

Development of proteomics strategies for the characterisation of endosomal TLR2 signalling

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Declaration

I hereby declare that the following thesis is based on the results and investigation conducted by myself and that this thesis is my own composition. Work other than my own is clearly identified in the text by reference to the researchers or publications. This dissertation has not in a whole, or in part, been previously presented for a higher degree.

Megan Sara Gant

i) Abstract

Liquid chromatography- mass spectrometry (LC-MS) is a highly sensitive analytical tool which can be instrumental for a wide range of applications, including use in the clinic to identify disease biomarkers in patient samples and in the laboratory to study the changing proteome from cells. Buffers and HPLC methods for protein separation were developed for the analysis of therapeutic monoclonal antibodies (MAbs) and human serum.

For serum analysis, protein separation methods were created using the MAbPac column with 0.02 % SDS added to the mobile phase. Coupled with basic reverse phase (BRp) chromatography, it is possible to identify >1000 proteins not present when BRp is used alone. This method was also applied to the analysis of serum, as an alternative to protein depletion and as a method of increasing the number of identifications of low-abundance disease biomarkers.

Toll-like receptors (TLRs) are one of the most widely studied groups of pathogen recognition receptors. plasma membrane (PM) TLRs initiate the pro-inflammatory cytokine pathway, and endosomal TLRs initiate the transcription of type I interferons (IFNs). An exception is TLR4, which is a PM TLR but is known to translocate to the endosome after activation, initiating the type I IFN transcription pathway. However, recent research suggests that TLR2 could also signal from the endosome.

The proteome of TLR2-activated TLR2^{-/-} (KO) and Wild-type (WT) cells were analysed by LC-MS/MS. Basal changes in endosomal proteins were found between KO and WT cells and key signalling differences detected upon TLR2 activation, indicating a role for TLR2 in endocytosis, endosomal signalling, and innate immune response.

The proteomes of phagosomes isolated from TLR2 and TLR4 stimulated WT cells were also analysed by LC-MS/MS. TLR2 was observed on the phagosome even in unstimulated cells, indicating that TLR2 is already present on the phagosome under basal conditions. Changes in phagosomal signalling between TLR2 and TLR4 activated cells shows that TLR2 and TLR4 have different roles in endocytosis and phagosomal signalling.

Biochemistry techniques were employed to investigate downstream endosomal TLR2 signalling, revealing that TLR2 initiation of type I IFN occurs via Interferon regulatory factors (IRF) 1 and 7.

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iv) List of Abbreviations

1D	1 dimension
2D	2 dimensions
ACN	Acetonitrile
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BAF	Bafilomycin A1
BCA	Bicinchoninic acid assay
Beta-ME	β -mercaptoethanol
BMDM	Bone marrow derived MΦs
BRp	Basic reversed phase
BSA	Bovine serum albumin
BSL	Biosafety Level
CDR	Cytosolic DNA receptor
CLR	C-type lectin
CpG	Cytosine phosphate guanine
CRD	Carbohydrate recognition domain
Cxcl10	C-X-C motif chemokine 10
Da	Dalton
DAMP	Danger-associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
DMP	Dimethyl pimelimidate
DMSO	Dimethyl sulphoxide
DNA	Desoxyribonucleic acid
DVB	Divinylbenzene
<i>E. coli</i>	<i>Escheria coli</i>
ECL	Enhanced chemiluminescence
EDTA	Sodium ethylenediaminetetraacetic acid
EEA	Early endosome antigen
EGFR	Epidermal growth factor receptor
ESCORT	Endosomal-sorting complex required for transport
ESI	Electrospray ionisation

EtOH	Ethanol
FA	Formic acid
FACS	Fluorescence-activated cell sorting
FBLN1	Fibulin-1
FBS	foetal bovine serum
Fc	Fragment crystallizable region
FDR	False discovery rate
FWHM	Full width at half maximum
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
GTP	Guanosine triphosphate
h	Hour
HB	Hypertonic buffer
HEK293	Human embryonic kidney
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
ID	Identification
IFN	Interferon
Ifnb1	Interferon beta
IKK β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
IMDM	Iscoe's Modified Dulbecco's Medium
CD11b/ITGAM	Integrin alpha-M
IP	Immunopurification
IRF	IFN regulatory factor
I κ B α	NF-kappa-B inhibitor alpha
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KO	Knock out

KRAS	GTPase KRas
L	Litre
LAMP	Lysosomal-associated protein
LB	Lysogeny broth
LC	Liquid chromatography
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
M	Molar
m/v	Mass/volume
m/z	Mass-to-charge ratio
MAbs	Monoclonal antibodies
MALDI	Matrix-assisted laser desorption/ionisation
MAMP	Microbe-associated molecular pattern
MAP	Mitogen activated protein
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
mg	Milligram
MHC	Major histocompatibility complex
µg	Microgram
µL	Microlitre
µm	Micrometre
min	Minute
MKK	MAP kinase kinase
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
MRC	Medical research council
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry/spectrometer
MS/MS	Tandem mass spectrometry
MSR	MΦ scavenger receptor
MyD88	Myeloid differentiation primary response gene 88

MΦ	MΦ
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NF-κB	Nuclear factor κB
NLR	Nod-like receptor
nM	Nanomolar
NOD	Nucleotide-binding oligomerization domain-containing protein
NP-40	Nonyl phenoxypolyethoxylethanol
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAM ₂ CSK ₄	S-[2,3-bis(palmitoyloxy)propyl]-Cys-Ser-Lys-Lys-Lys-Lys N-palmitoyl-S-(2,3-bis(palmitoyloxy)propyl) Cys-Ser-Lys-Lys-
PAM ₃ CSK ₄	Lys-Lys
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p-JNK	
(T183/Y185)	Phospho JNK Threonine 183 / Tyrosine 185
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pathogen recognition receptor
p-TBK1	Phospho TBK1
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time reverse transcription PCR
RBC	Red blood cells
rcf	Relative centrifugal force
RING	Really interesting new gene
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RNF115	RING finger protein 115
ROS	Reactive oxygen species
RP	Reverse phase

rpm	Revolution per minute
Rs18	30S ribosomal protein S18
RT	Room temperature
s	Second
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAX	Strong anion exchange
SCX	Strong cation exchange
SDS	Sodium dodecyl sulphate
SEAP	Secreted alkaline phosphatase
shRNA	Small interfering ribonucleic acid
SLAMF	Signalling lymphocytic activation molecule
SP3	Single-pot, solid-phase-enhanced sample preparation
TAB	TAK1-binding protein
TAK	Transforming growth factor beta-activated kinase
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-binding kinase
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TCEP	Tris(2-carboxyethyl)phosphine
TCL	Total cell lysate
TEAB	Tetraethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tnf α	Tumor necrosis factor alpha
TOF	Time of flight
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptic Soy Agar

Tween-20	Polyethylene glycol sorbitan monolaurate
UT	Unstimulated
V DC	Direct current
V RF	Alternative current
vATPase	Vacuolar-type H ⁺ -ATPase
WAX	Weak anion exchange
WCX	Weak cation exchange
WT	Wild type
YFP	Yellow fluorescent protein

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1. Chapter 1: Introduction

The innate immune system was previously considered to be a basic response to external pathogens (O'Neill, Golenbock, and Bowie 2013). Its purpose was thought to be limited to the recognition of invading microbes and initiation of a general, unspecified response which alerted the adaptive immune system to respond to the invasion (McDade, Georgiev, and Kuzawa 2016). However, as our knowledge of immunology deepened, so did our understanding of the increasing complexity and diversity of the innate immune system (Mayer et al. 2016).

The innate immune system is the first line of defence against pathogens and is vital for tissue injury healing (MacLeod and Mansbridge 2016; Jamieson et al. 2018). The key initiators of the innate immune response include epithelial cells, which create a physical barrier between the body and the pathogen, and intercept invading pathogens on contact. However, when pathogens penetrate epithelial defensive barriers, they are recognised by PRRs present on the surface of innate immune cells such as monocytes or macrophages (MΦs) (Alberts et al. 2002). MΦs have the dual function of phagocytosis and the initiation of the inflammatory response (Arts and Netea 2017; Daisuke Hirayama, Tomoya Iida, and Hiroshi Nakase 2017). These cells are the primary drivers of the innate immune response and are the first on the scene in the event of an infection. During an infection, they are responsible for the recognition of microbe-associated molecular patterns (MAMPs) found on invading pathogens (Figure 1.1) (McDonald et al. 2016; Martinez-Pomares and Gordon 2020). In addition, MΦs can also detect endogenous danger-associated molecular patterns (DAMPs) generated by either the host as bi-products of metabolic processes, or as a result of tissue damage (X. Liu et al. 2017; Serbulea et al. 2018). MΦs detect these molecular patterns via germline encoded PRRs (Akira, Uematsu, and Takeuchi 2006a) localised on the cell surface, in the cytosol, or on the membranes of intracellular compartments. The PRRs include transmembrane TLRs and C-type lectins, cytosolic Nod-like receptors (NLRs), RIG-I like receptors (RLRs) and cytosolic DNA receptor (CDRs) (Nosratabadi et al. 2017). By detecting MAMPs or DAMPs, PRRs trigger multiple signalling pathways that induce the synthesis and secretion of a range of cytokines that orchestrate early host defence against infection (Hung and Suzuki 2017).

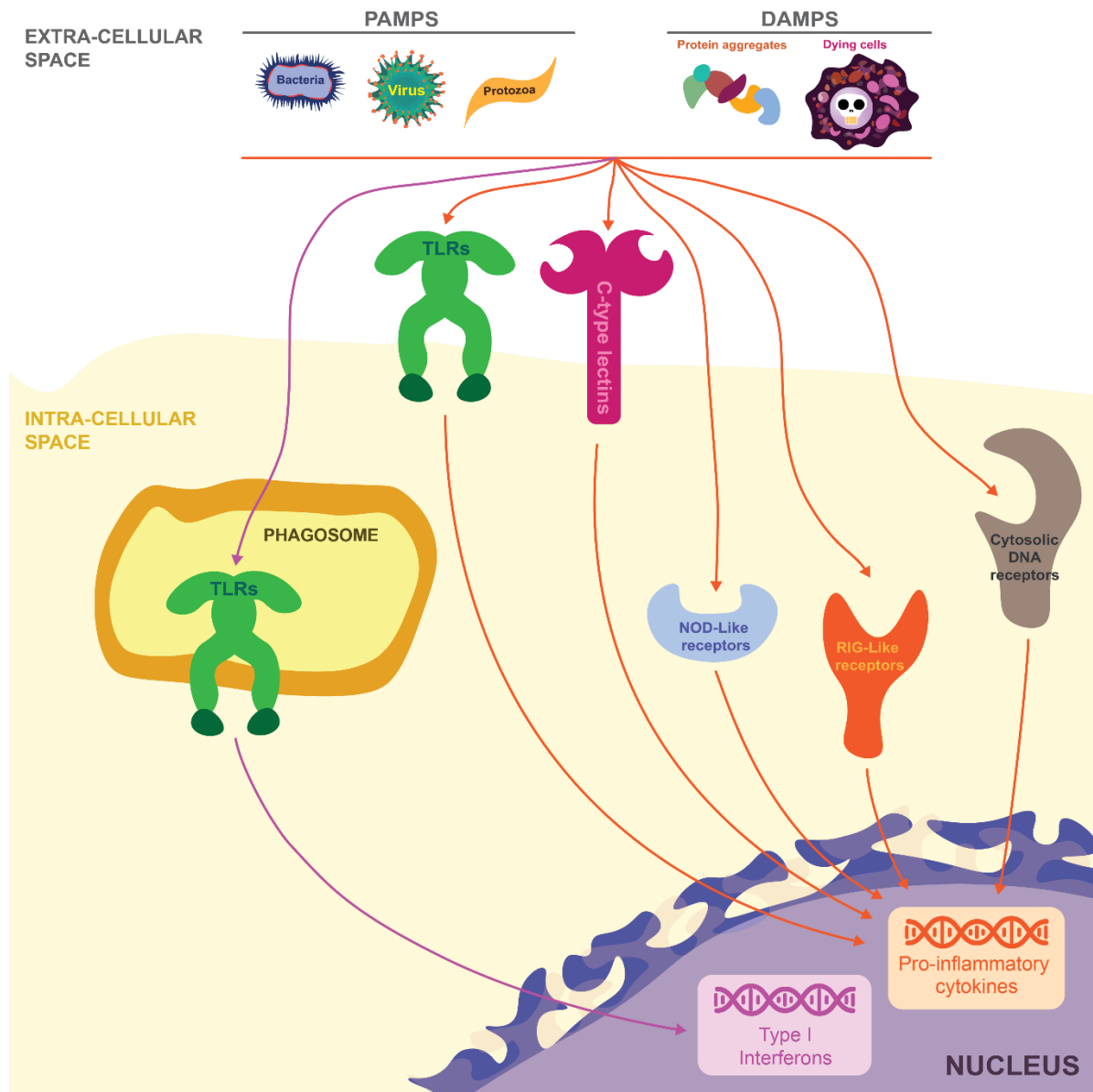


Figure 1.1: PRRs and the innate immune system. Pathogen recognition receptors are located at the PM, the phagosome/endosome, and the cytoplasm, where they detect PAMPs and DAMPs. Upon pathogen detection, PRRs initiate the release of pro-inflammatory cytokines (PM and cytosolic PRRs) or the induction of type I IFNs (endosomal PRRs).

1.1. Immunology

1.1.1. Phagocytosis

Immune cells such as MΦs and neutrophils are first on the scene in the event of an infection (Serbulea et al. 2018). They are found in almost all tissues within the body and have distinct functions relating to their resident tissue (Okabe and Medzhitov 2016). Their role in the innate

immune system is to detect the invading pathogen or endogenous DAMPs via their PRRs and destroy it via a process called phagocytosis (Freeman and Grinstein 2014).

Phagocytosis was first described by Élie Metchnikoff when he punctured the flesh of starfish larvae with thorns and observed specialised cells attacking the invading object as reported in Metchnikoff's book 'Immunity in infectious diseases' in 1901. His subsequent work in immunology birthed the concept of cellular immunity and eventually won him the Nobel prize in 1908, which he shared with Paul Ehrlich (Gordon 2016; Vikhanski 2016).

Phagocytosis is now understood to be an essential process of innate immune response by which large particles ($>0.5\ \mu\text{m}$) are internalised into the phagosome, and partially or wholly disabled or destroyed (Freeman and Grinstein 2014). Phagocytosis serves two key purposes: The first is to engulf, inactivate or kill and destroy the harmful pathogen or cell debris, thereby clearing it from the tissue and maintaining healthy homeostasis. The second is to present antigens from the degraded pathogen in order to activate T cells, thus linking the innate to the adaptive immune system (Lim, Grinstein, and Roth 2017b).

Professional phagocytes clear a wide range of particles from the body. They maintain homeostasis by removing apoptotic cells and protein aggregates and defend the body against pathogens such as bacteria, fungi and viruses (Shaw and Griffin 1981). Several cell types are capable of phagocytosis, including fibroblasts, epithelial cells, endothelial cells (non-professional phagocytes) as well as MΦs, neutrophils, dendritic cells, osteoclasts and eosinophils (professional phagocytes) (Savina and Amigorena 2007). Non-professional phagocytes cannot ingest microorganisms like their professional counterparts but play an important role for apoptotic cell clearance (C. Z. Han et al. 2016).

In order for a phagosome to form, the cell surface receptors must come into contact with a target particle and become activated, triggering cell signalling pathways that activate phagocytosis (Aderem and Underhill 1999). Once this occurs, the actin cytoskeleton is disrupted by coronins (F-actin debranching proteins) and gelsolin (F-actin severing proteins). Once the actin cytoskeleton is disrupted, the actin filaments undergo nucleation which polymerise F-actin filaments and allow the pseudopodia to extend around the particle targeted for phagocytosis (García-García and Rosales 2002). Pseudopods extend and prevent damage to the cell membrane (R. J. Botelho et al. 2000). F-actin is then eliminated from the base of the phagocytic cup and the pseudopodia fuse to completely enclose the particle and seal the nascent phagosome (Schiff et al. 1997). Once the pathogen is inside the cell, it is contained into its own organelle

– the phagosome – thus preventing it from coming into contact with the internal cellular environment and nutrients.

This early phagosome has similar properties to the plasma membrane but will quickly transform into a lethal microbe-killing organelle (Levin, Grinstein, and Canton 2016). The phagosome destroys its cargo by undergoing a process called phagosome maturation, where the membrane and internal environment of the phagosome change composition. This occurs via a cascade of fusion events with early and recycling endosomes and finally the lysosome to form the phagolysosome, where the particle is disabled or destroyed (Figure 1.2) (Rosales and Uribe-Querol 2017).

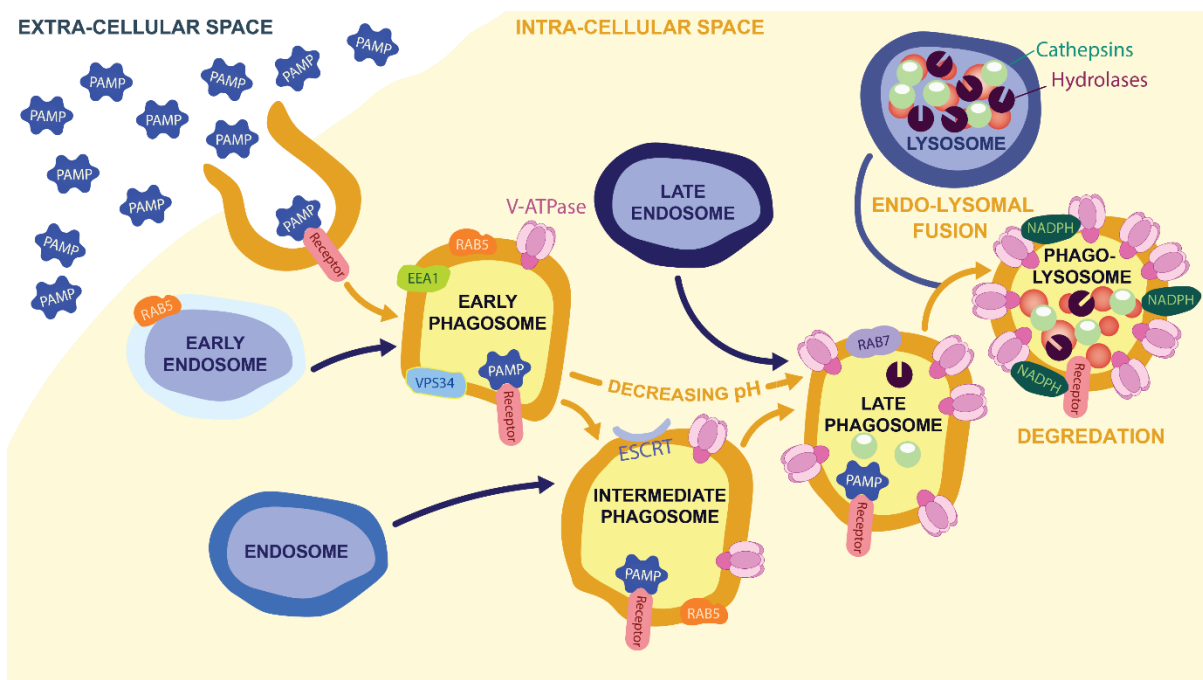


Figure 1.2: Phagocytosis. A PAMP is recognised by a PRR and internalised, forming the early phagosome. Early phagosomes are characterised by markers such as RAB5, EEA1 and VPS34. As the phagosome matures, it fuses with a sequence of endosomes and accumulates vATPases which cause the vacuole of the maturing phagosome to become increasingly acidic. The intermediate phagosome fuses with the endosome and late endosome, acquiring the ESCRT complex which is required for transport. RAB5 is eventually replaced by late endosomal marker RAB7 and finally fusion with the lysosome forms the phago-lysosome, which is full of cathepsins and hydrolases which contribute towards rendering the phagocytosed particle inactive and its full or partial degradation.

The early phagosome fuses with sorting and recycling endosomes and decreases slightly in pH, to around 6.1-6.5. Major histocompatibility complex (MHC) class I are delivered to the early

phagosome by the endoplasmic reticulum and then re-cycled to the cell surface where they send a danger signal to other immune cells (Chemali et al. 2011). Membrane fusion between the phagosome and early endosome is mediated by RAB5, a small GTPase which sits on the surface of the phagosome and is required for the transition from early to late phagosome. RAB5 facilitates the transition by other proteins, such as EEA1 (early endosome antigen 1) which promote phagosome-endosome fusion and RAB7, a facilitator of phagosomal fusion with late endosomes and a marker of a mature phagosome (Araki, Johnson, and Swanson 1996).

The phagosomal lumen becomes increasingly more acidic, due to the accumulation of vATPases delivered by endosomal fusion. The vATPase pumps protons (H^+) into the phagosomal lumen (Kinchen and Ravichandran 2008). This has the overall effect of decreasing the pH inside the lumen of the phagosome, gradually creating a more hostile environment for viruses and bacteria (Marshansky and Futai 2008). As the phagosome matures, RAB5 disappears and is replaced by RAB7 which mediates fusion of the phagosome with late endosomes (Vieira et al. 2003). At this stage of the phagosome maturation process, intraluminal vesicles are formed from inward pinching of the parts of the phagosomal membrane which contains proteins to be degraded. These proteins are ubiquitinated, allowing them to associate with ESCRT complex which is essential for transport of the vesicle to the phagosomal lumen. (Lee et al. 2005).

The phagosome continues to mature into a late-stage phagosome. Accumulation of vATPases on the phagosomal membrane further decrease the pH to 5.5-6.0 and fusion with the late endosome brings lysosomal-associated proteins (LAMPs) and cathepsins and hydrolases to the late phagosome (Schiff et al. 1997; Piplani et al. 2019). Finally, the late phagosome fuses with the lysosome to form the phagolysosome where the phagocytosed particle is finally destroyed in a very acidic (pH 4.5-5.0) environment containing a high concentration of hydrolytic enzymes (Hesketh et al. 2018). Scavenger receptors deplete the iron that is required for bacterial survival and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generates O_2^- which can either dismutate simultaneously or react to H^+ to form O_2 and H_2O_2 , which is a reactive oxygen species (ROS) (Cohen, Heikkila, and MacNamee 1974). ROS are free radicals (molecules containing one or more unpaired electrons) and are produced during metabolism. In the phagosome, they help to disable or destroy the phagocytosed particle (Babior 2004). After degradation, the indigestible content of the phagosome is expelled outside of the cell in a waste packet (Ballabio 2016; Nakamura and Yoshimori 2017).

The MΦ's ability to recognise such a wide range of pathogens is due to the broad spectrum of pathogen recognition receptors (PRRs) located on the cell surface and in the cytosol and endosome (Akira, Uematsu, and Takeuchi 2006a; Nosratabadi et al. 2017).

Phagocytosis requires physical contact with the target particle (Freeman and Grinstein 2014), and recognition of that particle by a surface receptor (Shaw and Griffin 1981). In MΦs, there are two types of surface receptors; the opsonin-dependent surface receptors, which recognise their target via binding to opsonins, such as antibodies, complement or fibronectin, or opsonin-independent surface receptors, which directly recognise conserved molecular patterns (PAMPs) at the surface the targeted particles (bacteria or other pathogens) (Rosales and Uribe-Querol 2013a; 2013b). The opsonin-independent receptor family include lectin-like recognition molecules (such as CD169 and CD33), C-type lectins and scavenger receptors and activation of these receptors is necessary to initiate phagocytosis (Canton, Neculai, and Grinstein 2013; Dambuza and Brown 2015) can also recognise PAMPs and DAMPs (danger-associated molecular patterns, such as those given off by dying cells- like Decorin, Fibrinogen and Heparin sulfate, all recognised by TLR4 (Roh and Sohn 2018)). Although TLRs do not directly initiate phagocytosis, they collaborate with other receptors to stimulate ingestion (Akira, Uematsu, and Takeuchi 2006a).

1.1.2. Pathogen recognition receptors

There are several groups of PRRs, each present at a specific location in the cell. Nod like receptors (NLRs), Rig-like receptors (RLRs) and cytoplasmic DNA receptors (CDRs) are found in the cytosol (Inohara et al. 1999). C-type lectins and TLRs are located at the plasma membrane, however the latter can also be found in the endosome (H. M. Kim et al. 2007; B. S. Park et al. 2009).

PRRs respond to different PAMPs and DAMPs (Akira, Uematsu, and Takeuchi 2006a). NLRs, CDRs and RLRs detect cytosolic danger signals from the cell, including intracellular pathogens and DAMPs (Kumar, Kawai, and Akira 2011). C-type lectins are characterised by their ability to recognise carbohydrates via their carbohydrate recognition domain (CRD), the number of which determines the specificity of the receptor to a microbial ligand (Cambi, Koopman, and Figdor 2005). TLRs respond to a wide range of both PAMPs and DAMPs (Mills 2011).

Just as different PRRs recognise different microbes, they also trigger different signalling pathways (Akira, Uematsu, and Takeuchi 2006a; Kawai and Akira 2010). The two branches of

the innate immune response initiated by MΦs are the pro-inflammatory immune response, characterised by the transduction of pro-inflammatory cytokines which work to promote inflammation in response to trauma, and the transduction of type I IFN, which attempts to prevent intracellular pathogens from replicating within the cell (O’Connell et al. 2005).

1.1.3. The innate immune system

In recent years, it has become apparent that the phagosome is not merely an organelle responsible for elimination of pathogens, but it is also involved in activation and shaping innate immune response (Aderem and Underhill 1999; Murray 2005; Herskovits, Auerbuch, and Portnoy 2007).

Innate immunity is the first branch of the immune system which is activated in the event of an infection (Tetreau et al. 2017; Jamieson et al. 2018). It is a non-specific, fast response which has two main functions: Firstly, the pathogen is engulfed and destroyed by phagocytosis, presenting antigens for the adaptive immune system, and secondly the cell releases pro-inflammatory cytokines and type I IFNs, which activate the JAK-STAT signalling pathway, which results in the induction of interferon-stimulated genes, such as antiviral proteins, chemokines, and antigen-presenting molecules. Hereby the cell recruits other cells to the infection site and initiates the adaptive immune response (S. H. Park et al. 2018).

Phagocytosis is essential for the induction of type I interferons (IFNs) (Garrido et al. 2018). For viral PAMPs to be detected by endosomal TLRs, the virus must be internalised by endocytosis. Therefore, without phagocytosis or endocytosis, the type I IFN innate immune response would be abrogated (Kouwaki et al. 2017). It has also been shown that blocking phagocytosis with cytochalasin D inhibits not only the induction of type I IFNs, but also the pro-inflammatory immune response (Lim, Grinstein, and Roth 2017a).

In order for the innate immune system to work effectively, it must be able to distinguish between the ‘self’ and ‘non-self’ (Hull and Bevilacqua 2016). For example, auto-immune diseases arise when innate immune cells are no longer able to distinguish between cells belonging to the host and foreign cells or pathogens (Connelly et al. 2016). When this happens, the immune system overproduces cytokines which attack its host which can have a variety of repercussions, from allergies to life threatening illnesses such as type 2 diabetes, myositis, multiple sclerosis, rheumatoid arthritis and other chronic inflammatory autoimmune diseases such as Chron’s disease (Duffy and O’Reilly 2016). PRRs play a vital role in distinguishing

which signals come from host cells and which originate from invading pathogens (Tartey and Takeuchi 2017). Many of the non-self signals are detected by TLRs, giving them an essential role in the overall functionality of the innate immune system (McDonald et al. 2016).

1.1.4. Toll-like receptors

TLRs are type I transmembrane glycoproteins composed of an extracellular binding domain made up of a leucine-rich repeat (LRR) motif and an intracellular toll/IL-1 (TIR) domain (Sandor et al. 2003).

Toll receptors were first identified by 1988 by Hashimoto *et. al.* (1988) in *Drosophila melanogaster* and were found to play a role in embryo development and polarisation. Gay and Keith (1991) found that *Drosophila* toll receptors play a role in fighting fungal pathogens by regulating a gene producing drosomycin- anti-fungal peptide. A human toll receptor homolog was identified by Charles Janeway (1997) and was originally named TLR for Toll-like receptor, but was later renamed TLR4 (Murdock and Núñez 2016).

Both TLR4 and the *Drosophila* toll receptor were found to signal via NF-KB and TLR4 was shown to be the receptor for LPS by Poltroak *et al.* in 1998 (1998). Together, these discoveries revolutionised the field of immunology by introducing the concept of PRRs as the initiators of the innate immune response (Akira, Uematsu, and Takeuchi 2006b).

TLRs are highly conserved in evolution, being present in simple organisms such as the nematode (*Caenorhabditis elegans*) (Deande et al. 2018). However, the number of TLRs possessed by different species varies. For example, amongst the animals most studied in science, the zebrafish has 17 (Irazoqui, Urbach, and Ausubel 2010), the mouse has 13 and humans have 10, named TLR1-10 (Bagheri and Zahmatkesh 2018).

In humans, TLRs are present on all innate immune cells (MΦs, dendritic cells, mast cells, NK cells, epithelial cells and endothelial cells (Kawai and Akira 2010; Sheen et al. 2017; Bo Huang, Dai, and Huang 2018)). Depending on the type of cell, TLRs can reside in different locations (McGettrick and O'Neill 2010). For example, in dendritic cells, the TLRs respond to pathogenic RNA and DNA and are present in the endosomes to prevent contact with the host's own RNA/DNA. This arrangement prevents an autoimmune reaction occurring from the activation of the TLRs (De Nardo 2015).

In MΦs, TLRs are produced in the endoplasmic reticulum (ER) and from there transported first to the Golgi complex and then to the plasma membrane (PM) or the endosome (Kawasaki and Kawai 2014). Upon activation, TLRs form either a homodimer (TLRs 3, 4, 5, 9 & 11) or a heterodimer (TLR1/2, TLR2/6, TLR7/8) (Jin and Lee 2008; Manavalan, Basith, and Choi 2011). The dimerisation forms the binding pocket between the horse-shoe extracellular domains and initiates the binding of the ligand and the subsequent internalisation of the receptor (Y.-M. Kim, Brinkmann, and Ploegh 2007). Each group of TLRs responds to a different set of ligands and initiates a distinct downstream signalling pathway (Wagner 2006; Duthie et al. 2011).

TLRs signalling from the PM respond to bacterial ligands such as LPS (TLR4), lipoteichoic acids and lipoproteins (TLRs 1, 2 and 6) or flagellin (TLR5) and adaptor proteins bind to the TIR domain and mediate the release of pro-inflammatory cytokines (Yamamoto, Takeda, and Akira 2004). TLRs that are activated from the endosome respond to viral and endogenous CpG DNA (TLR9), ssRNA (TLRs 7 & 8) and dsRNA (TLR3) from molecules which have been taken up by phagocytosis initiate the type I IFN immune response pathway (Kagan et al. 2008a).

The initiation of TLR innate immune responses occurs via one or more of the following adaptor proteins; MyD88, TIRAP (also known as Mal), TRAM and TRIF. MyD88 is used by all TLRs except for TLR3, which signals exclusively through TRIF (Kenny and O'Neill 2008). Generally, plasma membrane TLRs use MyD88/TIRAP except for TLRs 5, 7 and 9, which only signal via MyD88. Regardless of whether the plasma membrane TLR uses TIRAP or signals only via MyD88, the pro-inflammatory response is initiated via TRAF6 and later, TAB1/2 and NF-κB. The type I IFN response is more varied, utilising different IRFs depending on the activated receptor (Figure 1.3) (Janssens and Beyaert 2002).

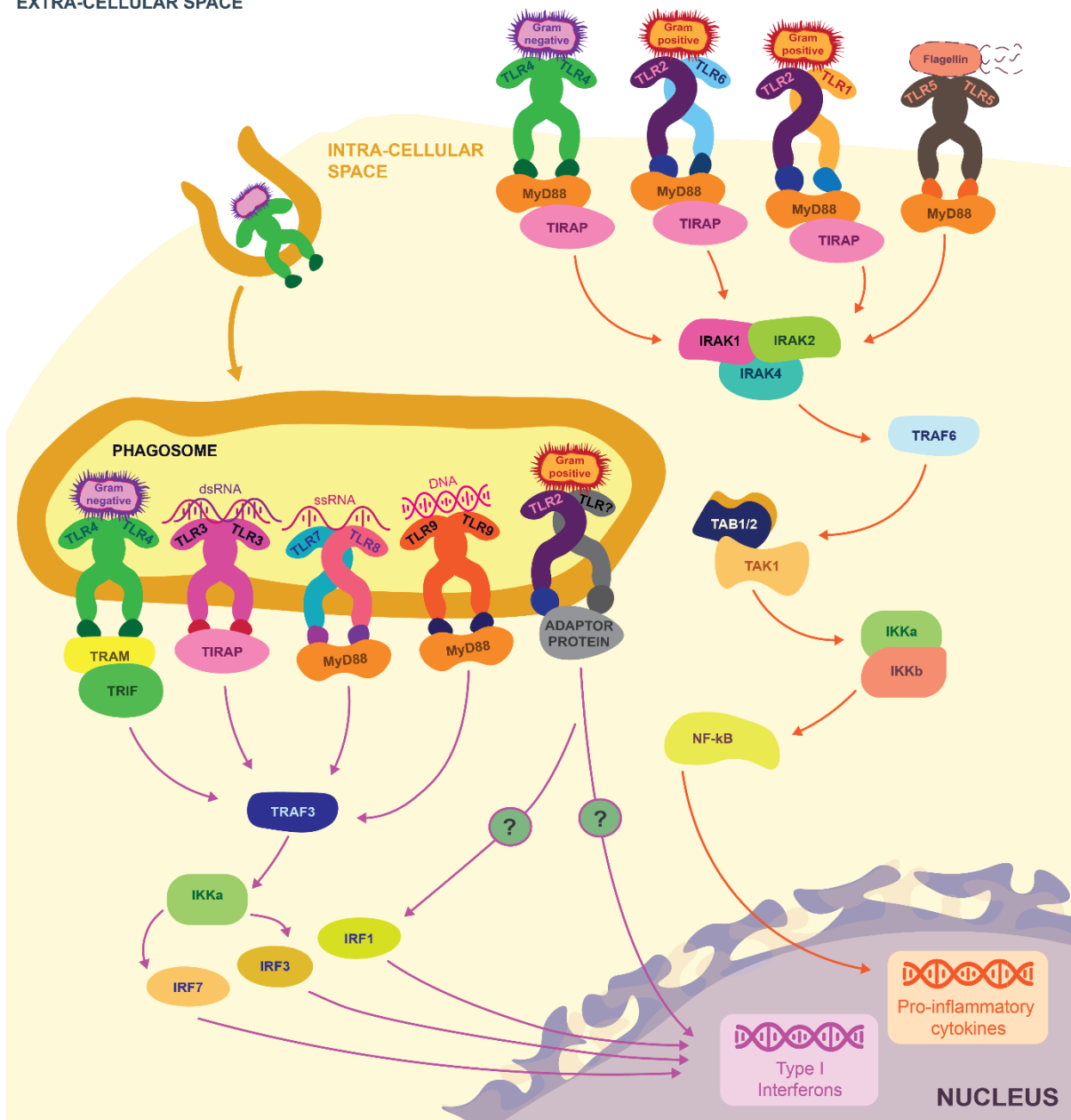


Figure 1.3: Induction of the innate immune response by Toll-like receptors signalling from the plasma membrane and from the phagosome. Plasma membrane TLRs signal via MyD88 and/or TIRAP, which activates IRAK4 which forms the IRAK complex with IRAK1 and 2. Signalling continues via Tab1/2 and TAK1 to activate Ikka and b. They work together to activate the transcription factor NF-κB, which results in the release of pro-inflammatory cytokines. Endosomal TLRs signal via a wider range of sorting and signalling adaptor proteins, including TRAM and TRIF as well as MyD88 and TIRAP. They all activate TRAF3 and then Ikka. Different IRFs are used depending on the pathway.

1.1.5. TLR4 signals from the plasma membrane and the endosome

More recent evidence has challenged the concept of two distinct TLR signalling groups as research suggested that TLR4 could initiate both possible signalling pathways resulting in cytokine production and type I IFN (Kagan and Medzhitov 2006; Kagan et al. 2008a). It has been previously documented that the adaptor protein TIRAP recruits the signalling adaptor protein MyD88 to TLR4 at the plasma membrane where they both bind and initiate the pro-inflammatory signalling pathway (Akira, Uematsu, and Takeuchi 2006b). It was also known that the type I IFN pathway was initiated via TLR signalling through another protein complex (TRAM/TRIF), but details of this mechanism were unknown. Kagan *et al.* (2008) reported that the TRAM/TRIF complex works as a means of recruiting MyD88, which initiates IRF7 pathway leading to activation of IFN β and, therefore, type I IFN production, and that TLR4 does not initiate both pathways simultaneously (Kagan et al. 2008). Instead, the pro-inflammatory pathway is initiated when TLR4 is located at a lipid-rich subdomain of the plasma membrane. TIRAP binds MyD88 and recruits it to TLR4 where TLR4 binds the MyD88/TIRAP complex via homologous TIR domains. TLR4 is then endocytosed. As it moves away from the lipid rich plasma membrane sub-domain, the MyD88/TIRAP complex dissociates and initiate the pro-inflammatory signalling pathway whilst TLR4 has moved to the endosome and therefore closer to the adaptor protein TRAM3 (Johnson, Li, and Pearlman 2008; McAllister et al. 2013). TRAM3 preferentially binds to proteins on the endosome and it also binds to TLR4 via a homologous TIR domain in a complex with another adaptor protein, TRIF (Palsson-McDermott et al. 2009). This activation of the TRAM/TRIF complex initiates interferon regulatory factors (IRFs) which leads to the regulation of type I IFN. TRAM-TLR interaction appears to be reliant on TRAM being physically close to the activated TLR in order to initiate type I IFN regulation from that TLR (Verstak et al. 2014). Bonham *et al.* (2014) showed that if TLR2 is moved closer to TRAM3 a type I IFN regulation from TLR2 can be initiated (Bonham et al. 2014). At the time of the study, this was the only useful information with regards to TRAM3 signalling mechanisms, but more recently it bears more significance with respect to TLR2 signalling.

1.1.6. The emerging role of TLR2 as a trans-locational TLR

TLR4 is now well documented as a trans-locational PRR, but recent work has demonstrated that TLR2 could also be capable of initiating both the pro-inflammatory and the type I IFN pathways from the PM and endosome, respectively (Gangloff 2012; Guijarro-Muñoz et al.

2014; Ruangkiattikul et al. 2019). The TLR2 signalling pathway from the plasma membrane is well characterised (Farhat, Riekenberg, Heine, Debarry, Lang, Mages, Buwitt-Beckmann, et al. 2008). TLR2 forms a dimer with TLR1 or TLR6 and signals via MyD88 and TIRAP to produce pro-inflammatory cytokines (Farhat, Riekenberg, Heine, Debarry, Lang, Mages, Buwitt-Beckmann, et al. 2008). A role for TLR2 as an inducer of type I IFNs was not known until work by Barbalat *et al.* (2009) showed that TLR2 can signal from the endosome. However, this study concluded that the TLR2-Type I IFN signalling was initiated only in response to viral ligands and that this signalling was only possible in inflammatory monocytes.

More recently Dietrich et al. (2010). showed that TLR2 also triggers the type I IFN pathway in response to bacterial ligands and that TLR2 re-locates to the endosome where it induces IFN β via two different signalling pathways- MyD88/IRF7 and MyD88/IRF1. This is reminiscent of TLR4 signalling from the endosome. Other studies by Nilsen et al. (2015a) and Stack et al. (2014) found roles for TRAM and TRIF in TLR2 endosomal signalling and uncovered a function for MyD88 in the TLR2 induced type I IFN pathway, where MyD88 acts as a transporter protein to facilitate signalling from the TRAM/TRIF complex at endosomal TLR2 to signalling via IRF1/2, IRF3 and IRF7.

However, whilst it is established that TLR2 localised at the endosome can initiate production of IFN β , the mechanisms of the TLR2-IRF signalling remains unclear. Unravelling the mystery behind TLR2 induced type I IFN induction is important because a new role for TLR2 would change our understanding of innate immune response pathways. This is especially important for drug development, as these pathways are integral for the development of several immune diseases, including type I diabetes and Chron's disease, where overexpression of PRRs are linked to disease (Farrugia and Baron 2017), Rheumatoid arthritis, where TLR3 is activated by RNA released from necrotic cells (Mohammad Hosseini et al. 2015) and myositis. TLRs 3, 7 and 9 are upregulated in tissue affected by myositis but their involvement in the disease remains unclear (Duffy and O'Reilly 2016). The ability to target parts of the innate immune pathway which contribute to the onset of illness is a key step in the drug development process (Jiménez-Dalmaroni, Gerswhin, and Adamopoulos 2016).

1.2. Mass Spectrometry

1.2.1. *Protein identification and quantification*

The study of the proteome can tell us a great deal about cellular functions on the protein level. Large-scale, untargeted experiments can provide swaths of data detailing thousands of proteins and is an effective way to identify novel roles for known proteins, as well as novel proteins that may play a role in a specific biological system (Aebersold and Goodlett 2001). Whilst several techniques are applied to the study of the proteome, the most widely used and effective is mass spectrometry (MS) (Aebersold and Mann 2003).

Mass spectrometry-based proteomics can be divided into two main strategies: top-down proteomics, which focus on analysing intact/undigested proteins, and bottom-up proteomics, where protein is digested using specific proteases and peptides are analysed (Catherman, Skinner, and Kelleher 2014).

The latter is the most widely used approach, as peptides are often simpler to analyse than proteins. Peptides are more stable and soluble than proteins at low pH without requiring any additive such as detergents (Bogdanov and Smith 2005; D. Botelho et al. 2010). These detergents are usually avoided in mass spectrometry-based experiments as they can contaminate both the liquid chromatography and the mass spectrometer. This is because salt is difficult to desolvate, making it likely that water will enter into the MS system. What does desolvate then competes with the analyte for ionisation, causing an ion suppression effect. As salt can be deposited in all areas of the MS system, it is very difficult to clean and remove. (X. Han, Aslanian, and Yates 2008a; Catherman et al. 2013). Peptides also present a higher ionisation efficiency than intact proteins- as more of the amino acid sequence is exposed due to the reduction and alkylation process- thus allowing for higher sensitivity, and MS/MS spectra of peptides are easier to interpret than intact proteins and often results in a higher sequence coverage and overall better identification rate (Steen and Mann 2004).

In top down proteomics, intact proteins are either pre-fractionated and injected or directly injected to the mass spectrometer (Catherman, Skinner, and Kelleher 2014). It has some advantages compared to the bottom up approach; namely that in bottom up proteomics, it is not uncommon to find several peptides that cannot be assigned to any one protein or that not all protein regions can be identified, which is an issue for post-translational modification (PTM) identification (D. Botelho et al. 2010). Analysis of intact proteins allows for 100 % sequence coverage and is more effective for identifying different proteoforms (Zhou et al. 2012; Savaryn

et al. 2013). However, complex mixtures cannot be analysed using the top-down approach and is therefore usually restricted to the analysis of simple mixtures or an analysis of a single protein of interest (Catherman, Skinner, and Kelleher 2014).

To extract peptides from a protein sample, the tertiary structure of the proteins must be broken to expose the protein backbone where cleavage occurs (Glatter et al. 2012). Exposure of more cleavage sites enables more complete digestion, which produces shorter peptides (Norrgran et al. 2009).

1.2.2. Proteomics sample preparation

1.2.2.1. Cell lysis

Before proteins can be analysed by mass spectrometry, they need to be extracted from the sample. Proteins can be extracted from cells or tissue in either denaturing or native conditions (Aebersold and Mann 2003).

For complete lysis, the solubilisation of the sample in SDS or urea will break down all membranes present within the sample, including those of organelles, and will denature proteins. Sonication can be used as an aid to lysis with surfactants as it will also shear DNA and is probably the most effective method of preventing further enzymatic reactions within the sample. To prevent further binding from DNA, a nuclease such as Benzonase can be added to the sample. However, it is impossible to isolate whole organelles using methods of complete lysis (X. Zhang 2015).

To achieve incomplete or 'gentle' lysis, mechanical lysis can be used, such as that achievable with a Dounce homogeniser. Hypertonic buffer (HB) is added to the cells or tissue before mechanical lysis, which aids lysis by permeating and weakening the cell membrane. Mechanical lysis is usually used when isolating specific organelles from a cell, as it will not affect the membranes of phagosomes or vacuoles. Organelles, for example phagosomes, can then be separated from the total cell lysis by phagosome isolation (Desjardins 2001).

1.2.2.2. Reduction and alkylation

Reducing agents are used to reduce the disulphide bonds between cysteine residues that hold the tertiary structure of the protein together. Once the protein has been reduced, there is still a

risk of the disulphide bonds re-forming. An alkylating agent, such as chloroacetamide or iodoacetamide, is then used to block the highly reactive and newly formed thiol groups, thus preventing the disulphide bonds from reforming (Person et al. 2005) (Figure 1.4).

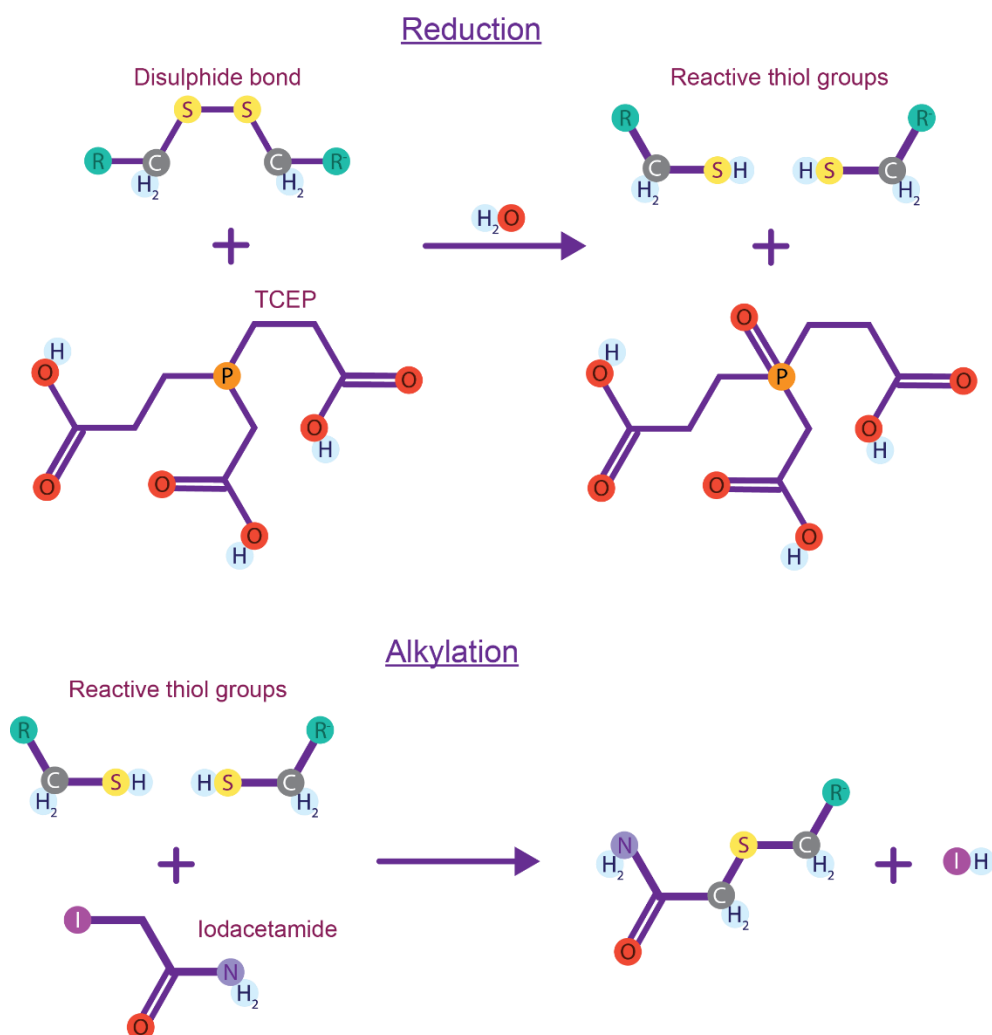


Figure 1.4: Reduction and alkylation with TCEP and iodoacetamide. Reduction of disulphide bonds with TCEP, leaving a sulphydryl group. Alkylation reacts with the sulphydryl group and ‘seals’ it, preventing re-formation of the original disulphide bridge.

1.2.2.3. Digestion

The digestion of proteins into peptides is the key to bottom up proteomics (Gundry et al. 2009). This approach is so popular because analysing peptides instead of the intact protein removes many problems involved with whole protein analysis. Digestion of proteins is usually achieved by use of a protease, such as trypsin (Norrgran et al. 2009).

1.2.2.3.1. *In solution digestion*

Trypsin is the most commonly used protease for digestion. It is an extremely stable enzyme, active in up to 2 M urea, 2 M guanidine or 0.1 % SDS, which cleaves proteins at the C-terminal side of arginine and lysine residues with high specificity (Vandermarliere, Mueller, and Martens 2013). This specific cleavage is desirable because it creates peptides with a basic residue at the C-terminal end of the peptide. At the low pH used in mass spectrometry based proteomics, the two amine groups resulting from the tryptic digestion (N terminal NH₂ and C terminal side chain from Lysine and Arginine residues) can easily be charged, thus facilitating the detection of the peptides and their fragments in the mass spectrometer (Steen and Mann 2004). Due to the specific abundance and distribution of Arginine and Lysine residues in the proteome, tryptic peptides are usually 8 to 20 amino acids long. This allows for the generation of proteotypic peptides (unique to a single protein) while shorter peptides can be assigned to different proteins than the ones they actually originated from (Lowenthal et al. 2014). On the other hand, longer peptides would generate more complex and challenging MS/MS spectra, resulting in lower identification rate (Gundry et al. 2009). Finally, trypsin is also a small protease than can easily penetrate a SDS PAGE gel, an attribute which allows for protein identification directly after 1D and 2D gel separation (X. Zhang 2015; Lowenthal et al. 2014; Wiśniewski and Rakus 2014; Groseclose et al. 2007).

Lys-C is another highly specific enzyme that can cleave at the C terminus of Lysine residues, with minor activity against Arginine residues. Lys-C is often used prior tryptic digestion as it can resist up to 8 M Urea and is stable at temperatures up to 70 °C. These conditions can be used to help digesting insoluble proteins that would precipitate in less stringent conditions (X. Zhang 2015).

