Effect of Arginine Glutamate on Protein Aggregation in Biopharmaceutical Formulation

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science & Engineering

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PRISCILLA KHEDDO
Monoclonal antibodies (mAbs) represent one of the fastest growing classes of therapeutic proteins. This success is due to a number of attractive properties such as high binding affinity, specificity, low immunogenicity and high aqueous solubility. Despite this, mAbs can suffer from undesirable physical instabilities, especially reversible self-association (RSA), which can lead to aggregation and phase separation. One aspect of formulation is therefore to find solution conditions which minimise mAb aggregation propensity during storage at high concentrations. Hence, the buffer, excipient and pH must be carefully considered to obtain the optimal formulation. Currently, if a platform formulation process is non-ideal for a particular candidate mAb, then an alternative strategy is to utilise high-throughput screening to measure various physical parameters indicative of physical stability. Arginine (in the form of hydrochloride salt Arg·HCl) is often used in formulations exhibiting high RSA and a propensity for aggregation. The interaction of Arg with the protein surface is complex and dependent on both the salt form and concentration. Here the focus was on the glutamate salt of arginine (Arg·Glu), to quantify its effect on mAb conformational and colloidal stability under different pH conditions. Arg·Glu was able to decrease the propensity of the mAbs to aggregate, particularly at pH values closer to their pI.

The work also included the use of in vitro cell culture models to examine cell viability in the presence of the various arginine salts over a range of osmolalities. Whilst Arg·Glu is composed of two naturally occurring amino acids and both of which are considered non-toxic individually, the effect of the increased concentrations of their combination, on cells has not been explored previously. In vitro cell lines were chosen to represent the subcutaneous tissue, the effect of Arg·Glu on cell viability was compared against NaCl, Arg·HCl and sodium glutamate (NaGlu). The work concluded there was no additional toxicity associated with the presence of Arg·Glu in the cell culture models studied, therefore Arg·Glu has the potential as an excipient as it reduces aggregation and is nontoxic.

Another aspect of the work was to assess the use of solution NMR spectroscopy as an orthogonal technique in mAb formulation characterisation. $^1$H NMR spectroscopy was used to measure a number of experimental parameters for high concentration mAb solution. The work proposed that $^1$H NMR spectroscopy can serve as a valuable orthogonal method for mAb characterization and formulation.
Declaration

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LIST OF ABBREVIATIONS

Δ  Diffusion time
δ  Chemical shift
δ'  Gradient length
1D  One-dimensional
2D  Two-dimensional
3D  Three-dimensional
1H  Proton nuclei
2H  Deuterium nuclei
13C  Carbon-13
15N  Nitrogen-15
2H2O  Deuterium water
A  Absorbance
ADA  Antibody-drug antigen
ANOVA  Analysis of variance
APC  Allophycocyanin
APCs  Antigen presenting cells
Arg·Glu  Arginine Glutamate
Arg·HCl  Arginine hydrochloride
B0  External magnetic field
B22  Second virial coefficient
BSA  Bovine serum albumin
c  Protein concentration
CD54/ICAM-1  Cluster of Differentiation 54/ Intercellular Adhesion Molecule 1
CD86  Cluster of Differentiation 86
CDR  Cluster of differentiation region
CP  Citrate-phosphate
CPMG  Carr-Purcell-Meiboom-Gill
CTD  Common technical document
D  Self-diffusion coefficient
DAMPs  Damp-associated molecular patterns
DC  Dendritic cell
DLS  Dynamic light scattering
Dm  Mutual diffusion coefficient
DOSY  Diffusion ordered spectroscopy
DSP  Downstream processing
e  Extinction coefficient
EBNA  Epstein–Barr nuclear antigen
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
EM  Exponential multiplication
EMA  European Medicines Agency
eNOS  Endothelial nitric oxide synthase
EU  European union
F  Fraction of protein remaining in solution
FCS  Fetal calf serum
FID  Free induction decay
FITC  Fluorescein isothiocyanate
FSC-H  Forward scatter
FT  Fourier transform
g  Gradient strength
GRAS  Generally recognised as safe
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human Leukocyte Antigen - antigen D Related</td>
</tr>
<tr>
<td>HRP</td>
<td>Streptavidin-horse radish peroxidase</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonisation</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Human interferon beta</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
</tr>
<tr>
<td>IPEC</td>
<td>International Pharmaceutical Excipients Council</td>
</tr>
<tr>
<td>L-Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>L-Glu</td>
<td>L-glutamate</td>
</tr>
<tr>
<td>LLPS</td>
<td>Liquid-liquid phase separation</td>
</tr>
<tr>
<td>MAA</td>
<td>Marketing authorisation application</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MALS</td>
<td>Multi Angle Light Scattering</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>mVROC</td>
<td>Micro viscometer-Rheometer on a chip</td>
</tr>
<tr>
<td>η</td>
<td>Viscosity</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaGlu</td>
<td>Sodium glutamate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PPI</td>
<td>PPIs</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>R₂</td>
<td>Transverse relaxation rate</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>r.t</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>Size exclusion-high performance liquid chromatography</td>
</tr>
<tr>
<td>SE-PFG</td>
<td>Stimulated gradient-pulsed field gradient</td>
</tr>
<tr>
<td>SLS</td>
<td>Static light scattering</td>
</tr>
<tr>
<td>SSC-H</td>
<td>Side scatter</td>
</tr>
<tr>
<td>T₂</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>T_{agg}</td>
<td>Onset of aggregation temperature</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocytic leukemia cell line</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>T_m</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>USP</td>
<td>Upstream processing</td>
</tr>
<tr>
<td>γ</td>
<td>Gyromagnetic ratio</td>
</tr>
<tr>
<td>τ_c</td>
<td>Rotational correlation time</td>
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Chapter One: Introduction

1.1 Protein-based pharmaceuticals

The pharmaceutical market has evolved considerably over the past 20 years, particularly with respect to the development of therapeutic moieties outside the small molecule arena (<900 Da) (Weiner 2015). Ongoing research in antibody therapeutics is on the rise with over 470 molecules in the clinical pipeline as of the beginning of 2016. Statistics from the year 2015 reported the marketing of 6 novel antibody therapeutics in the European union (EU) and United states (Reichert 2016). The development of monoclonal antibodies (mAbs) is primarily for diseases such as cancer and autoimmune diseases. Their growing success can be attributed to their high specificity, greater half-lives in humans in comparison to small molecules, the ability to produce conjugates with radioisotopes and other therapeutic entities and reduced toxicity in comparison with small molecule drugs as these do not elicit any intrinsic toxicity caused by harmful metabolites as they are converted into amino acids. However, mAbs and mAb-based proteins, such as antibody-drug antigens (ADAs), when formulated at very high concentrations are known to encounter problems with aggregation (Liu et al. 2005, Bang 2006, Wu et al. 2014, Nicoud et al. 2016). Aggregates can appear at any stage of protein production, such as during the upstream processes in fermentation and purification, to the downstream processes in formulation, fill-finish, storage and transportation (Obrezanova et al. 2015, Oyetayo and Kiefer 2016). Protein aggregation is the assembly of protein species into higher molecular weight species which is detrimental for industry as monomeric species are the desired protein form (Mahler et al. 2009).

The Introduction of the Thesis will begin with an overview of the problems associated with protein aggregation, followed by an outline of the structural biology of the Immunoglobulin G (IgG) protein. This will be continued with consideration of the importance of the conformational and colloidal stability of concentrated mAb solutions with focus on the external factors which give rise to aggregation. The Introduction continues with the techniques used to characterise aggregation in this Thesis and the necessity to carry out in vitro toxicity testing of new excipients intended for human use. As a potential solution to this problem, the equimolar mixture of arginine glutamate (Arg-Glu) will be discussed as a novel excipient combination to be incorporated in industrially relevant protocols to explore its ability in preventing aggregation. This Introduction finishes with the aims and objectives of the Thesis and how the current work fits into the pharmaceutical field.

1.1.1 The biological and industrial problems caused by protein aggregation

The complexity of protein molecules generally implies a greater number of challenges in regards to covalent and non-covalent instability-related changes in comparison to small-molecule drugs (This does not mean that small molecule drugs are any ‘easier’ to formulate into medicines). Briefly, proteins comprise of the primary structure encoding the linear
sequence of the amino acids; the secondary structure relates to the organisation of the polypeptide chain into regular structures such as α-helix and β-sheets followed by the tertiary structure which is the 3-dimensional (3D) arrangement of the polypeptide chain; lastly the quaternary structure involves the assembly of the polypeptide chains into multi-subunit structures (Berg JM 2002). The higher order structure of protein contributes specifically to their biological function.

A critical issue regarding the formulation of mAbs is the need to deliver high doses (>150 mg/mL) in relatively small volumes (Cheng et al. 2013). The preferred route of administration for mAbs for chronic diseases is subcutaneous, thus small volumes (1-2 ml) are required as this provides patient convenience. The drug can be self-administered into prefilled syringes with liquid formulations being substantially cheaper than lyophilised products (Walters et al. 2014). The propensity of proteins to aggregate and self-associate is a problematic issue for all proteins which is associated with their chemical and physical properties. In the early purification stages, aggregation can arise as the protocol involves varying environmental factors such as temperature and pH changes; others factors include surface interactions, ionic strength, mechanical and shear stresses (Cromwell et al. 2006). During drug storage and transportation, agitation can occur, causing the protein and container surfaces to interact resulting in alterations in the stability and efficacy of the drug (Rathore and Rajan 2008). The general agreement on the amount of soluble aggregates permitted in therapeutic proteins is between 5-10 % (Wang et al. 2012). Moreover, increases in the protein concentration often causes concentration-dependent aggregation and increased solution viscosity due to the large size and intricate structure of protein molecules causing shear stress on the protein (Liu et al. 2005). There are several proposed hypotheses to explain the increase in solution viscosity due to aggregation; one being the occupied volume fraction theory which is dependent on the aggregate size, morphology and concentration. This theory arises from colloid sciences as protein molecules lie within the colloidal region (Nicoud et al. 2015). A colloid is a solution consisting of particles in the size range 1-1000 nm which is evenly distributed within the solution. Protein molecules can be considered as colloids due to their ability to undergo Brownian motion and their larger size in comparison to the solvent molecules. The concept of occupied volume fraction has been applied previously in viscosity studies of aggregating colloidal dispersions thus can be related to protein molecules (Silbert and Melrose 1999, Castellanos et al. 2014, Nicoud et al. 2015).

Another important aspect that is still poorly understood is the environmental transition experienced by the mAb immediately following injection into the subcutaneous space. Recently a subcutaneous injection site simulator system (‘Scissor’) has been described (Kinnunen et al. 2015). The transition from the formulation to the subcutaneous environment can be detrimental to mAbs stability due to non-optimal (from the viewpoint of high concentration mAb solutions) pH and buffer ions. Known changes would involve a pH-shift from slightly acidic formulation pH 4-6 to neutral pH at the hypodermis (constituting of a
number of tissue components such as adipocytes, fibroblasts, macrophages) and buffer ion exchange between those used for formulation (e.g. histidine/histidine hydrochloride) and those present in the hypodermis (e.g. bicarbonate) for homeostatic control (Kinnunen and Mrsny 2014, Kinnunen et al. 2015). If the behaviour of the mAb is such that it undergoes extensive aggregation in the subcutaneous space then this may affect the pharmacokinetic outcome since insoluble mAb will not be rapidly cleared from the injection site.

Aggregation can be controlled quite successfully through protein modifications or by optimising the procedures during expression, purification and solubilisation. Previous studies have indicated that understanding the link between the stability of the secondary and tertiary structure of the protein and aggregation may allow the design of beneficial mutations within the 3D structure (Calloni et al. 2005, Idicula-Thomas and Balaji 2007). Even if these methods are successful, it may still be problematic to formulate highly concentrated samples without introducing aggregation and precipitation (Golovanov et al. 2004). Alternatively, excipients can be added to the final protein formulation. Excipients can be defined as ‘additives that are included in protein formulations to enhance the stability, shelf life and bioavailability of the drug’ (Kamerzell et al. 2011). Excipients function by weakening protein-protein interactions (PPIs) and stabilising the native protein structure by outcompeting any intermolecular interactions which may lead to aggregation (Bondos and Bicknell 2003). The pharmaceutical industry uses excipients from the generally recognised as safe (GRAS) category which are approved by the Food and Drug Administration (FDA). These established compounds are used to avoid complications due to possible toxicity and to speed up the approval process by the regulators (Ogaji IJ 2012). A novel excipient is defined by the European Medicines Agency (EMA) as ‘being used for the first time in a drug product, or by a new route of administration. It may be a new chemical entity or a well-established one which has not yet been used for human administration and/or for a particular human administration pathway in the EU and/or outside the EU’ (EMA 2007). In the development of clinically efficacious drug formulations the choice of excipient is important; both within and outside the United State the regulatory authorities prefer excipients to be selected from ones that are already approved (DeMerlis et al. 2009). Prior to 2007, novel excipients in pharmaceutical formulations were only approved within new drug applications as there was no independent regulatory approval process; however an independent excipient process was introduced by the International Pharmaceutical Excipients Council (IPEC)-America Safety Committee (IPEC 2014). Additionally appropriate clinical trial guidelines on medicinal products for human use need to be enforced to define harmonised rules and must be in accordance with the Good Manufacturing Practices for Medicinal Products. The use of a novel excipient in any medicinal product requires thorough details on their manufacturing process, characterisation and controls relevant to the safety of the product (EMA 2007).
1.1.2 Immunogenicity of aggregates

In addition to the quality-related consequences of aggregate formation, aggregates can cause concerns with respect to immunogenicity. Immunogenicity refers to the generation of an immune response due to the development of ADAs to human protein therapeutics (Kuriakose et al. 2016). One of many important reasons in developing therapeutic proteins which are almost identical to their endogenous protein counterparts was to preclude any response from the immune system; as such proteins should not be recognised as foreign. Humanised therapeutic proteins have been primarily developed to prevent immunogenic responses; however, this has not been entirely successful (Section 1.2 goes into more detail on the history of antibodies with Figure 1.2 illustrating the structure differences between the antibody types over the years). Immunogenic issues have been reported from even fully humanised therapeutic proteins which are believed to be due to factors such as the presence of aggregates, nonhuman sequences in the cluster of differentiation regions (CDRs), slight changes in the protein sequence or post-translational modifications (PTMs) (e.g. glycosylation) which may all increase the immunogenic risk (Wadhwa et al. 2015, Kuriakose et al. 2016). Previous experimental studies using animal models have shown a strong correlation between protein aggregation and immunogenicity (Wang et al. 2012). Aggregation is one of the main reasons in breaking of tolerance in humans to human proteins; immunological tolerance refers to the ability of the immune system to differentiate between self and non-self; the healthy response to self being “tolerance” or immune ignorance (Sauerborn et al. 2010). A well-known example of a human protein prone to aggregation resulting in immunogenicity is recombinant human interferon beta (IFN-β) which is used to treat patients suffering from multiple sclerosis. There are two therapeutic forms of IFN-β; the first is IFNβ-1a which is almost identical in its amino acid sequence and glycosylation patterns to the endogenous human protein due to its production in chinese hamster ovaries whereas the second, IFNβ-1b is unglycosylated due to its production in Escherichia coli. IFNβ-1b has been shown to increase ADA production in patients with multiple sclerosis much more than IFNβ-1a due to its lack of glycan chains which are required for protein stability thus resulting in aggregation and increased immunogenicity (Ratanji et al. 2014, Haji Abdolvahab et al. 2016).

The purpose of the immune system is to protect against infection by pathogenic organisms. The presence of molecules that are recognised as foreign (immunogenic) can generate an immune response within the host. The three main features of a healthy immune system are its selectivity, memory and ability to differentiate between self (host) and non-self (potentially harmful foreign material). Under normal circumstances, the immune system develops so called tolerance to self-proteins, to avoid the development of unwanted immune responses to these proteins. The selectivity and memory of the immune response, and thus the ability to discriminate between self and non-self, is conferred by the adaptive arm of the immune system, the cellular mediators of which are T and B lymphocytes. Both T and B-cells express
specific cell surface receptors that allow them to recognise a large number of different foreign molecules. B-cells are responsible for antibody production and T cells play a number of roles, including clearance of viruses but also regulatory roles, such as the provision of “help” for B-cells to make antibody (Wang et al. 2012, Jawa et al. 2013, Coico and Sunshine 2015). A number of adverse effects are associated with the development of ADAs due to the formation of immune complexes between the therapeutic protein and ADA causing hypersensitivity reactions and/or reduction in pharmacological activity (Krishna and Nadler 2016). At the very least, the formation of these immune complexes will prevent the therapeutic from reaching its target although in this situation antibodies will remove the therapeutic from the body resulting in no clinical problems or adverse effects. However, this reduces the drug efficacy and more importantly if an adaptive immune response is mounted (will be discussed below), the memory component will remember such an event thus subsequent treatments with the same drug will not be as effective (Ratanji et al. 2014). The subsequent exposure to the same pathogen causes these cells to become effector cells, and once the pathogen is eliminated the cells are removed whereas other memory cells remain in the circulation. This concept can be explained using vaccinations as an example as the exposure to a non-infectious antigen stimulates the immune system to develop protective immunity against the organism to produce memory cells (Vitetta et al. 1991, Janeway CA 2001).

The ability of a protein therapeutic to be immunogenic is dependent upon its interaction with immune cells via two main pathways (Figure 1.1); either via a classical response where the therapeutic drug must be recognised as a foreign material (i.e. an antigen) and induce an adaptive immune response resulting in the therapeutic being internalised, processed and presented on antigen-presenting cells (APCs) as peptide fragments which are recognised by CD4+ T cells that go on to stimulate cytokines resulting in B-cell activation to produce antibodies (Figure 1.1A); or the second pathway being the breakage of B-cell tolerance if there is resemblance between the drug (antigen) and an endogenous protein which may result in the breaking of B-cell tolerance inducing an antibody response (via a T cell independent pathway) (Ratanji et al. 2014, Yin et al. 2015) (Figure 1.1B). In order to thoroughly understand these pathways an understanding of the innate and adaptive immune system is necessary. Humans are prone to harmful pathogens on a daily basis; the ability of our body to prevent infection is dependent on the adaptive immune system due to memory from a previous attack. Although this is crucial, the adaptive immune response cannot respond as quickly to new pathogens due to prior B and T cell activation; therefore the human body employs the innate immune response as the first line of defence to new pathogens. The innate immune system has pattern recognition receptors (PPRs) which recognise common patterns associated with pathogens stimulating an inflammatory response (discussed in Section 1.9) (Lamond 2002). However, the ability to recognise a pathogen specifically and respond more rapidly during a second exposure is defined as the adaptive immune response due to the generation of memory components to remember subsequent attack from the same pathogen (Mogensen 2009). The adaptive response cannot occur without the initiation of the innate response and
release of signalling molecules which mounts an inflammatory response to initiate the adaptive immune system (Section 1.9). The T cell dependent pathway is the stimulation of a classic immune response against foreign antigens due to new epitopes that is dependent on T and B-cell interactions (Figure 1.1A). Although this is clearly the most common mechanism for immunogenicity provoked by non-human therapeutics (e.g bacterial streptokinase), which are non-host and result in T and B-cell activation (Schellekens 2002), there is less evidence that this is an important mechanism for the induction of immune responses to host (humanised) protein therapeutics or in response to other factors mentioned above. However, a classical adaptive T cell response has been shown to be induced by aggregated therapeutic proteins (humanised mAbs) in vitro, which was suggested to be due to more efficient phagocytosis by activation of APCs of the aggregated product (Joubert et al. 2012). The second, more widely accepted, mechanism is due to breaking of B-cell tolerance via T cell independent activation of B-cells. Aggregation results in the formation of repetitive and ordered antigens that can initiate cross-linking of B-cell receptors triggering B-cell activation independently of T cell help, thus breaking immune tolerance to the self-protein (Figure 1.1B). This theory has been suggested as many bacteria display repetitive elements, thus protein aggregation is thought to mimic this property of bacteria which is why it might overcome tolerance and activate the immune system (Wang et al. 2012, Ratanji et al. 2014).

Figure 1.1: Two pathways illustrating the mechanism protein therapeutics interact with immune cells to cause an immunogenic response. (A) classical immune response where the therapeutic drug i.e. antigen is internalised, processed and presented by APCs. CD4⁺ T cells recognise this to stimulate antibody production via B-cell activation; (B) breakage of B-cell tolerance as the therapeutic drug may resemble an endogenous protein which in the absence of T cells activates B-cells to produce antibodies. Figure taken from (Ratanji et al. 2014)

Although, this section has discussed the development of an immunogenic response to protein aggregates, another aspect is the ability of the components within the final drug product to
cause other tissue damage which can stimulate an unwanted immune response causing inflammation. This characteristic is relevant to this Thesis as a novel excipient combination will be discussed further in the introduction. Thus, the role of inflammation will be focussed on in Section 1.9.

1.2 The history of monoclonal antibodies

In 1975, Kohler and Milstein reported the \textit{in vitro} production of murine mAbs from hybridomas (Buss \textit{et al.} 2012). Three types of mAbs were developed during different time frames: mouse, mouse-human chimeras and, more recently, humanised mAbs. One of several memorable findings in research was the approval in the late 1980s by the FDA of the first murine-based mAb therapy, the anti-CD3 mAb, Muromonab, used in the treatment of organ transplant rejection. However, Muromonab was removed from the market by the FDA due to the major drawback in murine mAbs as a consequence of their short-half life in humans, induction of ADAs and reduced antibody efficacy (Liu 2014). Advances in recombinant DNA technology were made to overcome these problems where chimeric mouse-human antibodies were developed in 1984. Chimeric mouse-human antibodies use genetic engineering techniques based on grafting the total antigen-specific domain of a mouse antibody on the constant domains of a human antibody which is \( \approx \) 65 \% humanised (Ribatti 2014). In 1997, this lead to the first full length chimeric mAb, Rituximab, to be approved for the treatment of cancer; this is still to date a marketed therapeutic agent. Moreover, 2002 witnessed the approval of the first fully humanised mAb (i.e. no rodent sequences), Adalimumab by the US FDA (Nelson \textit{et al.} 2010).

1.3 The structure of immunoglobulins

Immunoglobulins (Ig) are heterodimeric proteins consisting of two identical light (25 kDa) and two identical heavy chains (50 kDa) which total to a molecular weight of 150 kDa. This structure forms the typical ‘Y’ shape molecule which contains two major functional groups, the two Fab fragments responsible for antigen binding and the Fc fragment which has the capability of triggering an immune response due to its effector function (Schroeder and Cavacini 2010) (Figure 1.2). The effector function is dependent on proper glycosylation patterns thus this requires careful consideration as this can also affect the antibody conformation (Wang \textit{et al.} 2007). Five classes of Ig exist based on the amino acid sequence of their constant region of the heavy chain, designated IgG, IgA, IgM, IgD and IgE (Goswami 2013). The current approved mAb therapeutics are mostly IgG’s, probably due to the high abundance in plasma (~80 \%), its propensity to remain monomeric and higher half-life in serum than other antibody classes. This structure is covalently stabilised through disulphide bonds in the hinge regions which links both the heavy chains together with a further link from each light chain to a heavy chain (Hanson and Barb 2015). The 3D structure of the protein depends on the unique organised amino acid sequence found on the variable regions on the tips of the light chains.
thus leading to the wide antibody specificity. The IgG class is further divided into four subclasses: IgG1, IgG2, IgG3 and IgG4 which differ from one another in the number of disulphide bonds and length of hinge region in the heavy chain (Schroeder and Cavacini 2010). The heavy chain has four domains: three in the constant regions (C_H1, C_H2 and C_H3) and one heavy chain variable (V_H) domain. The light chain is comprised of two domains called a light chain variable (V_L) and a constant light (C_L) domain which can consist of two types: lambda (λ) or kappa (κ) (Figure 1.2). Each respective domain consists of two β-pleated sheet structures which in a sandwiched like structure contain a hydrophobic interior and form the secondary structure called the Ig fold. The Fab region contains three distinct loops located on the sides called the CDRs. CDRs are crucial in the specific antigen-binding sites providing the greatest diversity for this section of the antibody (Goswami 2013).

Figure 1.2: Schematic diagram illustrating the antibody structure with the different components on the Fab and Fc regions.

1.4 Conformational and colloidal stability of concentrated antibody solutions

1.4.1 Mechanism of protein aggregation

As described in the previous section, mAbs have a complex structure, when the native protein structure is in solution it is in equilibrium with either the unfolding intermediate state or with the completely unfolded state under normal conditions which further highlights the delicate nature of proteins (W. Wang et al. 2010, Arzenšek et al. 2012). Antibodies undergo physical instabilities via two key routes: aggregation and denaturation (Wang et al. 2007). Aggregation can occur either physically or chemically. Physical aggregation refers to the direct association of native protein molecules with one another whereas chemical aggregation introduces newly formed covalent bonds (Wang 2005). The aggregate sizes can be classified as submicron
(<1 µm) particles referred to as soluble particles; sub-visible (1-100 µm) and visible particles (>100 µm) with the latter two being insoluble (Goswami 2013). Figure 1.3 provides a schematic representation of the aggregation pathways. The term aggregation has several interpretations; therefore for the clarity of this Thesis a number of definitions will be highlighted. Protein aggregation will be used to define any molecular associate present in the non-native state that has a larger molecular weight than the ‘normal’ native protein. Protein-self association refers to the direct native protein association in the absence of any intermediate unfolded state. Oligomers will be defined as the association of several monomers forming high molecular weight associates (W. Wang et al. 2010, Singla et al. 2016). Aggregates can be insoluble or soluble, irreversible or reversible and can be formed via covalent or non-covalent interactions. Non-covalent aggregation is a result of many weak forces: hydrogen bonding, electrostatic and hydrophobic interactions and van der Waals’. Covalent interactions on the other hand can occur through disulphide bond linkages. The potential mechanisms of protein aggregation have been described in several reviews (Philo and Arakawa 2009, Arosio et al. 2012). Five mechanisms of protein aggregation have been discussed in a review published by Philo and Arakawa (Philo and Arakawa 2009); two of these pathways will be discussed here which were focussed on in this Thesis.

![Figure 1.3: Schematic illustration of the basic aggregation pathway. The native protein structure can face a number of external stresses during protein production to the final delivery of the drug. The native protein can undergo partial unfolding leading to dimer and/or oligomer formation with the steps following depicted in the diagram ultimately leading to visible particulates. This figure has been taken from Ratanji et al (Ratanji et al. 2014).](image)

1.4.2 Reversible protein self-association

Reversible self-association occurs by the direct interaction of the native protein molecules without any conformational changes due to their self-complementary surface forming oligomers. It is known that the self-association of proteins is related to the colloidal stability. The colloidal stability refers to the intermolecular self-association of protein molecules whereas
the conformational stability refers to the solvent-shielded residues in the folded state (Pace et al. 1996, Arzenšek et al. 2012, Sule et al. 2012). Several studies have demonstrated that reversible self-association primarily occurs by dipole moments on the protein surface or through electrostatic interactions of the charged residues or by Van der Waals and hydrophobic forces. Although these interactions are reversible and generally weak these still pose a challenge in protein formulations at all stages especially at high protein concentrations. The increase in the total protein concentration overtime leads to larger oligomers and is a major factor which dictates the extent to which self-associates and aggregates form, leading to the formation of irreversible aggregates (Liu et al. 2005, Philo and Arakawa 2009). A common parameter used to assess the degree of protein self-association is through the osmotic second virial coefficient ($B_{22}$) (Arzenšek et al. 2012).

1.4.3 Partial unfolding of native protein exposing hydrophobic patches

This type of aggregation is mainly related to the conformational stability. In equilibrium, protein solutions normally contain partially unfolded intermediates that typically fold back into their native state. However, these intermediates can interact with each other due to the exposure of hydrophobic patches and greater flexibility causing protein aggregation. These hydrophobic regions are usually buried within the protein structure to exclude water molecules. The folding and unfolding of protein intermediates is thought to be the precursor of protein aggregation. Aggregation does not occur if the protein has completely unfolded as the hydrophobic regions are scattered (W. Wang et al. 2010). Thermodynamics and kinetics are important factors in aggregation. Thermodynamics is related to the stability describing the change in the free energy ($\Delta G$) either released or consumed during a reaction where the value is derived by the difference in the initial and final state independent of the pathway; this reaction occurs spontaneously. However, kinetics is related to reactivity where the reactants require enough activation energy to proceed forward towards the product. A study conducted by Patro and Przybycien (Patro and Przybycien 1996) showed that in the early stages of protein aggregation the overall free energy increases due to several factors such as conformational losses. As aggregation proceeds the overall free energy starts to decrease which thermodynamically favours protein aggregation. This occurs due to the exposure of hydrophobic regions (Wang 2005).

1.5 Phase separation phenomenon

MAbs at high (50-100 mg/mL) and ultra-high concentrations (> 150 mg/mL) often show opalescence due to the increased tendency of protein molecules to non-specifically interact with each other as a consequence of molecular crowding and excluded volume effects. Molecular crowding shifts the solution equilibria towards a more compact state as it is not possible for two molecules to occupy the same space hence; the molecule will exclude others within the same vicinity, giving rise to the excluded volume phenomenon. Thus molecular crowding is a powerful driving force in protein self-association (Snoussi and Halle 2005,
Kuznetsova et al. 2014). Overall, crowding increases the solution viscosity and slows down protein diffusion. The dynamics of fluids is closely related not only to the solution viscosity and diffusion but also the hydrodynamic radius; this relationship can be described by the Stokes-Einstein equation (Bancaud et al. 2009, Brillo et al. 2011):

\[ D = \frac{kT}{6\pi R_h \eta} \]  

(1.1)

Where \( D \) is the self-diffusion coefficient; \( T \) is the temperature in Kelvins; \( k \) is the Boltzmann constant; \( R_h \) is the hydrodynamic radius and \( \eta \) is the solution viscosity.

Self-diffusion describes the random Brownian motion of an individual protein molecule in the absence of any concentration (i.e. chemical potential) gradient which arises from the collisions between protein and other molecules in the solution (Gagnon and Lafleur 2009). It is quantified by \( D \) at infinite dilution which has proportionality with the mean-squared displacement of the protein per unit of time (Scalettar et al. 1988). Importantly, \( D \) describes the motion in a dilute solution (at zero protein concentration) and can be extrapolated from dynamic Light Scattering (DLS) data. However, at finite protein concentrations, protein diffusion is greatly affected by interprotein interactions (e.g. contributions from long-range electrostatic and PPIs) as mentioned above, and from the excluded volume effect. \( D \) significantly decreases with increasing protein concentrations due to PPIs, and the crowding effect restricting protein motion (Scalettar and Abney 1991). In contrast, mutual diffusion occurs due to concentration gradients which can be described through the Fick’s first and second law for example to give the mutual diffusion coefficient, \( D_m \) (Scalettar and Abney 1991). Fick’s first law (Equation 1.2) relates the diffusion flux to the concentration under steady state which describes the flow of the solute from a region of high concentration to one of low concentration. Fick’s second law (Equation 1.3) describes how the concentration changes over time (Milligen et al. 2005, la Barrera 2005).

\[ J = -D \frac{d\varphi}{dx} \]  

(1.2)

\[ J = D \frac{\partial^2 \varphi}{\partial x^2} \]  

(1.3)

Where \( J \) is the diffusion flux; \( D \) is the diffusion coefficient; \( \varphi \) is the concentration (dimension is amount of substance per unit volume); \( x \) is the position (dimension in length) and \( t \) is the time.

Both diffusion coefficients generally differ when the protein concentration is nonzero but are the same at infinite dilution (Abney et al. 1989a, Abney et al. 1989b, Scalettar and Abney 1991). The nonspecific attractive interactions involved in reversible self-association are a fundamental determinant of concentrated solution stability that is associated with aggregation, high solution viscosity, opalescence and phase separation (Raut and Kalonia 2015b). Opalescence can be defined as a type of dichroism (reflection of iridescent light, the name
referring to the appearance of opal), which in the context of the formulation of mAbs generally refers to a somewhat turbid solution exhibiting a blueish sheen under a light source. At high mAb concentrations in particular opalescence not only impacts the aesthetic appeal of the product but may act (not always) as a precursor for phase separation or indicates the presence of aggregates in solution (Raut and Kalonia 2015b).

Phase transitions relevant in protein liquid formulations are solid-liquid or liquid-liquid phase separation (LLPS). Solid-liquid phase separation is an aggregation mechanism resulting in particulate growth (protein crystals or amorphous precipitates). LLPS is a thermodynamic and kinetic process with protein unfolding not commonly observed. This phenomenon arises as each protein has a solubility limit: once a certain concentration is exceeded, the solution will no longer remain homogenous, and phase separation (either liquid-solid, or liquid-liquid) will occur. This process may be desirable, for example in protein crystallisation studies, to produce crystals when liquid-solid phase separation takes place (Asherie 2004). In LLPS, the non-homogeneity arises from the formation of concentrated liquid droplets which over time and below a specific temperature spontaneously segregate into two concomitant phases (Wang et al. 2011), with the upper phase being less dense and concentrated (protein lean) and the lower phase being more dense and very concentrated (protein rich). Although these phases have different concentrations, the chemical potential is the same, and the phases exist in thermodynamic equilibrium (Ying Wang et al. 2014). Wang et al. have reported that as LLPS is very sensitive to small changes in the average interprotein interaction it would be a useful tool to predict the propensity of antibodies to condense (Wang et al. 2011). LLPS normally arises from weak interactions between natively folded proteins (i.e. self-association) and is related to the colloidal stability rather than conformational stability (i.e. protein unfolding); it is mediated through hydrophobic and electrostatic interactions (Ying Wang et al. 2014). It has been suggested that the protein-rich phase can encourage aggregation (Raut and Kalonia 2015a). Opalescence is often associated with kinetically driven LLPS which may continue to increase; opalescence itself is caused by the presence of the mixture of different protein phases, causing multiple scattering of light. To further complicate the phase compositions, salts in the formulation would also partition between different phases according to the concentration gradient (Donnan effect) to maintain the chemical potential across the solution. Hence, this may further affect the stability of the protein biopharmaceutical due to pH shifts and changes in the ionic strength across the solution (Raut and Kalonia 2015a, Raut and Kalonia 2015b).

For therapeutic proteins, solution conditions such as the protein concentration and temperature may be prerequisites for phase separation (Wang et al. 2011) and are typically represented using a temperature-concentration phase diagram containing phase boundaries characterising the rich, lean and homogenous phases (Figure 1.4) (Asherie 2004). The liquid-liquid coexistence region is divided into two sections; a liquid-liquid region that is metastable (binodal) and another that is unstable (spinodal). The spinodal region refers to a region of
thermodynamic instability, with a single phase not existing due to the spontaneous split into two metastable liquid phases either side of this curve (lean and rich phases) corresponding to the binodal; these are in equilibrium with each other. These phases exhibit different physical properties such as density, concentration and refractive index (RI). The bimodal (also known as the coexistence) line illustrates how the protein concentration varies with temperature. This is a boundary between the homogenous protein solution and the region where droplets form. This region between the binodal and spinodal curves (i.e. metastable region) is where LLPS manifests slowly due to the formation of non-homogeneous protein droplets in this region (Ahamed et al. 2007). The critical point (which can lie anywhere on the binodal line) refers to the point where any extremely small change in the thermodynamic parameter (i.e. temperature) will result in phase separation of two distinct liquid phases; moreover extreme opalescence is observed here (Asherie 2004, Ahamed et al. 2007).

**Figure 1.4:** Simplified phase diagram representing the possible states of proteins. Adapted from (Raut and Kalonia 2015b).

### 1.6 External factors contributing to aggregation

Aggregation is a detrimental effect to the product quality. If the native protein is retained during aggregation (i.e. reversible aggregation) this can limit the solubility and alter the solution viscosity. With the latter, if the aggregates are condensed the resultant viscosity would decrease as aggregation manifests, however, if the aggregates assemble in to complexes with one another the viscosity would increase with aggregate formation (Roberts 2014).

Excipients are substances that are used to optimise the stability and solubility of the therapeutic drug. A number of biophysical techniques are available to understand the interactions between protein and excipient; in particular the use of high-throughput techniques
for screening numerous excipient conditions as each protein appears to behave differently with excipients thus this is a key requirement for the biopharmaceutical industry. The choice of excipients must meet certain standards set by the US or EU Pharmacopeia and should not impact on the safety, stability and efficacy of the therapeutic drug. However, these may cause immunogenic effects (Elvin et al. 2013). This is particularly important as even already approved excipients cause adverse reactions in some formulations; for example the presence of polysorbate in the formulation of the recombinant humanised anti-IgE mAb, Omalizumab, was associated with hypersensitivity reactions (Price and Hamilton 2007). Another example is the use of trifluoroethanol which was successful in preventing protein aggregation by the stabilisation of the alpha helices of the native structure; however chemicals such as trifluoroethanol can be toxic (Maggio 2008). The pharmaceutical industry is constantly looking for novel excipients to enhance the drug efficiency and allow the development of novel therapeutics.

1.6.1 Solution pH and buffering agents

Buffering agents are used to control the pH of solutions as this has a crucial role in sustaining the native structure of proteins and the chemical properties of its amino acids. The chemical stability of mAbs is highly pH-dependent with slightly acidic conditions being favourable. As chemical degradations such as deamidation and physicochemical properties (i.e. colloidal and conformational stability) are pH-dependent careful optimisation of buffer conditions for antibody solutions is critical (Karow et al. 2013). A suitable buffering system is one that has an appropriate overall ionic strength and favours the solubility and stability of the protein and any excipients within the formulation. Buffer concentrations are normally in the range of 10 and 100 mM. As alterations in the solution pH could result in aggregation it is important to keep the protein within a narrow pH range. It is imperative that the protein stability in the early formulation stages is studied in the range of pH 3-10 (Kamerzell et al. 2011). This range is important as a number of buffer ions can cause certain ion effects on the overall stability of the protein resulting not only in its stabilisation but destabilisation as well. Attention should also be given to crystallisation of the buffering system during freezing as this can create a drastic pH shift leading to the degradation of the therapeutic drug (Kamerzell et al. 2011).

1.6.2 Solubility

Protein solubility can be defined as the protein concentration in a saturated solution that is in equilibrium with a solid phase (either amorphous or crystalline) (Kramer et al. 2012). The necessity to formulate mAbs at very high concentrations (>150 mg/mL) is critical to meet the needs of administering these drugs in small volumes (1-2 mL) for subcutaneous injections. All proteins have an inherent solubility limit which cannot be crossed at a certain concentration. Solubility becomes a major problem if the protein remains folded upon aggregation (i.e. reversible self-association) resulting in lower protein concentrations to be used or changing the route of administration to intravenous delivery (Roberts 2014). A number of excipients are
added to increase solubility such as the amino acids arginine (Arg), aspartate and serine (Trevino et al. 2007). Moreover, solubility is influenced by extrinsic factors including pH, ionic strength, temperature and additives. There are difficulties in understanding the intrinsic factors which influence solubility due to the lack of quantitative solubility measurements. Currently, two methods are used: firstly by concentrating a protein by ultracentrifugation or secondly adding lyophilised protein to solvent; however limitations lie within both these methods. A more plausible method to address this is to use an external agent that quantitatively lowers the protein solubility resulting in precipitation, such as the molecular crowding agent polyethylene glycol (PEG) (Kramer et al. 2012). The mechanism of action is via an excluded volume effect where PEG is excluded from contact between the protein interacting areas (Asakura and Oosawa 1958, Arakawa and Timasheff 1985) resulting in protein precipitation (the principles are discussed further in Chapter 6).

1.6.3 Viscosity

MAbs formulated at high concentrations are faced with issues associated with increased solution viscosity. As high solution viscosities would hinder the development of a potential drug candidate, it is critical at the initial stages of product formulation the solution viscosity is investigated. The endeavour to develop new methods to determine solution viscosities is ongoing as a consequence of limited sample availability during early development (Nicoud et al. 2015). As with solubility, viscosity is greatly influenced by solution pH. Several diseases treated with biologics such as diabetes require regular treatment due to their chronic nature thus it is desirable to prepare highly concentrated therapeutics to prevent constant visits to the hospital and encourage self-administration of the drug (Cheng et al. 2013). However, it is not desirable to administer a viscous solution via the subcutaneous or intramuscular route. For subcutaneous administration the solution viscosity should be below 50 cP to reduce the time and force required for injection as above this can cause severe pain and discomfort at the injection site (Du and Klibanov 2011). At low concentrations the solution viscosity is proportionally dependent on protein concentration; however, this dependence becomes exponential at higher concentrations. It has been suggested that at high concentrations the increase in viscosity is not due to conformational changes but more towards reversible self-association through non-covalent PPIs such as electrostatic interactions leading to the formation of transient 3D protein networks which create a resistance to flow thus high solution viscosity (Guo et al. 2012). Common excipients that are added to highly viscous solutions include inorganic salts and hydrophobic salts. More recent studies showed the viscosity reduction capabilities of the amino acids, arginine hydrochloride (Arg·HCl) and lysine hydrochloride (Lys·HCl). It has been proposed their mechanism of action is similar to salts in weakening electrostatic interactions (Inoue et al. 2014). This is interesting as Arg·HCl is one of the most commonly used excipients involved in a number of varied roles including reducing aggregation and increasing the solubility of water-soluble drugs.
1.7 Classes of pharmaceutical excipients used in protein formulations

1.7.1 Hofmeister Series – Effect of salts

Salts can have a positive, negative or no effect on the stability of the protein; the effect largely depends on the protein type and concentration (ionic strength). Salts are normally added to concentrated mAb formulations as buffering agents, as well as viscosity and tonicity modifiers. Salts such as KCl and NaCl are used as excipients with the chloride ion being the most common (NaCl form) as it has proven to be highly effective in stabilising proteins such as Interleukin-1 receptor and in controlling solution viscosity in highly concentrated mAb formulations (Arosio et al. 2012). The ability of salts to precipitate protein generally follows a specified order which was discovered by Franz Hofmeister in 1888; this series ranks the relative ability of salts to either salt in or salt out proteins (Pegram et al. 2010; Cacace et al. 1997). The chloride ion is located at the centre of the Hofmeister series with chaotropic ions (at low concentrations) located on the left hand side that unfold the protein and increase protein solubility (e.g. thiocyanate, iodide) and on the right hand side are kosmotropes (at high ionic strength) which maintain the protein structure and cause proteins to salt out of solution thus decreasing solubility. In essence, the salting in effects stabilise the charged groups on the protein whereas as with salting out there is competition between the protein and salt for waters of hydration (Majumdar et al. 2013).

