al,* 2005). Though the data collected on acidification of (albeit a single phosphopeptide) samples with phosphoric acid prior to C_{18} concentration do not reflect these findings (see Figure 5.7), the addition of phosphoric acid and ammonium salts to the matrix for MALDI-TOF/MS may minimise ionic suppression of phopsphopeptides (Kjellsgtrom *et al*., 2004; Asara *et al*., 1999; Yang *et al*., 2004).

More recently it has been suggested that metal-ion adsorption onto analytical columns may lead to phosphopeptide retention in an IMAC-related manner, and washing columns with, or even inclusion of chelating agents may improve phosphopeptide mass spectra (Winter *et al*., 2009). This actually opens up the possibility of using a step-wise solvent against pH gradient on a reversed-phase or mixed mode resin charged with (e.g.) Fe^{3+}, to perform 2-D LC interfaced ESI-MS/MS for maximum fractionation of a phosphopeptide sample, though the

[7] Or collision activated disassociation (CAD)
[8] Not only is this not applicable to Y-phosphorylation, but it may also result in β-elimination of *O*-linked glycosylations, and the resulting intermediates may be falsely identified as phosphosites (D'Ambrosio et al., 2006).

effect of metal ion loss (especially co-ordinated by the phosphate group) on the spectrometry (and spectrometer) would require investigation.

To conclude, these results indicate the potential for peptides eluted from the MHC by way of the novel IMAC compatible class-I and class-II elution buffers to be characterised by mass spectrometry following chromatographic fractionation, revealing peptides which may be linked to the origins of the cell line in question, and in one instance may represent a known cancer epitope. Nevertheless the frequency of poor spectra suggest that further method development is required in this area (starting with increasing the concentration of samples loaded onto the LC-ESI-MS/MS).

Following on from mass spectrometric identification of a candidate peptide, the next logical stage would be validation of the antigen-of-interest's expression by a MS-independent approach (e.g. PCR, followed by western blotting) before investigation into confirmation of processing and immunogenicity. In the case of phosphopeptides, identification of the kinase responsible for peptide phosphorylation and determination of phosphorylation status in normal tissues are in all likelihood both desirable.

7.0 General Discussion

Previously of minor interest outside of immunology and hormone signalling, the burgeoning field of peptidomics is in no small way linked to the boom in mass-spectrometric approaches to the biomedical/proteomic sciences (as is partly evidenced by the relative under-use of 1D peptide PAGE compared to the standard protein-PAGE method). One has only to study the range of known tumour epitopes today, compared to that two decades ago to see the impact that mass spectrometric identification has had on the discovery of candidate antigens.

It is hoped that the modification of the BCA assay for improved accuracy in peptide measurement will make some modest contribution towards this field. Though the increasing drive towards miniaturisation may render the assay obsolete on its current scale, miniaturisation of colorimetric/absorbance based assays (e.g. NanoDrop® spectrophotometers) indicate that it may continue to play a role in a nano, or even pico-assay set-up (though whether the BCA assay is truly linearly scaleable is unknown). As it may be considered only semi-destructive (the reaction between peptide and reagent is ionic only, though it takes place in a pro-oxidative environment), the assay may also see continued use as a quantification method prior to chromatographic cleanup (though this would likely result in post-quantitative losses). In current usage the modified assay has allowed both the relative quantification of peptide/protein loss from MHC eluted cell lines, as well as improved accuracy in quantification of chromatographic fractions, and applicability to peptide quantification under conditions not suitable for UV absorbance.

While identification of cancer antigens may be by direct or reverse immunology, the prediction algorithms for MHC binding do not as yet take phosphorylation into account, and so the discovery of phosphopeptide epitopes is currently restricted to sampling of peptides from the MHC and sequencing by mass spectrometry.