Other enzymes, such as Glu-C, Chymotrypsin, Asp-N or Lys-N, can also be used (Giansanti et al. 2016). However, these enzymes are usually less specific, generate shorter peptides and are not as commonly available as Trypsin. As these peptides do not end with a basic residue, they can easily undergo internal fragmentation when analysed by MS/MS, rendering identification of such peptides much harder (Glatter et al. 2012; Norrgran et al. 2009; Turriziani et al. 2014).

1.2.2.3.2. *Precipitation based, solid-phase-enhanced sample preparation*

One method for combined de-salting and digestion is the precipitation based SP3 protocol, originally developed by Hughes *et al.* (2014). The columns work by conditioning the phase with an organic solvent such as methanol, which removes air bubbles and wets the phase. The sample is then introduced to the phase where the proteins bind via van-der-waals forces/hydrogen bonds, dipole-dipole ‘polar’ bonds or cation/anion interactions (ionic bonds). The proteins can then be washed with 50/50 v/v solvent and water which is enough to remove salts and detergents from the proteins but not enough to elute the proteins from the phase. Finally the proteins can be eluted from the column into a clean sample collection tube using water (Pucci *et al.* 2004). Proteins are digested overnight, and peptides eluted from the beads in 2% DMSO. Peptides prepared using SP3 are ready for immediate analysis by MS (Hughes *et al.* 2019).

1.2.2.3.3. *S-trap*

SP3 has been further developed by Protifi into the S-trap which is an all-in-one process (Figure 1.5), combining cleanup/ de-salting and digestion into one protocol. Proteins are bound to a solid phase, washed, and digested in a simple series of steps. Peptides are eluted from the column with trypsin and are ready for fractionation or direct injection to the mass spectrometer (HaileMariam *et al.* 2018). Advantages of the all-in-one workflows where proteins are cleaned and digested on the column include minimizing variation and sample loss between samples while providing a faster and more efficient workflow (Zougman, Selby, and Banks 2014).

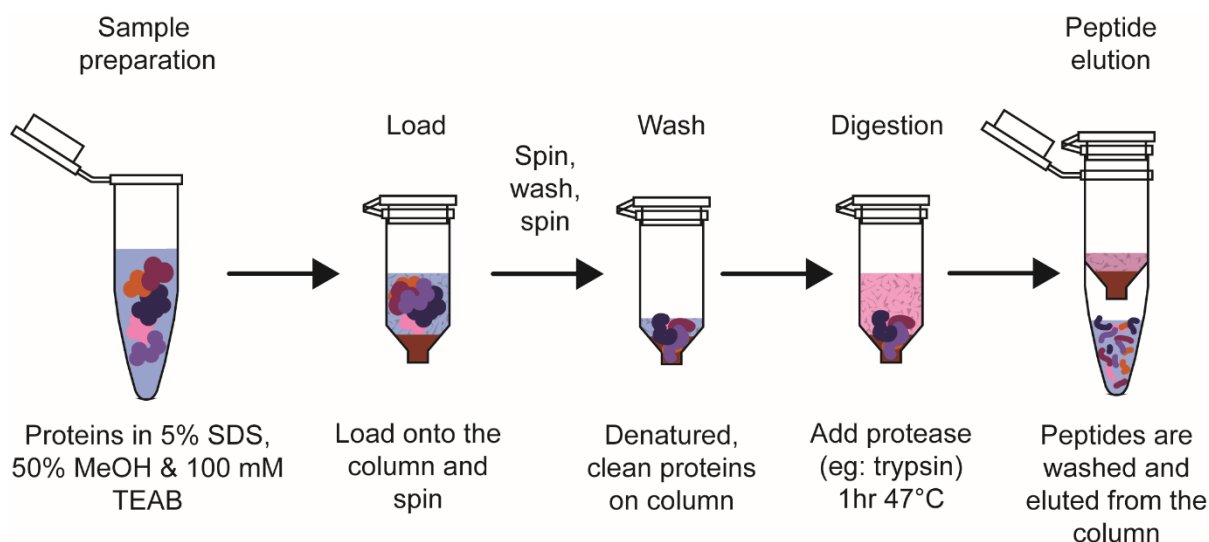


Figure 1.5: S-Trap workflow. Proteins are solubilised in 5 % SDS, reduced, alkylated, acidified, and loaded onto the column where they bind to the phase. Whilst bound, they are washed with organic solvent (90 % MeOH, 100 mM TEAB, pH 7.1) and digested with trypsin for 1 h at 47 °C. Once digested, peptides are eluted with 50 mM TEAB and 5 % formic acid, and 50 % acetonitrile and 5 % formic acid together for hydrophobic peptides. Samples can be injected directly to MS.

1.2.2.4. Sample De-salting (Cleanup)

Traditionally, cells are lysed in 8M Urea, reduced, alkylated, and digested. However, the more advanced HPLC-MS instruments are sensitive and unable to handle high amounts of salts or detergent (Gundry et al. 2009). Therefore, it is necessary to de-salt or clean up the sample before injection to the mass spectrometer (Jehmlich et al. 2014). There are various methods which can be used, including the use of solid-phase extraction (SPE) (Moggridge et al. 2018), such as the C18 columns which usually work by trapping peptides via hydrophobic interactions (Lehotay, Han, and Sapozhnikova 2016; Tubaon, Haddad, and Quirino 2017).

1.3. HPLC

Despite the high scanning rate of modern mass spectrometers, biological samples are usually too complex to achieve a deep proteome coverage using direct analysis/infusion. To overcome this problem, samples are usually gradually introduced into the mass spectrometer by fractionation. In proteomics, the most common technique in use today is high-performance liquid chromatography (HPLC) (Figure 1.6) (Mitulović 2015). The basis of HPLC is the separation of molecules according to their chemical properties- for example, hydrophobicity or

charge. During HPLC separation, the molecules are first loaded onto a chromatography column (the stationary phase) and are eluted by applying a solvent (mobile phase) onto the column (Gokce et al. 2011; Richard et al. 2017).

Standard MS-based proteomics usually uses a single on-line separation usually using reverse phase columns. However, if a deeper separation is required for a particularly complex sample, or fractionation by another chemical property is desired, the use of an off-line HPLC to pre-fractionate the sample can be helpful. Fractionating a sample using both an on-line and an off-line HPLC is termed 2D-LC (Figure 1.6). 2D-LC is most effective when the sample is fractionated using orthogonal methods to separate the peptides based on different chemical characteristics (Dwivedi et al. 2008; Richard et al. 2017). For example off-line fractionation using hydrophilic strong anion exchange (hSAX) phase which separates peptides by charge (Ritorto et al. 2013; Ruprecht et al. 2017; Giese, Ishihama, and Rappsilber 2018) and on-line fractionation using a reverse phase which separates by hydrophobicity (Yabré et al. 2018).

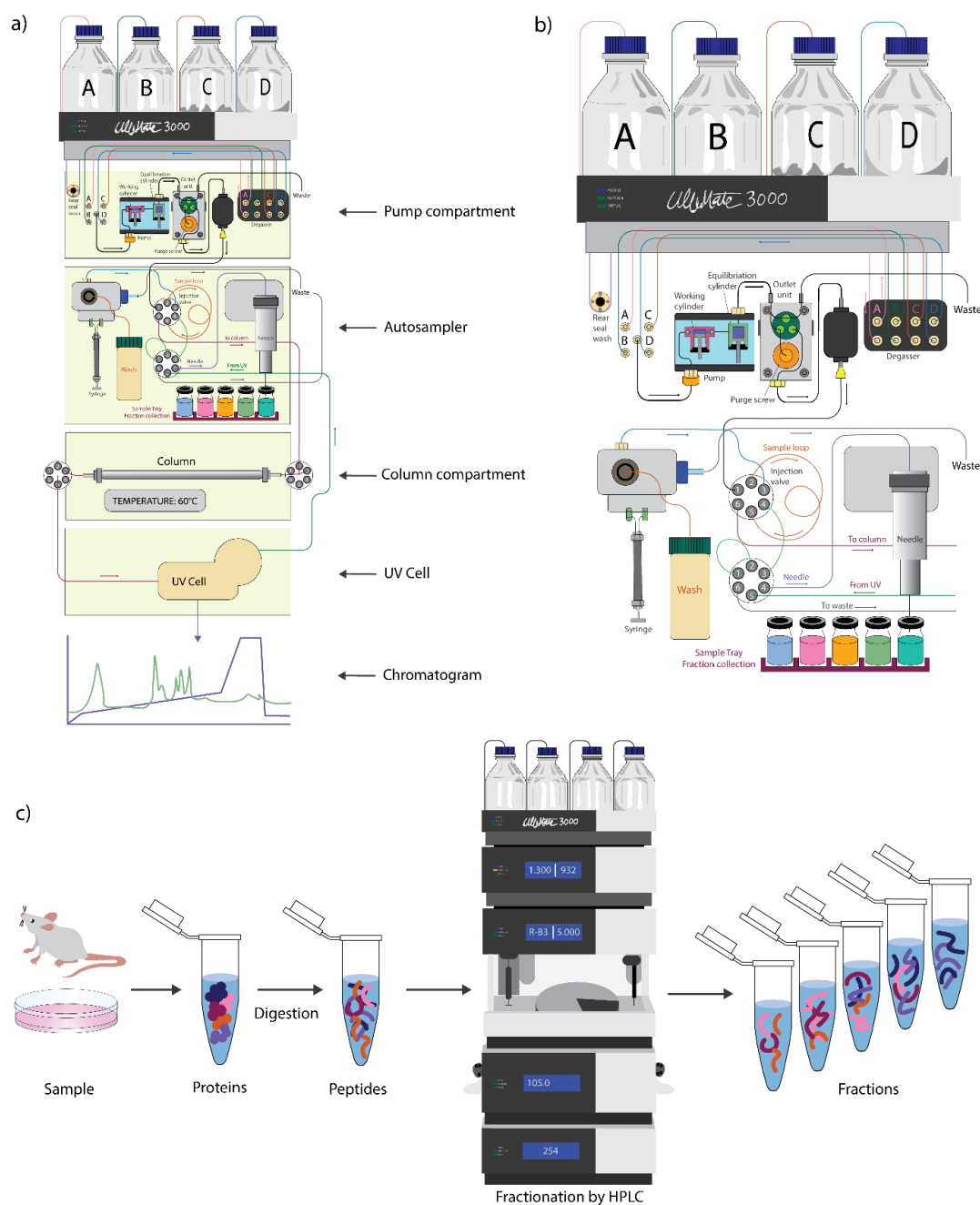


Figure 1.6: Fractionation of peptides by HPLC. a) Scheme showing an overview of each unit of the Thermo Fisher Scientific Ultimate 3000 HPLC. This system is made of five units: the degasser, pump module, autosampler, column oven and UV cell. Mobile phase is taken up by the pump and travels through a degasser before reaching the autosampler. The sample is stored in the autosampler and taken up by the needle and held in the sample loop. The mobile phase pushes the sample into the column (held in the column oven). The pump controls the gradient of mobile phase A: B. Once the sample leaves the column, it travels through the UV cell where the chromatogram is recorded and then back to the autosampler where it is deposited by the needle into vials in the sample tray that collect each fraction. b) Detail of the pump module and the autosampler, showing the mobile phase and sample path in the autosampler. c) Workflow for off-line HPLC fractionation of a peptide sample. Proteins are isolated from cells and digested into peptides. Peptides undergo fractionation by HPLC, where peptides of similar chemical properties are eluted together as indicated by the colours of the peptides shown.

1.3.1. Reversed phase HPLC

For reverse phase (RP) HPLC, the peptides are bound to a column containing a hydrophobic stationary phase. This is usually made of a range of alkyl chains, such as C18 (octadecyl) for peptide analysis or C4 (butyl) for proteins (Figure 1.7 a). The mobile phase is a mix of water, an organic solvent such as ACN or MeOH (Gokce et al. 2011; Mitulović 2015; Spicer et al. 2016). Peptides are loaded onto the stationary phase using low concentration of acetonitrile and then gradually eluted with increasing concentrations of acetonitrile. The more hydrophobic the peptide is, the later it will elute (Figure 1.7 b & c).

Buffers are usually supplemented with acids or bases to alter the retention of peptides onto the columns and/or their ionisation. The addition of ion pairing agents, such as FA or TFA, increases the hydrophobicity (and therefore retention) of the peptides, resulting in better chromatographic resolution (H. Wang et al. 2015). However, TFA is such a strong ion pairing agent it also induces an ion suppression effect when coupled directly to the MS, and therefore results in a loss of sensitivity. Consequently, TFA is reserved for offline separation or loading onto a trap, while the weaker ion pairing agent FA offers better ionization without compromising the chromatographic resolution.

Using weak bases (such as ammonium) offers different retention compared to FA and can be used in a 2D-LC strategy using as a first dimension basic reversed phase and as a second acidic reverse phase (Gilar et al. 2005). As the two separation methods are based on hydrophobic interaction, it is usually recommended to perform non-consecutive fraction concatenation to offer a higher peak capacity (Y. Wang et al. 2011). Such a strategy is usually only used for offline 2D-LC as weak bases will increase the pH of the buffer to basic condition and will result in poor ionisation and fragmentation of peptides in the MS.

A combination of two acidic and one basic reversed phases have even been used by Oleg Krokhin's team to develop a 3D HPLC workflow for bottom-up LC-MS (Spicer et al. 2016).

The reversed phase column is a flexible tool for proteomics. RP is not limited to peptides separation and other RP columns, such as the MAbPac™ columns from Thermo Fisher Scientific, have been specifically developed for separation of Antibodies. The MAbPac™ is a divinylbenzene (DVB) monolithic phase with a wide pore size of 1500 Å which allows proteins to bind.

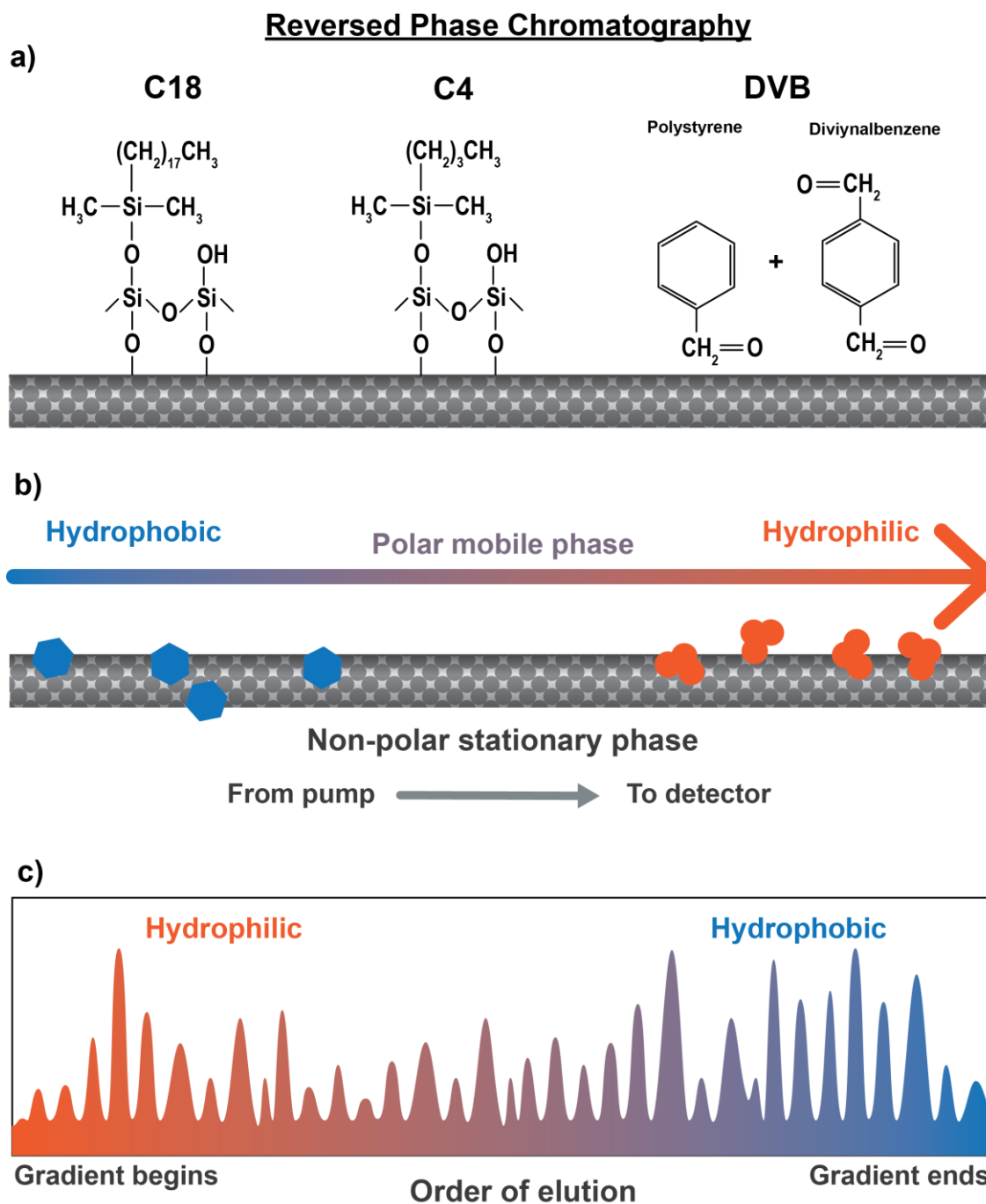


Figure 1.7: Reverse phase stationary phases. a) Chemical structure of C18/C4 reverse phase bonded to a silica base, and polystyrene DVB phase. b & c) Reverse phase order of elution according to hydrophobicity when polar solvents are used for the mobile phase.

1.3.2. *Ion exchange HPLC*

Ion exchange chromatography separates peptides by charge and is often used in conjunction with reversed phase for 2D-LC, as together these methods can provide excellent orthogonality. Ion exchange chromatography uses either a strong or weak cation (SCX/WCX) or anion (SAX/WAX) exchanger, which contains a silica phase cross-linked with a charged group (negatively charged phase for cation exchange, positively charged phase for anion exchange) (Ruprecht et al. 2017; Giese, Ishihama, and Rappsilber 2018). The mobile phase is usually a salt gradient such as NaCl and water. The Na⁺ or Cl⁻ ions compete with the positively charged analyte for binding space on the phase (Figure 1.8 a & b). At the beginning of the gradient, the mobile phase contains low concentration of salt which allows the analyte to interact strongly with the stationary phase. As the gradient progresses and the salt levels in the mobile phase increase, the analyte is pushed off the phase and eluted from the end of the column. When a salt gradient is used, several re-binding events can occur before the analyte finally exits the column. For this reason, columns and gradients need to be long -for example, a 30 cm column might be coupled with a 1 h long gradient. This is why ion exchange columns used with a salt gradient are not usually suitable for high-throughput separations or clinical applications where the aim is to save time (Floyd et al. 1986; Neill et al. 2015).

Although salt gradients can achieve excellent orthogonality with basic reversed phase on-line HPLC systems, the use of salt necessitates an extra clean-up step in the workflow, as these buffers are not MS friendly (Dakshinamurthy et al. 2017). This is a laborious and time-consuming process and will also inevitably introduce sample loss thus reducing the sensitivity of the overall approach.

pH gradients can also be used with ion exchange columns (Füssl et al. 2018). The gradient starts with a pH promoting binding of analytes to the column. As the gradient progresses, the pH will be altered to increase the eluotropic strength of the mobile phase. Once the protein is eluted from the stationary phase, it is unlikely that it will re-bind. For this reason, columns and gradients can be much shorter, saving time- however columns do need to be re-equilibrated after each run to restore the starting pH. This process can be made more efficient by using systems such as the Vanquish duo for Thermo Fisher Scientific, which can run two systems in parallel. In this set up, one column can run a sample whilst the other one re-equilibrates, halving the run time for any given sample. This system has proven effective in a clinical setting. It is also possible to make a pH gradient that is volatile and mass-spec friendly. Therefore, in the future it is plausible that high-throughput protein analysis could be achieved with the SCX with

a pH gradient by using a short column and coupling direct to MS for analysis. However, currently SCX/pH gradient is primarily being used for protein analysis and is not often used for peptide separation in commercial settings such as companies and clinics (Farnan and Moreno 2009)

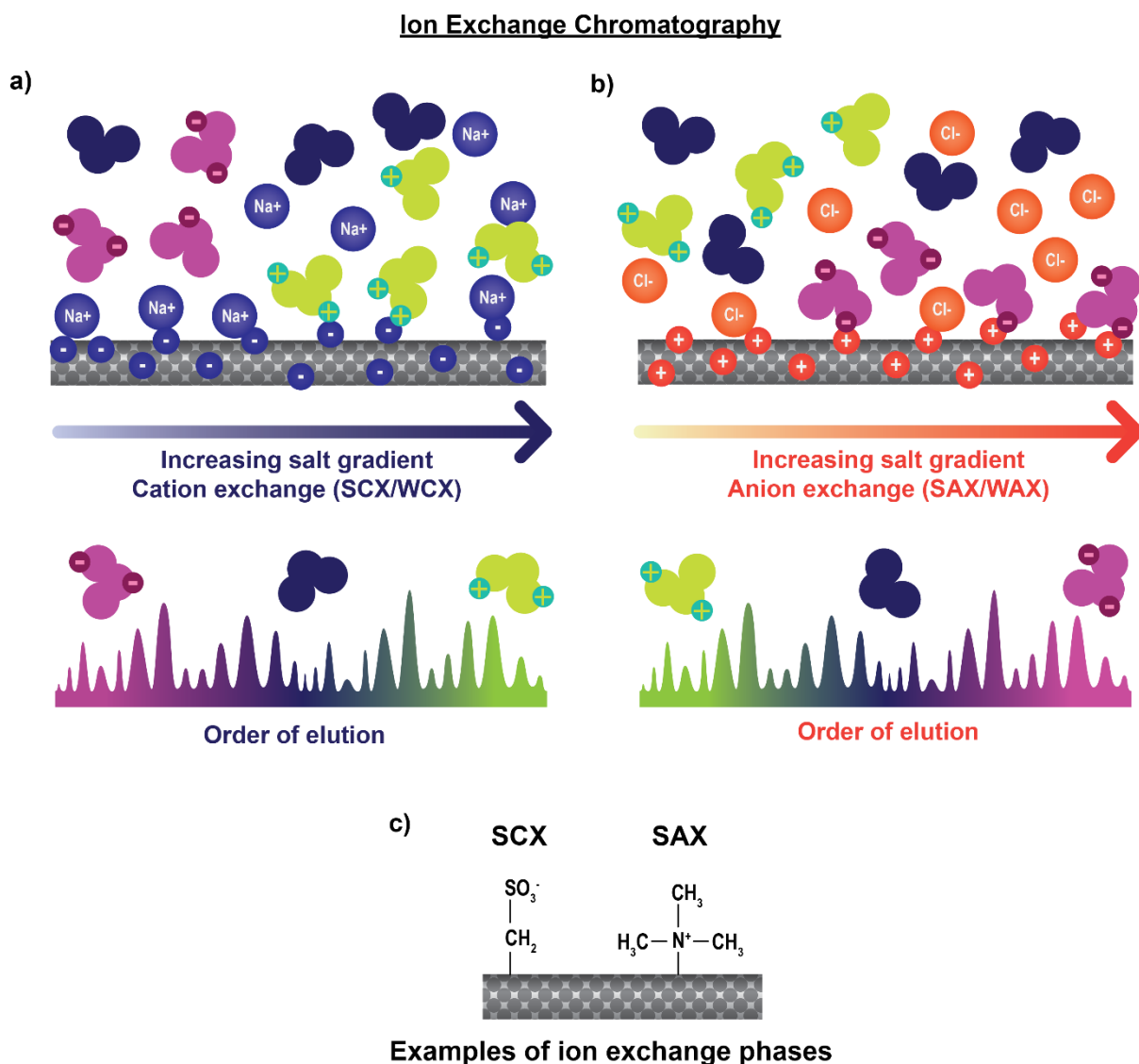


Figure 1.8: Ion exchange HPLC. a) When a cation exchanger is used with a salt gradient, positively charged salt molecules compete with the analyte for binding to the negatively charged residues on the phase. Negatively charged molecules are eluted first. b) When an anion exchanger is used with a salt gradient, negatively charged particles compete with the analyte for positively charged residues on the stationary phase. Positively charged molecules are eluted first. c) Examples of cation and anion exchangers.

1.3.3. Off-gel fractionation

Prior to modern HPLC, off-gel fractionation was preferred method for the separation of proteins or peptides for mass spectrometry (Chenau et al. 2008). Off-gel separates analytes on a pH gradient, where they migrate according to isoelectric point. It offers good orthogonality to RP-HPLC and works well for a relatively low-complexity protein sample. Off-gel works by using a gel strip with a linear pH gradient starting at pH 3 and increasing to pH 10. Peptides or proteins are loaded into wells which are situated above the pH strip. The electric current runs orthogonally to the pH strip and the direction of loading. Once a protein or peptide has been pushed into the region of its PI, it is effectively trapped and cannot migrate any further as it is pulled from both anode and cathode with the same strength (Figure 1.9) (Chenau et al. 2008; Ernoult, Gamelin, and Guette 2008).

Off-gel has worked well for both protein and peptide samples and can be modified into a 2D gel fractionation system, to further improve separation. However, this method is extremely low throughput, requires specialised equipment and skills and is overall less convenient than HPLC. For these reasons, many labs are now exclusively using HPLC for a faster, higher throughput analysis of complex mixtures which can be quickly injected to the mass spectrometer after fractionation.

Whilst HPLC for peptide analysis is well established, the field of protein separation lags behind. Despite holding some advantages over peptide separation, no method of protein separation can currently match the peptide separation techniques currently in use for complex samples.

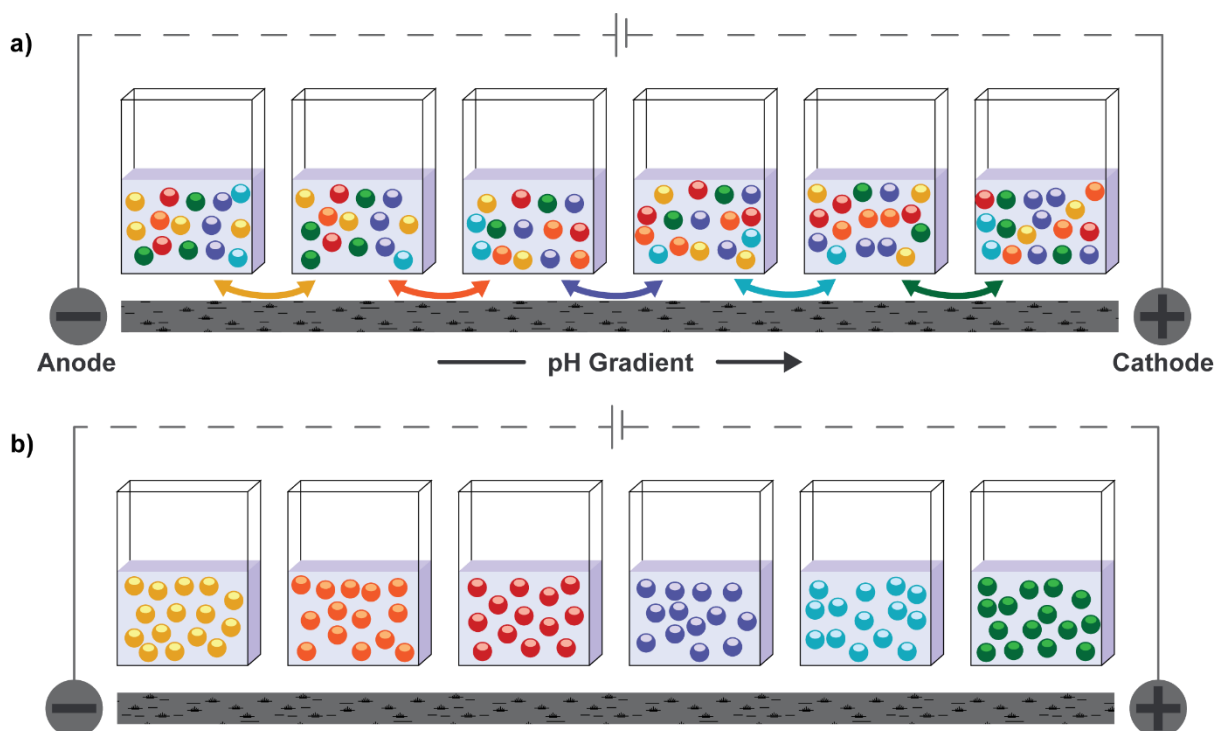


Figure 1.9: Off-gel fractionation. a) Off-gel fractionation set up. Sample is loaded into wells above a pH strip, which provides a pH gradient from pH 3-10. An electric charge is applied which causes the analyte to move along the pH gradient and stop when it reaches its isoelectric point. b) Analytes separated based on pH can be removed from the gel and processed for MS.

1.3.4. HPLC for Protein Fractionation

Proteomics of whole cell lysis can be challenging due to the complexity of the sample, as high abundance proteins or peptides can often mask lower abundance analytes. A complex mixture also increases the probability of co-elution, where peaks from different proteins or peptides are selected together in the mass spectrometer, which can lead to complications for quantification and identification (Baiwir et al. 2015a). Sample fractionation by HPLC increases the depth of analysis by separating complex samples into simpler fractions before analysis by mass spectrometry (Michel et al. 2003). By separating the analyte by its physical or chemical properties, a series of less complex samples can be analysed in tandem, increasing the likelihood of correctly identifying peptides from a target protein and increasing the detection of low-abundance analytes (Aguilar 2003).

In a regular proteomics workflow, complex protein samples are digested using a digestive enzyme such as trypsin to form a mix of peptides. The peptides are then separated into fractions,

which are analysed in tandem by the mass spectrometer (Aebersold and Goodlett 2001; Aebersold and Mann 2003).

One of the limiting factors of this analysis is the binding capacity of the analytical and/or trap column (Mitulović 2015). By performing a pre-fractionation of the sample and analysing individual fractions, the likelihood of identifying low-abundance peptides is greatly increased, thereby increasing proteome depth and sequence coverage. In a proteomics workflow, sample fractionation is usually achieved at the peptide level by high performance liquid chromatography (M. Bantscheff 2007). Peptide separation is usually performed using a reversed-phase C18 column.

To achieve optimal separation, as many as 40 fractions can be taken from a single sample. However, running each fraction individually would dramatically increase the time required to analyse the sample on the mass spectrometer. To avoid unnecessary time and expenditure, fractions can be pooled together to reduce the overall number of samples sent to the MS (Thammana 2016). To preserve the separation achieved by the reverse phase fractionation, fractions can be pooled non-consecutively to avoid pooling similar fractions together and thereby maintaining the separation of similar peptides. For example, from 40 fractions, fractions 1, 11, 22 and 33 can be concatenated together without interfering with the separation, as the peptides in these fractions would be sufficiently different as to elute separately when analysed by LC-MS (Cruz et al. 1997)

Pooling fractions non-consecutively is time consuming unless the process is automated. Even when automated, pooling fractions from multiple samples adds some time and an extra processing step to the proteomics workflow. Other methods of fractionation such as ion exchange, where peptides are separated by charge, or protein fractionation, do not need to be pooled non-consecutively and therefore the overall workflow is faster than off-line peptide reversed phase (Dwivedi et al. 2008).

There are other aspects for which peptide reversed phase fractionation can be sub-optimal for some samples. When HPLC is performed at the peptide level, peptides arising from a single protein will elute into different fractions. This can be problematic as peptides generated from high abundance proteins will co-elute with proteins present in trace amounts, thus impeding the identification of lower abundance proteins. For samples such as serum, which is hugely abundant in albumin, co-elution can be very detrimental for the identification of low abundance peptides which might be present in the sample (Anderle et al. 2004)

A way to overcome this issue is to perform the sample fractionation at the protein level. Whilst the columns previously mentioned are sub-optimal for protein fractionation, the MAbPac™ column from Thermo is well suited for protein separation (Michel et al. 2003; RAO et al. 2010).

The MAbPac™ is an HPLC column from Thermo Fisher Scientific, which was initially designed for the separation of monoclonal antibodies, giving rise to the ‘MAB’ part of the column name. It consists of a reversed phase macroporous polymeric column, with a stationary phase made of poly(divinylbenzene) (DVB) particles. Compared with many other commercially available HPLC columns, it can withstand very harsh conditions, having a pH range of 0-14 and able to withstand temperatures up to 110 °C. Used with an acidic organic solvent (pH 1-2) as the mobile phase, at 75 °C, it should theoretically be able to separate proteins in denatured conditions, which could aid protein separation and identification (Rao et al. 2011) (Figure 1.10)

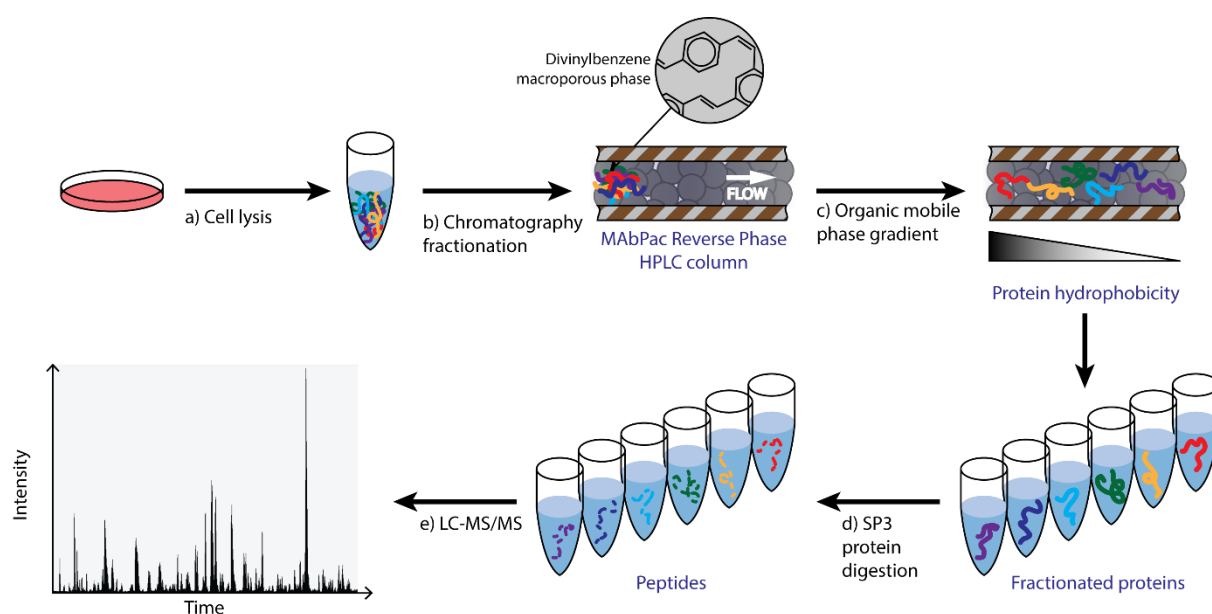


Figure 1.10: Method for MAbPac™ protein fractionation. a) Cell pellets are lysed in 5 % SDS buffer. Protein denaturation is performed using 5 mM TCEP, followed by cysteine alkylation using 20 mM iodoacetamide, incubated at room temperature, in the dark. b) Prior to injection, samples are diluted in water to achieve a final concentration of 2.5 % SDS. c) Samples are injected to the column where they bind to the DVB phase according to hydrophobicity and are eluted by using an organic mobile phase. d) Fractionated proteins are collected, and detergent removal and digestion is performed by SP3 using a modified protocol from (Hughes et al. 2014). e) Peptides are dried, re-suspended in TFA or formic acid and analysed by mass spectrometry.

1.3.5. HPLC for Charge Variant Analysis

Personalised medicine is an approach which works by separating patients into groups based on their genetics. Due to genetic variation, response to, or risk of developing, a particular disease can vary between groups. Therefore, each group has a different treatment plan which has been shown to be more effective for that group's genetic background. Usually, this means that different groups are given different drugs to combat the same disease. Advantages of personalised medicine are evident. Genotyping is faster than trialling a series of different drugs for each patient, to find the one that works- by immediately giving a patient the drug that is most likely to be effective for them saves time, suffering and expense. Other advantages include shorter clinical trials, a lower risk of adverse reaction to a prescribed drug and an overall reduction in the cost of treatment for the patient or healthcare system (Vogenberg, Isaacson Barash, and Pursel 2010).

However, there are still challenges for the development and use of personalised medicine. Drawbacks include ethical considerations relating to patient privacy and confidentiality and the analysis that must be done for genetic sequencing data, which requires extensive, computer-intensive processing (Klein, Parvez, and Shin 2017; Z. Liu, Li, and Zhou 2020). In addition, identifying and designing tests for all genetic variations that can affect a patient's likelihood of developing a particular disease, or their reaction to a drug, is an infinite task. In the future it is likely that problems such as these will be solved by using techniques such as whole genome sequencing combined and powerful machine learning and informatics. With these approaches, personalised medicine has enjoyed a recent surge in advancement thanks to the development of these techniques.

The adaptive immune system is an excellent basis for personalised medicine. B cells are lymphocytes which are created in the bone marrow and possess a unique antigen-binding receptor located on the cell membrane. This receptor is an antibody molecule, a glycoprotein which binds to PAMPs or DAMPs. Upon infection with a novel PAMP or DAMP, there is a period of infection and illness. Recovery depends on the generation of antibodies against the pathogen. When a new B cell is released into the blood stream, and it encounters a pathogen which matches its antigen-binding receptor, it divides rapidly. The new cells differentiate into memory and effector B cells. Effector B cells produce the antibody in a secreted form, and it binds to the pathogen, disabling it and targeting it for destruction. Memory B cells have a much longer life span than their parent B cell and retain the ability to make the same antibody. They intervene early in subsequent infections to prevent the host from becoming ill. Antibodies

specific to the invading PAMP are dispatched upon detection of the infection. They are so effective at finding and binding the PAMPs that often, one exposure to an illness such as chicken pox is enough to prevent further episodes for life.

Monoclonal antibodies (MAbs) are now widely used to tailor therapeutics to the patient. After genomic testing, MAbs and dosages are selected based on the patient's genetic profile. For example, patients identified as positive for the breast cancer biomarker human epidermal growth factor receptor 2 (HER2) will be more responsive to trastuzumab (Garattini, van de Vooren, and Curto 2015; Gajjar et al., n.d.; Dakshinamurthy et al. 2017), whereas patients expressing epidermal growth factor receptor (EGFR) or GTPase KRas (KRAS) may benefit more from treatment with cetuximab (Garattini, Curto, and Freemantle 2015). Diseases against which MAbs have been used effectively include cancer and numerous autoimmune diseases such as rheumatoid arthritis and Crohn's disease. They are synthetically produced and engineered to be targeted to a specific type of cell. Antibodies specific to a cancer cell, for example, can be conjugated to a drug designed to kill that cancer. The antibody can then be used to deliver the drug to the target cell (Linke et al. 2012). The primary benefit of this approach is that reduces binding events between the drug and non-target cells, reducing side effects, and treatment can be more powerful with a reduced dosage of the drug.

Therapeutic MAbs are produced in primary cells isolated from transgenic mice expressing human immunoglobulin genes and have been inoculated against the target disease. Once isolated, cells are cloned, and antibodies are produced and harvested. Antibodies are then injected into the human patient where they find and bind to the target cell flagging it for attention from the immune effector cells which engulf and kill the target cell (Figure 1.11).

Although MAbs represent a leap forward for personalised medicine, using antibodies as the therapy can also pose a medical risk to the patient. Rogue antibodies can also trigger an immune response from the patient (Millán, 2017.) by triggering a cytokine storm that can cause multiple organ failure, as was the case in the phase I pharmaceutical trial for TGN1412 (Attarwala 2010). Although such an extreme reaction is rare, other antibodies have also been known to trigger cytokine storms in patients- called cytokine release syndrome (CRS) (Shimabukuro-Vornhagen et al. 2018). Although CRS is a very rare occurrence, it has a very high mortality rate. MAbs associated with CRS include Rituximab which attaches to CD20 and is used for the treatment of chronic lymphocytic leukaemia and for some cases of non-Hodgkin's lymphoma (Kulkarni and Kasi 2012). However, as numbers of cases of CRS in response to Rituximab are low, and

the MAb is effective either as a standalone treatment or in conjunction with chemotherapy, extensive precautions are taken to prevent fatal CRS. Patients are given Tylenol and Benaryl 30 min prior to the infusion, nurses have emergency medical kits on hand and patients are monitored for a day. Infusions are administered in a slow titration. A large proportion of CRS cases occur during or after the first transfusion, enabling medical staff to be alert and intervene early should any signs of CRS arise. Therefore, the drawbacks of CRS-associated MAbs such as Rituximab can be mitigated so that the drug can still be beneficial to patients (Goodman 2015; Williams et al. 2016).

For MAbs to be effective, they need to be specific to the target cell. Post-translational modifications (PTMs) such as oxidation, deamination or sialylation can be induced by changes in the cells in which the antibody is produced. Fluctuations in the temperature of the cell culture medium, for example, is a key inducer of PTMs (Trappe et al. 2018). PTMs are a problem, as they will change the structure of the antibody, potentially rendering it unable to bind with the target cell, but also able to bind with non-target proteins, thus inducing off-target effects and reducing the efficacy of the treatment (Leblanc et al. 2017; Chung et al. 2018). Therefore, it is essential to be able to quickly detect PTMs in the clinic before administration to the patient.

PTMs change the structure of the antibody, and thus they also affect their charge. Antibodies usually have an overall negative charge, but induction of PTMs can cause the antibody to become more negatively charged (C-terminal lysine clipping, deamination of Asn or Gln and the sialylation of glycans will all do this), neutral or positively charged (succinimidation and disulphide conformational changes can be responsible for the positively charged antibody) (Füssl et al. 2019). Therefore, measuring changes in the overall charge of the antibody will give information on whether PTMs are present. This is called charge variant analysis and can be achieved by LC-MS.

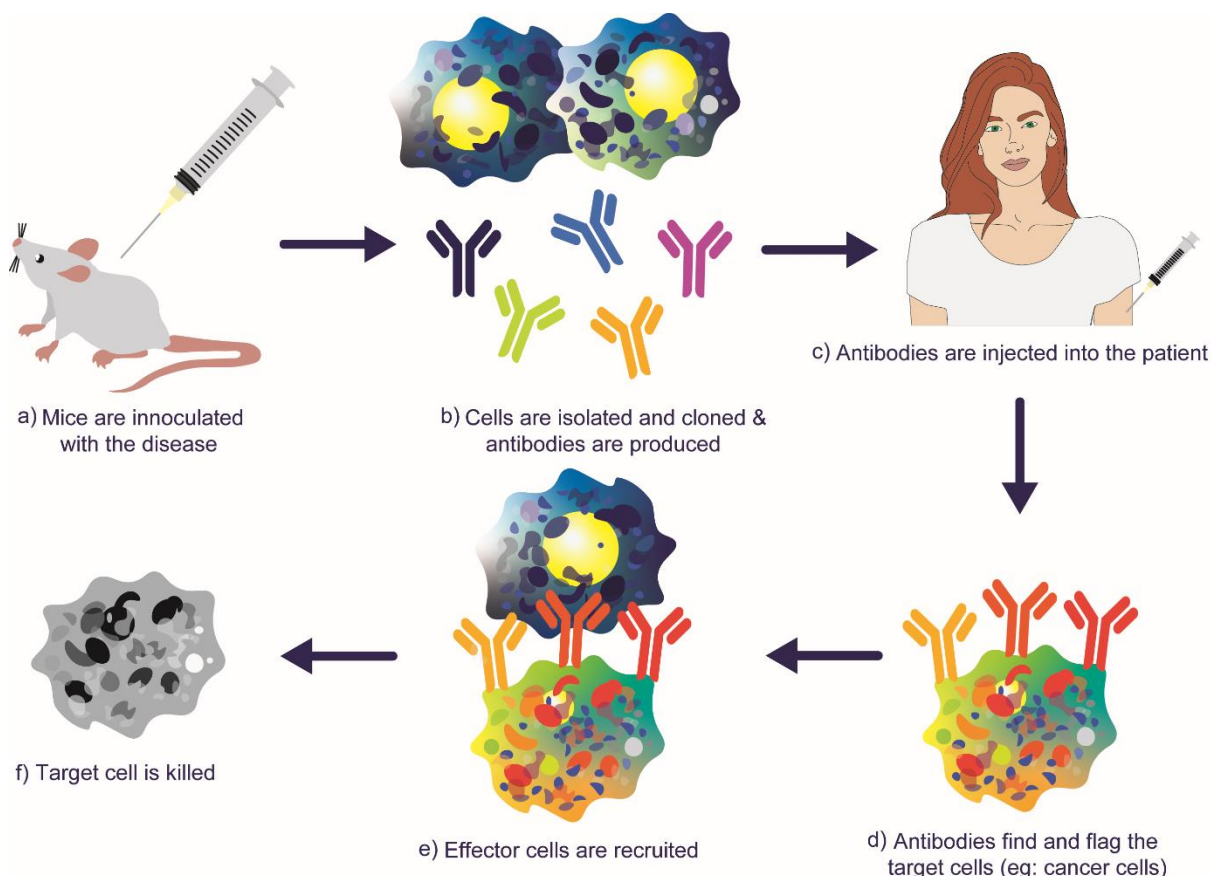


Figure 1.11: Use of MAbs as personalised medicine to treat disease. a) Transgenic mice expressing human monoclonal antibodies are inoculated with the target disease. b) Cells are isolated from the mice, cloned, and cultured where they express MAbs into the cell culture media. c) MAbs are injected into the patient. d) Antibodies specifically bind to the target cells, flagging them for destruction. e) Effector cells are recruited to the target cell. f) The target cell is killed.

1.3.5.1. SCX column - salt vs. pH gradient

LC-MS is routinely used to identify PTMs in antibodies because it is fast, accurate and suited to an automated, high-throughput analysis. Firstly, antibodies are separated using an off-line LC where they are separated by charge. Ion exchange columns are used, and thus charge variants can be identified and fractionated by the same method (Neill et al. 2015). Antibodies are typically eluted using a salt gradient (Lin et al., 2014.), where charged sites on the antibodies compete with salt ions in the mobile phase for access to binding sites on the stationary phase. The salt concentration starts low, but as the gradient continues, the salt concentration increases and begins to out-compete the antibodies for charged sites, pushing the antibodies off the column. Salt gradients require a long column and a long gradient- usually an hour- to fully separate antibodies. This is because, once an antibody has been outcompeted for a charged site

and displaced, it can re-bind to another vacant charge site further down the column. This repeated bind-and-elute pattern necessitates the longer length of both column and gradient (Figure 1.12 a). Cation exchange chromatography is excellent for robustness and resolving power but is time consuming and difficult to develop, with new gradients, methods, column type and mobile phase having to be adapted for each antibody (Rea et al. 2011).

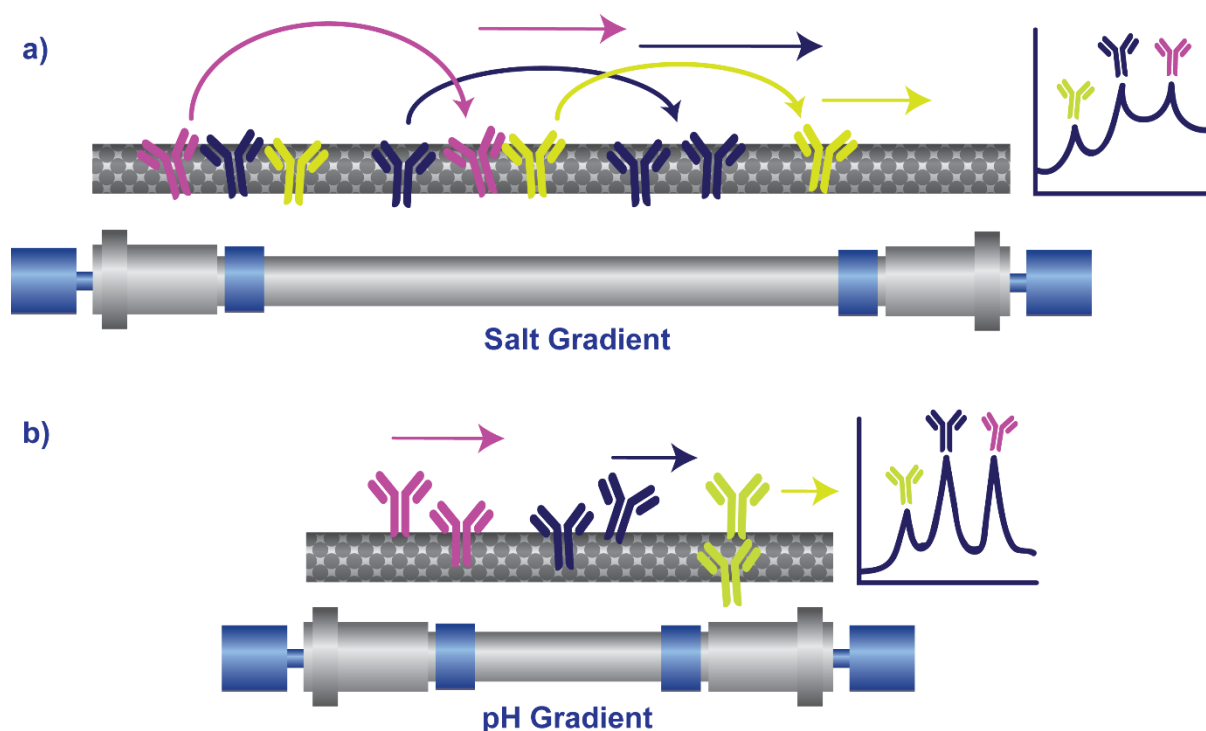


Figure 1.12: Antibody separation with an SCX column, comparing salt and pH gradients. a) Charged antibodies compete with salt for the charged sites on the stationary phase. Antibodies are outcompeted for the binding sites as the concentration of salt increases and can re-bind and re-elute until they finally exit the column, necessitating long columns and gradients. b) pH gradients elute antibodies according to isoelectric point, and no re-binding occurs. Therefore, columns and gradients can be much shorter.

More recently, pH gradients have been favoured over salt gradients for charge variant analysis using ion exchange columns. Rather than eluting with salt, a gradient from low to high pH is created. Once loaded onto the column, antibodies bind to all available charged sites. As the pH increases it becomes difficult for antibodies with the same pI as the mobile phase to stay bound to the stationary phase, and they are thus eluted from the column. Negatively charged antibodies have a low pI and thus eluted first, followed by neutral antibodies and finally by antibodies with a high pI (positively charged antibodies) at the end of the gradient. Once the pH of the

mobile phase is greater than the isoelectric point on the MAb, fewer interactions with the column occur, drastically reducing the likelihood of re-binding events (Figure 1.12 b). Therefore, both columns and gradients can be much shorter, some pH buffer gradients being as short as 10 min. This makes the overall workflow much faster by comparison, increasing the efficiency of the analysis for clinics. Another major advantage of the pH buffer is that they can be made to be MS-compatible, enabling, for the first time, charge variant analysis using on-line LC-MS as well as producing simplified mass spectra of proteins in native conditions (Phung et al. 2019). It has been shown that pH gradients have a superior resolving power (Trappe et al. 2018) and are more suited to faster, high-throughput workflows, compared with other ionic strength based ion separations (Farnan and Moreno 2009).