Extensive literature is available describing the Hofmeister series; however, the precise mechanism through which salts exert their action is still a mystery. Despite this, the series serves as an initial starting point in selecting salts to influence protein solubility, aggregation, denaturation and crystallisation (Baldwin 1996). Several research groups have classified these ions based on their relative abilities to constructively or destructively modifier the water structure (Tadeo et al. 2009). Based on this classification, kosmotropes are structure makers as a result of their strong hydration causing salting out whereas chaotropes are structure breakers. However, more specific interactions are thought to play a role due to the effects of the interactions between cosolvent and protein as well as the cosolvent on the solvent structure (Tadeo et al. 2009). This is important as cosolvents such as salts in protein formulations can impact the solubility and stability of the protein (Cacace et al. 1997). Salts can impact the protein solubility and stability based on their position in the series with the cations tending to be less effective in protein stability than anions due to their accumulation on the protein surface. Anions contribute more to the extent of protein aggregation caused from temperature changes and shaking. Anions and cations can elicit different effects from one another and alter the protein stability therefore careful selection and optimisation is required for each protein system (Maggio 2010).
1.7.2 Sugars/polyols/carbohydrates

Sugars/polyols are small neutral compounds which have a significant impact in inhibiting aggregation and stabilising the protein. The commonly used sugars are sucrose and trehalose which are osmolytes utilised in nature for microorganism stabilisation against harsh environmental conditions (Kamerzell et al. 2011). Sugars work more effectively with increasing concentrations as seen with sucrose for example (Maggio 2010) at higher concentrations in preventing surface adsorption (Wendorf et al. 2004, Maggio 2010). Sugars and carbohydrates have been found to stabilise proteins both in the liquid and lyophilised state although in the latter state crystallisation of sugars becomes a problem during the freezing process (mannitol for example) (Kamerzell et al. 2011). These classes of excipients are effective stabilisers due to preferential exclusion of the sugar molecules from the protein surface arising from their larger size than water molecules. This exclusion is highly repulsive and thermodynamically unfavourable thus in theory the excipient pushes the protein towards an equilibrium structure with the smallest solvent-exposed surface area hence the native structure is stabilised (Ohtake et al. 2011). Sugar alcohols such as sorbitol have been shown to increase the unfolding temperature and reduce aggregation. Moreover, studies have found that increasing polyol concentrations increased the folding temperatures with larger polyols conferring greater stability than smaller ones (e.g. glycerol and erythritol) (Ohtake et al. 2011). Additionally, sugars/polyols are common excipients used as cryoprotectants and lyoprotectants. Although sugars and carbohydrates have several beneficial effects as excipients they can have undesirable effects on the overall protein stability due to degradation and impurities (Kamerzell et al. 2011, Panzica et al. 2012). Surprisingly, sugars have been shown to increase mAb solution viscosity (Kamerzell et al. 2011).

1.7.3 Amino Acids

Sugars are one of the most widely used excipients; however, amino acids have proven to be as effective as or better than sugars especially for antibody therapeutics. Amino acids stabilise proteins by a number of mechanisms including preferential hydration (also known as preferential exclusion) or by direct interactions between the protein and ions. Preferential hydration refers to the excess water at the protein surface as opposed to preferential interaction where the cosolvent concentration is greater at the protein vicinity than in the bulk phase (Ohtake et al. 2011). There are several amino acids which are used as excipients for protein stability such as histidine, glycine and Arg·HCl. mAb formulations usually use histidine as a buffering agent due to its effective ability to control pH. Thus, amino acids have a number of roles in acting as a buffering agent, having antioxidant properties and their direct interactions with the protein (Kamerzell et al. 2011).

Arg·HCl is a widely used excipient in protein formulations for several reasons including preventing aggregation, promoting the refolding of proteins and as a solubilising agent in protein purification steps (Arakawa et al. 2007a). Other reported amino acids effective in
protein stabilisation include lysine and proline. The frequent use of arginine stems from its ability to increase protein solubility without compromising the stability. A complete mechanistic understanding of Arg·HCl has still not been determined thus ongoing studies are being conducted (Schneider et al. 2011).

Although, a range of different classes of excipients are available within the GRAS category, these are very much selected on a trial and error basis. As mentioned each protein behaves differently with excipients thus it would be ideal if a more ‘universal’ excipient were to be determined. The equimolar combination of the free L-amino acids L-Arg and L-Glutamate (L-Glu), which will be explored in detail in the current work, also belongs to the ‘amino acid’ class of excipients, and will be discussed in more detail in a dedicated section 1.10.

1.8 Methods utilised to study protein aggregation in this Thesis

Protein therapeutics entering clinical research and development require thorough characterisation and monitoring of their structural and biological integrity during the bioprocessing, formulation, manufacturing and storage stages. There is no single analytical method which effectively assesses protein aggregation (Joubert et al. 2012) and for this reason a variety of analytical techniques are used to detect any conformational changes or nucleation sites which may initiate aggregation (Gabrielson et al. 2007, den Engelsman et al. 2011). Analysing protein aggregates has been challenging due to their wide range of sizes and their complexity. The size of protein aggregates ranges from a few nm to µm and mm where they become visible and precipitate out of solution. A subset of these techniques was used in this Thesis; static light scattering (SLS), intrinsic fluorescence, size exclusion-high performance liquid chromatography (SE-HPLC), solution nuclear magnetic resonance (NMR) spectroscopy, viscometer and nephelometry. These will be discussed in more detail below.

1.8.1 Static light scattering and intrinsic fluorescence

Protein aggregation can be monitored using SLS and intrinsic fluorescence which reported on the colloidal and conformational stability of mAbs respectively. These were measured simultaneously using the Optim 2 (Avacta, Thorp Arch Estate, Wetherby). The data were processed using the standard manufacturing guidelines through the Optim analysis software package (Avacta 2013a). The changes in the SLS signal were indicative of changes in the weight averaged molecular mass manifesting due to the presence of protein aggregation; thus the signal is proportional to the aggregation state. The temperature-dependence of the SLS signal was recorded at 266 nm as an indicator for colloidal stability to give the onset of aggregation temperature, $T_{agg}$. $T_{agg}$ is defined as the temperature whereby the measured scatter reaches a threshold that is approximately 10% of its maximal value (Avacta 2013b).

The intrinsic fluorescence reported on the temperature of the on-set of melting, namely the mid-point temperature of the first unfolding transition, $T_{mi}$. An intrinsic fluorescence intensity ratio (350:330 nm) was determined from these experiments which is sensitive to the
tryptophan exposure as the protein unfolds (Avacta 2013b). Proteins contain three naturally occurring aromatic amino acid residues that contribute to the intrinsic fluorescence: tryptophan, tyrosine and phenylalanine. The fluorescence intensity of these aromatic amino acids can be ordered as tryptophan > tyrosine > phenylalanine. Tryptophan elicits the strongest fluorescence, tyrosine usually fluoresces when tryptophan is not present and phenylalanine is only fluorescent when both are absent. Fluorescence of proteins is usually detected between 300-400 nm with an excitation range within 250-300 nm (Printz and Friess 2012). These aromatic amino acids are particularly used for protein folding studies; as changes in the environment impact these hydrophobic residues. Briefly, in the native conformation these residues are buried in the core and during the unfolding they are exposed. Changes in their solvent exposure can provide information on conformational changes in proteins, in particular in their tertiary structure. This is useful for aggregation studies, as protein unfolding usually causes an increase in the fluorescence intensity. Fluorescence in the range of 350-355 nm indicates exposure of these residues whereas fluorescence between 325-330 nm can represent buried tryptophan (Bang 2006).

1.8.2 Size exclusion chromatography and Multiangle laser light scattering

SEC, often referred to as gel filtration or gel permeation chromatography, is the routine method used to detect and quantify protein aggregation and the quantity of monomer; typically this is an essential technique for regulatory approvals of all protein pharmaceuticals (Li et al. 2009). Generally, SEC is a non-denaturing method reporting on the apparent molecular weight, presence of aggregates and degradation products. However, nowadays SEC is primarily used to measure aggregation and degradation as the determination of the molecular weight requires calibrating the retention time against molecular standards which is not accurate unless the protein and the standards have similar conformations and interactions with the column. A downside to SEC is that highly concentrated protein solutions are diluted prior to SEC to maintain the resolution and prevent saturation during the process, therefore this would dissociate reversible aggregates (Mahler et al. 2009).

SEC separates proteins on the basis of their conformational size as the protein molecules travel through the column which incorporates particles of a given pore size and volume (Hong et al. 2012). The passage of molecules through the pores depends on the molecular size with the largest aggregates excluded first followed by subsequent protein molecules in order of decreasing size. The elution peaks for each molecule is determined relative to standards (den Engelsman et al. 2011). Additionally, aggregates large in size that are above the upper size limit of the column may be filtered out; this means the sample recovery needs to be checked constantly. It is known that the stationary phase of the SEC column matrix tends to nonspecifically bind to protein aggregates which possibly distort the end results. Therefore, prior to use of SEC, careful consideration needs to be made of the column type, buffer, pH, salt concentrations and even organic modifiers as optimising these parameters can improve separation (Gabrielson et al. 2007). In light of these problems it is advantageous to use
analytical techniques which avoid the use of columns (Arakawa et al. 2006). Thus, SEC is commonly coupled with molecular weight-sensitive detectors; particularly the combination of light scattering detectors such as multiangle light scattering (MALS) which determines the absolute measurement of molecular weight based on the radius of gyration from the angular dependence of the scattered light. SEC-MALS provides information on the size, shape and concentration of the sample (Tarazona and Saiz 2003). MALS is usually combined with two concentration detectors; ultraviolet (UV) and RI to accurately determine the molar mass of each component reporting on the oligomeric state and overall molar mass of the constituents (Challener 2014). The ‘actual’ molecular mass for each peak is proportional to the ratio of the light scattering and RI detectors. SEC-MALS can report on the monomer, dimer content as well as higher order aggregates (Hong et al. 2012).

1.8.3 Nuclear magnetic resonance spectroscopy

Solution NMR spectroscopy is a powerful technique that can be used to obtain information on macromolecules such as their conformation, structure and motions. This technique applied to large biomolecules came to light in the 1980’s when the first de novo structure of a globular protein was determined (Wuthrich et al. 1982), for which the Nobel Prize was later awarded in 2002 (Palmer and Patel 2002). NMR is unambiguously one of the most information-rich experimental techniques as it is capable of providing information on protein dynamics and structure on an atomic scale. It is a favourable technique for the exploration of protein motions due to its ability to study a wide range of dynamic processes on different timescales from rapid fluctuations (ns) to slower conformational transitions (μs) and global unfolding (ms-sec) (Sapienza and Lee 2010). Thus, this makes NMR spectroscopy a useful technique to characterise PPIs.

1.8.3.1 1D ¹H experiments

The nuclei of all elements have intrinsic properties such as charge, mass and spin. In the experiments described in this Thesis, the ¹H nucleus, which has a nuclear spin of ½, was used, as it is abundant in non-labelled proteins, such as commercially relevant mAbs produced in mammalian cell lines. Upon the application of an external magnetic field (B₀) in the z direction, the proton nucleus can align with B₀ either in the same direction (+ Z) or opposing (- Z) B₀ (i.e. two spin states, + ½ and - ½) (Shin et al. 2008) (Figure 1.5). At thermal equilibrium, the net magnetisation is energetically preferred along the Z axis. Hence, the spinning nucleus will precess around this magnetic field at a particular precession frequency called the resonance frequency. The external magnetic field that is applied along the Z axis determines the rate of proton precession. In these studies; the 800 MHz instrument was used; thus in this case if the external 18.8 Tesla magnetic field is applied this results in precession rate for the majority of the protons to be in proximity of 800 MHz. However, if the protons are exposed to different chemical environments then this results in slightly different precession frequencies. This is explained by the resonance frequency being affected by the electrons that surround the
nucleus, and in the vicinity, as this slightly shields \( B_0 \), hence the resonance frequency for each nucleus of the same type may differ depending on the electron density of the nucleus and its local environment (Marion 2013). These differences in the chemical environment give rise to the frequency of the peak in the spectra presented as the chemical shift (relative difference in frequency between the reference sample and sample being run). Chemical shifts (\( \delta \)) are recorded in parts per million (ppm) which is a normalised value that is not field dependent, and is calculated using Equation 1.4:

\[
\delta = \left[ \frac{v_{\text{sample}} - v_{\text{ref}}}{v_{\text{ref}}} \right] \times 10^6
\]  

(1.4)

where \( v_{\text{sample}} \) is the frequency of the sample and \( v_{\text{ref}} \) is the frequency of the reference standard (Harris et al. 2002).

**Figure 1.5:** Illustration of the precession of nuclei. Nuclei are subjected to an external magnetic field in the z direction (\( B_0 \)). The proton nucleus can align with \( B_0 \) either in the same (+ 1/2) or opposing (-1/2) direction.

In the magnet of an NMR spectrometer, in their equilibrium state, protons have a net magnetisation aligned along the z-axis, and the signal cannot be recorded. In order to measure the precession rate of the protons, a radiofrequency electromagnetic field (\( B_1 \)) is applied on the X-axis which rotates the net magnetisation 90° along the Y-axis. Once this 90° pulse is completed the magnetisation precesses in the x-y plane as only in this orientation can the signals from the oscillating spins be measured by the detector as a function of time which is known as the free induction decay (FID). However, this oscillation does not last forever as this magnetisation starts to decay to its equilibrium state along the z-axis; this phenomenon is known as relaxation which is described in the next section. For easier interpretation of the signals, a mathematical process known as Fourier Transform (FT) is used to translate this time-dependence of the detected signal into frequency spectra (i.e., distinct NMR peaks).
One of the key attributes of NMR is that certain signals can be monitored to study changes in the protein’s conformation. Using 1D $^1$H NMR experiments, aggregation can be monitored as the line width of peaks is associated to the molecular weight such that line broadening occurs with increasing protein size or protein self-association. Additionally, protein unfolding can be detected by monitoring characteristic signal changes in the methyl or amide regions (Tsai et al. 1998).

1.8.3.2 **Transverse relaxation experiments**

In NMR, relaxation describes the restoration process of the magnetisation to equilibrium after the magnetic moment has been disrupted by radiofrequency (RF) pulses. The two main relaxation processes are the spin-lattice (longitudinal) relaxation $T_1$ and spin-spin (transverse) relaxation $T_2$. $T_1$ relaxation refers to the reestablishment of the magnetisation to the direction of the external magnetic field which relies on the energy exchange occurring between the spins and the environment (lattice). Hence, this is the longitudinal relaxation rate in the z-direction referred to as $R_1(1/T_1)$. $T_2$ relaxation depends on the fluctuation of the local magnetic field strength leading to the dispersion of the phase coherence of the spins in the x-y plane. This gradual loss in x-y magnetisation follows an exponential decay in this plane. The rate at which this magnetisation decays to its equilibrium (zero) is described by the time constant, $T_2$, or the transverse relaxation rate constant, $R_2 (R_2 = 1/T_2)$. The signal detected in the x-y plane from the oscillating spins is measured as a function of time (Cavanagh et al. 2007).

In this Thesis, $T_2$ relaxation measurements were used to assess aggregation. Transverse relaxation measurement is based on spin echoes which is the refocussing of the magnetisation. An initial 90° pulse rotates the magnetisation into the x-y plane where the spins precess at slightly different rates due to their chemical shift, resulting in losses in their coherence and, additionally, relaxation occurs due to transient magnetic fields typically caused by molecular motion and chemical exchange processes. However, the loss in coherence due to chemical shift differences (but not due to relaxation) can be refocussed by applying a 180° refocussing pulse which flips the spins in the negative y axis and ultimately the spins regain phase coherence at time $2\tau$, resulting in the build-up of an echo which reaches a maximum where there is optimal coherence of the nuclei. In this Thesis, $T_2$ was measured using a standard Carr-Purcell-Meiboom-Gill (CPMG) pulse echo sequence. CPMG uses a train of successive 180° pulses which refocuses the spins allowing the whole decay process to be acquired. This process can be simply described as $90^\circ x \cdot [d20^\circ - 180^\circ y - d20^\circ - echo]_n; d20$ is a time delay which is repeated several times (Grzesiek 2003). Thus, the resulting signals from the spins are measured as the FID (Figure 1.6).
Figure 1.6: Schematic diagram of the pulse sequence used for the $^1$H $T_2$ measurements. These were acquired as 1D acquisition with presaturation (Carr-Purcell-Meiboom-Gill (cpmgr1d)). The refocusing element is repeated $n$ times. The CPMG $T_2$ experiment produces signal intensity dependence on $\tau$ where $\tau$ is the total evolution time (ca. $2\Delta n$) and $\Delta=d20$ is the refocusing delay time. The resulting signals from the spins are detected by the detector and measured as the FID.

The line widths of the signals in the NMR spectra are determined by $T_2$, a narrower line width indicates faster motion (longer $T_2$) in contrast to broader line widths indicating slower motion (shorter $T_2$); these are dependent on the molecular weight and conformational states of the molecule. This is useful for the studies here as factors such as changes in temperature, viscosity and molecule size can cause changes to the tumbling rate which results in either resonance broadening or sharpening (Jelinski and Melchior 2000). Within protein structures, there are several internal motions which occur on different time scales, from the literature it is known that $T_2$ is dependent on the molecular tumbling (i.e. rotational diffusion) occurring on the picosecond to nanosecond timescale (Markwick et al. 2008). This molecular tumbling can be quantified using $\tau_c$ which is defined as the time it takes a particle to rotate by one radian. This is a quantitative measure of the rate of molecular motion which describes the time taken for a nucleus to interact with a neighbouring nucleus before it rotates away by thermal motion of the corresponding molecules. Larger molecules (e.g. mAbs) will have a longer rotational correlation time due to slower tumbling. Figure 1.7 illustrates the fairly linear relationship between $R_2$ and $\tau_c$ in the range of $\tau_c$ between $10^{-9}$ and $10^{-6}$ s, which correspond to typical protein molecules or their aggregates. In the region we are interested in for larger proteins i.e. mAbs, $R_2$ is roughly proportional to $\tau_c$; in turn, $\tau_c$ is largely proportional to the molecular weight of the protein. These assumptions will be reiterated later on, to describe the apparent protein cluster size derived from the measured values of $R_2$, in the experiments conducted in the current study.
1.8.3.3 Diffusion experiments

NMR measurements of the translational motion of molecules in liquid provide useful information regarding the geometric properties of soluble proteins, such as determining their aggregation state. This translational motion is known as Brownian molecular motion and is usually referred to as self-diffusion. Stimulated echo Pulsed Field Gradient (SE-PFG) NMR spectroscopy was used here to measure the translational diffusion of molecules using the diffusion ordered spectroscopy (DOSY) method. This method allows the translational diffusion coefficient, $D$, to be determined (Stallmach and Galvosas 2007).

A schematic diagram of the SE-PFG pulse sequence is shown in Figure 1.8. This pulse program is based on a spin-echo pulse sequence where a 90° pulse is applied which rotates the magnetisation from the z plane into the x-y plane (transverse plane). Here the magnetisation starts to dephase, after a specified length of time, a 180° pulse is applied which rotates the magnetisation through 180° along the x plane. This 180° pulse causes the refocussing of the magnetisation which results in an echo signal. To allow diffusion measurements, in this pulsed gradient spin echo sequence, two gradient pulses are introduced before and after the 180° pulse which have the same amplitude ($g$) and width ($\delta$) and are separated by a specified time ($\Delta$) (Figure 1.9). The introduction of these gradient pulses along the z axis (longitudinal) dephases the magnetisation from spins which have diffused to a different position in $\Delta$; this pulse will not impact spins which have not moved from their original position. After the second gradient pulse, this spin will experience an equal but opposite phase which cancel each other out. Hence, the spins which have not moved come back in phase to produce an echo signal, however, the spins which have diffused to a new location do not come
back into phase causing the height of the echo signal to reduce (Figure 1.9) (Hrabe et al. 2007, Stallmach and Galvosas 2007).

**Figure 1.8:** Schematic of the SE-PFG NMR pulse sequence used to measure $D$. The diagram illustrates the gradient length ($\delta$) and the diffusion time ($\Delta$).

The signal measured is collected from the entire sample and integrated for selected spectral peaks of interest, with molecular diffusion leading to the attenuation of the NMR signal intensity, which depends on the diffusion time ($\Delta$) and the gradient parameters ($g, \delta$) (Dehner and Kessler 2005). The changes in the intensity can be described by Equation 1.5:

$$I = I_0 e^{-D\gamma^2 g^2 \delta^3 (\Delta - \frac{\delta}{3})}$$  \hspace{1cm} (1.5)

Where $I$ is the measured intensity, $I_0$ is the integral in the absence of the applied magnetic field, $D$ is the diffusion coefficient, $\gamma$ the gyromagnetic ratio, $g$ the gradient strength, $\delta$ the gradient length and $\Delta$ the diffusion time.
Figure 1.9: Schematic illustration of the effects of diffusion. The illustration shows the magnetic field gradient pulses and the rearrangements of the nuclei between this. The physical movement of the nuclei decreases the maximum capability of the refocussing pulse thus resulting in the reduction of the signal strength. The red arrows show the direction of the spins between each stage and the green double headed arrows show the changes in the original spin position after diffusion has occurred.

These experiments are valuable as protein aggregation, at least in a dilute solution, should cause an increase in $R_h$ leading to a decrease in $D$; this can be exploited here to measure if the presence of increasing Arg-Glu concentrations can prevent aggregation (Mansfield et al. 1999). Other factors, such as molecular crowding, or increase in solution viscosity, also slow down the diffusion (Rivero, S. and Witten, T. B., 1972, Nenninger et al. 2010).

1.8.4 Rheometry

Rheometry is used to measure the solution viscosity which is important as this attribute in highly concentrated samples can cause formulation delivery challenges. Also, parameters such as the $D$ and $R_h$ of the protein are greatly dependent on the solution viscosity thus this needs to be considered. There are a number of viscometers and Rheometers available to measure viscosity, in this Thesis the microfluidic device; Micro viscometer-Rheometer on a chip (mVROC) was used. Fluid flow is a complex phenomenon; fluids are usually termed as either Newtonian or non-Newtonian depending on the fluid viscosity behaviour as a function of shear rate, stress or deformation. Newtonian fluids describe the flow behaviour of fluids as a linear relationship between shear stress [mPa] and shear rate [1s] thus the proportionality constant $\eta$ is the solution viscosity [m Pas] with water as an example. The fluid viscosity of Newtonian samples are only dependent on temperature hence the solution viscosity is constant no matter how aggressively the solution is forced through a channel (Rheosense 2016b). The measuring cell (or ‘chip’) contains a rectangular slit flow channel made of borosilicate glass with a uniform cross-sectional area. Upon injecting the sample at a constant
flow rate through the flow channel several pressure sensors are found on the lower base of the instrument which records the drop in pressure as the sample is pushed from the inlet to the outlet. As a result the software plots the linear dependence of the measured pressures versus sensor positions where the slope is proportional to the solution viscosity (Rheosense 2016a). The use of rheometry in this Thesis was to assess the differences in solution viscosity between various mAb formulations; more viscous formulations would show an increased drop in the pressure when the sample is being pushed through the capillary.

1.8.5 Use of nephelometry to measure solution solubility

The relative solubility limits of different formulations can be assessed using the Nephelostar Plus which uses a laser diode at 635 nm. The decrease in solubility is induced by protein precipitation with a molecular ‘crowder’ for example polyethylene glycol (PEG). This particular method will be discussed further in Chapter 6. The nephelometer measures the turbidity recorded in Nephelometric Turbidity Units (NTU) (BMGLabtech 2016). The laser beam passes through the sample into an integrated sphere which provides uniform light scattering. If the solution is completely transparent the light passes directly through thus no signal measured. However, particulates will scatter light which is reflected within the sphere interior and the signal is recorded by a photodiode. In this Thesis, this technique was used to detect the increase in PEG-induced precipitation of mAb in different formulations reporting on the relative solubility limit; if the protein is heavily precipitated in the presence of PEG this would result in greater light scatter indicating this particular formulation is not relatively soluble (described in more detail in Section 6.3.2).

1.8.6 Controlled temperature storage stability studies

The ability for formulation scientists to predict the propensity of mAb formulations facing aggregation issues is commonly carried out via forced degradation studies. Developing safe therapeutics which are able to withstand the chemical, thermal and mechanical stress encountered during their early production through to formulation, storage, transport and administration is the primary goal in protein formulation (Weiss et al. 2009). The steps in optimising the formulation can be difficult and lengthy. During these stages, alterations in pH, temperature, or storage conditions can all affect the stability of the therapeutic. Long-term stability studies of the formulated sample can provide a valuable insight into the protein stability under different stress conditions reporting on the shelf-life of the therapeutic (Weiss et al. 2009). Both the FDA and the international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) clearly state the necessity to provide sufficient stability testing data to understand how the influences of various environmental factors affect the drug quality and product. The ICH guideline states that stress testing is required to report on the intrinsic stability of the protein as well as the potential degradation pathways (ICH guidelines 2003). Typically, long-term stability testing takes between 12 months to 2 years which is not a feasible length of time to monitor the stability of
every formulation condition; thus to screen an array of formulations in a faster manner, forced
degradation studies are conducted lasting up to 6 months which is a more manageable time
length (Sehrawat et al. 2010). During forced degradation studies the formulations are
subjected to extreme conditions which are known to cause degradation such as elevated
temperatures, freeze-thaw cycles and agitation. The use of accelerated temperatures is a
common approach to report on the protein thermal stability. The temperatures generally used
are refrigerator (5 °C), ambient (25 °C) and accelerated (40 °C); the former two temperatures
(5 and 25 °C) are conducted to observe the stability at real-time storage temperatures for at
least 12 months. The latter is an accelerated temperature which increases the degradation
rates and enhances the likelihood of observing significant differences among the mAb
formulations over a shorter period of time (Goldberg et al. 2011, Bajaj et al. 2012).

1.9 In vitro toxicity studies of excipients

1.9.1 Regulatory guidelines

There are strict guidelines in place from the US FDA when a novel excipient is proposed,
normally as part of a new drug substance for clinical trial (common technical document, CTD
or marketing authorisation application, MAA). These specific guidelines clearly state the
information required for excipients which are not part of the pharmacopeia; these include full
details of the excipients manufacture, characterisation and control with cross reference to
safety data. By definition excipients are inactive ingredients which are purposely incorporated
in the drug product for a number of reasons such as increasing product stability. For the
approval of a novel excipient, careful characterisation studies need to be executed and any
safety and efficacy concerns must be documented (Katdare 2006). There are a vast number
of excipients used for different purposes; for biological therapeutics commonly used excipients
include sugars and amino acids. Traditionally, these excipients were classified as being inert
by the FDA, however, with time pharmaceutical scientists realised excipients are in fact active
and can markedly impact the manufacture, safety and efficacy of the final dosage form
(Katdare 2006). In 1937, toxic effects were initially shown following an incident from
sulfanilamide causing deaths of thousands of children; this led to the FDA putting guidelines in
place for safety testing (Wax 1995). Nowadays, the FDA requires drug formulations to be
labelled with the content as well as clearly stating any safety issues which may be associated
with these additives (Richard A. Helms 2006). Another concern associated with excipients is
their ability to cause adjuvant activity on the immune system that may result in an inflammatory
response; therefore stringent regulatory guidelines need to be followed and met before
classifying any excipient as clinically safe for use.

1.9.2 Adjuvants and inflammation

When foreign substances enter the body, this can cause the direct simulation of the immune
system, however these substances are not always immunogenic (not capable of provoking a
specific response therefore no stimulation of specific B or T cells directed against that specific
foreign substance), instead these may act as adjuvants. Adjuvant comes from the Latin word *adjuvare*, meaning to aid which in immunology is a term coined for any material that can augment a specific immune response to other foreign materials (known as antigens) without causing detrimental toxicity or capable of stimulating an immune response in its own right (Hunter 2002, Reed *et al.* 2009). In the pharmaceutical industry, there are precedents for the use of adjuvants for vaccination formulations in order to enhance the immune response, resulting in an increase in antibody production, and hence, a more profound effect which mitigates the further need for multiple immunisation steps and reduces costs. This concept was first investigated by Raman who detected an increase in specific antibody titres when an abscess was present at the inoculation sites. A handful of adjuvants have been licensed for human use such as aluminium and calcium salts, however, due to toxicity implications no others have been licensed for routine clinical use (Sivakumar *et al.* 2011). Although being useful in this biological context (vaccination), materials acting as adjuvants can cause unwanted immune-enhancing effects in other therapeutic contexts such as during treatment with biologics by numerous functional pathways. Of particular concern in this Thesis is the ability of adjuvants to stimulate unwanted danger signals, for example reactions at the injection sites. In some instances, a material which is seen as foreign (non-self) is often not sufficient to mount a productive immune response; therefore, the foreign material needs to be recognised as a ‘danger’ signal (Gallucci and Matzinger 2001). The host is frequently exposed to non-self material such as gut commensal bacteria; an average human gut contains approximately $10^{14}$ foreign commensal bacteria that are symbiotes which the host will not want to react to as they are not causing any damage and are in fact beneficial thus only the foreign and potentially dangerous bacteria will be the ones the host responds (Malys *et al.* 2015).

The differentiation of naïve T cells into effector T cells (activated short-lived cells which defend the body during an immune response) requires multiple signals; these signals include the expression of co-stimulatory surface markers by APCs (DCs, macrophages and B-cells) and cytokine signals (Janeway CA 2001). This is a desirable response as the initiation of the adaptive immune response leads to the formation of memory B and T cells which are circulating in an inactive state; if, however, these cells are exposed to the same ‘danger’ these cells become activated to clear the therapeutic agent. These processes will be explained in detail below. Adjuvants can increase acute or sub-acute tissue damage around the injection site stimulating the release of ‘danger signals’ detected by immune cells. The ‘danger signal’ alarms the immune system of tissue damage which initiates an inflammatory response (i.e. innate immune response) (Allison and Byars 1991, Schijns 2000). In light of these effects, an understanding of the general principle of the inflammatory process is necessary.

### 1.9.3 The inflammatory response

The immune system consists of a complex network of cells with lymphocytes (white blood cells) being important immune cells. Almost all immune cells, except for neutrophils, monocytes and basophils are located in the blood under homeostatic conditions with small
numbers in resting tissues (Sokol and Luster 2015). The initial phase of the host response to common microorganisms and bacterial infections relies on the innate immune system which involves macrophages and neutrophils as a first line of defence. However, if these cells via their PRRs are unable to specifically recognise the pathogen, the adaptive immune system will be initiated (discussed in section 1.1.2). The T and B-cell specific receptors alone are able to recognise the diverse antigens however they need the ‘danger signal’ for the productive immune response thus their differentiation into effector cells (Murphey 2007). Moreover, memory T cells may be produced which have the capability to target a much more aggressive response on subsequent exposure (Janeway CA 2001). The on-set of inflammation is a process that primarily involves the innate immune system, triggered by any one of these harmful agents; infections (bacteria and viruses), irritants, physical trauma and extreme temperature. These stimuli cause the recruitment of a number of blood derived products including plasma proteins such as complement and leukocytes in to the injured area which causes the swelling associated with inflammation. These products initiate narrow blood vessels to expand encouraging blood flow resulting in redness and increased heat. The classical clinical symptoms of inflammation include pain, swelling, redness and heat. This may seem detrimental to the body, however, the increase in protein-rich fluids and extravasation of plasma and innate immune cells destroys the infection and prepares the repair processes (Harada et al. 1994, Zitvogel et al. 2010). Inflammation is a biological response with the primary roles to destroy or seclude the disruptive agent, eliminate damaged tissue and finally restore tissue homeostasis (Ashley et al. 2012). Inflammation was considered to be a disease for many years, however, over time this process has been accepted as a life preserving process, quoted by a surgeon in 1976 “Inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease” (Scarborough 1976). Moreover, previous studies have shown increased susceptibility to infection in individuals with genetic deficiencies in components involved in inflammation (e.g. neutrophils) and also knockout of proinflammatory cytokines (Bunting et al. 2002, Nathan 2002). The process of inflammation can be thought of as consisting of inducers, sensors, mediators and target tissues (Figure 1.10). The most common stimulus promoting inflammation is infection (Nathan 2002)
Figure 1.10: Schematic illustrating the inflammatory process. Firstly, inducers such as infection, initiate the inflammatory process that is detected by a number of sensors on sentinel cells including TLRs. These go on to stimulate the production of inflammatory mediators. These mediators elicit their effect on different target tissues altering their function to combat the pathogen. This final stage has been expanded showing the migration of circulating leukocytes by squeezing through gaps in the endothelium so that they can get into the endothelial cells (infected tissues) where further key mediators are upregulated such as the adhesion molecules, ICAM-1 and NO that are involved in the movement of leukocytes along the cell wall. Figures changed and combined from different sources (Medzhitov 2010, Navarro-Gonzalez et al. 2011).

The first line of defence against any infection or trauma is the skin and other barrier tissues. (Rock and Kono 2008); if these inducers succeed in penetrating these barriers, the resident immune cells sense pathogen invasion via the transmembrane receptor called germline-PRRs (Takeuchi and Akira 2010, Sokol and Luster 2015). These receptors recognise common molecular patterns found on majority of threats conserved in microbes called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS). Examples of PRRs are Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat containing receptors (NLRs). Moreover, evidence also suggests PRRs can recognise molecules released from damaged, stressed or malfunctioning cells undergoing necrosis (unprogrammed cell death) that act as endogenous danger signals, called damage-associated molecular patterns (DAMPs). These promote and exacerbate the inflammatory response with well-known examples such as high mobility group box-1 and uric acid (Medzhitov 2010, Schaefer 2014). TLRs are predominantly found on macrophages, mast cells and DCs, the three sentinel cells of the innate immune system. Once these receptors bind to their ligand, signals are transmitted to the nucleus where the transcription factor nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) is activated via numerous transcriptional and posttranscriptional mechanisms. This leads to the expression of
proinflammatory mediators such as histamine and cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). NF-κB is one of the most central regulators of the proinflammatory gene expression. Cytokines are small secreted proteins (~5-20 kDa) that function as signalling molecules to orchestrate the inflammatory and immune response. Along with these are another class of smaller cytokines called chemokines that regulate cell trafficking of immune effector cells to the site of inflammation i.e. chemo-attractants (Zlotnik and Yoshie 2000). Cytokines, along with chemokines (e.g IL-8), changes in expression of vascular adhesion molecules (e.g. Intercellular Adhesion Molecule-1, ICAM-1) and various costimulatory molecules work together for the recruitment of effector cells such as monocytes and neutrophils to the damaged tissue site. Leukocytes usually reside within blood vessels, however, at the site of inflammation; these mediators promote their entry into extravascular tissue. The activation of neutrophils leads to the release of toxic chemicals from their granules (i.e degranulation) such as reactive oxygen species (ROS) and reactive nitrogen species (NOS) which go on to the clear the infection.

1.9.4 Key inflammatory mediators

Leukocytes are constantly on surveillance in numerous cellular compartments, however, in order to fulfil their immune effector functions, they must have the capabilities of stopping, responding to stimulatory signals and moving on (Long 2011). The recruitment of leukocytes at the site of inflammation depends on the type, exposure and time intervals from the tissue injury indicating the presence of specific cell type leukocytes chemotactic factors (Harada et al. 1994). Chemokines are important mediators in the inflammatory pathway. One chemokine that has gained much attention is IL-8; it is an inducible chemokine whose production is stimulated in various cell types by other proinflammatory cytokines such as IL-1 and TNF-α. IL-8 plays a vital role in acute inflammation, being the main chemotactic factor for neutrophils, the most important phagocytic cell in many different types of inflammatory reactions and may also play a role in macrophage and fibroblast chemotaxis (Harada et al. 1994). IL-8 is secreted by many cells including monocytes, fibroblasts and epithelial, tumour and endothelial cells and belongs to the CXC subfamily of chemokines which bind to the IL-8 receptors, CXCR1 and CXCR2 expressed on these cells mentioned (de Oliveira et al. 2013, Sokol and Luster 2015). The key role of IL-8 in the inflammatory response is the chemotaxis of immune cells to the inflamed site; in particular its uniqueness arises from its ability to cause a massive accumulation of neutrophils that are usually circulating in the bloodstream (Qazi et al. 2011). Recent studies have confirmed the role of IL-8 in recruiting neutrophils under inflammatory conditions, hence confirming its importance (de Oliveira et al. 2013). This circulating innate cell becomes the major immune cell during inflammation which are attracted to sites of inflammation as a result of the concerted gradient actions of chemokines, with the most potent being IL-8. In addition to its role in neutrophil migration, IL-8 also promotes exocytosis of stored proteins and phagocytosis. First, the adhesion of neutrophils to the endothelial cell wall promotes migration along the endothelium; secondly, IL-8 stimulates the release of soluble
stored proteins for degranulation and lastly the engulfment of harmful substances (i.e. phagocytosis) aiding in the tissue repair process (Baggiolini and Clarklewis 1992). Moreover, it promotes the chemotaxis process, the release of ROS and expression of adhesion molecules. Non-pathological tissues have very low levels of IL-8 however this is augmented in response to pro-inflammatory stimuli such as LPS, TNF-α and IL-1 (Harada et al. 1994). As TNF-α and IL-1 levels are increased by the inducers of inflammation mentioned above, this causes an increase in IL-8 levels (Baggiolini and Clarklewis 1992, Jundi and Greene 2015).

As mentioned above, the recruitment of neutrophils to inflamed sites follows the sequential steps of rolling along the capillary vessel walls, their induced arrest on the vessel wall by chemokines and migration between the vascular endothelial cells (Long 2011). Another central component of the inflammatory response is ICAM-1 also known as CD54. ICAM-1 (90 kDa) is an adhesion molecule that is a member of the Ig superfamily with functions that relate to its role in cell adhesion and migration (Lawson and Wolf 2009). Its ligands include the matrix factor hyaluronan and the β2 integrin receptor lymphocyte function associated antigen-1 on T cells; interaction with its T cell ligand provides costimulation and therefore activation of the adaptive immune response (Simon et al. 1991). ICAM-1 is a cell-surface glycoprotein, constitutively expressed at basal levels on endothelial cells or on some lymphocytes and monocytes with its expression significantly increased by inflammatory mediators (IL-1, IFN-γ, LPS etc). In more detail, ICAM-1 facilitates a number of cell interactions; leukocyte-leukocyte, leukocyte-endothelial and leukocyte-epithelial as well as adhesion-dependent phagocytosis. ICAM-1 is important for the adherence of leukocytes on the endothelial cell wall and their migration out of blood vessels into the sites of inflammation (Hubbard and Rothlein 2000, Lawson and Wolf 2009). This trans-endothelial migration has been described as four sequential steps; in brief this involves (i) first the rolling of leukocytes along the endothelial cells managed by other selectins on the cell surface; (ii) secondly chemokines (as described above) activate integrins on the leukocyte cell surface increasing their adhesion to their ligands and aids in their migration; (iii) thirdly this strong interaction causes a spread of these activated leukocytes across the endothelium (e.g. LFA-1/ICAM-1) and (iv) finally these leukocytes can squeeze through the gaps in the endothelium to get into the endothelial cells (i.e. infected cells) (Lawson and Wolf 2009).

CD86 (or B7-2) is another costimulatory molecule expressed on a number of leukocytes, including DCs and B and T lymphocytes necessary for T cell activation and survival. Literature has shown its upregulation during DC maturation following inflammatory stimuli (Sansom et al. 2003). The antigen presenting molecule major histocompatibility complex (MHC) class II (or human leukocyte antigen (HLA)-DR) is a glycoprotein expressed on most immune cells that are upregulated in response to signalling. Its key role is its antigen presenting capability to the immune system to either elicit or suppress T helper cell responses leading to antibody production against the antigen. HLA-DR is a key inflammatory biomarker usually found in tissue in non-APCs and is used to indicate immune activation due to its

Another important signalling molecule in the pathogenesis of inflammation is nitric oxide (NO). NO does not bind to any specific receptor but enters cells depending on its concentration, association with other molecules and the location of the target cell (Coleman 2001). In normal physiological conditions, NO provides anti-inflammatory properties, however, during tissue disturbances it acts as a proinflammatory mediator inducing inflammation. NO is a paracrine mediator released by endothelial cells and neurons. It has a very short biological half-life (few seconds) due to its rapid oxidisation; hence, is only effective at the immediate locality it is produced. NO is synthesised by the conversation of the amino acid L-Arg with the cofactors oxygen and NADPH to L-citrulline and NO via the enzyme nitric oxide synthase (NOS). Three isoforms of NOS exist: neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS). nNOS plays a role in preventing leukocytes and platelets from adhering to the vascular walls; eNOS acts as a neuromodulator and iNOS plays a key roles in the inflammatory process (Wallace 2005, Sharma et al. 2007, Abramson 2008). The initial two are activated by different mechanisms to iNOS which is the most long lived with the production of NO in macrophages, monocytes and other cells induced by iNOS and L-Arg. iNOS is stimulated by inflammatory cytokines, TNF-α and LPS (Guzik et al. 2003) resulting in the production of very high and toxic amounts of NO. As mentioned previously, NO is a paracrine mediator although large amounts of NO can inhibit iNOS itself in a negative feedback mechanism (Guzik et al. 2003). The actual role of NO in inflammation is highly dependent on the environment as to whether inflammation is enhanced or retarded (Nathan 1997). The role of NO in inflammation is still ambiguous with it seeming to have a range of effects from apoptotic to anti-apoptotic during the inflammatory cascade (Coleman 2001). It is thought that NO can control the release of inflammatory mediators from immune cells such as leukocytes and macrophages, control blood flow, facilitate the adhesion of neutrophils to the endothelial wall and activate a number of enzymes all of which contribute to the inflammatory process. The role of NO can be summarised as a regulatory signal initiated by a cascade of proinflammatory events leading to a classical feedback cycle which then down-regulates inflammation (Kolb-Bachofen et al. 2006). This latter stage is vital during inflammation to shift from a tissue damaging response to tissue repair; experimental studies have shown that iNOS “knock-out” mice display higher risk of infections and decreased or excessive inflammation (Nathan 1997). Low concentrations of NO appear to show anti-apoptotic effects whereas higher concentrations cause cell death either apoptotic or necrotic although these effects are dependent on the cell type (Coleman 2001). Due to its production in many cells and participation in different areas of the inflammatory response; its mechanism of actions relies on its concentrations, cellular location and whether these cells have prior activation (Nathan 2002).
1.9.5 Injectable formulations

Final mAb formulations intended for subcutaneous administration requires optimal solubility, increased shelf-life and an acceptable tolerability upon injection. To achieve the first two requirements, excipients are usually added to the final formulation, however it is imperative this excipient does not elicit any unwanted immune effects when administered. Several factors can impact the tolerability of a drug with one imperative aspect being the solution osmolality, hence the importance for the formulation scientist to consider such parameters during product development. If any components of the drug product cause cellular stresses, such as an imbalance in the osmolality, this may lead to an adjuvant effect caused by the excipient resulting in an unwanted inflammatory response (Gallo and Gallucci 2013). As described above, this type of activation can be referred to as endogenous ‘danger signals’ which alert the immune cells due to changes in the steady state of the cellular environment. Maintaining osmolality is important as the administration of a drug into the body should have the same osmolality as the body’s fluids (blood is 280-295 mOsmol/kg) to maintain an isotonic environment; this is when the total molar concentration of the solutes is equal in two environments, hence, the movement of water out of a cell is balanced by movement of water into the cell. If the concentration of solutes outside the cell is more than inside the cell this causes more water movement into the cell resulting in cell swelling; this is a hypotonic environment. On the other hand, if the solute concentration is less outside the cell than inside which causes more water to move outside the cell resulting in shrinking of the cell; this is a hypertonic environment (Brocker C 2012). Hypotonicity has been shown to act as a danger signal in the context of immune and inflammatory responses (Compan et al. 2013) and it has also been reported that osmotic shock due to hypertonicity induced the production of the proinflammatory cytokine IL-8 by human peripheral blood mononuclear cells (Shapiro and Dinarello 1997). Therefore, it is imperative to investigate if novel excipients cause adjuvant effects leading to the onset of inflammation by monitoring the behaviour of these key inflammatory components and mediators discussed above. This is commonly carried out using in vitro cell culture systems to assess cell viability and markers of cellular stress in the presence of a range of osmolalities which are intended for drug use.