As discussed, the method for eluting MHC class-I peptides has changed little in two decades, apart from the addition of protease inhibitors, and a general drift away from a strictly isotonic formulation. Though historically contamination of cell surface eluates with non-MHC presented peptides has been the primary

weakness of the approach, more recent findings suggest that the alternative, lysis, immunoprecipitation and elution from immobilised MHC molecules may produce a different, but complimentary peptide population. Using a combination of novel, non-chelating elution buffers, supplemented to minimise cell lysis, and a prewash stage to encourage pH-dependent disassociation of non-MHC presented peptides from the cell surface, this research aimed to produce cleaner MHC class-I eluates, with less contamination by non MHC-presented peptides.

Progress in direct immunology for MHC class-II peptides is something of a younger field, and correspondingly fewer epitopes have been found. The methodology is restricted to elution of peptides from immunoprecipitated class-II molecules (Röhn *et al*., 2005), no viable cell surface method appears to exist in the literature.

Optimisation of a recently developed cell-surface elution buffer for MHC class-II was performed along similar lines as the class-I buffer above, producing levels of cell death/leakage comparable to the isotonic citrate-phosphate widely used for class-I elution. If, as with MHC class-I elution, cell surface elution of class-II reveals an only partially overlapping series of peptides, these may provide a range of new vaccines to stimulate $CD4^+$-mediated immunity by way of MHC class-II leukaemia cells themselves.

Given the relative similarities in structure between the MHC class-I and CD1 complex, another potential source of contamination lies with glycolipid material eluted during peptide elution. Like MHC class-I, β-2 microglobulin has been shown to disassociate from CD-1 in a pH-dependent manner, with CD1b showing the strongest binding, up to pH 3.0 (Polakova *et* al., 1993; Moody and Porcelli, 2003). While the amount of glycolipid contamination of class-I or class-II eluted peptides has not been assessed, the presence of such may affect quantification and chromatographic fractionation (and potentially also mass spectrometric analysis). Removal of these contaminants might be achievable through the addition of (e.g. 10 % v/v) chloroform to eluted peptide material, and would likely have the additional advantage of denaturing any protein contaminants (including proteases, peptidases, kinases and phosphatases). However the proportion of eluted peptide material which would partition into the chloroform, or to the interphase boundary is unknown, and may require investigation with model peptides and glycolipids prior to implementation.

While tumour-specific antigens are the gold-standard for immunotherapeutic agents, these are often difficult to identify. With the exception of novel proteomic entities such as frame-shifted proteins and gene-fusion products, it is difficult to be fully confident that what may appear to be a tumour-specific is not in fact expressed by somatic tissue under any other conditions. Furthermore, in the case of frame-shifts[1], expression of the protein may not be required for malignancy and an immune response may simply leave behind an immunosculptured tumour refractory to that antigen. As abberant kinase activity drives the proliferation of many cancers though signalling networks, downregulation of the proteins involved in these networks can compromise the malignant phenotype. Simultaneously, phosphorylation of proteins not normally co-expressed with these kinases can theoretically create novel tumour specific antigens which are immunologically distinct from their nonphosphorylated counterparts.

Identification of MHC-presented phosphopeptides by mass spectrometry combines the challenges of highly heterogenic samples with that of identifying a sub-stochiometric phosphopeptide population. Therefore sample fractionation and enrichment of the phosphopeptide subcomponent is key to successful characterisation. Both Fe^{3+} IMAC and TiO_2-MOAC have been shown as capable of MHC-presented phosphopeptide enrichment, though in a wider context it is accepted that the phosphopeptide populations they enrich only partly overlap (few options are available for the enrichment of non-*N* phosphorylated peptides). Various chromatographic approaches were investigated, primarily with regard to selective and nonselective phosphopeptide enrichment/retention. While the recently developed mixed-mode HiLIC resin HEA Hypercel showed some promise with regard to phosphoprotein/peptide retention (research ongoing), only Fe^{3+} IMAC showed true selectivity. Following minor method development of the running conditions; including a debuffering wash with a lower molarity version of the loading/wash buffer[2] (enabling a subsequent halving of the elution buffer

[1] Additionally mass spectrometric identification relies heavily on (though is not totally restricted to) proteomic databases. Novel frame-shifts and gene fusions may not appear in these databases unless they have been previously characterised, whereas the same is not true for the determination of a phosphosite.