However, the mobile phases used for pH-based separation have drawbacks. Using buffers where the ionic strength is too high can alter the charge variant profile and complicate analysis (Füssl et al. 2018). Due to absorption of carbon from the air, the pH changes over time, so these buffers lack the stability of their salt counterparts. As different MAbs have different isoelectric points, it is necessary to create a custom buffer recipe and gradient for each one. There are also some pH points which cannot be achieved in the context of the pH gradient- pH 7 is inconvenient for mass spectrometry, for example. Linear gradients are also challenging to create. As the acidic buffer A mixes with the basic buffer B, the pH is prone to resisting change and then leaping to a basic pH, eluting everything at once.

Clearly the pH ion exchange approach has potential but needs further development. Specifically, a one-gradient-fits-all would remove a vast amount of tedious and time-consuming optimisation. A single buffer recipe could then be used, which must be both reproducible and stable. If this could be achieved, MAbs could be analysed quickly in a high throughput manner with little to no optimisation required from the clinical labs.

1.4. Mass Spectrometry

1.4.1. Overview

Mass spectrometry measures the mass-to-charge ratio (m/z) of an analyte, in this case proteins or, more commonly, peptides. All mass spectrometers have three fundamental parts: the ion source, which introduces the ions into the MS; the mass analyser which separates the analyte

based on their m/z and the detector which measures the m/z of each analyte (Gygi and Aebersold 2000; Aebersold and Mann 2003).

1.4.2. Ion Sources

In order to be detected by the MS, analytes need to be ionised and in the gas phase. There are two main ion sources used for biomolecule analysis; electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) (Ho et al. 2003; Jurinke, Oeth, and van den Boom 2004). Of the two, the most commonly used in proteomics is ESI as it allows for direct coupling between the LC and the MS. In ESI, a high voltage applied between the LC and the MS. This high voltage will generate small droplets of liquid which evaporate within the source. As the evaporation progresses, the charge density in the droplets will increase until the charge density becomes greater than the surface tension of the droplets, resulting in the latter exploding in a process termed coulombic explosion thus releasing smaller droplets. The process continues until all that remains are desolvated ions which then travel to the mass analyser (Figure 1.13 a). ESI is considered a ‘soft ionisation’ technique as there is little fragmentation of the ions in the source (Fenn et al. 1989; Di Marco and Bombi 2006). MALDI is also a soft ionisation technique but differs in that the analyte is held in a matrix. The matrix molecules are excited by a laser shot which lead to desorption of the sample with transfer of proton(s) to the analyte molecules, thereby ionising them (Figure 1.13 b). (Jurinke, Oeth, and van den Boom 2004).

After ionisation, the analyte enters into the mass spectrometer where they can be isolated or fragmented by different mass analysers (Croxatto, Prod’hom, and Greub 2012).

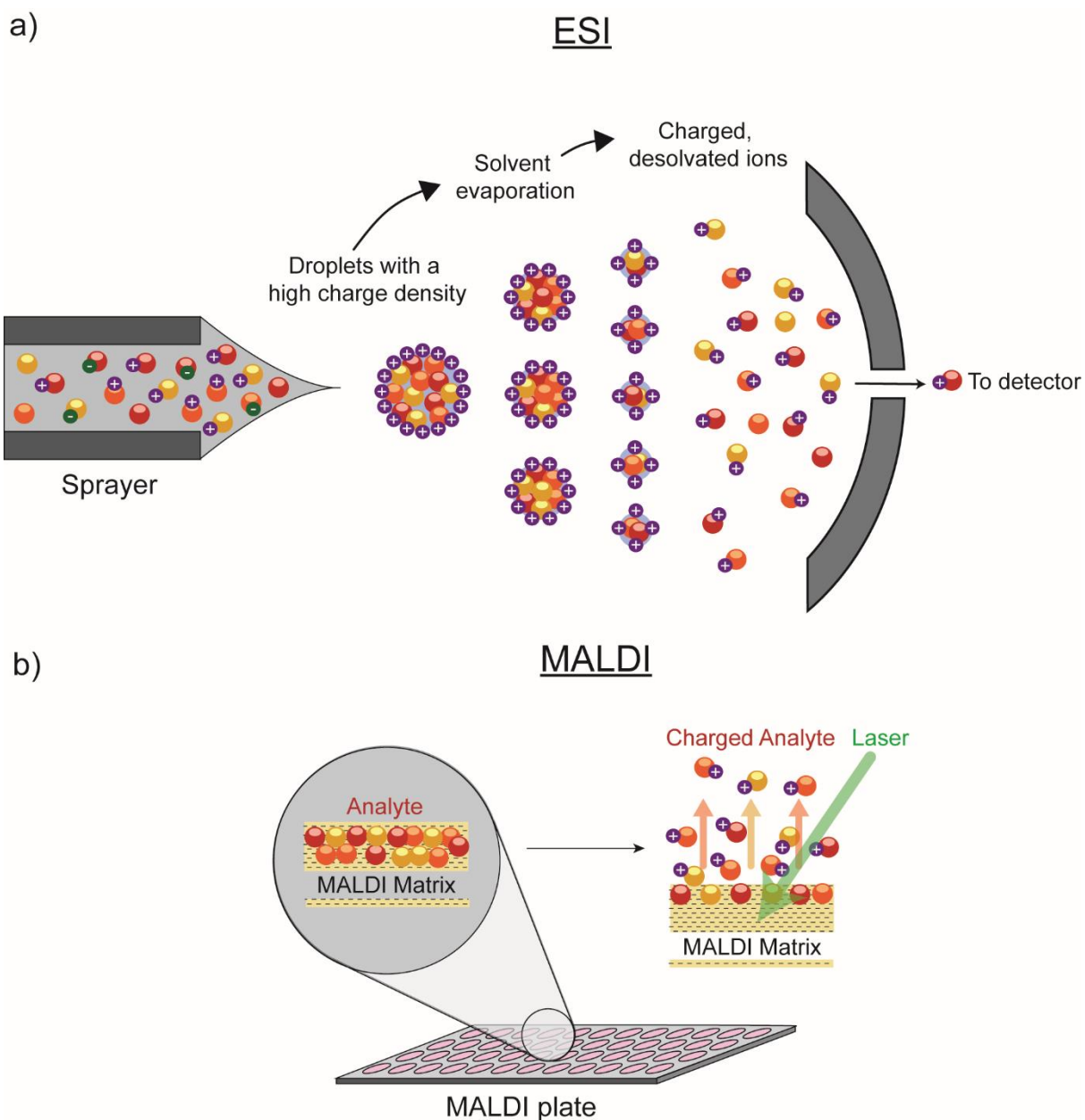


Figure 1.13: Ionisation sources. a) In electrospray ionisation (ESI), the analytes are dissolved in a solvent and introduced to the MS via a sprayer. Large droplets containing a mix of solvent and analyte are ejected from the sprayer and moved through the source by application of a high voltage. When the charge density of a droplet overcomes the surface tension, the droplet explodes, and smaller droplets re-form. This process is repeated until the analyte is fully desolvated and the naked ions enter the MS. b) In matrix-assisted laser desorption ionisation (MALDI), the analytes are mixed with a matrix and allowed to co-crystallise on a MALDI plate. A laser directed at the matrix is absorbed, resulting in charges being transferred from the matrix to the analyte, and the analyte and matrix moving away from the plate and towards the mass analyser.

1.4.3. Mass analysers

Mass analysers separate the ions by their m/z . The four most commonly used mass analysers in proteomics are the quadrupole, the time of flight (TOF), linear ion trap and the Orbitrap™ (X. Han, Aslanian, and Yates 2008b).

1.4.3.1. Quadrupole

The quadrupole is a versatile mass analyser that can be used to transmit, select, or fragment ions. In a quadrupole, ions oscillate between four conductive rods on which both a direct (V DC) and an alternative (V RF) voltage can be applied simultaneously (Figure 1.12 a). When only an RF voltage is applied, the quadrupole plays the role of an ion guide and all ions are transmitted through from one end to the other, no matter their m/z . In this instance, a high energy inert gas can be injected perpendicularly the ion trajectory in order to fragment the ions going through the quadrupole. Alternatively, when both RF and DC voltage are applied, the quadrupole plays the role of an ion filter and only ions with a specific m/z will have a stable trajectory and will be transmitted through the quadrupole. Due to their versatility, quadrupoles are the most common mass analyser and are often used in combination with other mass analysers such as time of flight, ion traps or even coupled to other quadrupoles in triple quadrupole instruments. In such case, the first quadrupole is used to select precursor of interest, the second is used as a fragmentation cell and the third is used to select the fragment ions for further detection. Whilst flexibility is the key advantage offered by the quadrupole, its drawback is its low resolution (1-10000 Full width at half maximum (FWHM), which is comparable with an ion trap and its working mass range (usually below 5000 Th for selection) (Michalski et al. 2011).

1.4.3.2. Time of Flight (TOF)

The time of flight (TOF) mass analyser separates ions according to the time they take to travel from an extraction grid to the detector in a field free region (Croxatto, Prod'homme, and Greub 2012). Firstly, the ions are injected in the acceleration zone and then sent into the TOF tube in packets. Ions with a lower m/z will fly faster than those with a higher m/z . The longer the TOF tube, the better the resolution (El-Aneed, Cohen, and Banoub 2009). The TOF can analyse a broad range of m/z and therefore it is a good choice for protein analysis. During the acceleration process, ions will receive a higher energy the closer they are to the extraction plate, which can

result in a loss of resolution as packets of ions separation the TOF tube (Jurinke, Oeth, and van den Boom 2004). To counteract this problem, a reflectron can be added between the acceleration plate and the detector. The reflectron effectively plays the role of an electrostatic mirror, refocussing and re-grouping ion packets before reflecting them onto the detector. The reflector effectively doubles the length of the TOF tube, which aids in separating packets of ions from one another, increasing resolution (Figure 1.14 a) (Cotter, Griffith, and Jelinek 2007). However, linear mode (without the reflectron) is better suited for high m/z ions, such as intact proteins, as they can be degraded in the reflectron. Therefore, a shorter flight path is desirable. In linear mode, the TOF can usually offer a unit mass accuracy. Using a reflectron, the TOF can offer ppm mass accuracy (Jurinke, Oeth, and van den Boom 2004).

1.4.3.3. *Linear Ion Trap*

The linear ion trap is similar in structure to the quadrupole but is able to hold ions thanks to an extra pair of electrodes stationed one at the entrance and one at the exit of the ion trap, which control the entrance and the exit of ions (Agthoven and O'Connor 2017) (Figure 1.14 a). One of the advantages of this system is that ions can be repeatedly selected and fragmented to acquire mass spectrums in the order MS^n . This has many uses, such as the determination of the structure of unknown compounds and is essential for tandem mass tag (TMT) experiments. Ion traps are extremely fast and have a high sensitivity but a poor resolution and limited m/z range (Makarov et al. 2006).

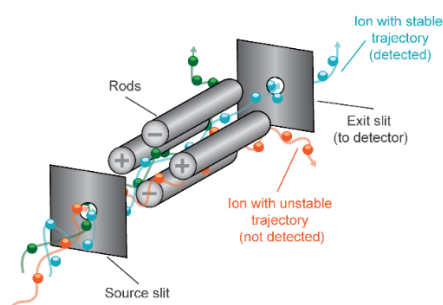
1.4.3.4. *Orbitrap™*

The Orbitrap™ is the most recent mass analyser to be available commercially and has the advantages of having a high mass resolving power, mass accuracy and wide dynamic range (Figure 1.14 a) (Hardman and Makarov 2003). It is a Fourier transform mass analyser and it consists of a spindle-like inner electrode and a larger barrel-like outer electrode, which both have a constant RF voltage applied. This creates a stable flight path which traps ions. The ions orbit around the inner electrode in a similar manner regardless of their m/z but oscillate in the axial dimension with a frequency determined by their m/z (Rosati et al. 2012). The resulting interferogram (relative intensity measured over time) is then converted into a mass spectra by Fourier transformation (Makarov et al. 2006).

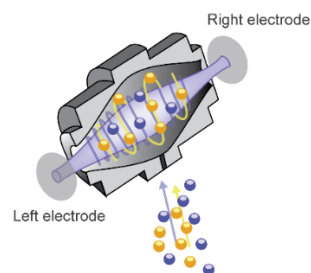
a)

Mass analysers

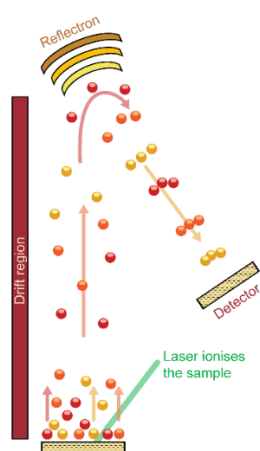
i) Quadrupole



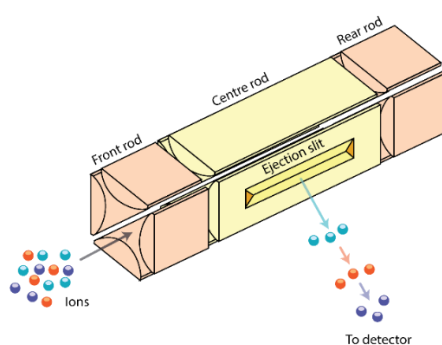
ii) Orbitrap



iii) TOF



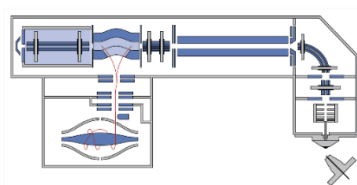
iv) Linear Ion Trap



b)

Tandem setups

i) Q-exactive



ii) Orbitrap Fusion Lumos

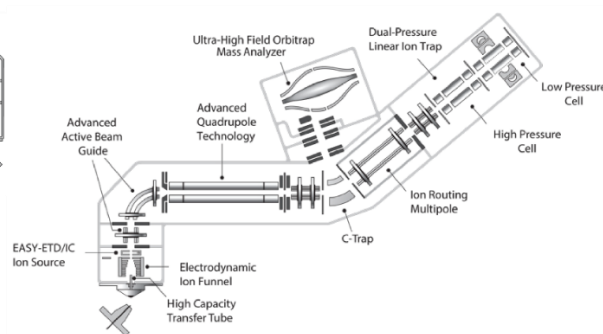


Figure 1.14: Commonly used mass analysers and tandem set-ups. a) Common mass analysers. From left to right: i) The quadrupole, ii) Orbitrap™, iii) TOF, iv) linear ion trap. b) i) The Q-Exactive uses a quadrupole linked to a C-trap. From the C-trap ions travel to the Orbitrap™ where they are analysed. ii) The Orbitrap™ Fusion Lumos Orbitrap™ is similar to the Q-Exactive but also includes a dual linear trap at the back of the instrument, thus giving it MSⁿ capabilities. Adapted from planetOrbitrap™.com

1.4.4. Common tandem set-ups: Orbitrap™ Fusion Lumos and Q-Exactive (Thermo Fisher Scientific)

As previously discussed, the power of the mass spectrometer comes from the flexibility achieved by coupling several mass analysers to one another in tandem, thereby harnessing the advantages of each whilst mitigating the disadvantages of individual analysers. The choice of ion source is also an important consideration. For example, MALDI is commonly coupled with the TOF and is used for the measurement of large biomolecules and can be very effective for high throughput assays due to its speed and sensitivity (Croxatto, Prod'hom, and Greub 2012). For smaller molecule and highly complex samples, LC separation coupled to the ESI ion source is usually better suited (for example, a quadrupole and an Orbitrap™) (Figure 1.14 b) (Croxatto, Prod'hom, and Greub 2012). The fragmentation of the ions allows for peptide mapping where the sequence of the peptide can be determined with the help of a computer database (Hale et al. 2000; Suckau et al. 2003).

Modern mass spectrometers used in proteomics are combining several mass analysers within a single instrument to take full advantage of their strength while overcoming their flaws. The most popular instruments used today are based around the Orbitrap™ technology from Thermo Fisher Scientific.

In the QE HF, ions first generated by electrospray ionisation as well as neutral molecules enter the instrument via a series of electrodes (S-lenses), which refocus the ions into a narrow stream. Ions are then guided through a bent quadrupole operating in RF mode only (no selection) while neutral molecules travel in a linear path and are eliminated in the instrument (Michalski et al. 2011). Ions travel through a second quadrupole which can be programmed to select specific ions. Selected ions can then be routed to the Orbitrap™ for detection of intact ions or can be sent to the ion routing multipole for fragmentation prior to their detection in the Orbitrap™. The C-trap main function is to condense the ions in a small packet in order to send them inside the Orbitrap™ (Rosati et al. 2012).

The Orbitrap™ Tribrid Lumos is based on the same architecture with only a few modifications: First, the ions enter in the mass spectrometer through a more advanced refocussing system termed ion funnel. This ion funnel allows for higher sensitivity compared to the older S-lenses. Then, after the second quadrupole, ions can be rerouted to either the Orbitrap™, the ion routing multipole or to the linear ion traps at the back of the instrument. Not only these ion traps offer MSⁿ capabilities, but they can also be used in parallel to the Orbitrap™. As the Orbitrap™ is

relatively slow compared to the linear ion traps, a first packet of ion can be used to generate a first MS spectrum. A second packet is then sent to the Orbitrap™ and analysed (El-Aneed, Cohen, and Banoub 2009) .

1.5. Data analysis

1.5.1. Identification

The mass spectrometer can only generate intact mass spectra and fragment mass spectra of different ions. It does not provide the sequence of the identified peptides. This last step can be carried out using different software depending on the type of analysis that was performed. Protein identification using a database search engine (such as Mascot, MaxQuant, Peaks Studio or Proteome discoverer) is commonly used for data dependant acquisition analysis. Library based searches (using Skyline, OpenMS or Spectronaut) are commonly used for data independent acquisition or for absolute quantification. Statistical analysis is usually performed using other software or packages and include Perseus, R or simply Excel (Gevaert and Vandekerckhove 2000).

Database search engines all operate in a similar fashion. First of all, the software is fed with a set of parameters and a protein database. These parameters relate to the type of mass spectrometer used (such as mass accuracy, type of fragmentation, type of ions and mass range) as well as the proteins expected to be in the sample (such as set of PTMs expected in the sample, artificial modification such as alkylation and type of quantification) and the conditions for the digestion (for example: enzyme used, number of allowed missed cleavages or expected size of the peptides) (Chamrad et al. 2004) . The software will then digest the database in silico and create a list of all the theoretical peptides obtainable using the set of parameters defined by the user.

In order to assess the quality of the score, the software will perform, in parallel, a search against a decoy database of sequences that are not expected to be in the sample. This second database is usually generated by shuffling the amino acids in all the proteins from the real database or, more commonly, by using the reversed database. This is a database made up of the reversed sequences of the same proteins that are in the real database. While both approaches will result in proteins with similar distribution of amino acids, only the second approach (reversed database) will generate peptides with the same length (with the exception of the peptides arising

from the C and N terminus of the protein) and similar chemical properties (Chamrad et al. 2004).

Artificial MS/MS spectra are then generated for all the peptides present in the database (target and decoy) and the database search engine will try to assign each experimental MS/MS spectra to one found in its database, then assign a score based on the probability/confidence the identification is correct. This score reflects how well the experiment MS/MS spectra fits with the database and usually depends on the mass error between experimental and theoretical data, the intensity of the matched ion versus unidentified ions or the distribution of fragment ions in the sequence. More advanced software will include features such as mass recalibration at the MS1 and/or MSⁿ level, identification of two or more peptides from chimeric MS/MS spectra, inclusion of multiple protein database (Cox and Mann 2008).

Peptide score distribution is then plotted individually for target and decoy identifications and a score cut off is applied to allow for a specific false discovery rate (FDR) defined by the user. Using an FDR, the user controls the level of false identifications included in the final peptide list. Peptides are then matched to their corresponding proteins (Zoun et al. 2018). For some peptides, protein matching is simple, as due to their sequence there is only one protein they can originate from. For other peptides, particularly shorter peptides, there are several candidate proteins which can produce these peptides after digestion. Therefore, identifying the correct candidate protein for such peptides presents a challenge. To overcome this issue, modern software uses the Occam razor approach. Simply, these peptides are usually assigned to the protein which already has the most evidence (most unique peptide identified, or if identical then with better score). This is especially important for protein quantification (Bielow, Mastrobuoni, and Kempa 2016).

Library search engines use a similar strategy, except a library of pre-recorded MS/MS spectra is given to the software instead of a protein database. Such software also relies on orthogonal information such as retention time or ion mobility. This can usually lead to better matches with higher confidence as the MS/MS spectra come from real experimental data, however this only allows for the identification of previously-identified proteins (Bittremieux et al. 2018).

1.5.2. Quantification

Mass spectrometry cannot only tell us which proteins are present in a sample but also how much of each protein or peptide is present. Intensity of a peptide can be determined by its sequence,

charge state, ionisation efficiency or even the pH of the buffer. As such, it is impossible to determine the absolute concentration of a peptide, or a protein, without using synthetic heavy peptides spiked with the sample. As such, most quantitative proteomics usually uses relative quantification and only consist in the comparison of two or more samples to one another (Norrgran et al. 2009).

1.5.2.1. Intensity-based label-free quantification

For this quantification, the intensity, or area, of every peptide identified during the run is extracted. Protein intensity is then calculated by summing the intensity of every peptide composing this protein for a given run. Protein intensities are then compared between samples. (Figure 1.15) (Arike and Peil 2014). Not only this quantification strategy does not require any extra sample preparation, but it can also be applied to any sample and this technique is easy to adapt to different quantities of analyte. However, as samples are injected sequentially, label free quantification can only be applied to stable peptides and on a system requiring minimal maintenance. It is also prone to error as every step of the sample preparation as do be done independently for each sample. While this approach does not add reagent cost, it is the slowest, requires the most instrument time and is the least reproducible between samples. Other quantification strategies use sample multiplexing to reduce sample-sample variability and instrument time required (Megger et al. 2013).

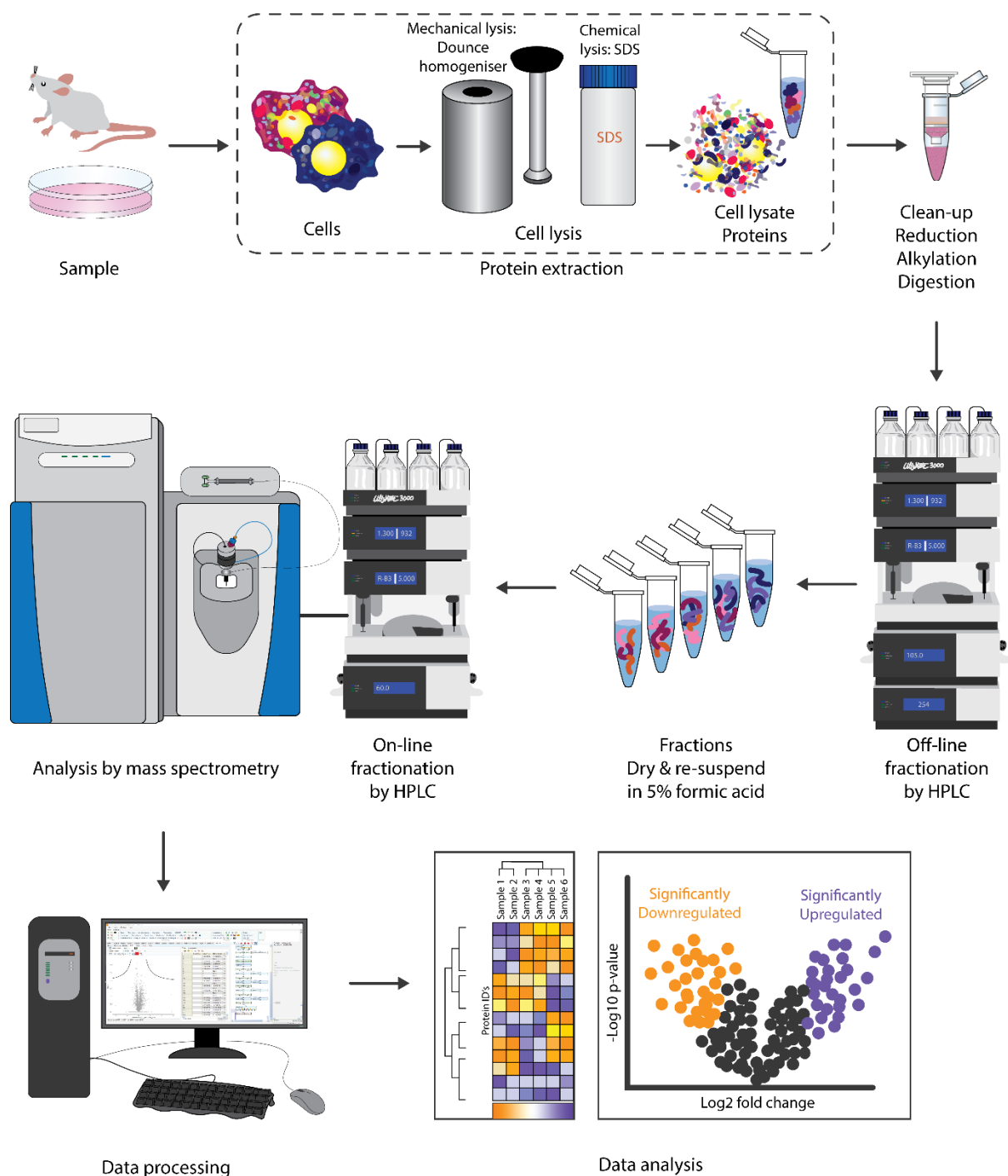


Figure 1.15: Workflow for a shotgun proteomics experiment with 2D fractionation. Cells are extracted from the sample and lysed. The sample is reduced, alkylated and de-salted before fractionation by HPLC. This step can be performed on proteins or peptides. Fractions are dried and re-suspended in FA or TFA and injected onto the on-line HPLC and then directly analysed by mass spectrometry.

1.5.2.2. *Isobaric labelling*

A common multiplexing strategy relies on isobaric labelling, usually at the peptide level. After trypsin digestion and sample cleanup, peptides from different conditions are labelled using isobars or isotopomers (or isotopic isomers) (Navarrete-Perea et al. 2018). These added tags have the same nominal or exact mass but will generate fragments with a different m/z . After tagging, peptides from different samples are mixed and can be differentiated at the MS2 level. A popular choice is tandem mass tag (TMT) (Figure 1.16) (Hunt et al. 1986). For TMT, each tag consists of three regions- the reporter region generating specific low mass fragments, the mass normalisation region which compensate for the different reporter and the reactive group which will attack reactive amine on the targeted peptide. As the quantification is done within a single injection, TMT labelling quantification is less sensitive to instrument variation over time, resulting in a more consistent quantification. The multiplexing also reduces the time required to inject the same number of samples (Tao and Aebersold 2003). Currently, TMT labelling kits are commercially available for multiplexing up to 16 samples (Lombardi et al. 2015; Y. Wu et al. 2019).

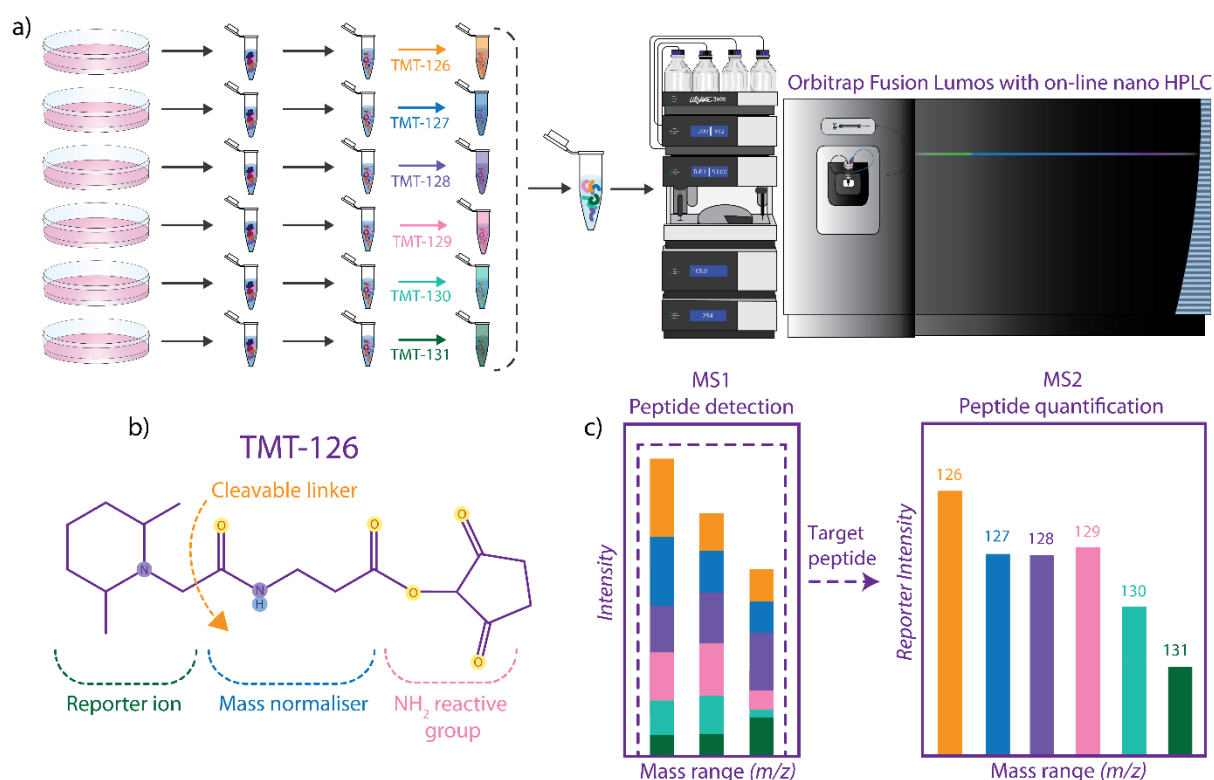


Figure 1.16: TMT workflow. a) Workflow for a TMT experiment (6-plex). b) The TMT-126 tag. c) Mechanism of peptide quantification by the mass spectrometer. a) Samples are reduced, alkylated, and digested and then labelled with TMT tags. Once labelled, they can be pooled and processed and injected together on the mass spectrometer. b) The TMT tags are composed of three parts: the reporter ion, which identifies the tagged sample; the mass normaliser, which is responsible for keeping the tags the same mass (before fragmentation) and the NH₂ reactive group, which binds to the peptide. c) TMT detection. The MS1 scan is pre-fragmentation when the peptides are detected. At this stage, the TMT tags are unfractionated and therefore all have the same mass and are detected as once large peak which is selected and fragmented in the MS2 scan. The TMT tags are fractionated at this stage and are either identified in MS2.

The main disadvantage associated with TMT is ratio compression, where the difference between two samples can appear to be reduced due to co-eluting peptides from different proteins which are isolated along with the ion of interest (Figure 1.17). Ratio compression can interfere with quantitation. Synchronous precursor selection (SPS) can help mitigate much of the ratio compression problem. In a TMT experiment without SPS, co-eluting peptides are selected during the MS1 run and fragmented in MS2 where they add to the signal of the TMT labels. In the case of wild type (WT) vs. knock out (KO) experiments, this can give the impression that the protein of interest is present in the KO, when in fact, it is a contaminant ion. SPS used MS3 to select the most abundant ions from the MS2 scan and only fragment those ions. This greatly improves the selectivity for the ions of interest and reduces ratio compression

very effectively. However, being more selective will reduce overall number of identifications in the MS run (Ow et al. 2011).

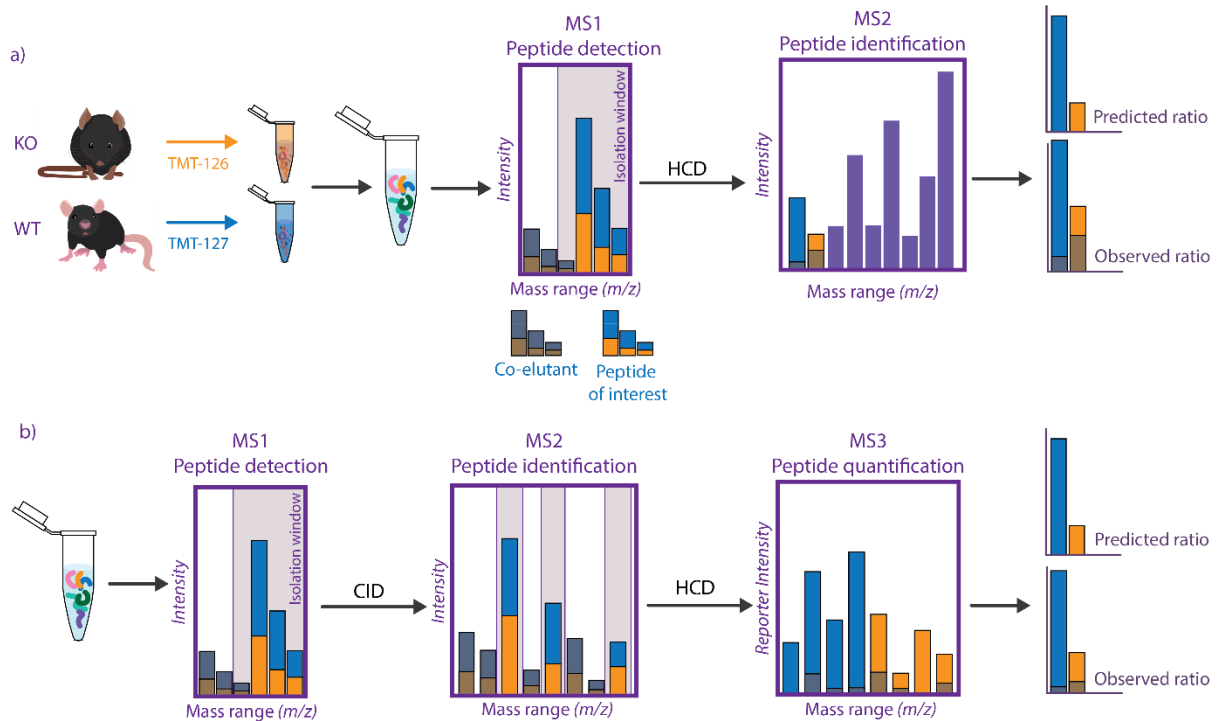


Figure 1.17: SPS as a solution to ratio compression. a) TMT using MS2, without SPS. Samples are labelled with TMT reagents and combined. Peptide detection is performed in the MS1 scan. All ions within a pre-set isolation window are selected for HCD fragmentation, which fragments ions of both high and low mass range. selected ions can include the peptide of interest, and any other peptides which are in a similar mass range. Quantification is performed on the TMT tags, the intensity of which is a combination of tags from the peptide of interest, and any other co-eluting peptides. b) TMT using MS3 SPS. Ions selected from the MS1 scan and first fragmented using CID, which only fragments ions at a high mass range. Peaks from the peptide of interest are selected from the MS2 spectra and fragmented using HCD. This second fragmentation step reduces the relative input of co-eluting ions to reporter intensity, resulting in decreased ratio compression compared with TMT samples analysed using MS2 only.

1.5.2.3. Other quantification strategies

Other techniques are available for quantifying peptides in a sample. These include SILAC (Stable isotope labelling with amino acids in cell culture) or dimethyl labelling, each allowing a form of multiplexing. Using SILAC, cells are labelled with light or heavy amino acids which are incorporated as the cell grows and the quantification is done at the MS1 level. This technique is advantageous for quantification as samples can be mixed as soon as they are lysed. This

reduces variability between samples. However, SILAC also requires using special, amino acid free (or dialyzed) foetal bovine serum and special culture media (FBS) which can change cellular metabolism or cell signalling. SILAC is usually limited to a maximum of 3-plex and, while SILAC animals have been reported in the literature, the cost involved in a SILAC project usually limit it to cells in culture (Geiger et al. 2010).

Dimethyl labelling is a chemical labelling done at the peptide level and quantified at the MS1 level and is one of the most cost-effective labelling techniques. Dimethyl labelling is less popular than other techniques as it uses toxic chemicals (formaldehyde) and relies on the mass difference between deuterium and hydrogen, resulting in shift in retention time between the different labels (Gibson, Large, and Bamforth 1985).

1.6. Thesis Hypothesis

The primary aim of this project was to create a novel 2D-LC/MS3 workflow, where samples are fractionated at the protein level before digestion and MS3 analysis. This workflow will be applied for the proteomics analysis of phagosomes extracted from cells where TLR2 has been activated.

The hypotheses and project aims were:

1. The hypothesis was that TLR2 play a regulatory role in phagocytosis, and the aim was to use proteomics to measure the changing proteome of phagosomes and TCL in response to TLR2 activation.
2. The hypothesis was that TLR2 signals from the phagosome and initiates Type I IFN, and the aim is to use biochemistry techniques to investigate downstream signalling from activated phagosomal TLR2.
3. The hypothesis was that pH buffers could be used to identify CVAs in commercial MABs. The aim was to develop pH buffers to be used in a clinical setting for MAB analysis.
4. The hypothesis was that the MABPac column could be re-purposed as part of a novel method for protein separation by HPLC. The aim was to develop and use 2D-LC/MS3 proteomics workflow to investigate biological samples such as serum.

2. Chapter 2: Materials and Methods

2.1. Materials

Buffers for SDS-PAGE and Western Blot analyses are shown in Table 2.1, Buffers for Silver Staining and Colloidal Coomassie are shown in Table 2.2. Buffers for cell lysis are shown in Table 2.3 and Buffers for cell culture are shown in Table 2.4. Buffers for HPLC analyses are shown in Table 2.5. Antibodies are shown in Table 2.6, Ligands are shown in Table 2.7 and inhibitors and nucleases are shown in Table 2.8.

Table 2.1: Buffers for SDS-PAGE and Western blot

Buffer	Usage	Supplier	Composition
NuPAGE™ 3-(N-morpholino)propanesulfonic acid (MOPS) SDS Running Buffer	Running pre-cast gels	Invitrogen	50 mM MOPS, 10 mM NaH ₂ PO ₄ , 50 mM NaCl, pH 7.5
Tris Buffer	pH buffer	Home-made	1.5 M Tris pH 6.8 and 8.6
1 x Laemmli Buffer	Gel loading	Home-made	2 % (w/v) SDS, 0.1 % (v/v) beta- βME, 0.0005 % (v/v) Bromophenol blue, 10 % (w/w) Glycerol, 63 mM Tris-HCl, pH 6.8
Tris-glycine SDS/PAGE running buffer	SDS-PAGE	Home-made	25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3
Tris-glycine transfer buffer	SDS-PAGE	Home-made	48 mM Tris base, 39 mM glycine, 20 % (v/v) MeOHMeOH
PVDF blocking buffer	Protein blocking	Home-made	5 % (w/v) milk powder in 1x TBS-T
TBS-Tbuffer (TBS-T)	Membrane washing	Home-made	50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.2 % (v/v) Tween 20
Poncaeu S	Protein staining	Sigma-Aldrich	5 % (w/v) acetic acid, 0.1 % (w/v) Ponceau S
10 % acrylamide resolving gel	Protein separation	Home-made	8.55 mL MilliQ water, 4.5 ml 40 % (w/w) acrylamide, 4.5 mL

			1.5 M TRIS pH 8.6, 180 μ L 10 % (v/v) APS, 18 μ L TEMED
Stacking gel	Protein separation	Home-made	3.1 mL MilliQ water, 0.5 mL 40 % (w/w) acrylamide, 1.25 mL 1.5 M Tris pH 6.8, 50 μ L 10 % (w/v) APS, 5 μ L TEMED
Mild stripping buffer	Membrane stripping	Home-made	15 g glycine, 1 g SDS, 10 mL Tween-20, pH 2.2

Table 2.2: Buffers for silver staining and colloidal Coomassie

Buffer	Usage	Supplier	Composition
Fixing solution	Silver staining	Home-made	50 % (w/w) Water, 40 % (w/w) EtOH, 10 % (w/w) Acetic Acid
Washing solution	Silver staining	Home-made	50 % (w/w) Water, 50 % (w/w) EtOH
Sensitisation solution	Silver staining	Home-made	0.02 % (w/v) Sodium Thiosulfate in water
Developing solution	Silver staining	Home-made	0.05 % (v/v) Formaldehyde, 3 % (w/v) Sodium carbonate
Quenching solution	Silver staining	Home-made	95 % (v/v) Water, 5 % (v/v) Acetic Acid
Coomassie staining solution	Coomassie	Home-made	50 % (v/v) Water, 40 % (v/v) EtOH, 10 % (v/v) Acetic Acid, 0.1 % (v/v) Coomassie R250
Coomassie destaining solution	Coomassie	Home-made	50 % (v/v) Water, 40 % (v/v) EtOH, 10 % (v/v) Acetic Acid

Table 2.3: Buffers for cell lysis

Buffer	Usage	Supplier	Composition
Non-denaturing lysis buffer stock solution	Lysis buffer	Home-made	50 mM Tris, 250 mM NaCl, 10 % (v/v) Glycerol, 1 % (v/v) NP40, pH 7.4
Non-denaturing lysis buffer	Cell lysis	Home-made	Non denaturing lysis Buffer stock solution + 5 mM β ME, 100 mM N-ethylmaleimide (NEM), protease and phosphatase inhibitors

Cell Lysis Buffer	Cell lysis	Home-made	50 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 % (v/v) Triton X-100, 50 mM NaF, 5 mM sodiumpyrophosphate, 1 mM sodium , orthovanadate, 10 mM sodium b-glycerophosphate, 100 mM NEM, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 % (v/v) , 2-βME, 0.27 M sucrose and cOmplete p rotease inhibitor
HB Buffer	Cell lysis	Home-made	25 g sucrose in 500 ml ddH2O with 5 ml 300 mM imidazol, pH 7.4

Table 2.4: Buffers for cell culture

Buffer	Usage	Supplier	Composition
Phosphate buffered saline (PBS)	Cell stability	Thermo Fisher Scientific	10 mM NaH ₂ PO ₄ , 1.8 mM KH ₂ PO ₄ , 137 mM NaCl, 2.7 mM KCl, pH 7.5
Red Blood Cells (RBC) lysis buffer (10x)	Cell Culture	Home-made	154 mM Ammonium chloride, 12 mM sodium bicarbonate, 0.1 mM EDTA

Table 2.5: Buffers for HPLC analysis

Buffer	Usage	Supplier	Composition
MES buffer	Column regeneration	Home-made	20mM MES hydrate, 300mM NaCl pH to 5.6 with NaOH)

Table 2.6: Antibodies

Antibody	Supplier	Catalogue Number	Host
Integrin alpha-M (CD11b/ITGAM)	Cell Signalling Technology (CST)	49420	Rabbit

NF-kappa-B inhibitor alpha (I κ B α)	CST	4814	Rabbit
TANK-binding kinase 1 (TBK1)	CST	3504	Rabbit
p-TBK1	CST	5483	Rabbit
TLR2	Invitrogen	14-9021-82	Rabbit

Table 2.7: Ligands

Ligand	Receptor	Supplier
Lipopolysaccharide (LPS)	TLR4	Enzo Life Sciences
S-[2,3-bis(palmitoyloxy)propyl]-Cys-Ser-Lys-Lys-Lys-Lys (PAM ₂ CSK ₄)	TLR2/6	Enzo Life Sciences
N-palmitoyl-S-(2,3-bis(palmitoyloxy)propyl)-Cys-Ser-Lys-Lys-Lys-Lys (PAM ₃ CSK ₄)	TLR1/2	Enzo Life Sciences

Table 2.8: Inhibitors and nucleases

Inhibitor	Function	Supplier
Bafilomycin A1 (BAF)	Vacuolar-type H ⁺ -ATPase (vATPase) - prevents endosome acidification	Invivogen
Benzamidine	Protease inhibitor	Sigma-Aldrich
Benzonase Enzyme	Nuclease - degrades with RNA/DNA	Pierce
cOmplete™ Protease inhibitor cocktail	Protease inhibitor	Roche
Dynasore	Interferes with dynamin function	Sigma-Aldrich
Glycerophosphate	Phosphatase inhibitor	Sigma-Aldrich
PMSF	Protease inhibitor	Sigma-Aldrich
Sodium ethylenediaminetetraacetic acid (EDTA)	Protease inhibitor	Sigma-Aldrich
Sodium molybdate	Phosphatase inhibitor	Sigma-Aldrich
Sodium orthovanadate	Phosphatase inhibitor	Sigma-Aldrich
Sodium tartrate dihydrate	Phosphatase inhibitor	Sigma-Aldrich
Pierce™ Universal Nuclease for Cell Lysis	Nuclease - interferes with RNA/DNA	Pierce
BIX02514	Inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β) inhibitor	Kindly donated by Neil Perkins

2.1. Cells

Bone marrow-derived MΦs were used, along with the following cell lines: BMA3.1A7 immortalised BMDM cell line were kindly provided by Kenneth Rock (Kovacsovics-Bankowski and Rock 1995) RAW 264.7 MΦ-like cell line, L929 (NCTC clone 929 [L cell, L-929, derivative of Strain L]) and J774A.1, a MΦ-like cell line from BALB/C mice were purchased from ATCC. BMDMs were differentiated from bone marrow stem cells taken from C57BL/6 mice femurs and tibias, as described in Chapter 2 section 2.2.6. L929 cells were obtained from ATCC, derived from C3H/An mice.

2.2. Cell culture

2.2.2. Cell passaging

BMA cells were grown in 10 or 15 cm Corning® BioCoat™ Poly-D-Lysine Culture Dishes plates to 80-90 % confluency. Cells were removed from the plates by scraping and were centrifuged along with the old medium at 1200 rpm (645 rcf) for 5 min. Old medium was aspirated, and cells re-suspended in fresh medium which had been previously warmed to 37 °C in a water bath. Cells were counted using a haemocytometer, and a cell suspension of $1-2 \times 10^6$ were transferred to a new cell culture dish and new medium was added to a total volume of 10-15 mL.

2.2.3. Culture of MΦ cell lines

All cell culture was performed under aseptic conditions in a class II biological safety cabinet. Cells were maintained in DMEM (for BMA (gifted by Kenneth Rock, see Chapter 2, section i.), J774.2 and RAW cells, purchased from ATCC) or IMDM (bone marrow derived MΦs (BMDM, extracted from mouse femurs)), supplemented with 10 % (v/v) foetal bovine serum (FBS), 1 mM L-Glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin. Cells were incubated under 5 % CO₂ at 37 °C in a water saturated incubator.

2.2.4. Freezing and thawing cells

Cells were removed from the cell culture plate by scraping and centrifuged at 1200 rpm (645 rcf) for 5 min to pellet the cells. Old medium was removed, and cells were re-suspended in DMEM medium containing 10 % DMSO. Approximately 5×10^5 cells suspended in 1.5 mL

medium are added to each cryogenic vial (Corning Incorporated) and placed into a Mr Frosty™ Freezing Container (Thermo Scientific) at -80 °C for 48 h, after which vials were transferred to liquid nitrogen.

For thawing cell, cryogenic vials were defrosted in a water bath set to 37 °C and cells were removed by pipette and added to a falcon (brand) tube containing 10 mL pre-warmed (to 37 °C) DMEM medium. Cells were centrifuged at 1200 rpm (645 rcf) for 5 min, and all medium was removed by aspiration. Cells were re-suspended in 10 mL new DMEM and transferred to a 10 cm cell culture plate and transferred to the incubator.

2.2.5. *Generation of L929 medium*

L929 cells from mouse connective tissue (ATCC) were maintained in T-75 cm flasks in 20 mL DMEM supplemented with 1 % Penicillin/Streptomycin, 1 % L-Glutamine and 10 % heat-inactivated Fetal Bovine Serum (all Sigma-Aldrich). Cells were passaged every week by removing cell culture medium, washing cells in the flask by adding 10 mL PBS (Thermo Fisher Scientific) and rotating the flask in a circular motion for approximately 5 minutes. The PBS was removed and 1 mL of Trypsin EDTA (Sigma-Aldrich) was added to the flask. Cells were incubated at 37 °C 5% CO₂ for 5 min before addition of 10 mL DMEM to wash the cells from the surface of the flask. Cell suspension was then added to a 15 mL falcon tube and centrifuged at 1200 rpm (645 rcf) for 5 min at RT. After centrifugation, supernatant was removed, and cell pellet was re-suspended in 10 mL fresh DMEM medium. 0.5 mL was added to each of two new flasks, each prepared with 20 mL fresh DMEM supplemented as described earlier.

To produce L929 medium for differentiating bone marrow-derived stem cells into MΦs, T-150 flasks (Nunc) were used, each with 150 mL DMEM cell culture medium (supplemented as previously described). Cells were counted in fresh DMEM after trypsinisation using a counting chamber. To count the cells, 10 µL of cell suspension was mixed with 10 µL Trypan Blue (Sigma-Aldrich) and added to the counting chamber. The number of cells in each square was counted, and the number of cells in each square were added together and divided by 9 (the number of squares counted) for an average value and multiplied by 1000. This was the number of cells per mL of suspension. To seed the cells, 0.2×10^6 cells in 1 mL of suspension were added to each flask and mixed by gentle rotation for an even suspension. Cells were incubated at 37 °C, 5% CO₂ for 7 days. After 7 days, the old medium was poured from the flasks and collected in 50 mL falcon tubes. It was filtered through 0.2 µM syringe filter and stored at -20

°C. Fresh DMEM (150 mL) was added to the cells which were incubated for another 7 days. At the end of the second 7 days, medium was collected, filtered, and stored as before and the cells were discarded. This medium was L929 medium, which was used for BMDM differentiation.