1.10 Arginine glutamate as a novel pharmaceutical excipient

New and safe excipient combinations working synergistically, such as Arg-Glu, have been recently endorsed (Golovanov et al. 2004, Hautbergue and Golovanov 2008, Blobel et al. 2011, Kheddo et al. 2014, Kheddo et al. 2016b), suggesting that the search for new excipient combinations even within the GRAS category can significantly improve the storage stability and injectability of mAbs (Pifferi and Restani 2003). As mentioned in Section 1.7.3, Arg is one of the most frequently used excipients in protein formulation in the hydrochloride form (Arg-HCl). The mechanism of action of Arg-HCl and sodium glutamate (NaGlu) was previously investigated where the authors found both these amino acids could stabilise proteins via a preferential hydration mechanism which was also compared to the salt LysHCl. The authors
found that the three charged groups on the lysine and glutamate ions caused a strong cohesive force on water molecules providing the energy required for the salts to be excluded from the protein vicinity into the bulk solution (Arakawa and Timasheff 1984, Kita et al. 1994). Conclusions drawn from a number of similar studies indicated at relatively high concentrations (>100 M) Arg or Glu preferentially hydrates proteins, although at low concentrations (<100 M) the overall protein charge of the protein governs the specific binding of the ions which dominates over preferential hydration effects with the protein thermodynamic stability not affected (Golovanov et al. 2004).

The equimolar combination of the free L-amino acids L-Arg and L-Glu was first described by Golovanov et al to substantially decrease protein aggregation in highly concentrated formulations. For example, using equimolar concentrations of L-Arg and L-Glu (50 mM) increased the solubility of selected test proteins by a factor between 4 and 8 (Golovanov et al. 2004). Adding these amino acids individually only increased the solubility by less than 1.5 times. The shelf-life, stability and solubility were increased with a reduction in protein precipitation and degradation in the presence of this mixture (Golovanov et al. 2004). An increase in the $B_{22}$ (Valente et al. 2005), enhanced native oligomer (Blobel et al. 2007) and thermal stability (Vedadi et al. 2006) have also been observed in other studies. Other amino acid combinations have been reported such as Arg and aspartate (Fukuda et al. 2014) (further discussion will be provided in Chapter 3).

1.10.1 Mechanism of action

The authors from these combined studies (mentioned in 1.10) have speculated that these charged amino acids interact with oppositely charged residues on the protein surface (Golovanov et al. 2004); more specifically L-Arg interacts with the protein through its charged or aromatic residues while the presence of other charged groups can interact with other Arg or Glu molecules in the vicinity. In a similar manner, Glu interacts via hydrogen bonding through its positively charged residues to the protein surface with their other carboxylate groups being free to interact with Arg molecules nearby (Blobel et al. 2007, Shukla and Trout 2011). Thus, the majority of exposed hydrophobic patches are covered hence reducing aggregation. The combination of hydrophobic and electrostatic components would probably provide more effective counterion binding than other monovalent ions such as Na+ and Cl− typically added to increase protein solubility (Golovanov et al. 2004).
1.11 Aims of the Thesis

The main aim of this Thesis was to assess the ability of Arg-Glu in preventing aggregation and increasing stability and solubility in industrially-relevant mAbs. This was investigated using a number of biophysical techniques, and the potential issues with toxicity of these excipients were investigated in cell culture models. The findings obtained from this Thesis are building on previous studies where Arg-Glu has increased solubility and stability in several diverse aggregation-prone proteins mainly used in structural and functional studies (Golovanov et al. 2004). Hence, it was of interest to assess the applicability of Arg-Glu in protein therapeutics which also require these optimal characteristics.
Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Monoclonal antibodies

Five different test mAbs were used in this Thesis as these represented the structure and behaviour of mAbs of industrial significance. The identities of any of the antibodies used were not disclosed for reasons of confidentiality, and so were designated “mAb1”, “mAb2”, “mAb3”, “mAb4” and “mAb5” (Table 2.1).

Table 2.1: Tabulated properties for the formulations of four different test mAbs supplied.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Initial formulation buffer</th>
<th>pI</th>
<th>Extinction coefficient (mg\textsuperscript{-1}cm\textsuperscript{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb1</td>
<td>10 mM histidine, 150 mM NaCl, pH 6.0</td>
<td>7.9-8.3</td>
<td>1.61</td>
</tr>
<tr>
<td>mAb2</td>
<td>25 mM histidine, 7 % sucrose, pH 6.0</td>
<td>8.44</td>
<td>1.42</td>
</tr>
<tr>
<td>mAb 3</td>
<td>10 mM histidine.HCl, 6% trehalose dihydrate, 2% Arg·HCl, 0.025% PS80, pH 6.0</td>
<td>8.56</td>
<td>1.47</td>
</tr>
<tr>
<td>mAb4</td>
<td>25 mM histidine, pH 6.0</td>
<td>8.53</td>
<td>1.38</td>
</tr>
<tr>
<td>mAb5</td>
<td>20 mM Succinate, 95 mM Arg, 180 mM mannitol, 20 mM NaCl, pH 6.0</td>
<td>8.1-8.6</td>
<td>1.66</td>
</tr>
</tbody>
</table>

2.1.2 Chemicals and Reagents

All chemical and reagents used for the biophysical, NMR and in vitro studies were purchased from Sigma-Aldrich (Poole Dorset, UK) unless otherwise stated. Arg·HCl was purchased from J.T.Baker (USP-FCC grade).

2.1.3 General buffer solutions for biophysical and NMR studies

The common buffers used for all experiments are given in Table 2.2. In the case of citrate-phosphate (CP) buffer, pH was adjusted using either citric acid or sodium phosphate. For acetate buffer, pH was adjusted with either acetic acid or sodium acetate.
Table 2.2: Buffer compositions for biophysical and NMR experiments

<table>
<thead>
<tr>
<th>mAb</th>
<th>Base</th>
<th>Concentration (mM)</th>
<th>pH</th>
<th>Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb1-mAb4</td>
<td>Sodium phosphate</td>
<td>10</td>
<td>5.6 and 7</td>
<td>Citric acid</td>
</tr>
<tr>
<td>mAb5</td>
<td>Sodium acetate</td>
<td>20</td>
<td>5.5</td>
<td>Glacial acetic acid</td>
</tr>
</tbody>
</table>

The mass of the base salts and acid were calculated from the molar mass in g/mol. These components were dissolved in MilliQ H₂O at ~70 % of the total required volume. The pH was adjusted where necessary with either stock solutions of the acid or base to generate the target pH. Once, pH was achieved the total volume was topped up with the remaining 30 % MilliQ H₂O.

2.2 Methods

The methods for this Thesis have been sectioned separately for each results Chapter.

2.2.1 Biophysical and accelerated stability studies (Chapter 3)

2.2.1.1 Sample preparation

For the SLS and intrinsic fluorescence measurements, these four mAbs were dialysed in 10 mM CP buffer at pH 5.0, 6.0 and 7.0 and were diluted to 1 mg/mL in the respective buffers. These solutions were supplemented with varying concentrations of Arg·Glu (50 to 200 mM) as required, Arg·Glu was prepared from a 1 M stock solution containing an equimolar mixture of the free amino acids L-Arg and L-Glu in MilliQ water (18.2 MΩ.cm), with pH adjusted where necessary to pH 7.0.

For the accelerated stability studies, mAb1, mAb3 and mAb4 were concentrated to concentrations between 30-50 mg/mL in the respective dialysis buffer (10 mM CP buffer) at each pH. Each of these mAbs were prepared in two formulations (Table 2.3) to compare the effects of Arg·Glu to Arg·HCl at different pH’s. Arg·HCl was added from a 1 M stock solution using the hydrochloride salt of L-Arg in MilliQ water (18.2 MΩ.cm), with pH adjusted where necessary. The final mAb conditions were transferred in to glass vials and stored between 6-10 weeks in controlled temperature incubators of 5, 25 and 40 °C; with 40 °C representing extreme stressed conditions.
Table 2.3: Formulation conditions chosen for comparative accelerated stability studies of the three selected mAbs

<table>
<thead>
<tr>
<th>Base Buffer</th>
<th>mAb1 10 mM citrate--phosphate + 200 mM Arg·Glu</th>
<th>mAb2 10 mM citrate--phosphate, pH 5.5</th>
<th>mAb4 10 mM citrate--phosphate, pH 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation 1</td>
<td>pH 5.5 + 200 mM Arg·Glu</td>
<td>+ 200 mM Arg·Glu</td>
<td>+ 200 mM Arg·Glu</td>
</tr>
<tr>
<td>Formulation 2</td>
<td>pH 7.0 + 200 mM Arg·HCl</td>
<td>+ 200 mM Arg·HCl</td>
<td>+ 200 mM Arg·HCl</td>
</tr>
</tbody>
</table>

2.2.1.2 Protein concentration determination

Protein concentrations were determined by diluting samples to 0.5 mg/mL and measuring absorbance at 280 nm. The protein concentration was calculated using the Beer-Lambert equation:

\[ A = e \cdot c \cdot l \]  \hspace{1cm} (2.1)

Where \( l = 1 \text{ cm}, c = \text{protein concentrations}, e = \text{extinction coefficient for each mAb} \) (Table 2.1).

2.2.1.3 Determining solution osmolality of arginine glutamate

The Osmomat 030-D Cryoscopic Osmometer (Gonotec GmbH, Berlin, Germany) was used to determine the osmolarity of Arg·Glu solutions in the presence and absence of mAb2 only, due to sample limitations of the other mAbs. The instrument uses the freezing point depression method to determine the total osmolarity of aqueous solutions. In the absence of mAb2, Arg·Glu concentrations 5, 10, 25, 50, 100 and 150 mM were prepared from the 1 M stock solution prepared above in MilliQ water. For measurements with protein, mAb2 was dialysed overnight into 10 mM CP buffer, pH 6.0 supplemented with Arg·Glu concentrations of 50, 150 and 200 mM. The protein concentration was adjusted to 30 mg/mL by dilution with the appropriate buffer solution containing Arg·Glu. Final antibody sample was collected and the concentrations were verified using the Nano-Drop 2000 (Thermoscientific, Stafford House, Hertfordshire) in triplicates.

2.2.1.4 Static light scattering and intrinsic fluorescence

The instrument Optim 2 (Avacta, Thorp Arch Estate, Wetherby) was used to simultaneously determine the protein melting \( (T_m) \) and onset of aggregation \( (T_{agg}) \) temperatures. \( T_m \) reported on the conformational stability and was measured as the mid-point temperature of the first unfolding transition, \( T_{m1} \) (i.e. the onset of melting); this was derived using the intensity ratio of the fluorescence emission of 350 and 330 nm where tryptophan fluoresces in its unfolded and folded state respectively. The colloidal stability was measured using the SLS at 266 nm reporting on the onset of aggregation \( (T_{agg}) \); this is derived as the temperature at which the scattered intensity reaches a threshold that is 10 % of the maximum value. \( T_{agg} \) reported on
the weight averaged changes in the molecular mass as a consequence of protein aggregation. In principle this instrument measures light scattering at two wavelengths; 266 and 473 nm, however only data at 266 nm was reported as this was more sensitive to smaller aggregates than at 473 nm which looks at larger aggregate with less obvious differences seen between formulations. Sample volumes of 9 µL were loaded in triplicates on to Micro-Cuvette Array sample holders. The temperature profile was set the same for each screening condition with an increase in temperature from 20 to 90 °C in 1 °C increments; with an initial 10 second incubation hold when each start temperature was reached. The plate was held for 60 seconds between each measurement point before the SLS and fluorescence signal was recorded. These conditions were selected for the sample preparation and instrument as these would apply stress to the protein making these less stable. The sample measurements were made in triplicate and statistical analysis made using GraphPad Prism v6.

2.2.1.5 Size Exclusion-High Performance Liquid Chromatography

The samples set up for the accelerated stability studies were monitored on a weekly basis, using a Tosoh TSKgel column (with 5 mm beads) attached to Agilent HPLC 1200 system, monitored at 280 nm. Injection volumes of 25 mL were used for each sample in duplicates, with a running buffer of 100 mM sodium phosphate, 100 mM NaCl, pH 6.8, and monitoring the absorbance profile at 280 nm. The peaks on the chromatograms were analysed to determine the percentage of mAb monomer present in solution at each time point.

2.2.2 In vitro cytotoxicity studies (Chapter 4)

2.2.2.1 Cell line maintenance: THP-1 cells and fibroblasts

The THP-1 human monocytic leukaemia cell line was cultured in RPMI-1640 medium supplemented with 400 µg/mL streptomycin, 400 µg/mL penicillin, 2 mM L-glutamine (GIBCO; Paisley, Renfrewshire, UK) and 10 % fetal calf serum (FCS; GE Healthcare, Cambridge, UK). THP-1 cells were maintained in vented T75 flasks at 37 °C in an atmosphere of 5 % CO₂ and split every 3-4 days when confluent (<2 x 10⁶ cells/mL). Primary fibroblasts from human skin were cultured in DMEM medium (high glucose [4.5 g/L] with 2 mM L-Glutamine; GIBCO) supplemented with 400 µg/mL streptomycin, 400 µg/mL penicillin, 2 mM GlutaMax (GIBCO), 0.25 µg/mL amphotericin B and 10 % FCS. Fibroblasts were maintained in 10 cm culture dishes at 37 °C/5 % CO₂ and passaged every 3-4 days when >80 % confluent. Cell number was assessed by exclusion of 0.5 % trypan blue using a haemocytometer.

2.2.2.2 Salts used for generating changes in osmolality

Stock solutions of cell culture grade Arg-Glu from equimolar mixtures of L-Arg (CAS number 74–79-3) and L-Glu (CAS number 142–47-2), NaCl (CAS number 7647–14-5), Arg-HCl (CAS number 1119–34-2) and sodium glutamate (NaGlu; CAS number 56–86-0) were prepared at 2.24 M, 4.96 M, 4.32 M and 3.9 M, respectively, in RPMI-1640 medium supplemented as
described above without FCS. The salts of the amino acids (rather than the free bases) were used to keep the pH of L-Arg and L-Glu solutions within physiological range, whereas to prepare Arg-Glu, free bases of these amino acids were mixed together. Solutions were filtered using a 0.22 μm syringe filter and stored at 4 °C until use.

2.2.2.3 Determination of osmolality of salt solutions

The osmolality of Arg·Glu, NaCl, Arg·HCl and NaGlu solutions was measured using an Osmomat 030-D Cryoscopic Osmometer (Gonotec GmbH, Berlin, Germany). A 1 M stock solution formulated in RPMI-1640 media was prepared for each salt and final concentration of 50, 100, 150 and 200 mM were prepared for each compound. Method described in Section 2.2.1.3.

2.2.2.4 Cell treatments

Confluent THP-1 cells were harvested by centrifugation (1000 g at r.t for 5 min) and re-suspended at 1 x 10^6 cells/mL in RPMI-1640 medium without FCS in flat-bottomed 24 well tissue culture plates. Salts were prepared in medium at stock concentrations and added to cell cultures to achieve the required osmolalities (280-680 mOsM); various amounts of water were also added to achieve the required osmolalites (0-230 mOsM). Control cells were treated with medium alone. In initial experiments dose responses were conducted, in subsequent experiments cells were treated with Arg-Glu, NaCl, Arg-HCl or NaGlu to achieve the osmolality range (280-680 mOsM) or the equivalent concentration range 50-200 mM. Cells were also treated with 0.1 μg/mL LPS from Escherichia coli 055:B5. Cells were incubated for 24 h at 37 °C in an atmosphere of 5 % CO₂. Following the incubation, the cells were spun at 1000 g at r.t for 5 min and re-suspended in 100 μL phosphate buffered saline (PBS) without calcium and magnesium for determination of cell viability. For phenotypic marker expression the cells were re-suspended in 2 % bovine serum albumin (BSA) in PBS. Supernatants and lysates were also harvested for cytokine and NO determination. Lysates were obtained by lysing the cell pellets in 100 μL 0.01 % Triton X 100.

Confluent fibroblast cells were washed once with PBS and trypsinised with 3 mL 0.05 % trypsin-EDTA (EDTA) for 3-4 min at 37 °C until the cells detached from the plate. Cells were re-suspended in complete DMEM medium and were centrifuged at 1000 g r.t for 5 min. Cells were re-suspended at 2 x 10^5 cells/mL in complete DMEM medium in flat-bottomed 24 well tissue culture plates for 6 h at 37 °C/5 % CO₂. Following incubation, the media was discarded and cells were washed with PBS and treated with the salts as mentioned above in DMEM medium without FCS to achieve the required osmolalities for 24 h. Following the overnight incubation, media was collected and cells were washed twice with PBS and collected with the media. Cells were trypsinised with 500 μL 0.05 % trypsin-EDTA and re-suspended in complete DMEM medium; cells were collected and added to the previous media collection. Finally, a further 500 μL complete DMEM medium was added to each well and this was
collected and again added to the previous media and cells collected. This was centrifuged at 1000 g for 5 min and the media was gently aspirated and cells were re-suspended in 5 % FCS/PBS to determine cell viability.

2.2.2.5 Measurement of cell viability

Cell viability was routinely determined by staining of cells with 5 μg/mL propidium iodide (PI) immediately prior to analysis. Cells (10⁴) were analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.2.2.6 Measurement of phenotypic marker expression of THP-1 cells

Following treatment, phenotypic marker expression was assessed. Cells were re-suspended in 2 % BSA in PBS. Approximately 2 x 10⁵ cells were transferred to individual wells in round bottomed 96 well tissue culture plates and incubated at 4 °C for 15 min. The cells were washed at 1000 g for 5 min and incubated with the following mAbs at 4 °C for 30 min: anti-human leukocyte antigen antibody (HLA-DR; DAKO, Glostrup, Denmark), anti-human CD54 antibody and allophycocyanin (APC)-conjugated anti-human CD86 antibody (BD PharMingen, Oxford, UK) at a 1 in 50 dilution. Isotype controls used were mouse IgG2ak for anti-human HLA-DR and IgG1k (BD PharMingen) for anti-human CD54 antibody and anti-human CD86 antibody. After incubation, cells were washed twice with PBS (1000 g for 5 min) followed by a further 30 min incubation at 4 °C with fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ goat anti-mouse IgG at a 1 in 50 dilution (DAKO) for anti-human CD54 and anti-human HLA-DR antibody stained samples; cells stained with APC-conjugated anti-human CD86 antibody were incubated with 2 % BSA in PBS. Cells were washed as previously described and finally re-suspended in 5 % FCS/PBS, and analysed by FACSCalibur. Dead cells were excluded from all analyses by staining with 5 μg/mL PI immediately prior to analysis for cells stained for CD54 and HLA-DR; for CD86 staining dead cells were excluded following 7 min incubation with 2 μg/mL of 7-Aminoactinomycin D (7-AAD; BD PharMingen). For each sample, a total of 10⁴ viable cells were analysed.

Flow cytometry data were analysed using FlowJo v10. Cell debris was eliminated by gating on the forward scatter (FSC-H) and side scatter (SSC-H) parameters and gates for marker expression were defined on the basis of isotype control staining. The mean fluorescent intensity (MFI) and the percentage positive cells were both used as separate indicators of the extent of surface marker expression.

2.2.2.7 Flow cytometric analyses for apoptosis and cytotoxicity of THP-1 cells

The levels of apoptosis/necrosis induced were investigated by staining of THP-1 cells for Annexin V and PI (TACS™ Annexin V- FITC Apoptosis Detection kits, R&D Systems Europe, Abingdon, UK). Following 4 h or 24 h incubation at 37 °C, the cells were centrifuged at 1000
g for 5 min at r.t. The media was discarded and the cells were washed with 500 µL of cold 1 X PBS (4 °C) and further centrifuged at 1000 g for 5 min to pellet the cells. The PBS was discarded and the cells were gently re-suspended in 100 µL Annexin V incubation reagent (10 µl 10 X Binding Buffer, 10 µl PI, 1 µL Annexin V-FITC, 79 µL MilliQ water) and incubated in the dark for 15 min at r.t. Subsequently, 400 µl 1X Binding Buffer was added to each sample and 10^4 cells were analysed by FACSCalibur and FlowJo. The combination of Annexin V-FITC and PI staining allows for the differentiation between early apoptotic cells (annexin V+/PI−), late apoptotic/necrotic cells (annexin V+/PI+), dead cells (annexin V−/PI+) and viable cells (annexin V−/PI−) (van Engeland et al. 1998).

2.2.2.8 Analysis of IL-8 expression by ELISA of THP-1 cells

The IL-8 content of culture supernatants harvested 24 h after initiation of culture was measured by sandwich ELISA (Duoset mouse IL-8 kit; R & D Systems). Maxisorb plastic microtiter plates (Nunc, Copenhagen, Denmark) were used for these assays. Briefly, 96-well plates were coated with 0.1 µg/mL mouse anti-human IL-8 and incubated overnight at 4 °C. The following day, the plates were washed three times (3 min wash step) with 0.05 % PBS/Tween-20. 1 % BSA in PBS was added to all wells to prevent nonspecific binding and the plates were placed on an orbital shaker (300 rpm) for 1 h at r.t and then washed a further three times. A standard series of diluted recombinant human IL-8 standard (2 to 0.008 ng/mL) was added to triplicate wells and supernatant samples were diluted and added in duplicate and plates were incubated for a further 2 h on the shaker. The plates were washed a further three times and goat anti-human IL-8 antibody diluted 1 in 5000 times was added to each well and the plates were incubated for 2 h. A further wash step was performed and streptavidin-horseradish peroxidase (HRP) diluted 1 in 1000 times was added to each well and incubated for 30 min in the dark. Optical density at 450 nm was measured using an automated reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). A standard curve derived with murine recombinant cytokine and associated computer software for microplate-based assays (Genesis, Life Sciences International Ltd, Basingstoke, UK) was used to calculate the cytokine concentration in supernatants.

2.2.2.9 Analysis of nitric oxide release of THP-1 cells by Griess assay

The nitrite concentration from supernatants and lysates harvested at 24 h after initiation of culture was measured by the Griess assay (Miranda et al. 2001). Initially, a nitrite standard reference curve was prepared by diluting the provided 0.1 M nitrite standard 1 in 1,000 times in RPMI media without FCS to make a 100 µM nitrite solution. Then, a 6-fold dilution series using the prepared nitrite standard (100-1.56 µM) was prepared and added to Maxisorb plastic microtiter 96 well plates. Supernatants and lysate samples were added to the plate and 1 % sulfanilamide in 5 % phosphoric acid was added to every well. This was incubated for 10 min in the dark. N-1-naphthylethylene diamine dihydrochloride (0.1 %) in MilliQ water was then added to every well and incubated for a further 10 min in the dark. Optical density at 550 nm
was measured using an automated reader (Multiskan). The nitrite concentrations were determined by plotting the nitrite standard reference curve and using the associated computer software for microplate-based assays (Genesis).

2.2.2.10 Cell line maintenance and treatment for HEK293 cells

The adherently growing transfected human embryonic kidney (HEK) 293 EBNA (Epstein–Barr virus nuclear-antigen) cells expressing recombinant histidine tagged OLFD were cultured in DMEM medium (high glucose, 4.5 g/L, with 2 mM L-Glutamine) supplemented with 400 μg/mL streptomycin, 400 μg/mL penicillin and 10 % FCS. Cells were maintained in T75 flasks until ~70 % confluent and replaced with fresh DMEM medium containing 1 μg/mL puromycin. Cells were passaged once >70-80 % confluent in to T225 flasks. Once HEK293 cells were ready for protein expression, three conditions were set up; firstly protein containing 50 mM Arg+Glu from the expression stage, protein expressed in media alone and protein expressed in media alone with 50 mM Arg+Glu introduced in the elution buffer. These conditions are illustrated in Figure 2.1. Growth media was removed and cells were washed with 30 mL PBS. 50 mL expression media (1:1 mixture of DMEM medium and Ham’s F12 nutrient mixture supplemented with 400 μg/mL streptomycin, 400 μg/mL penicillin) was added to each T225 flask in the presence or absence of 50 mM Arg-Glu and incubated at 37 °C/5 % CO₂. Media was collected every 2-3 days for each condition and replaced with fresh expression media. Collected media was centrifuged at 1000 g for 5 min, filtered using a 0.22 micron syringe filter and stored at 4 °C until use. 1 mL aliquots from each collection were stored at -80 °C for Western blot.
2.2.2.11 **Expression and purification of transfected HEK293 cells**

Prior to purification, the collected media was pooled together and dialysed overnight in 20 mM Tris, 0.4 M NaCl, 0.5 mM CaCl$_2$, pH 7, either in the presence or absence of 50 mM Arg·Glu. Histidine tagged purification was performed using 1 mL HisTrap columns according the manufacturer’s instructions (GE Healthcare Life Sciences, Buckinghamshire UK). Briefly, the column flow rate was set to ~ 1 mL/min and equilibrated with water and the appropriate running buffer. The protein was eluted in several 1 mL fractions in the appropriate elution buffer composed of 20 mM Tris, 0.4 M NaCl, 0.5 mM CaCl$_2$, 500 mM imidazole in the presence or absence of 50 mM Arg·Glu. The eluted protein was further dialysed overnight in to buffer described above to remove the imidazole.

2.2.2.12 **SDS-PAGE electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the eluted protein fractions under reducing conditions, using 4-20 % tris-glycine gel with tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS. Samples were diluted 1:1 with sample buffer (65 mM Tris-HCl [pH 6.8], 10% [w/v] SDS, 0.5% [w/v] bromophenol blue, 25% [v/v] glycerol, 5% [v/v] β-mercaptoethanol) and at 95 °C for 5 min. Molecular weight standards (Thermoscientific, Stafford House, Hertfordshire) and samples were loaded on the gel using the mini-gel electrophoresis apparatus (Biorad) and ran for 45 min at 200 V. Gel was stained with instant blue overnight. Protein concentration was confirmed using a Nano-Drop 2000 (Thermoscientific), by measuring optical absorption at 280 nm.
2.2.2.13 Western blot

The samples were electrophoresed on a 10 % SDS polyacrylamide gel, and electro-blotted over to a Polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in Western blot blocking buffer (4 % milk powder in PBS with 1 % Tween) for 30 min. After blocking, the primary anti-histidine-tagged antibody was diluted 1:1000 in PBS/1 % Tween and incubated overnight at 4 °C with gentle shaking. Following incubation, the membrane was washed three times with PBS/1 % Tween, each wash step consisting of gentle shaking for 10 min. The membrane was then incubated with sheep anti-Mouse IgG-HRP diluted 1:2000 in PBS/1 % Tween for 45 min. The membrane was washed again as above, and incubated with chemiluminescent substrate for 5 min before exposing to photographic film.

2.2.2.14 Multi-Angle Light Scattering (MALS)

Samples (1 mL at ~30-50 µg/mL) were loaded onto a Superdex200 10/300GL column (GE Healthcare) running at a flow rate of 0.75 mL/min in 10 mM Hepes (pH 7.4) containing 150 mM NaCl, 2 mM CaCl₂ with or without 50 mM Arg·Glu. Samples eluting from the column passed through a DAWN Wyatt EOS 18-angle laser photometer. This was coupled to a Wyatt Optilab rEX RI detector, and the molecular mass and concentrations of the resulting peaks were analysed using Astra (version 6).

2.2.2.15 Statistical analyses

The statistical significance of differences in membrane marker expression, cytokine secretion and cell viability between experimental and control groups were calculated using one-way analysis of variance (ANOVA). To test for synergy of combinations of Arg·Glu treated cells and LPS with predicted effects due to the addition of the treatments alone, two-way ANOVA analysis was performed. The reasoning for this is that it allows analysis of whether each treatment alone significantly affects the membrane marker expression, but also whether these treatments behave independently (additively) or interact together to influence this response. Antagonism would be referred to as a negative interaction whereas synergy would indicate positive interactions i.e. non-additively (Slinker 1998).

2.2.3 NMR studies (Chapter 5)

2.2.3.1 Sample preparation

For the NMR studies conducted here, mAb2 (MW 145 kDa, pI 7.9-8.3) was chosen as a test mAb and was identical to mAb2 used in Chapter 3. mAb2 samples were dialysed in CP buffer at pH 6.0 and 7.0 and concentrated to 40, 100 and 200 mg/mL, each to a final volume of 1 mL. Samples were supplemented with 5 % D₂O and 500 µL was transferred to a 5 mm NMR tube with another 500 µL kept for Arg·Glu titrations. To these latter conditions, pre-measured aliquots of freeze-dried Arg·Glu were successively reconstituted with 500 µL mAb2 to achieve a range of concentrations 5 to 200 mM. The freeze-dried aliquots of Arg·Glu were prepared
from a 0.5 M stock solution containing an equimolar mixture of the free amino acids L-Arg and L-Glu in MilliQ water, with pH adjusted where necessary to pH 7.0. At each Arg-Glu concentration, a set of NMR experiments were performed (discussed in the next section) after which the sample was removed from the NMR tube and reconstituted with the next dry aliquot of Arg-Glu to achieve the following desired concentration, this was repeated until total of 200 mM Arg-Glu was added.

For the long-term stability studies, four formulations were prepared with mAb2 dialysed in appropriate diluted formulations (Table 2.4). These were freeze-dried as described above and finally reconstituted in an eighth of the original volume in 180 µL D2O to obtain a final mAb2 concentration of 300 mg/mL. Samples were supplemented with 0.01 % NaN3 to prevent bacterial growth, and were transferred to 3 mm NMR tubes, sealed and stored in a 40 ºC incubator for the duration of the study. Final mAb2 concentrations were determined based on their absorbance at 280 nm. For SE-HPLC, similar experiments were set up using a different preparation batch where mAbs were diluted to 10 mg/mL in the appropriate buffer, with the monomer content quantified as described previously.

Table 2.4: Formulation conditions chosen for mAb2 for comparative accelerated stability studies using NMR and SE-HPLC

<table>
<thead>
<tr>
<th>Formulation</th>
<th>mAb2 10 mM CP buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH 6.0</td>
</tr>
<tr>
<td>2</td>
<td>+200 mM Arg·Glu, pH 6.0</td>
</tr>
<tr>
<td>3</td>
<td>+200 mM Arg·Glu, pH 7.0</td>
</tr>
<tr>
<td>4</td>
<td>+200 mM Arg·HCl, pH 6.0</td>
</tr>
</tbody>
</table>

2.2.3.2 General NMR experiments

NMR spectroscopy was used for mAbs formulation characterization as an orthogonal approach to assess stability. A Bruker Avance DRX-800 MHz spectrometer with 5 mm TXI cryoprobe and temperature control unit was used for all NMR experiments, controlled by Topspin 3.1. All experiments were conducted at either 20 ºC or 40 ºC unless specified otherwise. The following key experiments were conducted; standard Bruker excitation sculpting ¹H experiments, Carr-Purcell-Meiboom-Gill sequence (CPMG) experiment for T₂ measurements and SE-PFG measurements for 2D translational diffusion measurements. Proton 1D spectra were recorded using p3919gp pulse program using 16.0194 ppm spectral width and applying the common exponential multiplication (EM) window function with typical 10 Hz broadening. The proton pulse length was recalibrated each time with the addition of increasing Arg-Glu concentration in the sample. Spectra were processed and analyzed using Topspin 3.1 and Dynamics Centre 2.2.4 (Bruker).
2.2.3.3 Use of 1D $^1$H NMR spectra to determine viscosity-corrected signal intensities

Due to the increase in buffer viscosity with the addition of Arg·Glu, it was necessary to compensate for this as this results in slower molecular tumbling and reduces apparent spectral intensities. Therefore, the viscosity-corrected normalized signal intensities from the NMR spectra $I_{\eta}^N$ were calculated using Equation 2.2:

$$I_{\eta}^N = \frac{l_{[RE]} \cdot \eta_{[RE]}}{l_{[RE=0]} \cdot \eta_{[RE=0]}}$$  \hspace{1cm} (2.2)

where $l_{[RE]}$ and $l_{[RE=0]}$ are signal intensities and $\eta_{[RE]}$ and $\eta_{[RE=0]}$ are buffer viscosities in the presence and absence of Arg·Glu, respectively. The buffer viscosity values were derived from the diffusion coefficients of citrate ions measured using SE-PFG measurements (see below). Flat dependencies of $I_{\eta}^N$ with increasing Arg·Glu concentrations would indicate that the concentration of soluble monomeric or lower-oligomeric protein species is not affected by the addition of Arg·Glu.

2.2.3.4 Investigating the short and long-term storage stability of mAb2 using NMR temperature dependent experiments

mAb2 samples that were transferred into NMR tubes after concentrating to 40, 100 and 200 mg/mL and samples at the end of the Arg·Glu titration experiments containing a final concentration of 200 mM were subjected to increased temperatures between 40 to 75 °C, incremented with 5 °C intervals, with 10 min equilibration after each temperature increase. A pair of 1D NMR spectra (p3919gp pulse program) was acquired at each temperature with ca 45 min interval. The 1D NMR spectra acquired at each temperature increment was analysed to assess the structural stability of mAb2 by determining the melting temperature. The integral of two well defined peaks from the methyl region were selected from the 1D spectrum at each temperature. The temperature-dependent viscosity-corrected normalized signal integrals $L_{\eta}^N(T)$ were calculated using Equation 2.3:

$$L_{\eta}^N(T) = \frac{L^T}{L_{[RE=0]}^{40}} \cdot \frac{\eta_{[RE]}}{\eta_{[RE=0]}}$$  \hspace{1cm} (2.3)

where $L^T$ is the signal integral at a particular temperature $T$, $L_{[RE=0]}^{40}$ is the integral measured at 40 °C in the absence of Arg·Glu, and $\frac{\eta_{[RE]}}{\eta_{[RE=0]}}$ is the ratio of the buffer viscosity (with or without 200 mM Arg·Glu, as appropriate) to the viscosity without Arg·Glu. Again a constant flat dependence of $L_{\eta}^N$ over $T$ would indicate that there is no temperature-dependent change in the population of monomeric or lower-oligomeric species. For the short term stability studies, from the 1D spectra, eight well-resolved methyl signals between -0.5 to 0.5 ppm were selected
which reflect the amount of folded protein in solution and marked as ‘soluble folded protein’ and four signals from the strongest overlapped peak in the methyl region at 1 ppm, which reflect the total amount of soluble protein staying in solution before and after the temperature exposure. Normalized signal intensity ratios, $F$, were calculated, to obtain the fraction of protein preserved in solution after high temperature exposure for a fixed amount of time, represented as (Equation 2.4):

$$F = \frac{I^t}{I^0}$$

where $I^t$ is the intensity after the 45 min temperature exposure and $I^0$ is the initial signal intensity. $F$ was used as a parameter to measure the short-term stability of mAb2 at increased temperature reporting on the fractional loss of monomeric or lower-oligomeric species in solution over a specified time period.

For the long-term stability studies, the formulations were stored at 40 °C and was analysed by acquiring 1D NMR spectra at 40 °C for each time point over 10 weeks. Samples were removed from the temperature controlled incubator for the duration of the experiment and put back once these were completed. The fraction of soluble monomeric or lower-oligomeric protein preserved in solution after time exposure was calculated using the equation above for a number of peaks integrated in the aromatic ($F_{AR}$) and amide ($F_{NH}$) regions (7 and 9 ppm respectively).

### 2.2.3.5 Use of PFG-NMR spectroscopy to measure diffusion rates

The translational diffusion coefficient, $D$, was measured at each Arg-Glu concentration using a pseudo 2D SE-PFG for diffusion measurements with bipolar gradients and WATERGATE (stebpp1s19) from Bruker. The diffusion time ($\Delta$) and the gradient length ($\delta$) were set to 250 ms and 4.0 ms, respectively, with a gradient pulse of 45 G/cm. The acquisition time and relaxation delay were set to 640 ms and 2.0 sec respectively. Data were accumulated by linearly varying the diffusion encoding gradients over a range from 10-98 % over 16 linear gradient steps. Each sample was allowed to equilibrate in the NMR spectrometer for 5 min before tuning and shimming. DOSY analysis was used to derive $D$ by following the standard processing parameters in Topspin. The standard errors reported for each $D$ was calculated based on the upper and lower error limits for each peak. The data was plotted in GraphPad V 6.0.

$D$ is dependent on several parameters including the size and shape of the molecule and for diluted solutions can be described by the Stokes-Einstein Equation:

$$D = \frac{kT}{6\pi R_h \eta}$$

(2.5)
where $D$ is the diffusion coefficient; $T$ is the temperature in Kelvins; $k$ is the Boltzmann constant; $R_h$ is the hydrodynamic radius and $\eta$ is the solution viscosity.

As it is standard practice for diffusion NMR measurements (Yao et al. 2008), an internal reference probe molecule, dioxane, with a known and solution-independent $R_h$, was introduced. Measuring the translational diffusion of this molecule first in water, and then in solutions in the absence of mAbs, enabled the calibration of the gradients for SE-PFG diffusion NMR experiments, to measure the absolute values of $D$ accurately using Equation 2.6 and 2.7. Unfortunately, the dioxane signal was overlapping with the signals of Arg·Glu, therefore a secondary reference probe molecule present in the buffer, citrate ion, was used to measure the values of its $D$ with increasing Arg·Glu concentrations. Equation 2.7 was transformed to Equation 2.8 to first approximate the radius of citrate ion which was then transformed in to Equation 2.9 to obtain the apparent solution viscosity $\eta_{[RE]}$ as a function of Arg·Glu concentration added in CP buffer alone.

\[
D_{\text{Dioxane}} = \frac{kT}{R_h^{\text{Dioxane}} \eta_{[0]}} \tag{2.6}
\]

\[
D_{\text{Citrate}} = \frac{kT}{R_h^{\text{Citrate}} \eta_{[0]}} \tag{2.7}
\]

\[
R_h^{\text{Citrate}} = \frac{kT}{D_{\text{Citrate}} \eta_{[0]}} \tag{2.8}
\]

\[
\eta_{[RE]} = f([RE]) = \frac{kT}{D_{[RE]}^{\text{Citrate}} R_h^{\text{Citrate}}} \tag{2.9}
\]

The values in the formulae were: $T$, temperature at which the measurements were made (313 K), $\eta_{[0]} = 0.653$ mPas (dynamic viscosity of water at this temperature in the absence of Arg·Glu), $k$ is Boltzmann constant, and $R_h^{\text{Dioxane}}$ and $R_h^{\text{Citrate}}$ are the apparent radii of the small probe molecules, dioxane and citrate, respectively. $D$ for dioxane were measured in the absence of Arg·Glu (RE).

Using the measured $D$ and the calculated viscosities of the mAb2 solutions, Equation 2.5 was rearranged to estimate the hydrodynamic radius ($R_h$) of mAb2 as a function of solution conditions (Equation 2.10):

\[
R_h^{\text{mAb2}} = \frac{kT}{D_{\text{mAb2}}^{\text{RE}} \eta_{[RE]}} \tag{2.10}
\]
where $D^{mAb2}$ was measured by monitoring the strongest overlapping peak in the methyl region at 1 ppm, which represents the total amount of mAb2 staying in solution; $\eta_{[\text{EE}]}$ was calculated above.

### 2.2.3.6 Long-term storage stability studies using SE-HPLC

Accelerated stability studies were set up as described in Section 2.2.3.1 for mAb2 conditions tabulated in Table 2.4. These four formulations were chosen in order to compare the stabilizing effect of Arg-Glu versus Arg-HCl and the effect of pH. The final mAb formulations were transferred to 3 mL glass vials and stored for 16 weeks at 40 °C (40 °C was considered to represent stress conditions). The samples were tested every week by SE-HPLC for the first month and then monthly up to four months, using a Tosoh TSKgel column (with 5 μm beads) attached to Agilent HPLC 1200 system, monitored at 280 nm. Samples of 25 μL were injected each time, with a running buffer of 100 mM sodium phosphate, 100 mM NaCl, pH 6.8, and monitoring the absorbance profile at 280 nm. The peaks on the chromatograms were analysed to determine the percentage of mAb monomer present in solution, with each experiment carried out in duplicate.

### 2.2.3.7 Viscosity measurements

The viscosity of the mAb solutions was determined using the mVROC viscometer (RheoSense Inc., San Ramon, CA, USA). mAb2 at concentrations 40 and 100 mg/mL were measured with a B05-chip at a shear rate of 6000 1/s for 30 sec and mAb2 at 300 mg/mL used a D05-chip at a shear rate of 5000 1/s for 15 sec. Measurement temperature was set at 40°C and was controlled by an external water bath. Samples were filled in a 1 mL syringe and triplicate measurements were acquired where possible due to the limitation of sample volumes. Between each measurements the system was washed with 1 % tergazyme followed by 1 % aquet and then water each with 750 μL/min flow rate for 60 sec (for B05-Chip) or 1000 μL/min for 45 sec (D05-chip). Triplicate measurements were recorded for each sample (mean and SD).

### 2.2.3.8 Use of NMR to measure transverse relaxation rates, $R_2$

Non-selective proton transverse relaxation rates $R_2$ were measured using a series of standard CPMG pulse echo sequence with the number of echos varied (pulse program cpmgr1D from Bruker). The relaxation delay was 5 sec. Data was processed using the standard $T_1/T_2$ relaxation analysis tool in Topspin and Dynamic Center 2.2.4. To obtain the relaxation rate, $R_2=1/T_2$, the signal amplitude (defined as fixed-width area under the selected peak) was fitted to the following exponential function shown in Equation 2.11:

$$I_{xy}(t) = I_0 e^{-t/T_2}$$ (2.11)
Where, $I_0$ is the initial amplitude, $T_2$ the transverse relaxation time and $I_{xy}$ the magnetisation in the x-y plane.

During the Arg·Glu titration, 12 well-defined signals representative of the protein conformation in the methyl region (-0.5 to 0.5 ppm) and the total soluble protein remaining in solution (1 ppm) were integrated. Further analysis was done, based on the assumptions that $R_2$ is more or less proportional to the rotational correlation time $\tau_c$ (ignoring contributions from internal motions and chemical exchange) (Anglister et al. 1993) and moreover proportional to the volume $V$ (and hence size) of the protein shown in Equation 2.12 (Yao et al. 2008):

$$R_2 \sim \tau_c = \frac{V \eta}{kT} \quad (2.12)$$

From this, further assumptions were made that the lowest $R_2$ value, $R_2^m$, for mAb2 at 40 mg/mL in the presence of 200 mM Arg·Glu (with microscopic viscosity $\eta^m$), corresponds to mAb2 monomer species $V^m$ (i.e., minimum cluster volume). Using this parameter, the most appropriate illustration of this data was the effective number of mAb2 in a cluster, $N$ i.e. the apparent aggregation number. This was estimated from $R_2$ and known microscopic viscosity $\eta$ as:

$$N = \frac{V}{V^m} = \frac{R_2 \eta^m}{R_2^m \eta} \quad (2.13)$$

This parameter was thought to be most appropriate as $N$ indicates the expected change in the apparent size of the mAb2 cluster as an alternative way to interpret the changes seen with the $R_2$ values.