[2] The use of an isotonic saline wash before treatment with MHC class-I and class-II elution buffers follows the same logic; by debuffering the void volume/intercellular media, achievement of MHC peptide elution may be confidently achieved without concern that the wash buffers may alter the final pH. This may also allow reduction in elution buffer volume, though in the case of suspension cells there is a trade-off, as smaller elution volumes result in loss of a greater proportion of eluted material into the (relatively constant) intercellular space.

molarity / doubling of downstream compatibility, while still rendering a sharp increase in peak fraction concentration) IMAC was applied as a preparative chromatographic stage to concentrate phosphorylated MHC-presented peptides prior to (RP)LC-ESI-MS/MS.

As can be seen, only a limited population of MHC phosphopeptides were sequenced with any confidence (especially true with regard to MHC class-II phosphopeptides), though the predicted protein sources for many of these can be tied back to leukaemia, and in the case of the JY cell line, EBV-linked malignancies. Subsequent research has also indicated that poor chromatographic binding of Fe^{3+} IMAC-isolated MHC-phosphopeptides to C_{18} resins may be at least partly responsible for this, with effective underloading of the C_{18} analytical column as a result. Continuing research aims to increase the loading concentration of peptides and determine if there is a corresponding improvement in the number of confidently identified peptides. Depending on these results the use of RP SPE to concentrate/retain C_{18} binding peptides from the IMAC eluates may also be utilised, though chromatographic losses may prove prohibitive.

Of course phosphorylation is not the only post-translational modification presented by the MHC. Glycosylation, disulphide bridges, deamidation, trifluroacetylation and many other PTMs have been reported (reviewed in Engelhard *et al*., 2006; Petersen e*t al*., 2009). However, in terms of cancer immunotherapy and antigen recognition only glycosylation has received significant attention; both *O*- and *N*-linked glycopeptides have been found to be presented by both MHC class-I and –II (Haurum *et al*., 1999; Xu, *et al*., 2004b; Dengjel *et al*., 2005; Ostankovitch *et al*., 2009), and like phosphorylation, changes in glycosylation has known associations with cancer (Brooks *et al*., 2008) (though the complexity of the carbohydrate chains can significantly complicate analysis) (Freeze, 2003). This is not to imply that changes in other post-translationally modified peptide expression is not a likely outcome of some cancers, but that for most part these modifications lack both the impact and the direct and obvious links to malignancy that phosphorylation has.

Following on from peptide identification by mass spectrometry, it is recommended that the presence of the target gene/protein is confirmed by PCR, western blotting or ELISA, first in the cell line of origin, and then the level of expression in leukaemic patient samples. In the case of phosphopeptides the picture is more complicated; if the kinase responsible is known then its

expression must also be assessed, however if, as is the case for many of the phosphopeptides encountered, the phosphorylation site is not in the literature, this may require confirmation by proteomic means, including immunoaffinity and mass spectrometry of tryptic digests.

Existence and presentation do not however necessarily mean that the (phospho)peptide is immunogenic. While there are efforts to develop *in silico* models to predict peptide immunogenicity (e.g. Tung and Ho, 2007), inmmunogenicity is a product of peptide-TCR interactions and peptide-MHC interactions (as well as the resulting TCR-MHC interactions) and requires experimental confirmation (Yu *et al*., 2004). IF a CTL population specific for the peptide(s) in question can be generated, the immunogenicity of the peptides may be assessed by CTL mediated killing of the cell line of origin (e.g. K562-A3). Again in the case of phosphorylated peptides, the immunogenicity of the non-phosphorylated form should also be assessed.