2.2.6. Isolation, differentiation, and culture of BMDMs from mice

Femurs and tibias were dissected from C57BL/6 male mice at 6 – 8 months old and cleaned of tissue. Bone marrow was extracted by cutting away the ends of the bones and flushing out the marrow with ice-cold PBS, using a syringe and a needle. The collected marrow was homogenised and passed through a cell strainer to remove any remaining pieces of tissue and collected in a 50 mL falcon tube. The homogenised marrow/PBS mixture was centrifuged at 1300 rpm (747 rcf) for 3 min to pellet cells. The supernatant was removed, and cells were re-suspended in RBC lysis buffer (Table 1.ii) (2 mL per mouse) and left on ice for 2 min to lyse RBC. After 2 min, the RBC lysis buffer was quenched with ice-cold DMEM or IMDM (5 mL) and the mixture was centrifuged as before and supernatant containing red blood cell debris was removed. The remaining cells were washed twice in PBS and plated into a 10 cm cell culture plate (Corning) with 10 mL IMDM medium supplemented with 10 % FBS, 20 % L929 medium, 1 % L-Glutamate and 1 % Pen/Strep (all Sigma). After 24-48 h the cells were removed by scraping. The cells and medium were centrifuged as before, and the supernatant removed. Cells were then re-suspended in fresh medium and counted. Cells were then plated onto 10 cm bacterial cell culture plates (Thermo Fisher Scientific) at a cell density of around 4×10^6 cells per plate in 10 mL of fresh medium and incubated at 37 °C, 5 % CO₂ for six days until use, with 2 mL of fresh medium being added every two days.

2.2.7. Stimulation of cells with ligand-coated beads

For magnetic beads, EM2 100/40 magnetic beads (Etapor) were sonicated in the original container for 5 min and 333 µL beads were removed and washed three times in 1 mL ice-cold sterile PBS on the magnetic rack. Beads were then incubated with 50 µg PAM₃CSK₄ or 5 µg LPS in 500 mL PBS, or the equivalent of DMSO in 500 mL PBS as the control, overnight on rotation at 4 °C.

For latex beads, carboxylated polystyrene (0.8 μ m) beads were sonicated in the bottle for 5 min and 1 mL beads were removed and washed three times in 1 mL ice-cold PBS (centrifuged at 1000 rpm (112 rcf) for 2 min at 4 °C). Beads were then incubated with 150 μ g PAM₃CSK₄, 15 μ g LPS or the equivalent of DMSO as a control, overnight on rotation at 4 °C.

2.2.8. Stimulation of cells with soluble ligands

Cells were stimulated with 1 μ g/mL PAM₃CSK₄ or 1 ng/mL LPS in 10 mL medium for 30 min before being washed three times with ice-cold PBS to stop any further cellular response and to remove traces of medium. Cells were then frozen or lysed.

2.2.9. Treatment of cells with inhibitors

BAF and Dynasore (Dyn) were added to cell media at a concentration of 1 μ M for 1 h prior to cell stimulation, and for the duration of cell stimulation (30 min for western blot, FACS or proteomics, 3 h for qRT-PCR). BIX02514 was re-suspended in DMSO for a concentration of 10 mM and added to cell media at a concentration of 0.5 μ L per 1 mL of solution (2 μ M) for 1 h prior to cell stimulation and for the duration of cell stimulation.

2.3. MS Proteomics

2.3.2. Cell lysis

Cells were moved onto ice and washed twice with ice-cold PBS before the addition of Cell lysis buffer (Table 2.3, Buffers for cell lysis).

Lysis buffers were supplemented with protease inhibitors (cOmplete™, Roche), metalloprotease inhibitors (EDTA and EGTA), phosphatase inhibitors (1.15 mM sodium molybdate, 4 mM sodium tartrate dihydrate, 1 mM freshly prepared sodium orthovanadate, 5 mM glycerophosphate, all Sigma) and nucleases (Universal Nuclease or Benzonase® from Sigma.)

Lysate was clarified by centrifugation at 4 °C for 20 min at 14,000 rpm (21952 rcf)). Protein concentration was determined by Bradford (Bradford, 1976), Bicinchoninic acid assay (BCA) or EZQ assay.

2.3.3. *Bicinchoninic acid assay*

BCA protein quantification was performed using the BCA protein assay kit (Pierce). A standard curve was created from BSA serial dilutions ranging from 2000 µg/mL to 25 µg/mL. Serial dilutions of the protein samples were aliquoted into a 96-well plate in triplicate, with the standards and a protein-free blank. Reagent A was mixed with reagent B for a ratio of 50 parts A to one part B to make the working reagent. 200 µL of the working reagent was added to each well and the plate was incubated at 37 °C for 30 min. Reagent colour change was measured by fluorescent plate reader at 562 nm and measurements were given against the standard curve prepared from BSA protein standards.

2.3.4. *Bradford protein assay*

Proteins were quantified by Bradford assay which uses Coomassie dye that binds to basic amino acid residues, causing the dye to turn from brown to blue. Serial dilutions of cell lysate and corresponding dilutions of a protein standard (such as BSA) were created in a 96-well plate, as well as a blank. Absorbance is measured at 595 nm and protein concentration is determined against a standard curve prepared from BSA protein standards.

2.3.5. *EZQ protein assay*

As some components of cell lysis buffers, such as SDS or other denaturing agents, can interfere with the Bradford assay, some protein samples were quantified using EZQ (Invitrogen). A standard curve was created against BSA protein standards ranging from 0.02-5 mg/mL. Assay paper was inserted into an EZQ cassette, with 96 open spaces. A serial dilution of protein samples was made and 2 µL of each dilution and each standard was pipetted into a separate space on the paper, indicated by the cassette with 96 holes. Care was be taken not to touch the assay paper with the pipette tip. Samples were then dried for 10 min before the assay paper was removed from the cassette. Samples were fixed to the assay paper by washing it in 40 % MeOH with agitation for 5 min. This step also removed interfering buffer components such as in Laemmli buffer. After fixing with MeOH, the assay paper was left on the bench until completely dry. Proteins were stained by addition of 40 ml EZQ protein quantitation reagent to

the assay paper and incubating for 30 min at RT with gentle agitation. After staining, the assay paper was rinsed with 10 % MeOH, 7 % acetic acid for 1-2 min for a total of three washes. The assay paper was then left to completely dry before analysis by fluorescence plate reader. Fluorescence was measured at 618 nm using excitation at 450 nm.

2.3.6. Reduction and alkylation

Proteins were reduced by addition of 5 mM TCEP in milliQ water to the total cell lysate. Samples were incubated at 37 °C on a shaker for 30 min. Samples were immediately alkylated by addition of 20 mM iodoacetamide and incubated on the bench for 30 min, in the dark, (achieved by wrapping the samples in aluminium foil).

2.3.7. In-gel digestion

Proteins were resolved by SDS-PAGE and stained by colloidal Coomassie. Bands were excised by using a scalpel and placed into 1.5 mL Eppendorf tubes. To detain the gel, 100 µL of 100% ACN was added to the gel, which was followed by a 10 min incubation period on a shaker at RT, after which the CN was removed and discarded. Gel pieces were incubated with 50 µL 50 mM Tris HCL pH 8.0 for 5 minutes on a shaker at RT. Supernatant was removed and discarded. These steps were repeated three times, or until gel pieces were no longer blue. Proteins within the gel were reduced by addition of 5 mM TCEP in 50 mM Tris HCL pH 8.0, followed by incubation on a shaker at 37 °C for 30 minutes. Proteins were alkylated by addition of 20 mM iodoacetamide and incubation at RT, in the dark for 30 minutes. After alkylation, gel pieces were shrunk by addition of 100 µL ACN and shaken at RT for 5 min, and the ACN discarded. Gel pieces were incubated with 50 µL 50 mM triethylammonium bicarbonate, pH 8.0 for 5 min at RT, after which supernatant was discarded. Gels pieces were incubated again with 100 µL ACN and shaken for 5 min at RT before discarding supernatant. Proteins were digested by addition of 0.2 µg trypsin in 50 µL triethylammonium bicarbonate, pH 8.0 and incubated overnight at 37 °C on a shaker. For protein extraction, the supernatant was removed from the gel and 100 µL ACN added to the gel. The gel was incubated for 5 min at room temperature on a shaker, and the supernatant was removed and kept. T re-swell the gel, 50 µL of 0.1 % TFA was added, and the extraction process was repeated twice more: 100 µL ACN was added, and

after a 5 min incubation, removed and combined with the other extractions. Extractions were then lyophilised in a speedvac and stored at -80 °C before MS analysis.

2.3.8. Single-pot, solid-phase-enhanced sample preparation

The single-pot, solid-phase-enhanced sample preparation (SP3) protocol (Hughes et al. 2014), (Hughes et al. 2019) was used to de-salt and digest protein samples. Before starting, 1 µL/mL nucleases such as universal nuclease (Pierce) was added to the sample to prevent agglomeration due to DNA, and the stock of encapsulated magnetic microspheres (beads) stock was gently sonicated to re-suspend the beads. Magnetic beads were washed in PBS and a preparation of 10000 µg beads/mL was created by adding the beads to a magnetic rack to separate them from the PBS, removing the PBS and weighing the dry beads. PBS was added according to the dry weight for a 10 mg/mL concentration. After reduction and alkylation, the sample was diluted to 0.1 - 1 mg/mL in SDS lysis buffer. Beads were added to the sample at a concentration of 1:5 (v: w) (beads: proteins) and the sample was gently vortexed before 500 µL of 100 % acetonitrile and 16 µL of 10 % formic acid were added to the sample. The sample was gently vortexed again before an incubation period of 8 min on the bench.

The samples were placed on the magnetic rack for 2 min at RT. The beads were washed twice on the rack with 200 µL 70 % EtOH and incubated for 30 s before the supernatant was discarded. The beads were washed for the third time with 180 µL ACN, incubated for 30 s and the supernatant discarded. The beads were allowed to air dry on the rack. Once dried, beads were re-constituted in 200 µL 100 mM HEPES and Trypsin was added at a ratio of 1:100 (w/w) protein: trypsin. Proteins were incubated with trypsin for 4 h at 37 °C on a shaker. The digestion step was repeated twice.

After digestion, 700 µL 100 % acetonitrile was added to the sample and samples were incubated for 8 min. Samples were placed on the magnetic racks and the supernatant was removed. Beads were washed with 180 µL of 100 % acetonitrile and left to dry for 10 min. Peptides were eluted from the beads with 12 µL 2 % DMSO and beads were completely removed from the sample with a final centrifugation step, where samples were centrifuged at 200 rcf at 4 °C for 10 min. Supernatant containing the peptides was collected and 20 µL 20 % formic acid was added before analysis by MS.

2.3.9. S-Trap

The S-trap (Protifi) is a dual SDS-removal and digestion technique, which uses S-Trap columns. Cells were lysed in 5 % SDS, sonicated for 3 rounds of 30 s burst at 4 °C. The sample was clarified by centrifugation for 8 min at 16000 rcf and reduced and alkylated. 2.5 µL of 12 % phosphoric acid was added to the cell lysate, and 165 µL of S-trap binding buffer (90 % MeOH, 100 mM TEAB, pH 7.1) was added to the lysate before it was added to the S-trap micro column. The column was spun in a benchtop centrifuge at 4000 rcf to force the solution through the phase. The column was washed three times with 150 µL S-trap binding buffer. All flow through was discarded. For digestion, trypsin was added to the column at a concentration of 1:10 in 25 µL of 50 mM TEAB. The column was loosely capped and transferred to a clean tube for incubation at 47 °C for 1 h. Peptides were eluted with 40 µL of 50 mM TEAB and with 0.2 % aqueous formic acid, both of which were centrifuged through the column as before. A further elution for hydrophobic peptides was performed by adding 35 µL 50 % acetonitrile and 0.2 % formic acid to the column and centrifuging. The flow through containing the peptides was collected and lyophilised before being re-suspended for analysis by MS.

2.3.10. TMT labelling

TMT reagents (TMTsixplex™ Isobaric Label Reagents from Thermo Fisher Scientific) were warmed to RT before use. After protein digestion, the TMT reagent was re-suspended in anhydrous acetonitrile (41 µL for the 0.8 mg vials). 40 µL of the dissolved TMT reagent was added to each peptide sample and incubated for 1 h. Each experimental condition was labelled with a different tag. The reaction was quenched by addition of 8 µL 5 % hydroxylamine followed by an incubation period of 15 min at RT. Samples from the same replicate (for example, replicate 1 from each experimental condition) were combined and stored at -80 °C until analysis.

2.3.11. Preparation of samples for mass spectrometry

Cells were lysed and the protein sample clarified by centrifugation at 4 °C for 20 min at 15000 rcf. Supernatant was transferred to a clean 1.5 mL centrifuge tube and either separated by SDS-PAGE gel (for in-gel digestion) or reduced and alkylated. Digestion was performed by either S-Trap or SP3, or in-gel digestion if the proteins were separated by SDS-PAGE gel. Peptides were fractionated by basic reversed phase HPLC, fractions were concatenated and lyophilised. Lyophilised samples were re-suspended in 5 % formic acid and up to 2 µg of peptides were

injected on the LC-MS for analysis. The LC-MS analyses were performed on an Ultimate 3000 RSLCnano HPLC (Thermo) coupled to an Orbitrap™ Fusion™ Lumos™ Tribrid™ Mass Spectrometer, an Orbitrap™ Fusion™ Tribrid™ Mass Spectrometer (Thermo), or a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ (Thermo).

2.3.12. HiPPR™ Detergent Removal Spin Column Kit

HiPPR™ Kit from Thermo Fisher Scientific (Cat. No: 88305). 200 µL of removal resin was added to the spin column and column was centrifuged for 1 min at 15000 rcf. The flow through was discarded. 200 µL of equilibration buffer was added to the column and the column was centrifuged again at 1500 rcf for 1 min. The flow through was discarded and the equilibration buffer step was repeated two more times. 200 µL of the sample containing 0.2 % SDS in water was added to the column and the column was incubated for 10 minutes at RT. After incubation, the column was centrifuged at 1500 rcf for 1 min and the detergent-free sample was collected from the lower section of the spin column.

2.4. High performance liquid chromatography (HPLC)

2.4.2. Off-line reverse phase fractionation of peptides

Peptide samples were lyophilised and re-suspended in buffer A (0.1 % TFA in water) at a concentration of 1 µg/µL and loaded on C18 reverse phased column (Phenomenex), (100 mm x 2.1 mm with a particle size of 5 µm). Peptides were eluted using a gradient of buffer B (80 % ACN, 0.1 % TFA in water) from 2 to 98 % over 21 min. Fractions were collected every 43 s for 16 min and non-consecutive fractions were concatenated in order to obtain a good orthogonality with the second dimension of separation.

2.4.3. Off-line MAbPac™ reverse phase fractionation of proteins

Protein samples were lyophilised and re-suspended in Buffer A (2 % ACN, 0.1 % FA in water) at a concentration of 1 µg/µL and fractionated using a MAbPac™ RP column (Thermo Fisher Scientific; 100 mm x 3 mm with a particle size of 4 µm). Proteins were eluted using a gradient of buffer B (45 % ACN, 45 % Isopropanol and 0.1 % FA in water) from 2 % to 100 % over 32 min. Fractions were collected every 82 s for 32 min.

2.4.4. Off-line MAbPac™ SCX fractionation of antibodies

Antibody samples were lyophilised and re-suspended in water at a concentration of 5 µg/µL and 0.025 µL of each antibody was fractionated using a MAbPac™ SCX-10 RS (Thermo Fisher Scientific; 50 mm x 2.1 mm with a particle size of 5 µm). Rituximab was eluted using a gradient of buffer B (10 mM ammonium hydroxide) from 85 % to 100 % over 10 min, with 25 mM ammonium bicarbonate, 30 mM acetic acid in water as buffer A. Bevacizumab was eluted using a gradient of 35 % to 60 % buffer B over 10 min.

2.4.5. On-line reversed phase

Lyophilised peptides were re-constituted in 5 % formic acid before being loaded on the LC-MS. First samples were injected onto a trap using loading buffer (2 % ACN, 0.1 % TFA in water), then separated on the analytical column (an easyspray C18 reverse phase column with 1.9 µm particle size, diameter of 75 µm) using a linear gradient of buffer A (0.1 % TFA in water) and B (80 % ACN, 0.1 % TFA in water) starting at 3 % B and increasing to 80 % B over 160 min.

2.5. Bioinformatics

2.5.2. MAXQUANT search

Peptide and protein identification and quantification were performed using MaxQuant (version 1.5.7.4) using relevant search strategies (LFQ for label free experiment and MS2 or MS3 reporter ion for TMT performed on the Q-Exactive HF or Fusion Lumos respectively). NEM was set up as fixed modification when experiments involved using NEM, while Carbamidomethyl (C) was used otherwise. Protein N terminal acetylation, methionine oxidation and asparagine/glutamine deamination were set as variable modifications. The maximum number of modifications per peptide was set to 5 and maximum missed cleavages to 2. Searches were conducted against a murine Uniprot database with isoforms (downloaded 2018) and a list of common contaminants. Identifications were filtered at a 1 % false-discovery rate (FDR). Quantification used only razor and unique peptides with a minimum ratio count of 2. “Re-quantify” was enabled. “Match between runs” was used with alignment time window

20 min and match time window 0.7 min. LFQ intensities were used for data analyses. For TMT, corrected intensities were used for data analysis.

2.5.3. Analysis of MS data using Perseus

MaxQuant output files were used for the analysis of MS data, which was performed using Persues (version 1.6.2.1). For label-free experiments, LFQ intensities were used for analysis and for TMT experiments, normalised intensities were used. Rows were filtered to remove hits only identified by reverse, those labelled as potential contaminants and those which have a score of below 2. Data was log2 transformed were and visualised as multi-scatter plots and histograms as a quality control. The matrix was reduced based on valid values and multi-sample or two-sample tests were performed on the data. Statistical tests were carried out based on permutation-based FDR of 5 % or a p-value of 0.05. Median based imputation, which can be used to replace missing values in a dataset, was not used unless clearly mentioned in the text.

2.5.4. GO terms assignment using DAVID

Significant hits obtained from Perseus analysis were clustered based on hierarchical clustering. UniProt accession numbers of these hits were entered into DAVID (<https://david.ncifcrf.GOV/>) (D. W. Huang, Sherman, and Lempicki 2009) and submitted as a gene list. Functional annotation clustering was performed against a murine database and results for CC, BP and MF (cellular signalling, biological process and molecular function) were downloaded and processed in EXCEL.

2.5.5. Analysis of MS data using R

Volcano plots, bar charts, violin plots, chromatograms and box plots were plotted using R version 3.6.2 (R Development Core Team 2008) with packages ggplot2 (Wickham 2016), dplyr (Wickham et al. 2019) and reshape (Wickham 2007).

2.5.6. Figure creation using Adobe suite

All Figures unless otherwise stated were drawn or assembled by Megan Gant in Adobe Illustrator CS6. Adobe Photoshop CS6 was used for the cropping of raw Western blot films.

2.6. Biochemistry

2.6.2. Phagosome isolation using carboxylated polystyrene beads

Phagosomes were isolated from BMA cells according to previous methods (Desjardins and Griffiths 1994; Trost et al. 2009; Boulais et al. 2010; Dill et al. 2015; Härtlova et al. 2017) (Figure 2.1). The beads used for phagosome isolation were 0.8 μm carboxylated polystyrene beads (Millipore Estapor® Microspheres #K1 080) and they were prepared according to 'Coating of carboxylated beads with ligands'.

To induce phagocytosis, prepared beads were added to aliquots of pre-warmed serum-free DMDM medium. Medium was removed from cells and 4 ml of the medium/beads mixture was added to each plate. Cells were incubated with beads at 37 °C, 5 % CO₂ for 30 min to allow phagocytosis to occur. After incubation, cells were washed with ice-cold PBS and scraped into 50 mL falcon tubes on ice. Non-internalised beads were removed by centrifugation, 1500 rpm (527 rcf) for 5 min at 4 °C. Cells were re-suspended in ice-cold PBS and transferred to 15 mL falcon tubes (7 dishes, or one condition per falcon tube) and re-centrifuged at 1500 rpm (527 rcf), 5 min at 4 °C. The cell pellet was gently re-suspended in 1 mL hypertonic buffer (HB, 8.55 % (w/w) sucrose, 2.5 mM imidazole, pH 7.4) by briefly and gently vortexing the falcon tube. After re-suspension, cells were centrifuged as before, and the supernatant removed. The pellet was re-suspended in 1 mL HB supplemented with inhibitors of proteases (cOmplete™, Roche) and phosphatases (1.15 mM sodium molybdate, 4 mM sodium tartrate dihydrate, 1 mM freshly prepared sodium orthovanadate, 5 mM glycerophosphate, all Sigma) and 100 mM NEM. Cells were homogenized in a pre-cooled metal Dounce homogeniser (Wheaton, Millville, NJ) until 80 % of cells were broken without major breakage of the nucleus, as verified with trypan blue under a microscope. Unbroken cells and nuclei were pelleted in 15 mL Falcon tubes at 2000 rpm (1792 g) for 5 min at 4 °C. The supernatants were collected into 2 mL Eppendorf tubes (1 mL per tube).

Phagosomes were separated from the total cell lysate by sucrose gradient. In an ultracentrifuge tube (Beckman Coulter), 1 mL of 68 % sucrose solution (68 % (w/w) sucrose, 2.5 mM imidazole, pH 7.4) was added using a Pasteur pipette. This layer was followed by 1 mL TCL mixed with 1 mL 68 % sucrose solution with inhibitors of proteases (cOmplete™, Roche) and phosphatases (1.15 mM sodium molybdate, 4 mM sodium tartrate dihydrate, 1 mM freshly prepared sodium orthovanadate, 5 mM glycerophosphate, all Sigma) and 100 mM NEM. The

second layer was created using 2 mL of 35 % sucrose solution (35 % sucrose, 2.5 mM imidazole, pH 7.4), followed by a third layer consisting of 2 mL of 25 % sucrose solution (25 % sucrose, 2.5 mM imidazole, pH 7.4). The final layer consisted of 2 mL of 10 % sucrose solution (10 % sucrose, 2.5 mM imidazole, pH 7.4). Creation of the sucrose gradients was performed on ice, in a cold room set to 4 °C using Pasteur pipettes to prevent any mixing between the layers. After the gradients were complete, each ultracentrifuge tube was weighed, and the weights were adjusted with 10 % sucrose solution so that the weight of tubes placed in opposite positions on the rotor weighed the same (within ≤ 10 mg).

The tubes containing the samples and gradients were centrifuged in an ultracentrifuge (Beckman Coulter) with a SW41Ti swinging-bucket rotor (Beckman Coulter) at 24000 rpm (72300 rcf) for 1 h at 4 °C. After the centrifugation, the latex beads containing phagosomes were visible by showing a blue band between the 10 % and the 25 % sucrose layers. The phagosomes were collected into new ultracentrifuge tubes using a thin-tipped transfer pipette. Phagosomes from two gradients were combined into one ultracentrifuge tube and were washed by adding cold PBS until the tube was three quarters full. After mixing, phagosomes were pelleted by centrifugation at 15000 rpm (28400 rcf) for 15 min at 4 °C. After removing the supernatant, the phagosomes were either lysed for experiments or stored at -80 °C.

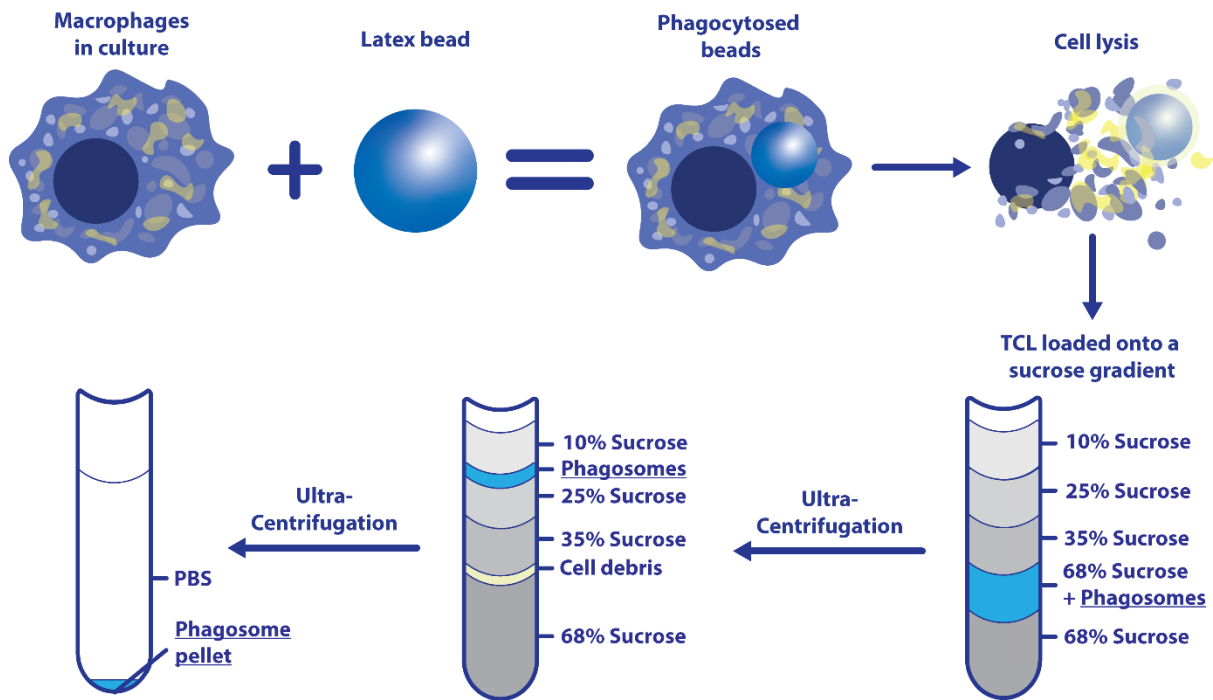


Figure 2.1: Phagosome isolation. Latex beads are fed to MΦs in culture, who phagocytose the beads. Cells are mechanically lysed that leaves organelles intact, including the phagosomes with the beads still inside. The TCL is then loaded onto a sucrose gradient and the various components of the cell are separated by ultracentrifugation. Pure phagosomes float between the 10 % and 15 % sucrose layers where they can be siphoned off, washed with PBS and pelleted by another round of ultracentrifugation.

2.6.3. Preparation of ligand-coated carboxylated beads

To create ligand-coated beads, 0.8 μm carboxylated polystyrene beads (Millipore Estapor® Microspheres #K1 080) were sonicated for 5 min in the original bottle before use to re-suspend beads. For each 10cm dish, 100 μL of beads were used. Beads were washed 3 times in PBS before being re-suspended in 1 mL PBS. Ligands PAM_3CSK_4 or LPS were added to the beads/PBS mixture at a concentration of 10 μL (10 μg) PAM_3CSK_4 or LPS per 100 μL beads. Beads and ligand were rotated at 4 $^\circ\text{C}$ for 24 h. After binding had occurred, beads were washed 3 times in PBS before being introduced to cells. For washing, beads were centrifuged at 527 rcf for 2 min. To preserve the ligands, all PBS was cooled to 4 $^\circ\text{C}$.

2.6.4. Protein stripping for PVDF membranes

Membranes were stripped by washing 6 times with TBS-T for 5 min per wash before incubating membranes with 5 mL mild stripping buffer (Table 2) and 50 μL β -mercaptoethanol for 15 min.

This process was repeated twice. Membranes were washed 6 times with TBS-T and re-blocked with 5 % milk powder in TBS-T for 30 min. After a further 6 washes in TBS-T, the membrane was ready for incubation with the primary antibody.

2.6.5. Measurement of RNA concentration

RNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific) in RNase-free water. The absorbance of isolated RNA was measured at 260 nm. Measurements were taken from the average measurement of three replicates per condition.

2.6.6. Mouse primers for qRT-PCR

Primers GAPDH, Cxcl10, Ifnb1, RS18 and Tnf α were all Validated QuantiTect primer assays from Qiagen.

2.6.7. qRT-PCR

BMA or BMDM cells were seeded at a final concentration of 0.2×10^6 cells per well into a 12-well plate and left to settle for 2 h or overnight at 37 °C. Cells were treated and/or stimulated before being placed on ice and washed three times in ice cold PBS. RNA was extracted from the cells using RNeasy Purification Kit (Qiagen, Cat No. 74104). 5 μ g RNA was reverse transcribed using the iScript cDNA synthesis kit from Bio-Rad (107-8891). PCR was performed in a 96 or 384-well plate, each containing a 10 μ L reaction. Each reaction consisted of 0.5 % cDNA, 0.5 μ M primer and 5 μ L SYBRTM Green from ThermoFisher. mRNA levels were normalised to RS18 and the relative gene expression levels compared to unstimulated cells and were calculated according to the comparative cycle threshold (Livak and Schmittgen 2001). Levels of mouse RS18, Tnf α , IL-6, Ifnb1 and Cxcl10 mRNA was detected by QuantiTect primers (Qiagen).

2.6.8. SDS-PAGE protein separation and PVDF membrane transfer

Gels were either homemade (Table 1.ii) or pre-cast (4-12 % Bis-Tris gels, 10, 15 or 20 well, from NuPAGETM)

Home-made gels were cast between glass plates of 1.0- or 1.5-mm depth, with the separating gel being cast first and the resolving gel being cast on top. After each casting, gels were left on the bench, levelled and upright, at RT for 45 min. Wells were created in the stacking gel using a comb.

Protein samples were quantified by Bradford assay and mixed 1:1 with 3x Laemmli buffer with 16 % β ME to aid protein denaturation. 25 μ g of each protein sample and 5 μ L of molecular weight marker were loaded into the wells at the top of the gels and the gels were electrophoresed at RT, at 80 V for 30-45 min and 120 V until the largest proteins had reached the bottom of the gel (1.5 - 2 h).

Commercial gels were electrophoresed in 1x MOPS buffer and home-made gels were electrophoresed using running buffer (Table 1.2). All gels were electrophoresed using The Mini Trans-Blot Cell apparatus (Bio-Rad).

After proteins had been resolved within the gel, gels were removed from the casing, washed in deionised water, and transferred to a PVDF membrane. Membranes were cut to size and pre-activated for 1 min in 100 % MeOH and subsequently washed in deionised water. Membranes and gels were layered together in a gel holder cassette with blotting paper and sponge in the following order, from Cathode side to anode side: sponge, blotting paper, gel, membrane, blotting paper, sponge. The layers were sandwiched inside the transfer cassette and placed in the transfer tub in ice-cold transfer buffer (Table 2.2). Transfer using The Mini Trans-Blot Cell apparatus (Bio-Rad) took place on ice at 100 V for 1 h.

After transfer, membranes were removed and washed in deionised water. Membranes were stained with Ponceau Red (Sigma) for approximately 30 s on a shaker at RT. Membranes were de-stained and washed in deionised water before being scanned and processed by western blotting.

2.6.9. *Western blot*

The PVDF membranes from the SDS-PAGE transfer were washed in deionised water and blocked for 30 min to 1 h at RT on a rotator to reduce non-specific background antibody signal using 10 mL of 5 % milk dissolved in 0.1 % TBS-T. After blocking, membranes were rinsed in 5 mL TBS-T for 1 min on a rocker. Membranes were incubated with the primary antibody at a ratio of 1:1000 in TBS-T. Incubation was either at RT for 1 h or overnight at 4 °C. After

incubation with the primary antibody, a further 6 TBS-T washes were performed before incubation with the secondary antibody at 1:5000 in TBS-T at RT for 40 min – 1 h. Membranes were washed a further 6 times in TBS-T and enhanced chemiluminescence (ECL) reagent (Biorad) was mixed 1:1 and added to the membrane, where they were incubated together on the bench for 5 min. After ECL application, membranes were washed once in TBS-T and visualised by Hyperfilm (GE Healthcare Amersham™ Hyperfilm™ ECL, GE Healthcare) film in a dark room. Developed films were scanned by a digital scanner. The same conditions were used for all antibodies.

Membranes were stored in TBS-T at 4 °C for up to a week for eventual re-visualisation or stripping.

2.6.10. Colloidal Coomassie protein staining of SDS-PAGE gels

Colloidal Coomassie staining solution was made by mixing 80 % MilliQ Water, 10 % Phosphoric Acid, 10 % Ammonium Sulphate, 0.1 % Coomassie G250 and left to stir at RT overnight before filtering. Proteins were fixed in the SDS-PAGE gel was then incubated for 30 min at RT in a glass container which was thoroughly cleaned with 50 % Water, 40 % Ethanol, 10 % Acetic Acid. After fixing, the gel was washed for 10 min with MilliQ water at RT for a total of 2 washes. The gel was then stained with 50 mL 80 % Colloidal Coomassie staining solution freshly supplemented with 20 % MeOH. Staining took place on rotation, at RT, overnight. After the gel was stained, the gel was removed to a new glass container which had been cleaned with EtOH. The gel was covered with 1 % acetic acid. Destaining took place on rotation at RT for 30 min, and the 1 % acetic acid solution was replaced if it turned blue. The destained gel was then scanned by digital scanner and either discarded or processed for in-gel digestion.

2.6.11. Silver staining of SDS-PAGE gels

The SDS-PAGE gel was covered in 50 mL 40 % EtOH, 10 % Acetic acid and incubated at RT for 30 min, with agitation. The gel was removed from the solution and incubated in 50 mL 30 % EtOH for 30 min at RT with agitation. The gel was washed three times in 50 mL MilliQ water for 10 min per wash, at RT with agitation. During the washes, the following solutions were made fresh: 0.02 % Sodium thiosulfate pentahydrate and 0.1 % Silver nitrate (cooled to 4

°C) (both in MilliQ water). After washing, the gel was incubated with 50 mL 0.02 % Sodium thiosulfate pentahydrate for 1 min at RT with agitation. The gel was washed twice for 20 s. The gel was covered with 50 mL 0.1 % Silver nitrate and the container wrapped in aluminium foil and incubated at 4 °C for 2 h, with agitation. During incubation, the development solution (0.05 % formaldehyde (stock is 37 % formaldehyde solution in MilliQ water), 3 % sodium carbonate) and stopping solution (95 % MilliQ water, 5 % acetic acid) were made fresh. After incubation with silver nitrate, the gel was washed twice in MilliQ water for 20 s per wash and incubated with 25 mL development solution. The gel was agitated at RT, under a bright light, until protein bands became clear or until the development solution became yellow, at which point the developing solution was replaced with another 25 mL of development solution. Once bands were visible, the reaction was stopped by removal of development solution and incubation of the gel with 50 mL stopping solution. Gels were scanned with a digital scanner and either discarded or destained for in-gel digestion.

2.7. Microbiology

2.7.2. Culture of Escherichia coli

For the culture of *E. coli*, which was used for HPLC optimisation, all procedures were carried out in sterile conditions next to a Bunsen burner. To grow *the E. coli*, 5 ml lysogeny broth (LB) media was added to a 15 mL falcon tube. *E. coli* came from *E. coli* DHB10 competent frozen stocks in glycerol which were stored at -80 °C. One aliquot was added to the LB media in the falcon tube and incubated at 37 °C on a shaker overnight. After shaking, the *E. coli* was amplified by adding 5 mL of the overnight growth to 500 mL of LB media in a conical flask with aluminium foil loosely covering the opening. The stock was again incubated overnight, on a shaker at 37 °C. The stock was separated into four flasks, each containing 500 mL LB. OD readings were taken at 600 nm. Once in the growth phase (with an OD at 600 nm between 0.5 and 1), the bacteria were centrifuged at 4000 rpm (7168 rcf) for 10 min and pellets were re-suspended in water. Lysis was performed using a French press and clarified by further centrifugation. Supernatant was quantified by Bradford assay and frozen at -80 °C in 2 mg/mL aliquots.

2.7.3. *Culture of Staphylococcus aureus*

The SH1000 strain of *S. aureus*, which had been transfected with yellow fluorescent protein (YFP), was obtained from the lab of Dr. Sarah Coulthurst at the University of Dundee and arrived as a frozen stock which was kept at -80 °C. All work with these bacteria was carried out in a laboratory with Biosafety Level 2 (BSL-2), under sterile conditions. Bacteria were taken from the frozen stock and established on a plate containing Tryptic Soy Agar (TSA) supplemented with 10 µg/mL chloramphenicol and left to grow overnight at 37 °C. The next day, 3-5 colonies were selected from the agar plate and added to 5 mL TSA in a 15 mL falcon tube, which was incubated overnight at 37 °C on a rotating shaker. The density of the *S. aureus* was measured at OD₆₀₀ and used for infection of cells and phagocytosis assay.

2.7.4. *Infection of cells with S. aureus & phagocytosis assay*

For the phagocytosis assay, BMA cells were grown in 96-well plates to 80 % confluency. *S. aureus* was added to the cells in 500 µL medium at a MOI of 1:10. Cells were allowed to phagocytose the bacteria at 37 °C for 30 min before being washed with trypan blue to quench signal from any un-phagocytosed bacteria. Phagocytosis was measured at 514 nm over 16.5 h using a plate reader (SpectraMax iD3 from Molecular Devices).

3. Chapter 3: Development of the MAbPac™ columns for charge variant analysis and protein separation

3.1. Introduction

This Ph.D was co-sponsored by Thermo Fisher Scientific, which, in addition to other projects, required collaboration in research and method development of some Thermo Fisher Scientific products, particularly HPLC columns.

In a clinical setting, SCX and WCX columns have been used for CVA (charge variant analysis). Therapeutic antibodies which are used to treat diseases such as auto-immune diseases and cancer are tested for PTMs which could potentially interfere with their medicinal function, by inducing off-target effects or being rendered unable to bind with the target protein. Presence of PTMs can be detected by identification of a change in the overall charge of the protein. Charge variants produce a shift in the chromatogram of proteins separated by ion exchange HPLC or MS (Chung et al. 2018; Dakshinamurthy et al. 2017; Farnan and Moreno 2009; Füssl et al. 2018). Ion exchange columns elute analytes by an increasing salt concentration, where the positively (or negatively) charged salt particles compete with the analyte for binding sites on the charge stationary phase. Therefore, ion exchange chromatography requires long columns and gradients of up to an hour to achieve a good separation. In the clinical setting, a faster, higher throughput method is more desirable. Using a pH buffer instead of a salt buffer has been shown to achieve comparable separation using a shorter column and a much shorter gradient (10 min) and could therefore be a potential candidate for high-throughput methods for clinics. This is only possible if the buffers can be made 'MS-friendly' (without salts or detergents) as to enable samples to be immediately analysed by MS. However, MS-compatible pH buffers are difficult to reproduce and keep stable for long periods of time (Füssl et al. 2018).

The MAbPac™ range of columns were initially developed for the separation of antibodies but could be applied for the separation of any whole protein sample. Separating a sample at the protein level could have many advantages, including increased orthogonality for 2D-LC, and the isolation of high abundance proteins from a sample, enabling identification of lower-abundance proteins which could otherwise have been masked.

3.2. Aims

1. *To develop pH buffers for the separation of antibodies using the MAbPac™ SCX column.*
2. *To develop protein separation methods for total cell lysate using the MAbPac™ RP column.*

With the objectives of applying the MAbPac protein separation method to a biological sample and increasing the number of low-abundance proteins identified compared with basic reverse phase chromatography.

3.3. Results

3.3.1. Development of pH buffers for charge variant analysis

As part of a collaboration with Thermo Fisher Scientific, it was a requirement to undertake a placement in industry, which was completed in September 2019. The placement took place at the research and development site in Sunnyvale, California, USA, where the role was to develop a mobile phase for a MAbPac™ SCX column which was to be used in a clinical setting to separate therapeutic antibodies. The buffer recipe had to be reproducible and suitable for larger-scale production. Primarily, the project required development of pH buffers for chromatography which fit the following criteria:

1. Chromatogram reproducibility: Elution peaks from different runs must be identical.
2. Stability: Buffers must be stable enough to be shipped and stored until use.

Previous work on this project had shown that the SCX column was favourable compared with the WCX, as WCX was shown to bind the buffers, reducing the linearity of the gradient. Although the SCX column had the least effect on buffer pH, it still produced a noticeable shift in the pH as the buffers were eluted from the column. This had to be considered during gradient and buffer design (Figure 3.1). As it was necessary to use volatile buffers in order to be MS compatible, it was previously determined that the best chemicals for buffer A were ammonium bicarbonate, which acts as a buffer, and acetic acid which is required to achieve an acidic pH. For buffer B, only ammonium hydroxide was used. Another key reason for choosing these chemicals for the mobile phase was that, theoretically, buffers could be made to the desired pH without needing to adjust with HCl or NaOH. Other volatile buffers were previously shown to require additional pH adjustment which could have an adverse effect on the reproducibility of

the buffers when made commercially. Without adjustment, buffers could remain stable for longer and be more reproducible between batches.

Buffer composition was optimised using the MAbPac™ SCX-10 RS (2.1 x 50 mm) which has a particle size of 5 µm and was run on a Vanquish Flex system coupled with an on-line pH meter.

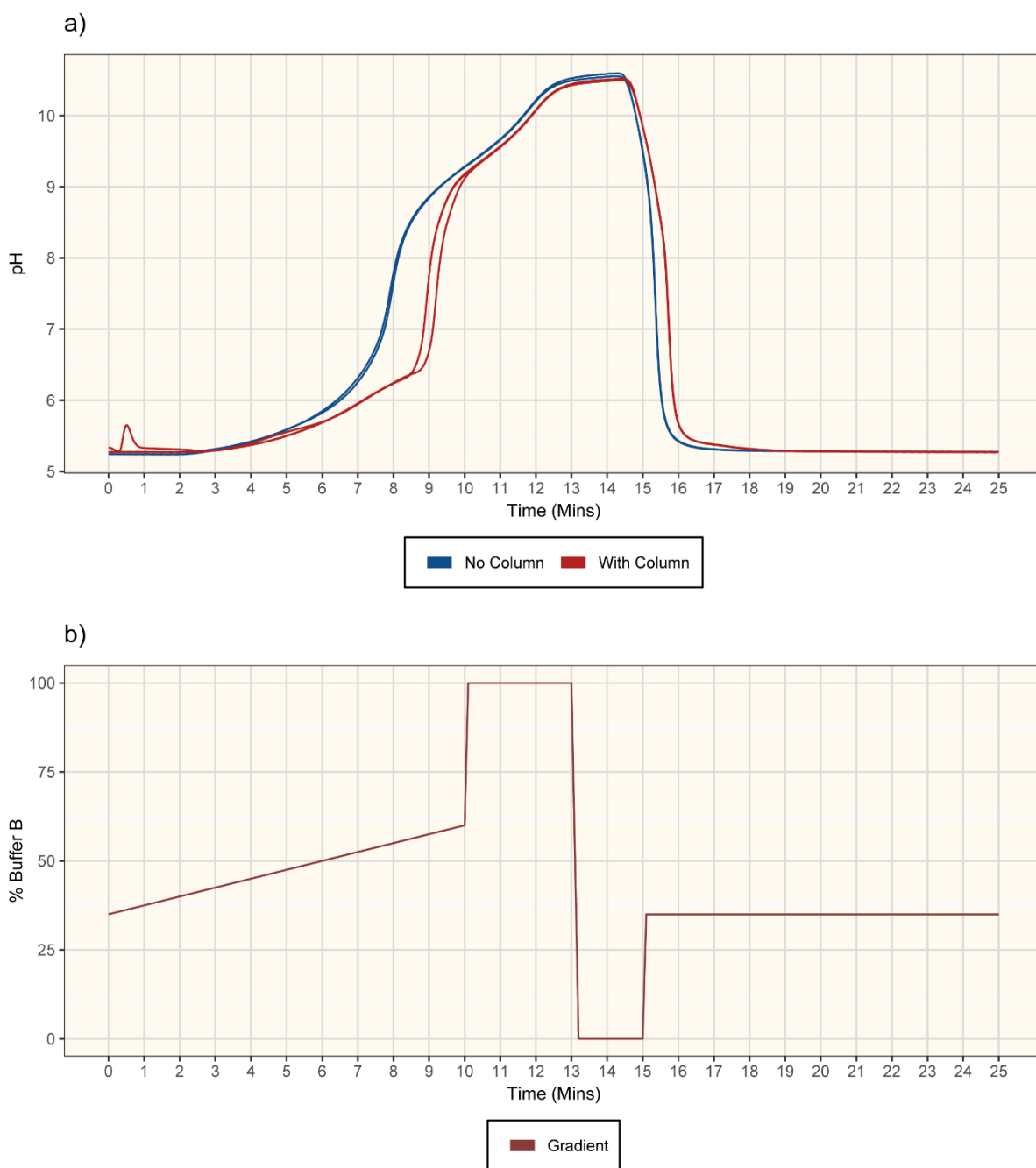


Figure 3.1: Buffering effect of the column on the pH gradient. a) pH readings taken with a pH meter of the mobile phase after elution from the column is shown in red and eluted without the column is shown in blue. b) The gradient. For all blanks, the gradient was linear, 0-100 % buffer B in 10 min. Buffer A was 30 mM acetic acid and 25 mM ammonium bicarbonate in water, pH 5.25. Buffer B was 10 mM ammonium hydroxide in water, pH 10.69. Banks were run with a MAbPac™ SCX-10 5 μ m 2.1 x 50 mm column at 30 °C, flow rate was 0.4 mL/min. Samples were run on a Vanquish Flex system coupled with an on-line pH meter.

Buffers were previously created at 5x solutions. Drawbacks to using a 5x concentration included the possibility that the dilution of the buffers by the customer could induce variability in the composition and therefore the results. Buffers at 5x and 1x concentration were compared to determine which could achieve the best reproducibility. Firstly, the pH of the newly created 5x buffers was measured before dilution, the same buffers after dilution to 1x and the pH values of buffers created directly at 1x concentration. Replicates of each buffer were made in triplicate, and it was observed that overall buffers made at 1x concentration were more reproducible in terms of pH than those diluted from a 5x concentration. When these buffers were injected on the HPLC, the chromatograms were more reproducible with the 1x buffers. Therefore, the buffer optimisation was continued using a 1x concentration (Figure 3.2).

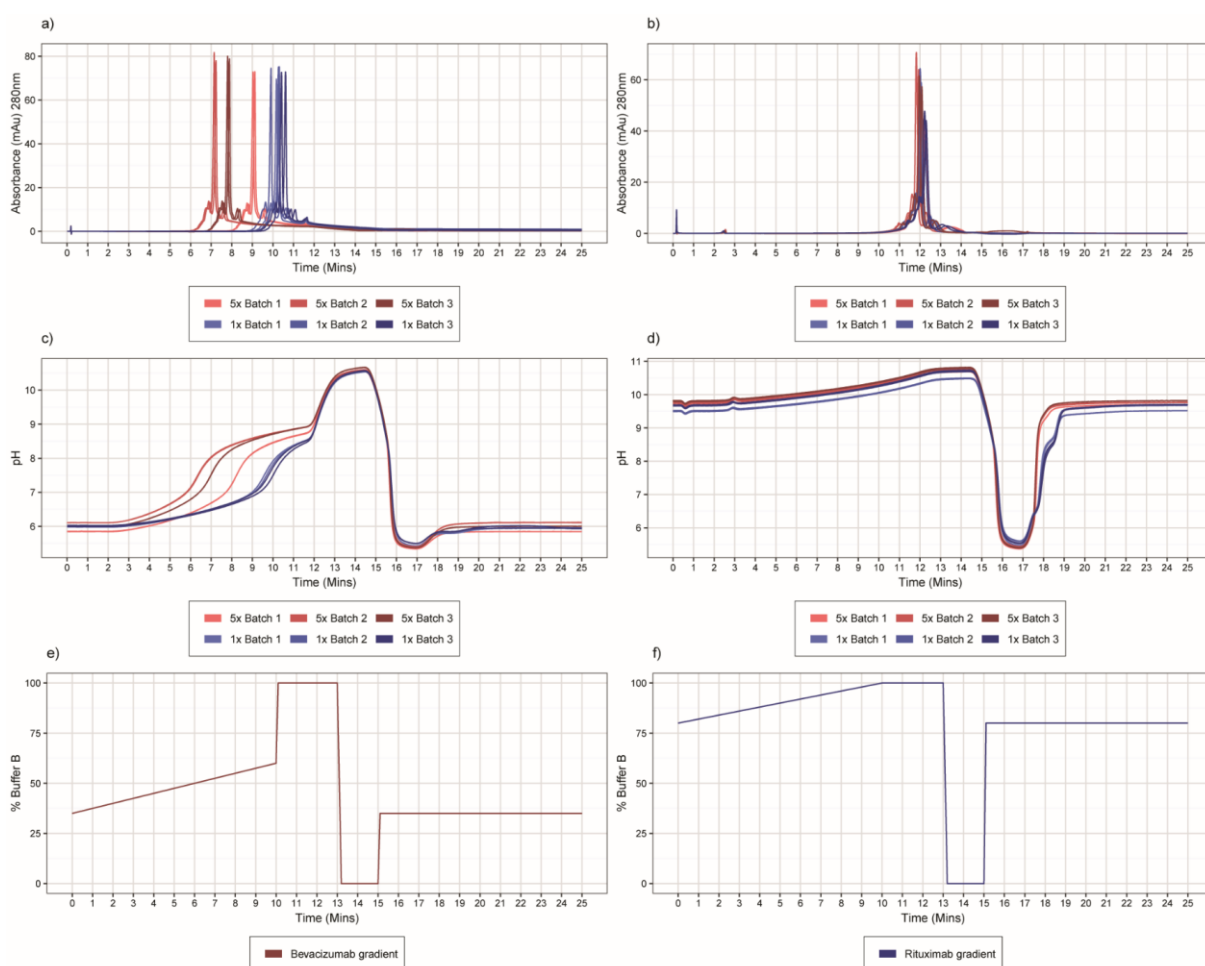


Figure 3.2: Buffering effect of the column on the pH gradient. a) Chromatogram of Bevacizumab elution with three independent batches of buffer made at 5x (red) and 1x (blue) concentration. B) Chromatogram of Rituximab. C) pH trace from Bevacizumab sample. D) pH trace of Rituximab. E) Gradient for Bevacizumab. f) Gradient for Rituximab.

The next step was to determine the optimal pH which could achieve the best separation/identification for two MAb's Rituximab and Bevacizumab, the mAbs at the centre of this study. Both are MAb's used for the treatment of cancer. Rituximab is an anti-CD20 antibody which is used for the treatment of non-Hodgkin's lymphoma and, more commonly, chronic lymphocytic leukaemia (Kulkarni and Kasi 2012; Williams et al. 2016). Bevacizumab is an antibody against Vascular endothelial growth factor A (VEGF), an important protein expressed on cancer cells (Seo et al. 2018). It is used for the treatment of advanced colorectal cancer, metastatic breast cancer, advanced non-small cell lung cancer and advanced renal cell cancer (Kazazi-Hyseni, Beijnen, and Schellens 2010). Both Rituximab and Bevacizumab are known to be glycosylated (Planinc et al. 2017) and the N-glycosylation site of Rituximab has been confirmed (Montacir et al. 2017).