### 2.2.4 Phase separation studies using NMR (Chapter 6)

#### 2.2.4.1 Sample preparation

For these studies, mAb5 (MW 149; pl 8.1-8.6) was chosen as the test mAb as historical data at MedImmune showed this mAb was susceptible to LLPS. The default formulation of mAb5 is described in Table 2.1. mAb5 was dialysed overnight in 20 mM acetate buffer at pH 5.5; acetate buffer was prepared using sodium acetate trihydrate (USP-FCC grade, J.T. Baker) in MilliQ water which was titrated with glacial acetic acid (USP-FCC grade, J.T. Baker) to obtain the required pH 5.5. After dialysis mAb5 was concentrated to 70 mg/mL using a centrifugal concentrator (Amicon, MWCO 10k) in ~20 mL volume. Sample volumes of 1 mL were distributed in to 1.5 mL clear glass vials with screw caps and were supplemented with NaCl to achieve final concentrations between 0-30 mM; these were used as the control samples. A 0.3 M NaCl stock solution was prepared in 20 mM acetate buffer, pH 5.5, with pH adjusted with glacial acetic acid or sodium acetate. A 1 M stock solution of Arg-Glu was prepared from
the equimolar mixture of the free amino acids L-Arg and L-Glu in 20 mM acetate buffer, pH 5.5. Similarly, a 1 M stock solution of Arg-HCl was also prepared in the same formulations using the hydrochloride salt of L-Arg; pH was adjusted where necessary. This same sample set up (mAb with 0-30 mM NaCl) was prepared; where each set was spiked with either Arg-Glu or Arg-HCl stock solutions to a final concentration between 50 to 200 mM. Table 2.5 describes the final samples prepared. These samples were placed in the -80 °C freezer and thawed when required. These samples were used for the visual inspections.

Table 2.5: mAb formulations set up for the visual inspection.

<table>
<thead>
<tr>
<th>NaCl [mM]</th>
<th>Arg-Glu [mM]</th>
<th>Arg-HCl [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50 100 150 200</td>
<td>50 100 150 200</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Above formulations with increasing Arg-Glu concentrations were repeated for each NaCl concentration</td>
<td>Above formulations with increasing Arg-HCl concentrations were repeated for each NaCl concentration</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
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<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For the PEG solubility assay and all the NMR studies except for the analysis of the two phases, selected mAb5 (Table 2.6) was prepared separately in exactly the same manner as described above however the samples were used immediately and not stored in the -80 °C freezer. Prior to all these experiments, samples were inverted several times ensuring a close to homogenous sample was achieved. To obtain samples of two separate phases, the phase separated sample was left to fully thaw on the bench without any disturbances to allow the phases to completely partition. The two phases were separated by carefully withdrawing the upper (protein lean) phase using a Pasteur pipette just before the sharp meniscus separating the phases; this ‘middle’ phase was withdrawn and transferred in to a second Eppendorf and finally the lower (protein rich) phase was completely withdrawn in to a third Eppendorf. These were stored at 4 °C until the NMR experiments were ready. For the investigations with the two phases, samples were transferred into 2 separate 3 mm NMR insert tubes which were placed into 5 mm tubes with the appropriate level of D₂O (as external lock solution, not mixing with the mAb sample).

2.2.4.2 Visual inspections

mAb5 samples (Table 2.5) were removed from the -80 °C freezer the following day and left on the bench top to thaw. Initial visual inspections were conducted by placing these samples under a light box against a dark background to observe opalescence or phase separation. Images were taken with a standard digital camera. During the time course of these studies, these samples were regularly examined under the light box.
2.2.4.3 PEG solubility assay sample preparation and measurement using Nephelometry

The solubility limit of 15 selected mAb5 solutions (Table 2.6) was examined using PEG (8000 Da, Alfa Aesar). Other mAb formulations (Table 2.5) were not studied due to sample limitations. Firstly, 16 g of PEG 8000 was weighed into 15 separate 50 mL falcon tubes, then the 15 buffer formations (prepared in the absence of mAb5) were added separately to each falcon tube to give a final 40 % PEG solution. This was vortexed and stored in a 40 °C incubator for 3-4 hours to allow the PEG to dissolve. Subsequently the pH was verified and adjusted accordingly using 4 M acetic acid to back to pH 5.5. The mAb5 formulations were diluted to 10 mg/mL in each respective formulation buffer without PEG. The final 40 % PEG solutions, 10 mg/mL mAb solutions and buffer alone were transferred to Bijou tubes which were placed on to the robotics systems for automated liquid handling (Tecan). A 96-well UV-Star microplate (Greiner Bio-one) was placed next to these tubes. The Tecan was used to automatically prepare the gradient of PEG (0-16%) with mAb samples at a final concentration of 1mg/mL in 200 µL in the 96-well plate for each formulation in triplicates. Immediately after the plate preparation this was transferred to the NEPHELOstar® Plus microplate nephelometer (BMGlabtech) which uses a laser diode at 635 nm for the turbidity measurements. were performed and data was analysed using the MARS-Omega analysis software. The nephelometer reports the turbidity of each sample as NTU. The laser intensity was set to 30 %. The sharp increase in turbidity indicated the percent PEG required causing gross precipitation of mAb in that particularly formulation, thus reporting on the relative solubility limits. Data was analysed using the MARS-Omega analysis software. For each data set a standard curve fit with a 4-parameter curve fitting was applied.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Arg·Glu (mM)</th>
<th>Arg·HCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>200</td>
</tr>
</tbody>
</table>

2.2.4.4 General NMR experiments

Solution NMR spectroscopy was used for selected mAb formulations to assess if events associated with opalescence and LLPS could be characterised, depending on formulation. Lowering temperature ramp experiments were performed between 20 to 2 °C with 5 °C decrements from 20 to 10 °C followed by 2 °C decrements. The temperature was calibrated using an external thermocouple placed in the NMR probe, and with methanol test sample, following the standard NMR procedure (Findeisen et al. 2007). All other experiments were conducted at either 20 °C or 40 °C unless specified otherwise. All NMR experiments were
similarly run as described in Section 2.2.3.2 unless otherwise mentioned. The protein sample in the lean and rich phase was transferred into separate 3 mm NMR tubes which were placed in 5 mm tubes with D₂O. For the other experiments D₂O was transferred into NMR insert tubes placed in 5 mm tubes containing the protein sample.

2.2.4.5 Use of 1D ¹H NMR spectra to observe spectral changes associated with LLPS and opalescence

Selected mAb formulations were chosen for these NMR studies (Table 2.7). These formulations were selected as these displayed diverse degrees of opalescence and LLPS. These samples were placed in 5 mm NMR tubes with an insert tube containing D₂O.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Formulations</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Default formulation</td>
</tr>
<tr>
<td>2</td>
<td>Buffer alone</td>
</tr>
<tr>
<td>3</td>
<td>Acetate buffer, pH 5.5</td>
</tr>
<tr>
<td>4</td>
<td>+30 mM NaCl</td>
</tr>
<tr>
<td>5</td>
<td>+30 mM NaCl, 100 mM Arg-Glu</td>
</tr>
</tbody>
</table>

1D ¹H NMR spectra were acquired for each of these formulations at the specified temperatures. Due to the difference in protein concentrations in the default formulation to the other formulations and the change in water viscosity with decreasing temperatures, concentration-normalized and viscosity-corrected signal intensities in NMR spectra $I[^{[A]}]{\eta}$ were calculated using Equation 2.14:

$$I[^{[A]}]{\eta} = \frac{I^T}{[A]}\eta_T$$

(2.14)

where $I$ is the measured signal intensity measured at a particular temperature $T$, $[A]$ is mAb concentration (either 56 mg/mL for the default formulation or 46 mg/mL for all other formulations), and $\eta_T$ is the water viscosity at a particular temperature $T$.

Normalised signal intensities were plotted as a function of temperature.

2.2.4.6 Use of PFG-NMR spectroscopy to measure diffusion rates

$D$ was measured for the 5 mAb formulations in Table 2.7 as described in Section 2.2.3.5 with the exceptional change of the $\Delta$ set to 150 ms. The acquisition time and relaxation delay were set to 640 ms and 2.0 sec respectively. $D$ for acetate and Arg-Glu were also measured for the protein-lean and rich phases of phase separated samples. The standard errors reported for
each $D$ was calculated based on the upper and lower error limits for each DOSY peak. The data was plotted in GraphPad V 6.0.

The Stokes Einstein equation (Equation 2.5) was rearranged to determine $R_h$ for mAb5 under the 5 selected formulations using the measured $D$ and the viscosity of water at each temperature using Equation 2.15:

$$R_h^{mAb5} = \frac{kT}{D^{mAb5}\eta T} \quad (2.15)$$

where $D^{mAb5}$ was measured by monitoring the strongest overlapping peak in the methyl region at 1 ppm, which represents the total amount of mAb5 staying in solution; $\eta_T$ was water viscosity at temperature $T$.

2.2.4.7 Use of NMR to measure transverse relaxation rates, $R_2$ in the lean and rich phases of phase-separated samples

$T_2$ was measured using a series of standard CPMG pulse echo sequences as described in Section 2.2.3.8. $T_2$ was measured only for acetate and Arg-Glu signals as the relaxation of mAb5 signals was too fast to measure using the existing method.
Chapter Three: The effect of arginine glutamate on the stability of monoclonal antibodies in solution using SLS and intrinsic fluorescence

The work presented in this Chapter has been published:


3.1 Abstract

During the formulation of mAbs as high concentration liquids (≥ 100 mg/mL), the buffer, excipients and pH must be carefully considered in order to attenuate potential PPIs that may otherwise lead to aggregation. This Chapter investigated the use of Arg·Glu, in the concentration range 50-200 mM, as an excipient that may attenuate mAb aggregation. In addition, it was reassuring to find that the maximal effects of Arg·Glu lie within this concentration range which is probably in the pharmaceutically-acceptable osmolality range. Molecular modelling experiments suggest that Arg·Glu clusters at specific protein surface patches through ionic interaction, but it remains unclear if this mechanism is widely applicable during mAb formulation. Here a medium throughput method has been described to quantify the effect of Arg·Glu on mAbs (using the Optim 2), and tested the predictions of these experiments in long-term storage stability studies (using temperature controlled storage in conjunction with size exclusion high performance liquid chromatography (SE-HPLC)). The effect of the equimolar mixture of Arg·Glu was investigated on the colloidal and conformational stability for four mAbs under different pH conditions (5-7), where the $T_{agg}$ and $T_{m1}$ were measured simultaneously using SLS and intrinsic fluorescence, respectively. The findings showed that these four mAbs increased the $T_{agg}$ in a concentration-dependent manner, particularly with an increase in pH to neutral. Arg·Glu only had an impact on the least thermally stable mAb3 with increases seen in $T_{m1}$ however did not significantly impact the $T_{m1}$ of the others mAbs in a similar manner. Generally, raising the solution pH towards neutral caused an increase in $T_{m1}$ for most of the mAbs. The long-term storage stability was explored for selected mAb conditions for the percentage of monomer still remaining in the solution in the presence of 200 mM Arg·Glu with comparison to a reference formulation (Arg·HCl). Under accelerated stability conditions, Arg·Glu was able to suppress the aggregation of mAb3 to a greater degree by than by Arg·Glu. Moreover, the aggregation of mAb1 was reduced by Arg·Glu at neutral pH in a manner that the percentage monomer was near to that at the more typical formulation pH of 5.5. In conclusion, Arg·Glu can suppress mAb aggregation under the conditions tested (increasing temperature/pH) and, importantly, under accelerated stability conditions at weakly acidic to neutral pH.
3.2 Introduction

Native PPIs generally arise due to a number of molecular and structural properties such as net charge, charge distribution, surface hydrophobicity (Esfandiary et al. 2015) and the self-complementary nature of protein monomer (Philo and Arakawa 2009). Intermolecular interactions may be governed by the distribution of long-range repulsive versus attractive electrostatics between protein molecules or short-range van der Waal forces. The colloidal stability of a protein may be dominated by reversible self-association or involve essentially irreversible aggregation pathway, both of which can be assessed by a number of methods (Ota et al. 2016). Aggregation associated with partial protein unfolding may be driven by the exposure of hydrophobic regions or ‘patches’ to the solvent and its investigation would require methods able to discern protein conformational stability and change. In this Chapter, mAb stability as a function of temperature was assessed simultaneously by SLS (reporting on the colloidal stability) and intrinsic fluorescence (reporting on protein unfolding, or ‘melting’). Optimisation of colloidal and conformational stability could be anticipated to yield a stable and robust formulation, however, the analytical technique may report a proxy parameter which is not fully indicative of long-term stability and more than one complementary measure of colloidal/conformational stability is general required.

The process of protein aggregation is driven by various factors such as the amino acid composition and sequence, the stress conditions encountered during protein production and the solution conditions. When considering approaches in minimising aggregation, the solution conditions are usually optimised (Obrezanova et al. 2015). Most therapeutic mAbs are formulated at pHs between 5 and 7 as this is usually away from the mAb isoelectric point (pI), therefore mAb should possess strong electrostatic repulsion here. Thus slight drifts in pH can cause detrimental effects to the structure, stability and biological implication of the product (Thakkar et al. 2012, Wu et al. 2014). To avoid issues associated with aggregation and/or self-association and other degradation events it is essential to optimise solution conditions however difficulties are encountered due to these different solution conditions being closely affected by each other, thus controlled temperature storage stability studies are conducted following ICH stability testing guidelines (Food and Drug Administration 2003) where drug substances intended for storage in a refrigerator are exposed to defined temperatures (5, 25 and 40 °C). The temperatures generally used are refrigerated (5 °C), ambient (25 °C) and forced-degradation (40 °C) (Goldberg et al. 2011, Bajaj et al. 2012) (discussed in Section 1.8.6). A common method used to supress aggregation is the addition of protein-stabilising excipients to the final formulation (Shukla et al. 2011a). At present there is no ‘universal excipient’ which can suit every protein formulation. Furthermore, the selection of excipients approved for formulation use listed in the GRAS category is limited (Pifferi and Restani 2003, Ogaji IJ 2012). To successfully select suitable excipients for each mAb formulation high-throughput screening methods are employed. To further simplify this, it would be ideal if a combination of excipients could work together in targeting a wider range of mAb formulations.
In this context, the equimolar combination of the free amino acid L-Arg and L-Glu may be employed as a ‘novel’ excipient for mAb formulation. L-Arg is already a well-established excipient in protein formulations in functions such as preventing aggregation, promoting the refolding of proteins and decreasing solution viscosity (Arakawa et al. 2007b, Vagenende et al. 2013, Fukuda et al. 2014, Miyatake et al. 2016) as well as in certain affinity chromatography processes to increase protein recovery (Shukla and Trout 2011). Previous work in protein structural and functional studies have demonstrated that the addition of the equimolar mixture of L-Arg and L-Glu (Arg·Glu) can increase the protein solubility and decrease protein aggregation (Golovanov et al. 2004, Valente et al. 2005, Blobel et al. 2007, Hautbergue and Golovanov 2008, Blobel et al. 2011) For example, using Arg·Glu at 50 mM increased the protein solubility by a factor between 4 and 8 (Golovanov et al. 2004). On a mole-per-mole basis, Arg·Glu noticeably decreases intermolecular attractions and aggregation in comparison to L-Arg alone (Golovanov et al. 2004, Vedadi et al. 2006) with its additional benefit of increasing the protein shelf-life, stability and solubility (Golovanov et al. 2004). Arg-Glu has also been shown to increase the second virial coefficient ($B_{22}$) (Valente et al. 2005), enhanced native oligomer (Blobel et al. 2007) and thermal stability in other studies (Schneider et al. 2011).

Since the first published work reporting on the mechanism of Arg-Glu in 2004 (Golovanov et al. 2004), several other authors have assessed this mechanism experimentally (Blobel et al. 2007) and using in silico (Shukla and Trout 2011) models where all these studies have confirmed the synergistic behaviour between these two amino acids. Golovanov et al first showed this synergistic effect resulted in maximal aggregation suppression and increase in protein solubility (Golovanov et al. 2004). The authors from these combined studies have speculated that these charged amino acids interact with oppositely charged residues on the protein surface (Golovanov et al. 2004); more specifically L-Arg interacts with the protein through their charged or aromatic residues while the presence of other charged groups can interact with other Arg or Glu acid molecules in the vicinity. In a similar manner, Glu interacts via hydrogen bonding through their positively charged residues to the protein surface with their other carboxylate groups being free to interact with Arg molecules nearby (Blobel et al. 2007, Shukla and Trout 2011). Thus, the majority of exposed hydrophobic patches are covered hence reducing aggregation. The combination of hydrophobic and electrostatic components would probably provide more effective counterion binding than other monovalent ions such as Na$^+$ and Cl$^-$ typically added to increase protein solubility (Golovanov et al. 2004). Arg-Glu at 50 mM already showed decreases in protein aggregation and from the in silico simulations optimum concentrations were predicted at 100-200 mM. Although, these observations have been seen there has been no thorough studies for Arg-Glu in the context of mAb formulations which was investigated in this Chapter.

Furthermore, several imperative considerations need to be assessed before the final therapeutic product is approved; amongst these is the osmolality of the excipient. This is
important as the osmolality of the administered drug into the body ideally should have the same osmolality as the body's fluids (blood is 280-295 mOsmol/kg) to maintain an isotonic environment (Brocker C 2012). This concept has been described in detail in the introduction of the Thesis (Section 1.9.5).

3.3 Aims of this Chapter

The aim of this Chapter was to explore the applicability of Arg-Glu in industrially-relevant protocols and procedures for reducing protein aggregation. As targets, four mAbs supplied by Medimmune, mAb1, mAb2, mAb3 and mAb4 were used in these studies. Initially, the osmolarity measurements of Arg-Glu in the proposed dosage range (50-200 mM) was conducted to ensure these were within the pharmaceutically-acceptable ranges. Once establishing the osmolarities, high-throughput analysis was used to systematically study the colloidal and conformational stability for these mAbs at different pH's as a function of increasing Arg-Glu concentrations by using SLS and intrinsic fluorescence. Accelerated stability studies were conducted for selected mAb conditions to investigate how the presence of Arg-Glu would affected the overall long-term stability of mAb formulated at higher concentrations with comparisons made to mAb in the presence of Arg-HCl. The percentage of monomer was recorded over the stability studies using SE-HPLC.
3.4 Results

3.4.1 Determination of the osmolarity of arginine glutamate solutions

Prior to conducting experiments with Arg·Glu, it was essential to measure the osmolarity of Arg·Glu within the concentration ranges expected to be used in all subsequent studies (50-200 mM) (Figure 3.1). The reason for using Arg·Glu concentrations in this range was based on two main published studies (Golovanov et al. 2004, Shukla and Trout 2011). Experimental studies showed that the equimolar mixture of L-Arg and L-Glu at only 50 mM was capable of increasing the solubility of proteins susceptible to aggregation in a concentration-dependent manner (Golovanov et al. 2004); moreover, Shukla et al. conducted theoretical in silico studies providing evidence that Arg·Glu may have its maximum effect at 150 mM (Shukla and Trout 2011), thus this concentration range was chosen and extended to 200 mM. With increasing Arg·Glu concentrations prepared in MilliQ water the osmolarity was linear. In the presence of protein in the buffer there were minimal changes in the osmolarity. Therefore, these findings showed that Arg·Glu in this range agrees with the acceptable osmolality’s used in protein formulations for subcutaneous injections. As osmolarity determination is critical for therapeutic formulations it was ideal to use a representative mAb to obtain results from at least one therapeutically relevant protein: mAb2 was chosen due to its availability.

![Figure 3.1: Osmolality of increasing Arg-Glu concentrations in different solution conditions. Arg-Glu solutions were prepared in distilled water, 10mM CP buffer pH 6.0, and 10 mM CP buffer containing 30 mg/mL mAb2, as indicated. Duplicate measurements of independent samples were plotted and the variation between measurements was < 5% (Kheddo et al. 2014).]
3.4.2 Use of SLS to investigate the effect of Arg-Glu on mAb colloidal stability

The colloidal stability of four test mAbs was assessed using SLS by observing the temperature dependence of the SLS signal as a function of pH (in the range 5-7) and Arg-Glu concentrations (0-200 mM). This method derives the onset of aggregation temperature ($T_{agg}$) using the Avacta Optim 2. An attractive aspect of this instrument is the use of small volumes with high-throughput analyses capabilities to investigate different formulation conditions. The pH range (5-7) was chosen as it allowed mAb conditions to be investigated in the slightly acidic region (pH 5-6) commonly used in mAb formulations, and at neutral pH which is close to the pI of the mAbs thus a region where aggregation is likely to occur.

Prior to starting experiments with mAbs, the scattered light for Arg-Glu in the absence of mAb was measured to ensure there was no cluster formation contribution from Arg-Glu in subsequent studies. Figure 3.2 shows representative raw SLS traces of increasing Arg-Glu concentrations in the presence of mAb (as labelled) and secondly Arg-Glu alone in CP buffer, pH 5 (50 and 200 mM). Focusing on Arg-Glu alone in buffer, there was a flat dependence of light scattering with increasing temperatures thus confirming there was no light scattering contribution from Arg-Glu in the absence of mAb measured at 266 nm, similar results were observed at pH 6, 7 and 8 (data not shown). Moreover, the traces for Arg-Glu in the presence of mAb represented how the original light scattering traces are obtained for any molecule at 266 nm to derive the $T_{agg}$. $T_{agg}$ was determined when a sudden, sharp increase in scattered intensity was seen as a result of protein molecules associating into higher order aggregates. In this Thesis only results from 266 nm were recorded as the data was more consistent and reliable on reporting on smaller aggregates whereas at 473 nm the larger aggregates obscured the data. Previous experiments conducted at MedImmune have led to a preliminary conclusion that a 5-10 °C change in the $T_{agg}$ is considered significant. For ease of data interpretation, the $T_{agg}$ were recorded and plotted in an alternative manner in subsequent experiments.
Figure 3.2: Example raw SLS traces at 266 nm used to measure $T_{agg}$. The amount of light scatter for Arg-Glu solutions in CP buffer pH 5 without mAb1 was relatively insignificant, such that the SLS signal for Arg-Glu solutions containing mAb1 represent light scatter from mAb aggregation. The decrease in SLS signal at temperatures above ~ 70 °C for mAb1 samples was due to the gross precipitation of the protein sample and consequent light obscuration. Similar traces were obtained for the others mAbs and different pH CP buffer. Data were analyzed using GraphPad Prism v6, plotting averages of measurements from three independently prepared samples with error bars representing the standard deviation (Kheddo et al. 2014).

The varied intrinsic $T_{agg}$ measurements for the four mAbs at pH 5 (where the pI was furthest away) in the absence of Arg-Glu indicated the mAbs chosen represented a wide range of inherent colloidal stabilities (between 54 to 83 °C) (Figure 3.3). Several key findings were observed from these data; in the absence of Arg-Glu the $T_{agg}$ progressively decreased with increasing pH seen with all mAbs. For all mAbs at pH 5 except for mAb1, the $T_{agg}$ values were unaffected by the addition of increasing Arg-Glu concentrations. mAb1 at pH 5 showed the lowest $T_{agg}$ in the absence of Arg-Glu (55 °C) however a systematic increase in $T_{agg}$ was seen with increasing Arg-Glu concentrations up to 9 °C. At pH 6 the $T_{agg}$ increased by ~ 10 °C with the addition of 200 mM Arg-Glu; and at pH 7 even more remarkable increases in $T_{agg}$ were observed with 200 mM Arg-Glu (20 °C) to levels similar to the $T_{agg}$ at pH 5 with the maximum Arg-Glu concentration. For all the mAbs Arg-Glu showed remarkable increases in the $T_{agg}$ at pH 6 and 7 where the maximum $T_{agg}$ increases were surprisingly observed at neutral pH. At pH 6, all mAbs showed increases in the $T_{agg}$ between 10 to 20 °C with the maximal Arg-Glu concentrations. The same $T_{agg}$ values at pH 7 for all the mAbs was analysed more closely by plotting $T_{agg}$ as a function of Arg-Glu concentrations (Figure 3.4); mAb1 and mAb2 showed a continual increase in $T_{agg}$ upon approaching the maximum Arg-Glu concentrations whereas mAb3 and mAb4 reached a plateau at 100 mM Arg-Glu although the addition of just 50 mM displayed a significant stabilising effect.
In summary, the addition of Arg·Glu increased the $T_{agg}$ in a concentration-dependent manner for all the mAbs. For mAb1 with the optimal effect from Arg·Glu was seen at 200 mM at pH 7 which brought the $T_{agg}$ close to what was observed at pH 5. For all the mAbs except mAb1, Arg·Glu did not have any effect on $T_{agg}$ at pH 5. Focussing on the effect of Arg·Glu at pH 7, all the mAbs showed increases in $T_{agg}$ even at concentrations of only 50 mM, indicating the increased stabilising effect of Arg·Glu when the solution pH is closer to the pI of the mAbs.

![Figure 3.3](image)

**Figure 3.3:** Changes in $T_{agg}$ as a function of pH for different concentrations of Arg·Glu for four mAbs as labelled. This is the Interpretation of the light scattering data to obtain $T_{agg}$ at 266 nm. The data have been reanalysed on GraphPad Prism v6 where the averages from triplicate measurements from independently prepared sample for each data point has been plotted; the error bars represent the standard deviation (Kheddo et al. 2014).
3.4.3 Use of intrinsic fluorescence to investigate the effect of Arg-Glu on mAb conformational stability

The conformational stability of mAb was assessed for exactly the same samples and conditions used for SLS measurements to observe how mAb conformational stabilities are affected as a function of pH (5-7) and addition of Arg-Glu (0-200 mM). This was simultaneously measured with the SLS measurements from the temperature dependence of the intrinsic fluorescence signal to derive the protein melting temperature $T_m$. A typical trace of how the conformational stability of mAbs was determined is shown in Figure 3.5. As tryptophan shifts between two extreme wavelengths 350 nm and 330 nm depending on the environment it is exposed to, calculating the ratio between these wavelengths provides a reliable measure of the degree of protein unfolding. MAbs are multidomain proteins comprising of 3 different domains ($F_{ab}$ fragment, $C_{H2}$ and $C_{H3}$ domain). The unfolding of these domains occurs independently at different temperatures. The first transition usually represents the unfolding of the least conformationally stable $C_{H2}$ domain; this is due to the large area of hydrophobic surfaces which are solvent accessible whereas in the $C_{H3}$ domain these are mostly hidden (Mehta et al. 2014). In this Chapter, the first protein melting temperature $T_{m1}$ was reported. An increase in $T_{m1}$ indicates the protein is conformationally stabilized whereas a decrease indicates destabilisation.
Figure 3.5: Example intrinsic fluorescence data showing the ratio of the intensities at 350 and 330 nm, used to measure $T_{m1}$, mAb3 in CP buffer, pH 5 is shown here. Two unfolding transitions can be observed. The first transition is most clearly seen and occurs between ca. 55 and 75 °C, corresponding to the approximate locations of the lower and upper baselines respectively, giving $T_{m1}$ values of ~65 °C. The subsequent transition(s) are weaker and more variable but can be seen to occur above 75-80 °C. Similar traces were obtained for the others mAbs and different pH CP buffers. Data were analyzed using the Avacta Optim 2 software and GraphPad Prism v6, plotting averages of measurements from independently prepared samples with error bars representing the standard deviation (Kheddo et al. 2014).

The findings showed that on the whole $T_{m1}$ mostly increased with an increase in pH from 5 to 7 except with mAb3 where a slight decrease in $T_{m1}$ was observed in the absence of Arg-Glu (Figure 3.6). In the absence of Arg-Glu at pH 5, mAb1, mAb2 and mAb4 exhibited $T_{m1}$ values in the range of 60 to 65 °C which noticeably increased to 70 °C at pH 7. However, $T_{m1}$ did not significantly increase with the addition of Arg-Glu as a function of pH. mAb3 was the least conformationally stable (~ 46 °C) which only slightly increased to ~ 50 °C in the presence of 200 mM Arg-Glu. There was no obvious effect on $T_{m1}$ with the addition of Arg-Glu for mAb2 at any of the pH’s; moreover Arg-Glu seemed to marginally destabilise mAb1 and mAb4. This different behaviour from mAb3 indicated that Arg-Glu had more of a stabilising effect with the least stable mAb particularly at pH 7 with a 5 °C increase in the absence of Arg-Glu to 200 mM.

To summarise, the addition of increasing Arg-Glu concentrations did not show any considerable increases in $T_{m1}$. Although, by simply increasing the pH towards neutral pH, $T_{m1}$ increased considerably with the highest conformational stability observed at pH 7 whether in the presence or absence of Arg-Glu.
3.4.4 Accelerated stability studies of mAbs in the presence of Arg·Glu

Following from monitoring the colloidal and conformational stability of these mAbs as a function of different solution conditions; it was interesting to investigate the long-term stability of selected mAbs formulated at medium concentrations (30-50 mg/mL). Three mAbs from the previous experiments were selected that were representative of different inherent behaviours formulated at differing pH's and additives. These three mAb were placed into two formulations each making a total of six formulations (Table 2.3) which were stored at different temperatures (5, 25 and 40 °C); the percent monomeric protein species remaining in solution was monitored over 2 months using SE-HPLC. MAbl was formulated at pH 5.5 and 7 in the presence of 200 mM Arg·Glu; this condition was chosen as the \( T_{agg} \) was not affected with increasing pH in the presence of Arg·Glu. Figure 3.7 showed at 5 and 25 °C MAbl at both pH's did not show any significant changes in the % of protein monomer remaining in solution over the 6 weeks (remaining at ~98 %). MAbl at pH 5.5 in the presence of Arg·Glu only showed < 2 % difference in monomer loss in comparison to pH 7 at the accelerated temperature of 40 °C; this was surprising as MAbl showed better colloidal stability at low pH in the absence of Arg·Glu (Figure 3.2). The greater loss in % monomer of MAbl at pH 7 is most likely due to this pH being closer to the pl (7.9–8.3). The accelerated stability temperature of 40 °C was very close to the \( T_{agg} \) of MAbl (43 °C) at pH 7 in the absence of Arg·Glu; despite this it was interesting to observe that the presence of 200 mM Arg-Glu maintained the stability of MAbl for a number of weeks at 40 °C with >93 % protein monomer remaining in solution after the 6 weeks storage.
Figure 3.7: Monomer loss determined from SE-HPLC data for mAb1. mAb1 was stored over 6 weeks at 5, 25 and 40 °C in 10 mM CP buffer, 200 mM Arg-Glu pH 5.5 or 7, as labelled. The percent monomer at time = 0 for both formulations was 99.0%. Data reported were derived from two independent measurements which differed by <5% (Kheddo et al. 2014).

The conditions selected for mAb3 was based from this mAb being the least conformationally stable at pH 5 (Figure 3.8), thus the ability of Arg-Glu to increase long-term stability was compared to Arg-HCl at pH 5.5. At 5 and 25 °C under both conditions there was < 5% losses in the monomer content over the studies similarly seen for mAb1. However, at 40 °C there was a noticeable difference in the stability of mAb3 between Arg-Glu and Arg-HCl. After only the first week of the studies, there was a ~ 30% decline in the monomer content in the presence of Arg-HCl whereas in the presence of Arg-Glu there was only a 15% loss. This decrease in monomer loss continued over the two months where at the end of the studies there was 15% more monomer population remaining in the presence of Arg-Glu (55%) in comparison to Arg-HCl (40%). This rapid decline in the monomer population could be attributed to this mAb displaying its intrinsic $T_m$ at 45 °C (Figure 3.6) which is very close to the storage temperature use here at 40 °C.
Figure 3.8: Monomer loss determined from SE-HPLC data for mAb3. mAb3 was stored over 8 weeks at 5, 25 and 40 °C in 10 mM CP buffer pH 5.5, with addition of arginine salts Arg·Glu or Arg·HCl, as labelled. The percent monomer at time = 0 for both formulations was 99.1%. Data reported were derived from two independent measurements which differed by <5% (Kheddo et al. 2014).

Finally, mAb4 was also investigated under these same accelerated conditions (Figure 3.9). mAb4 was the most stable mAb both conformationally and colloidally displaying the highest $T_{agg}$ and $T_{m1}$ values (Figure 3.3, 3.6). The ability of Arg·Glu to increase the long-term stability of mAb4 was compared to Arg·HCl. Although longer storage time of up to 10 weeks was assessed with mAb4, there was no distinguishable differences in the monomer losses between Arg·Glu and Arg·HCl at 5, 25 and 40 °C (Figure 3.9). As with mAb1 and mAb3, the monomer loss with mAb4 remained almost constant at 5 and 25 °C (<1 % loss) with the extent of monomer loss at 40 °C being similar to other mAbs over the storage period (~1 0%); however mAb4 did not show any distinguishable differences in monomer loss at 40 °C between Arg·Glu and Arg·HCl. Thus, there was no noticeable difference in the performance of Arg-Glu in comparison to Arg-HCl.
Figure 3.9: Monomer loss determined from SE-HPLC data for mAb4. mAb4 was stored over 10 weeks at 5, 25 and 40 °C in 10 mM CP buffer pH 5.5, with addition of arginine salts Arg·Glu or Arg·HCl, as labelled. The percent monomer at time = 0 for both formulations was 98.9%. Data reported were derived from two independent measurements which differed by <5%. Only data from week 3 onwards is shown on the graph as there was no significant change in the percent monomer before that (data for weeks 1 and 2 are very similar to week 3) (Kheddo et al. 2014).

In summary, as obvious differences were not seen between 5 and 25 °C for all mAbs, longer stability studies would be required to pull out any distinguishable differences at these storage temperatures. mAb1 presented surprising data as this was a mAb inherently prone to aggregation (Figure 2.3) however in the presence of 200 mM Arg·Glu the monomer loss profile was comparable (Figure 2.7) to what was observed with the more stable mAb4 (Figure 2.9). MAb3 at pH 5.5 with Arg-Glu showed much less monomer losses in comparison to Arg-HCl despite Arg-Glu not showing any obvious changes in $T_{agg}$ at pH 5 accompanied by a marginal increase in $T_{m1}$. The long-term stability performances of Arg-Glu and Arg-HCl could not be distinguished at any of the temperatures with mAb4. The results suggested that the incorporation of Arg-Glu in less stable mAbs that are aggregation prone could be beneficial. It seems more extreme stress conditions would be required (increasing the degradation rate of these mAbs) to explore the maximum effects of Arg-Glu in comparison to Arg-HCl for example at room temperature for a long time period.
3.5 Discussion

The key aims of this Chapter were to investigate the physical stability of four representative mAbs (mAb1-4) as a function of different solution pH and increasing concentrations of Arg·Glu. The main degradation pathway encountered by mAbs is aggregation with the rate and extent being dependent on the mAbs colloidal and conformational stability (Goldberg et al. 2011). To assess the conformational stability of the native protein under different formulation conditions, the intrinsic fluorescent signal is measured as a function of temperature to determine $T_{m}$. The self-association of the native protein monomer and aggregation (i.e. colloidal stability) can be assessed by measuring $T_{agg}$; this arises from the increase in the scattered light which is sensitive to aggregate formation. The conformational stability of mAbs is important as models of protein aggregation have proposed that partial unfolding leads to dimer formation due to the exposure of hydrophobic residues acting as a nucleation site for further aggregation. Therefore, it is desirable to shift the conformational equilibria to a more native state (Goldberg et al. 2011).

Initial studies were carried out to investigate the colloidal stability of four test mAbs as a function of solution pH and Arg·Glu concentrations. The findings showed the greatest stabilising effect from Arg·Glu was at pH 7 followed by pH 6 and pH 5. At pH 5 the $T_{agg}$ was insensitive to the addition of Arg·Glu, with the exception of mAb1 showing increases in the $T_{agg}$. This can be explained by this mAb being the most prone to aggregation. The greater mAb stability at pH 5 which was unaffected by the presence of Arg·Glu can be explained by the strong electrostatic repulsion between the positively-charged proteins molecules as pH 5 is furthest away from the mAb pI’s (Roberts et al. 2015). On the other hand, mAb3 and mAb4 displayed increasing $T_{agg}$ values with the addition of only 50 mM Arg·Glu which reached a plateau after 100 mM; this is in agreement with earlier studies also showing the increased protein stability from Arg·Glu at this relatively low concentration (Golovanov et al. 2004). These are interesting findings as in support with other earlier studies, it seems an optimal Arg·Glu concentration does exist within the concentration range tested here (Shukla and Trout 2011). These are promising observations as these optimal concentrations probably lie within the pharmaceutically-acceptable osmolality range for subcutaneous administration. Ideally the osmolality of the therapeutic drug should be as close to or as near to the osmolality of the body’s fluid (blood is 280-295 mOsmol/kg) as to maintain an isotonic environment (described in more detail in Chapter 4). It was also an interesting observation that at pH 6 and 7, there was a continual increase in $T_{agg}$, predominantly seen with mAb1 and mAb2, up to 200 mM Arg·Glu suggesting the optimal concentration may not have been reached in these cases. This indicates even higher Arg·Glu concentrations may prevent aggregation further with the optimal Arg·Glu concentrations differing between mAbs. Moreover, the increases in $T_{agg}$ particularly at pH 6 and 7 did not exceed the optimal $T_{agg}$ achieved at pH 5 for mAb1 and mAb2 suggesting there is most probably a saturation limit for the effect of Arg·Glu above 200 mM. mAb therapeutics are usually formulated at least 1 pH unit away from the pI which increases the
net charge of the protein, aggregation is favourable when the pH is close to the pI due to the total protein charge equalling zero, thus the repulsion between molecules is at a minimum (Roberts et al. 2015). In theory, when the pH is equal to the pI the electrostatic contributions to the conformational stability of individual molecule should be optimal (Ugwu 2004). Arg-Glu seemed to significantly increase the $T_{agg}$ of less colloidal stability mAbs especially at pH 7, therefore, as the stability of the mAb’s are clearly enhanced at pH’s closer to neutral it could be an advantageous consideration to the biopharmaceutical industry to formulate at these conditions (Bang 2006).

mAbs are susceptible to conformational destabilisation or partial-to-complete unfolding particularly at elevated temperatures (Goswami 2013) which can lead to aggregate formation. The addition of Arg-Glu did not greatly influence $T_{m1}$ for the mAbs studied here except for mAb3 which was the least conformationally stable. This indicates less stable molecules may be conformationally stabilised by Arg-Glu. A similar increase in mAb thermal stability was reported by other studies for less conformational stability mAbs (Vedadi et al. 2006). This observation can be due to mAb3 having a greater propensity to undergo slight structural perturbations thus will have a relatively large number of partially folded intermediates at any one time within the sample. Arg-HCl is known to be used to refold and stabilise structural intermediates by association at the interface of poorly hydrated regions (specifically hydrophobic regions that are transiently exposed to the solvent) hence this excipient is expected to stabilise less stable mAbs. Moreover, the reason that Arg-HCl stabilises the mAb and does not promote further unfolding can be closely related to the guanidine moiety which experiences a larger volume of exclusion. The kinetics of protein folding and unfolding is an important consideration. Electrostatic interactions can affect protein stability, as the number of charges on the protein is increased there is increased charged repulsion leading to protein destabilisation as a consequence of the greater charge density on the folded protein than the unfolded (Chi et al. 2003). Experimental work recently published investigated the conformational and colloidal stability of mAbs at similar pH’s to this Thesis where the author also found formulating mAbs at pH’s away from the pI destabilised the protein structure due to monomolecular protein unfolding (i.e. decreases conformational stability) however on the other hand this same situation decreased protein intermolecular repulsion thus reducing protein aggregation (increases colloidal stability) (Nicoud et al. 2016). Thus, there is an intricate relationship between these two stabilities to obtain the optimal formulation.

Previous studies have investigated the role of these amino acids independently on preventing aggregation; an example study reported that Arg-HCl inhibited heat-induced aggregation by preventing protein self-association without impacting the conformational stability; whereas, NaGlu stabilised proteins by enhancing the conformational stability via preferential exclusion of Glu from the protein surface (Shukla and Trout 2011). To understand this mechanism of Arg-Glu it is important to consider the molecular interactions between these two amino acids and the protein surface. Extensive research has been carried out on Arg-HCl as it is a well-
established excipient used for several purposes including protein refolding, elution of antibodies from Protein A resin and decreasing protein aggregation where these effects seen by Arg-HCl occur at different concentrations (Tsumoto et al. 2004, Fukuda et al. 2014). Arg is more effective at much higher concentrations (> 0.1 M); for example 2 M Arg increased antibody recovery from Protein-A columns. The use of high concentrations indicates Arg weakly interacts with the protein (Arakawa et al. 2007b). A unique feature of Arg is the guanidinium functional group which has been used as the starting point for many years in explaining the molecular mechanism of Arg. Guanidinium is a well-known protein denaturant (Monera et al. 1994, Qasim and Taha 2013); however some salt forms (e.g. guanidinium sulphate) stabilises proteins rather than denature them due to guanidiniums strong attractive interactions with similar sized anions resulting in the clustering of the ions in solution as opposed to binding directly to the protein surface and causing denaturation. The difference between guanidinium and Arg is the presence of the carbon chain and charged groups (N-terminal amino group and C-terminal carboxylate group) on Arg which appears to augment cluster formation (Schneider et al. 2011). Molecular simulations of the mechanism of Arg have revealed that the guanidinium group interacts with the protein surface with it additionally forming attractive interactions with other Arg molecules. This self-association Arg prohibits it to strongly bind to the protein surface hence denaturation is prevented however this self-association successfully prevents PPIs (Shukla and Trout 2010, Shukla et al. 2011a, Schneider et al. 2011). Other studies have reported more specifically that the guanidinium group interacts via a cation–π interaction with the aromatic ring of tryptophan (Fukuda et al. 2014) on the protein indicating Arg may have some specific weak affinity to side chains involved in protein aggregation (Tsumoto et al. 2004). Preferential interaction measurements confirmed GdnHCl at concentrations > 1 M interacts with the protein surface at a higher affinity than Arg-HCl thus probably explaining the non-denaturing effect of Arg-HCl (Arakawa et al. 2007b). In contrary to this, recent studies by Wen et al concluded Arg at high concentrations between 0.25 to 1 M disturbs the conformational and colloidal stabilities of protein via preferential binding effects altering the protein charge and enhancing aggregation (Wen et al. 2015). The abundant literature available on Arg suggests it is used on a trial and error basis in protein formulation with the concentration playing an important role; moreover the effective concentrations of Arg-HCl are much higher than the concentrations studied for the equimolar Arg-Glu mixture. The ‘negative’ effects reported for Arg has been mitigated with the addition of counter-ions reported by a number of studies at concentrations between 50 to 200 mM (e.g. Glu, Asp and Gly) (Shukla et al. 2011b, Fukuda et al. 2014, Ajmer and Scherliess 2014). The enhanced effects of Arg at lower concentrations in the equimolar mixture with Glu at concentrations between 50 to 200 mM have been successfully shown in this Thesis compared to these published studies which require much greater concentrations of Arg-HCl alone. The studies in this Chapter showed the increases in the $T_{agg}$ with the addition of Arg-Glu directly increasing the mAbs colloidal stability whereas the $T_{m1}$ results showed a comparatively small effect on the conformational stability; this correlated well with previous studies which also
showed the decrease in protein self-association without compromising the native protein conformation (Golovanov et al. 2004). A recent study by Fukuda et al investigated the thermodynamic stability of proteins using Arg-Glu (at concentrations 100, 300 and 500 mM) rather than the colloidal stability (mainly investigated in other published studies); they found the Arg counterions Asp and Glu prevented the destabilising effects of L-Arg alone (Fukuda et al. 2014). This is encouraging results as the results in this Chapter also showed decreases in aggregation with increasing concentrations of Arg-Glu up to 200 mM; it seems even higher concentrations may further stabilise proteins to a greater extent than Arg alone.