When activated, the immune system is capable of rapidly and effectively destroying cells and tissues that are recognised as non-self, as evidenced by the reaction against mismatched transplants, or by GvHD, where tissue measuring in kilograms may be rejected (Robson *et al*., 2010). However, the history of T cell-mediated cancer immunotherapy contains many vaccines which appeared highly promising in the laboratory, but which failed to deliver significant effects during clinical trial (Rosenberg *et al*., 2004). This, combined with the relatively recent discovery of a regulatory cell population within each effector immune cell class, and our slowly improving understanding of the role stem cells play in malignancies, suggest that new antigens alone will not be the key to successful cancer immunotherapy. Instead improved understanding of immuno-modulation by cytokines and regulatory cells; how cross-presentation effects immune activity; the immunosuppressive effects of tumours; and the role of cancer stem cells in tumour growth (and how tolerance/immunoprotection of these stem cells operates) are also in all probability required if current efforts into cancer immunotherapy are to be improved upon. As several studies have investigated, *in vivo* priming of cells may be less effective than vaccination of *ex vivo* cell preparations (including allogenic bone marrow transplants) (Barrett and Rezvani, 2007), and multiple peptide or single-peptide-multiple-epitope vaccines may be required to generate large numbers of anti-leukaemic $CD4^+$ and $CD8^+$ lymphocytes, though an effective peptide-only vaccine would have the

advantages of relative stability, lower complexity/risk of pathogen transmission, shorter lead times and far lower cost per patient.

The increasing awareness of the benefits to successful therapy of patient stratification, and classification of tumours based on their genetic and proteomic characteristics (as well as that of the patient), rather than simple morphology or tissue of origin suggest a potential role for cell surface-elution in personalised medicine. It has been shown that responses to peptide vaccines are highly individual (Reynolds *et al*., 1998), and it is possible that even in MHC allele-matched patients all suffering an early stage malignancy with a consistent mutation (e.g. bcr:abl) multiple peptides may be required to elicit a response in all patients. It is conceivable that in the not too distant future the MHC peptide repertoire of leukaemic patients might be sampled using cell surface elution upon presentation (ideally prior to the prescription of kinase inhibitors), and in conjunction with (e.g.) microarray data, potential immunotherapeutic peptides may be identified and used in conjunction with imatinib/dasatinib (etc)[3] (which despite not being capable of clearing a leukaemia when used in isolation, may inhibit bcr:abl dependent recruitment of $CD4^+$, $CD25^+$ T_{reg} cells) (Larmonier *et al*., 2008). Though obviously such an approach would be significantly aided by further improvements in mass spectrometric sensitivity and reductions in computational analysis time. Additionally, recent years have seen a large increase in the range of software that may be used to analyse MS/MS data (including some specifically for phosphopeptide identification), either in isolation or integrated with data from other (often high-throughput) methods (Han *et al*., 2008). These may be aided by alterations to method workflow, such as that of Imanishi *et al*. (2007) who divided their phosphopeptide enriched fraction into two, and dephosphorylated one, significantly increasing the number of correctly identified phosphopeptides. Such approaches may of course be aided by improvements in the selectivity of existing chromatographic techniques, and conversely, improvements in mass spectrometry also allow the use of additional chromatographic dimensions, improving sample fractionation and selectivity.

There is therefore still much work to accomplish and many false leads to be eliminated before immunotherapy for cancer becomes an effective and routine aspect of patient treatment. MHC-presented class-I and class-II peptides appear

[3] A WT-1 vaccine was recently shown to render a previously imatinib-resistant patient sensitive to imatinib, and lead to a complete molecular response (Narita *et al*., 2010 (in a timeframe consistent with an effect at the stem cell level (Rojas *et al*., 2007).

to be a strong candidate for achieving total remission in patients, ideally without the necessity of a stem-cell transplant, and the aberrant phosphorylation patterns found in all cancers may generate tumour specific antigens at the epigenetic level. It is hoped that the methods developed herein aid in the discovery of successful therapeutic agents.

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