Experiments to determine the optimal pH began by modifying the pH of buffer B, continuing the optimisation by testing different combinations of buffers (Figure 3.3) until finding the combination that produced the best separation: Buffer A at pH 5.38 and buffer B at pH 10.76. These buffers were reproduced three times. Although the buffers were prepared in an identical manner, on the same day, at the same time, there was still variability in the resulting pH. It would be necessary to test buffers before leaving production and those that vary from the ideal pH should be discarded. It is worth noting that absolute reproducibility is only necessary for standalone LC analysis. For on-line LC-MS, identification will be achieved by the mass spectrometer, and therefore the exact elution time of the MAb from the column is inconsequential.

Once the buffer recipe was optimised, MES buffer (0.5 M MES adjusted to pH 6.0 with NaOH) was used to regenerate the column after every run and compared runs with and without the regeneration. The results showed a noticeable improvement in stability when MES was used, and an increase in signal for Rituximab, suggesting that some of the sample had been trapped on the column (Figure 3.4).

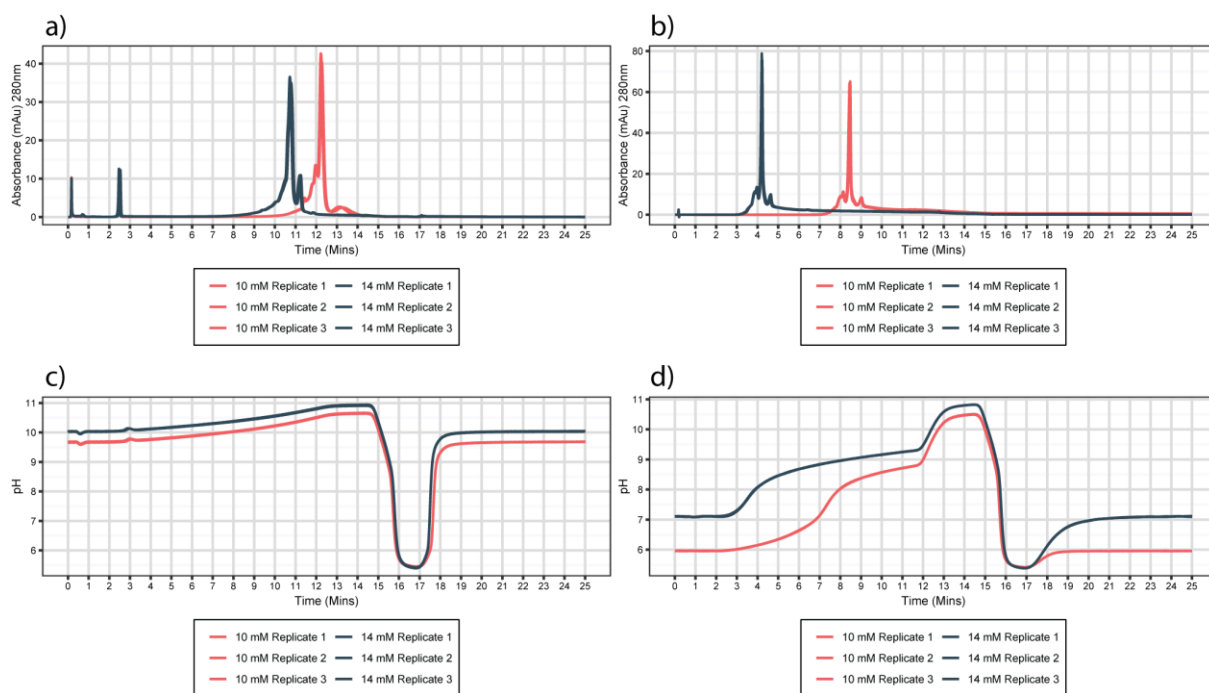


Figure 3.3: UV chromatogram and pH trace of Rituximab and Bevacizumab in triplicate. a) UV trace of Rituximab analysed with 10 mM and 14 mM Ammonium hydroxide in Buffer B. b) pH trace of Rituximab. c) pH trace of Bevacizumab. d) pH trace of Bevacizumab, all showing buffers made and run in triplicate. Buffers were as follows: Buffer A: 25 mM ammonium carbonate, 26.35 mM of acetic acid in water at pH 5.38 (Rep 1), 5.36 (Rep 2) and 5.37 (Rep 3). Buffer B: 10 mM or 14 mM ammonium hydroxide in water, pH 10.76 (Rep 1), 10.68 (Rep 2) and 10.71 (Rep 3). Rituximab and Bevacizumab were both prepared at a concentration of 5 $\mu\text{L}/\text{mL}$ and 5 μL of each sample was injected to the HPLC (for 0.025 μL MAb) in triplicate. MABs were analysed using a linear 10 min gradient: Rituximab – 0mins at 85 % B, 10 min at 100 % B; Bevacizumab- 0 min at 35 % B, 10 min at 60 % B with a MABPacTM SCX-10 5 μm 2.1 x 50 mm column at 30 $^{\circ}\text{C}$, flow rate was 0.4 mL/min. MABs were detected by the UV cell at 280 nm.

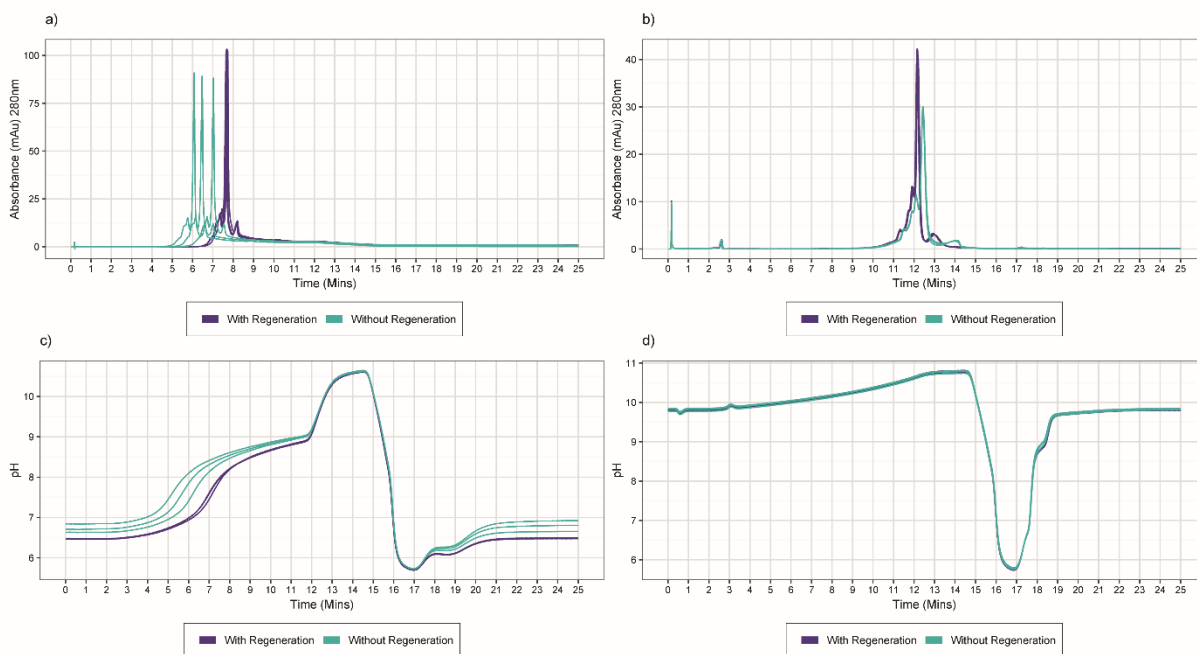


Figure 3.4: Regeneration of the analytical column. a) UV trace of Bevacizumab with (purple) and without (blue) column regeneration. b) UV trace of Rituximab with and without column regeneration. c) pH trace of Bevacizumab with and without column regeneration. d) pH trace of Rituximab with and without column regeneration, all showing buffers made and run in triplicate. Buffers were as follows: Buffer A: 25 mM ammonium carbonate, 26.35 mM of acetic acid in water at pH 5.38 (Rep 1), 5.36 (Rep 2) and 5.37 (Rep 3). Buffer B: 10 mM ammonium hydroxide in water, pH 10.76 (Rep 1), 10.68 (Rep 2) and 10.71 (Rep 3). Rituximab and Bevacizumab were both prepared at a concentration of 5 $\mu\text{L}/\text{mL}$ and 5 μL of each sample was injected to the HPLC (for 0.025 μL MAb) in triplicate. MAbs were analysed using a linear 10 min gradient: Rituximab – 0 mins at 85 % B, 10 min at 100 % B; Bevacizumab- 0 min at 35 % B, 10 min at 60 % B with a MAbPac™ SCX-10 5 μm 2.1 x 50 mm column at 30 °C, flow rate was 0.4 mL/min. MAbs were detected by the UV cell at 280 nm.

Buffer stability over time and reproducibility batch-batch were compared using three batches of the same buffer recipe, made on different days. UV Chromatograms and pH traces of Rituximab and Bevacizumab run using these buffers showed perfect reproducibility for Rituximab, but some batch to batch variation was apparent for Bevacizumab (Figure 3.5). This could be due to the buffering effects of the column. However, although reasonable run-run reproducibility was achieved (Figure 3.5 a & b) there was obvious batch-batch variability between batches of buffer, evident also in the pH traces (Figure 3.5 c & d).

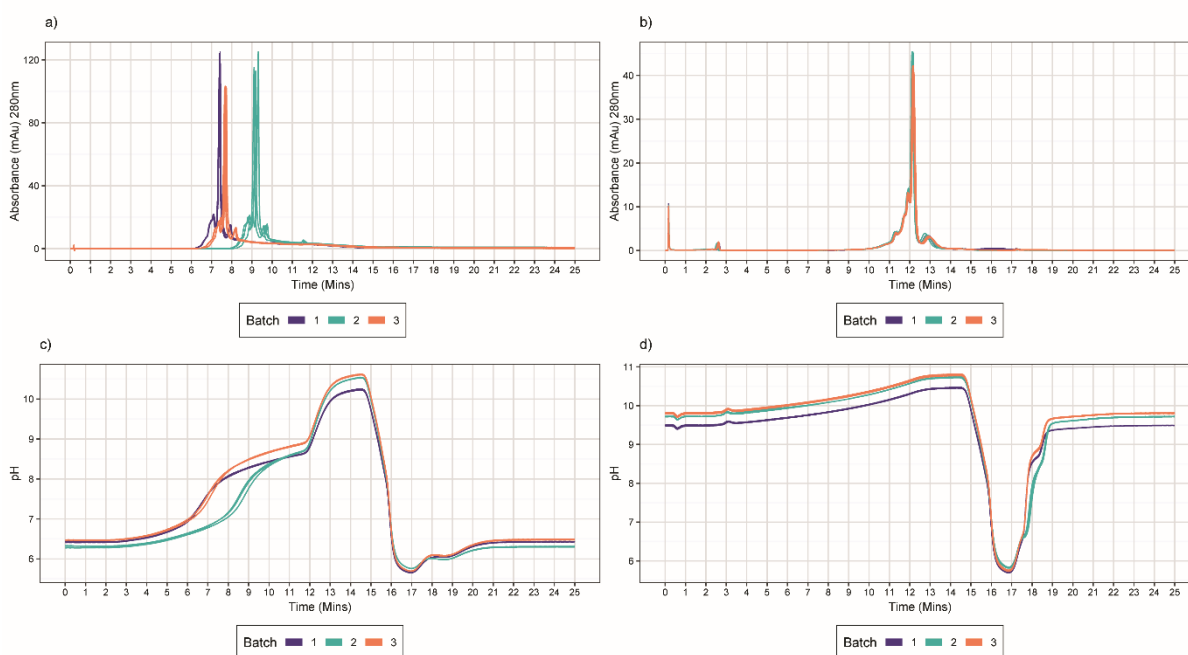


Figure 3. 5: Reproduction of the optimised buffers. a) UV trace of Bevacizumab. b) UV trace of Rituximab. c) pH trace of Bevacizumab. d) pH trace of Rituximab, all showing buffers made and run in triplicate. Buffers were as follows: Buffer A: 25 mM ammonium carbonate, 26.35 mM of acetic acid in water at pH 5.38 (Rep 1), 5.36 (Rep 2) and 5.37 (Rep 3). Buffer B: 10 mM ammonium hydroxide in water, pH 10.76 (Rep 1), 10.68 (Rep 2) and 10.71 (Rep 3). Rituximab and Bevacizumab were both prepared at a concentration of 5 µl/mL and 5 µL of each sample was injected to the HPLC (for 0.025 µL MAb) in triplicate. MABs were analysed using a linear 10 min gradient: Rituximab – 0mins at 85 % B, 10 min at 100 % B; Bevacizumab- 0 min at 35 % B, 10 min at 60 % B with a MAbPac™ SCX-10 5 µm 2.1 x 50 mm column at 30 °C, flow rate was 0.4 mL/min. MABs were detected by the UV cell at 280 nm.

Variation in buffer pH after manufacture could be due to lack of stability, or subsequent effects from the HPLC system.

Although the buffers were made as identically as possible, there were disproportionately large differences between the resulting chromatograms. It was evident that there was another factor contributing to differences between runs and batches of buffer, aside from the buffers themselves. The sample, column and HPLC were kept constant throughout the optimisation process, so the variability must have been arising from one of these.

Removal of the column caused a small shift in pH (Figure 3.1 b). However, as the shift was consistent between runs and batches of buffers, which had been considered during gradient and buffer optimisation, it is likely that the column was not the source of run-run irreproducibility. It was hypothesised that the degasser could be removing carbonate from the buffers as they pass

through. Inconsistent removal of carbonate would adversely affect the pH of the buffers. To investigate the effect of the degasser on the pH of the buffers, Rituximab, Bevacizumab, and blanks were run with the same batch of buffers with and without the degasser. The results (Figure 3.6 e & f) show that the degasser clearly increases the pH of the buffer. Therefore, gradient re-optimisation was necessary to adjust for the new pH with the aim of improving the batch-batch reproducibility of the buffers.

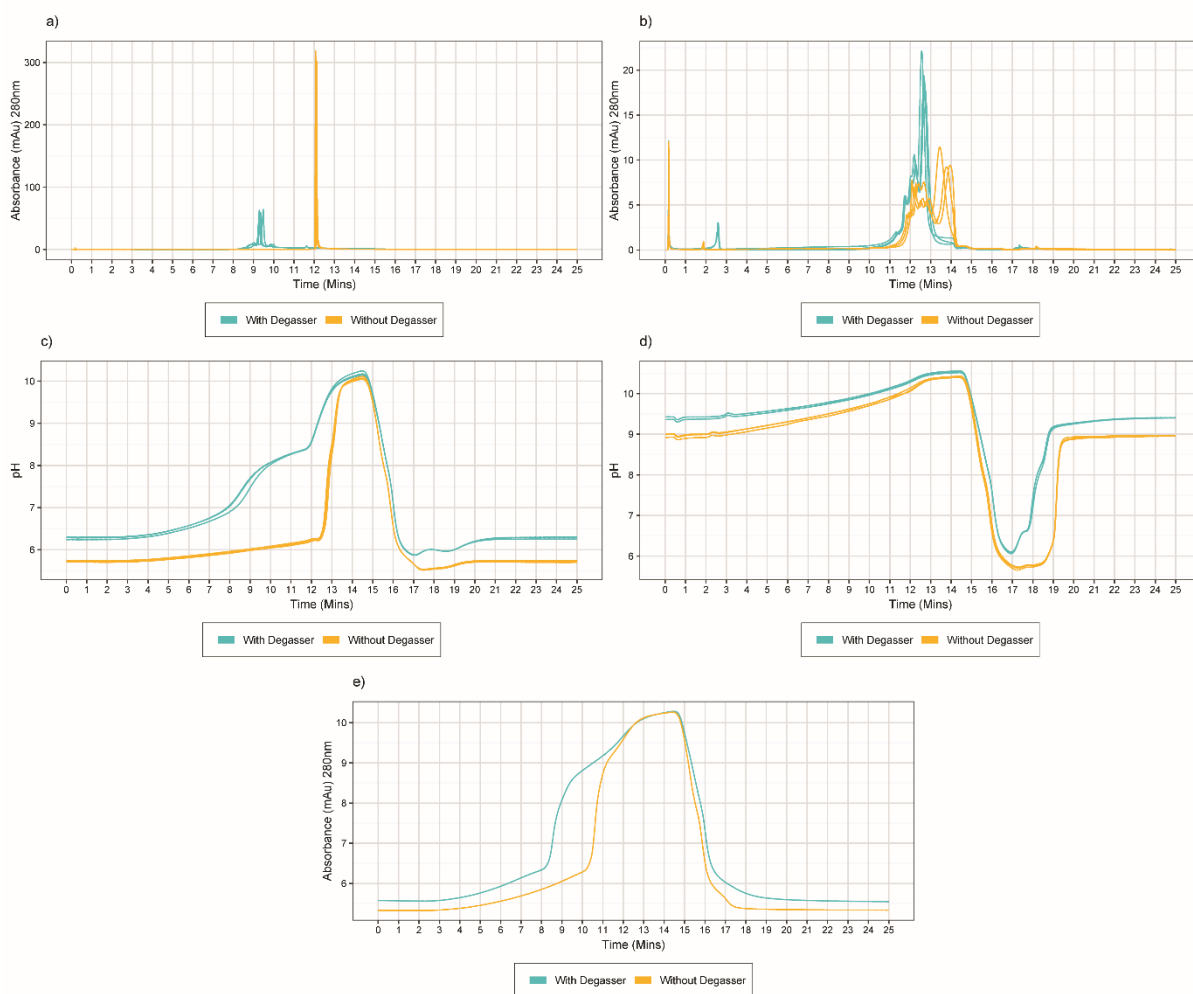


Figure 3.6: Removal of the degasser affects pH and chromatography. a) UV trace of Bevacizumab with and without the degasser. b) UV trace of Rituximab with and without the degasser. c) pH trace of Bevacizumab with and without the degasser. d) pH trace of Rituximab with and without the degasser. e) pH trace of blanks, with the column, with and without the degasser. Blanks were run with a linear gradient 0-100 % buffer B in 10 min. Buffers were as follows: Buffer A: 25 mM ammonium carbonate, 26.35 mM of acetic acid in water at pH 5.43. Buffer B: 7.5 mM in water, pH 10.55. Rituximab and Bevacizumab were both prepared at a concentration of 5 µl/mL and 5 µL of each sample was injected to the HPLC (for 0.025 µL MAb) in triplicate. MABs were analysed using a linear 10 min gradient: Rituximab – 0 min at 85 % B, 10 min at 100 % B; Bevacizumab- 0 min at 35 % B, 10 min at 60 % B with a MAbPac™ SCX-10 5 µm 2.1 x 50 mm column at 30 °C, flow rate was 0.4 mL/min. MABs were detected by the UV cell at 280 nm.

3.3.2. Development of protein separation methods for TCL using the MAbPac™ RP column

3.3.2.1. MAbPac™ Optimisation

The MAbPac™ RP column is usually used for the separation of antibodies. However, it is well suited for the separation of other protein samples, such as total cell lysate (TCL). 2D-LC protein fractionation at both the protein and the peptide level could be highly orthogonal, potentially resulting in higher ID numbers from 2D-LC/MS experiments.

The primary aim was to develop methods for this column and apply them for the separation of proteomics samples. Ideally, these methods could be applied to the study of the downstream biology of Toll-like Receptors (TLRs) using a proteomics approach. Firstly, to determine the optimal conditions for the MAbPac™ RP column. A number of protein standards were created (Figure 3.7). Proteins used for standards were bovine serum albumin (BSA), Carbonic anhydrase, Cytochrome C and Lysozyme from egg white. Lyophilised protein powder was re-suspended at 1 mg/ml in water before being injected to the MAbPac™. Standards were injected in different volumes ranging from 5-100 µg, with 10 µg of protein producing the sharpest peaks (Figure 3.7 a-e). When all four proteins were mixed together, proteins still eluted at the same time compared with the individual injections (Figure 3.7 f).

The most effective gradients are shown in Figure 3.8. Of these three, the gradient that produced the most reproducible peaks was gradient 3 (Figure 3.8 c), but the intensity of gradients 1 and 2 (Figure 3.8 a & b) were much greater. For the mobile phase, 45 % Isopropanol and 45 % Acetonitrile were used instead of 80 % Acetonitrile to aid elution of proteins from the column.

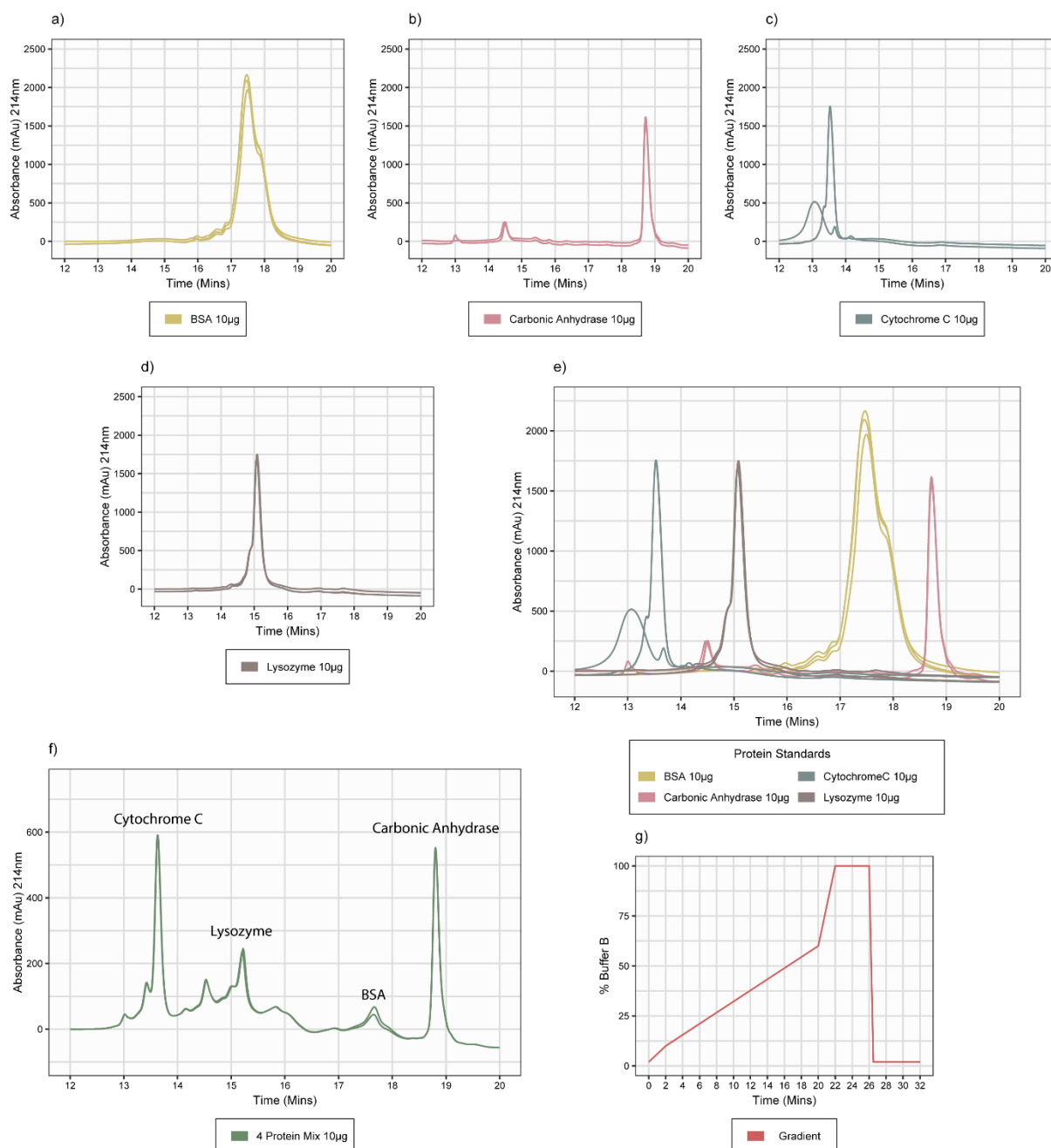


Figure 3.7: Optimisation with 4 protein standards. a) MAbPacTM chromatogram of three replicates of 10 μ g BSA re-suspended in water, b) Three replicates of 10 μ g Carbonic anhydrase re-suspended in water, c) Three replicates of 10 μ g Cytochrome C in water and d) Three replicates of 10 μ g Lysozyme in water. e) Overlay of all individual protein standards, in triplicate. f) Chromatogram of all protein standards injected into a mix in triplicate; 10 μ g injected. g) Gradient of Buffer B. MAbPacTM was run at 80 $^{\circ}$ C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPacTM RP 2.1 x100 nm column with 4 μ m particle size and 1500 $\text{\AA}$ pore size.

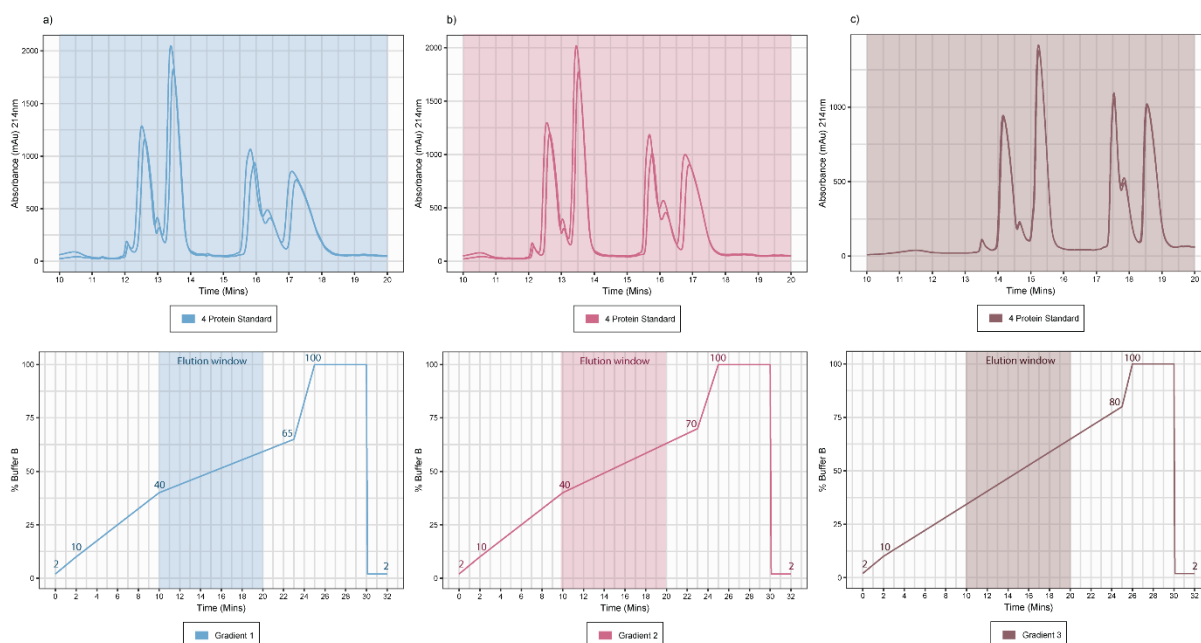


Figure 3.8: Gradient optimisation. UV trace and gradient plot of 4 protein standard injected using gradient 1 (a), gradient 2 (b) and gradient 3 (c). All protein standards injected into a mix; 10 μ g injected. Gradients of Buffer B for the MAbPac™ were run at 80 °C, 0.250 μ l/min flow rate with buffer A as 0.1 % TFA, 2 % acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPac™ RP 2.1 x 100 nm column with 4 μ m particle size and 1500 Å pore size.

The MAbPac™ column differs from many other commercially available columns in its ability to withstand higher temperatures. To investigate the effects of running samples at different temperatures, four protein standards were tested at 30 °C (the usual running temperature for most HPLC samples), 37 °C (body temperature), 40 °C, 50 °C, 60 °C, 70 °C and 80 °C, the upper limit of the column compartment (Figure 3.9). As temperature increased, so did peak intensity. Samples run at 30 °C achieved a higher intensity (Figure 3.9 a). Samples run at 30 °C and 80 °C both produced perfect reproducibility, although samples run at 80 °C suffered a minor loss of resolution (Figure 3.9 f).

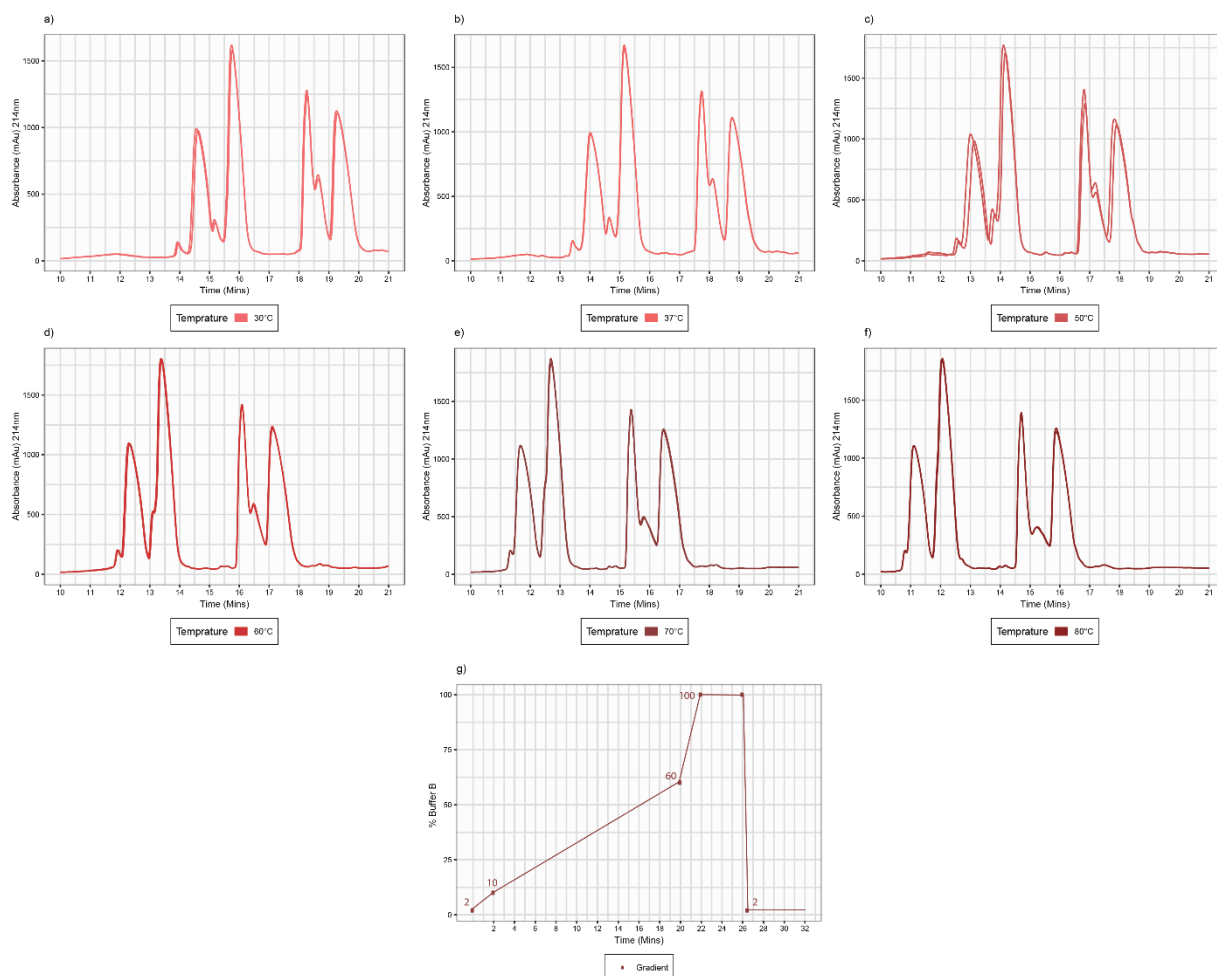


Figure 3.9: Temperature optimisation. a - f) Four Protein standard run at temperatures ranging from 30 °C to 80 °C. g) Gradient. MAbPac™ was run at 80 °C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPac™ RP 2.1 x 100 nm column with 4 µm particle size and 1500 Å pore size.

3.3.2.2. Reduction and alkylation results in loss of intensity

Reduction is a process by which the disulphide bonds, which are essential for the formation of a protein's 3D structure, are broken, leaving free cysteine residues. Alkylation is the process which blocks the reactive cysteine residues to prevent re-formation. Simply, reducing and alkylating a protein unfolds it. This is useful for the tryptic digestion as an unfolded protein has more Arginine and Lysine residues exposed where trypsin cuts. Intact and non-reduced and non-alkylated proteins can have different conformations in solution that could elute at a different retention time. Therefore, it was hypothesized that reduction and alkylation might be useful for protein separation by MAbPac™, as it would hold the proteins in a unique conformation, theoretically improving the separation of proteins, allowing them to be eluted

separately. Reducing disulphide bonds would also expose more hydrophobic sites and therefore could increase protein interaction with the column. A five-protein standard mixture composed of equal amounts of BSA, ovalbumin, cytochrome C, lysozyme, and carbonic anhydrase with and without reduction and alkylation was injected to the HPLC and the UV trace of each protein eluted from the column (Figure 3.10) were compared. The overall peak shape was the same for each protein regardless of reduction and alkylation. These data suggests that reduction and alkylation is not a necessary step for MAbPac™ sample preparation, meaning that this step can be eliminated, and the overall workflow can be streamlined. Error bars were larger for smaller proteins, suggesting that smaller proteins where the addition of Rapigest had the strongest effect. This suggests that the MAbpac protein fractionation method may not be perfectly reproducible for small proteins.

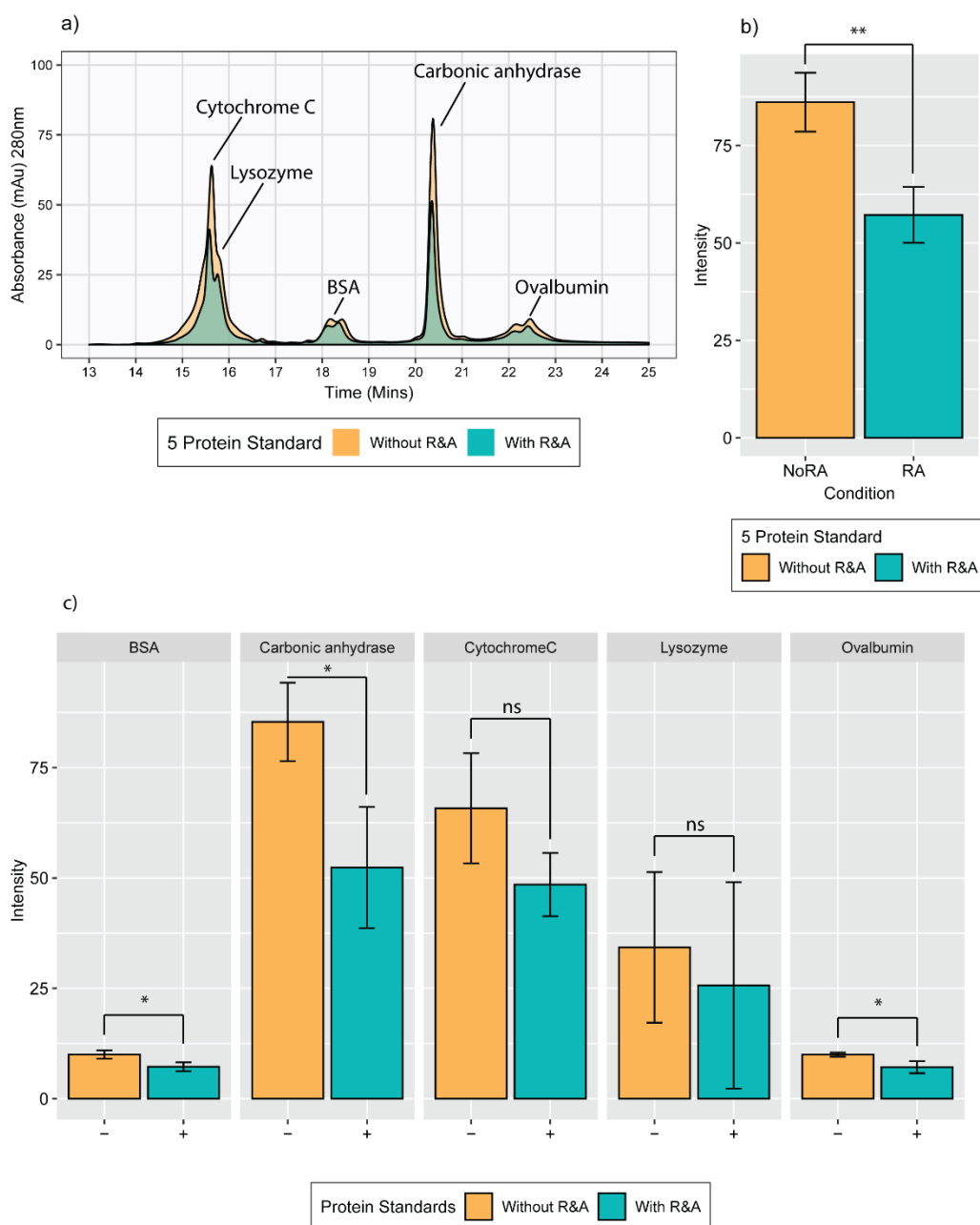


Figure 3.10: Testing reduction and alkylation for 5 protein standards. Buffer A is 0.1 % TFA in water and buffer B is 0.1 % TFA in 45 % acetonitrile and 45 % isopropanol. Chromatograms were run with a gradient of 0 min – 2 % buffer B, 2 min – 10 % buffer B, 20 min – 60 % buffer B, 22 min – 100 % buffer B, 27 min – 100 % buffer B, 28 min – 2 % buffer B. a) Chromatograms of a 5-protein standard with and without reduction and alkylation. b) Bar chart showing mean intensities of protein with and without reduction and alkylation (p-value 0.008) c) Bar charts showing the relative intensities of each of the five proteins present in the standard. P-value for BSA was 0.02, p-value for Carbonic anhydrase was 0.02, p-value for Ovalbumin was 0.02, Cytochrome C and Lysozyme were not significant. MABPac™ was run at 80 °C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MABPac™ RP 2.1 x100 nm column with 4µm particle size and 1500 Å pore size. Error bars were calculated using standard deviation. Asterix indicate significance: * indicates >0.05 p value, ** indicates <0.01 p-value, ns indicates > 0.05 p-value, or not significant (Student's T-test).

3.3.3. Application of MAbPac™ for human serum analysis

Although the development of protein separation methods for the MAbPac™ RP columns were originally intended for analysis of TCL, the necessity of desalting each fraction after separation rendered this technique time-inefficient for individual experiments. It was felt that the MAbPac™ would be better suited to the high-throughput application where the cleanup could be automated by a robot such as a Kingfisher (Thermo Fisher Scientific). The MAbPac™ RP protein fractionation methods were therefore modified for a clinical application more suited for the technique. Serum contains many biomarkers that can be highly informative. Unfortunately, it also contains a number of high abundance proteins such as albumin, which, if not removed, can mask lower-abundance proteins, and prevent identification by MS. Protein fractionation could be a viable method of separating albumin from other proteins in the sample, and thereby improving the identification of lower-abundance proteins.

3.3.3.1. Gradient optimisation for human serum

After optimisation, it was important to test the suitability of the column to effectively separate protein groups without albumin depletion.

Different gradients were tested with human serum (Figure 3.11) to establish which gradient was most suitable for resolving different protein groups in the serum sample. Of these five gradients, it was determined that Gradient 5 was the most effective for the separation of serum proteins because it produced the best separation (Figure 3.11 e).

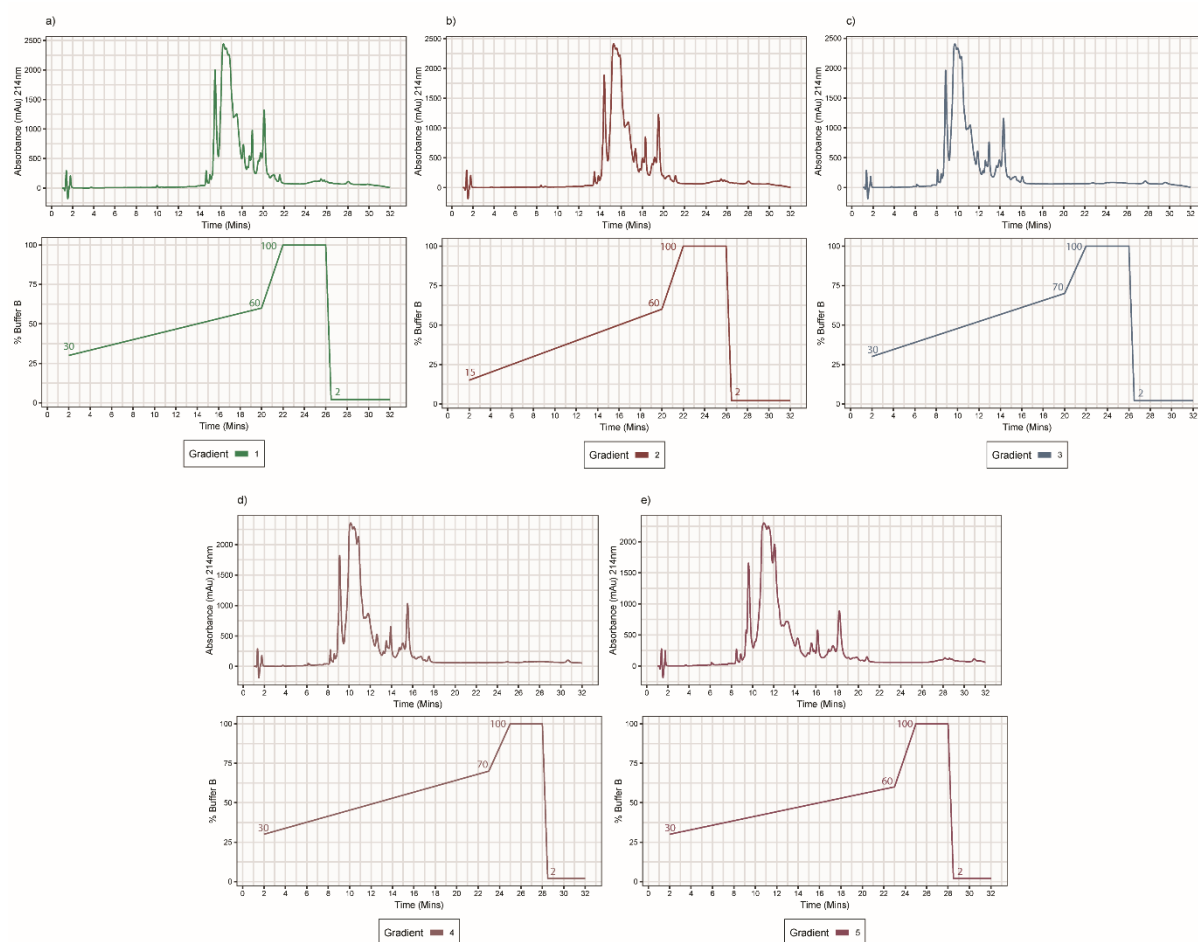


Figure 3.11: Human serum gradient optimisation. 20 μ g of human serum protein was injected into the HPLC using a range of gradients (a-e). MAbPacTM was run at 80 $^{\circ}$ C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPacTM RP 2.1 x100 nm column with 4 μ m particle size and 1500 $\text{\AA}$ pore size.

3.3.3.2. Human serum protein identification

After gradient optimisation, the aim was to test the MAbPacTM on a biological sample. Serum was chosen because it is a complex mixture of proteins which is difficult to fractionate due to the high abundance of several proteins such as albumin. It also contains important disease biomarkers and is therefore valuable for clinical analysis. Because of the highly abundant proteins present in serum, protein depletion is often used to remove them. However, this process can also remove lower abundance proteins, including disease biomarkers (Bellei et al. 2011). The aims were to compare MAbPacTM fractions of human serum which had been depleted using a classical albumin depletion method (depletion columns) and un-depleted serum, and to

establish how many unique proteins could be identified from un-depleted human serum using the MAbPac™ workflow.

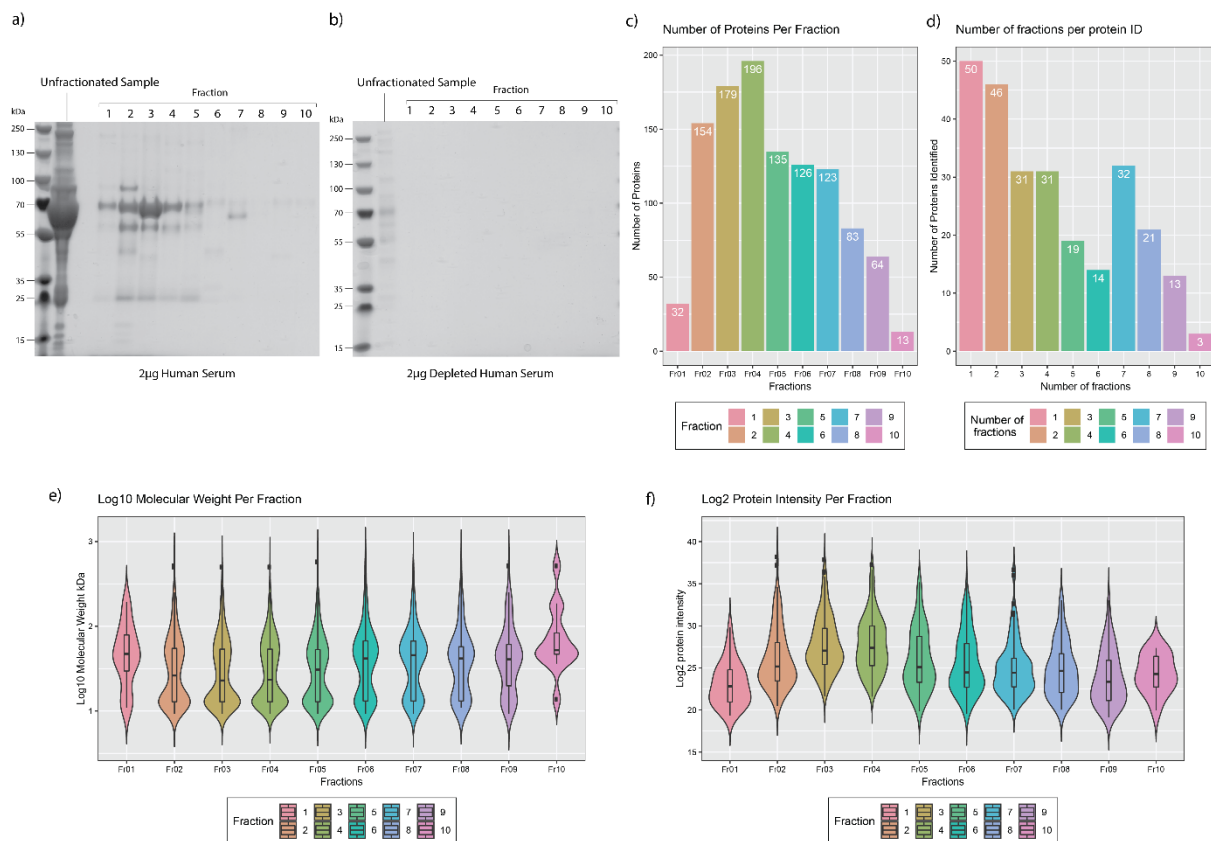


Figure 3.12: Human serum protein identification. a) Colloidal Coomassie of human serum fractionated without protein depletion. b) Number of proteins per fraction for serum with protein depletion. c) Number of proteins identified per fraction for serum without depletion. d) Number of fractions single proteins were identified in (50 proteins were identified in only 1 fraction, 46 were identified in 2 fractions etc...). e) Log10 of the molecular weight (kDa) for the proteins identified in each fraction. f) Log2 for the intensities for the proteins identified in each fraction. For off-line fractionation, the samples were run at 80 °C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPac™ RP 2.1 x100 nm column with 4µm particle size and 1500 Å pore size. LC-MS analyses was performed on an Orbitrap™ Fusion Lumos Tribrid mass spectrometer (Thermo) coupled to an Ultimate 3000 RSLCano HPLC (Dionex). Each fraction was separated over a 60 min gradient on an EASY-spray pepmap C18 column (50 cm x 75 µm, 2 µm particle size, 100 Å pore size).

As shown in Figure 3.12, 2 µg of human serum was compared +/- albumin depletion. Fractions were loaded onto an SDS-PAGE gel and the proteins were stained using colloidal Coomassie.

These results show that albumin depletion removes a many of the proteins present in the serum sample (Figure 3.12 a & b). Whilst it is expected that albumin would be absent from the depleted sample, this could also suggest that the low-abundance proteins are being removed by the depletion columns. Therefore, using the columns would necessitate that larger quantities of serum would need to be depleted and analysed in order to observe remaining proteins in the sample. Using protein depletion columns would be more time-consuming and expensive compared with fractionation by MAbPac™, which would not require the depletion step.

MS analysis of proteins from serum show that over 10 fractions, high abundance proteins such as complement elute in fractions 4-7. Figure 3.12 c showed counts of different proteins eluting in each fraction. Most proteins elute in fraction 4 (196 distinct proteins). Proteins in this fraction were largely identified as various forms of complement, many of which were also found in fraction 3 (complement component C6, C6 and C8), and others which were also found in fractions 5-7 (complement C3, C4-A and C4-B). Fibronectin and vitamin D-binding protein were largely found in fraction 4. Haemoglobin-related and ion-binding proteins, such as haptoglobin, hemopexin and histidine-rich glycoprotein, were mostly identified in fractions 2-4.

As these proteins are likely to be found in un-depleted serum and are among the top 14 most abundant proteins, it is promising that they can be largely fractionated into two fractions along with other high-abundance proteins such as immunoglobulin (found in fractions 2-7). Albumin was identified in fractions 4-7, although a vast majority was identified in fractions 5 & 6.

Alipoprotein was less successfully isolated, being identified in all fractions except fraction 10, although a vast majority was confined to fractions 4-9. This is likely due to many different proteoforms of this protein. For example, Alipoprotein B100 appears in vast quantities in fraction 9, whilst is largely unidentified in other fractions. Conversely, Alipoprotein H is mainly found in fraction 2.

However, reducing the number of high abundance proteins in many of the fractions enabled identification of several very low-abundance proteins which may otherwise have been masked. Fibulin-1 (FBLN1), an IgG Fragment crystallisable region (Fc)-binding protein was identified in low amounts in two fractions, whilst two insulin-like growth factors were identified only in fractions 1 and 2, respectively. Several glycoproteins were also uniquely identified in one fraction. This is important as various glycoproteins are often used as biomarkers for disease (Cavalcante et al. 2016; Connelly et al. 2016; Lan et al. 2016; Shinzaki et al. 2017; D. Zhang et

al. 2017). For example, Alpha-1-acid is a serum biomarker for lung and other cancers (Ayyub et al. 2016) and two forms (Alpha-1-acid glycoprotein 1 and Alpha-1-acid glycoprotein 2) were respectively uniquely identified in fraction 2 and fraction 3 in this dataset.