More recently, Reslan et al (Reslan et al. 2016) investigated the ability of Arg-Glu in preventing aggregation and increasing stability in spray-dried bovine serum albumin (BSA). They concluded Arg-Glu at 25 mM did not show synergistic effects on the stabilisation of spray-dried BSA. They found that L-Arg alone significantly reduced monomer loss measured by SE-HPLC however when combined with L-Glu, it was ineffective in preventing monomer loss (Reslan et al. 2016). In this Thesis, liquid formulations were studied thus it would not be feasible to directly compare the results. There is currently no other studies assessing the performance of Arg-Glu in stabilising proteins in the dry state. Speculations can be made on the authors results; firstly a much lower concentration of Arg-Glu (25 mM) was used in these studies in comparison to others where optimal Arg-Glu concentrations to prevent aggregation were found to lie between 100 to 200 mM (Shukla and Trout 2011). Secondly, for their accelerated stability studies extremely high accelerated temperatures of 65 and 70 °C were used whereas typically in liquid formulations the accelerated temperature used is 40 °C. Moreover, protein concentrations of < 1mg/mL are probably not ideal to show the optimal synergistic effect from Arg-Glu as self-association would in theory be minimal thus no added benefit or even destabilising effects may be observed from Arg-Glu (Reslan et al. 2016). These points may have affected the ability of Arg-Glu in effectively preventing monomer loss in their studies. Another aspect is whether Arg-Glu is stable in the solid form. Protein formulations in the dry state involves removal of water thus any excipient which acts through excipient-water interactions will be affected (Ohtake et al. 2011). A number of excipients are already used in stabilising proteins in the dry state such as trehalose and sucrose; moreover, Arg has been reported to be effective in preventing aggregation in lyophilised formulations (Katdare and Chaubal 2006). Other studies have reported that salts in the dry state are not expected to provide stability due to their propensity to crystallise i.e excipient phase separates from the protein; excipients which maintain their amorphous state provide optimal protein stability with Arg being one of these (Ohtake et al. 2011). It can be anticipated that in the dry form L-Arg effectively co-crystallizes with L-Glu (Bhat and Vijayan 1977). It could be also speculated that Arg-Glu is not stable enough in the solid crystalline form, especially at higher temperature. The study in this Thesis and by Reslan et al looked at the applicability of Arg-Glu in different formulation states; these findings stress the earlier message that firstly ‘universal’ excipients do not exist for every protein. However, the studies conducted in this Thesis strongly shows the stabilising effects of Arg-Glu on four different IgG1 mAbs of different intrinsic stabilities.
Moreover, it is obvious that the ability of Arg-Glu to successfully work depends on the mechanism of the amino acid side chains to interact with the protein; slight differences in the amino acid structure play a critical role in the effective prevention of aggregation; which could account for L-Glu synergistically working with L-Arg and even L-Asp with L-Arg \cite{Fukuda2014} whereas other counterions may not be as successful. Moreover, differences have been observed between charged and hydrophobic amino acids in their extent to reduce protein aggregation most likely due to the side chain structures and the nature of the equilibrium between the native and unfolded state which determines the effect of the amino acid on protein stability \cite{Shiraki2002, Reslan2016}.

It is desirable to formulate mAbs at high concentrations where protein self-association is more pronounced; thus additionally to assessing the effect of Arg-Glu on the colloidal and conformational stability of mAbs, it is imperative to assess if these improvements correlate with the overall storage stability in concentrated conditions, particularly under stressed conditions. The three mAbs selected represented differing characteristic inherent behaviours; a low $T_{agg}$ (mAb1), a low $T_{m1}$ (mAb3) and a relatively stable mAb4 in comparison to the former two mAbs. These mAbs were formulated in the presence of 200 mM Arg-Glu and were compared to a relevant reference sample (described in Section 2.2.1.1). Accelerated stability studies are routinely performed in protein formulations which are monitored by measuring the percentage of monomer remaining in solution during the course of the stability studies. From the $T_{agg}$ results, mAb1 at pH 7 was heavily susceptible to temperature-induced aggregation which was colloidally stabilised with 200 mM Arg-Glu; remarkably under thermal stress during the long-term stability studies the overall storage stability at 40 °C of mAb1 at pH 7 in the presence of 200 mM Arg-Glu was very comparative to mAb1 at pH 5.5 in the presence of 200 mM Arg-Glu despite pH 7 being close to the pI and the mAb concentration being relatively high. The colloidal and conformational stability assessment of mAb1 was performed at very dilute mAb concentrations (1 mg/mL) where the results showed mAb1 was colloidally stabilised with the addition of increasing Arg-Glu concentrations at all pH’s. Moreover, the findings from the long-term stability results of mAb1 correlated well with its colloidal and conformational stability where the overall stability of mAb1 at pH 7 was very similar to pH 5.5, indicating these are valuable parameters used in the assessment of different formulation conditions.

With mAb3 and mAb4 the performance of Arg-Glu in supressing aggregation was compared to Arg-HCl. mAb3 at pH 5.5 showed a greater decrease in aggregation in the presence of 200 mM Arg-Glu in comparison to Arg-HCl regardless of the intrinsic $T_{m1}$ of mAb3 being 45 °C which was very close to the accelerated temperature used. It would be ideal in future experiments to compare mAb3 in the same conditions at pH 7 as it would be expected that the intrinsic $T_{m1}$ for mAb3 at pH 7 with Arg-Glu would be greater than at pH 5.5 as seen from Figure 3.3. Finally, the most stable mAb4 did not show noticeable differences between Arg-Glu and Arg-HCl over the stability studies thus more challenging stress conditions may be required.
to pull out differences. Referring back to the studies by Fukuda et al where they looked at preventing aggregation in mAb liquid formulations initially with Arg-HCl and L-Arg in combination with L-Glu or L-Asp, the authors found increased stabilising effects from Arg-Glu in comparison Arg-HCl (concentrations between 50 to 200 mM) similarly seen in this Chapter (Fukuda et al. 2014). The different conclusions obtained by Reslan et al were discussed above; more thorough analysis would be required to understand the performance of Arg-Glu in spray-dried formulations (Reslan et al. 2016).

In summary, the findings from this Chapter have illustrated the addition of Arg-Glu to mAbs formulated at mildly acid to neutral pH clearly increases mAb stability. Four mAbs with different inherent physical stabilities were selected; the colloidal stability ($T_{agg}$) was significantly enhanced for all mAbs with increasing Arg-Glu concentrations. It was positive to see in line with other studies that even 50 mM Arg-Glu increased $T_{agg}$. These results provide reassurance that the maximal effects of Arg-Glu probably lie within this concentration range which is probably in the pharmaceutically-acceptable osmolality range. In terms of $T_{m1}$ Arg-Glu did not cause any significant increases however by simply shifting the pH towards neutral increased $T_{m1}$. The findings from the accelerated stability studies clearly showed for the least stable mAb that Arg-Glu was able to prevent monomer loss much more than Arg-HCl and additionally prevent aggregation to a comparable level observed at pH 5.5. As previously mentioned, the common wisdom is that the buffer pH of the formulation should be away from the pI, however this Chapter suggests that the presence of Arg-Glu increased protein stability against aggregation in the pH range very close to pI. This could open new routes in to moving away from the typical formulation at ~ pH 6 to pH’s closer to the pI of the molecule, where the protein molecule is inherently more stable conformationally.
Chapter Four: *In vitro* cell culture studies of arginine glutamate

The work presented in this Chapter has been published:


4.1 Abstract

The search for novel excipients is an ongoing process for mAb formulations, particularly, in preventing aggregation at high concentrations (≥ 100 mg/mL). Excipients used in mAb formulation are selected from GRAS’ by the regulatory bodies. Whilst being composed of naturally occurring amino acids which belong to the GRAS category, the effect of the equimolar mixture of increasing concentrations of Arg·Glu on cells as a salt has not been explored previously. Here the effects of Arg·Glu on cell viability and cellular stress were examined using *in vitro* cell culture systems with reference to an already established excipient NaCl, in the perspective of mAb formulations. Cell culture modals were selected based on representative cells founds in the subcutaneous administration route: the human monocyte cell line THP-1, grown as a single cell suspension, and adherent human primary fibroblasts. The changes in cell viability of THP-1 cells in the presence of Arg·Glu, the individual components Arg·HCl and NaGlu and NaCl over a range of osmolalities (well above isotonicity) was tested. Furthermore, the mechanism of cell death was investigated by high salt concentrations as well as their effects on alterations on membrane markers and cytokine expression. The findings displayed the expected decline in cell viability with increasing osmolalities with a similar drop in cell viability between Arg·Glu and NaCl, moreover both salts at higher concentrations triggered cell death by apoptosis (programmed cell death). This indicates the impact of Arg·Glu on cell viability is entirely attributable to its osmolality and thus there is no additional toxicity associated with this excipient beyond what was observed with NaCl. Also, the mechanism of toxicity indicated that cell death is unlikely to illicit an inflammatory response via subcutaneous injection *in vivo*. Additionally, these salts did not significantly effect membrane marker activation or cytokine expression from THP-1 cells. In comparison to the adherent cells, Arg·Glu and NaCl did cause significant toxicity with a steeper decline in cell viability observed with Arg·Glu. In conclusion, Arg·Glu concentrations added to the cell culture media below 100 mM (mild hypertonicity) did not noticeably impact cell viability indicating Arg·Glu is as safe for cell cultures as NaCl at equivalent osmolalities.
4.2 Introduction

4.2.1 Toxicity studies of Arg·Glu in downstream bioprocessing

As described in the introduction section of the Thesis, there is a limited choice of excipients available for formulation of pharmaceutical mAbs as only those listed as GRAS by the regulatory bodies are used in practice for patient-injected formulations, including L-Arg and L-Glu (Ogaji et al., 2011; Pifferi and Restani, 2003). Although these amino acids are a natural constituent of organisms and cells, they are part of human diet, and therefore inherently are non-toxic; this novel combination has no reported data illustrating this and cannot be included in the GRAS category. The approval process for novel excipients was discussed in detail in the introduction.

Excipients are known to cause increases in solution osmolality. Therefore, it is vital for formulation scientist to consider the osmolality of the drug product during the development process. Ideally, the osmolality of the final formulation should be equivalent to the osmolality of blood. Hypotonicity has been shown to act as a danger signal (Compan et al. 2013) and it has also been reported that osmotic shock due to hypertonicity induced the production of the proinflammatory cytokine IL-8 by human peripheral blood mononuclear cells (Shapiro and Dinarello 1997). Homeostatic control mechanisms are constantly operating in maintaining the body’s internal environmental factors (such as osmolality and oxygen levels) within an acceptable range. However, the introduction of any agent into the body which disrupts these acceptable ranges can cause stress to the cells (Medzhitov 2008). Immune cells can be activated by this so-called “danger signal” derived from these perturbations of tissue/cell homeostasis to potentially go on to onset the inflammatory response. The use of Arg-Glu may disrupt normal homeostasis, acting as an adjuvant to initiate an inflammatory response. The release of numerous inflammatory mediators are largely responsible in coordinating the inflammatory process in particular the cytokine IL-8, costimulatory molecules CD54, CD86 and HLA-DR and the release of NO; these can be used as markers of inflammation.

During the exposure of cells to these stress responses, it is also of interest to understand the mechanism of cell death. Cells can die via two major mechanisms either by apoptosis or necrosis. Apoptosis, also known as programmed cell death generally occurs as part of physiological events such as during normal development processes. However, necrosis is considered to be a pathological process arising from insults such as trauma that is identified with cell swelling and loss of membrane integrity. Necrotic cell death usually stimulates an inflammatory response as these expel their contents when they lyse, including inflammatory cytokines that stimulate further pro-inflammatory responses (Rock and Kono 2008). In contrast, cells that die by apoptosis are packaged into apoptotic bodies which escape immune activation and instead are phagocytosed (Biermann et al. 2013). One of the early indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This molecule is recognized by
phagocytes, triggering phagocytosis and also the production of anti-inflammatory cytokines, resulting in efficient removal of the dying cell without inflammation (Medzhitov 2008, Rock and Kono 2008). Therefore, it is necessary to confirm Arg-Glu (as well as any other novel excipient) does not cause any adjuvant effects on stimulating the inflammatory response and understand the type of cell death occurring, in vitro methods can be used to assess the impact of a range of osmolalities on cell viability and cellular stress. Based on these in vitro methods, it is possible to extrapolate to in vivo effects.

4.2.2 Arg-Glu as a tissue culture supplement in upstream processing

The key aim of this Thesis was to investigate the use of Arg-Glu in reducing protein aggregation and increasing stability in primarily downstream processes (DSP). As an extension (small focused study), it was of interest to also investigate the impact of Arg-Glu in upstream processing (USP) in the culture mediums during protein expression and purification where protein aggregation is also a frequently encountered problem which is a challenge faced in industrial and academic applications (Liu et al. 2010, Saraswat et al. 2013). DSP is generally the area which offers most opportunity to mitigate aggregates; however aggregation encountered in UPS can result in lower protein yield (Paul et al. 2014). The large-scale production of recombinant mAbs is in mammalian cells, commonly HEK, Chinese hamster ovary, NSO, Sp2/0 and PER-C6 cells; with the latter three approved for human therapy (Kunert and Reinhart 2016). The studies conducted here used the HEK-293 cell model system; this cell line is most commonly used for transient gene expression to produce protein within a few days preceding the delivery of DNA. The test protein of interest, OlFd was expressed by these cells due to its propensity to aggregate. There is already precedent for the use of the basic amino acids L-Arg, L-lysine, and L-ornithine in preventing aggregation (Shiraki et al. 2002). This leads to the potential benefit of incorporating the equimolar mixture of Arg-Glu throughout protein expression and purification. The incorporation of Arg-Glu at the post purification stage has started to be used within laboratories which has shown a decrease in aggregation.

4.3 Aims of this Chapter

The aim of the work under taken in this Chapter was to examine the effects of increased osmolality of Arg-Glu on cell viability and cellular stress using in vitro cell culture systems. From the studies in Chapter 3, higher Arg-Glu concentrations (e.g. ≥200 mM in the final formulation, Kheddo et al., 2014), may be necessary for optimal protein solubility, this increases osmolality and hence may affect cell viability in vitro or in vivo or cause cellular stress (Vazquez-Rey and Lang, 2011). Specific cells were selected which are of relevance to the subcutaneous route of administration (Kagan 2014): the human monocyte cell line THP-1 (a surrogate DC line; (Megherbi et al. 2009)), grown as a single cell suspension, and human primary fibroblasts, cultured as an adherent monolayer have been selected here. The impact on cell viability of increasing osmolalities of L-Arg and L-Glu solutions (together as the
equimolar mixture and separately) has been examined in comparison with the reference standard NaCl. Furthermore, for THP-1 cells, the mechanism of cell death which these different salts cause at relatively high concentrations has been investigated by flow cytometry, allowing differentiation between death by necrosis and apoptosis (Kabakov et al. 2011). More subtle effects of the presence of Arg-Glu on cell activation have been assessed as a function of changes in membrane marker expression (CD54, CD86 and HLA-DR) on activated THP-1 cells (Megherbi et al. 2009). To further assess if Arg-Glu causes an unwanted inflammatory response, the upregulation of a key proinflammatory cytokine; IL-8 was also investigated. Finally, to examine the impact of Arg-Glu as a tissue culture supplement in the protein expression and purification stages, HEK 293-EBNA system was used due to its high transfection efficiency as a vehicle to produce exogenous proteins (Black and Vos 2002).
4.4 Results

4.4.1 Characterisation of control THP-1 cells by flow cytometry: phenotypic marker expression

To ensure that the THP-1 cells were expressing the appropriate cell surface markers, the cells were stained with primary antibody (anti-CD54, anti-CD86 or anti-HLA-DR antibody) or specific isotype control antibody and the viability dye, PI, to exclude dead cells. The isotype control was used as a negative control to account for background signal caused by nonspecific binding of the antibodies. Figure 4.1A-D shows the gating strategy to determine surface marker expression where the data are illustrated as the analysis of the marker of interest versus forward scatter (a measure of cell size). Figure 4.1A shows the isotype control staining where the gates were set so that >95 % of the cells fall in the lower quadrant (are negative for the marker of interest), with <5 % of the cells appearing in the upper quadrant (positive for the marker of interest). In the presence of anti-CD54 and anti-HLA-DR antibodies, there was an increase in the percentage of cells in the upper quadrant, whereas there was little change in the presence of anti-CD86 antibody (Figure 4.1B, C, D). From these flow cytometric data, two different types of measurements can be obtained; mean fluorescence intensity (MFI) and percentage positive cells. The MFI is a measure of the intensity of expression of the marker of interest on a per cell basis expressed in arbitrary units [au] as illustrated in Figure 4.1E. The percentage of cells positive for the marker of interest is illustrated in Figure 4.1F. The MFI analysis revealed that the cells expressed high levels of HLA-DR and relatively high levels of CD54 (approximately 90 au and 40 au, respectively). In contrast, the levels of membrane CD86 were considerably lower (approximately 20 au) in comparison to the other markers. As shown in Figure 4.1F, approximately 70 % of cells express HLA-DR constitutively whereas 37 % of cells expressed CD54 and CD86 was fairly low (<5 % of cells).
Figure 4.1: Analysis of THP-1 cells by flow cytometry for constitutive expression of specific cell surface markers of interest. Following antibody staining, cells were incubated with PI to exclude dead cells and 10,000 cells were counted using a flow cytometer. Results are displayed as representative dot plots for PI staining (FL2 channel; viability) against forward scatter (FSC; size) for (A) isotype control, (B) CD54, (C) HLA-DR and (D) CD86; positive cells are located in Q1 and negative cells are located in Q4 (for this analysis Q2 and Q3 are not utilised). Results are also displayed as bar graphs for (E) mean fluorescence intensity (MFI) and (F) % positive cells for each marker of interest for n=3 experiments (mean and SEM).

4.4.2 Effect of changes in osmolality on cell viability of THP-1 cells: comparisons of Arg-Glu with other salts

From the initial experiments, it was shown that the THP-1 cells have the expected phenotype. The next objective was to examine the effects of Arg-Glu on cell viability. The use of Arg-Glu in formulations will impact on osmolality and this is likely to affect cell viability. Therefore, as osmolality is an important part of drug formulation, in vitro experiments were conducted to investigate the impact of changes in osmolality on cell viability using the additives Arg-Glu, NaCl, Arg-HCl and NaGlu. The first aim was to compare the impact of Arg-Glu on cell viability with NaCl as this is already an established excipient used in therapeutic formulations; therefore, NaCl was used as a positive control. It was also important to investigate how the individual salts in the Arg-Glu mixture impacted cell viability. Firstly, THP-1 cells were cultured with medium spiked with varying amounts of water to create a range of hypotonic osmolalities (0-230 mOsM) and varying concentrations of NaCl spanning a range of hypertonic osmolalities (280-680 mOsM) for 24 h and the cell viability was determined by PI staining measured using flow cytometry. The impact of osmolality changes on cell viability is illustrated in Figure 4.2. The cell population was gated for PI staining (FL2-channel) versus forward scatter (cell size) showing viable cells in the lower quadrant and dead (PI positive) cells in the upper quadrant. Figure 4.2B shows the viability of cells in an isotonic environment (medium alone), the cells
were mainly found in the lower quadrant as they were viable, but as osmolality either decreased to a hypotonic (Figure 4.2A) increased to a hypertonic (Figure 4.2C) environment, in both cases this resulted in an increase in dead cells, which are PI positive and therefore were found in the upper quadrant.

From the raw data plots, the percentage of viable cells following each treatment was determined and displayed in Figure 4.2D. The increase in osmolality from 0-680 mOsM resulted in a bell shaped curve for cytotoxicity. The middle of the curve at 280 mOsM represents cells that have been incubated in media alone (isotonic environment); although these cells were cultured at isotonicity, they were not 100% viable, with a small decrement in cell viability (10% drop to 90%) recorded. This is the baseline with which the various osmolalities have been compared. The expected dose dependent decreases in cell viability caused by decreasing osmolality with addition of water (hypotonic) and increasing osmolality (hypertonic) with NaCl were observed. Statistical significance (compared with medium-treated cells) for cytotoxicity was reached at 80 mOsM in the hypotonic and 480 mOsM in the hypertonic environment indicating that these osmolality changes had a significant impact on cell viability.
Figure 4.2: Representative data plots of viable and dead cells and graph showing % cell viability versus additive with respect to cumulative osmolality for THP-1 cells. THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with varying amounts of water or with various concentrations of NaCl in order to provide for a range of osmolalities from 0 to 680 mOsm. Following the incubation 10,000 cells per treatment group were analysed by flow cytometry for PI staining. Data are displayed as representative dot plots; (A) hypotonic environment using water at 100 mOsm; (B) isotonic environment in medium alone at 280 mOsm and (C) hypertonic environment using NaCl at 600 mOsm all treated for 24 h with the lower and upper quadrants of each dot plot representing the viable and dead populations respectively. Data are displayed as (D) % cell viability for n=3 experiments (mean and SE) versus additive (water or NaCl) with respect to cumulative osmolality. The statistical significance of differences between cells cultured in medium alone (isotonicity) and cells treated with various amounts of water or NaCl was assessed by one way ANOVA (*p<0.05).

The next objective was to compare the impact of osmolality changes caused by Arg-Glu on cell viability with the effects of NaCl using the PI endpoint. Furthermore, the effects of individual components of the equimolar mixture of Arg-Glu, Arg-HCl and NaGlu, at equivalent osmolalities were also investigated (Figure 4.3). THP-1 cells were cultured with varying concentrations of these additives for 24 h spanning a range of osmolalities (280-525 mOsM); 280 mOsM represents the cells cultured in media alone and the addition of varying amounts of salts constituted the range of osmolalities up to 525 mOsM for each of the salts individually. The cell viability was determined by PI staining using flow cytometry as reported previously and is illustrated as percentage viable cells for each treatment with respect to both solution
osmolality (Figure 4.3A) and solution concentration (Figure 4.3B). Figure 4.3A shows a similar
dose dependent drop in cell viability for Arg·Glu, NaCl and NaGlu as the profiles are virtually
superimposed. Thus cell viability remained at approximately baseline levels (280 mOsM; 90%
until statistically significant decreases in cell viability were recorded at osmolalities of 410
mOsM for Arg·Glu, 460 mOsM for NaCl and 468 mOsm for NaGlu, respectively. At 450–475
mOsM, there was complete loss of cell viability for all three salts. However, Arg-HCl caused a
more marked loss in cell viability at much lower osmolalities, with a significant increase in
toxicity at 382 mOsM (from ~90% to ~40% viable compared with medium alone). Compared
with the other salts there was a residual population of cells (~15%) that appeared to be
resistant to the cytotoxic effects of Arg-HCl (from 400 to 525 mOsM). The same cytotoxicity
data were displayed as a function of the concentrations of the individual additives needed to
achieve each osmolality for each salt (Figure 4.3B). When comparisons were made with
respect to concentrations instead of osmolalities, the data revealed that the profiles of Arg-Glu
and NaGlu remained superimposed whereas the profiles for NaCl and Arg-HCl were shifted
towards the left of the other salts. This demonstrates that lower concentrations of Arg-HCl (80
mM) and NaCl (74 mM) impacted more markedly on cell viability. Figure 4.3C illustrates the
direct relationship between the concentrations of the salts required to achieve each osmolality.
The data showed that much higher concentrations of Arg-Glu, Arg-HCl and NaGlu are needed
to achieve equivalent osmolalities to NaCl; importantly, this indicates that the use of higher
concentrations of Arg-Glu would not cause a loss of cell viability as higher amounts are
required to achieve the same impact on cell viability in comparison to NaCl (Figure 4.2B).
Figure 4.3: % cell viability versus additive with respect to cumulative osmolality and concentration for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10^6 cells/mL in serum free RPMI media and were cultured for 24 h at 37°C with varying concentrations of NaCl (○), Arg·Glu (●), Arg·HCl (■) and NaGlu (△) spanning a range of osmolalities from 280-525 mOsm. Control cells were cultured with medium alone (280 mOsm). Following culture, cell viability was determined using PI and 10,000 cells were analyzed by flow cytometry. Data are displayed as (A) % cell viability for n=3-6 experiments (mean and SE) versus additive with respect to cumulative osmolality and (B) the concentrations of each additive required to achieve the required osmolality. The statistical significance of differences between cells cultured in medium alone (isotonicity) and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05). For clarity of presentation, only the first concentration of each salt preparation at which there was a significant loss of cell viability is illustrated with *. The relationship between each individual salt concentration as formulated in RPMI media and cumulative osmolality as measured using an osmometer is illustrated in (C) (Kheddo et al. 2016b).

In summary, these initial experiments revealed that the THP-1 cells used here have the expected phenotype of baseline cell surface CD54, HLA-DR and CD86 expression. Furthermore, it was shown that changes in osmolality can be achieved with varying amounts of water and salts and that this impacted on cell viability. The key findings here were that the effects on cell viability caused by Arg·Glu, NaCl and NaGlu are consistent with changes in osmolality, however, a different profile was seen with Arg·HCl indicating unlike the other salts these effects are not due solely to osmolality changes.
4.4.3 Investigating the release of nitric oxide in the presence of Arg-Glu and other salts

The next objective was to examine a potential mechanism for the additional impact of the Arg-HCl salt on cell viability, given that the effects of this salt could not be reconciled simply on the basis of osmolality changes (cf Figure 4.3A). It was hypothesised that the more profound cytotoxic effect of Arg-HCl could be due to the release of NO by the cells in the presence of Arg-HCl as Arg is a substrate for the formation of NO (Tsikas 2007). THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in cell culture media alone and in the presence of 50 or 100 mM Arg-Glu, NaCl, Arg-HCl or NaGlu for 24 h; concentrations of salts that resulted in significant cytotoxicity for Arg-HCl and NaCl (cf Figure 4.3). The supernatants and lysates were collected after the incubation and the nitrite concentration was determined by the Griess assay. The nitrite content of treated samples were normalised to the levels in control samples (cells cultured alone in media) and are presented as normalised NO, with levels in control cells (which ranged from 0.9 to 1 µM in supernatants and from 1 to 1.2 µM in cell lysates) (Figure 4.4). The NO content in medium alone in the absence of cells was also measured; these levels were routinely <5 % of the control cell levels, demonstrating that the presence of THP-1 cells was required for detectable NO production in both supernatants and lysates. The NO content of supernatants derived from cells following treatment with the various salts was variable; ranging from approximately 60 % to 140 % of control levels; however, such differences were not statistically significant (Figure 4.4A). In contrast, statistically significant differences were recorded for all treatments with all salts with respect to NO content of cell lysates (Figure 4.4B). In all cases, there was a significant drop in NO content of cell lysates, with levels of between 30 - 40 % of levels recorded for medium-treated control cells. Thus, the different cytotoxicity profile recorded for Arg-HCl could not be reconciled on the basis of NO production.
Figure 4.4: Nitrate concentration versus additive for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10^6 cells/mL in serum free RPMI media and were cultured for 24 h at 37°C in medium alone (represented by the dashed line) or in the presence of 50 mM (open column) or 100 mM (closed column) Arg·Glu, NaCl, Arg·HCl or NaGlu. (A) supernatants and (B) lysates were collected after the 24 h incubation and the nitrite concentration was determined by the Griess assay. The nitric oxide content of the media (with supplements) in the absence of cells was also determined (dotted line). The nitrite concentrations were determined using a nitrite standard reference curve and the associated computer software for microplate-based assays. For each individual experiment (n=3), the nitrite content of treated samples was normalized to the concurrent control (cells in medium alone; 1 µM for supernatants and 1.2 µM for lysates) and data are displayed as percentage of control (mean and SE). The statistically significance of differences in the nitrite content of samples from cells cultured in medium alone and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05) (Kheddo et al. 2016b).

4.4.4 Investigation of the mechanism of cytotoxicity for Arg·Glu and other salts using Annexin V-FITC/PI staining

In the previous experiments cell viability was assessed using PI staining which distinguishes dead (necrotic) cells from live (viable) cells. In those experiments, Arg·Glu and NaGlu showed very similar toxicity profiles with respect to solution osmolality and cell death to that which was observed for the reference excipient, NaCl. However, to further characterise the type of cell death occurring, and to investigate whether the mechanism of cell death was indeed similar between Arg·Glu and NaCl, a combination of PI and Annexin V-FITC staining was used. Annexins are a family of proteins that are Ca^{2+}-regulated phospholipid-binding molecules. Annexin V is used as a marker to identify apoptotic cells as it binds to PS (van Genderen et al. 2006). In healthy cells, PS is mostly situated on the cytoplasmic surface of the cell membrane. However, during the beginning of apoptosis, PS loses its even distribution in the phospholipid bilayer and is translocated to the outer part of the membrane. This movement of PS to the outer membrane can be detected with fluorescently labelled Annexin V (van Genderen et al. 2006, Yen et al. 2010). During early apoptosis, PI is excluded from the plasma membrane and the cells are only positive for Annexin V staining (Annexin V+ve/PI-ve); late
stage apoptosis occurs when the integrity of the cell membrane is damaged enabling both access for Annexin V to bind to PS and PI to be taken up (Annexin V+ve/PI+ve) (van Engeland et al. 1998, Rieger et al. 2011). Once the cells are necrotic, they will have lost PS from the membrane but will maintain staining for PI (Annexin V-ve/PI+ve).

THP-1 cells were seeded into 24-well plates at 10^6 cells/mL and cultured for 4 h or 24 h in serum free RPMI media alone or with varying concentrations (50-200 mM) of Arg-Glu, NaCl, Arg-HCl or NaGlu. These concentrations were chosen as this is the normal biophysical dose range used in formulation which is acceptable in terms of osmolality (Arakawa et al. 2007c). Following incubation, cells were stained with Annexin V-FITC and PI and analysed using flow cytometry. Figure 4.5 shows representative quadrant analyses for the gating strategy used to determine the type of cell death that was occurring, with PI staining detected in the FL-2 channel versus Annexin V-FITC in the FL-1 channel. Figure 4.5A illustrates viable cells (Annexin V-ve/PI-ve cells) found in the lower left quadrant (Q4) after culture of THP-1 cells in serum free media for 24 h. As the cells are largely viable (approximately 95 %) the majority of the cells reside in the lower left quadrant, with <3% in the lower right which represents cells entering the early apoptosis stage, <3 % in the upper right representing late apoptotic cells and lastly <2 % in the upper left representing necrotic cells. Figure 4.5B illustrates cells entering the early apoptotic stage (Annexin V+ve/PI-ve) found in the lower right quadrant (Q3) following culture of THP-1 cells in 150 mM NaGlu in serum free media for 4 h. Here, the cells are moving from the lower left (viable; 24 %) to both the lower right (early apoptosis; 33 %) and upper right quadrant (late apoptosis; 42 %). Figure 4.5C shows cells entering late apoptosis (Annexin V+ve/PI+ve) found in the upper right quadrant (Q2) after culture of THP-1 cells in 200 mM Arg-Glu in serum free media for 24 h. At this point, the cells are moving from the lower right (early apoptosis; <2 %) to the upper right quadrant (late apoptosis; 86 %). Figure 4.5D shows the last stage of cytotoxicity with cells undergoing necrosis (Annexin V-ve/PI+ve) found in the upper left quadrant (Q1) after treatment of THP-1 cells with 200 mM NaCl in serum free media for 24 h. Here the cells are moving from the upper right (late apoptosis; 72 %) to the upper left quadrant (necrotic cells; 15 %).
Figure 4.5: Representative quadrant analyses showing the pattern of cytotoxicity for each treatment group for THP-1 cells. In each case the lower left quadrant represents Annexin V-ve/PI-ve (viable) cells, the lower right quadrant represents Annexin V+ve/PI-ve (early apoptotic) cells, the upper right quadrant represents Annexin V+ve/PI+ve (late apoptotic) cells and the upper left quadrant represents Annexin V-ve/PI+ve (necrotic) cells. The percentage of cells in each quadrant is indicated.

THP-1 cells were seeded into 24-well plates at 10^6 cells/mL and cultured for 24 h (A) in serum free RPMI media alone, (B) 150 mM NaGlu, (C) 200 mM Arg-Glu, (D) 200 mM NaCl all treated for 24 h. Following incubation, cells were stained with Annexin V-FITC (FL-1 channel) and PI (FL-2 channel) and 10,000 cells were analysed using a FACSCalibur flow cytometer (Kheddo et al. 2016b).

The flow cytometric data acquired and the gating strategy shown in Figure 4.5 were used to determine the percentages of the cell population in each apoptotic stage for THP-1 cells that had been treated with varying concentrations (50-200 mM) of Arg-Glu, NaCl, Arg-HCl or NaGlu for 4 h or for 24 h. As described above, it is possible using this gating strategy to define 4 distinct populations of cells. However, in order to first compare these data with the data shown previously (cf Figure 4.3) in which PI staining only was used to define dead cells, in the first instance the analysis of this second data set has been confined to characterisation according to PI staining only. Thus, viable cells are defined for the purpose of this initial analysis as those cells that are PI-ve and therefore cumulative data for Annexin V-ve/PI-ve and Annexin V+ve/PI-ve cells have been calculated. These data are shown in Figure 4.6A and Figure 4.6B for treatment of THP-1 cells with Arg-Glu, NaCl, Arg-HCl and NaGlu (50-200 mM) for 4 h and 24 h, respectively. There was no significant cytotoxicity after 4 h incubations at low
concentrations for any of the salts (up to 100 mM), although there was a significant loss in viability for all 4 salts at concentrations of 150 mM (from ~90 % to between 70 and 80 %). The extent of cytotoxicity was different at the highest concentrations, with a substantial decrease in cell viability seen with NaGlu (~40 %), followed by both Arg-Glu and Arg·HCl (~60 %) whereas the least marked effect was seen with NaCl (84 %) followed by NaGlu (~80 % viable). More profound effects on cytotoxicity were observed following 24 h treatment with the salts; at concentrations of 100 mM and above significant toxicity was recorded for all salts. The cytotoxicity profiles for Arg·HCl and NaCl were superimposable, with an almost complete loss of viability observed at 150 mM. In contrast, Arg·Glu and NaGlu caused less marked effects on cell viability. Importantly, comparisons between Figure 4.6B and 4.3A reveal that the two methods for assessing cell viability are similar as both result in more marked cell death being recorded at lower Arg·HCl and NaCl concentrations compared with Arg·Glu and NaGlu and the cytotoxicity profiles observed for each salt are very similar for the two methods of analysis.

**Figure 4.6:** % cell viability for viable cells for cell treatment with additives at 4 h and 24 h for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10⁶ cells/mL in serum free RPMI media and were cultured for 4 h at 37 °C in the presence of 50-200 mM NaCl (○), Arg·Glu (●), Arg·HCl (■) or NaGlu (△). Control cells were cultured with medium alone. To characterise the extent and pattern of induced cytotoxicity, cells were stained with Annexin V-FITC (FL-1 channel) and PI (FL-2 channel) and 10,000 cells were analysed using a flow cytometer. For comparison with previous experiments where PI staining alone was used to define viability, cumulative data for Annexin V-ve/PI-ve (viable) cells are displayed in (A) and (B) for cell treatment at 4 h and 24 h respectively. In each case, data are shown as % total cells in each category (mean and SE for n=3 experiments, where for clarity SE > 2 % only are shown). The statistical significance of differences between cells cultured in medium alone and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05).

A more detailed analysis of the type of cell death occurring as a result of treatment with the salts is shown in Figure 4.7 for the 4 h incubation of THP-1 cells where the four distinct stages of cell death (viable, early apoptotic, late apoptotic and necrotic stages) were examined for each of the treatments. Here, viable cells are more stringently defined as those that are both PI and Annexin V negative. Whilst the dose dependent effects of the salts on cell viability
showed similar trends, the extent of cytotoxicity was different particularly at the 200 mM concentration, with the most substantial decrease recorded for NaGlu (40% viability), with less marked effects on Arg-Glu and Arg-HCl (60% viable) and NaCl treatment having the least effect on viability (80%) (Figure 4.7A). The differential effect on cell viability of NaGlu was paralleled by a corresponding increase in the frequency of early apoptotic cells, reaching maximal levels of approximately 35%; the other salts did not cause any marked changes in this cell population (Figure 4.7B). All treatments resulted in small increases in the percentage of late apoptotic and necrotic cells, although such were below 10% with the exception of Arg-HCl, where maximal levels of 30% late apoptotic cells were recorded (Figure 4.7C and D). In general the pattern of changes in the different populations were similar for NaCl and Arg-Glu, with the drop in viable cells being paralleled with relatively small increases in early and late apoptotic cells and necrotic cells.

Figure 4.7: % cell viability for the different cytotoxicity categories for cell treatment with additives at 4 h for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10^6 cells/mL in serum free RPMI media and were cultured for 4 h at 37 °C in the presence of 50-200 mM NaCl (○), Arg-Glu (●), Arg-HCl (■) or NaGlu (∆). Control cells were cultured with medium alone. To characterise the extent and pattern of induced cytotoxicity, cells were stained with Annexin V-FITC (FL-1 channel) and PI (FL-2 channel) and 10,000 cells were analysed using a flow cytometer. The results are displayed as the % of cells that are (A) Annexin V-ve/PI-ve (viable), (B) Annexin V+ve/PI-ve (early apoptotic), (C) Annexin V+ve/PI+ve (late apoptotic), (D) Annexin V-ve/PI+ve (necrotic). In each case, data are shown as % total cells in each category (mean and SE for n=3 experiments, where for clarity SE > 2% only are shown). The statistical significance of differences between cells cultured in medium alone and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05) (Kheddo et al. 2016b).
In subsequent experiments, the incubation period was increased to 24 h with each salt and the frequency of cells in each population was assessed by flow cytometry as described previously (Figure 4.8). The overall pattern of cytotoxicity was very similar for each of the salts. Thus, there was a dose dependent drop in the number of viable cells, approaching 0 % at 200 mM (Figure 4.8A), very little increase in early apoptotic cells (Figure 4.8B; <10 %) or necrotic cells (Figure 4.8D; <10 %) but a corresponding increase (approaching 95 %) in late apoptotic cells, indicating that all four salts are killing the cells by a similar mechanism. Differences were apparent at the intermediate doses; with more marked decreases in cell viability (Figure 4.8A) and corresponding increases in late apoptotic cell frequencies (Figure 4.8C) recorded for Arg·HCl and NaCl than for equivalent doses of Arg·Glu and NaGlu.

Figure 4.8: % cell viability for the different cytotoxicity categories for cell treatment with additives at 24 h for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10⁶ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C in the presence of 50-200 mM NaCl (○), Arg-Glu (●), Arg·HCl (■) or NaGlu (Δ). Control cells were cultured with medium alone. To characterise the extent and pattern of induced cytotoxicity, cells were stained with Annexin V-FITC (FL-1 channel) and PI (FL-2 channel) and 10,000 cells were analysed using a flow cytometer. The results are displayed as the % of cells that are (A) Annexin V-ve/PI-ve (viable), (B) Annexin V+ve/PI-ve (early apoptotic), (C) Annexin V+ve/PI+ve (late apoptotic), (D) Annexin V-ve/PI+ve (necrotic). In each case, data are shown as % total cells in each category (mean and SE for n=3 experiments, where for clarity SE > 2% only are shown). The statistical significance of differences between cells cultured in medium alone and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05) (Kheddo et al. 2016b).
In summary, overall at both time points the four salts showed similar mechanisms of cell death, with a small proportion of cells going into early and late apoptosis and necrosis at 4 h whilst at 24 h the loss in viability results in a corresponding increase in late apoptotic cells. The main differences are that NaGlu causes a more rapid (4 h) and marked increase in early apoptotic cells and that after 24 h, Arg-Glu and NaGlu have less marked effects than Arg-HCl and NaCl on cell viability at intermediate doses.

4.4.5 Phenotypic changes in THP-1 cells following LPS activation with Arg-Glu and other salts

Having investigated the effects of Arg-Glu and other salts on responses of resting (inactivated) THP-1 cells, the next series of investigations employed THP-1 cells under activating conditions. The toll-like receptor (TLR)4 ligand LPS, a bacterial product which acts as a PRR molecule and is known to stimulate monocytes, DCs and B-cells as well as other immune cell populations was utilised (Schildberger et al. 2013, Awada et al. 2014). The gating strategy to determine surface marker expression upon activation of the cells using LPS (0.1 µg/mL) is illustrated in Figure 4.9A-C showing the analysis of the marker of interest versus forward scatter (size) for the markers CD54, HLA-DR and CD86. If the cells are positive for the marker of interest they will appear in the upper quadrant. As described previously, the flow cytometric data were processed and are displayed as both MFI (Figure 4.9D) and percentage positive cells (Figure 4.9E). With respect to MFI, resting THP-1 cells expressed relatively high levels of HLA-DR and CD54 but little CD86; culture with LPS significantly upregulated CD54 expression (from ~ 60 au to 360 au) but had little effect on levels of the other two markers. With respect to the percentage of positive cells, approximately 20 % of resting THP-1 cells were CD54 positive, 70 % were HLA-DR positive and less than 5 % expressed CD86. Treatment with LPS resulted in a small upregulation in the frequency of HLA-DR positive cells (90 %), was without effect on the CD86 population and significantly increased the frequency of CD54 positive cells (to 70 %).
Figure 4.9: Representative data plots and bar charts of cell surface marker expression for THP-1 cells. THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with media alone or with LPS (0.1 µg/L). Cells were analysed for CD54, HLA-DR and CD86 expression by flow cytometry; dead cells were excluded by PI staining and 10,000 cells counted using a flow cytometer. Results are displayed as representative dot plots for (A) CD54, (B) HLA-DR and (C) CD86; positive cells are located in Q1 and negative cells are located in Q4 (for this analysis Q2 and Q3 are not utilised). Results are also displayed as bar charts as (D) mean fluorescent intensity (MFI) and (E) % positive cells in the absence of LPS (open column) or presence of LPS (closed column) for each marker of interest for n=3 experiments (mean and SEM). The statistical significance of differences between cells cultured in medium alone or between cells cultured in the presence or absence of LPS was assessed by one way ANOVA (*p<0.05).

Subsequently, the impact of Arg·Glu and NaCl on LPS-induced changes in membrane marker expression was investigated. THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in cell culture media alone or in the presence or absence of 50 or 100 mM Arg·Glu or NaCl for 24 h each in the presence or absence of LPS (0.1 µg/mL). Expression of HLA-DR, CD86 and CD54 under these conditions is displayed with respect to both changes in MFI and frequency of positive cells (Figures 4.10, 4.11 and 4.12, respectively). As reported previously (cf Figure 4.9), THP-1 cells expressed HLA-DR constitutively (~100 au and 60 % of cells positive) and LPS treatment resulted in a small increase in both MFI and the frequency of positive cells; however such did not reach statistical significance (Figure 4.10). Treatment with either Arg·Glu or NaCl at 50 or 100 mM was without statistically significant effects on either parameter. Identical results were seen when expression of the costimulatory molecule CD86 was examined, although here both the frequency of positive cells (<5 %) and MFI (~20 au) in resting THP-1 cells was considerably lower than HLA-DR expression (Figure 4.11). A different pattern was observed when CD54 expression was examined (Figure 4.12). Culture with LPS resulted
in an increase in both parameters compared with medium alone treated cells (from ~30 % to 90 % and from ~50 au to 400 au). Interestingly, culture with 50 and 100 mM Arg-Glu and 100 mM NaCl in the absence of LPS also caused a slight increase in the percentage of positive cells (~20 %) but such did not reach statistical significance and there was no enhancement of the LPS-induced effect on CD54 positive cell frequency. However, there was a significant effect of co-culture of THP-1 cells with LPS and 100mM Arg-Glu with respect to the intensity of CD54 expression, with a significant increase in levels of this molecule in LPS/100 mM Arg-Glu treated cells compared with LPS alone (from ~400 au to ~550 au) (additive effect; Figure 4.12Aii). Lower concentrations of Arg-Glu and all concentrations of NaCl were without significant effect. In subsequent experiments, the impact of Arg-HCl and NaGlu on the same endpoints was examined. As observed for Arg-Glu and NaCl, there was no significant effect of either salt on the frequency of CD54 positive cells regardless of the presence or absence of LPS. A similar pattern was seen with Arg-HCl and NaGlu, although with respect to the enhancement of MFI in the presence of both LPS and 100 mM Arg-HCl and Arg-Glu these reached statistical significance in comparison to cells treated with LPS. Synergistic effects were not observed between the co-culture of LPS with salts in comparison to these individually indicating the effects seen on CD54 marker expression were additive.

Figure 4.10: Graph showing % positive cells and MFI for HLA-DR expression versus additive for THP-1 cells. THP-1 cells were seeded into 24-well plates at 10^6 cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with medium alone or in the presence of 50 or 100 mM (A) Arg-Glu; (B) NaCl each in the presence (closed column) or absence (open column) of LPS (0.1 µg/mL). At the end of the culture period, cells were stained for expression of HLA-DR or with isotype control antibody and dead cells were excluded with PI. Data (10,000 cells) were acquired using a flow cytometer and results are displayed as (i) % positive cells and (ii) mean fluorescent intensity (MFI) (mean and SE for six independent experiments). There were no statistically significance differences between cells cultured in medium alone and cells treated with various concentrations of salts, or between cells cultured in the presence or absence of LPS (assessed by one way ANOVA)
Figure 4.11: Graph showing % positive cells and MFI for CD86 expression versus additive for THP-1 cells. THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with medium alone or in the presence of 50 or 100 mM (A) Arg-Glu; (B) NaCl each in the presence (closed column) or absence (open column) of LPS (0.1 µg/mL). At the end of the culture period, cells were stained for expression of CD86 or with isotype control (mouse IgG1κ) antibody and dead cells were excluded with PI. Data (10,000 cells) were acquired using flow cytometer and results are displayed as (i) % positive cells and (ii) mean fluorescent intensity (MFI) (mean and SE for six independent experiments). There were no statistically significant differences between cells cultured in medium alone and cells treated with various concentrations of salts, or between cells cultured in the presence or absence of LPS (assessed by one way ANOVA).
Figure 4.12: Graph showing % positive cells and MFI for CD54 expression versus additive for THP-1 cells. THP-1 cells were seeded into 24-well plates at $10^6$ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with medium alone or in the presence of 50 or 100 mM (A) Arg·Glu; (B) NaCl; (C) Arg·HCl; (D) NaGlu each in the presence (closed column) or absence (open column) of LPS (0.1 µg/mL). At the end of the culture period, cells were stained for expression of CD54 or with isotype control antibody and dead cells were excluded with PI. Data (10,000 cells) were acquired using flow cytometer and results are displayed as (i) % positive cells and (ii) mean fluorescent intensity (MFI) (mean and SE for six independent experiments). The statistical significance of differences between cells cultured in medium alone and cells treated with various concentrations of salts, or between cells cultured in the presence or absence of LPS was assessed by one way ANOVA (*p<0.05). Part of this Figure has been published (Kheddo et al. 2016b).
In summary, these experiments demonstrated LPS activation of THP-1 cells resulted in marked upregulation of the costimulatory molecule CD54 (both with respect to positive cell frequency and the intensity of expression of the marker on a per cell basis) showing additive effects between the co-culture of LPS with salts in comparison to these individually and that co-culture of THP-1 cells with LPS and 100 mM of Arg-Glu or Arg-HCl results in a more vigorous increase in expression of CD54 on a per cell basis.

4.4.6 Effect of LPS-induced IL-8 expression

Another marker of LPS-induced activation is the production of inflammatory cytokines such as IL-8 (Awada et al. 2014). THP-1 cells were seeded into 24-well plates at 10^6 cells/mL in cell culture media alone or with LPS or in the presence of 50-200 mM Arg-Glu, NaCl, Arg-HCl or NaGlu for 24 h. The supernatants were collected after the incubation and the IL-8 content was analysed by sandwich ELISA (Figure 4.13). Co-culture of LPS with the salts was not investigated here as there was generally no statistical significance in membrane marker expression observed between LPS and LPS with salts. Cells cultured with medium alone did not secrete detectable levels of IL-8 (<0.1 ng/mL) whereas culture in the presence of LPS induced detectable cytokine expression (~0.7 ng/mL). Treatment with the various salts in the absence of LPS generally resulted in a small increase in cytokine production, (approaching ~0.4 ng/mL), however such did not reach statistical significance and refers to limit of accuracy. The various salts showed inter-experimental variation which did not result in significant cytokine production for any of the conditions in comparison to LPS-induced cells. Looking at concentrations 50 and 100 mM for all the salts alone; there was an increase in IL-8 production with increasing concentration, however, this decreased at higher concentration where cell viability was impacted. Despite these observations, the increases were not beyond LPS induced cytokine production. It was also noticeable that Arg-HCl and NaGlu caused higher amounts of IL-8 at all concentrations in comparison to Arg-Glu and NaCl. To summarise, hypertonic concentrations of all the salts that impact cell viability failed to impact significantly on monocyte IL-8 production.
Figure 4.13: Graph showing IL-8 content versus additive for THP-1 cells. THP-1 cells were seeded into 24-well plates at $10^5$ cells/mL in serum free RPMI media and were cultured for 24 h at 37 °C with medium alone or in the presence of 50–200 mM of Arg-Glu, NaCl, Arg-HCl or NaGlu. Positive control cells were cultured with LPS (0.1 μg/mL). Following culture, samples were centrifuged at 1000 rpm for 5 min and supernatants were collected and analyzed for IL-8 content by ELISA. Results are displayed as mean and SEM of 3 independent experiments. The statistical significance of differences between cells cultured in medium alone and cells treated with various concentrations of salts or LPS was assessed by one way ANOVA (*p<0.05). Part of this Figure has been published (Kheddo et al. 2016b).

4.4.7 Effect of changes in osmolality on cell viability with fibroblasts: comparisons of Arg-Glu with NaCl

In order to extend these observations beyond a single cell line and one grown in single cell suspension (THP-1 cells), in subsequent experiments the impact of Arg-Glu and NaCl were investigated using a different cell line, adherent human fibroblasts. Fibroblast cells were cultured at $2 \times 10^5$ cells/mL in medium with water to create a range of osmolalities (0-230 mOsM) and varying concentrations of NaCl spanning a range of osmolalities (280-620 mOsM) for 6 h and the cell viability was determined by PI staining using flow cytometry. At 280 mOsM this represents the cells cultured in media alone (isotonic). It is important to note that as this cell line grows as an adherent monolayer, in order to remove the cells from the tissue culture plates for flow cytometric analyses it is necessary to treat the cells with trypsin and EDTA, this may impact on cell viability. Similar to that observed for THP-1 cells, a bell shaped curve for cytotoxicity was observed with hypotonic conditions (240mOsM and below) and hypertonic conditions (334mOsM and above) resulting in significant decreases in viability compared with isotonicity (medium alone ~90% viable) (Figure 4.14A). Even at maximal hypertonic and hypotonic osmolalities, there was a residual population of cells (~ 10 % and 10 %, respectively) that were relatively resistant to cell death. Additionally, the effects of the individual components of the Arg-Glu mixture were also investigated at equivalent osmolalities.

Fibroblast cells were cultured at $2 \times 10^5$ cells/mL in medium with varying concentrations of Arg-Glu, NaCl, Arg-HCl and NaGlu spanning a range of osmolalities (280-620 mOsM) for 6 h
and the cell viability was determined by PI staining using flow cytometry; data are illustrated with respect to osmolality (Figure 4.14B) and concentration (Figure 4.14C) of the solutions. For each salt there was a dose dependent decrease in cell viability. With respect to osmolality changes, NaCl and NaGlu profiles were virtually superimposable, with a relatively slow decline in viability until ~525 mOsM, then a very rapid drop in viability. However the residual resistant population was not observed for NaGlu treated cells. In contrast, again with respect to osmolality, the cytotoxicity of profiles of Arg·Glu and Arg·HCl were also largely superimposable but showed a much more rapid drop in viability with significant cell death recorded at concentrations of 400 mOsM and above. These data indicated that for the adherent fibroblast cell line, Arg·Glu and Arg·HCl have effects on cell viability due to factors in addition to osmolality. When the cytotoxicity profiles are considered with respect to salt concentration, then the profiles are superimposable for all the salts with the exception of NaGlu.
Figure 4.14: % cell viability versus additive with respect to cumulative osmolality and concentration for human primary fibroblasts. Fibroblasts were seeded at 2 x 10^5 cells/mL in complete DMEM medium in flat-bottomed 24 well plates for 6 h at 37 °C. After the incubation, medium was replaced with serum free DMEM medium supplemented with varying amounts of water or with various concentrations of NaCl spanning a range of osmolalities from 0-620 mOsm; or with varying concentrations of NaCl (○), Arg·Glu (●), Arg·HCl (■) and NaGlu (▲) spanning a range of osmolalities from 280-525 mOsm. Control cells were cultured with medium alone (280 mOsm). Cells were cultured for a further 24 h at 37 °C. Following the incubation cells were trypsinised and 10,000 cells per treatment group were analysed by flow cytometry for PI staining (FL2 channel; viability) against forward scatter (size). Data are displayed as (A) % cell viability versus additive (water or NaCl) with respect to cumulative osmolality; (B) % cell viability versus additive with respect to cumulative osmolality and the (C) concentrations of each additive required to achieve the required osmolality. The statistical significance of differences between cells cultured in medium alone (isotonicity) and cells treated with varying amounts of water or NaCl was assessed by one way ANOVA (*p<0.05) (A) and differences between cells cultured in medium alone (isotonicity) and cells treated with various concentrations of salts was assessed by one way ANOVA (*p<0.05) (B,C). For clarity of presentation, only the first concentration of each salt preparation at which there was a significant loss of cell viability is illustrated with *. Part B and C of this Figure has been published (Kheddo et al. 2016b).

In summary these experiments have demonstrated that Arg-Glu does indeed impact on cell viability, but at least with respect to resting cells cultured in suspension (THP-1 cells), the effects appear to be related to changes in osmolality and are equivalent to those caused by parallel treatment with the reference excipient NaCl. With respect to molar concentrations, on a mole for mole basis Arg-Glu is somewhat less toxic than NaCl for cells grown in suspension.
A slightly different pattern was observed for the adherent fibroblast cell line, here cytotoxic effects of Arg·Glu and Arg·HCl were more marked at lower osmolalities than those of NaCl or NaGlu. However, on a mole for mole basis NaCl and Arg·Glu displayed equivalent toxicity over the physiological range of concentrations examined (50-200 mM).

4.4.8 Effect of Arg·Glu as a tissue culture supplement: using HEK293 cells as a model system

The cell line chosen for these experiments was the HEK293-EBNA system. HEK 293 cells have been derived from the transformation of HEK cells by exposure to fragments of the human adenovirus type 5 DNA. For the studies carried out here, the HEK 293 cells have been transformed with the EBNA1 virus resulting in another variant, the HEK293-EBNA cell line (Thomas and Smart 2005). This was the preferred cell line as it was possible to insert a gene of interest in to this EBNA vector that cannot be disturbed, moreover, due to its high transfection efficiency it was used as a vehicle to produce exogenous proteins (Black and Vos 2002). Therefore these cells were selected as a platform to express the protein of interest; recombinant C-terminal histidine tagged OLFd protein, which was known to be prone to aggregation (personal communication; Richard Tunnicliffe). With this cell line, three experimental conditions were set up; firstly the cells were grown in standard culture media and the resulting recombinant protein was purified in buffers containing 50 mM Arg·Glu, secondly cells were grown in culture medium containing 50 mM Arg·Glu and 50 mM Arg·Glu was also introduced in to the buffers during protein purification so that the recombinant protein was expressed and purified in the presence of Arg·Glu, and finally cells grown in standard culture media and recombinant protein purified in buffers without Arg·Glu (Figure 2.1). HEK-293 EBNA cells expressing recombinant histidine tagged OLFd were initially cultured in growth medium, once these were ready for protein expression, the growth media was removed and replaced with expression media according to the three conditions described above and the media was collected every 2-3 days to obtain four collections in total for each of the conditions. After the histidine-tagged purification of proteins derived from each of the three experimental set-ups, it was necessary to identify which eluted fractions contained the protein. This was achieved using SDS-PAGE analysis which illustrated that the OLFd protein was predominately present in the second elution (E2) for the three conditions as a single band at ~35 kDa which was the expected molecular weight (Figure 4.15).
Figure 4.15: Gel showing protein bands for different protein expression and purification conditions using HEK293 cells. Stably transfected HEK293-EBNA cells expressing recombinant histidine tagged OLFd were initially cultured in growth medium. When the cells were 70-80 % confluent, growth medium was replaced with serum free expression media in two conditions; expression media in the presence or absence of 50 mM Arg-Glu. The expression media was collected every 3-4 days (a total of 4 times). The media collected from the same treatment conditions were pooled, dialysed and concentrated using tangential flow filtration followed by histidine-tagged purification. In the elution stage of the purification, a third experimental condition was introduced where samples from cells grown in expression media in the absence of 50 mM Arg-Glu were divided equally and 50 mM Arg-Glu was utilised in the downstream preparation of one of the two batches. For each of the three preparations elution fractions (E1-E4) from histidine-tagged OLFd purification were collected and quantified using SDS-PAGE under reducing conditions (4-20% Tris-glycine gel) with a Tris-glycine buffer system. Bands were stained with coomassie. The representative gel shows protein bands from fractions E1-E4 for each of the three different protein expression conditions: 50 mM Arg-Glu throughout the protein expression and purification (= “+”; lanes 2-5), in the absence of 50 mM Arg-Glu throughout the protein expression and purification (= “-”; lanes 7-10) or in presence of 50 mM Arg-Glu only in the elution stage of the purification (= “++”; lanes 12-15). The molecular weight marker lane (M) is shown in lanes 1, 6 and 11 ranging from 10-250kDa.

Furthermore, the cell morphology of the cells during the initial protein expression stage in the absence and presence of 50 mM Arg-Glu was examined by light microscopy (Figure 4.16A-B). In the absence of Arg-Glu the cells reached confluence (Figure 4.16A) whereas cells seeded at the same number in the presence of Arg-Glu did not reach the same cell density (Figure 4.16B). Given that there was a marked impact in cell number following culture in the presence of Arg-Glu, it was important to determine if similar amounts of OLFd protein were expressed during each media collection, therefore a Western blot was performed on the four individual media collections obtained following culture of cells in the absence and presence of Arg-Glu (Figure 4.16C). In the absence of Arg-Glu, the OLFd protein has been expressed in all collections except collection 2, however, in the presence of Arg-Glu, OLFd was only expressed in the first media collection and subsequently there was no further expression.
Figure 4.16: Microscopic images of the HEK-293 cells. Stably transfected HEK293-EBNA cells expressing recombinant histidine tagged OLFd were cultured in serum free expression media in two conditions; expression media in the presence or absence of 50 mM Arg-Glu. Microscopic images of the cells were taken at the time of media collection in the absence (A) and presence (B) of 50 mM Arg-Glu. Western blot analysis was used to measure the amount of OLFd protein expressed after each media collection (C); aliquots from the individual four collections in the presence (lanes 1-4) and absence of Arg-Glu (lanes 5-8) were loaded on to 10% SDS-PAGE, and transferred to a PVDF membrane. The membrane was incubated with primary histidine-tagged antibody. Finally, the membrane was incubated with chemiluminescent substrate.

Although the presence of Arg-Glu in the culture medium decreased the overall protein expression and total yield, it was still important to determine if the presence of Arg-Glu decreased the amount of aggregation; in order to achieve this, MALS-SEC was employed. Figure 4.17A illustrates the relative UV absorbance measured from the tryptophan residues in the protein for the eluted proteins. The first peak at 8 mL is eluted in the void volume; this represented the higher order aggregates which were too large for separation in the column, thus eluted the fastest. The subsequent peak at 13 mL represented dimer formation, followed by the largest peak at 16 mL which consisted of monomer. The highest levels of monomer were recorded in the conditions containing Arg-Glu at the purification stage, intermediate levels were found following Arg-Glu incorporation at the protein expression stage and the lowest levels of monomer were observed in the absence of Arg-Glu. The preceding peak at 13 mL revealed a greater dimer content in the absence of Arg-Glu in comparison to both the conditions in the presence of Arg-Glu which showed smaller peaks. Examination of peak at 8 kDa
mL also revealed higher levels of high order aggregates in the absence of Arg·Glu followed by a smaller peak with Arg·Glu at the post purification stage and very little higher order aggregate content when Arg·Glu was present from the protein expression stage. Furthermore, it was confirmed from the light scattering analysis, the first peak that eluted at 8 mL in all conditions represented the high molecular weight aggregates followed by smaller peaks at 13 mL and 16 mL. Here, it was prominently seen that in the absence of Arg·Glu there is a greater amount of high molecular weight aggregates in comparison to the other two conditions containing Arg·Glu. The incorporation of Arg·Glu at the elution stage contained considerably less aggregate, however the least aggregate formation was seen when Arg·Glu was incorporated from the protein expression stage (Figure 4.17B). It was important to note that the peaks at approximately 8 mL and 13 mL did not scatter enough light as there was not enough signal from the monomer (signal to noise ratio was too low); however, the addition of Arg·Glu in both conditions showed a smaller peak than in the absence of Arg·Glu along with an improved signal to noise ratio. The percentage mass of protein retained in each condition was calculated from these data (Figure 4.17C). As indicated from the raw data, the conditions with Arg·Glu at the protein expression and at the elution stage there was a greater percentage of monomer retained (93 %) than in the absence of Arg·Glu (56 %). Also, in the absence of Arg·Glu there is protein present as dimer and higher order aggregates in the void volume (30 % and 8 % respectively). In the presence of Arg·Glu in the other two conditions, there is considerably less dimer and higher order aggregates, however, the least aggregates was seen in the condition containing Arg·Glu from the protein expression stage (<2 % ).
Figure 4.17: Analysis of protein aggregation in HEK293 cells using MALS-SEC. The presence of aggregates was determined for the three conditions. Representative chromatograms are shown for the three conditions where the expression media contained either 50 mM Arg·Glu throughout the protein expression and purification (+), in the complete absence of 50 mM Arg·Glu (-) or in presence of 50 mM Arg·Glu in the elution stage of the purification only (+*) for UV traces (A) and light scattering (B) for OIFd separated using a Superdex200 10/300GL column. The percentage mass of protein retained for each peak is illustrated in (C).

In summary, these experiments have demonstrated that Arg·Glu does have the potential to reduce protein aggregation in USP. In the absence of Arg·Glu, there was considerably more aggregate formation than in the other two conditions which contained Arg·Glu. Importantly, the incorporation of Arg·Glu from the protein expression stage showed the least aggregate formation. With regards to the percentages of protein mass retained, both the Arg·Glu conditions showed similar percentages in the first peak whereas the addition of Arg·Glu from the protein expression stage mostly eliminated the formation of higher order aggregates. However, due to the limited availability of the protein expressed it was not possible to calculate the molecular weight of the fractions.
4.5 Discussion

Although the studies conducted in this Chapter are not the classical toxicity tests, they have provided an initial insight into the toxicity assessment of the novel excipient combination Arg-Glu which has not been performed previously. Prior cytotoxicity testing is required as any novel excipient intended to be administered for human use needs evidence of safety at clinically administered doses. Moreover, the applicability of Arg-Glu in USP during protein expression and purification was also investigated. From Chapter 3, Arg-Glu has shown the ability to reduce protein aggregation and increase protein stability at a range of concentrations (50-200 mM) in the test mAb’s provided; however, for any novel excipient it is necessary to ensure that these proposed concentrations do not cause any off-target effects. Firstly, these should not be directly toxic as this would cause cell death and tissue damage. An additional concern is that excipients may cause adjuvanticity, this is of serious toxicological concern as therapeutic mAb’s can be immunogenic; this is the ability of a substance to induce an adaptive immune response if it is recognized as a ‘foreign’ entity to the body. Extrinsic factors such as adjuvant-like additives/contaminants or aggregates in the formulation or intrinsic factors such as PTMs are key examples of causing immunogenicity from the production of ADAs. These can ultimately affect both the safety and pharmacokinetic properties of the mAb causing the efficacy of the mAb to be impacted (Harding et al. 2010, Foltz et al. 2013, Gao et al. 2013, Ratanji et al. 2014) due to ADA binding to the drug and neutralising its activity or even worse speeding its biological elimination (Kelley et al. 2013). Finally, the incorporation of excipients within mAb formulations administered subcutaneously may cause adjuvant effects that would stimulate the innate immune response due to the excipient acting as a ‘danger signal’ or causing localised tissue damage enhancing immunogenicity to the mAb. Adjuvants are known for causing and prolonging an inflammatory response, this has been thoroughly discussed in Section 1.9.2. Adjuvants, such as aluminium, are already used in vaccine formulations (e.g. tetanus and diphtheria) where there is a desire to manipulate the immune response to be more effective towards an antigen (vaccine) (Petrovsky and Aguilar 2004, Sivakumar et al. 2011); this is thought to be typically achieved through the innate immune response inducing up regulation of pro-inflammatory cytokines, chemokines and cell recruitment; particularly the activation and maturation of DCs which has been found to be necessary in HIV infections (Lindblad 2004, Marrack et al. 2009, Barroca et al. 2014). For these reasons in vitro cytotoxicity testing were employed to examine any toxic or adjuvant-like effects of Arg-Glu that may provide an understanding of any underlying mechanisms which could then be later confirmed by in vivo studies.

Two cell lines - monocyte THP-1 cells (cell suspension) and human primary fibroblasts (adherent) were exposed to various osmolalities of the equimolar mixture Arg-Glu, and as the individual components Arg-HCl and NaGlu and compared to a reference excipient NaCl. THP-1 cells are commonly used as a cell model to study immune responses as these cells are considered to be DC-like (sentinels of the immune system), hence, it would be expected that
these cells would respond to these “danger” signals that might make them react to the excipient in an adjuvant-like manner. Furthermore, THP-1 cells are an immortalised cell line that can withstand long cell culture periods without changes in their cell sensitivity and activity, their homogenous genetic background restricting cell phenotype variabilities, hence reproducibility and the faster ability in upregulating inflammatory markers and cell surface receptors (Chanput et al. 2014, Chanput et al. 2015). Previous studies have demonstrated the suitability of using THP-1 cells in screening compounds before going on to using cells obtained from humans; further results from these cells have shown good agreement with effects observed in peripheral blood mononuclear cells (Chanput et al. 2010). The other cell line chosen was the adherent human primary fibroblast. The use of adherent and non-adherent cells would provide an idea of how different cell types would react to the test compound as other cellular and molecular cascades may be activated which need to be investigated. Also, as the fibroblasts are primary cell types their morphology and other characteristics are similar to the original tissue (Thrivikraman et al. 2014). The use of human skin fibroblast for in vitro cytotoxicity testing for surfactants and nanoparticles has been reported (Cornelis et al. 1991, Dechsakulthorn 2007). The impact of increasing osmolality is of key relevance and was investigated here as the osmolality of the administered drug needs to be the same or as near osmolality to the body’s fluid (blood is 280-295 mOsmol/kg) as to maintain an isotonic environment. An increase in osmolality would cause hypertonicity in the THP-1 cells which arises from the solute concentration being more outside the cell than inside resulting in shrinking of the cell and rupture of the cell membrane hence cell death (Brocker C 2012). This can lead to associated pain and discomfort for the patient. The addition of Arg·Glu to the THP-1 cells did not cause any further detrimental effects on the cell viability in comparison to NaCl at equivalent osmolalities. This was a promising finding as NaCl is already an established excipient in formulation. However, the noticeably more marked loss in cell viability observed with Arg·HCl suggested that the presence of Glu, ameliorated the toxic effects of Arg alone which seems to be due to other factors beyond osmolality changes. This could be due to the equimolar mixture of Arg·Glu providing an equi-osmolality. On the other hand, with the adherent cell line (fibroblasts), the salts Arg·Glu and Arg·HCl caused a more detrimental viability loss than NaCl and NaGlu. The results suggested that the presence of Arg caused a more pronounced loss in cell viability which is due to other factors beyond hypertonicity as with the THP-1 cells the equimolar mixture showed similar toxicity profiles with NaCl and with the fibroblasts Arg·HCl was still one of the components causing the most toxicity. Furthermore, this result could be a consequence of the adherent nature of this cell line with preliminary cell morphology data using immunofluorescence indicating that the incubation of Arg-Glu in the tissue culture media with the fibroblast cells caused interference with the cells ability to adhere to the wells of the plate observed under the microscopy, however due to time constraints this study was not completed (data not shown). The cytoskeleton is a structure in cells that plays a major role in the cells movement and internal organisation; it consists of different filaments such as actin and microtubules (Alberts 2010). Hypertonicity has been reported to induce
abnormalities in cytoskeletal rearrangement and interference with microtubule network organisation with adherent human monocytes and macrophages; with the latter showing this effect with NaCl at 400 mOsmol/kg. Moreover, mannitol, NaCl and urea (final osmolalities of 400 or 600 mOsm) have shown loss of their adherent nature in other cell types such as cells that line the surface of blood vessels (i.e. vascular endothelial cells) due to changes in their normal cell architecture (Malek et al. 1998). Although these effects have been seen in in vitro systems it is important to note that these hypertonic effects are highly dependent on the cell type and vary differently between in vitro and in vivo systems; for example the renal inner medullary cells (found in the kidney) in vivo can withstand high interstitial NaCl and urea of total osmolality of 600-1,700 mOsmol/kg/H2O whereas much lower osmolalities kill these cells in vitro (up to 600 mOsmol/kg/H2O). This has also been seen in other continuous cell lines which explains why most osmotic stress studies are restricted to this lower limit (Burg et al. 2007, Alfieri and Petronini 2007, Nunes et al. 2013). In light of these other studies, it may not be unusual to see that the additives studied in this Chapter can interfere with the functioning of adherent cell lines especially at hypertonic ranges; although this has already been reported in the literature with other established excipients (NaCl and mannitol), at appropriate concentrations these are used in formulation.

This increased toxicity observed by Arg·HCl in THP-1 cells was speculated to be a consequence from the formation of NO which is produced through the conversion of L-Arg to NO and L-citrulline through the action of endothelial NO synthase (discussed in Section 1.9.4) (Feng Chen et al. 2013, Pierini and Bryan 2015, Lepetsos and Papavassiliou 2016). The other speculation was that the presence of Glu in the equimolar mixture ameliorated the toxicity of Arg·HCl alone; the reasons for this are still unclear although a possible explanation could be the interactions between the negatively charged Glu and positively charged Arg preventing direct binding of Arg to the cells which seems to be causing more toxicity itself and cell death. Although NO is a key regulatory signalling molecule playing a role in endothelial function and vascular relaxation, it also has a role as a precursor cytotoxic effector molecule of the immune system. NO can react with superoxide radicals forming peroxynitrile causing oxidative stress in a number of cells (Valko et al. 2007, Sarmento Rios et al. 2011, Calcerrada et al. 2011). This as well as oxidative stress could account for the increased toxicity profile seen in the presence with Arg·HCl. Contradictory literature have been reported on the ability of THP-1 cells to secrete any detectable amount of NO (Fang 2002); however, Garg et al. have reported ~2.5 µM NO in unstimulated THP-1 cells which is not too far off what was observed in the studies here (~1 µM) (Garg et al. 2005). All salts demonstrated variable results in the supernatant and a decreased production of NO in the lysates even in conditions where cell viability was not effected (50 mM salt concentrations). Reduced levels of NO and eNOS have been shown in other cell types such as muscle cells in the presence of NaCl or sucrose in hypertonic amount (Pingle et al. 2003, Le et al. 2006). The more profound effects of Arg·HCl could not be reconciled with NO release; however, speculation can be made to the differences in the cellular uptake of Arg and Glu in cells. A previous study has reported the increased
uptake of L-Arg in lung inflammatory cells (such as macrophages) exposed to silica as well as an increased production in NO (Schapira et al. 1998); moreover, another study by Bogle et al has also shown increased L-Arg uptake in activated macrophages (Bogle et al. 1992). Literature has stated that the glutamate transport in different cell lines is dependent on preformed Glu which is at an inadequate physiological concentration (10–30 µM) to sustain glutamate transport (Meade et al. 1998). These could suggest that stressed cells (i.e. hypertonic insult here from L-Arg) may cause increase in cellular uptake in comparison to glutamate resulting in greater loss of viability. The mechanism of cell death observed from all salts was via apoptosis (programmed cell death), particularly, cells undergoing late apoptotic cell death after 24 hours with very few cells appearing necrotic. Necrosis can be initiated by a number of insults (trauma, infection etc) where there is total cell disruption resulting in the loss of cell membrane integrity and the expulsion of intracellular contents in an uncontrolled manner provoking an inflammatory response from the release of inflammatory cytokines as well as other danger molecules such as uric acid, ATP and heat shock proteins that act to stimulate the immune system further stimulating pro-inflammatory cytokines. The process of cell death and the content expelled by necrotic cells also causes the activation of other immune cells such as DC’s by triggering a number of receptors (e.g PRRs) stimulating both the innate and adaptive immune response (Biermann et al. 2013, Gallo and Gallucci 2013, Rock and Kono 2008). On the other hand, cell death via apoptosis proceeds in an anti-inflammatory manner, occurring in a more chronological fashion; the cells maintain their cell membrane integrity and avoid activating the immune system. These cells are encapsulated into apoptotic bodies and undergo phagocytosis (Rock and Kono 2008, Biermann et al. 2013). Several physical features of apoptotic cells have been suggested to aid this process; a key feature is the presentation of so called ‘eat me’ signals on the cell surface, primarily PS exposure. The recognition and clearance of apoptotic cells needs to be rapid to prevent the onset of an inflammatory response; however a delay in this process can cause phagocytes to instead release pro-inflammatory cytokines or go on to secondary necrosis which is associated with loss of cell membrane integrity. At this point, apoptotic cells display inflammatory features and go on to behave similarly to necrotic cells (Munoz et al. 2010, Biermann et al. 2013). Thus, as the cells in this study displayed apoptotic behaviour with hypertonic levels of the salts it is unlikely that these salts will cause any adjuvant effects to result in an inflammatory response. Published literature showed that hypoosmotic stress was a weaker inducer of apoptosis in comparison to hyperosmotic stress; with hyperosmotic concentrations mannitol, NaCl and urea showed apoptotic behaviour in both endothelial and smooth muscle cells (Malek et al. 1998). More evidence has shown that very hypertonic solutions of NaCl (>600 mOsm/l) induced apoptosis through mitochondrial dysfunction causing cellular damage (Michea et al. 2002, Kojima et al. 2010). Another study using the sugar trehalose as an excipient indicated hyperosmotic stress leads to cell apoptosis (Onitsuka et al. 2014). Not many studies have used monocytic or DC-derived cells for cell death studies, although, ones that are available suggest NaCl and mannitol at 500 mOsm/kg induced apoptosis (Gastaldello et al. 2008). This has revealed that
these results are not a characteristic feature of THP-1 cells as other studies mentioned above show similar effects in different cell lines; moreover, it seems that hypertonic solutions are likely to induce apoptosis rather than necrosis.

To further understand the impact of all these salts, the THP-1 cells were studied to investigate how these cells impacted cell marker expression representative of cellular stress under activated conditions (stimulated by TLR4 ligand LPS) and how this compared to inactivated THP-1 cells challenged with the salts alone and finally the effect of the combination of activated cells with salts (co-culture effect). Furthermore, statistical analysis was performed to see if any synergistic effects on membrane marker were occurring. Here, inactivated THP-1 cells (not stimulated by LPS) challenged with the salts did not show any further stimulation (or downregulation) on HLA-DR, CD86 or CD54 markers with the levels expressed by cells cultured with medium alone remaining similar to the presence of all the salts at all concentrations. The classical activation of macrophages is exposure to LPS synthesised by gram-negative bacteria. LPS is a natural adjuvant in stimulating cells through TLR4 which results in increased release of inflammatory cytokines and upregulation of costimulatory molecules on the cell surface of APCs (McAleer and Vella 2008), THP-1 cells cultured with LPS alone was used as a positive control as under activated conditions; these cells are expected to upregulate their membrane marker expression, however, in the THP-1 cell line used here only CD54 marker was significantly upregulated under activated conditions with LPS. Moving on to the co-culture of LPS and salt, these only showed statistically significant increases in CD54 marker expression in comparison to cells cultured with salts alone which may suggest the THP-1 cells are sensing the different degree of cell death by these salts as ‘danger signals’ and in response upregulating this marker which is involved in cell adhesion. Furthermore, the co-culture of LPS and salt in comparison to LPS activated cells generally did not show further stimulation for any of the markers tested, with the exception of cells cultured with LPS and 100 mM Arg·Glu and LPS with 100 mM Arg·HCl causing a significant increase in CD54 expression which could be attributed to the significant loss in viability at 100 mM Arg·HCl and initial loss of viability for Arg·Glu which are again from speculation acting as potent ‘danger signals’. Synergistic versus additive effects was analysed to see how LPS and salt were causing the increase in membrane marker expression. If additive effects were observed then the combination of LPS and salt in the experiment would be no greater than what would be expected from the addition of the two values that would be obtained experimentally if LPS and salt were independently analysed. In the case of synergy, the actual effect of the combination of LPS and salt would be greater than simply the sum of the two independent effects together (i.e greater than the additive effect) (Dearman et al. 2008, Tallarida 2011). The co-culture of LPS and all salts displayed additive effects indicating that these do not experience any specific interactions when together, therefore independently impact CD54 expression. As synergy was not found with membrane marker expression, synergy was not investigated with IL-8 expression, cells were cultured with salts alone and this was compared to activated THP-1 cells by LPS; cytokine expression was not significantly
impacted with salts alone over the hypertonic salt ranges tested. Synergy is often a rare situation to see however the use of two or more agents intentionally as a synergistic combination has been used for many reasons such as overcoming toxicity or avoiding the use of higher drug dosages. An example of this is the use of drugs targeted to cancer (multi therapy targets), some drug combinations cause unwanted side-effects such as hypertension therefore the addition of a third drug which can synergistically elevate this by acting on different mechanisms and receptors would be beneficial. On the other hand, unwanted synergistic effects can be harmful as other receptors may be activated which can influence the biological response (Dearman et al. 2008, Singleton et al. 2008, Tallarida 2011).

It is interesting to note, in regard to toxicity issues, that Arg·Glu is used as a drug in humans, in its own right, and marketed in some countries as Glutargin (also called Glutepar, Modumate and Dynamisan). Experimental studies as early as 1972, found that the use of N-carbamoyl-L-glutamate plus L-Arg in rats was effective in treating hyperammonemia in clinical conditions of liver disease (Kim et al. 1972). Arg-Glu, known as Glutargin, was developed in Ukraine as a hepatoprotective drug in 2001 and is used since 2005 (KUTKO I.I. 2006). Glutargin’s pharmacological action arises from the ability of Arg and Glu to rapidly neutralise ammonia, which in turn is a highly toxic metabolite, and excreting this from the circulation (Degtyaryova et al. 2012). Also, Glutargin has been shown to play a role in membrane stabilisation thus providing a beneficial influence on hepatocytes providing constant energy supply in them (Symptomsis 2016). Moreover, Glutargin has been reported to be very beneficial to patients suffering with delirium tremens (acute psychotic condition) due to ammonia build up. The drug is particularly effective in the removal of alcohol from the liver by removing and inactivating ethanol metabolites. Glutargin’s daily intake lies between 6-10 g and depends on the form it is administered as: either as a tablet, powder or solution injection (DINNIK 2012, HealthTips 2016). Relevant to this Thesis is the administration as a solution via infusion; Glutargin is available in ampules of 2 g (for a single dose) which is reconstituted in 250 mL isotonic NaCl which is administered twice daily. The treatment length is an average of 10-11 days with a maximum daily dose of 8 g. It has been reported that this drug has no mutagenic, embryotoxic or gonadotoxic effects with no immunotoxic effects on patients (Symptomsis 2016). However, it has been mentioned Glutargin should not be used for patients with severe disruption in the functioning of the urinary system and kidneys (HealthTips 2016). As Arg-Glu is already administered in human use via an infusion route, its general toxicity even at these higher doses is low. However, there is no indication of any studies where the subcutaneous route of administration for Arg-Glu is explored. Its use as an excipient in subcutaneous injectables would probably not cause any further cytotoxic effects as long as the final concentration maintains the isotonic range in the human body. The dosage of Arg-Glu administered as excipient will be significantly lower than single typical dosage of Glutargin. As an estimate, if 200 mM Arg-Glu would be included in the final mAb formulation in a 1.5 mL volume, this would equate to 0.3 g which is much less than the administered dose of Glutargin. In regards to storage, Glutargin can be stored for 2 years at room temperature (should not be stored at
temperatures exceeding 25 °C) (Symptomsis 2016). MAb formulations are usually stored at refrigerated temperatures (~5 °C); although from the results shown in Chapter 3 from the accelerated stability studies at 25 °C there were no significant changes in the percent monomer loss over the duration in the presence of Arg·Glu thus it would be expected that Arg·Glu is likely to be suitable as an excipient for subcutaneous injection. Moreover, from the results obtained in this Chapter, Arg·Glu does not have any detrimental effects on cell viability beyond what is seen by the existing excipient NaCl which has been reconciled to osmolality changes.

The generation of a novel excipient to prevent aggregation in DSP and USP is a desirable attribute for the biopharmaceutical industry. For example chromatography issues faced in DSP due to interactions between the protein and stationary phase is a common problem in SEC which yields low protein recovery. In this case, moderate NaCl concentrations have been used to suppress electrostatic interactions and 0.2 M Arg to suppress this interaction (Saraswat et al. 2013). A number of other literature examples have reported on the various roles of Arg in protein refolding, solubilisation and purification all of which stem from the fact that Arg can suppress protein aggregation (Arakawa et al. 2004, Shukla et al. 2005, Liu et al. 2010). A study by Tsumoto et al using green fluorescent protein expressed in E. coli reported the use of 0.1-1 M Arg in the media buffer improved protein expression with increased soluble protein at even higher concentrations (~2 M); the same study also used Arg to reduce PPIs during antibody elution (i.e. increase in monomeric state) from Protein A columns using both IgG1 and IgG4 mAbs which showed very sharp elution peaks with 0.5 M and 1 M Arg of 82 and 84 % recovery respectively (Tsumoto et al. 2004). This complements the findings in this Chapter as there was still an appreciable decrease in aggregated species using our model protein when Arg·Glu was only incorporated at the purification step. Furthermore, a number of other excipients such as NaCl and trehalose have been used in the cell culture media to prevent protein aggregation (Onitsuka et al. 2014, Townsend et al. 2015). The incorporation of Arg·Glu during the entire protein expression and purification stages or only at the latter stage clearly showed a decrease in aggregated protein species and a significant increase in protein mass with encouraging effects seen at only 50 mM Arg·Glu where no significant loss in cell viability observed in THP-1. Unfortunately cell viability was not measured for the HEK cells but as these have a similar growth characteristic to fibroblast (adherent), the results showed a significant decrease in cell viability for Arg·Glu at 56 mM which may be a problem here. Any changes in the USP of mAb expression needs to be carefully optimised to ensure any other cell culture attributes are not compromised (Jing et al. 2012) for example lower protein yields. Despite this positive effect seen by Arg·Glu, a decreased cell density and adherence of the HEK293 cells was observed supporting the findings seen with the preliminary cell morphology data carried out with the fibroblasts; both indicating Arg·Glu disrupted the cells adherent capabilities which has been shown to occur with other excipients (e.g. mannitol) impacting on the cells cytoskeleton (Malek et al. 1998) As cell viability was not investigated with these HEK293 cells, the work does require repeating in a more systemic manner with cell viability
measurements alongside measurements of protein yield and aggregation in order to optimise conditions. It would be acceptable to conclude that the greater loss in cell viability with the fibroblast cells can be reconciled to disruption in the cells cytoskeleton due to their similar growth features.

From the work in this Chapter, the data showed similar toxicity profiles for Arg-Glu in both the adherent and non-adherent cell lines. The toxicity observed by the non-adherent cell line can be attributed to osmolality changes whereas the effect in the adherent cell line seems to be more complex. The mechanism of cell death triggered by high concentrations of both Arg-Glu and NaCl is via apoptosis and there was no further impact on cellular stress markers, hence there was no evidence that Arg-Glu would have adjuvant-like properties in mounting an inflammatory response. To summarise, Arg-Glu and NaCl showed equivalent toxicity profiles thus indicating that Arg-Glu is likely to be a safe excipient to use in subcutaneous injections.
Chapter Five: The use of NMR as a tool to investigate mAb formulations

The work presented in this Chapter has been published:


5.1 Abstract

In order to assess how excipients affect mAb self-association, a number of complementary analytical techniques are currently used however many of these suffer from observable signals out of scale, thus the need for sample dilution. It would be beneficial if direct *in situ* measurements were available for highly concentrated solutions without the need to dilute or perturb the original sample. One of the analytical techniques underused for the formulation characterisation of mAbs is solution NMR spectroscopy. NMR is a powerful technique due to its ability to monitor signals from individual groups and types of atoms in a protein molecule as well as reporting on the structure and dynamics of proteins. The evident complications in using NMR for mAbs is their large molecular size (ca 145 kDa) resulting in spectral broadening and significant signal overlap. Despite these issues, two favourable properties were exploited with mAbs: they are highly concentrated and can withstand higher temperatures which are both parameters leading to sufficiently good NMR spectra. As a next step, it was interesting to assess mAb self-association in different formulations containing Arg·Glu using solution NMR spectroscopy as an orthogonal analytical technique. In the initial experiments, the findings showed that by monitoring changes in signal intensities from 1D $^1$H NMR spectra, when compensated for changes in buffer viscosity, it was possible to explore the sensitivity of mAbs to changes in the solution environment by looking at changes in the peak properties. Furthermore, a number of other experimental parameters were measured such as molecular translational diffusion and transverse relaxation rates. The molecular translational diffusion rates were not as informative as the transverse relaxation rates for these concentrated samples in pointing towards optimal formulations, primarily due to the crowding effect. Comparative accelerated stability studies were conducted between NMR and SE-HPLC with both generating consistent data, furthermore NMR could report on the solution viscosity and mAb aggregation. The addition of increasing concentrations of Arg·Glu significantly increased peak intensities with a remarkable decrease in the molecular transverse relaxation rate. Overall, the pragmatic approach developed here indicates NMR spectroscopy could be used as a complementary tool in selecting for optimal mAb formulations as well as other large therapeutic proteins.
5.2 Introduction

As described in the introduction, there are a number of analytical techniques that are currently used to select the optimal formulation for mAbs in situ including light scattering and analytical ultracentrifugation. These techniques allow the determination of formulations with the greatest stability while minimising other unwanted attributes such as self-association, aggregation and increased viscosity (Sule et al. 2012, Bhambhani et al. 2012, Saito et al. 2013, Kamerzell et al. 2013). Despite the fact that existing platform techniques have been put in place to select optimal mAb formulations, many limitations lie within existing methods such as the requirement of sample dilution to get a meaningful detection (which may distort the results, e.g. change the self-association). Monitoring such physical parameters as a function of excipient type and concentration in situ, at the target mAb concentration and temperature (e.g. during the accelerated stability studies), would allow a direct and undistorted way of choosing the best excipients and buffer conditions. An orthogonal analytical method, which is greatly underused for mAbs formulation characterization, is solution NMR spectroscopy. NMR is a very powerful method, capable of detecting and tracking signals from individual groups and types of atoms in a protein molecule, this is a very sensitive technique that can report on the structure and dynamics of proteins in solution (Cavanagh et al. 2007, Marion 2013, G. Wang et al. 2014). It is well-known that the application of NMR to whole mAbs faces difficulties due to their large molecular size (ca 150 kDa) leading to broad and overlapping spectral peaks. The usual protocols to overcome these issues such as using deuteration and/or the introduction of isotopic labels, are not feasible for the production of whole native mAbs due to the expression systems used (typically, mammalian cells). Despite these issues, two favourable properties of mAbs can be exploited here: the high concentration of their formulations (> 100 mg/mL) and their thermal stability/resistance to thermal denaturation. High temperature and concentrations together result in appreciably good quality NMR spectra due to decreased water viscosity and increased molecular tumbling. For soluble proteins, the use of high mAb concentrations improves the signal-to-noise ratio thus sharper signals however if aggregates are present due to self-association this increases the effective molecular tumbling correlation time resulting in signal broadening or even their disappearance (Karamanos et al. 2015). Recent literature has reported the use of $^1$H NMR as well as $^1$H-$^{13}$C correlation spectra in fingerprinting mAbs (Poppe et al. 2013, Poppe et al. 2015, Chen et al. 2015, Arbogast et al. 2015). In order to obtain optimal spectral quality, solution conditions with minimal aggregation, high stability and low viscosity are desirable. Thus, by finding optimal mAb solution conditions would lead to high quality NMR spectra. Such affected measureable parameters include translational and rotational diffusion, transverse relaxation times, deuterium exchange rates and observed signal intensities (Yao et al. 2000).