A majority of proteins were uniquely identified in one or two fractions (Figure 3.12 d), including proteins such as Serpin peptidase inhibitor (SERPINA4) which is associated with the acute inflammatory response (Gómez 2017). Only three proteins were identified in all 10 fractions. These proteins were Complement C3, another serpin peptidase inhibitor (SEPP1) and an unidentified protein. The distribution of protein molecular weight was relatively even between fractions (Figure 3.12 e), with lighter proteins eluting in the first and last fractions, with a slight shift in size from fractions 2-5, where slightly heavier proteins eluted, to fractions 6-9 which contained slightly lighter proteins. Protein intensity (Figure 3.12 f) was greatest in fractions 2-5, probably due to the higher number of highly abundant proteins that elute here. Promisingly, lower-abundance proteins that elute in the peripheral fractions were still identified at high intensities.

3.3.3.3. *Addition of Rapigest improves number of IDs from serum fractionation*

Rapigest is an acid-labile surfactant, which could improve interaction between the protein sample and the column, providing better separation. To further improve the MAbPac™ workflow and achieve a higher number of unique proteins from serum, Rapigest was added to the sample prior to fractionation. The addition of a detergent could improve protein solubility and prevent protein precipitation on the column. Injecting the serum mixed with Rapigest could also improve the fractionation. To test this hypothesis, samples were collected by fractionating the serum by peak and by time. The HPLC can be programmed to collect fractions in two ways: firstly, each fraction can be collected for a set period of time, for example 40 s, or fraction collection can be triggered when a peak is detected on the UV trace. By testing both methods, it appears that proteins can be more reliably separated by fractionating by time (Figure 3.13 a & b). Protein orthogonality and separation are improved with the addition of 0.1 % Rapigest (Figure 3.13 c).

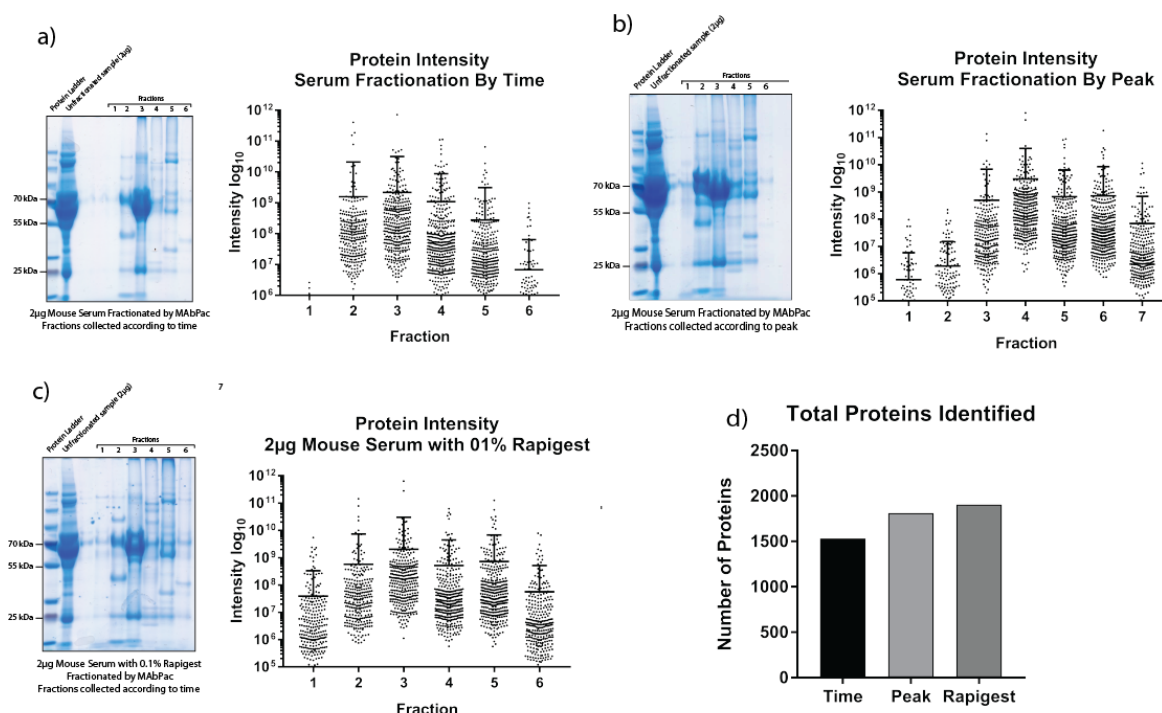


Figure 3.13: Addition of Rapigest enhances identification of serum proteins. a) 2 µg mouse serum with fractions collected according to time. b) 2 µg mouse serum fractions collected according to peak. c) 2 µg mouse serum with 0.1 % Rapigest added in basic conditions with fractions collected every 41 seconds. d) Total number of proteins identified in 2 µg mouse serum collected by time, peak and with 0.1 % Rapigest fractionated by time. MAbPac™ was run at 80 °C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPac™ RP 2.1 x100 nm column with 4 µm particle size and 1500 Å pore size. LC-MS analyses was performed on an Orbitrap™ Fusion mass spectrometer (Thermo) coupled to an Ultimate 3000 RSLCano HPLC (Dionex). Each fraction was separated over a 60 min gradient on an EASY-spray pepmap C18 column (50 cm x 75 µm, 2 µm particle size, 100 Å pore size). Error bars were calculated using standard deviation.

3.3.3.4. Addition of SDS to the sample shows little improvement for chromatogram reproducibility

SDS is a surfactant usually used for protein denaturation and has been previously compared with Rapigest for HPLC sample preparation. Researchers have been able to achieve a higher protein yield with SDS compared with Rapigest (Tanca et al. 2013). SDS is a powerful detergent most commonly used to aid solubilisation of proteins in solution and can achieve complete denaturation of cell and organelle membranes.

To investigate the effect of adding SDS to HPLC samples before fractionation, SDS was added directly to the sample buffer for a final concentration of 2.5 % SDS. Identical samples of 50 µg

of *E. coli* protein digest were run with and without SDS (Figure 3.14) but the running buffers were kept SDS-free. Serum samples analysed without SDS had a higher degree of reproducibility compared to those run with SDS. Proteins without SDS also had a better elution distribution (Figure 3.14 a & c) compared with those lysed in SDS (Figure 3.14 b & d).

It is known that SDS can reduce the separation power of the HPLC (Tanca et al. 2013). The lack of reproducibility of the chromatograms for samples with added SDS could also be due to proteins precipitating on column once the SDS was washed away, or because the SDS injected with the sample accumulated over time on the column and modified the properties of the column. While the MAbPac™ is a reversed-phase column, the accumulation of SDS could give properties similar to ion pairing. Another complication of adding SDS to a sample could be that introducing SDS by injection results in different amounts of SDS in the columns at different stages of the run. As SDS elution could vary injection-injection, it was necessary to add SDS to the buffers in which would help keep the concentration of SDS the same over time and would be predicted to improve reproducibility protein recovery from the column.

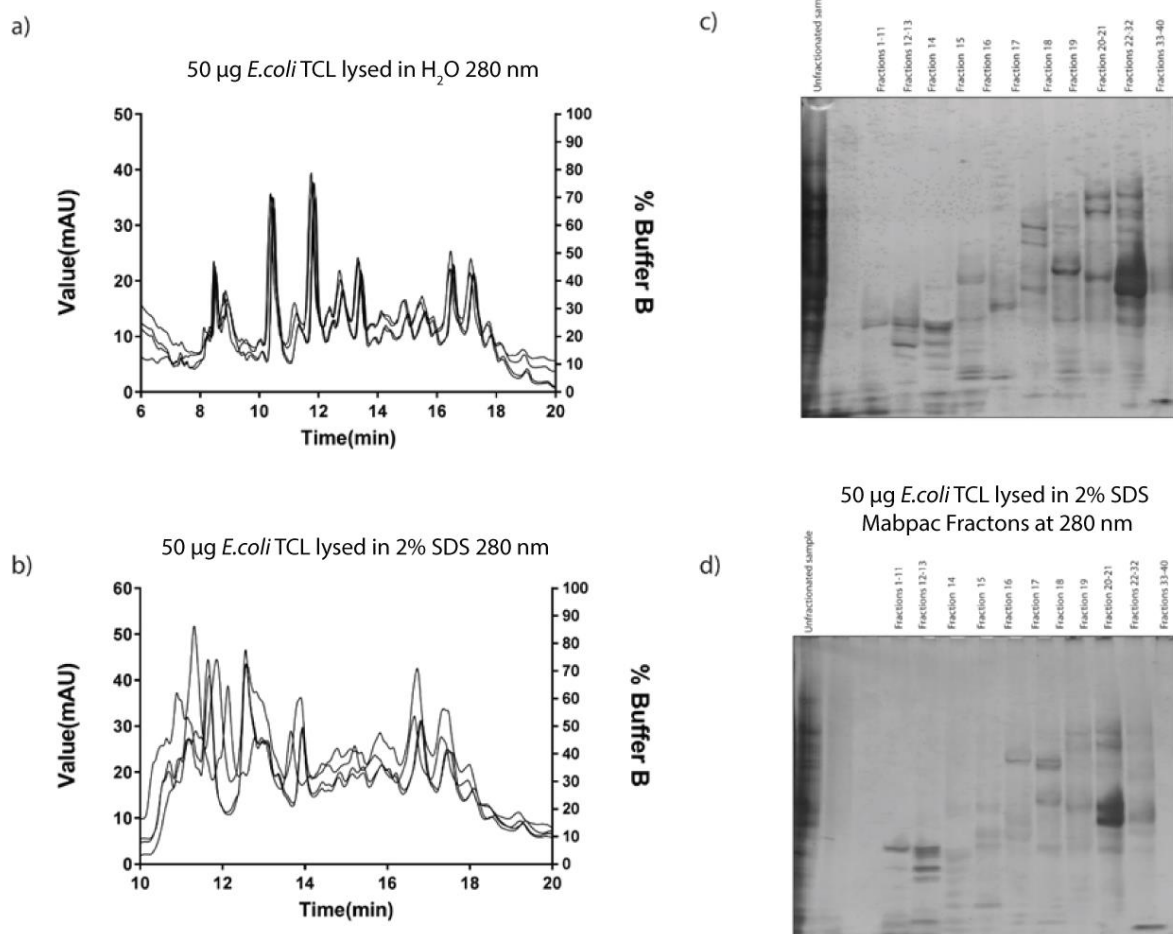


Figure 3.14: Addition of 2.5 % SDS decreases chromatogram reproducibility between samples. a & b) Chromatograms of 4 replicates of *E. coli* total cell extract lysed in H_2O or 2.5 % SDS. c & d) SDS-PAGE gels silver stained to show protein separation for *E. coli* samples lysed in H_2O or 2.5 % SDS. Mobile phase A was 2 % acetonitrile, 0.1 % TFA in water. Mobile phase B was 45 % acetonitrile, 45 % isopropanol and 0.1 % TFA in water. Chromatograms were run with a gradient of 0 min – 2 % buffer B, 2 min – 10 % buffer B, 20 min – 60 % buffer B, 22 min – 100 % buffer B, 27 min – 100 % buffer B, 28 min – 2 % buffer B. Samples were lysed by mechanical lysis in water or 2.5 % SDS and injected directly onto the column. Samples were run at 80 °C, 0.250 ml/min flow rate with buffer A as 0.1 % TFA, 2 % Acetonitrile in water and buffer B as 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA in water. The column was a MAbPac™ RP 2.1 x100 nm column with 4 μm particle size and 1500 Å pore size.

3.3.3.5. Addition of SDS to the buffers improves separation and chromatogram reproducibility

Adding SDS to the protein sample only to improve sample reproducibility was unsuccessful (Figure 3.15). Reasons for this include inconsistent levels of SDS; once inside the column with

the mobile phase the levels of SDS were not consistent enough to maintain the denaturation of the protein, and the SDS in the sample could interfere with the gradient. A potential solution would be to add SDS into the buffers, which would keep the levels of SDS constant and therefore reduce inference.

SDS is a detergent and is highly undesirable inside the mass spectrometer. Therefore, to ensure that the MS was not contaminated, the MAbPac™ workflow was modified to ensure the removal of any detergent before samples were analysed. Once the fractions were eluted from the HPLC, they were loaded onto an SDS-PAGE gel and silver stained to observe the distribution of the proteins (Figure 3.15). This had the added benefit that protein separation could be observed, and although the process was time consuming and not suited to high throughput analysis, it was nevertheless a useful tool for optimisation.

SDS was added into the buffers to a final concentration of 0.02 % and the chromatograms of RAW264.7 murine MΦ TCL with and without added SDS were compared (Figure 3.15 a & c). Fourteen fractions from each run were collected and loaded onto an SDS-PAGE gel, along with 10 µg of the sample for comparison. Gels were silver stained to observe the distribution of proteins with and without SDS (Figure 3.15 b & d).

The elution of the proteins was more concentrated at the beginning of the gradient when SDS was not used, and that overall, the sample without SDS (Figure 3.15 a & b) achieved a poorer separation compared to those run with SDS (Figure 3.15 c & d). The chromatograms for the samples with added SDS show a less reproducible elution compared to those without added SDS, and that overall, the proteins were spread more evenly between fractions without the addition of SDS. It was therefore concluded that the addition of SDS into the samples but not the mobile phase destabilises the proteins within the sample and contributes to a poorer overall separation of proteins in the MAbPac™ column.

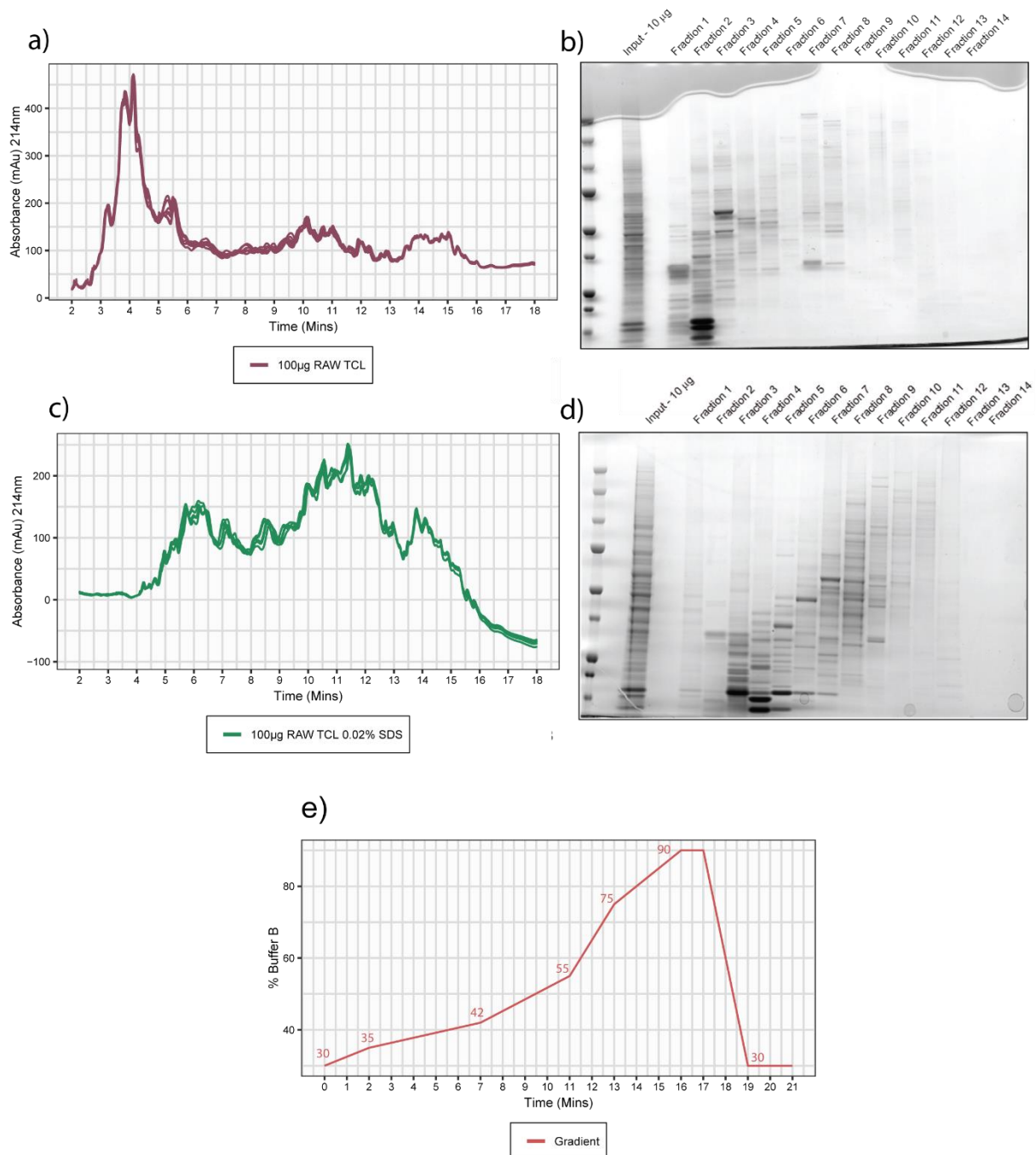


Figure 3.15: Addition of 0.02 % SDS to the mobile phase improves protein separation. a) Six replicates of 100 µg RAW264.7 cell lysate was fractionated by MAbPac™. The organic mobile phase B was 45 % acetonitrile, 45 % isopropanol and 0.01 % TFA in water. Mobile phase A was 2 % acetonitrile, 0.1 % TFA in water. The gradient was 30-36 % B in 2 min, 36-55 % B in 9 min 55-75 % B in 0.1 min and 75-90 % B in 3 min. Flow rate was 0.250 ml/min and the column temperature was set at 75 °C. Fractions were collected over 18 min for 14 fractions each collected over a time period of 84 s. b) Silver stained SDS-PAGE gel of fractions from A, with a 10 µg unfractionated sample for reference. c) Three replicates of 100 µg RAW264.7 cell lysate was fractionated by MAbPac™. d) Silver stained SDS-PAGE gel of fractions from C, with a 10 µg unfractionated sample for reference. e) The gradient of buffer B. The organic mobile phase B was 45 % acetonitrile, 45 % isopropanol, 0.1 % TFA and 0.02 % SDS in water. Mobile phase A was 2 % acetonitrile, 0.1 % TFA and 0.02 % SDS in water.

After concluding that it was beneficial to add SDS to the mobile phase, it was necessary to demonstrate the functionality of the MAbPac™ compared to the most commonly utilised method of HPLC fractionation, basic reversed phase chromatography. Three replicates of RAW264.7 cell lysate were compared using the MAbPac™/SDS method against classical basic reversed phase, where samples were lysed in 5 % SDS, diluted to 2.5 % SDS before being reduced and alkylated. SDS was removed from the sample using a modified version of the SP3 protocol from (Hughes et al. 2014), and the sample was digested with trypsin prior to fractionation with basic reversed phase. The samples intended for analysis by MAbPac™ were also denatured in 5 % SDS, which was diluted to 2.5 % SDS before fractionation by HPLC, using the MAbPac™ column. Fractions were collected and loaded onto a 15-well SDS page gel, which subsequently underwent silver staining. Bands were digested using in-gel digestion and subsequently analysed by mass spectrometry (Figure 3.16). Although there was a greater number of proteins identified by BRp (Figure 3.16 b), there were 1656 proteins that were exclusively identified in the MAbPac™ samples (Figure 3.16 a), and the MAbPac™ achieved a better protein coverage (Figure 3.16 c). The ability of the MAbPac™ to identify unique proteins is in part due to its ability to withstand very high temperatures (75 °C compared with the basic reversed phase column, which is kept at 30 °C) and harsh conditions. These characteristics mean that the MAbPac™ can achieve a separation environment unlike any other column. This is promising evidence that the MAbPac™ could be a valuable tool in MS proteomics.

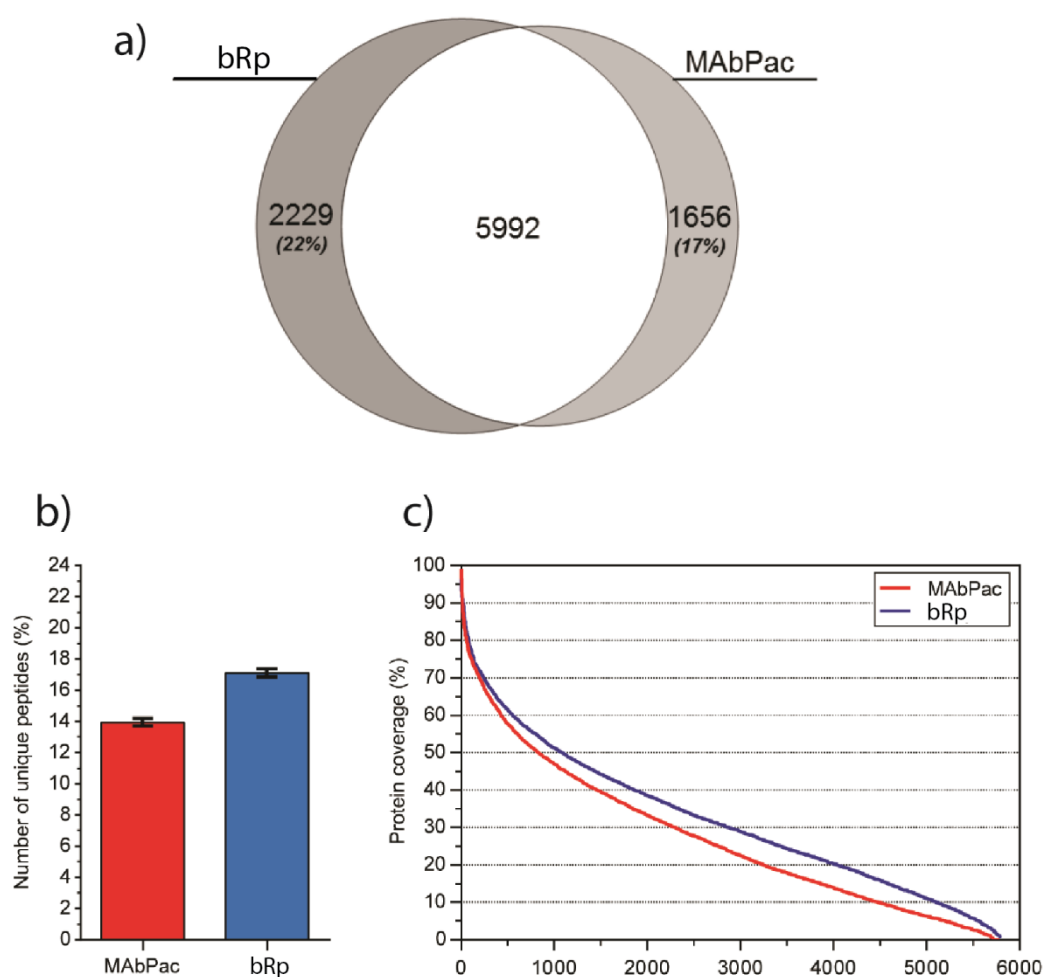


Figure 3.16: MS analysis of RAW 264.7 total cell lysate by peptide basic reversed-phase and Protein separation by MAbPac™ with SDS. a) Venn diagram showing number of proteins identified in both basic reverse and MAbPac (middle, white), and uniquely by basic reversed phase and MAbPac™ with 0.02 % SDS in the mobile phase (left and right, grey). B) Total numbers of proteins identified by the MAbPac™ and basic reversed phase (BRp). Error bars are calculated using standard deviation. C) Total protein coverage achieved by MAbPac™ and basic reversed phase. RAW264.7 cells were lysed in 5 % SDS and subsequently diluted to 2.5 % SDS. Samples intended for fractionation by basic reversed phase were reduced by 100 mM TCEP and alkylated by 100 mM iodoacetamide both for 30 min. Basic reverse samples were then cleaned of SDS by a modified version of the SP3 protocol from Hughes et al. 2014, before digestion with trypsin and fractionation by basic reversed phase HPLC. Samples intended for fractionation by MAbPac™ were denatured in 5 % SDS and diluted to 2.5 % SDS using buffer A and fractionated by MAbPac™. The organic mobile phase B was 45 % acetonitrile, 45 % isopropanol and 0.01 % TFA in water. Mobile phase A was 2 % acetonitrile, 0.1 % TFA in water. The gradient was 30-36 % B in 2 min, 36-55 % B in 9 min 55-75 % B in 0.1 min and 75-90 % B in 3 min. Flow rate was 0.250 ml/min and column temperature was set at 75 °C. Fractions were collected over 18 min for 14 fractions each collected over a time period of 84s. Fractions were run on an SDS-PAGE gel before undergoing in-gel digestion and analysis by HPLC. The LC-MS analyses were performed on an Orbitrap™ Fusion mass spectrometer (Thermo) coupled to an Ultimate 3000 RSLCano HPLC (Dionex). Each fraction was separated over a 60min gradient in an EASY-spray pepmap c18 column (50 cm x 75 µm ID, 2 µm particle size, 100 Å pore size).

3.3.4. Optimising protein recovery and digest from MAbPac™ fractions

3.3.4.1. Single-Pot Solid-Phase-enhanced Sample Preparation

It is necessary to remove SDS from samples before they enter the MS, and this is usually done by in-gel digestion for samples fractionated by MAbPac™. However, this is a laborious and time-consuming process which has the additional drawbacks insofar that it is impossible to automate and induces a 50-60 % sample loss at the stage of the in-gel digestion. Therefore, it was necessary to explore alternative methods of removing SDS.

A modified version of the SP3 protocol from (Hughes et al. 2014) offers a number of advantages over in-gel digestion. The principles behind SP3 are as follows: proteins are bound to paramagnetic beads in the presence of an organic solvent, such as 70 % acetonitrile at acidic pH. The organic solvent promotes binding, and therefore detergents such as SDS can be removed during the subsequent washing steps. After washing, proteins can be digested by addition of trypsin (at basic pH, achieved by re-suspending beads in 100 mM HEPES at pH 8). Washing steps do not require a centrifuge. The sample can easily be separated from the beads as a magnetic rack can be used to attract the beads to the side of the tube allowing the supernatant to be removed without disturbing the proteins. After further washes in organic solvent, the beads are left to dry to remove all traces of solvent from the sample. Peptides are eluted from the beads with DMSO. Once removed from the beads, the peptides can be acidified with formic acid or TFA and injected directly to the MS. SP3 is a time-efficient method for the clean-up and digestion of a protein sample and has been shown to produce a high protein recovery, making it ideal for small samples. Resulting peptides are ready for further MS processing, such as labelling with TMT or fractionation by HPLC (Ly et al. 2017).

The SP3 method can be combined with further methods of purification (Yoshikawa et al. 2018), and the paramagnetic beads and the magnetic sample rack mean that the separation of beads and supernatant can be achieved without a centrifuge. This makes it suitable for automated workflows which can use a robotic liquid handling platform, such as the Kingfisher (Thermo Fisher Scientific) (Müller et al. 2019). Samples in plates which can be lifted on and off a magnetic rack mean that large numbers of samples can be processed using a fully automated workflow. As it is a long-term ambition for the MAbPac™ to be optimised for serum analysis in a clinical setting, incorporation of an automatable protein cleanup and digestion workflow is highly desirable. However, whilst SP3 is usually an effective method of detergent removal,

digestion takes two rounds of 4 h, or overnight, so the process is still relatively long. Using SP3 does not guarantee that 0 % SDS will be left in the sample. It was therefore necessary to investigate other options for detergent removal.

3.3.4.2. *Using the HiPPRTM Detergent Removal Spin Column Kit to remove SDS*

HiPPR from Thermo Fisher Scientific is a detergent removal method, which utilises a spin column to remove detergents from HPLC fractions. It is designed for samples with low protein or peptide concentrations, suggesting that it could be effective for reducing sample loss. It is therefore a favourable alternative to in-gel digestion, which has a high level of sample loss. Use of the HiPPR columns is fast- the protocol is 15 min long- and also has the potential to be optimised for automation. It was therefore possible that use of the HiPPR columns was a viable alternative for in-gel digestion for the MAbPa workflow.

To investigate the possibility of HiPPR columns as an alternative for in-gel digestion, a standard curve was created using known concentrations of SDS in water, measured by plate reader at 650 nm (Figure 3.17 a). The HiPPR columns (Section 2.1.3.11) were then used to remove SDS from standards and MAbPa fractions containing 0.2 % SDS in water. An SDS assay was used to measure SDS concentration once the samples had eluted from the column. The HiPPR columns were found to be effective for the removal of SDS for a final concentration of 0.025 - 0.05 % SDS (Figure 3.17 b & c).

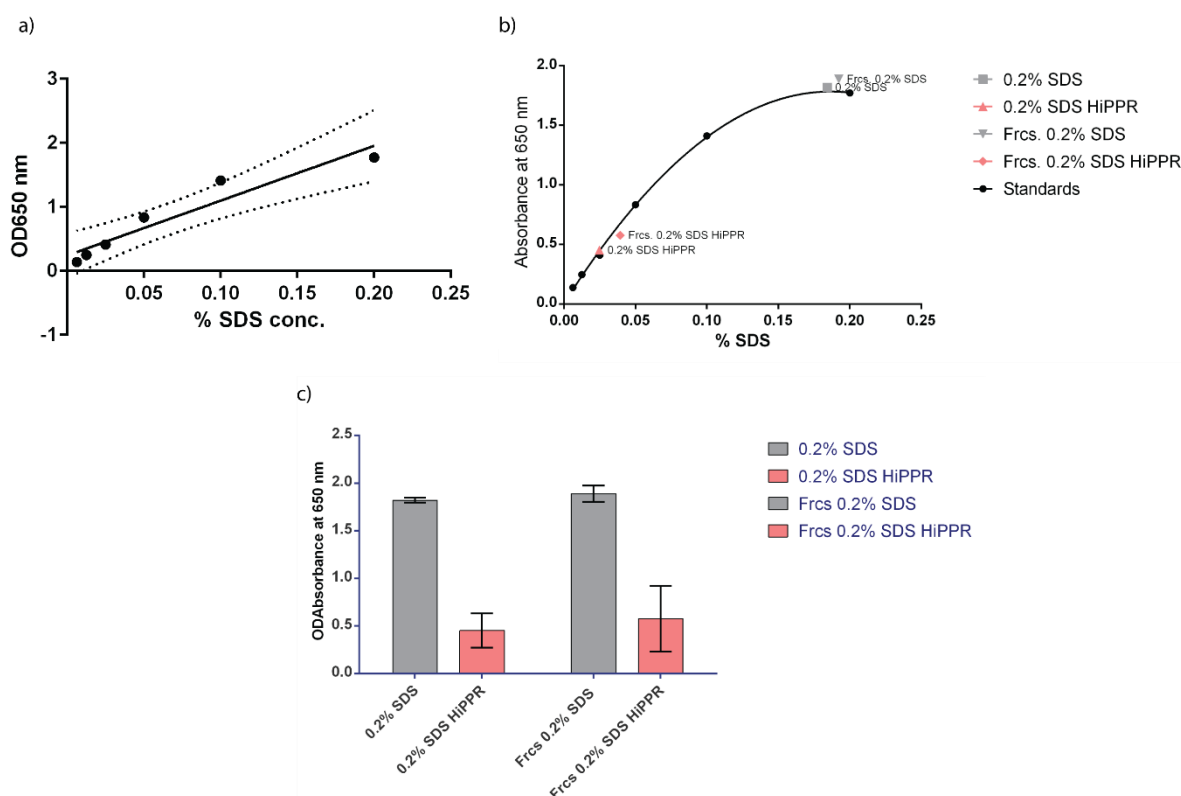


Figure 3.17: Use of HiPPR columns from Thermo Fisher Scientific for the removal of detergent from MAbPac fractions. a) Development of a standard curve for identification of SDS by plate reader. b) SDS standards and MAbPacTM fractions with and without detergent removal by HiPPR measured by plate reader at 650 nm and plotted against the standard curve. Error bars were calculated by standard deviation. c) Plotted SDS levels in standards and MAbPacTM fractions with and without detergent removal by HiPPR.

3.3.4.3. S-Trap

SP3 and HiPPR both left trace amounts of SDS in the sample. However, SP3 is overall a more convenient protocol as the digestion and clean-up can all be done on the beads. This makes the workflow easier to automate and less expensive. However, for both approaches, digestion time is long. The S-trap was developed by ProtiFi and can reduce digestion time from overnight to 1 h. Like the SP3 workflow, the clean-up and digestion process are performed with the proteins bound- in the case of S-trap, to a hydrophilic phase. Digestion takes 1-2 h at an elevated temperature (47 °C), which removes the need for reduction and alkylation of the sample prior to digestion- the higher temperature holds proteins in a denatured form in the phase. The entire workflow is notably faster than the previously mentioned methods and has been shown to be effective for removing up to 5 % SDS from samples. As this was the fastest and most efficient SDS removal technique tested, it was coupled to the MAbPac workflow. This set-up was found

to mitigate the extra time required to clean and digest many fractions instead of one sample. This method therefore has the potential to be a fast, accurate method of analysing complex samples such as serum, without the need for expensive albumin depletion or lengthy clean-up of fractions.

3.4. Discussion

The analysis of therapeutic MABs is traditionally performed using an SCX column and a salt gradient, a protocol that takes an hour. Using a pH instead of a salt gradient reduces the gradient length to 10 min. This workflow is faster and is therefore better suited to high throughput analysis. Fast analysis of therapeutic MABs is desirable in clinical settings.

In this project, the aim was to develop a pH mobile phase for the SCX column to use in MAB charge variant analysis. These buffers were challenging to create, as they needed to be both volatile enough for use with MS, and stable enough for storage. Acetic acid and ammonium hydroxide were chosen for the mobile phases because, with these reagents, buffers could be made to the desired pH without additional adjustment. This could improve reproducibility and stability, whilst also being MS-compatible.

During this project, it was found that although the buffers were made in an identical manner, they were adversely affected by conditions within the HPLC. It was determined that the degasser was affecting the pH of the buffers, which contributed to the variations between chromatograms over time. Running blanks and samples without the degasser showed a clear pH change compared with previous runs. This was because the degasser was removing uneven amounts of carbonate from the buffers as they passed through, thereby changing the pH.

However, to bypass the degasser, all experimental conditions would have to be re-optimised to reflect the new HPLC set-up. This would require the user to remove the degasser for every experiment performed with these buffers. Therefore, these buffers could not be used on an LC system where the buffers are not readily accessible, such as the widely used Ultimate 3000 series.

Removal of the degasser may not be in the interests of clinical laboratories, where time and ease of use are priorities. Reproducibility is important, but for MAB studies, it may not be necessary for chromatograms to be perfectly reproducible if the samples will be analysed by

MS. Charge variants are identified by the MS. For MS analysis, exact chromatographic replication is unnecessary as the mass shift recorded by the MS is still the same, regardless of chromatographic elution time. Therefore, in a clinical setting, the SCX-pH method of charge variant analysis could still offer a time-efficient alternative to the current approach.

Alongside the development of the MAbPac SCX column there was a second project in collaboration with Thermo Fisher Scientific. The objective of this project was to re-purpose the MAbPac RP column, usually used for MAb analysis, for the separation of whole protein samples such as TCL. For the mobile phase, it was found that the combination of isopropanol and acetonitrile produced a more reproducible separation. The presence of isopropanol changed the selectivity of the column, which influenced the elution in this case. Isopropanol is more hydrophobic than acetonitrile, and this aided the elution of very hydrophobic analytes from the column. Desai *et al.* (2011) used isopropanol as an organic modifier also found that, with the same sample, isopropanol produced comparable results to acetonitrile. Therefore, this buffer recipe provided an alternative method for protein separation using the MAbPac column.

To further improve the separation of proteins, reduction and alkylation was performed prior to fractionation. Reducing and alkylating a protein prior to whole protein fractionation would be unusual- normally, this step would be performed after protein fractionation, before digestion and analysis by MS. However, it was theorised that reducing the protein into its linear state could expose hydrophobic residues and therefore enhance the binding of the protein to the column, thereby aiding separation. When tested, a similar separation was observed, but the intensity for standards which had been reduced and alkylated was lower. It is probable that linear proteins precipitate on the column, but this does not explain the comparable elution times between both sets of standards. It is also possible that reduction and alkylation could interfere with the absorption of the proteins by the UV cell, resulting in a lower rate of detection. Therefore, it was concluded that reduction and alkylation of proteins before fractionation did not improve separation of proteins on the column.

To test the capabilities of protein fractionation using the MAbPac column against classical peptide basic reversed phase fractionation, TCL was analysed using both columns as part of 2D-LC/MS workflows. The off-line fractionation was either protein fractionation using the MAbPac or peptide fractionation using an RP column. The on-line fractionation was always peptide basic reversed phase.

These initial experiments showed that classical 2D basic reversed phase separation on the peptide level produced a higher number of IDs for TCL. This could be because of the increased resolution of the peptide RP column, which can separate peptides which differ by as little as one amino acid residue (Herrero et al. 2017). However, the unique proteins identified in both the MAbPac and the RP experiments shows that the MAbPac is valuable for 2D-LC/MS workflows, particularly with the right biological application.

Serum analysis was chosen as a potential application for MAbPac separation as is primarily composed of a low number of very high abundance proteins, also contains many lower-abundance proteins. Several of these are important biomarkers. For serum analysis, separation of proteins into different fractions could be highly desirable as it would allow identification of serum biomarkers by separating them from highly abundant proteins such as albumin, which often masks less abundant proteins, making them difficult to identify.

If highly abundant proteins could be separated from low abundance proteins using HPLC, the protein depletion step could be eliminated, streamlining the workflow. This would also minimise the loss of lower abundance proteins and biomarkers from the sample.

To explore this, human serum samples which had been depleted using albumin depletion and separated by MAbPac. As expected, the samples separated by MAbPac contained a greater number of proteins compared to the depleted samples as shown by a clear difference in silver-stained gels. MS analysis of the MAbPac -separated samples, however, showed a separation of high abundance proteins and low abundance proteins into different fractions.

Human serum has been fractionated before, but usually with depletion using albumin depletion columns (Zolotarjova et al. 2008) which makes the method unsuitable for high-throughput clinical analysis. As the MAbPac workflow has the potential to be automated and adapted for a high-throughput workflow and was also able to separate high abundance proteins and identify key biomarkers, this is a novel method which is well-placed for development in a clinical setting.

In whole (un-depleted) human serum separated by MAbPac, 301 proteins were identified from 2 µg of human serum. This is an improvement from other methods such as the ProteoPrep20 and ProteoMiner where 191 and 130 proteins were identified from albumin-depleted serum, respectively (Baiwir et al. 2015b).

The MAbPac method differs from those used by Baiwir *et. al.* (2015a) because, by using the MAbPac to fractionate whole serum, it is more effective at minimising sample loss. By separating the sample rather than reducing it, low-abundance proteins can be retained, such as the potential glycoprotein biomarkers. Even a method such as ProteoMiner, which aims to enrich low abundance proteins, also removes high abundance proteins from the sample. Removal of any protein group risks simultaneous loss of low abundance proteins, which strengthens the argument for using a depletion-free method of serum analysis.

To investigate whether addition of a surfactant would improve serum analysis by MAbPac, Rapigest was added to samples prior to fractionation. The conclusions were that the optimal method of serum fractionation was with 0.1 % Rapigest added to the sample. This method offers the advantage of increased control over fraction collection. In an area where many proteins elute, methods can be programmed to collect fractions more frequently. It also offers a defined number of fractions for each LC run, which can be useful for comparisons between different samples or replicates.

Addition of Rapigest to the sample improves the protein recovery from the column because it increases the solubility of hydrophobic proteins, facilitating their elution. However, even though Rapigest proved to be an effective method for increasing the number of protein IDs from a set of MAbPac fractions, Rapigest is expensive and therefore not suitable to add to a large volume of samples.

As an alternative, the effects of denaturing the proteins with SDS before fractionation was investigated. Addition of a harsh detergent such as SDS is usually impossible for a sensitive HPLC workflow, but the MAbPac is more robust than many of its counterparts and will not be damaged by low levels of SDS.

To further optimise methods for other biological samples, *E. coli* TCL was used as a complex protein standard. To establish which method was superior in regard to reproducibility between replicates and distribution of proteins. *E. coli* TCL (50 µg) which had been mechanically lysed either in water or in 2.5 % SDS and immediately injected onto the MAbPac™ column were compared.

Proteins were recovered more successfully in the water-lysed samples, and the chromatograms were more reproducible compared to those samples lysed in SDS. Theoretically, the increasing concentrations of SDS being introduced into the column could interfere with the ion pairing once the sample was in contact with the mobile phase. As the SDS is pushed through the column

as the gradient progresses, levels of SDS accumulated and were then eluted at different rates. These changing levels of SDS interfere with the binding of the proteins in the column and therefore the time of elution for each protein. If SDS was to be present in the column, the concentration of SDS would need to remain constant to mitigate any effects on protein binding and to keep conditions reproducible between samples.

To investigate the effect of SDS on protein elution from MΦs, 100 µg RAW264.7 TCL with SDS added to the mobile phase (for a final concentration of 0.02 %) were compared to samples without added SDS. It was hypothesised that addition of SDS to the buffers aids the distribution of proteins along the gradient. These findings are in line with the conclusions that addition of the surfactant Rapigest aids protein distribution and elution in HPLC analysis, but SDS may have a stronger effect as it is a stronger surfactant. Thus, addition of SDS to the sample alone was disruptive. Addition of SDS to the buffers themselves was necessary to keep SDS levels constant during the run and minimise the variable effects of SDS which was introduced only by injection.

Whilst SDS was clearly effective for improving protein separation in off-line HPLC, there is the major drawback that the presence of SDS in the buffers will subsequently distribute SDS into the tubing, pumps, switch valves and any other part of the HPLC machinery. This would adversely affect future analyses as SDS would contaminate future samples. It was therefore necessary to have a dedicated column and machine for SDS runs, which is not possible in every laboratory due to the high cost of the HPLC system. Subsequently, for this method to advance MAbPac protein separation, it would need to be adapted for a high-throughput application and ideally be developed for fast, automated analysis of a specific sample, such as serum or antibody analysis in a clinical laboratory.

The MAbPac fractionates samples at the protein level, which offers a degree of orthogonality for 2D-LC/MS experiments that offers an improvement on current 2D-LC methods which often use peptide-level fractionation. A comparison of MAbPac-fractionated samples with C18 basic-reverse fractionated samples, showed that, whilst the basic reversed phase C18 column achieved a higher number of IDs, the MAbPac can still identify 1656 unique proteins and can achieve a better protein coverage- the numbers of amino acids identified from each protein.

The poorer number of proteins IDs achieved by the MAbPac can be attributed to the in-gel digestion, which was not performed for the directly injected samples analysed using the C18

column only. In-gel digestion results in sample loss (Speicher et al. 2000), and this extra experimental stage could also have been a point of protein loss for the MAbPac samples.

Therefore, to gain a better understanding of how the MAbPac compares with the classical shotgun approach of 1D on-line HPLC, the comparison of both techniques would need to be repeated where either both samples undergo in-gel digestion, or preferably an alternative method of SDS removal can be used to minimise sample loss. However, the presence of unique peptides identified in the MAbPac cohort strongly indicated that the MAbPac is a valuable tool for the 2D-LC/MS workflow. Currently, this is the only study where SDS is used for RP-HPLC protein fractionation.

Overall, the MAbPac reverse phase DVB column offers a unique and orthogonal method of protein separation. It can be utilised effectively as a method of 2D-LC, especially for samples which contain very hydrophobic proteins. Compared to peptide-based chromatography, the MAbPac can be used to identify a large cohort of unique proteins which contributes to its suitability for 2D-LC, combined with an on-line peptide separation method such as a reverse-phase C18 column. It is suitable for the analysis of serum, which presents various challenges to peptide separation techniques.

Use of detergent has been shown to improve protein coverage and protein recoverability, although there are many challenges associated with the addition of SDS. Alternatively, Rapigest could be used for a similar, if lower-level benefit without necessitating a different machine.

Finally, the MAbPac is unlikely to replace the C18 basic reversed phase column for standard proteomics experiments due to difficulties with SDS removal from both the HPLC fractions and machinery. However, its unique properties, such as being able to withstand very high temperatures, operating on a protein level, and having a robust, monolithic phase which can withstand harsh detergents and salts, could give it an edge for analysis of the right sample. This could include serum analysis, analysis of antibodies, biomarker identification, protein purification or sample fractionation, or top-down proteomics experiments. Working towards automation would further improve this method.

4. Chapter 4: Proteomics investigation into TLR2 signalling

4.1. Introduction

This section is centred on the hypothesis that TLR2 plays a role in phagosomal signalling, maturation and particle uptake. Quantitative proteomics techniques were used to investigate the effects of TLR2 and TLR4 ligands on the MΦ proteome. *Staphylococcus aureus* (*S. aureus*) was used as a ligand for TLR2 dimers in BMDMs. BMDMs incubated with *S. aureus* at two time points were analysed by quantitative mass spectrometry using TMT labelling. Specific ligands were used to target TLR2/1, TLR2/6 and TLR4/4 dimers and the vATPase inhibitor Bafilomycin (BAF) was used to differentiate endosome dependent and independent downstream signalling. This section is centred on the hypothesis that TLR2 plays a role in phagosomal signalling, maturation and particle uptake.

4.2. Aims

1. Investigate the proteome changes induced by activated TLR2 in MΦs.
2. Use *S. aureus* as a 'biologically relevant' bacterial ligand for TLR2 and measure proteome changes over two time points.
3. Target TLR2 and TLR4 receptors using specific ligands and isolate phagosomes to specifically measure the phagosome proteome.
4. Use BAF to inhibit endosomal signalling so that endosome-dependent processes can be identified.

4.3. Results

4.3.1. TLR2 KO BMDM cells have a higher rate of particle uptake compared with WT cells.

To investigate the effect of the loss of TLR2 on phagocytosis, the particle uptake of *S. aureus* was measured in TLR2 KO and WT BMDMs infected with YFP-transfected *S. aureus*, prepared as described in Section 2.1.3.5.

A plate reader (Molecular devices, model: SpectraMax iD3) was used to measure particle uptake of YFP-transfected *S. aureus* by TLR2 KO and WT BMDMs which had previously been treated for 1 h with 1 µg/mL BAF (to inhibit lysosomal acidification and, therefore, maturation) or 1 µg/mL DMSO. *S. aureus* (harvested at OD₆₀₀) was added to cell media for 30 min before

cells were washed and extracellular bacteria were quenched with trypan blue. YFP fluorescence from phagocytosed bacteria was measured over a period of 60000 s (16.5 h). Results (Figure 4.1) show that treated TLR2 KO cells phagocytosed higher numbers of *S. aureus* particles compared to WT cells incubated with the same bacteria (Figure 4.1). Use of BAF reduced but did not completely inhibit particle uptake. Taken together, this indicates a role for TLR2 in the negative regulation of phagocytic processes.

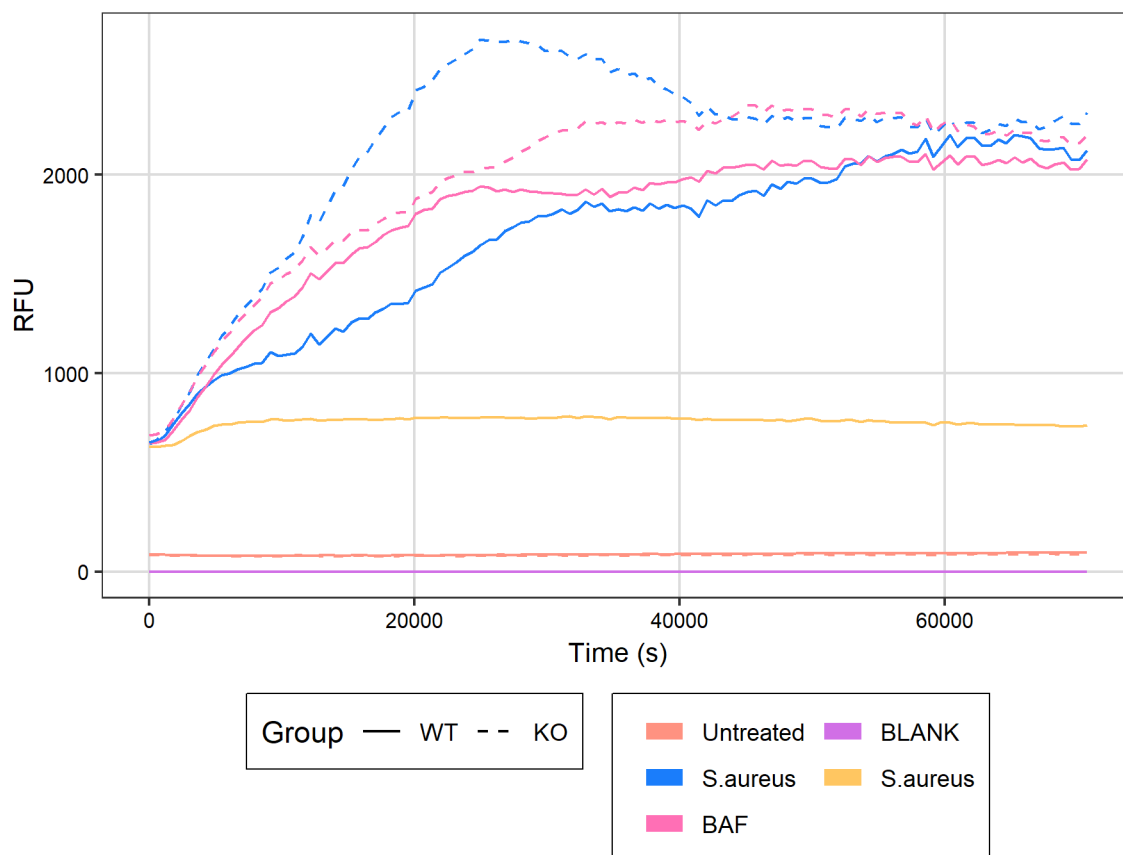


Figure 4.1: RF units measured over time for *S. aureus* particle uptake by WT and TLR2 KO BMDMs. Cells were incubated with a YFP strain of *S. aureus* for 30 min and pre- treated for 1 h with 1 μ g/mL BAF or 1 μ g/mL DMSO.

4.3.2. TLR2 and TLR4 elicit distinct proteome changes on the phagosome

In order to understand the impact on the phagosomal proteome when endosomal TLR2 or TLR4 is activated, a bottom-up proteomics approach with TMT labelling was employed to investigate proteome changes in the phagosomes of BMA cells. To focus on selected signalling pathways, the synthetic TLR2/1 ligand PAM₃CSK₄ was used to activate the TLR2 receptor, and LPS used to target the TLR4/4 active homodimer. BAF inhibits endosomal vATPases and therefore acts as an inhibitor for phagosome maturation and endosomal signalling.

The cells had been pre-treated with 1 µg/mL BAF or DMSO for 1 h as a control. Following pre-treatment, cells were stimulated with 10 µg/mL PAM₃CSK₄-coated, LPS-coated or plain 1 µm carboxylated polystyrene beads for 30 min. Bradford assay was used for protein quantitation (Section 2.3.4).

Stimulation using the carboxylated beads helps to deliver the ligand to endosomal receptors. The beads, which are coated with -COOH (carboxyl) groups trigger MΦ surface receptors such as scavenger receptors and are phagocytosed. The physical presence of the beads induces phagocytosis, and in this respect provokes a more realistic response from the cell compared to stimulation by soluble ligands by mimicking bacteria. Using a specific ligand, however, enables the targeting of specific receptors and subsequently activated pathways. Carboxylated beads enable the extraction of the phagosomes from the total cell lysate (TCL).

Four replicates were created per condition, but the three most reproducible were used to produce the results. Outliers are shown in Figure 4.2 c). After treatment and stimulation, cells were harvested and mechanically lysed in HB buffer. Phagosomes extracted using the standard phagosome isolation protocol as described in Section 2.6.1. Three replicates per condition were taken for analysis.

The p-value fold changes between different conditions with a cut-off value of 0.58 (which is the -LOG₂ of 1.5) shown in Figure 4.2 and Figure 4.3.

The data show that coating beads with either LPS or PAM₃CSK₄ induced upregulation of lysosomal proteins, ubiquitin associated proteins and interferon-associated proteins, all indicative of innate immune signalling (Figure 4.2 a & b). Treating the cells with BAF also induced considerable changes on the phagosome, particularly for vATPases, lysosomal proteins and cathepsins (Figure 4.2 c – e). When BMA cells were treated with PAM₃CSK₄, an increase in cathepsins O, B, D & L1 was observed compared with cells stimulated with inert beads (Figure 4.2 a). This is similar to the response seen in BAF -treated cells.

RAB7, a late endosomal marker which plays a role in the maturation and acidification of the endosome as well as being a regulator of late endosomal trafficking (Vitelli et al. 1997), was found to be significantly upregulated upon LPS stimulation (Figure 4.2 b) but unregulated in cells stimulated with PAM₃CSK₄ (Figure 4.2 a).

Other proteins of interest identified in these data were SLAMF1 and SLAMF5, which regulate the connection between the innate and adaptive immune responses and boost LPS-induced induction of MAPKs and NF- κ B. They also help moderate LPS-induced cytokine secretion and were found to be significantly decreased in samples treated with BAF compared with stimulated samples (Figure 4.2 c & d). NADPH, which is required for the oxidative killing of pathogens, was predictably upregulated upon stimulation with LPS. However, no upregulation was observed upon PAM₃CSK₄ stimulation. LAMTOR5, which is involved in receptor recycling, was upregulated upon treatment with BAF, suggesting that, as with the cathepsins, the proteins are not being activated and degraded in the absence of endosomal acidification. The IL-1 receptor, which binds IL-1 to activate NF- κ B and MAPK pathways, signals via the TIR domains of MyD88, IRAK1 and IRAK2, as do many PM TLRs, and was found to be significantly upregulated upon LPS stimulation but not PAM₃CSK₄ stimulation.

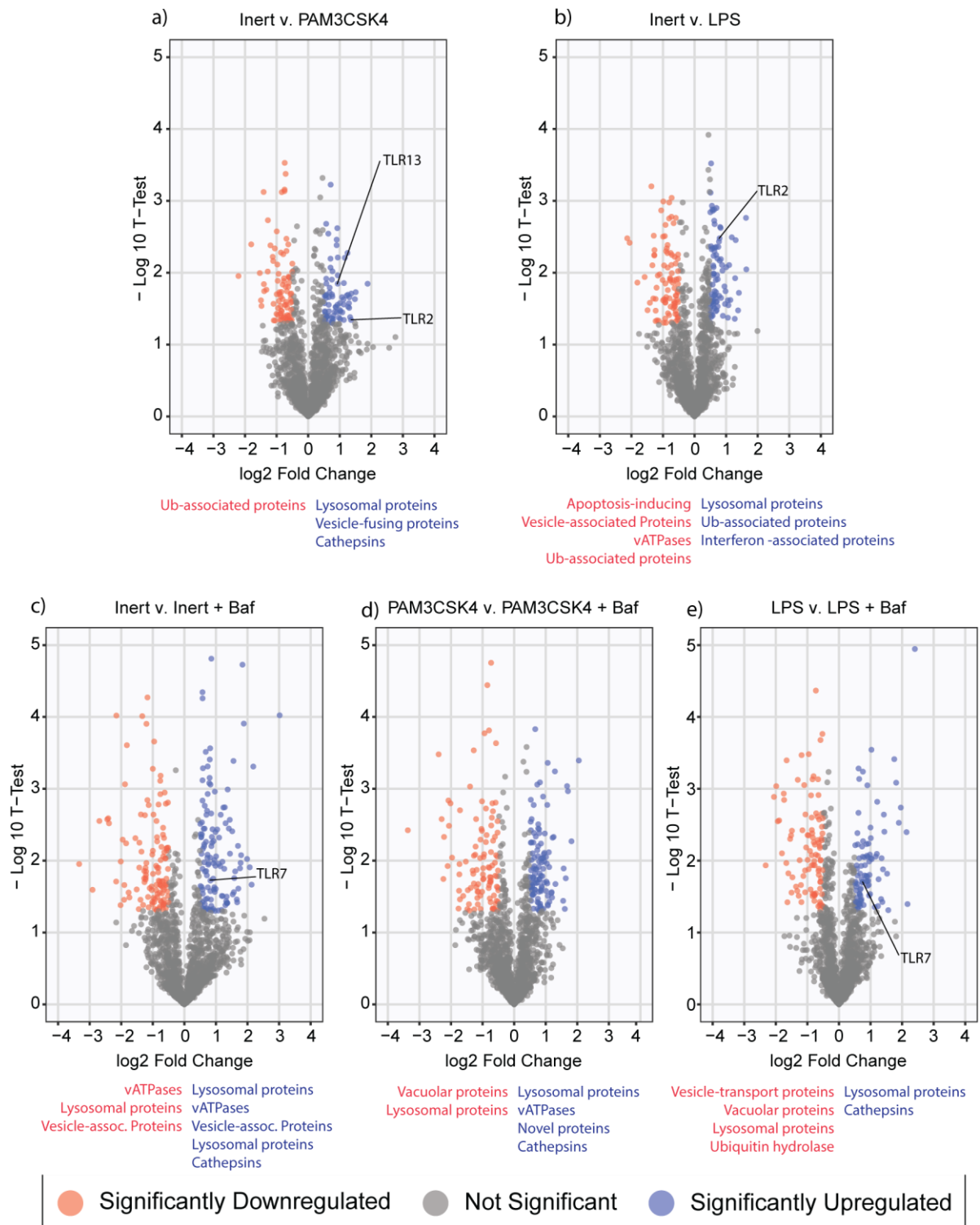


Figure 4.2: Volcano plot distribution of all phagosomal proteins extracted from BMA cells treated with DMSO (UT) or BAF and stimulated with PAM₃CSK₄ or LPS. The x axis represents the log₁₀ fold change distribution calculated in Perseus using two sample t-test. Cells stimulated with PAM₃CSK₄ or LPS were compare with unstimulated cells, and cells treated with BAF were compared with untreated cells. (a), LPS compared with Inert (b), Inert + BAF compared with BAF (c), PAM₃CSK₄ + BAF compared with PAM₃CSK₄ (d), LPS + BAF compared with LPS (e). The y axis represents the p-value across 3 biological replicates.

The effect of BAF on the phagosomal proteome can clearly be seen in the hierarchical clustering performed on significant hits from the p-value ANOVA results (Figure 4.3). The extreme effect of BAF overrides the more subtle differences between stimulated and unstimulated cells, and the BAF samples cluster firmly together. Cluster 1 shows proteins which are downregulated in response to BAF treatment. Proteins in this group are part of the TLR2 and TLR4 signalling pathways, and also play a role in the NF- κ B signalling pathway. Cluster 4 shows proteins which are upregulated in response to BAF treatment. These proteins are primarily involved in lysosome degradation and proteolysis. Proteins in each cluster were analysed using DAVID functional annotation and clustering tool to find GO! Terms and pathway analysis for each cluster represented on the heatmap (Figure 4.3 a).

Cluster 2 shows proteins which are downregulated in response to stimulation with PAM₃CSK₄, but not in response to PAM₃CSK₄ + BAF. Therefore, these proteins are likely to be activated in response to PAM₃CSK₄ in an endosome-dependent manner. There is no change in response to LPS, which shows that these proteins are changing specifically to TLR2. DAVID functional annotation clustering analysis showed that proteins in this group are part of the TNF signalling, MAPK, I κ B α and TNF α pathways and are also involved in endocytosis. The I κ B α and TNF α pathways are key initiators of the innate immune response, and the TNF α pathway is initiated by TLR4 from the endosome. The response shown in figure 4.3 a suggests that TLR2 is an initiator of the TNF α pathway, though through different mechanisms compared with initiation downstream of TLR4.

Cluster 3 shows proteins that are downregulated in phagosomes from cells treated with LPS, but not in LPS + BAF or PAM₃CSK₄-treated cohorts. This shows that these proteins are responding to TLR4 stimulation in an endosome-dependent manner. Proteins in this groups were found to be involved in many TLR pathways, and this group included TLR2, TLR4, CD14 (a co-activator of TLR4) and TLR6.

Figure 4.3 c shows experimental cohorts treated with BAF clustering away from other groups. This highlights the overriding effect of BAF on the phagosome. There is no difference between the samples treated with BAF, showing complete eradication of endosomal signalling. PAM₃CSK₄-stimulated replicates cluster together with the exception of one, which is marked by an arrow. Outliers marked by arrows were removed for quantitative analysis and is not represented in any other figure. Inert and LPS-treated cohorts clustered together, showing that

a higher number of phagosomal protein changes happened in response to TLR2 activation compared to TLR4 activation.

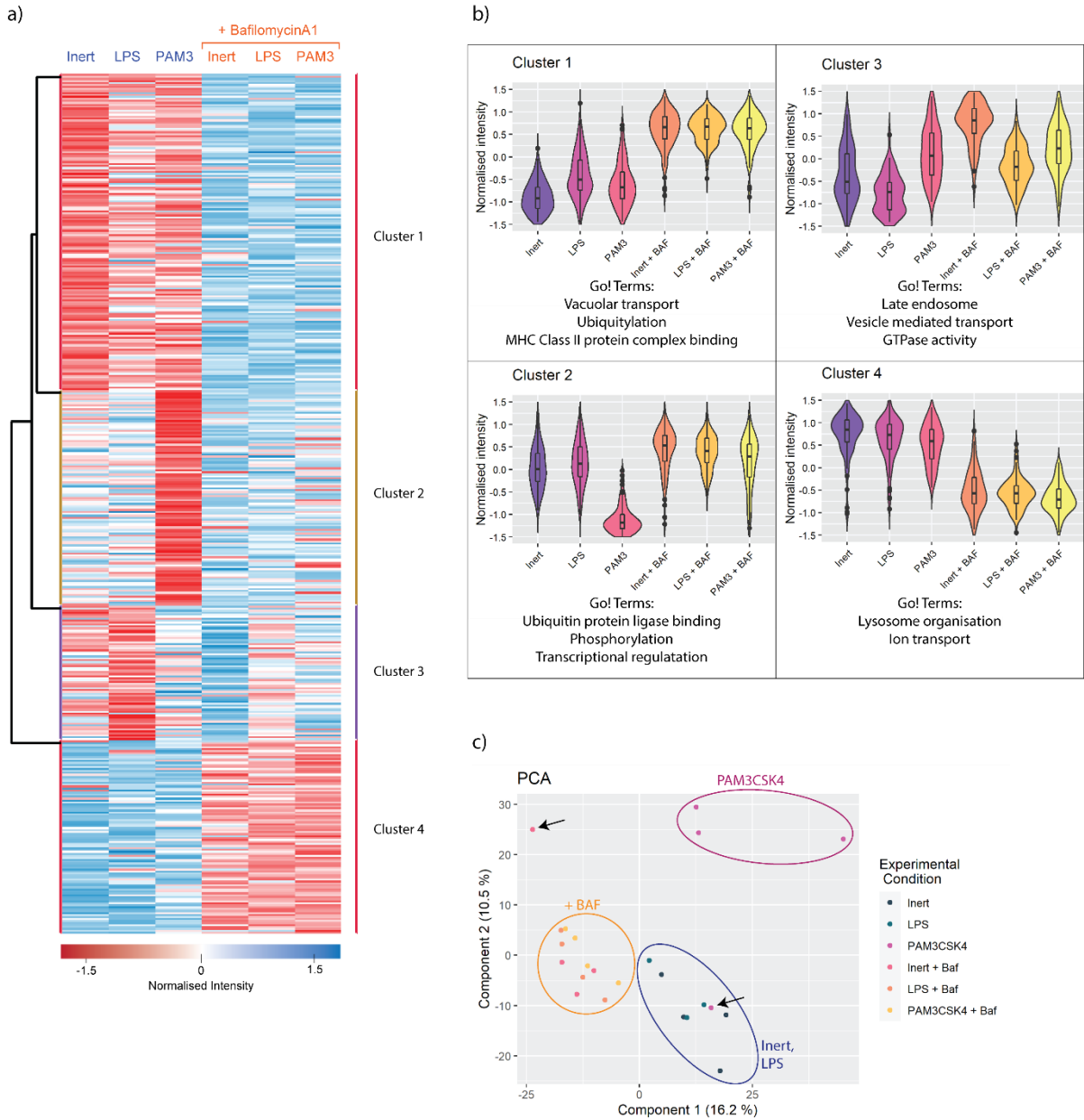


Figure 4.3: Heatmap, intensity plots PCA analysis showing normalised intensity and clustering between phagosome proteomes from BMA cells. a) Heatmap, showing normalised intensity (z score) for proteins identified from phagosomes isolated from BMA cells stimulated with PAM₃CSK₄ or LPS and treated with DMSO or BAF. b) Cluster analysis showing fold change for each identified protein between different conditions with Go! Term results for each cluster. c) PCA plot with clusters highlighted by circles and annotated by group. Outlying replicates marked by arrows were removed for quantification.

4.3.3. Phagosomal changes in WT and TLR2 KO BMDM cells challenged with *S. aureus*

To investigate the effects of TLR2 stimulation on the proteome, BMDMs isolated from TLR2 KO and WT mice were used for a proteomics study. To provoke a biologically relevant response from the proteome, *S. aureus* was used as a stimulus. *S. aureus* is a gram-positive bacterium whose cell wall components are a natural ligand for TLR2 dimers. TMT MS/MS analysis was used to compare WT and TLR2 KO cells infected with *S. aureus* over two time points, which were chosen to represent early and late stage phagocytosis. Firstly, it was necessary to determine how much bacteria to use to achieve a high rate of uptake in BMDMs without causing cells to die. WT BMDMs were infected with a YFP transfected strain of *S. aureus* at a multiplicity of infection (MOI) of 1:5 – 1:300 (cells: *S. aureus*) at OD₆₀₀ and the infection was by microscopy after 3 h (Figure 4.4). Cells infected at 1:50 remained alive and phagocytosed an average of 5 bacteria per cell whereas cells infected at lower MOIs phagocytosed fewer bacteria. Cells began to die when infected at MOIs 1:100 and 1:300. Therefore 1:50 was chosen as the ideal MOI to use for future experiments.

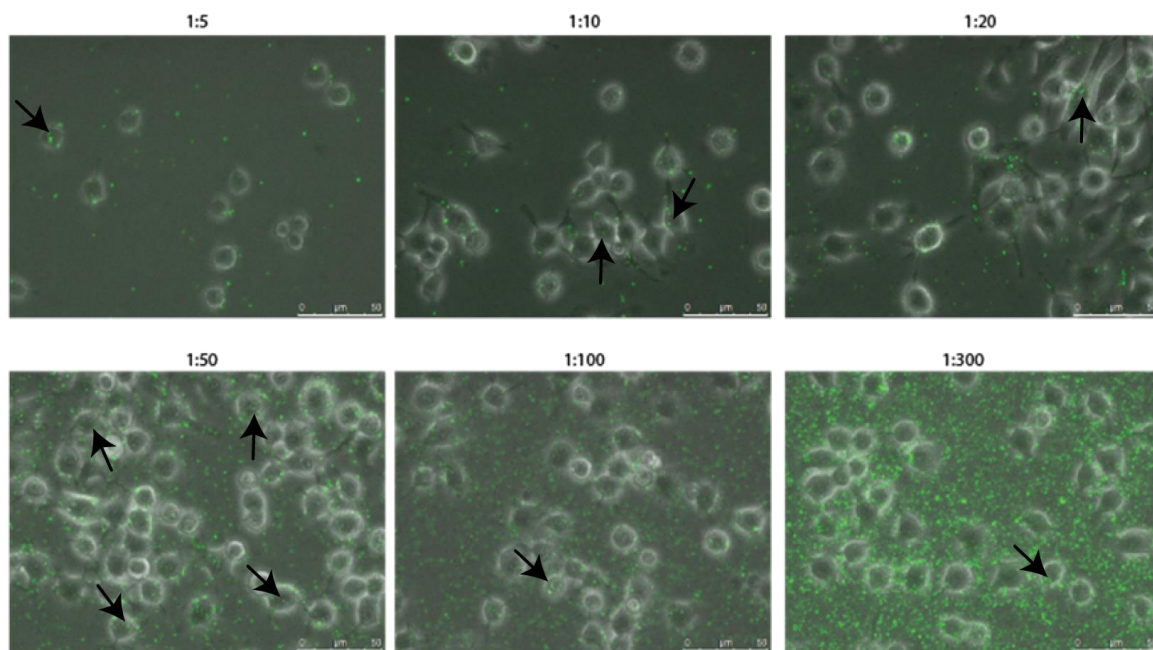


Figure 4.4: *S. aureus* uptake by WT BMDMs. YFP-transfected *S. aureus* at OD₆₀₀ incubated for 3 h with cells at MOI of 1:5 (one cell to five *S. aureus* bacteria), to 1:300. Arrows indicate examples of *S. aureus* uptake.

To measure the whole proteome changes in BMDMs challenged with *S. aureus*, WT and TLR2 KO BMDMs were infected with live *S. aureus* at OD₆₀₀, at a ratio of 1:50 for 15 and 60 min to represent early and late stages of phagocytosis. These time points were chosen to measure earlier and later responses to bacteria, without inducing cell death. Cells were harvested and proteins from the total cell lysate (4 biological replicates per condition) were fractionated using basic reversed phase HPLC before being analysed by MS/MS, as shown in Figure 4.5. BCA assay was used for protein quantitation (Section 2.3.3).

Whole proteome changes in response to *S. aureus* at 15 min were small with between 3 - 27 proteins significantly up or down regulated between samples (Figure 4.6), which was likely due to the short infection period. After 15 min, the main protein changes in WT cells consisted of proteins involved in the membrane, vesicle, or lysosome (highlighted in WT UT vs. WT 15 min, Figure 4.6 a), but also ATPases and Mitogen-activated protein kinase (MAPK) activation, all of which indicated initiation the immune response (Figure 4.6 a). These proteins have been highlighted because they represent phagosomal signalling. Compared to TLR2 KO cells (Figure 4.6 c), fewer proteins were significantly regulated at this time point. At 15 min, TLR2 KO cells showed significant upregulation of vATPases and Ubiquitin E3 ligases compared to WT cells. Overall, the results suggested that, at 15 min, TLR2 KO cells showed a faster progression in immune signalling, supporting the hypothesised inhibitory role for TLR2 in the innate immune response.

After 60 min, Ubiquitin-associated proteins and proteins associated with the NF- κ B pathway became upregulated in WT cells (Figure 4.6 b). At 60 min, there were fewer upregulated proteins in the KO compared to the WT samples. This is indicated a faster, shorter response in the KO compared with the WT, which was not sustained to 60 min (Figure 4.6 d).

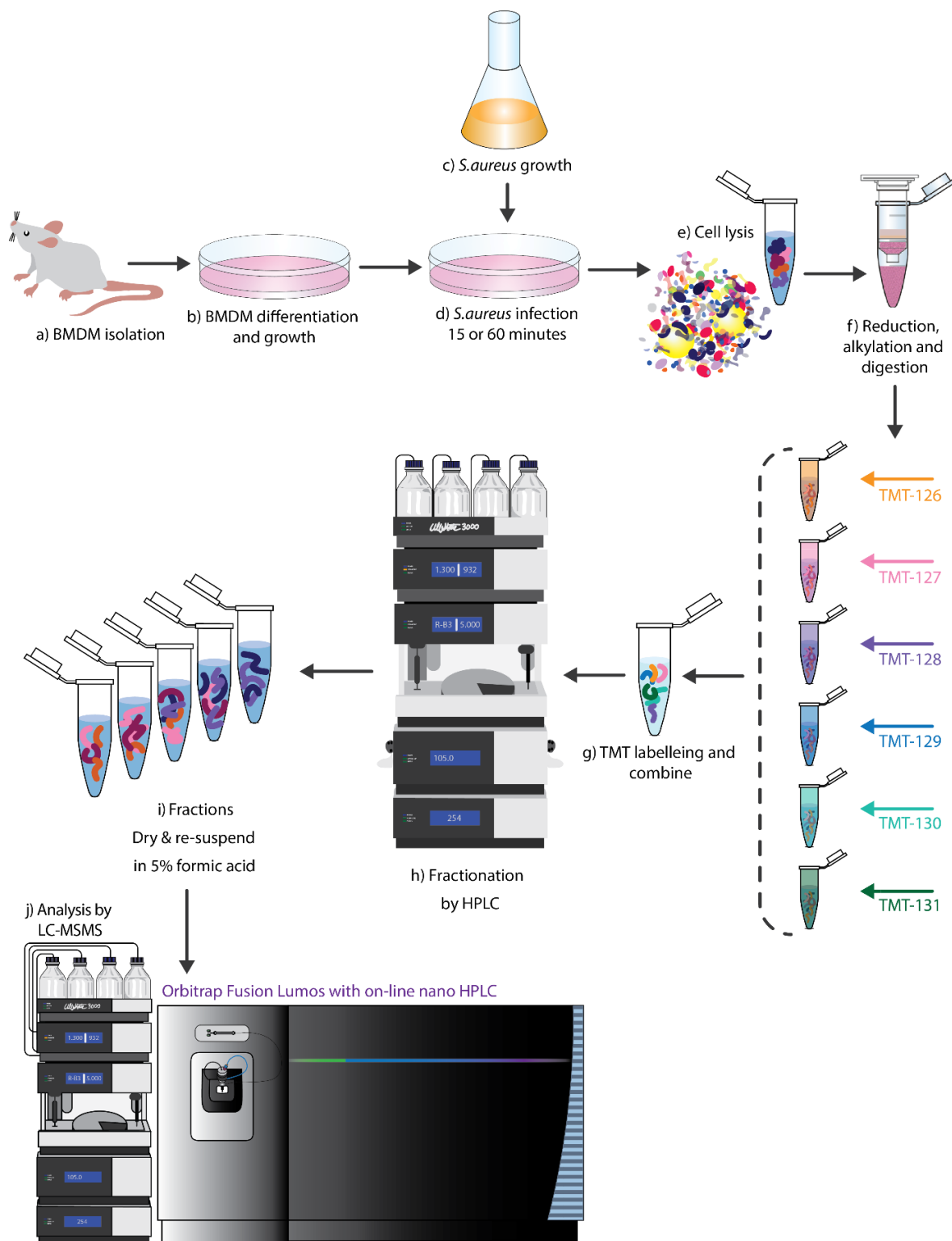


Figure 4.5: Experimental set up for *S. aureus* TMT experiment. a) WT or TLR2 KO BMDMs were isolated from mouse femurs. b) BMDMs were grown and differentiated for 7 days. d) *S. aureus* was grown to OD₆₀₀. e) *S. aureus* was incubated with BMDMs for 15 or 60 min. f) Cells were lysed with 5 % SDS and de-salted and digested using S-trap (vi). g) Samples were labelled with TMT and samples were combined. h) Samples were fractionated by off-line HPLC (basic reversed phase), dried and re-suspended in 5 % formic acid (i) and analysed by LC-MS/MS (j).

Differences between the proteomes of resting WT and TLR2 KO BMDMs show that TLR2 plays a role in cellular processing in the absence of infection. Levels of membrane and interferon-related proteins were downregulated in WT cells compared with TLR2 KO (Figure 4.6 e). Compared to WT cells treated for 15 min, KO cells had increased levels of proteins involved in RNA binding. In contrast, WT cells showed higher levels of interferon-related and transmembrane proteins (Figure 4.6). At 60 min, WT cells showed continued signs of activation compared to TLR2 KO, as the WT cells showed elevated levels of cathepsins, interferon-induced proteins and CD180 antigen, all of which are activated in response to pathogenic infection (Figure 4.6 g).

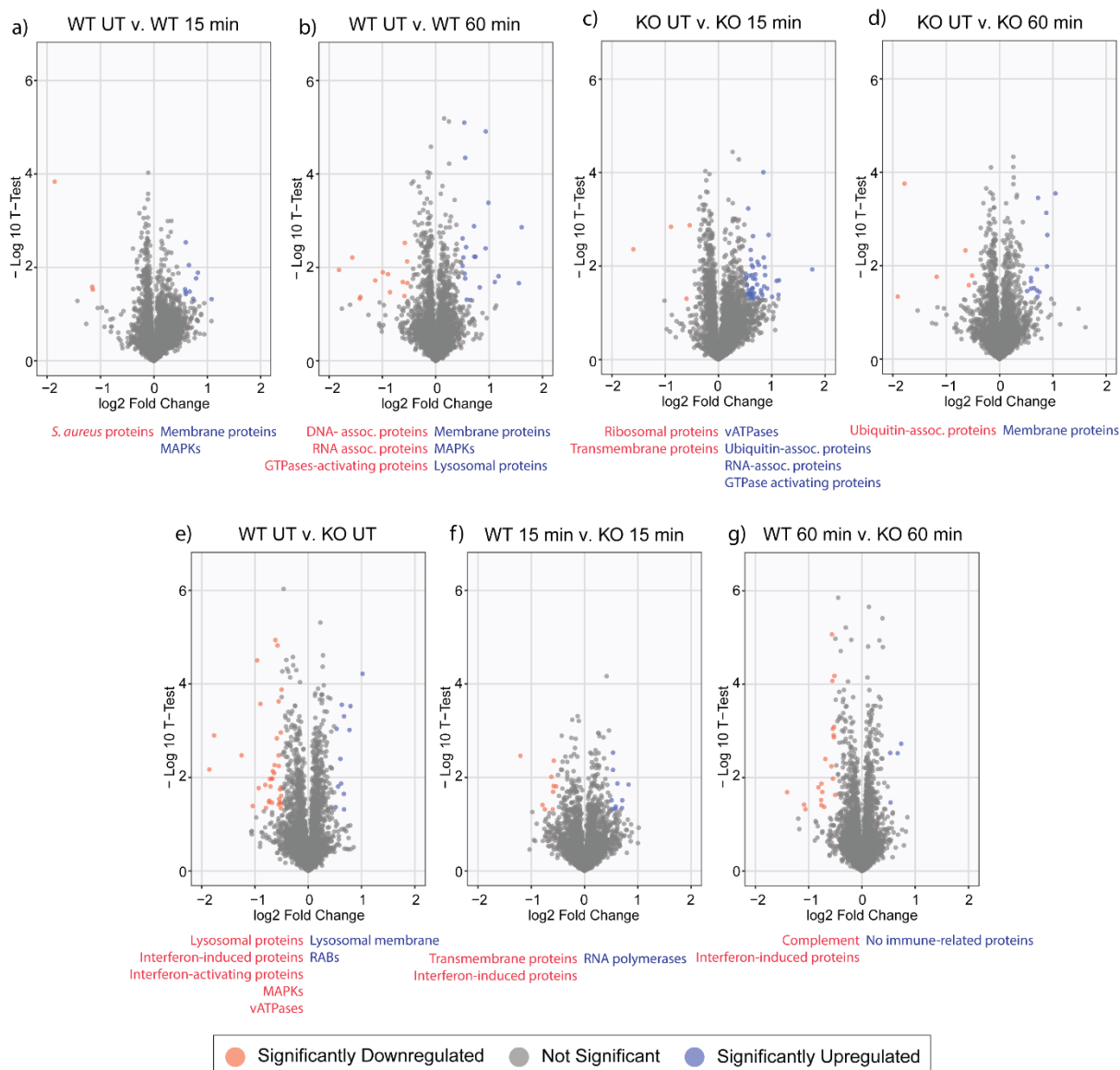


Figure 4.6: Volcano plot distribution of all phagosomal proteins extracted from BMDM cells isolated from WT or TLR2 KO mice. Cells were with *S. aureus* for 15 or 60 min or left unstimulated (UT). The x axis represents the $\log_{10}$ fold change distribution calculated in Perseus using two sample *t*-test. Cells stimulated with *S. aureus* were compare with unstimulated cells. KO cells were compared with WT cells. The x axis represents the $\log_{10}$ fold change distribution of condition1 / condition2. (a), WT 15 min compared with WT UT. (b), WT 60 min compared with WT UT (c), KO 15 min compared with KO UT (d) KO 60 min compared with KO UT. (e), KO UT compared with WT UT (f). KO 15 min compared with WT 15 min (g) KO 60 min compared with WT 60 min. The y axis represents the *p*-value across 3 biological replicates.

Hierarchal clustering (Figure 4.7 a) of the significant ANOVA FDR hits shows the differences between experimental cohorts. Large changes were observed between WT and TLR2 KO unstimulated (UT) cells. There was little difference between UT TLR2 KO cells and TLR2 KO cells stimulated for 60 min, but for TLR2 KO cells treated for 15 min groups of proteins in

clusters 1, 2 6, 7 and 8 were significantly regulated, indicating that TLR2 is active in early endosomal signalling but not late endosomal signalling. At 15 min, TLR2 KO cells and WT cells activate different groups of proteins as shown in Figure 4.7 a, Clusters 2, 7 and 8. Proteins in cluster 2 were associated with ubiquitin binding and phosphorylation.

Cluster 7 highlights differences between WT and TLR2 KO cells in early endosomal signalling. This group of proteins include RABs, Ubiquitin-related proteins, GTPases, Cathepsins, CD180, CD36 and interferon-induced proteins, which are proteins associated with the early endosome and protein transport. All were reduced at 15 min after stimulation in the TLR2 KO cells, before returning to original levels after 60 min. In contrast, levels do not appear to change at any time point in the WT cohorts., further indicating a regulatory role for TLR2. Cluster 8 contained proteins involved in innate immune signalling pathways. Taken together, this indicates that TLR2 has a regulatory role in early endosomal signalling.

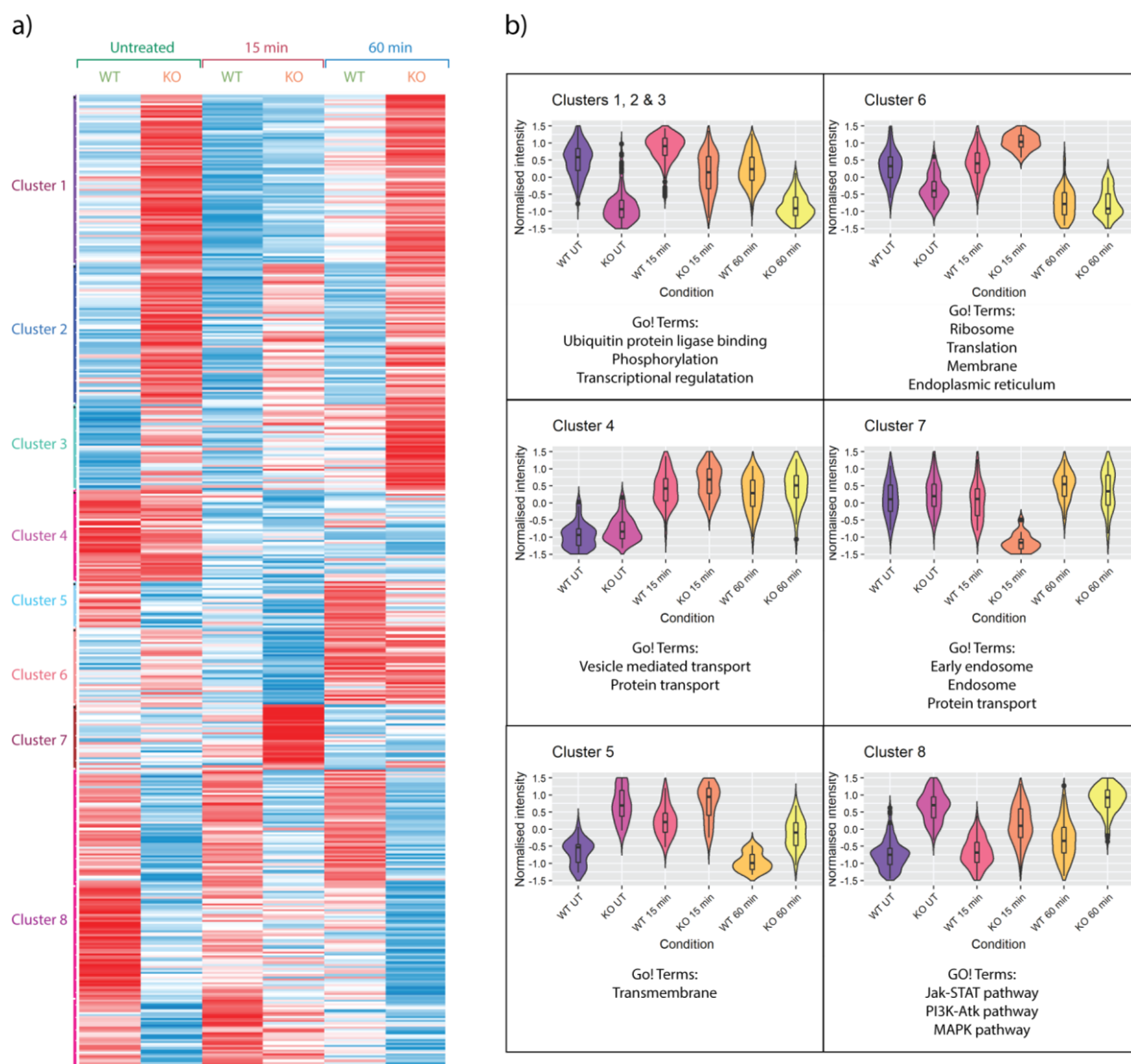


Figure 4.7: Heatmap and intensity plots showing normalised intensity for proteomes of WT and TLR2 KO BMDM cells. a) Heatmap, showing normalised intensity (z score) for proteins identified from TCL from BMDM cells stimulated with *S. aureus* for 15 or 60 min, or left unstimulated (UT). b) Cluster analysis showing fold change for each identified protein between different conditions and Go! Terms results for each cluster.

4.4. Discussion

TLR2 is a known activator of the innate immune response, but its effects on phagocytosis and endosomal maturation were previously largely unknown. A phagocytosis assay (Figure 4.1) showed that TLR2 KO BMDMs phagocytosed *S. aureus* particles at a faster rate compared with WT BMDMs, and that they also phagocytose more particles compared with the WT. This suggests that TLR2 plays a negative regulatory role in phagocytic uptake.

TLR2 is known to play a role in the response to *S. aureus* in RAW264.7 cells, by mediating phagocytosis and activating the JNK signalling pathway (Fang et al. 2014), but this had not been shown in BMDMs. However, this study by Fang *et al.* found that TLR2 knock-out RAW cells had a decreased rate of phagocytosis. Conversely, activation of TLR2 in bovine mammary epithelial cells by *S. aureus* is associated with a reduction in phagocytic uptake, a phenomenon which is linked to activation of the TLR2/P38 pathway (Alva-Murillo et al. 2015). Therefore, it is possible that this is a cell-line specific response.

Proteomics analysis of phagosomes showed that TLR2 play a role in early endosomal signalling. To more specifically target TLR2 dimers, a synthetic ligand for the TLR2/1 dimer, PAM₃CSK₄, was used. As a comparison, LPS was used to target TLR4, and to distinguish endosome-dependent signalling, BAF was used to inhibit endosome maturation. The MS analysis identified several proteins and protein groups which are involved or affected by TLR2 downstream signalling. Cathepsins, which are endosomal and lysosomal proteases and are most abundant in MΦs, play a role in inflammatory disease and cancer (Creasy and McCoy 2011). They were upregulated upon activation and increased upon treatment with BAF in BMA cells, because without endosomal maturation they could not be activated and degraded. Their upregulation upon activation was in line with their role as regulators of TLR2 and TLR4 expression (Cathepsin S), (H. Wu et al. 2016) and involvement in chronic inflammation (Cathepsin B), (Li et al. 2016). Cathepsin S and Cathepsin B upregulation suggests NALP3 inflammasome activation which induces the innate immune response (Maitra et al. 2009). Together with the results, this confirmed the activation of the cells by PAM₃CSK₄ and LPS. It also showed that without endosomal maturation, a build-up of cathepsins could contribute to increased inflammasome activation as seen in inflammatory disease. Use of BAF resulting in an upregulation of inflammatory proteins can be compared with the upregulation of cathepsins in TLR2 KO cells. This suggests that TLR2 protects against hyper-inflammatory response by limiting phagosome maturation and signalling.

Cathepsins are known to be linked with TLR2 and TLR4 signalling, and levels of Cathepsin B and Cathepsin S decrease when endosomal TLR2 signalling is inhibited by BAF (Figure 4.3). TLR4-induced cytokines also increase the proteolytic activity of cathepsins, suggesting a feedback loop between TLR activation and cathepsin upregulation. Induction of TNF α , IFN β 1 and IL- β 1 by cathepsins, which in turn further increases cathepsin upregulation, can result in hyper-inflammation and auto-immune disease without proper regulation (Creasy and McCoy

2011). Therefore, the observed upregulation of cathepsin activity when BAF was used to inhibit endosomal maturation suggests that cathepsin levels were controlled by endosomal maturation. After activation, which occurs at an acidic pH (around pH 5.5), cathepsins are degraded, preventing further excessive activity in downstream signalling processes. It is also known that cytokines which are induced during downstream processes from endosomal TLRs regulate cathepsin activity. Without endosomal TLR activation, this regulatory effect does not exist, causing upregulation of the type that might contribute to auto-immune disease (Creasy and McCoy 2011; Tsukuba et al. 2006).

The data showed an increase in cathepsins O, B, D & L1 in BMA cells treated with PAM₃CSK₄, compared with cells treated with inert beads. It is possible that the increase in cathepsins in PAM₃CSK₄ -stimulated cells is a consequence of TLR2 activation of the pro-inflammatory signalling pathway from the PM. Cathepsins are also increased in cells all stimulated samples treated with BAF, compared with the stimulated but un-treated samples. This is consistent with the observations of Shafer & Onucka, (1989).

It may appear strange that cathepsins were upregulated in the presence of BAF, which prevents the acidification of the endosome on which cathepsin activity depends. However, BAF specifically blocks fusion of the late endosome to the lysosome, which occurs at pH 4.5- 5.5. Cathepsins are mostly active at this pH range, but some can be active at a pH as high as 7. Without the transition of the late endosome to the lysosome, cathepsins cannot be fully activated and degraded. Without normal acidification and degradation, cathepsins accumulated at the endosome and were not able to catalyse the activation of TLRs 3, 7 and 9. It is therefore interesting that cathepsins also accumulated upon TLR2/1 activation with PAM₃CSK₄, but not upon treatment with LPS (a TLR4 agonist). This suggests that TLR2, but not TLR4, plays a role in the negative regulation of endosome maturation.

Proteome changes induced by the use of BAF included MSR I & II, Integrin β -1, RABs 14, 32 & 7 and CD81 antigen. In this dataset, these proteins were induced by stimulation with PAM₃CSK₄ or LPS but significantly reduced by BAF treatment, indicating that their activity is enhanced by endosomal maturation and signalling. Integrin β -1 is regulated by TLR activation and is associated with cell motility and enhance pro-inflammatory cytokine production, two functions which aid the M Φ in finding and fighting pathogens (B. Huang et al. 2008). Inhibition of endosome maturation by BAF would not affect the internalisation of integrins at the PM but integrins would not undergo normal trafficking and signalling at the endosome and would not

be regulated by endosomal TLRs. CD81 activates the MAP kinases and is associated with β -integrin activity. Its presence shows activation of the pro-inflammatory pathway, and its downregulation in the presence of BAF could be due to the decrease in integrin activity, which in turn regulates CD81. It is important to remember, however, that the use of BAF will affect receptor recycling as endosomal processes will be inhibited when the endosome cannot mature. RABs are late endosomal markers and play a role in phagosome maturation, and their downregulation upon BAF treatment fits with their role as endosomal signalling molecules.

PAM₃CSK₄ and LPS are synthetic ligands used to specifically target one TLR dimer. However, to measure a realistic and complex response, *S. aureus* (a natural TLR2 ligand) was used to stimulate TLR2 in BMDM cells harvested from WT and TLR2 KO mice. The TCL was analysed by TMT LC-MS3.

BMDM cells treated with *S. aureus* for 15 and 60 min showed differences between the proteins of TLR2 KO and WT cells at both time points. The experiment was designed to capture early and late responses to stimuli and showed that WT and TLR2 KO cells have different response rates when challenged with bacteria.

TLR2 KO cells responded faster to stimulation, with a faster rate of particle uptake. Although at 15 min, both cohorts showed activation of the pro-inflammatory pathways, TLR2 KO cells revealed protein changes which suggested a more mature response. These include regulation of E3 ligases, such as Ltn1, which are required for ubiquitylation, vATPases, which suggest a maturing, more acidic phagosome and RNA-binding proteins which suggest transcription.

At 15 min stimulation, levels of CD166 and CD81 antigens, Ubiquitin-associated proteins, ribosomal proteins and RABs increased in the TLR2 KO cells and WT cohorts. Together, this shows that in TLR2 KO cells, the response to *S. aureus* was in progress at 15 min after stimulation, but the response was largely completed by 60 min. In contrast, WT cells appeared to possess more endosomal signalling proteins at the basal level but their upregulation upon stimulation is less pronounced and the overall response was slower.

At 15 minutes stimulation, a number of proteins were upregulated in KO cells compared to WT. Amongst these proteins was RAB5A, which has a role in phagosome maturation by regulating transport vesicle formation. It is required for the fusion of the plasma membrane with the early endosome and regulates the maturation of apoptotic cell-containing phagosomes (Kitano et al. 2008, 5). RAB5A is a GTPase which cycles between its active GTP-bound form and its inactive GDP bound form. It has been shown that increased RAB5A expression directly correlates with

the accelerated maturation of phagosomes containing *Listeria monocytogenes* (Alvarez-Dominguez and Stahl 1999). At 15 min, RAB5A transcription levels are unlikely to be affected – therefore this change may have been the result of a lack of specific degradation in the absence of TLR2.

Lysozyme was decreased at 15 min, but levels recovered at 60 min in both WT and KO cells, although levels in KO cells were higher. Lysozyme is essential for the degradation of ingested pathogens, and its expression has been linked to levels of TLR2. Deng *et al.* (2012) found that overexpression of TLR2 resulted in elevated levels of lysozyme, and Kluter *et al.* (2014) found that lysozyme levels were increased in cells stimulated with *S. aureus* compared with cells where TLR2 receptors had been blocked using an inhibitor. This is further evidence that TLR2 has an inhibitory role in pathogen degradation.

Cathepsin D plays a role in the breakdown of proteins in an acidic environment and helps to break down ingested pathogens. Increase in Cathepsin D levels correlate to a mature, active phagosome and increased pathogen degradation. The presence of ubiquitin-activating enzymes in TLR2 KO cells suggests ubiquitylation and signalling from the phagosome, and its upregulation in the absence of TLR2 is consistent with the hypothesis of TLR2 being a negative regulator of phagosome signalling.

At 60 min, WT cells showed a more pronounced upregulation of membrane proteins, suggesting ongoing signalling, compared with TLR2 KO cells, which showed lower proteome change at 60 min. Less change in the KO compared to the WT samples supports the hypothesis of a faster, shorter response in the KO compared with the WT, which is not sustained to 60 min. This suggests that by 60 min, the response in the TLR2 KO cells was largely completed.

Overall, the results suggest that, at 15 min, TLR2 KO cells show a faster progression in immune signalling, supporting the hypothesised role for TLR2 as a regulator of phagosomal signalling.

There has already been some controversy surrounding the role of TLR2 and TLR4 in phagosome maturation. Previous have found that absence of TLR2 results in a decrease in phagocytosis, but these studies were conducted in other cell lines (Fang *et al.* 2014; Assinger *et al.* 2011). Blander and Medzhitov (2004) published data showing that TLR2 and TLR4 had a positive impact on the rate of phagosome maturation and concluded that the mechanism could be MyD88-dependent activation of p38 downstream of TLR2 activation. They followed up their study with several papers confirming their results (Blander and Medzhitov 2006b; 2006a; Blander 2007) These conclusions were directly disputed by Russell and Yates, who used three

types of assays to measure phagosome maturation, phagosome/lysosome fusion and lysosomal degradation. They concluded that phagosome maturation proceeds independently of TLRs (Russell and Yates 2007; Yates and Russell 2005).

Both results conflict with these findings that TLR2 KO cells have an increased rate of phagocytosis and degradation. The most likely explanation for the discrepancy between these data and their work is differences in experimental design. Blander and Medzhitov used FACS and microscopy as their readouts. FACS was used to measure the rate of phagocytosis of fluorescent *E. coli* and inert microspheres in BMDMs isolated from WT, MyD88 KO and TLR2x4 KO mice (the latter being the heterogenous offspring of TLR2 KO and TLR4 KO mice).

Microscopy was used to measure the kinetics of phagosome maturation, determined by the engagement of TLRs. Their methods can be called into question on a number of points: Firstly, by using TLR2x4 heterogenous mice, they cannot specifically measure for TLR2 or TLR4 activity. Secondly, the use of inert microspheres as a control for live fluorescent *E. coli* may induce changes in phagocytosis which are a result of the particle being ingested, rather than due to the TLR activity. In these experiments, WT and TLR2 KO BMDMs were used, which can be directly compared for TLR2 activity. *S. aureus*-treated cells were also compared with untreated cells as a control, to compare the proteome of activated and inactivated TLR2. When microspheres were used to stimulate the cells, inert and coated microspheres were compared, which provides a better control/treated experimental setup compared with inert microspheres/bacteria.

The fluorescent assay used to measure phagosomal degradation in this study (Figure 4.1) is a modified version of that developed by Russell and Yates (Jin et al. 2007; Farhat, Riekenberg, Heine, Debarry, Lang, Mages, Buwitt-Beckmann, et al. 2008; Oosting et al. 2011; Triantafilou et al. 2006) In these experiments, fluorescent *S. aureus* was used to stimulate WT and TLR2 KO BMDMs. Stimulated cells without treatment were compared to stimulated cells treated with inhibitors for phagocytic uptake and phagosome maturation.

Russell and Yates used microspheres covered in antibodies which bind fluorescent particles to stimulate their cells. Once the beads are in the phago-lysosome, the outer fluorophore is degraded, revealing the inner fluorophore which is measured as a readout for degradation. A concern raised by Blander and Medzhitov concerning the use of these beads was that the antibody coating could initiate opsonin-dependent phagocytosis. TLRs are opsonin-

independent PRRs. Therefore, the addition of opsonin-dependent phagocytosis could ‘mask’ the differences observed between the WT and TLR2 KO BMDMs.

Aside from the assays and Western blots confirming the KO cells, Russell and Yates did not provide any other evidence for their claim that phagosome maturation is independent of TLRs. In my experiments, serum-free media was used for phagocytosis, removing the effect of opsonin-dependent phagocytosis but also reducing the overall rate of phagocytosis, for all experimental conditions (D. L. Gordon and Rice 1988).

Data from both of these studies cannot be directly compared to the studies using BMDMs. The proteomics approach used here provides a sea of complex information which cannot be observed by microscopy, FACS or fluorescence/absorbance assays. The aim was not to directly measure the rate of phagocytosis or phago-lysosomal degradation, but to map the changing proteome after TLR2 stimulation. Therefore, it may be more productive to focus on the protein groups that are affected downstream of TLR2, rather than make concrete claims regarding the effect of TLR2 on phagocytosis and phagosome maturation.

Overall, these data suggest that TLR2 initiates an immune response, but also has a negative regulatory role in phagocytosis and endosomal signalling. The negative regulatory aspect of TLR2 activity may serve to limit induction of the pro-inflammatory phenotype, which aids in the prevention of auto-immune diseases. Therefore, TLR2 could be self-regulatory, with signalling mechanisms in place to limit the feedback signalling loop and control the induction of the innate immune system. In light of contradictory data from other researchers, validation of proteomics data by biochemical techniques such as IP, qRT-PCR, Western blot, FACS or microscopy is required for a clearer picture of the role of TLR2 in *S. aureus* infection.

5. Chapter 5: Type I IFN is induced downstream of endosomal TLR2

5.1. Introduction

Toll-like receptor 2 (TLR2) is a PRR which is well characterised as an inducer of the pro-inflammatory innate immune response upon detection of gram-positive bacteria. Upon activation, it forms a heterodimer with either TLR1 or TLR6 (Jin et al. 2007; Farhat, Riekenberg, Heine, Debarry, Lang, Mages, Buwitt-Beckmann, et al. 2008; Oosting et al. 2011; Triantafilou et al. 2006). Once the dimer is formed, MyD88 recruits TIRAP and binds to the intracellular TIR domain of TLR2/1/6 via its death domain. TRAF6 is recruited with IRAK1/2 and IRAK4 (Keating et al. 2007) and phosphorylated, activating downstream signalling via Ikka/Ikkb, Ikb α , NF- κ B and eventually the induction of pro-inflammatory cytokines (Ozinsky et al. 2000; Kobayashi, Walsh, and Choi 2004; Matsumura et al. 2003).