Monitoring the observed signal intensities and line width can provide information on the state of the protein. Sharp and narrow peaks indicate the presence of non-aggregated folded soluble protein in solution; whereas less intense and broad peaks can be indicative of aggregated
species. These simple but powerful observations were exploited in these studies with concentrated mAb solutions and used to monitor how the presence of Arg·Glu influences the solution conditions. Another parameter of interest is the translational diffusion of molecules (molecular self-diffusion) in solution, which is dependent on a number of factors, all of which are important for choosing the optimal formulation conditions for mAbs. The dependence of the translational diffusion on $\eta$ and $R_h$ can be characterized by the Stokes-Einstein equation (see Materials and Methods 2.2.3.5). The formation of soluble aggregates or clusters of proteins would cause an increase in the apparent $R_h$, and correspondent decrease in the observed $D$. Furthermore, NMR relaxation was used reporting on the dynamics of proteins. This is most commonly investigated in the form $R_1$ or $R_2$ describing the overall rate of tumbling and internal motions of the protein. In the studies conducted here, the transverse relaxation was measured as this is impacted by protein aggregation. The rate of tumbling can be related to the $\tau_c$ as described in Section 1.8.3.2. Therefore, these measurements can be used to further examine the aggregation state of the protein.

### 5.3 Aims of this Chapter

To use solution NMR spectroscopy to measure a number of experimental parameters for a selected mAb, mAb2, exploring their sensitivity to changes in the buffer environment. The excipient investigated was Arg·Glu which was previously shown to reduce protein aggregation and increase mAb solubility and stability in the concentration range 50-200 mM; this also matches the typical osmolarities used in the formulation of proteins for subcutaneous injection (Golovanov et al. 2004, Blobel et al. 2011, Shukla and Trout 2011, Kheddo et al. 2014). Derived NMR measurements from apparent solution viscosities and accelerated stability studies were compared to conventional techniques using the mVROC viscometer and SE-HPLC respectively. The overall aim of this study was to determine whether NMR could be a suitable method for monitoring a typical industrially-relevant IgG1 mAb and if so, could this approach be used as an alternative or additional technique in understanding the behaviour of mAbs in different formulation conditions.
5.4 Results

5.4.1 Using 1D $^1$H NMR spectroscopy to assess the aggregation state of mAb2 in different formulations upon addition of Arg-Glu

As a first step, a series of 1D proton NMR experiments were carried out to investigate the applicability of this technique in assessing the aggregation states of mAbs in selected conditions and at the same time observe spectral changes from the presence of Arg-Glu. 1D proton experiments were chosen as this is a quick and simple method in monitoring the native and unfolded state of the protein (Hu et al. 2012).

Figure 5.1 shows a representative 1D proton spectrum of a full length folded antibody. All NMR experiments were conducted at 40 °C (unless stated otherwise), as this temperature offers a good compromise between spectral quality (increased molecular tumbling at higher temperatures) and the protein structure not being significantly perturbed as the mAb2 melting transition temperature is ~70 °C (refer to Chapter 3). Within the NMR spectra, certain signals were monitored as measurable parameters to study changes in the aggregation state and conformation of proteins. Thus the aggregation state of proteins was monitored via the line width of peaks as this is associated to the molecular weight, for example signal broadening and a reduction in peak height can be caused by an increased rate of transverse relaxation, due to protein aggregation (hence increase in protein size) (Williamson 2013). Moreover, the signal intensities or integrals are generally proportional to the soluble protein concentration (Akoka et al. 1999, Pauli et al. 2005, Hu et al. 2012). The smaller the protein is, and the lower the viscosity, the sharper (and hence more intense) are the observable signals. Hence, by measuring signal intensities of protein signals in different formulation conditions, we can obtain a better understanding of the aggregation state. Additionally, protein unfolding can be detected by monitoring characteristic signal changes in the methyl or amide regions (Tsai et al. 1998). The unfolding of protein leads to decreased dispersion of peaks with the possibility that signals may become sharper (Dyson and Wright 2004). Signals found in the methyl region are characteristic signals belonging to the soluble natively-folded protein (~0.5 to 0.5 ppm) and the sharp peak at 1 ppm represents the total amount of protein in solution including contribution from partially-thermally-unfolded species in solution, if any.
Figure 5.1 Example of a 1D $^1$H NMR spectrum of a full length IgG1. The spectra is representative of mAb2 showing the chemical shift values on the x-axis and the regions on the spectra reporting on the different parts of the protein (Kheddo et al. 2016a).

In the first set of experiments, the effects of solvent conditions on 1D $^1$H NMR spectra were investigated. A series of 1D $^1$H spectra were acquired for mAb2 prepared at concentrations 40, 100 and 200 mg/mL at pH 6 and 7 to monitor mAb2 stability as a function of increasing Arg-Glu concentration (0-200 mM). In all experiments, defined amounts of dry Arg-Glu were added, to achieve the required range of concentrations, while avoiding protein dilution and change in its concentration (Materials and Method section 2.2.3.1). To closely investigate the changes in signal intensities, these 1D spectra were overlaid focussing on two clearly observable methyl signals (Figure 5.2D; labelled peak 2 and 3) representative of soluble, folded and mostly monomeric protein. Comparisons of the spectra in all conditions showed that the addition of Arg-Glu caused changes in the observed signal intensities of mAb2. The decrease in signal intensities observed with mAb2 at 40 mg/mL at both pH 6 and 7 with the addition of Arg-Glu concentrations greater than 20 mM can be explained by the minimal protein self-association expected as this was a relatively low mAb concentration, hence the presence of Arg-Glu had no further benefit. However, increasing mAb2 concentration from 100 to 200 mg/mL at both pH 6 and 7 showed remarkable increases in signal intensities with increasing Arg-Glu concentrations. Another important consideration is the increased buffer viscosity with increasing Arg-Glu concentrations leading to slower molecular tumbling hence signal broadening and reduction in signal intensities. The viscosity values for the buffer containing increasing Arg-Glu concentrations were calculated from translational diffusion measurements.
which will be described later in the chapter (Section 5.3.4). The viscosity-corrected normalised signal intensities (parameter $I^N_\eta$; see Materials and Methods Section 2.2.3.2), for mAb2 at 40 mg/mL at pH 6 and 7 remained reasonably flat as expected from the low self-association at this concentration (Figure 5.3A,D). However, at 100 and 200 mg/mL at both pH 6 and 7 there was a stronger increase in signal intensities with increasing Arg-Glu concentrations (Figure 5.2B,C,E,F) and increases in the viscosity-corrected normalised signal intensities, $I^N_\eta$ (Figure 5.3B,C,E,F). Strikingly at 200 mg/mL, $I^N_\eta$ displayed a 3-fold and 6-fold increase at pH 6 and pH 7 respectively (Figure 5.3E,F). At 200 mg/mL only one signal in the 1 ppm region was observable across all conditions (Figure 5.2D; labelled as peak 1). In order to further understand the differences in signal intensities seen between the raw spectra and viscosity-corrected normalised signal intensities, it was of interest to see if such effects were due to buffer type and ionic strength. Previous studies have shown that citrate ions binding to the protein surface lead to protein charge inversion and the formation of attractive electrostatic interactions causing PPIs (Roberts et al. 2015). Therefore, to ensure that the citrate ions present in the buffer were not contributing to the increased aggregation of mAb2 (which was apparently decreased by the presence of Arg-Glu), a control experiment was carried out: mAb2 at 100 mg/mL was prepared in only MilliQ water (no buffer) at pH 7 (also the pH was checked to ensure this did not deviate overtime). As the electrostatic repulsion between the protein molecules is not screened by salt in this solution condition it should be at its maximum (Roberts et al. 2015). The results showed that both the signal intensities (Figure 5.2H) and the viscosity-corrected normalised signal intensities, $I^N_\eta$, (Figure 5.3H) increased significantly upon addition of Arg-Glu. These findings support previous data indicating Arg-Glu shifts the equilibrium mixture of aggregated state (monomer-dimer/oligomers) towards lower molecular weight and monomeric species in solution, with the increase in signal intensities beyond what was observed with mAb2 prepared in 10 mM CP buffer alone.

In summary, the addition of increasing Arg-Glu concentrations caused an increase in the population of monomeric species, therefore countering concentration-dependent protein-self association.
Figure 5.2: Effect of Arg-Glu addition on NMR signal intensities of mAb2 in different solutions, as labelled. Panels A-G displays overlays of selected high-field region of \(^1\)H NMR spectra of mAb2, with concentrations of components as labelled. In (A)-(F) 10 mM CP buffer was present. Panel (G) includes spectra of mAb2 at 100 mg/mL recorded in the absence of any salt apart from Arg-Glu added as indicated (Kheddo et al. 2016a).
Figure 5.3: Dependences of viscosity-corrected normalised signal intensities $I_N^\eta$. This was measured for peak 1 (labelled in Figure 5.2) upon increase in Arg·Glu concentrations are shown in panels (A)-(G) (Kheddo et al. 2016a).

Another interesting observation identified in these 1D $^1$H spectra with increasing Arg-Glu concentrations were a concentration-dependent relative peak shift between peak 2 and 3 (labelled on Figure 5.2D) in the high-field methyl region (Figure 5.4A-E). Several NMR parameters can be used to measure protein-ligand binding which allows the dissociation constant, $K_d$, to be derived. Commonly, this is monitored by measuring the chemical shift ($\Delta\delta$) changes between peaks and is most useful when there is fast chemical exchange (Fielding 2007, Su et al. 2007). As all solution conditions were controlled, it can be assumed that the relative $\Delta\delta$ changes between these two peaks was due to the weak transient binding of Arg-Glu to mAb2. It is important to point out that peak 3 clearly seems to be an overlap of two peaks (shoulder at 0 mM Arg-Glu) which would affect the peak position and $K_d$; however, here, this was treated as a single proton contributing to this peak. This shift (i.e., change in peak
separation) can be used to estimate the apparent $K_d$ for this binding as approximately $90 \pm 15$ Mm (Figure 5.4E).

**Figure 5.4**: Chemical shift changes (changes in peak separation) between two well-resolved high-field mAb2 signals. These are marked peak 2 and peak 3 on Figure 5.2. Panels (A)-(D) display representative 1D $^1$H NMR spectra with increasing Arg-Glu concentrations. The disassociation constant $K_d$ for this interaction was derived by using a one site specific binding equation in GraphPad prism shown in (E). Results are displayed as mean and SD from 6 independent measurements of the chemical shift differences. Part of this Figure has been published (Kheddo *et al.* 2016a).

### 5.4.2 Temperature dependence of 1D $^1$H NMR spectra in mAb2 solutions

Having successfully shown that the normalised and viscosity-corrected signal intensities can effectively report on the protein state in solution, the temperature dependent effects on this parameter was investigated. Traditionally, the conformational and colloidal stability of mAbs at increased temperatures under various conditions is assessed by independently monitoring
signals reporting indirectly on whether the protein is in a folded conformation (such as solvent exposure of fluorescent groups), and signals reporting on appearance of larger-order aggregates (such as light scattering). One of these methods has been reported in Chapter 3 using Optim 2 which simultaneously measures the fluorescence intensity (reporting on conformational stability) and SLS (reporting on colloidal stability) (Kheddo et al. 2014). Here, NMR spectroscopy was used as an orthogonal approach to monitor the combined process of protein melting (unfolding) and aggregation at higher temperatures under different formulations, with the best formulation conditions expected to maximise both parameters. mAb2 under selected conditions at pH 6 and 7 was tested, with and without 200 mM Arg·Glu present at increasing temperatures from 40 to 75 °C. 1D 1H spectra were acquired immediately after each temperature increase. A series of 1D 1H spectra of mAb2 at increasing temperatures (from bottom to top) is shown in Figure 5.5. Comparison of the spectra showed that with increasing temperatures the sharp signal at ~1 ppm remained fairly consistent until a point where the signal dispersion decreased followed by signal broadening and reduction in signal intensity. This was most probably due to protein unfolding at higher temperatures and ultimately precipitating out of solution, hence indicating, this method may be used to determine the protein melting temperature. In these experiments the signal intensity of 1 ppm (Figure 5.2D; labelled as peak 1) was monitored, representative of all monomeric and lower-oligomeric protein species, both folded and unfolded, remaining in solution. Figure 5.6 displays the parameter, \( L^H \eta N \) (normalized signal integral corrected by the viscosity change due to Arg·Glu addition) vs temperature. It was evident that in the absence of 200 mM Arg·Glu the signal intensities were lower in comparison to the presence of Arg·Glu, most probably due to more protein self-association in the absence of Arg·Glu. As previously observed, mAb2 formulated at 40 mg/mL, pH 6 was a relatively low starting concentration hence the addition of 200 mM Arg·Glu did not significantly affect soluble non-associated protein. Nevertheless, on approaching 75 °C, when the protein starts to unfold, the quantity of protein remaining in solution was at least twice higher than without 200 mM Arg·Glu (Figure 5.6A). mAb2 at 40 mg/mL, pH 7 where the buffer pH approaches the pI of mAb2 (pI ~8.1) the presence of 200 mM Arg·Glu reduced protein self-association across all the temperatures with ~ 2.5 times more protein staying in solution at 75 °C despite reaching the protein melting temperature (Figure 5.6B). At even higher mAb2 concentrations of 100 mg/mL at both pH 6 and 7 in the presence of 200 mM Arg·Glu the starting integral intensity was 1.5 times greater than in the absence (Figure 5.6C,D). mAb2 at 100 mg/mL with 200 mM Arg·Glu showed a similar trend to 40 mg/mL highlighting the stabilising effects provided by Arg·Glu which can be attributed to the reduction in protein self-association particularly prominent at higher concentrations. Another observation was the slight apparent increase in \( L^H \eta N \) for mAb2 (peaking at 60-65 °C) in the absence of Arg·Glu (particularly prominent for mAb2 at 100 mg/mL, pH 7), this can be attributed to the presence of clusters that are being dissolved at elevated temperatures (Yearley et al. 2014), with concomitant increase in population of monomeric species.
In summary, the overall decrease in $\eta$ with increased temperatures can be due to protein loss from irreversible aggregation. This suggested parameter ($L_N^0$) was useful in assessing how different formulation conditions or excipients affect the melting temperature and amount of soluble mAbs.

Figure 5.5: Overlay of 1D $^1$H NMR spectra at increasing temperatures. These were acquired at 5 °C increments between 40 and 75 °C. Signals characteristic to the protein have been selected here with the sharpest peak at ~1 ppm representing the total amount of protein in solution (both folded and unfolded).
Figure 5.6: Assessing structural stability of mAb2 at increased temperature, without and with 200 mM Arg-Glu added. The viscosity-corrected integrals of peaks 2 and 3 (Figure 5.2D) were additionally normalized to the integral intensities at 40 °C in the spectra without Arg-Glu, to yield relative normalized integral parameter $L_{N}$. The measurements were done at pH 6 formulated at (A) 40 and (C) 100 mg/mL, and pH 7 formulated at (A) 40 and (C) 100 mg/mL (Kheddo et al. 2016a).

As useful information was obtained from the temperature-dependent experiments above reporting on the protein melting temperature and amount of soluble mAb remaining in solution, it was interesting to further investigate the use of NMR to monitor mAb2 stability at higher temperatures. The same set of mAb2 experiments used above were assessed at the same time by monitoring preservation of the protein signals in the spectra after a temperature stress lasting for 45 minutes at each temperature increment. The representative parameter $F$ represents the fraction of protein (in monomeric or lower-oligomeric form) remaining in solution following the temperature exposure. $F$ was derived from two groups of characteristic mAb2 signal; firstly at 1 ppm representing the total soluble protein species (unfolded and folded) and secondly well-resolved mAb2 signals from high-field shifted methyl signals (in the range 0.5 to -0.5 ppm) representing soluble natively folded protein mostly in monomeric form. Figure 5.7A-D showed that the increase in temperature to around 65 °C for both mAb2 at 40 and 100 mg/mL at pH 6 and 7 did not significantly cause protein destabilization hence no significant protein loss, therefore the value of $F$ remained close to 1. However, at 70 °C and above (i.e. protein melting temperature (refer to Chapter 3)), a sharp decrease in parameter $F$ was
observed across all conditions in the absence of Arg-Glu. Looking more closely at parameter $F$ at 70 and 75 °C as a function of pH (Figure 5.7E-H); the fraction of soluble protein in the absence of Arg-Glu mostly remained constant or slightly decreased as pH was increased from 6 to 7, however there was a significant increase in the fraction of soluble protein in the presence of Arg-Glu, which was prominent at 70 °C. Moreover, mAb2 at 40 mg/mL further showed the stabilizing effects from Arg-Glu particularly at 75 °C; despite mAb2 being heavily precipitated (Figure 5.7G).

In summary, these results demonstrate that Arg-Glu can reduce mAb aggregation and improve stability on approaching the melting temperature, with this becoming more apparent at pH 7 (closer to the mAb pI). These temperature experiments revealed that the sensitivity of NMR signals to both aggregation and viscosity provided a convenient and informative way to finding optimal solution conditions with increased stability and the least viscous solutions. Also, the temperature stress period selected here was fairly short however this can be increased to any length of time to obtain more robust information on different formulations.
**Figure 5.7:** Short-term mAbs stability at increased temperatures in the presence (+) and absence (−) of Arg·Glu. Short-term temperature stability factors $F$ (calculated as ratio of characteristic signal intensities after/before 45 min temperature stress) are plotted vs temperature (A)-(F), with highest temperature data points marked in dotted boxes expanded further for clarity and redrawn vs pH in panels (E)-(H). Panels on the left-hand side represent total soluble protein present in solution as measured from characteristic peak 1 (cf Figure 5.2D), whereas the right-hand panels represent natively-folded protein present in solution, as measured from characteristic peaks 2 and 3 (cf Figure 5.2D) (Kheddo et al. 2016a).
5.4.3 Long-term stability studies of mAb using NMR and SE-HPLC

The studies conducted so far have shown that proton NMR signals could be representative of the amount of protein remaining in solution as either monomeric or lower-oligomeric states, therefore, it was interesting to investigate the applicability of NMR to monitor protein degradation (i.e. long-term stability) of mAb2 at very high concentrations (300 mg/mL) at accelerated temperatures (40 °C). A recent study by Reslan et al reported that Arg·Glu did not show protein stabilising effects (Reslan et al. 2016); hence Arg·Glu stabilising effects were also monitored during these stability studies with other literature supporting the ability of Arg-Glu to stabilise mAbs (Kheddo et al. 2014). Sample preparation of mAb2 differed here as final formulations were reconstituted in 2H2O to assess the extent of amide group exposure found on the protein backbone to the solvent by examining the deuterium exchange, such NMR studies provide a rich source of protein structural information (Czerski et al. 2000). SE-HPLC, a well-established and understood technique in formulation to monitor monomer content in stability studies, was also repeated with these samples and the fraction of monomeric protein remaining in solution \( F_{\text{mono}} \) was calculated. Due to these formulations experiencing strong signal overlapping between protein (in high field) and buffer and excipient signals, well defined signals in the amide region ~8-10.5 ppm (region mainly affected by proton exchange with deuterons) and aromatic region ~6-8 ppm regions were selected (some contribution of deuterium exchange). Raw 1D \(^1\)H NMR spectra at 40°C were acquired at each time point across the stability study illustrated in Figure 5.8A-D. Overall, the spectra showed the signal intensities decreased over time at different extents between formulations. Signal integrals were measured and represented as the fraction of the initial signal integral of the aromatic signals \( F_{\text{AR}} \), amide signals \( F_{\text{NH}} \) both from NMR and monomer in solution \( F_{\text{mono}} \) from the SE-HPLC all reporting on the protein degradation rate as a consequence of aggregation and precipitation (Figure 5.8E-H). There were clear differences in the rate of signal loss from the aromatic groups \( F_{\text{AR}} \), in comparison to the monomer loss \( F_{\text{mono}} \), with the former being faster and the latter showing a steadier decline. The faster decrease in \( F_{\text{AR}} \) can be due to deuterium exchange occurring in the amide region which is overlapping in the aromatic region. Direct comparisons of \( F_{\text{AR}} \) between formulations was not possible here as the rate of deuterium exchange is highly pH-dependent, therefore the only comparison possible was at pH 6 in the presence and absence of 200 mM Arg-Glu; the presence of Arg-Glu showed a significant increase in the storage stability reflected by \( F_{\text{mono}} \) and \( F_{\text{AR}} \) and the rate of deuterium exchange \( F_{\text{NH}} \) also being reduced which could be a consequence of a more stable folded structure (Figure 5.8E,F). At pH 7, comparisons were made between Arg-Glu and Arg-HCl, where in the presence of Arg-HCl, \( F_{\text{AR}} \) and \( F_{\text{NH}} \) showed increased stabilising effects than Arg-Glu whereas \( F_{\text{mono}} \) did not show any notable difference in the long-term stability after 16 weeks. From this part of the work, the comparison between SE-HPLC and NMR was somewhat similar with NMR providing extra information during the stability studies.
Figure 5.8: Assessing by NMR and SEC the long-term storage stability of mAb2 at 40°C in selected formulations. The 1D $^1$H NMR spectral overlays (amide and aromatic region) for four different formulations of mAb2 at 300 mg/mL in 10 mM CP buffer are shown, as a function of time: at pH 6 (A) in the absence of additives; (B) in the presence of 200 mM Arg-Glu; at pH 7 (C) in the presence of 200 mM Arg-Glu; and (D) in the presence of 200 mM Arg-HCl. Correspondent panels (E)-(H) show for the same four formulations the time-dependence of relative fractions of aromatic ($F_{AR}$) and amide ($F_{NH}$) signals remaining in the spectra vs time, reporting on soluble protein loss. Independently, the fraction of monomeric protein ($F_{mono}$) was assessed using SEC and plotted (Kheddo et al. 2016a).

The differences observed between $F_{AR}$ measured by NMR and $F_{mono}$ obtained from SE-HPLC was further studied by taking into account the solution viscosity. It was hypothesised that an
increase in viscosity with time may contribute to the decay in signals which may be reflected in \( P^{AR} \). Therefore, the macroscopic viscosities of these formulations were measured using the mVROC. Although increases in the solution viscosity were observed for all formulations over the course of the study, the extent of these increases were very different which is most probably due to the formation of aggregates (Figure 5.9). The least viscous sample was observed with Arg-Glu at pH 6, followed by the solution at pH 6 only, then pH 7 with Arg-Glu and finally the highest viscosity at pH 7 with Arg-HCl. This was an interesting observation as the accelerated stability studies above indicated the mAb2 formulation at pH 7 with 200 mM Arg-HCl lead to the maximum preservation of monomer in solution. Taking all this into account, we conclude that the additional signal decay depicted by \( P^{AR} \) in comparison to \( F^{mono} \) (benchmark technique) is most probably due to the increased formulation viscosity over the time course of storage. This is extremely valuable information as solution viscosity is an important consideration in developing injectables. Hence, by measuring another parameter such as \( P^{AR} \) can be a valuable orthogonal criterion in obtaining the optimal formulations, with formulations that have the greatest soluble monomer in solution with the least viscosity.

![Figure 5.9: Assessing the macroscopic solution viscosity, using mVROC, during long-term accelerated mAb2 stability studies. mAb2 was formulated at 300 mg/mL in 10 mM CP buffer at pH 6 or 7, and further additives, as labelled, and stored at 40°C for 18 weeks (Kheddo et al. 2016a).](image)

### 5.4.4 Investigating the effect of viscosity and aggregation of mAb2 solutions using SE-PFG NMR and rheometry

Following the preliminary NMR based observations that Arg-Glu had a stabilising effect on mAb2, the following experiments focused on determining if the excipients could prevent protein aggregation and also alter viscosity. In subsequent experiments, the ability of Arg-Glu to

Molecules in solution will undergo Brownian motion either as rotational or translational motion. In this section, the translational motion was measured which determines the diffusion coefficients, $D$. Several physical parameters are dependent on this motion, particularly the solution viscosity, size and shape of the molecule and temperature. These parameters are linked to $D$ through the Stokes-Einstein equation (Equation 1.1) based on the assumption that the molecules have a spherical shape and solution is very dilute.

Firstly, the gradients were calibrated using the small-reference probe dioxane following normal NMR procedures to allow the measurement of $D$ (Materials and Methods 2.2.3.5). However, due to strong signal overlap from Arg·Glu with dioxane, a secondary reference molecule present in the buffer, citrate ion, was used to measure the values of $D$ as a function of increasing Arg·Glu concentrations (Figure 5.10). The results showed the expected decrease in $D$ with increasing Arg·Glu concentrations, most likely due to the increased crowding from the Arg·Glu salt in the buffer solution causing the citrate ions to diffuse slower. Using these measurements of $D$ it was possible to calculate the solution viscosity using the Stokes-Einstein equation (Figure 5.11). The solution viscosity increased with the increase in Arg·Glu concentrations; with almost a two-fold increase in the apparent viscosity at 200 mM Arg·Glu. The measured $D$ for citrate ion in the absence of Arg·Glu at 40 °C was in good agreement with the literature value for water viscosity at 40 °C (Figure 5.11; dashed line) although the slightly higher viscosity from the measured $D$ for citrate ion can be explained by the presence of both buffer along with 5% $^2$H$_2$O for the frequency lock signal. These parameters were used in subsequent analysis to account for the concentration-dependence of the apparent viscosity of the buffer itself, as well as for mAb2 solutions.
Figure 5.10: Changes in the experimentally-determined $D$ of citrate ion versus the increasing Arg·Glu concentrations. The sample contained 10 mM CP buffer, pH 6, and $D$ was measured at 40°C using DOSY NMR experiments. Mean and SEM shown for four signals selected in the region 2.7-2.8 ppm.

Subsequently, $D$ was measured for mAb2 formulated at 40, 100 and 200 mg/mL, at pH 6 and 7, in the presence of increasing Arg·Glu concentrations (5-200 mM) (Figure 5.13). DOSY is a technique used to separate different species in a mixture based on their differing $D$. $D$ is measured from the signal decay, i.e. $D$ is proportional to the signal decay (Poppe et al. 2013). Figure 5.12 shows a representative example of a 2D DOSY spectra of mAb2, citrate ion and Arg·Glu signals after processing with the chemical shift values (ppm) on the F2 axis and the logarithm of $D$ measured in $log(m^2/s)$ (i.e. $log(D)$) on the F1 axis. Figure 5.13A illustrates the raw DOSY spectra for each mAb2 condition with increasing concentrations of Arg-Glu; the dashed line at $log(D) = -10$ is the boundary set as a guide where the protein signals are expected to appear; thus, any shifts in the signal up or down from this boundary was easily identified. Measured $D$ were plotted as a function of Arg·Glu concentrations (Figure 5.13B,C),

Figure 5.11: Changes in the apparent $\eta$ with increasing Arg·Glu concentrations. This was calculated using the Stokes-Einstein equation based on NMR diffusion data. Dashed line represents the expected dynamic viscosity of water at 40°C. Mean and SEM shown for four signals selected in the region 2.7-2.8 ppm.
or as a function of mAb2 concentration (Fig. 5.13D,E). The results showed that the expected decrease in \( D \) with increasing protein concentrations (Riverosm.V and Wittenbe.Jb 1972) at both pH 6 and 7 can be explained by a crowding effect from the protein, increased protein self-association at higher concentrations and excluded volume effects (Nenninger et al. 2010). mAb2 at 40 and 100 mg/mL displayed very similar decreases in the \( D \) at pH 6 and 7 as the Arg·Glu concentration increased; although mAb2 at 100 mg/mL (pH 6) with 200 mM Arg·Glu showed a prominent increase in \( D \). On the other hand, at 200 mg/mL at pH 6, there was a steady increase in \( D \), up to 100 mM Arg-Glu followed by a drop at the highest concentration, whereas at pH 7, there was a slight overall increase in \( D \) when reaching 200 mM Arg-Glu. Alternatively, \( D \) plotted as a function of mAb concentrations evidently showed the increase in mAb concentrations decreased \( D \), whereas the effect of Arg·Glu on \( D \) is more complex (Figure 5.13D, E).

**Figure 5.12:** Example of a 2D DOSY spectrum of mAb2. The chemical shift values are on the x-axis and logarithm diffusion coefficients on the y-axis. The areas highlighted point to the protein signals from mAb2 and the signals from citrate ions and Arg-Glu.
Figure 5.13: Changes in $D$ of mAb2, measured using SE-PFG NMR spectroscopy, versus the increasing Arg·Glu concentrations. Data are displayed as mAb2 at 40, 100 and 200 mg/mL in 10 mM CP buffer at both pH (A) 6 and (B) 7 are shown vs the concentration of Arg-Glu added. The same data is also presented in different coordinates on panels (D) and (E), respectively. Mean and SEM shown for four signals selected in the region 1-1.5 ppm (Kheddo et al. 2016a).
Thus, to obtain a more in depth understanding of these results, it is imperative to consider the changes in solution viscosity as this strongly influences $D$ (cf. Equation 1.1). Figure 5.14 A,B shows the solution viscosities ($\eta$) measured by NMR (microscopic viscosity) for buffer alone (measured by following the diffusion of a small probe molecule, citrate ion) and for mAb2 at concentrations 40, 100 and 200 mg/mL at pH 6 and pH 7 as a function of increasing Arg-Glu concentration. This was compared to the viscosity measured using the mVROC viscometer reporting on the macroscopic viscosity (Figure 5.14C,D). Limitations on sample availability prevented the viscosity at 200 mg/mL to be measured with viscometer. First, the viscosity of the buffer alone measured by NMR and viscometer were in very good agreement (Figure 5.14A,C). Focussing on the NMR data, Figure 5.14A,B showed the expected apparent viscosities of mAb in the absence of Arg-Glu, with the least viscous sample at 40 mg/mL and the most viscous sample at 200 mg/mL. The viscosity of mAb2 at 40 and 100 mg/mL at pH 6 are almost similar in the absence of Arg-Glu (~1-1.2 mPa-s). With increasing Arg-Glu concentrations the viscosity at both concentrations remains fairly consistent. However, at pH 7, the differences between the concentrations were more obvious especially at 100 and 200 mg/mL (1.5 and 3 mPas respectively) than at pH 6 (1.2 and 2 mPas respectively) (Figure 5.14B). At 200 mg/mL, increasing Arg-Glu concentrations at both pH’s resulted in a decrease in viscosity particularly at pH 7 with 200 mM Arg-Glu with a slight increase in viscosity seen at pH 6 when approaching 200 mM Arg-Glu. Overall, similar trends were observed between the two methods although the microscopic viscosities measured by NMR seemed to be systematically smaller than the macroscopic viscosity measured by viscometer. The re-presentation of these same data as a function of mAb concentration evidently showed higher Arg-Glu concentrations noticeably decreased the solution viscosity for the microscopic (Figure 5.14E,F) and macroscopic measurements (Figure 5.14G,H).

In summary, the addition of increasing Arg-Glu concentrations decreased the overall viscosity of mAb2 in the formulations studied here, particularly at higher mAb2 concentrations (200 mg/mL) despite the underlying buffer viscosity. Also, the optimum Arg-Glu concentration was found to be between 100-150 mM where the least viscous mAb2 solution was mostly seen. A reasonable agreement was observed between NMR derived viscosity measurements and the viscometer.
Figure 5.14: Apparent $\eta$ of the mAb2 solutions as function of Arg·Glu concentrations, measured by NMR and Rheometry. First, microscopic $\eta$ was calculated based on the Stokes-Einstein equation using the measured $D$ for citrate ion. Data are displayed as changes in the apparent $\eta$ of the solution with mAb2 at 40 and 100 mg/mL at pH 6 (A) and pH 7 (D). The macroscopic bulk $\eta$ was also measured using the mVROC at pH 6 (C) and pH 7 (D). The solution $\eta$ without mAb2 was only measured at pH 6 (A,C). Data are displayed as changes in mAb2 $\eta$ at 40 and 100 mg/mL at pH (C) 6 and (D) 7. Mean and SEM are from three measurements; error bars are hidden within the symbol (Kheddo et al. 2016a).
Thus far in this chapter, the breadth of information that can be obtained by accurately measuring the translational diffusion is evident; another parameter closely related to $D$ is the apparent size of the molecule. These measured $D$ and calculated viscosities can be interpreted in to physical parameters relating the aggregation states of the mAb by using the Stokes-Einstein equation again to assess the apparent $R_h$ of the protein. Although this is a oversimplified approximation of $R_h$, it can provide an idea of the apparent changes in the cluster sizes of mAbs especially at higher concentrations and how these are influenced by the addition of Arg·Glu. Figure 5.15A,B shows the $R_h$ values plotted as a function of increasing Arg·Glu concentrations or as a function of mAb concentration (Figure 5.15C,D). Again, the expected increase in $R_h$ was observed with increasing mAb concentration at both pH in the absence of Arg·Glu with mAb2 at 40 and 100 mg/mL having similar $R_h$ ~8 nm, whereas at 200 mg/mL there was a two fold increase in the $R_h$, ~20 nm. mAb2 at 40 mg/mL at pH 6 and 7 showed the $R_h$ values remained steady with a slight decrease with increasing Arg·Glu concentrations to ~4 nm which is the expected monomeric mAb size; this can be explained by the fairly low mAb concentration thus a minimal level of self-association is expected (Figure 5.15A,B). A similar picture was shown at 100 mg/mL for both pH's. The largest drop in $R_h$ was seen with mAb2 at 200 mg/mL with the optimal drop in $R_h$ seen at 50 and 100 mM Arg·Glu at pH 6 and 200 mM Arg·Glu at pH 7.

In summary, by measuring the translational diffusion, increasing concentrations of Arg·Glu were revealed to effectively decrease the apparent $R_h$ which was supported by the ability of Arg·Glu to reduce the solution viscosity as well as correlating well with the increases in the signal intensities from the 1D $^1$H experiments.
Figure 5.15: Assessing the changes in the apparent $R_h$ of mAb2 upon addition of Arg-Glu. The values of $R_h$ were assessed vs concentration of Arg-Glu added, using Stokes-Einstein equation (Equation 2.6) for solutions with different concentrations of mAb2 (as labelled) formulated at (A) pH 6 and (B) pH 7. Same dependences are also presented vs mAb2 concentrations, with concentrations of Arg-Glu added color-coded, for (C) pH 6 and (D) pH 7 (Kheddo et al. 2016a).

5.4.5 Assessing the transverse relaxation rate of mAb2 in different formulations upon addition of Arg-Glu

Molecules can undergo two types of Brownian motion, either translational or rotational. In the previous experiments, the translational diffusion was studied. Here, the rotational diffusion of the molecules was assessed, which strongly affects the transverse relaxation rates of protons, $R_2$. This type of motion describes the molecular tumbling of the molecule which is dependent on the particle size and therefore can report on protein self-association; hence the larger the particle size the faster $R_2$ will be (Inoue and Akasaka 1987, Anglister et al. 1993, Yao et al. 2008). Signal broadening and decrease in signal intensities would be resultant of an increase in $R_2$. Thus, to investigate further the changes in mAb2 signal intensities as well as the apparent $R_h$ for mAb2 in the presence of Arg-Glu; the $R_2$ values were measured for mAb2 prepared at 40 and 100 mg/mL at pH 6 and 7 with the addition of increasing Arg-Glu concentrations. A number of signals were monitored as there was strong overlap in these individual proton signals making it difficult to consistently track these throughout the titration series between conditions, hence, several proton signals in the aliphatic part of the spectra (1 to -0.5 ppm) were monitored (Figure 5.16). The results showed that $R_2$ significantly decreased around 3-fold (~100-200 sec$^{-1}$ to 10-90 sec$^{-1}$) across all conditions with increasing Arg-Glu concentrations except for mAb2 at 100 mg/mL at pH 6 with $R_2$ values remaining fairly
consistent (Figure 5.16C). This was somewhat an unexpected result as the additional increase in buffer viscosity in the presence of increasing Arg·Glu concentrations would logically slow down molecular tumbling rather than causing such a dramatic decrease in $R_2$. Also, this effect is probably not due to any destabilization of the mAb2 structure or increased polypeptide chain flexibility in the presence of Arg·Glu as mAb2 has been shown to be thermally stable (refer to Chapter 3).

Figure 5.16: Changes in the $R_2$ of mAb2, measured at 40°C using spin-echo CPMG NMR experiments, versus the increasing Arg·Glu concentrations. Data are displayed as $R_2$ of mAb2 at 40 mg/mL at pH (A) 6, (C) 7 and at 100 mg/mL at pH (B) 6 and (D) 7. Mean and SEM shown for several protein signals integrated in the region 1 to -0.5 ppm (Kheddo et al. 2016a).

To further interpret these $R_2$ values, an empirical formulae in the literature was used which describes the proportional relationship of $R_2$ values of this protein size to the molecular mass and hence the molecular volume of mAb2, as well as the solution viscosity (as monomer or cluster) (Nenninger et al. 2010) (refer to Materials and Methods 2.2.3.8). Based on this simple estimation, we estimated the relative effective volume, displayed as the averaged aggregation number, $N$, of the mAb2 cluster at each Arg·Glu concentration (Figure 5.17). The results show an apparent 6-fold decrease in $N$, across all conditions; this relative value gives an indication of the extent of changes required in the apparent aggregation state of the rigid protein to give the observed decline in $R_2$ values. It is not clear why this 6-fold reduction in $N$ is occurring but
this may suggest that on the addition of Arg-Glu any self-associated mAb2 clusters undergo
dissociation, leading to the following increase in the overall molecular tumbling of mAb, \( R_2 \).
Another possible reason for the rapid decrease in \( R_2 \) could be that Arg-Glu is causing
destabilising effects of the mAb conformation, hence increasing side chain flexibility; although
we can speculate this, data presented from the temperature-dependent experiments (Figure 5.2 and 5.6) did not corroborate any conformational disturbances.

Figure 5.17: Changes in the apparent aggregation number \( N \) for mAb2 versus increasing
Arg-Glu concentrations. This was calculated using the \( R_2 \) measured by NMR spectroscopy
at 40°C. Data are displayed as \( N \) of mAb2 at 40 mg/mL at pH (A) 6, (B) 7 and at 100
mg/mL at pH (C) 6 and (D) 7. Mean and SEM shown for several protein signals integrated
in the region 1 to -0.5 ppm (Kheddo et al. 2016a).
5.5 Discussion

The overall aim of this study was to explore the applicability of solution NMR spectroscopy as an orthogonal method in choosing optimal mAb formulations, particularly at high concentrations (> 100 mg/mL) that display maximum stability with minimal self-association and viscosity. Solution NMR spectroscopy is a powerful technique which has emerged as the desired method in structural biology to study both protein dynamics and structure. Generally NMR spectroscopy is suited to study small proteins and fragments (< 30 kDa) via labelling with isotopes such as $^{15}$N, $^{13}$C and $^2$H; however advances in pulse sequences and effects from relaxation has enabled NMR to be considered as a tool to investigate much larger proteins (Lee et al. 2006). Although this technique is mainly used in structural biology, the versatility of the technique can be applied in a broader manner to understand other areas such as characterising molecular interactions and conformational changes (Dehner and Kessler 2005). As bio macromolecules are extremely flexible systems, their broad range of dynamics on time-scales from picoseconds to seconds can be explored by NMR (Markwick et al. 2008).

To our knowledge there is no ‘standard’ procedure available which states experimental strategies or documents the sensitivity of NMR-measured parameters in mAb formulation screening. The use of NMR as a tool in quality assessment in biological formulations, are associated with limitations arising from other components within the formulation (e.g. strong interferences from buffer components) (Jeannerat and Furrer 2012, Narhi 2013, Poppe et al. 2015). Even though there are a number of existing analytical methods available to study protein self-association such as DLS, SE-HPLC and analytical centrifugation, these require sample dilution and it is difficult to detect small population of aggregates (<5 %) particularly when the monomer and aggregates are in a dynamic equilibrium (G. Wang et al. 2014). Coincidentally, to achieve high quality NMR spectra, the initial standard procedures in setting up protein NMR experiments require thorough sample optimisation; thus, solution conditions are carefully selected (pH, excipients, temperature) to minimise aggregation and increase monomer content (Anglister et al. 1993, Dingley et al. 1995). Additionally, higher protein concentrations (above mM range) provide good quality spectra although this immediately faces problems with aggregation and increased viscosity. Hence, in these studies, the criteria used throughout all the experiments to determine the optimal mAb conditions were achieved based on spectral quality, stability and reproducibility. Several well-known assumptions based on current practices and protein NMR have been used to interpret the data; the signal linewidth (proton transverse relaxation rate $R_2$) is proportional to the apparent weight-averaged molecular mass (Inoue and Akasaka 1987, Anglister et al. 1993, Skinner and Laurence 2008); the observed signal intensities for folded stable protein are proportional to the concentration of monomeric and lower-oligomeric species and presence of larger aggregates broaden signals. Hence, solution properties such as increased viscosity or short-lived aggregates will slow the molecular tumbling rate, increase $R_2$ resulting in signal broadening followed by decrease in
signal intensity. These observable signal changes are complementary to methods such as SLS, in this case the observable signal increases in line with aggregation, but monomers or lower order protein clusters are difficult to observe on the background containing larger aggregates. An additional aspect to consider is the general running of NMR samples in several NMR tubes and the analysis of resultant spectra can be easily automated which is an important criteria for formulation. Moreover, during the initial stages of formulation screening the more high-throughput traditional assays would probably be more convenient however NMR can be incorporated further down in understanding the differences in the solution behaviour between formulations and validating the final formulation.