Previous literature has reported differently with regards to TLR2 signalling from the endosome, and downstream activation of the type I IFN pathway. Work by Barbalat *et al.* (2009) showed that TLR2 also induces the type I IFN response, but only when stimulated with viral ligands, and only in monocytes. Although the study by Barbalat *et al.* (2009) could only find that TLR2 can induce the type I IFN response when stimulated with viral ligands, a study by Dietrich *et al.* (2010) successfully demonstrated induction of type I IFNs by TLR2 in response to bacterial ligands. However, the downstream signalling mechanism from endosomal TLR2 remains unclear.

In this chapter, a new method of TLR2 stimulation was used, where latex beads coated with TLR2 ligand are phagocytosed by cells. Inhibitors were used to block endocytosis as a control. Using this experimental setup, it was shown that induction of type I IFN results from activation of TLR2 and that this process is dependent on endosomal maturation. The downstream signalling of endosomal TLR2 was then investigated, revealing that endosomal TLR2 induces type I IFN pathway via IRF1 and IRF7, and that downstream endosomal TLR2 signalling is influenced by the process of phagocytosis.

5.2. Aims

1. *Define TLR2-dependent type I IFN induction in response to bacterial ligands*

The current discrepancies in the present TLR2 literature were addressed by establishing that TLR2 does initiate the type I IFN pathway from the endosomal compartment in response to bacterial ligands.

2. *Define novel regulators of TLR2-dependent type I IFN induction*

The downstream signalling pathway from endosomal TLR2 was unknown. To investigate downstream signalling, distinct TLR2 signalling complexes from phagosomes were analysed using 1 µm carboxylated beads coated with ligands for TLR2 and TLR4. Using the inhibitor BAF to block endosomal signalling gained insight into the molecular composition of pro-inflammatory (BAF treated) and type I IFN (BAF non-treated) TLR2 complexes.

5.3. Results

5.3.1. *TLR2 initiates type I IFN pathway from the endosome in response to bacterial ligands*

To identify whether endosomal TLR2 initiates a type I IFN response, two inhibitors were used, BAF and Dynasore. BAF is an inhibitor of an endosomal vATPase, which prevents the acidification and maturation of the endosome thus resulting in a complete eradication of endosomal signalling. Dynasore is an inhibitor of dynamin, a GTPase that interacts with actin and the cytoskeleton at the plasma membrane. Addition of Dynasore prevents endocytosis of plasma membrane receptors, which is an essential process for the initiation of signalling pathways. Specific ligands for TLR2/1 and TLR2/6 dimers were used (Figure 5.1). BMDMs were stimulated with the TLR2/1 ligand PAM₃CSK₄ for 3 h and analysed for induction of pro-inflammatory response (Tnfα) and type I IFN response (Ifnb1, Cxcl10) (Figure 5.2). EZQ assay was used for quantitation (Section 2.3.5). The inhibition of dynamin by Dynasore caused a reduction in the transcription of Tnfα, Ifnb1 and Cxcl10. This indicated an abolition of signalling from the plasma membrane and from the endosome, and of a lack of initiation of both the pro-inflammatory and the type I IFN immune responses.

BAF addition reduced the transcription of Ifnb1 and of Cxcl10, which suggested that the initiation of the type I IFN by TLR2 was dependent of the maturation of the endosome. Unlike

type I IFN response, transcription of $Tnf\alpha$ was significantly increased in response to BAF treatment, indicating that the initiation of the pro-inflammatory response from TLR2 was not dependent on endosomal maturation. Moreover, enhanced gene expression of $Tnf\alpha$ upon BAF treatment indicated that the signalling was sustained and therefore suggests that in the absence of endosomal maturation, pro-inflammatory signalling is prolonged. These results suggested that TLR2 initiates the pro-inflammatory response from the plasma membrane and the type I IFN response from the endosome (Figure 5.2).

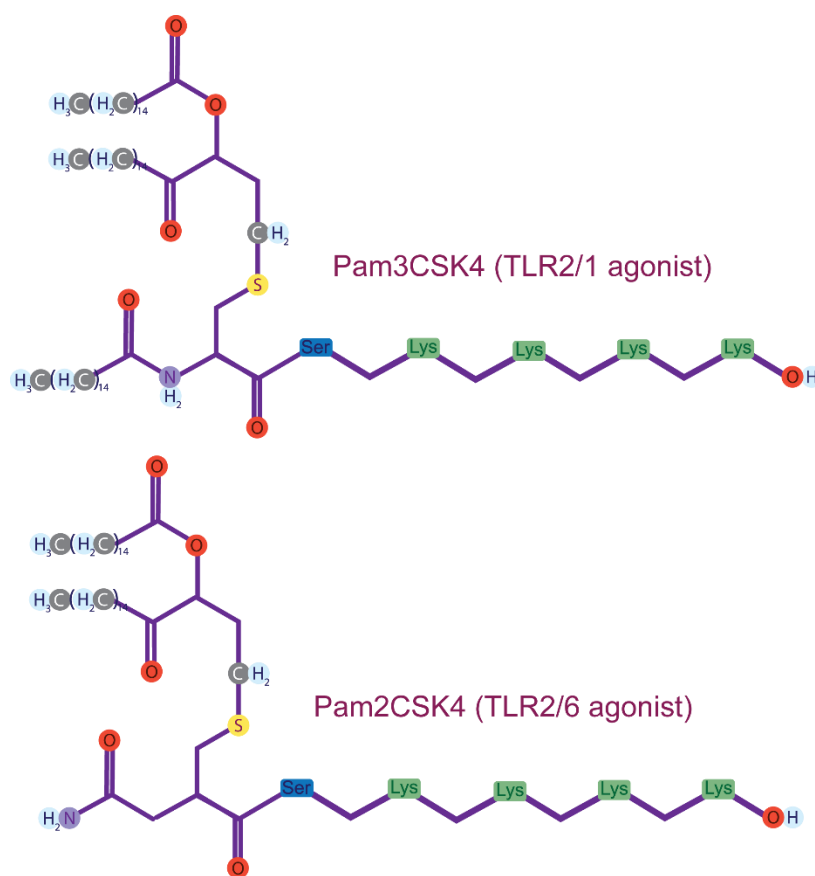


Figure 5.1: Structure of PAM₂CSK₄ and PAM₃CSK₄.

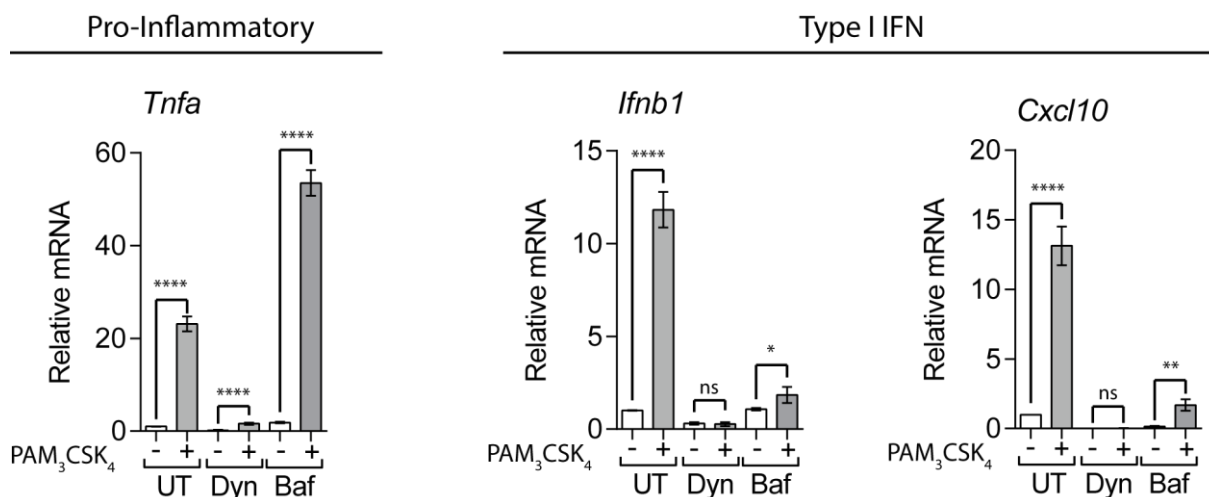


Figure 5.2: qRT-PCR data showing pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*, *Cxcl10*) mRNA levels of BMDMs. Cells stimulated with 0.1 μ g/ml BAF or 1 μ g/ml Dynasore for 1 hr prior to treatment with 10 μ g/ml PAM₃CSK₄ for 3 h. Three replicates were used per condition, error bars were calculated using standard deviation. Asterisk indicate *p*-value significance: **** indicates *P*-value of ≤ 0.0001 , *** indicates *P*-value of ≤ 0.001 , ** indicates *P*-value of ≤ 0.01 and * indicates *P*-value of ≤ 0.05 . ns indicates non-significance (Student's *T*-test).

5.3.2. Optimisation of TLR2 stimulation by ligand-coated carboxylated beads

Latex beads mimic bacteria by inducing phagocytosis (soluble ligand is too small are only endocytosed). The beads also aid endosomal TLR stimulation. They are densely coated with ligand, which comes into direct contact with endosomal TLRs once the bead is inside the phagosome. Beads were therefore used to ensure a higher concentration of ligand was present within the phagosome to induce a stronger signalling response. Carboxylated latex beads (1 μ m) were used. The beads were phagocytosed by the cells and could be extracted from cell lysis by ultracentrifugation to yield highly enriched phagosomes as described in Section 2.6.2 To ensure that endosomal receptors are activated from inside the phagosome, the beads were coated with TLR2 ligands (Figure 5.3).

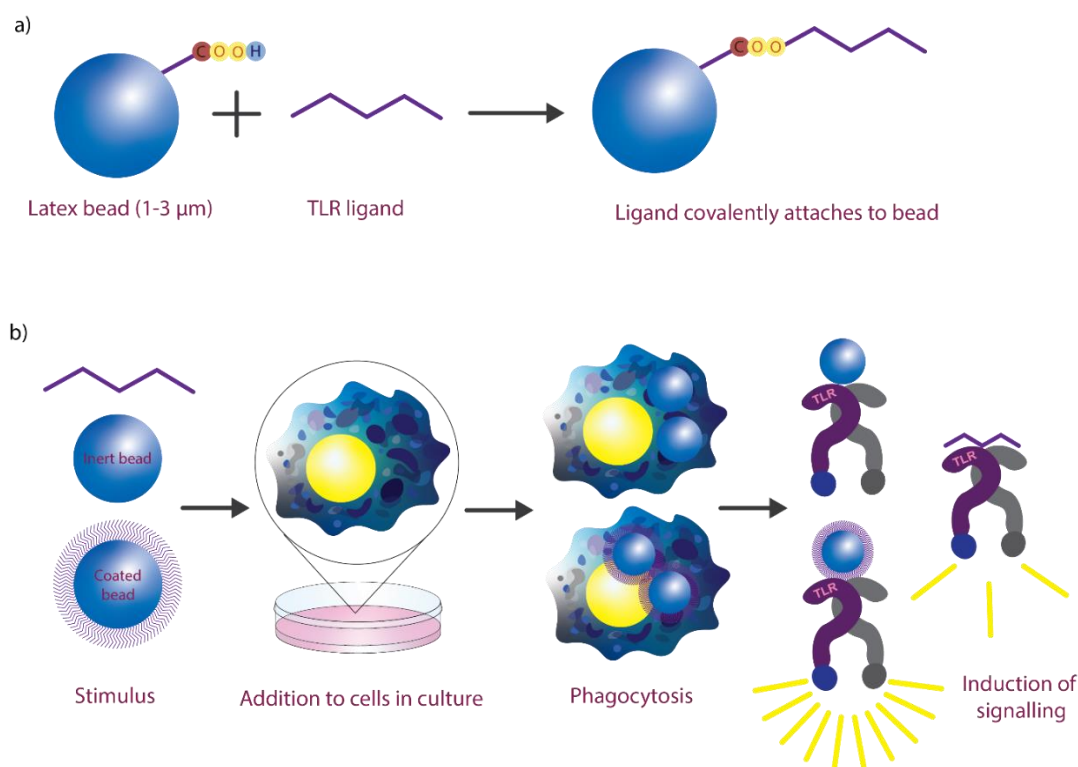


Figure 5.3: Activation of TLRs using carboxylated latex beads. a) Latex beads come in two sizes, 1 μm and 3 μm , and can covalently bind to TLR ligands via a carboxyl group attached to the surface of the bead. b) Scheme showing the advantages of using carboxylated beads to induce phagocytosis and deliver higher quantities of ligand to the endosome compared to soluble ligand.

It was necessary to first confirm that an innate immune response can be initiated using PAM₃CSK₄ (TL2 ligand)-coated carboxylated beads. RAW-BLUE cells were used, which are derivatives of the murine M Φ -like cell line RAW 264.7. RAW-BLUE cells produce a secreted embryonic alkaline phosphatase (SEAP) in response to activation of the NF- κ B pathway. SEAP can be detected and quantified by QUANTI-Blue cell culture medium that changes colour depending on levels of secreted alkaline phosphatase (SEAP) secreted by the cells (Lewis et al. 2014). The results (Figure 5.4 a) show that there is a strong NF- κ B (pro-inflammatory) response when cells are incubated with PAM₃CSK₄ coated carboxylated beads as opposed to uncoated carboxylated beads. This demonstrated that there was no induction of the innate immune system in response to the beads themselves.

Once it had been established that the pro-inflammatory pathway was initiated downstream of TLR2, the conditions were optimised for the observation of the induction of type I IFN from TLR2. Two types of stimulation were used to induce an inflammatory response- the addition of a soluble ligand to the cell media or the addition to latex beads coated with the ligand to the media. A comparison of soluble ligand to ligand-coated beads is shown in Figure 5.4 b. The

Western blot bands indicating presence of p-TBK1 (type I IFN pathway) and degradation of I κ B α (indicative of induction of the pro-inflammatory response) are more pronounced when beads are used compared to soluble ligand.

The concentration of the beads was optimised (Figure 5.4 c) with 1 μ g beads in 10 mL PBS or cell culture medium being the optimal concentration for activation of both the pro-inflammatory and the type I IFN response.

The pro-inflammatory and type I IFN pathways were measured in BMDMs in response to soluble ligand and ligand-coated beads (Figure 5.4 d). These data show that for Tnf α and Cxcl10, the pro-inflammatory and type I IFN responses, respectively, are stronger when BMDMs are stimulated using ligand-coated beads.

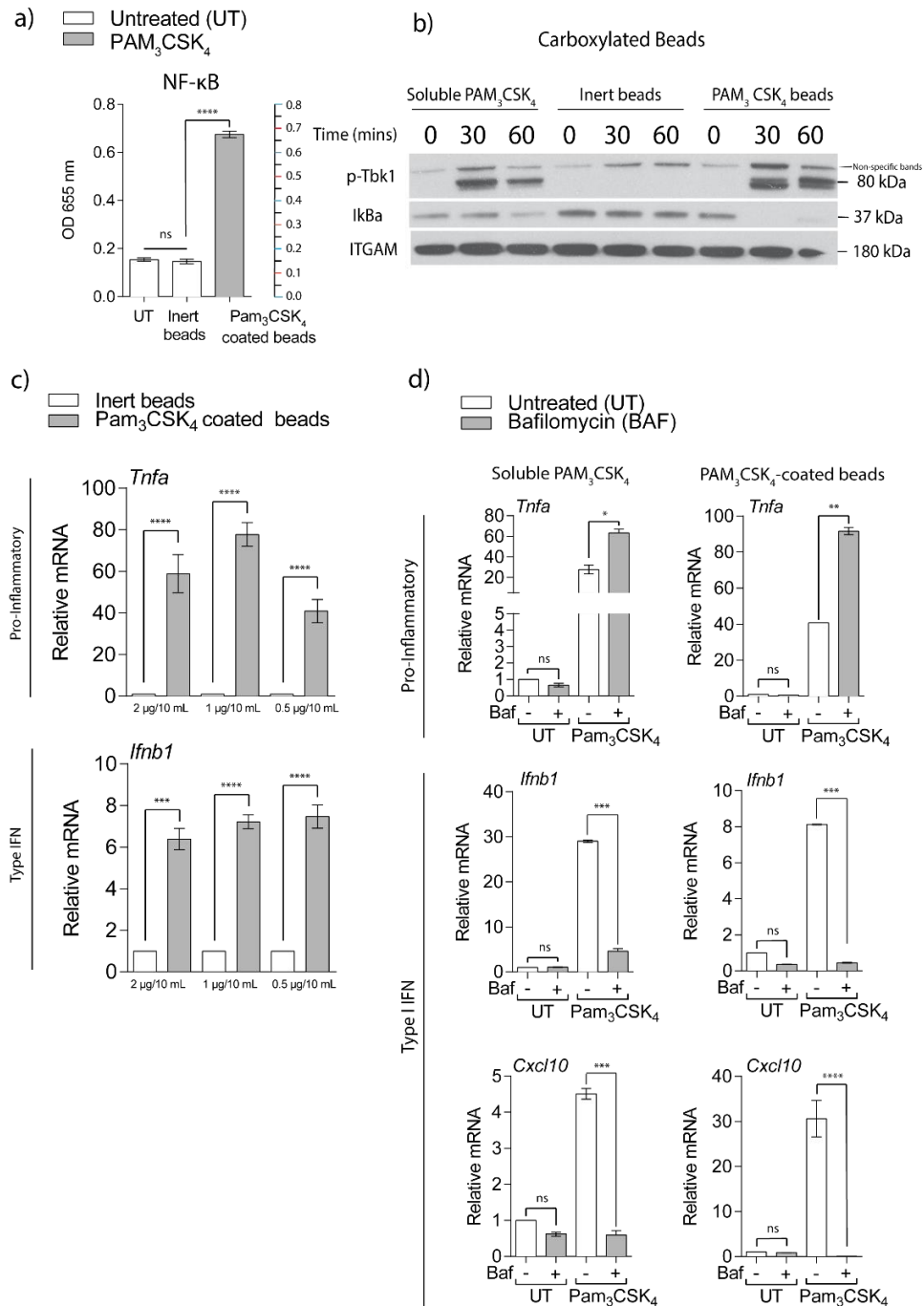


Figure 5.4: Optimisation of BMA stimulation using latex beads. a) NF-κB activation plotted as SEAP quantitation in RAW-BLUE cells b) Western blots showing pro-inflammatory (IκBa) and type I IFN (p-TBK1) activation in response to soluble PAM₃CSK₄, inert and PAM₃CSK₄ coated carboxylated beads in BMDMs c) qRT-PCR data showing pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*) activation in response to inert and PAM₃CSK₄ coated carboxylated beads in BMDMs (2 μg beads, 1 μg beads and 0.5 μg beads in 10 mL PBS, respectively) d) qRT-PCR data showing pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*, *Cxcl10*) activation in response to soluble PAM₃CSK₄, inert and PAM₃CSK₄-coated carboxylated beads in BMDMs. Each graph represents three biological replicates. Asterix indicate p-value significance: **** indicates P-value of ≤ 0.0001 , *** indicates P-value of ≤ 0.001 , ** indicates P-value of ≤ 0.01 and * indicates P-value of ≤ 0.05 . ns indicates non-significance (Student's T-test).

5.3.3. *Comparison of cell lines*

BMDMs require seven days to fully differentiate, and several mice have to be sacrificed in order to gain enough cells for phagosome isolation. Three immortalised MΦ cell lines were tested to determine whether it could produce a comparable response to BMDMs with TLR2 ligands (Figure 5.5). First, the phagocytic ability of BMDM, BMA (a murine MΦ cell line from C57BL/6 mice), J774.A1 (a murine monocyte cell line from BALB/C mice purchased from ATCC) and RAW264.7 cells were compared for phagocytic ability by green fluorescence assay. This was used to compare the phagocytosis rate of each cell line (Figure 5.5 a). The results showed that the RAW264.7 and J774.A1 cell lines produced a lower response (at least 50 RFU lower) compared with the BMDMs, the BMA and BMDM cells showed no significant difference in their rate of phagocytosis. It was therefore decided to continue with further work using BMA cells and BMDMs.

To test the pro-inflammatory and type I IFN responses of BMDMs and BMAs to PAM₃CSK₄, qRT-PCR was used to measure the response of each cell line with read-outs of Tnfα for the pro-inflammatory response and Ifnb1 and Cxcl10 for the type I IFN response. The results (Figure 5.5 b) showed that both BMDMs and BMAs produce both comparable immune responses when stimulated with PAM₃CSK₄, and that BMDMs react similarly to PAM₂CSK₄ and to PAM₃CSK₄.

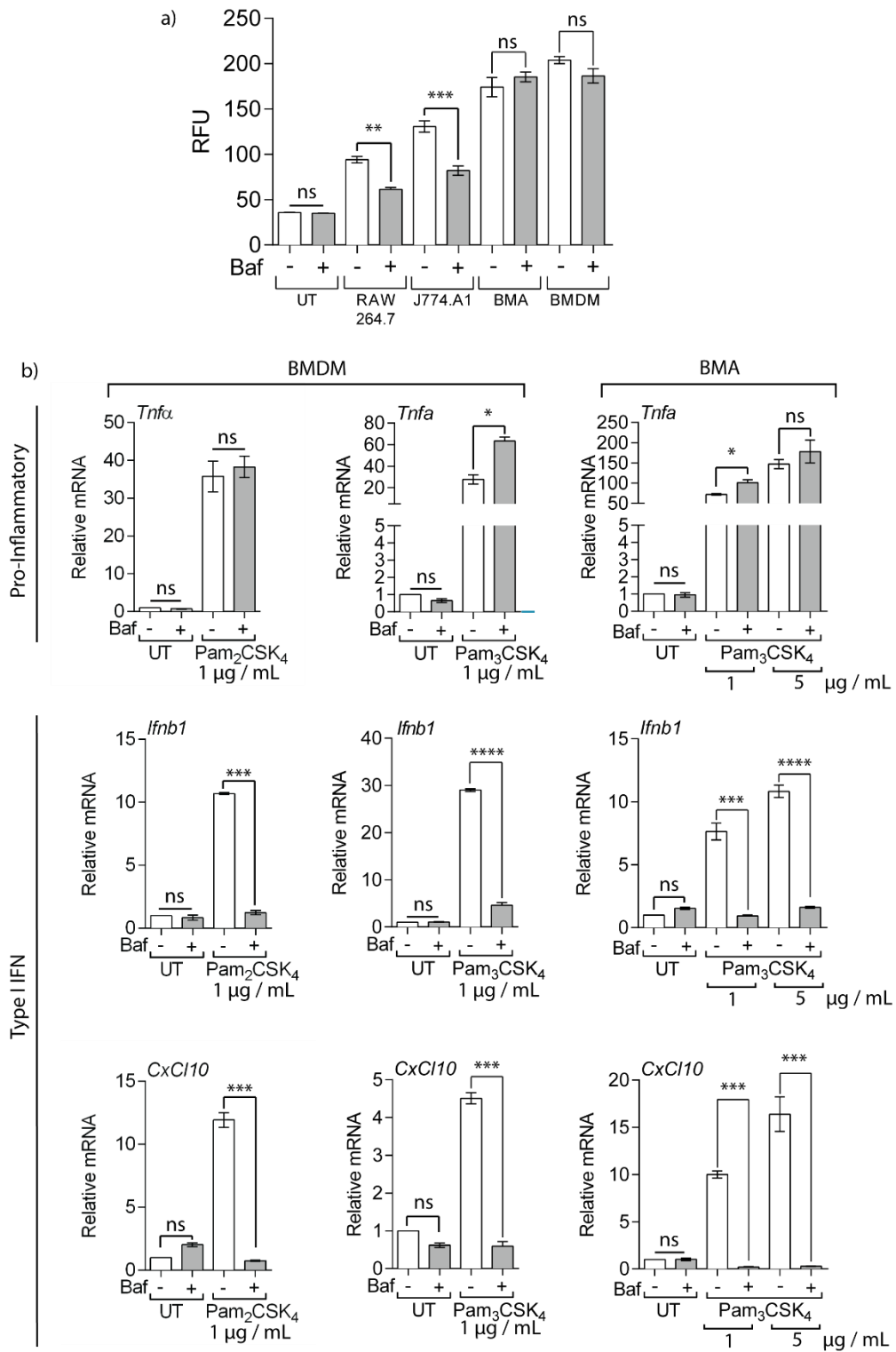


Figure 5.5: Type I IFN response induced by TLR2 activation. a) Uptake assay showing rates for phagocytosis of AF488 coated beads for three cell lines and BMDMs b) qRT-PCR data showing the pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*, *Cxcl10*) immune responses in response to PAM₂CSK₄ and PAM₃CSK₄ in BMDMs and BMAs. Each graph represents three biological replicates. Asterix indicate *p*-value significance: **** indicates *P*-value of ≤ 0.0001 , *** indicates *P*-value of ≤ 0.001 , ** indicates *P*-value of ≤ 0.01 and * indicates *P*-value of ≤ 0.05 . ns indicates non-significance (Student's *T*-test).

5.3.4. *Signalling complexes utilised by activated endosomal TLR2*

The next aim was to compare signalling complexes utilised by endosomal TLR2 by stimulating BMAs and BMDMs. Soluble ligand and ligand-coated beads were used to determine whether endosomal TLR2 signals through IRF1, IRF3 (like endosomal TLR4 and TLR3) or IRF7 (like TLR9 and TLR7/8 complex). To do this, BMDMs and BMAs were incubated with soluble PAM₃CSK₄ for 3 h. Half of the cells were pre-treated for 1hr with BAF. Quantitative RT-PCR was used to measure the transcription of IRF1, IRF3 and IRF7 (Figure 5.6 a). The preliminary results showed that when TLR2 was stimulated with a soluble ligand, there was no significant change in IRF3 transcription between the treated and untreated cells. These data suggest that IRF3 is unaffected by stimulated TLR2 and that TLR2 does not signal through IRF3. There was a significant increase in IRF1 when stimulated with PAM₃CSK₄ in BMA. Interestingly, IRF7 was significantly increased in cells stimulated with soluble PAM₃CSK₄ and significantly decreased with the addition of BAF. These data indicate that endosomal TLR2 is signalling through IRF7. In contrast, when using beads coated with PAM₃CSK₄ and PAM₂CSK₄, (Figure 5.6 b) this effect was reversed. IRF1 was induced by PAM₃CSK₄ and PAM₂CSK₄. Levels were significantly reduced when treated with BAF, whereas IRF7 was unaffected when TLR2 is stimulated by PAM₂CSK₄.

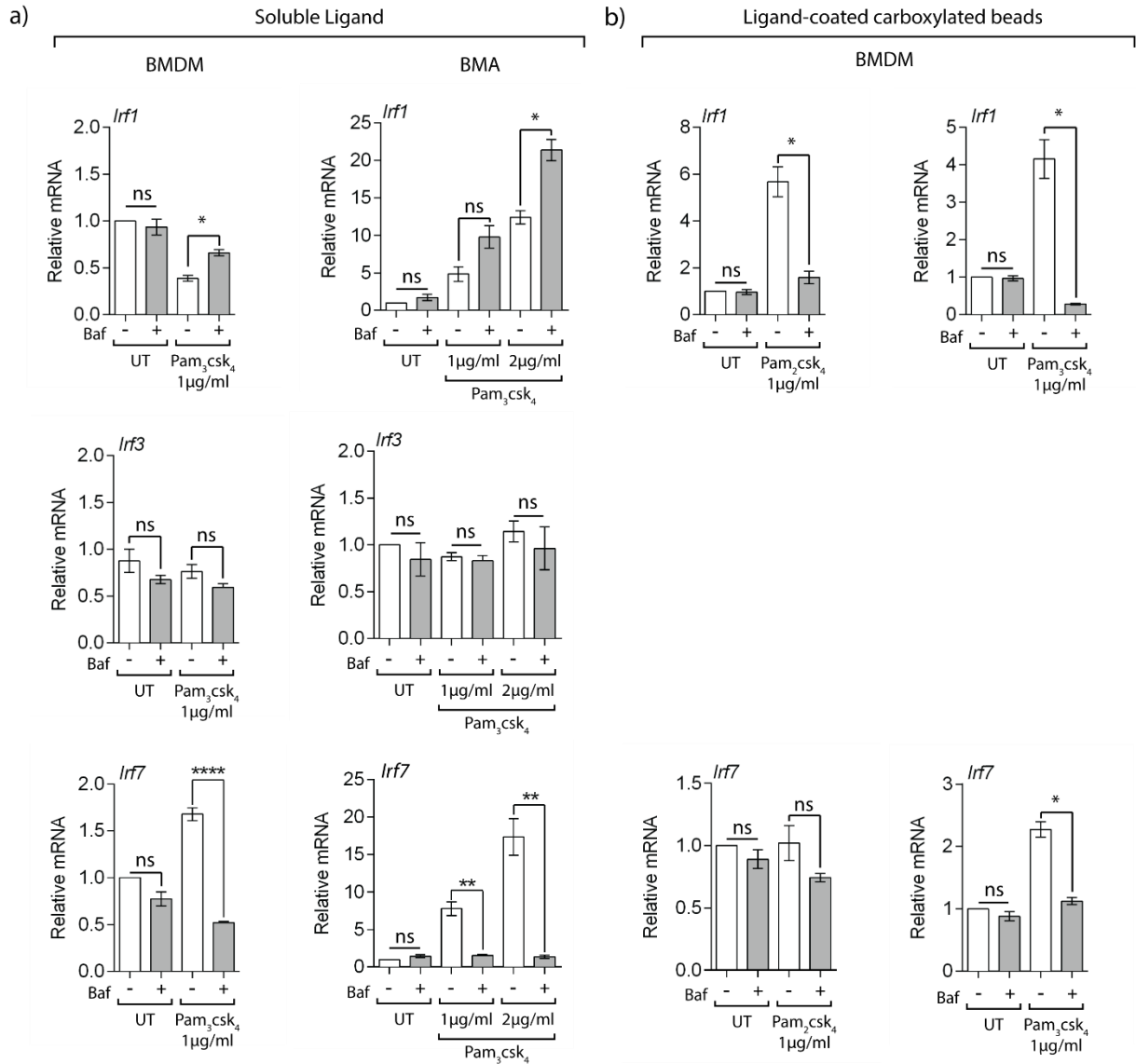


Figure 5.6: IRF activation downstream of activated TLR2. a) qRT-PCR data showing the pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*, *Cxcl10*) immune responses in response to soluble PAM₂CSK₄/ PAM₃CSK₄ in BMAs and BMDMs. b) qRT-PCR data showing the pro-inflammatory (*Tnfa*) and type I IFN (*Ifnb1*, *Cxcl10*) immune responses in response to PAM₂CSK₄/ PAM₃CSK₄ - coated beads in BMDMs. Each graph represents three biological replicates. Asterix indicate p-value significance: **** indicates P-value of ≤ 0.0001 , *** indicates P-value of ≤ 0.001 , ** indicates P-value of ≤ 0.01 and * indicates P-value of ≤ 0.05 . ns indicates non-significance (Student's T-test).

5.4. Discussion

The first aim was to establish whether TLR2 initiates the type I IFN pathway from the endosome in response to bacterial ligands. In the first study to identify endosomal TLR2 signalling, Barbalat *et al* (2009) tested monocytes, pDCs and MΦs by measuring induction of the pro-inflammatory and type I IFN pathways in response to a viral TLR2 agonist (Vaccina virus) and a bacterial TLR2 agonist (CpG oligonucleotides). They found that TLR2 induces the production of type I IFNs, but only in monocytes, and only in response to the vaccina virus. One explanation as to why Barbalat *et al* (2009) did not observe the type I IFN response in cells stimulated by the bacterial ligand is that CpG oligonucleotides are recognised by TLR9 on the endosome. Therefore, the lack of specificity for TLR2 could be responsible for the lack of type I IFN response from TLR2 in MΦs when stimulated with CpG.

In the results presented in this chapter, TLR2 is shown to activate type I IFN induction in response to the bacterial ligands PAM₃CSK₄ and PAM₂CSK₄. PAM₂CSK₄ and PAM₃CSK₄ are highly specific ligands for TLR2/6 and TLR2/1 dimers, respectively. These results were corroborated by Dietrich *et al.* (2010) who was also able to demonstrate initiation of the type I IFN response in response to TLR2 activation in MΦs in response to PAM₃CSK₄ and PAM₂CSK₄. Furthermore, Dietrich *et al.* (2010) concluded that TLR2 activates the pro-inflammatory cytokine pathway from the plasma membrane and induction of type I IFNs from the endosomal compartment.

After demonstrating that endosomal TLR2 initiates the type I IFN pathway in response to bacterial ligands, the next aim was to probe the downstream signalling mechanisms from endosomal TLR2, specifically whether the type I IFN pathway is activated via IRF1, IRF3 or IRF7. Whilst there are some studies that claim that endosomal TLR2 signals via IRF1, many more have claimed a larger role for IRF3 or IRF7 (Sweet *et al.* 2007; Bozza *et al.* 2006; Honda *et al.* 2005; Zhong *et al.* 2015). Dietrich *et al.* (2010) proposed that IRF1 and IRF7 are activated downstream of activated endosomal TLR2, whereas Barbalat *et al.* (2009) demonstrated that endosomal TLR2 induced type I IFN via IRF3 and IRF7 and found no involvement of IRF1. However, there were some limitations in the study, namely that the bioassays (qRT-PCR, FACS) used to identify type I IFNs were performed after a 24 hr stimulation, which is now thought to be far past the optimal time to identify a full response (Petnicki-Ocwieja *et al.* 2013). Another study by Nilsen *et al.* (2015b) showed that IRF1 and IRF7 are activated downstream of endosomal TLR2, although LPS was used as the stimulation, which is primarily a ligand for TLR4. The Nilsen study was disputed by Stack *et al.* (2014) who demonstrated that endosomal

TLR2 signals via IRF7, endosomal TLR4 signals via IRF3 and not IRF1- however these results were produced human embryonic kidney (HEK293) cells which may not fully reflect human or murine MΦs. Whilst useful, the discrepancies between these studies show the current conflicted state of the TLR2-type I IFN signalling pathway and the need for a different, more sensitive model to study this system in more detail.

The results presented in this chapter were obtained using a combination of soluble ligand and ligand-coated latex beads to stimulate BMDM and BMA cells. The beads mimic bacteria by inducing phagocytosis, producing a more realistic signalling response whilst still targeting specific signalling pathways. The use of beads influenced downstream endosomal TLR2 signalling. When a soluble ligand was used, the type I IFN pathway was activated via IRF7. However, when ligand-coated beads were used for stimulation, type I IFN activation was achieved primarily through IRF1. These data show that the process of phagocytosis is linked to endosomal TLR2 signalling which has not previously been demonstrated. This research is the beginning of a new investigation into the link between phagocytosis and TLR2 endosomal signalling.

It was also observed that TLR1/2 activates IRF1 and IRF7, but TLR2/6 only appears to signal via IRF1. This is an interesting discovery, because it suggests that the two dimers have distinct functions and signalling mechanisms which has previously not been shown in the literature. This research invites a future study to compare the responses of different TLR2 ligands and to investigate the distinct roles of TLR1/2 and TLR2/6 dimers. Interestingly, when phagosomes are analysed by MS (as shown in Chapter 4), neither TLR1 nor TLR6 has ever been observed on the phagosome, yet the data presented in Chapter 5 demonstrates signalling functionality from endosomal TLR2. In the future it would be interesting to investigate the mechanism of TLR2 dimerisation and activation on the endosome, with the hypothesis that endosomal TLR2 forms a homodimer in response to bacterial ligands. To observe specific proteins which might be unique to either the TLR2 or TLR4 signalling pathways, the use of immunoprecipitation techniques could be a useful approach to extract specific signalling complexes from the phagosome. Although this technique has already been attempted in this project, and although it has been shown that TLR2 is a challenging protein to extract by IP, further optimisation could yield exciting results for identifying phagosomal TLR2 binding partners.

Another future study would be to investigate TLR2 translocation to the endosome after activation at the PM. In previous literature, TLR2 is assumed to behave in the same way as

TLR4, with sequential activation, first at the phagosome, followed by translocation to the endosome. However, MS data (Chapter 4) shows that TLR2 is present on unstimulated phagosomes, and the western blot from Figure 5.4 shows that at 30 and 60 minutes, the type I IFN response is just as evident as the pro-inflammatory response. This indicates that TLR2 may not signal sequentially but may be present on the PM and on the phagosome in resting cells, ready to signal simultaneously. This could be shown using fluorescent live microscopy to observe the location of TLR2 during phagocytosis of ligand-coated beads.

In summary, the data presented in this chapter open new avenues of research into the links between TLR2 and phagocytosis, differential signalling between TLR2/1 and TLR2/6 dimers, TLR2 translocation and TLR2 dimerisation on the endosome.

6. Summary and Future Directions

The overall Scientific aim for this thesis was to develop and apply proteomic methods to better understand endosomal TLR2 signalling induction of the type I IFN pathway. This Ph.D was also sponsored by Thermo Fisher Scientific, and therefore involved projects associated with Thermo research and development.

In Chapter 3, The aim was to develop pH buffers for MAb analysis by HPLC. The buffers had to be stable, MS compatible and produce reproducible chromatograms for the same MAb. Firstly, the buffer recipe and pH were optimised to achieve a reproducible result before different tests were performed to increase the stability of the buffers.

There were various challenges associated with this project, particularly external factors changing the pH of the buffers. Buffers can absorb carbonate from the air, which changes the pH. The column was shown to have a buffering effect on the column, decreasing the pH and changing the shape of the gradient. However, the most challenging aspect of the HPLC set-up was the degasser, which removed carbonate from the buffers in uneven amounts, changing the pH directly before analysis. As removing the degasser was not an option, the next step in the project should be to improve the stability of the buffers.

Blanketing buffers with nitrogen gas was one method of preventing pH change caused by absorption of carbonate from the air and prolonging the shelf life of the buffers. Nitrogen is an inert gas which is readily available and inexpensive. However, it is lighter than oxygen, so rather than completely shielding the buffer from contact with oxygen, blanketing with nitrogen would only reduce the exposure of the buffer to oxygen. Argon would be more effective as it is heavier than oxygen and would therefore displace any remaining oxygen to the top of the container, away from the buffer. However, argon is expensive and less readily available compared to nitrogen and therefore is not economical to use for a readily consumable product such as HPLC buffers.

As it was shown in Chapter 3 that buffers could be made reproducibly and were more stable at 4 °C, the next stage would be to test their suitability as a product. Fresh reagents, particularly ammonium hydroxide which can change pH quickly once opened and exposed to air. Buffers should be made in single-use bottles and shipped and stored at 4 °C. Produced and used in this way, the buffer recipe developed in Chapter 3 can be produced for commercial use.

The buffers will benefit clinical work testing batches of therapeutic MABs using a high throughput workflow. The ability to identify charge variants using HPLC with pH buffers is a faster approach compared to analysis using MS, but as the buffers are MS compatible, LC-MS can be used for a more detailed analysis if required.

In addition to developing HPLC buffers for MAB analysis, MABPac RP columns were used to develop a method of protein fractionation by HPLC that could be applied to a biological sample.

The MABPac, which was designed for the separation of antibodies, was re-purposed to serum analysis. By separating highly abundant proteins from other proteins using HPLC fractionation, low-abundance proteins, including biomarkers, could be identified from un-depleted serum. Such a method is not possible using classical HPLC methods, such as peptide basic reverse phase. Therefore, the MABPac protein fractionation method offers a valuable alternative to peptide fractionation and protein depletion for serum samples.

Separation of serum by MABPac can be further developed by automating the workflow. There are challenges associated with protein fractionation. The main challenge is the MS processing of many protein fractions. During the peptide fractionation workflow, one protein sample is processed before digestion and peptide fractionation. Fractionating protein samples necessitates processing after fractionation, where 40 or more samples require desalting, reduction, alkylation, and digestion before MS analysis.

A robot such as a Kingfisher (Thermo Fisher Scientific) could automate protein fraction processing by using magnetic beads to bind the proteins during the reduction, alkylation, desalting and digestion processes- simply, an automated version of the SP3 or S-trap protocols described in material and methods sections 1.2.2.3.2 and 1.2.2.3.3, respectively. Robotic processing saves labour, time and ensures homogeneity in sample processing, reducing variability between samples. Biomarker identification using an automated MABPac protein fractionation workflow would benefit clinics interested in identifying low-abundance biomarkers which can easily be removed by protein depletion methods. Results from Chapter 3 showed that protein fractionation by MABPac and peptide basic reverse phase fractionation can each be used to identify a unique set of proteins. Therefore, both the use of both techniques together as part of a 2D-LC/MS workflow would increase overall protein identification.

The automated workflow would be cost effective and can be used as part of a 2D-LC/MS set-up for enhanced analysis, which would be beneficial to both research and clinical laboratories.

In addition to developing HPLC methods in collaborations with Thermo Fisher Scientific, MS proteomics techniques were used to investigate endosomal TLR2 signalling.

TLR2 had been reported in previous literature as a trans-locational TLR, but whether TLR2 relocates and signals from the endosome in MΦs was disputed. In Chapter 4, proteomics techniques were used to investigate the effect on the phagosomal proteome when TLR2 was activated by TLR2/1 ligand PAM₃CSK₄. PAM₃CSK₄ stimulation was compared to LPS, a TLR4 agonist, as TLR4 was already well established in the literature as a trans-locational TLR which signals from the phagosome to initiate the pro-inflammatory pathway and then translocates to the endosome where it initiates activation of the type I IFN pathway.

This is the first study to use phagosome isolation to specifically investigate the changing phagosomal proteome in response to TLR2 or TLR4 stimulation. BAF was used to inhibit endosomal maturation. However, BAF is an inhibitor of vATPases, which are essential for the maturation of the phagosome. Phagosomes of unstimulated (UT), PAM₃CSK₄-stimulated or LPS-stimulated cells treated with BAF were indistinguishable from one another by PCA and overall, differences attributable to the cell stimulation were lost. Other endosomal inhibitors may have been more suitable, such as chloroquine, which is a lysosomothrophic weak base. Unlike BAF, it is not a protein inhibitor. Instead, it diffuses across the membranes of the endosomes or phagosomes and neutralizes the acidic environment inside (Fredericksen et al. 2002). As it does not inhibit specific proteins, it is more likely that it will preserve distinct protein interactions initiated downstream of TLR2 and TLR4 activation. In future studies, proteins of interest identified in the proteomics methods would be validated using a targeted method such as Western blotting.

Previous literature has often presented results based on the assumption that, if TLR2 is a trans-locational TLR which signals from the endosome, the signalling mechanism would be comparable to that of endosomal TLR4. However, the results presented in Chapter 4 show that TLR2 and TLR4 initiate different signalling mechanisms. Interestingly, TLR2 binding partners TLR1 and TLR6 were never identified on the phagosome by MS, whereas TLR2 was identified in every replicate. TLR2 was also found on the phagosomes of unstimulated cells, indicating that TLR2 may not move to the phagosome after PM activation but is endogenously present on the phagosome.

These findings implications for TLR2 dimerisation on the endosome. Future direction for this project would include use of microscopy and fluorescent tagging, as well as IP and western blot

to observe TLR2 dimerisation on the phagosome. The absence of TLR2 dimerisation partners suggests that TLR2 may form a hetero dimer with TLR1 or TLR6 at the PM but form a homodimer on the endosome.

TLR4 initiates the type I IFN response from the PM and subsequently translocates to the endosome to initiate the type I IFN response. It is well established that TLR2 also initiates the pro-inflammatory pathway from the PM. TLR2 has also been shown to induce the type I IFN response, however, the signalling mechanism downstream of endosomal TLR2 remains unclear and literature is disputed.

Synthetic ligands can be used to target a specific receptor, but they do not represent a realistic immune response such as that which occurs in response to a bacterial infection. To investigate how the proteome changes in the event of pathogenic infection, gram positive *S. aureus*, a natural TLR2 ligand, was used to stimulate BMDM cells isolated from WT and TLR2 KO mice.

S. aureus proteins identified at 15 min timepoints had disappeared by 60 min indicating that the MΦs had at least partly cleared some bacteria by this time. *S. aureus* proteins were more abundant in the TLR2 KO cells at 60 minutes, indicating faster bacterial clearance in the absence of TLR2. These data indicate a regulatory role for TLR2 in the clearing of the bacteria. A phagocytosis assay measuring the uptake of *S. aureus* by TLR2 KO and WT BMDMs showed that TLR2 KO cells engulf more particles compared with WT cells. Overall, the data from this chapter suggest that the phagosome an essential component of the response pathway for MΦs responding to *S. aureus*. This experiment could be repeated to include different time points to get clearer picture of the timed response. For example, to create a snapshot of early changes after stimulation, a range of earlier timepoints could be used, such as 2, 5- or 10-min. *S. aureus* begins to replicate after 3 h. Therefore, a later 3 h timepoint could be included to compare the proteomes at the beginning and at the end of the infection. Proteins of interest identified in the proteomics methods would then be validated using an orthogonal method such as Western blotting.

Data presented in Chapter 5 shows that TLR2 requires endosomal acidification to induce type I IFNs. TLR2 was shown to induce type I IFN in response to bacterial ligands and that this activation is blocked when acidification of the endosome is inhibited (Figure 5.2). This discovery contradicted earlier studies claiming that endosomal TLR2 can only induce type I IFNs in response to viral ligands (Barbalat et al. 2009), but these findings were corroborated by more recent studies (Stack et al. 2014; Dietrich et al. 2010).

Key findings from Chapter 5 include the discovery that endosomal TLR2 initiates the type I IFN pathway via IRF7 and IRF1, and that the downstream signalling pathway changes from IRF7 to IRF1 when phagocytosis is initiated. TLR2/1 and TLR2/6 dimers were also shown to primarily signal via IRF7 and IRF1 respectively, indicating that the dimerisation partner of TLR2 affects downstream signalling. Further work could investigate the biological significance of TLR2/1 and TLR2/6 dimers by testing natural TLR2 ligands against KO of TLR1 and TLR6. In Chapter 4, it has been suggested that TLR1 and TLR6 may not be present on the phagosome, and that TLR2 may form a homodimer to initiate type I IFN. Therefore, the initiation of IRF1 or IRF7 from the endosome may be influenced by the initiation of signalling pathways from the PM. However, it is unlikely that TLR2 initiates IRF1 or IRF7 from the PM, as when phagosomal maturation was blocked by BAF, induction of IRF1 and IRF7 was also inhibited. Overall, data presented in Chapter 5 show a link between endosomal TLR2 signalling and phagocytosis, different roles for each TLR2 dimer and possible cross-talk between PM and endosomal TLR2 pathways.

TLR2 is found on the phagosomes of unstimulated cells, indicating that TLR2 may not move to the phagosome after PM activation but is endogenously present on the phagosome.

In summary, several different projects have been presented in this thesis, including the development of pH buffers for charge variant analysis of therapeutic MAbs and development of a protein fractionation method that could be automated to provide fast, effective biomarker identification from human serum in Chapter 3. Chapters 4 and 5 present novel signalling mechanisms for TLR2. These findings have implications for clinical analysis of biofluids by LC-MS and further the field of immunology by proposing a different mechanism for TLR2 activation on the endosome.

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vii) Appendix

Table 8.1: Chemicals

Chemical	Usage	Supplier
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Cell culture, MS	Sigma-Aldrich
Acetic acid	High performance liquid chromatography (HPLC) & MS	Thermo Fisher Scientific
Acetonitrile (CAN)	HPLC & MS	Millipore
Acrylamide	SDS-Polyacrylamide gel electrophoresis (PAGE)	Sigma-Aldrich
Ammonium bicarbonate	HPLC & MS	Sigma-Aldrich
Ammonium chloride	Cell culture	Sigma-Aldrich
Ammonium hydroxide	HPLC & MS	Thermo Fisher Scientific
Ammonium persulfate solution (APS)	SDS-PAGE	Thermo Fisher Scientific
Bovine serum albumin (BSA)	Protein standard	Sigma-Aldrich
Bradford reagent	Protein quantitation	Bio-Rad
Carbonic anhydrase	Protein standard	Sigma-Aldrich
Chicken egg lysozyme	Protein standard	Sigma-Aldrich
Chloramphenicol	Bacterial culture	Sigma-Aldrich
Cytochrome C	Protein standard	Sigma-Aldrich
Dimethyl pimelimidate (DMP)	Cross-linking buffer	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Cell culture	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Cell culture	Merck
Ethanol (EtOH)	Cleaning	Millipore
Formalin solution	Silver staining	Sigma-Aldrich
Formic Acid (FA)	HPLC & MS	Millipore
Glycerol	Reduce hydrophobic non-specific binders	Sigma-Aldrich
Glycine	Tris-glycine SDS-PAGE Running bBuffer	Sigma-Aldrich
InstantBlue Coomassie stain	Protein staining	Expedeon
Iscove's Modified Dulbecco's Medium (IMDM)	Cell culture	Merck
Isopropanol	HPLC & MS	Millipore
L-Glutamine	Cell culture	Sigma-Aldrich

Methanol (MEtOHMeOH)	EZQ/ Polyvinylidene fluoride (PVDF) transfer	Millipore
Milk powder	Protein blocking agent Western blotting	Tesco
Nonyl phenoxypolyethoxylethanolEtOH (NP-40)	Detergent	Sigma-Aldrich
Ovalbumin	Protein standard	Sigma-Aldrich
Penicillin-Streptomycin	Cell culture	Sigma-Aldrich
Polyethylene glycol sorbitan monolaurate (Tween-20)	Detergent	Sigma-Aldrich
Ponceau S	PDVF Protein membrane staining	Sigma-Aldrich
Precision Plus protein markers	Protein marker	Bio-Rad
Rapigest	Detergent	Waters
Saccharose	Saccharose gradient	Sigma-Aldrich
Silver nitrate	Silver staining	Sigma-Aldrich
Sodium azide	Preservation of antibody aliquots	Sigma-Aldrich
Sodium borate	Cross-linking buffer	Sigma-Aldrich
Sodium carbonate	Silver staining	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Cell lysis, buffer, SDS-PAGE	Sigma-Aldrich
Sodium thiosulfate pentahydrate	Silver staining	Sigma-Aldrich
Trifluoroacetic acid (TFA)	HPLC & MS	Sigma-Aldrich
Tetraethylammonium bicarbonate (TEAB)	Trypsin digestion buffer	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	SDS-PAGE	Sigma-Aldrich
TriethanolEtOHamine	Cross-linking buffer	Sigma-Aldrich
Tris(2-carboxyethyl)phosphine (TCEP)	Reducing agent	Sigma-Aldrich
Tris(hydroxymethyl)aminomethane (Tris)	Buffering agent	BDH
Trypan Blue	Cell staining	Sigma-Aldrich
Trypsin	Protein digestion	Pierce/Sigma-Aldrich
Tryptic Soy Agar	Bacterial culture	Merck
Tryptic Soy Broth	Bacterial culture	Merck
β-mercaptoEtOH (βME)	Reducing agent, Cell culture, MS	Sigma-Aldrich