The common approaches applied in protein NMR, such as using isotopic labelling and/or deuteration are not feasible for whole native mAbs mainly due to complications with production of such labelled material in the standard mammalian expression systems. Thus, only $^1$H NMR spectra were conducted (1D and 2D) as recording other experimental types such as 2D NMR methods poses several challenges in data interpretation due to spectral complexities which cannot be easily resolved in two dimensions as well as causing impracticality (Clore and Gronenborn 1998, Poppe et al. 2015). As mAbs can withstand elevated temperatures, all NMR experiments were conducted at 40 °C as this resulted in more rapid tumbling and narrower linewidths hence better spectral resolution (Kwan et al. 2011); moreover, this temperature is coincidently relevant to mAb formulation for accelerated stability studies. The acquisition of a series of 1D $^1$H spectra from various formulations provided an efficient and simple way to understand how different mAb formulations differ from each other, by simply observing any spectral changes. These results were interpreted based on the reasonable assumption that the increases in the peak/signal intensities for folded mAb are proportional to the concentration of monomeric and lower-oligomeric species, whereas, broader signals indicated presence of larger aggregates. The signals monitored were from the methyl region which is a fingerprinting region indicative of the natively-folded protein; commonly these signals are selected for labelling largely due to methyl groups exhibiting high mobility associated with slow relaxation properties (hence, producing good quality spectra) (Serber et al. 2004). Thus, these findings showed by simply looking for conditions which maximised signal intensities of mAb meant that the size of the mAb cluster is driven towards a monomeric form with minimal viscosity, this was demonstrated by the addition of increasing Arg-Glu concentrations. The observed decreases in signal intensities with increasing Arg-Glu concentrations seen in the lower mAb concentrations can be explained by an opposing effect from the increasing concentration of Arg-Glu causing crowding in the solution which in turn increases the solution viscosity which appears as a decrease in the peak intensities. However, this process is counter balanced by the decrease in mAb self-association upon addition of Arg-Glu leading to sharper and more intense signals: particularly dominant at very high mAb concentration (200mg/mL). Therefore, removing this viscosity factor takes into account the viscosity contribution from Arg-Glu. After taking into account the viscosity-corrected normalized intensity, the less obvious effects of Arg-Glu on mAb at 40 mg/mL is most likely due to less protein self-association at this fairly low
mAb concentration, thus, Arg-Glu has no further benefit in breaking any transient mAb clusters, whereas at higher mAb concentrations (200 mg/mL) the significant increase in signal intensities despite the underlying increase in buffer viscosity indicated that mAb is shifting to a more monomeric state from a state of aggregation. This data complements the results in Chapter 3 which showed that increasing Arg-Glu concentrations increased the colloidal stability of mAb ($T_{agg}$).

An important aspect to consider if using NMR as a tool in mAb formulation screening is the addition of concentrated excipients or other buffer components as these cause very strong signals which may obscure the area of interest in the spectrum (Poppe et al. 2013). Arg-Glu concentrations above 100 mM did produce strong signals causing some difficulties in monitoring signals in the methyl region, which is a fingerprinting region indicative of the natively-folded protein. A recent study by Frank et al demonstrated that the incorporation of spin-diffusion for the protons in the proteins structured region minimised excipient signals and enhanced protein signals (Franks et al. 2016). Similar issues have been addressed by Poppe et al suggesting an alternative method in separating the protein and excipient signals by a procedure referred to as protein fingerprint by line shape enhancement (PROFILE). This is based on exploiting the diffusion properties between mAb and buffer components to derive highly resolved 1D $^1$H NMR spectra (Poppe et al. 2013, Poppe et al. 2015, Franks et al. 2016). Although these published studies highlight the attractiveness of using 1D $^1$H NMR by emphasising this technique has plenty to offer which is similar to the main message described in this Chapter, the aims were slightly different; with the authors aiming to find the simplest NMR tool which will identify the subtle differences between prepared batches of mAbs or between different mAbs (mAb profiling), whereas in this Chapter ‘traditional’ NMR approaches were followed in finding the simplest ways to identify the optimal mAb formulations associated with the least self-association.

From the determination that viscosity-corrected normalized intensity of NMR signal intensity can report on the state of protein in solution, this parameter was explored further to observe its dependence on temperature, and hence whether it can report on both protein melting and aggregation at higher temperatures under different formulation conditions. Depending on the view point one takes to interpret parameter, $L_{\eta}^N$; two considerations can be made. First, this parameter can report on the structural stability of mAbs in different formulations as at higher temperatures (approaching mAb unfolding temperature) protein begins to fall out of solution due to precipitation. On the other hand, this parameter can be a measure of aggregation as in the absence of Arg-Glu there is a slight peak at 60-65 °C which approaches the values of $L_{\eta}^N$ seen in the samples with Arg-Glu; this can be explained by the breaking of protein clusters due to increased temperatures hence causing an increase in the amount of monomeric and lower-oligomeric species present in solution. Furthermore, the stabilising effects of Arg-Glu were clearly seen particularly for mAb at 100 mg/mL at pH 6 and 7 which showed that Arg-Glu was successfully reducing protein self-association. Thus, parameter $L_{\eta}^N$ is multifaceted.
Another useful parameter derived was from the short-term stability studies, stability factor, $F$, which can be linked to the colloidal stability of mAbs. $F$ reports on the rate of protein loss per unit time at elevated temperatures which can be related to the onset of aggregation temperature ($T_{agg}$) derived from Chapter 3. These results emphasised the stabilising effects and ability of Arg·Glu to reduce aggregation near the melting temperature point of mAb2 (70-75 °C) with pH 7 providing slightly more stability compared to pH 6. The advantage of using NMR to perform these temperature dependent experiments is more diverse information can be obtained about the sample, *in situ* as formulated and is complementary to other well-known methods.

It is well known from the literature that monitoring chemical shift changes can describe protein-ligand binding (Fielding 2007, Su *et al.* 2007, Skinner and Laurence 2008). Visible chemical shift changes were detected with increasing Arg·Glu concentrations under all conditions, it is reasonable to assume the observed chemical shifts were due to the binding effects of Arg·Glu to the mAb. Although the simple 1D $^1$H NMR spectra do not directly reveal the mechanism why an addition of excipient causes increases in the observed signal intensities, it can still be used as a modest criteria in finding the best condition for shifting the solution equilibrium towards soluble monomeric state.

Two types of self-diffusion methods were measured using NMR spectroscopy in these studies: either using relaxation or SE-PFG, with the former reporting on rotational diffusion in the picosecond to nanosecond timescale and the latter reporting on translational diffusion on the millisecond to second timescale (Dehner and Kessler 2005). The spectral properties of proteins differ due to differences in hydrodynamic properties that affect the rotational and translational mobility of molecules in solution (Skinner and Laurence 2008). The translation diffusion of molecules is commonly used in characterising protein (and peptide) self-association, conformational changes, amide exchange as well as ligand binding (Price *et al.* 1999, Yao *et al.* 2000, Dehner and Kessler 2005, Brand *et al.* 2007, Yao *et al.* 2008). Through the Stokes-Einstein equation, this parameter is related to the $R_h$ and the solution viscosity (Y. Q. Wang *et al.* 2010). It is well-known from the literature that protein self-diffusion is limited in crowded solutions arising from concentrated protein solutions and buffer components (excipients) causing close collisions between protein molecules (i.e. crowding effect) resulting in the diffusion to be attenuated (Price *et al.* 1999, Price 2009, Balbo *et al.* 2013). The clustering caused in these crowded solutions would increase solution viscosity thus lowering the protein diffusion in solution (Schneider *et al.* 2011). Thus, protein diffusion under these conditions would no longer follow the dependence on its mass and size as described by the Stokes-Einstein equation. Although, this equation has inadequate validity at high protein concentrations, relative comparisons can be made between comparable solution behaviours (Inoue and Akasaka 1987, Y. Q. Wang *et al.* 2010). Moreover, the translational diffusion has been reported to be affected by other factors such as long-distance electrostatic repulsion (McGuffee and Elcock 2006); uniformly charged protein molecules do not want to be in close
proximity with each other so they maintain the furthest possible distance from one another thus leading to the apparent protein size seeming larger and therefore indicating slower diffusion. Difficulties were encountered in interpreting $D$ measurements in a quantitative manner as there is no established theory which clearly explains the events causing self-association and crowding at very high concentrations. Although the findings obtained here were quite complex to interpret, monitoring the self-diffusion of small probe molecules, such as citrate ion in the buffer, was useful as these ions seem to be noticeably sensitive to the apparent solution viscosity despite the presence of mAb; this correlated well with the macroscopic viscosity measurements from the viscometer. Based on the findings, the translational diffusion was not a straight-forward reporter of self-association. Also, the results showed that $D$ was strongly dependent on mAb concentration with little obvious benefit seen on addition of Arg·Glu, as Arg·Glu also increased the solution viscosity. It was reassuring to note that $D$ of mAb2 at 40 mg/mL in the absence of Arg·Glu ($5.5\cdot10^{-11}$ m$^2$/s) was in good agreement with the theoretical value of $6.3\cdot10^{-11}$ m$^2$/s calculated using HYDRONMR software (Ortega et al. 2011) based on the structural model of mAb2 and NMR-derived buffer viscosity of 0.65 cP (Figure 5.11). Previous studies have used diffusion experiments to study lysozyme aggregation where they also observed a complex behaviour from increasing salt concentrations (Price et al. 1999). To conclude, selecting the ‘best’ formulation based on $D$ would not be sufficient as the other findings showed Arg·Glu has promising effects on reducing self-association.

A different scenario was seen when measuring the rotational diffusion. This parameter does not experience problems associated with the ‘crowding effect’ as the protein can tumble even in enclosed environments. Protein NMR spectroscopy is dependent on its rotational dynamics which is represented as the proteins $\tau_c$ which is proportional to the molecular mass (G. Wang et al. 2014). $\tau_c$ can report on interactions with neighbouring molecules therefore can be an informative measure in assessing aggregation (Lee et al. 2006). This would be useful in formulation as the influence of excipients in these conditions can be observed. With an increase in size or solution viscosity, $\tau_c$ would increase resulting in a shorter $T_2$ hence a longer $R_2$ ($R_2=1/T_2$) (Y. Q. Wang et al. 2010). It was surprising to see that in all conditions tested here, the molecular tumbling of mAb showed a dramatic decrease in $R_2$ upon addition of Arg·Glu. This can be explained by the ‘breaking’ of transient mAb clusters upon the addition of increasing Arg·Glu hence increasing the molecular tumbling resulting in a decrease in $R_2$.

In conclusion, informative and unexpected findings were derived from these NMR-observable parameters. It was remarkable to see such differences in mAb2 behaviour in different solvent conditions at such high concentrations as based on the findings in Chapter 3 (using the same mAb2), mAb2 is inherently a highly stably and tolerant mAb to range of conditions. This has further emphasised the high sensitivity of NMR to subtle changes in molecular self-association. Looking forward at the application of solution NMR as an orthogonal technique in formulation, it would be expected that with antibodies prone to self-association, the selection of optimal
formulations would be even more challenging, and even larger differences in measurable parameters may be observed. It was also expected that such high excipient (200 mM) concentrations would cause severe distortions in the spectra due to strong overlapping signals, masking any weaker signals seen from protein (0.26 to 2.0 mM used here); however this was not the case due to the optimisation and advances in spectral parameters allowed sufficient separation. Referring back to the aims of this Chapter, NMR data has successfully shown that protein self-association found particularly at highly concentrated mAb solutions can be significantly reduced using increasing concentrations of Arg-Glu, especially when the pI of mAb2 approaches the solution pH.
Chapter Six: Investigating the role of arginine glutamate in preventing phase separation

6.1 Abstract

Opalescence and LLPS are physical instabilities which are unacceptable in the final drug product of protein-based pharmaceuticals, such as mAbs. LLPS manifests as an inhomogeneous solution which spontaneously results in the formation of a protein-rich phase at the bottom and protein-lean phase at the top, commonly arising at lower temperatures. Generally, opalescence and LLPS are dependent on the nature of a protein (i.e., charge distributions and hydrophobic patches), as well as numerous formulation factors such as ionic strength, pH, concentration, excipient and temperature. Thus, developing a better understanding of the factors leading to LLPS with methods to predict and mitigate it would be ideal. This chapter focussed on investigating a mAb (mAb5), which was extremely sensitive to changes in solution conditions causing LLPS. The link between different mAb5 formulations and its propensity to LLPS were investigated using several methods; by visual inspection of the sample, looking at its solubility using PEG precipitation assay and by extracting useful parameters using $^1$H NMR spectroscopy, based on findings from Chapter 5. The finding from the visual inspection clearly showed opalescence in the control samples leading to LLPS however this was mitigated with increasing concentration of Arg·Glu which was greater than what was seen with Arg·HCl. The results from the NMR experiments showed that NMR signal intensities were highest with the formulations least prone to LLPS whereas the lowest signal intensities were attributed to mAb5 formulations with greater propensity to phase-separate. The addition of Arg·Glu in the phase-separated samples resulted in significant increases in signal intensities in comparison to marginal effects from Arg·HCl. Moreover, the protein-rich and protein-lean phases were examined using NMR spectroscopy to further characterise these regions; the results showed that the addition of Arg·Glu remarkable increased the signal intensities in the protein-rich phase indicating significantly reduced PPIs. To conclude, these studies showed that NMR spectroscopy can be used to assess the propensity of mAb solutions to phase-separate; moreover the use to Arg-Glu significantly reduces PPIs found in the protein-rich phase.
6.2 Introduction

Clinical doses often require mAbs to be formulated at high (50-100 mg/mL) and ultra-high (~150 mg/mL) concentrations, which can subsequently lead to formulation challenges. Under these formulations, interactions between mAb molecules impacts not only on the stability of the formulation during its shelf-life but also hinders manufacturing (e.g. fill-finish) and patient delivery. Aggregation and increased solution viscosity has been discussed extensively in this Thesis; other issues which can arise in protein formulations are increased solution opalescence, turbidity and the tendency for the protein to undergo phase transitions. Opalescence and turbidity are not desirable due to aesthetic appeal as well as being a possible precursor to aggregation thus decreased product stability (Woods and Nesta 2010). The regulatory guidelines state it is mandatory for mAb solutions to be practically clear of visible particulates (Mathonet et al. 2016) and/or with a low degree of opalescence (in comparison to a set of standards) throughout their life time. Furthermore, if the original drug product showed opalescence it would be challenging to recreate this in placebo formulation for clinical studies (Salinas et al. 2010). Phase transition leading to LLPS may be prevalent at higher protein concentrations and lower temperatures which are both generally necessary for therapeutic use and refrigerated storage, respectively (Zhang 2011, Raut and Kalonia 2015a). LLPS is unacceptable in the final drug product since it presents an inhomogeneous formulation with different layer concentrations of protein and buffer components (Meyer 2012). From a biopharmaceutical perspective, it has now become common knowledge that LLPS occurs with globular proteins such as mAbs. LLPS is dependent on a number of formulation factors including ionic strength, pH, concentration, excipient and temperature (Chow et al. 2016).

It is important to distinguish between opalescent and turbid solutions as it is likely that both of these occur through different mechanisms (Salinas et al. 2010), this will be discussed in this Section. The descriptive terms ‘turbidity’ and ‘opalescence’ are used in other scientific fields outside formulation in very different contexts. A review of the literature reveals that some authors (e.g. (Woods and Nesta 2010, Raut and Kalonia 2015b)) use these terms interchangeably to describe a translucent solution which has the appearance of a white/cloudy solution; in both cases due to increased light scattering attributed to the presence of aggregates or to density/concentration fluctuations. Although aggregation and LLPS both result in opalescence the mechanisms contributing to the increased light scattering are different (Raut and Kalonia 2016b). Other authors state different definitions, with only turbid solutions arising from aggregation or particle formation and opalescence arising in the absence of aggregation but retaining a Rayleigh scattering property, which mAbs inherently possess due to their diameter being < 30 nm (Salinas et al. 2010). It is imperative to clearly define these terms from an industrial (MedImmune) perspective, in order to understand the reasoning behind the data presented in this Chapter. Opalescence was described for samples that to the human eye are slightly white/cloudy with a blue sheen under an artificial light source. The term ‘turbid’ at MedImmune is distinguished from opalescent and would refer to a more
extensive degree of phase separation/aggregation, measured against defined standards. The term ‘precipitation’ is usually not used by MedImmune unless describing a gross phase separation resulting in an extremely turbid sample. If precipitation was seen in the early formulation stages then this would likely result in the failure of the mAb formulation. However, in this Thesis, to experimentally measure the upper limit of solubility of a model mAb, referred to as mAb5, a molecular crowding method was used where the extent of precipitation was measured by nephelometry. Therefore, where the term precipitation is mentioned in this section, we refer to the precipitation induced artificially by the molecular crowding method. mAb5 was selected for these studies as historical data within MedImmune showed this mAb was prone to LLPS under certain formulations.

Phase transitions can be either solid-liquid or liquid-liquid phase separation. The former causes particulate growth leading to visible or sub-visible particulate formation which at this point can either cause turbidity or opalescence. At MedImmune, high concentration mAb products may fall in the opalescence space and if underlying aggregation or LLPS progresses then this may manifest in to turbid samples. Different sets of opalescent and particulate standards are used to ensure samples are thoroughly and consistently analysed throughout all formulation development. The solubility limit of mAb formulations is also critical as this can impact the ability to formulate at very high concentrations. PEG induced precipitation is commonly used to determine the relative solubility limit of mAb formulations in different formulations. This assay is performed by preparing a gradient of protein/PEG concentrations and the relative solubility is determined from the increase in turbidity measured by a nephelometer (Li et al. 2013). This assay is of interest here as a mAb with very high solubility is not expected to show opalescence which is a good predictor of what may be seen in the formulation (Li et al. 2013). The mechanism of action of PEG is via an excluded volume effect where PEG is excluded from the regions where there is contact between the protein interacting areas (Asakura and Oosawa 1958, Arakawa and Timasheff 1985) thus there is an increase in protein self-association resulting in protein precipitation. Hydrophilic polymers used as molecular crowding agents induce PPIs due to the protein being excluded from the solvation shell of the polymer, the result of which is a reduced solvation shell for the protein molecules up to and beyond their solubility limit (with increasing polymer concentration) (Atha and Ingham 1981, Arakawa and Timasheff 1985). It should be noted that there are several theoretical explanations (osmotic, thermodynamic/entropic, water activity) for the molecular crowding phenomenon which are outside the scope of this section (Arakawa and Timasheff 1985, Shulgin and Ruckenstein 2006, Kuznetsova et al. 2015). In addition to the use of PEG in solubility testing, literature has reported the use of PEG in inducing LLPS at temperatures above the freezing point thus allowing this phenomenon to be studied and also for systemic studies in comparing the effects of excipients on colloidal stability (Ying Wang et al. 2014). A link between opalescence and LLPS has been suggested however there is a lack of experimental data supporting this, particularly an understanding of the PPIs under different solution formulations and associated changes through the phase transitions (Mason et al.)
Thus, $^1$H NMR spectroscopy would be valuable in these investigations since it is a very powerful and sensitive technique to molecular motions. The conclusions from Chapter 5 showed that self-association could be investigated by monitoring the observed signal intensities, by measuring $D$ which is dependent on $R_h$ and $\eta$ and also relaxation measurements can report on the rate of molecular tumbling; thus, it was logical to see if this technique could report on subtle changes occurring during these transitions by looking at these same measureable parameters.

### 6.3 Aim of this Chapter

To investigate the utility of Arg·Glu as an excipient capable of resolving challenges with opalescence and LLPS in high concentration mAb solutions, using NMR spectroscopy to understand this phenomenon at a molecular level.
6.4 Results

6.4.1 Investigating the opalescence of mAb5 as a function of salt concentration: comparison of Arg-Glu and Arg-HCl

The first step in these studies was to obtain a mAb system that was prone to phase separation. Historical data within the formulation group at MedImmune showed the propensity of one particular mAb, mAb5, to turn opalescent and phase separate in 20 mM acetate buffer, pH 5.5 with increasing ionic strength up to 30 mM provided by NaCl. Concentrations beyond 30 mM were not tested as phase separation was already observed at this formulation. Upon attaining these formulations, the effect of Arg-Glu at concentrations previously shown to reduce aggregation and increase stability were added in these formulations. The objective was to observe if this excipient was able to mitigate or at least reduce opalescence and the onset of phase separation; this was compared to Arg-HCl. mAb5 samples prepared in 20 mM acetate, pH 5.5 with increasing NaCl concentrations (0-30 mM) alone or additionally prepared in the presence of Arg-Glu or Arg-HCl (50 to 200 mM) were subjected to ~9 freeze-thaw cycles.

Visual inspection of the product (Figure 6.1) illustrates images taken under a light box after the first freeze-thaw cycle. In the control samples, increasing salt concentration resulted in an increase in opalescence visually. Increasing concentrations of Arg-Glu caused a visible reduction in opalescence. With increasing Arg-HCl concentrations there was still a degree of opalescence remaining particularly at the bottom of vials.

Stress applied to the mAb solution via freeze-thawing cycles resulted in phase separation in the control sample at the highest salt concentration (30 mM). Figure 6.2 illustrated selected images of the different formulations; phase separation was seen in the second and third vial from the left as an upper clear, less concentrated phase and a lower cloudy, more concentrated ‘dense’ phase. Samples containing Arg-Glu did not show any opalescence or phase separation. However, with comparable concentrations of Arg-HCl, phase separation was observed with opalescence visible at the bottom of the vials. The additional mAb5 sample displaying phase separation was prepared as larger volumes, these were required for more detailed studies of the protein-rich and lean phases with several repeats needed (section 6.3.7). The 4 other samples illustrated in Figure 6.2 with the smaller volumes were sufficient for the initial NMR experiments which will be discussed in section 6.3.3.

In summary, the increase in NaCl concentration resulted in the expected opalescence leading to phase separation in the control sample of mAb5. Furthermore, the addition of increasing concentrations of Arg-Glu both decreased opalescence and prevented the onset of phase separation. The presence of Arg-HCl showed opalescence at the bottom of the vials indicating some degree of phase separation was still occurring.
Figure 6.1: Photos for the mAb5 solutions under different formulations. Photos taken after samples were removed from -80 °C and thawed at room temperature for 1-2 hours. mAb5 was prepared at 46 mg/mL in 20 mM acetate, pH 5.5 with increasing concentrations of NaCl salt (0-30 mM) and visual images were taken of these formulations with (A) salt alone; salt with Arg-Glu at (B) 50 mM; (C) 100 mM; (D) 150 mM (last sample lost during set-up) and (E) 200 mM; or salt with Arg-HCl at (F) 50 mM; (G) 100 mM; (H) 150 mM and (I) 200 mM.

Figure 6.2: Photo of selected mAb5 solutions in different formulations after ~8 freeze-thaw cycles. Phase separation (2nd and 3rd image from the left) was successfully achieved with 30 mM NaCl and this was compared to the default buffer, acetate buffer only and 30 mM NaCl with 100 mM Arg-Glu and 100 mM Arg-HCl.
6.4.2 Investigating the solubility limit of mAb5 using PEG-induced precipitation

Highly concentrated mAb formulations impose challenges due to solubility issues; an early insight into the solubility limits can provide an indication of the long-term stability of the formulation over its shelf-life and also upon delivery in the subcutaneous space (Pindrus et al. 2015). PEG induced precipitation is performed to determine the relative solubility of a mAb as a function of PEG concentration, so calculating the theoretical upper limit of solubility in the absence of PEG. The extent of protein precipitation scales over a linear region with the concentration of PEG, or other hydrophilic polymeric ‘macromolecular crowding agents’. These polymers essentially promote PPIs and aggregation-precipitation through short range weak attractive forces (e.g. van der Waals’ forces). The extent of aggregation/precipitation is measured as turbidity using nephelometry. Increasing concentrations of PEG (0-16 %) were incorporated into 15 selected mAb formulations (Table 2.6) and the turbidity of these was measured; the point where a sudden increase in turbidity occurred indicated that the solubility limit of mAb in that particularly solution has been exceeded. This method may predict opalescence because protein solubility is affected by PPIs which are also seen in opalescence (Li et al. 2013); it was of interest to see if the turbidity data were in agreement with the visual images (Figure 6.1; 6.2). Figure 6.3 showed the 15 mAb formulations selected represented different degrees of opalescence based on the visual inspections observed above. In the control experiments, mAb5 in the absence of NaCl was relatively soluble as an increase in turbidity was only seen at > 10 % PEG. When mAb5 was formulated in the presence of 15 mM NaCl its relative solubility decreased, with 7 % PEG causing turbidity. MAb5 in the presence of 30 mM NaCl had the lowest relative solubility with only 5 % PEG causing turbidity (Figure 6.3A). These results suggest that the formulations with NaCl had limited solubility as even a small percentage of PEG was sufficient to promote PPIs, reaching the solubility limit for mAb5. These data corresponded with the increase in opalescence seen from the visual inspections. All formulations tested reached their respective maximum solubility limit (sharp increase in turbidity) followed by an eventual plateauing in the turbidity, however, this was not seen with mAb5 in the absence of salt (Figure 6.3A) indicating the solubility limit of the solution is much greater. A characteristic feature seen in the data for this PEG precipitation assay is the gradual decline in measured turbidity following the sharp increase with increasing PEG concentrations. This is due to the assay being performed in a well-format (‘high-throughput’) such that as protein precipitates the immediate increase in the turbidity is followed by a settling out of the precipitant, conversely leading to a clarification of the upper well contents and decrease in turbidity as measured by the nephelometer geometry.

To directly compare the differences in the relative solubility with Arg-Glu and Arg-HCl an alternative way of representing the data for comparative purposes was to determine the half maximal effective concentration (EC50) for each formulation (Figure 6.4). EC50 in this context refers to the percentage of PEG required to induce 50 % maximal increase in the turbidity of mAb5 (a NTU response midway between the baseline and maximum). The EC50 values
confirmed the decreases in the relative solubility of the control formulations with increasing NaCl concentrations. A high EC50 value was reported in the absence of NaCl (18.9 % PEG) due to no plateau being reached consequential of the sharp increase in turbidity as shown in the raw traces (Figure 6.3A). The EC50 values for mAb5 sample which started to show opalescence (15 mM NaCl) showed decreased relative solubility with increasing Arg-Glu concentrations; however, a remarkable increase in the relative solubility was seen with the sample that phase separated (30 mM NaCl) with increasing Arg-Glu concentrations (5.7 % PEG with 30 mM salt to 9.4 % PEG with 200 mM Arg·Glu). EC50 values for Arg·HCl either reported a decrease in the relative solubility with increasing Arg-Glu concentrations (15 mM NaCl) or remained fairly consistent (30 mM NaCl).

To summarise, the PEG solubility results showed that mAb5 in the presence of 30 mM NaCl was most readily crowded out of solution, seen as a rapid increase in turbidity. Moreover, it was clearly shown that Arg-Glu increased the solubility of mAb5 in formulations where LLPS was seen, whereas Arg·HCl showed a general propensity to further reduce the relative solubility of mAb5.
Figure 6.3: Opalescence measured by nephelometric turbidity, as a function of PEG concentration (0-16 %). mAb5 was prepared in 20 mM acetate, pH 5.5 with increasing concentrations of NaCl (0-30 mM) alone and in the presence of Arg·Glu or Arg·HCl. Selected formulations were chosen to investigate mAb5 solubility. Solution formulations are (A) salt alone; (b) 50 and 200 mM Arg·Glu with 15 mM NaCl salt or (C) 30 mM salt; (D) 50 and 200 mM Arg·HCl with 15 mM NaCl salt or (E) 30 mM salt. Mean and SD (n=3) represent the error bars.
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Figure 6.4: Graph displaying EC50 (% PEG) values for each mAb formulation. EC50 in this context refers to the concentration of PEG required to induce 50% precipitation of mAb5 (response midway between the baseline and maximum). Formulations selected were mAb5 prepared in the absence, 15 mM and 30 mM salt each in the presence of 50 or 200 mM Arg·Glu or Arg·HCl. Mean and SD from n=3 experiments.

6.4.3 Using NMR to investigate LLPS: comparison of Arg·Glu to Arg·HCl

NMR spectroscopy was used to provide an insight into the events associated with LLPS in mAb formulations. NMR has the potential to uniquely provide an understanding of the different molecular events occurring in the protein-rich and protein-lean phases and how they are affected by the addition of Arg·Glu. It was also of interest to investigate if any changes in mAb5 structure or solution behaviour (e.g., self-association) could be observed by NMR in different mAb5 solution formulations with the addition of Arg·Glu or Arg·HCl.

The temperature selected for subsequent NMR experiments were chosen based on previous literature which has also investigated phase transitions. It is well-known from the literature that phase separation is likely to occur at lower temperatures with the system being homogenous at higher temperatures. Previous studies have started their temperature ramp from ~ 35 °C with a downward ramp to ~ 2 °C (or even lower temperatures in some cases (Chow et al. 2016) where phase separation was captured (Raut and Kalonia 2015a). Thus, the NMR studies in this Chapter were conducted from 20 °C to 2 °C as these generated spectra which were manageable to analyse and interpret.

Initial 1D 1H NMR experiments were used to observe if meaningful spectra would be attainable that would report on events associated with phase separation. A selection of mAb5 formulations representing different extent of opalescence and LLPS were selected for these studies (Figure 6.5); additionally, 100 mM Arg·Glu and Arg·HCl was chosen here as test excipients which potentially can modulate LLPS. The chosen concentration, 100 mM, was a
compromise between the drive to reduce NMR signal intensity to minimize spectral distortions (see Chapter 5), and the concentration sufficient to affect significantly protein self-association, as seen from NMR spectra (see Chapter 5). Samples prepared in the different formulations described in Table 2.7 were subjected to decreasing temperatures from 20 °C to 2 °C. 1D $^1$H spectra were acquired for the four mAb5 samples at each temperature. Figure 6.5A shows the overlays of the raw spectra. The relative peak intensity from a trackable signal in the protein methyl region (~0.5 ppm) was recorded. The concentration-normalized and viscosity-corrected signal intensities $I_\eta^{[A]}$ (normalised by measured mAb5 concentration and multiplied by the water viscosity at each temperature, refer to section 2.2.4.5) were plotted versus the temperature (Figure 6.5B). All formulations showed a reduction in the signal intensities with decreasing temperature although the rate at which these occurred varied. At 20 °C, mAb5 formulated with 30 mM NaCl (sample exhibiting LLPS at low temperature) showed the lowest $I_\eta^{[A]}$ intensity despite no phase separation seen at this temperature. This was followed by comparable trends in $I_\eta^{[A]}$ between mAb5 formulated in 30 mM NaCl with the addition of Arg·HCl and in its default buffer. The highest values of $I_\eta^{[A]}$ at 20 °C were observed with mAb5 formulated in acetate buffer alone and in 30 mM NaCl with the addition of Arg·Glu. Although mAb5 formulated in only acetate buffer displayed the greatest $I_\eta^{[A]}$ intensity at 20 °C, it showed the fastest decline with decreasing temperatures which was most probably due to increased self-association and the absence of any excipient. However, mAb5 samples formulated with 30 mM NaCl with the addition of Arg·Glu displayed the second greatest signal intensity at 20 °C which progressively decreased with decreasing temperatures. On the other hand, this same mAb5 sample with Arg·HCl displayed much lower signal intensities across all temperatures. These results suggest that the increased self-association seen with mAb5 formulated with 30 mM NaCl is indeed reduced in the presence of Arg·HCl although the greatest reduction in self-association was provided by Arg·Glu. Arg·Glu may be reducing protein self-association more efficiently than Arg·HCl leading to sharper and more intense peaks whereas broader and less intense peaks were observed with mAb5 sample formulated with 30 mM NaCl, suggesting that self-association in this formulation is more prominent.

To conclude, the mAb5 formulation which phase separated in the presence of 30 mM NaCl showed the lowest concentration-normalized and viscosity-corrected NMR signal intensity $I_\eta^{[A]}$ even at temperatures where phase separation was not yet observed (i.e., room temperature 20 °C); this indicates this measureable parameter should be explored further, whether it can be used as an early predictor of whether formulations would phase separate at lower temperature. Moreover, Arg·Glu was able to reduce self-association to a greater extent than Arg·HCl, which correlated with the effect of these excipients in the PEG solubility experiments, and their effect on LLPS.
6.4.4 Investigating LLPS using SE-PFG NMR

DOSY was used to measure the translational motion $D$ of the buffer components (buffer ion and excipient) in these same selected mAb formulations (Table 2.7). $D$ was measured as a function of decreasing temperatures (20 °C to 2 °C). An overlay of a representative example of the raw DOSY spectra for each formulation at 20 °C is shown in Figure 6.6A. The signals from the buffer components for each of the formulations are shown here using the dotted lines commencing from the left hand side corresponding to the sharpest peak in each formulation. The signals monitored with mAb5 formulated in acetate buffer alone and with 30 mM NaCl was from acetate; for mAb5 prepared with Arg·Glu and Arg·HCl and in the default formulation the Arg signals were measured (regions labelled on Figure 6.6A). It was not possible to measure mAb signals due to the fast decay of the signals during the pulse train sequence used during the DOSY acquisition. The data showed that with decreasing temperatures $D$ for the acetate and Arg signals decreased across all formulations (Figure 6.6B). The formulations which displayed the highest $D$ at 20 °C ($\sim9\times10^{-10}$ m$^2$/s) were mAb5 samples formulated with 30 mM NaCl (LLPS sample) and acetate buffer alone. Moreover, the decrease in $D$ with decreasing temperatures for both of these samples showed a superimposable trend. Arg·Glu and Arg·HCl in the presence of salt also showed superimposable trends however the starting $D$ at 20 °C was much lower ($\sim4\times10^{-10}$ m$^2$/s) indicating these samples were diffusing faster in these buffer solutions with comparable effects from both excipients. The default buffer showed the fastest diffusion which can be expected as there are already stabilising excipients within this formulation which are preventing protein self-association or any instabilities. Using the Stokes-Einstein equation, the normalised relative viscosity for the buffer and excipient signals was calculated for the five selected mAb5 formulations and plotted versus temperature (Figure 6.7). With decreasing temperatures there was no significant deviation from the expected
viscosity dependence of water with any of the formulations tested. Therefore, if phase transitions occur at lower temperature, it does not affect the microscopic viscosity of the solution.

In summary, $D$ declined for all the conditions with decreasing temperatures. The slowest $D$ was observed with mAb5 formulated in 30 mM NaCl however in the presence of both Arg·Glu and Arg·HCl $D$ was faster indicating these excipients are reducing the mAb self-association which is increasing the diffusion of mAb.

Figure 6.6: $D$ of small molecules present in mAb5 solution measured by DOSY. (A) Overlay of DOSY spectra for different mAb5 samples at 20 °C; (B) $D$ for mAb5 in these formulations are shown vs temperature. Error bars represent the upper and lower limits for each $D$. 
6.4.5 Determining the protein concentrations in the protein-lean and rich phases

The next step of the studies was to understand the molecular mechanisms causing phase separation by investigating the protein-lean and rich phase, to identify molecular changes brought upon by Arg·Glu, as from the visual inspections Arg·Glu mitigated phase separation and affects PEG-derived solubility (Figure 6.1;6.2). Using the only formulation which showed phase separation (mAb5 with 30 mM NaCl) (image in Figure 6.2), the protein lean and rich phases were carefully separated. The protein concentrations were measured in each of the phases along with the other selected mAb5 formulations used in the above NMR experiments which did not phase separate (Figure 6.8). The overall sample-averaged mAb concentrations in all the formulations did not change after the freeze-thaw cycles (~43-46 mg/mL). The concentrations reported for the two phases firstly showed the lean phase was much less concentrated (8.6 mg/mL) than the rich phase (114 mg/mL). From the visual images there were clear differences in the volumes of the two phases with the volume in the lean phase being ~0.75 mL and the rich phase ~0.25 mL, when starting from an initial 1 mL sample volume. Taking this into account, only a total of ~28 mg was in the rich phase and ~7 mg in the lean phase (~35 mg total, as compared to expected ~46 mg per 1 mL sample); the losses here could be attributed to sample loss during separation of the phases, mAb binding to the vials or possibly aggregation on the interface of the protein-rich and protein-lean phase.
Figure 6.8: Determination of protein concentration after phase separation of mAb2. This was recorded after 9 freeze-thawing cycles. mAb5 was prepared in 20 mM acetate, 30 mM NaCl, pH 5.5 which phase separated; aliquots from the lean and rich phase were taken. Serial dilutions were performed to accurately measure the protein concentration using UV spectrophotometer and known extinction coefficient at 280 nm. mAb concentrations were also verified for the formulations containing additional 100 mM Arg·Glu or Arg·HCl, the default sample and mAb in acetate buffer only. Mean and SD are shown for n=3 measurements.

6.4.6 Using 1D $^1$H NMR to investigate the molecular differences between the lean and rich phase in the presence of Arg-Glu

Following from verifying the protein concentrations for each sample, experiments were conducted to see if it was possible to characterise the protein behaviour in each phase using NMR spectroscopy. First, 1D $^1$H spectra were acquired, in the first instance, at 20 °C which showed the lean phase had a higher peak intensity (at ~1 ppm) than the rich phase, despite being ~13-times less concentrated (Figure 6.9C). In the subsequent experiments, spectra of both fractions were acquired at 40 °C to obtain better spectral quality. Similarly, the lean phase of the phase-separated sample showed higher peak intensity than the rich phase (Figure 6.9D; first two bar graphs) which could be attributed to the rich phase being significantly more prone to self-association. To observe if the self-association can be reduced and NMR signal intensities increased, dry defined aliquots of Arg-Glu was re-suspended in these phases (with a final concentration of 100 mM) to see if this could affect the spectra and hence protein self-association. Figure 6.9A,B show overlays of the protein-rich and lean phase in the absence and presence of 100 mM Arg-Glu. The changes in signal intensity are also shown in Figure 6.9D (second two bar graphs). It was clearly seen that in the lean phase there was no further change or increase of signal intensity with the addition of Arg-Glu. However in the rich phase there was a significant increase in the signal intensity in the presence of Arg-Glu, although signals did not recover in proportion to the expected protein concentration. This can be
explained by Arg·Glu breaking some transient interactions and reducing self-association of mAb5 in the rich phase sample, although not completely, whereas because in the lean phase there was no noticeable self-association in the first place, due to a fairly low protein concentration there, there was no effect of Arg·Glu on the protein signal intensities.

In summary, monitoring the changes in the signal intensities can report on the state of the mAb which has strengthened the findings from Chapter 5. Meaningful spectra were acquired from both phases and the addition of Arg·Glu caused a noticeable increase in the signal intensity for the rich phase whereas there was not much change for the lean phase.

![Graphs showing signal intensities for rich and lean phases with and without Arg·Glu](image)

**Figure 6.9:** Overlay of 1D $^1$H spectra of the rich and lean phase at 40°C. mAb5 was prepared at 46 mg/mL in 20 mM acetate, 30 mM NaCl, pH 5.5. These phases were subsequently spiked with dry aliquots of Arg·Glu at a final concentration of 100 mM. Spectral overlay in the presence and absence of Arg·Glu for the (A) rich and (B) lean phases. The measured intensity at 1 ppm for the rich and lean phases at (C) 20 °C and (D) 40 °C.

6.4.7 **Assessing the translation motion of mAb5 in the lean and rich phase in the presence of Arg·Glu**

To assess the translational self-diffusion of mAb in these two phases, DOSY experiments were performed to diligently measure $D$ for acetate, Arg·Glu and protein signals; selected regions
of raw DOSY spectra's for these formulations are shown in Figure 6.10. In the absence of Arg-Glu, $D$ for acetate ion was measured which remained the same in the rich and lean phase (Figure 6.11A). In the presence of Arg-Glu, acetate signals could not be measured due to signal overlapping hence Arg-Glu signals were tracked; $D$ for Arg-Glu was greater in the lean phase in comparison to the rich phase which could be attributed to the increased molecular crowding from Arg-Glu and the high mAb concentration thus reducing the translation motion of Arg-Glu (Figure 6.11B). With the mAb signals in the absence of Arg-Glu, mAb diffused faster in the lean phase in comparison to the rich which is expected due to this being less concentrated. With the addition of Arg-Glu $D$ in the lean phase noticeably reduced (probably due to increased viscosity due to Arg-Glu) however in the rich phase there was no difference in $D$. This was surprising as the measurements of the signal intensities (Figure 6.11C) for the two phases showed that in the rich phase there was a significant increase in the mAb5 signal intensity with the presence of Arg-Glu. Overall, the findings fit well with the earlier conclusions from Chapter 5 that for highly-concentrated protein solutions $D$ is not a good measure of protein self-association, due to diffusion limited by extreme molecular crowding effects.
Figure 6.10: $D$ of mAb5 measured by DOSY. Collection of DOSY spectra for mAb5 in the lean and rich phase highlighting the region for either the buffer components or mAb5 signals in the absence and presence of 100 mM Arg·Glu, as labelled.
6.4.8 Assessing the transverse relaxation rate of mAb5 in the lean and rich phase in the presence of Arg-Glu

Furthermore $R_2$ was measured as proteins in solution undergo molecular tumbling. The rate of molecular tumbling depends on the size of the cluster, and therefore can report on the protein self-association state. Relaxation was initially measured at 20 °C which showed the expected faster molecular tumbling of the protein in the lean phase than in the rich phase due to the less concentrated mAb solution and more monomeric species in this phase (Figure 6.12A). Again, to improve spectral quality experiments were repeated at 40 °C which showed sharper signals here hence reducing $R_2$ in the rich phase (Figure 6.12B). $R_2$ was measured for protein signals but the data was not reliable particularly for the rich phase due to extremely fast relaxation of protein signals using the chosen pulse sequence. 

![Figure 6.11](image.png)

**Figure 6.11**: $D$ of buffer components and mAb5 measured by DOSY. Data are displayed as $D$ for the lean and rich phase for (A) acetate; (B) Arg·Glu and (C) mAb signals as labelled on the (y-) axis.

Furthermore, $R_2$ was measured as proteins in solution undergo molecular tumbling. The rate of molecular tumbling depends on the size of the cluster, and therefore can report on the protein self-association state. Relaxation was initially measured at 20 °C which showed the expected faster molecular tumbling of the protein in the lean phase than in the rich phase due to the less concentrated mAb solution and more monomeric species in this phase (Figure 6.12A). Again, to improve spectral quality experiments were repeated at 40 °C which showed sharper signals here hence reducing $R_2$ in the rich phase (Figure 6.12B). $R_2$ was measured for protein signals but the data was not reliable particularly for the rich phase due to extremely fast relaxation of protein signals using the chosen pulse sequence.
Figure 6.12: Changes in the $R_2$ of acetate molecule. This was measured at (A) 20 and (B) 40°C using spin-echo CPMG NMR experiments, in the lean and rich phases of the phase-separated formulation.

To conclude, the results from this Chapter showed that Arg·Glu could be used as an excipient to mitigate issues associated with opalescence and LLPS. The expanded visual inspections clearly displayed this; moreover, Arg·HCl did not successfully prevent LLPS as opalescence was still visible at the bottom of the vial. NMR spectroscopy did report on events associated with opalescence and LLPS on a molecular level; the sample which showed LLPS presented the lowest signal intensity with broader signals whereas Arg·Glu and Arg·HCl both showed higher signal intensities indicating these excipients are breaking transient PPIs with more pronounced effects from Arg·Glu. Thus, NMR could be used as a predictive tool for samples that may phase separate during its lifetime. From the diffusion and relaxation data it seemed more informative to monitor both these parameters using small molecular probes, acetate ion in these studies. This could be useful in mAb formulation screening, as the incorporation of a reporter molecule or monitoring the buffer ion species can be reliably tracked due to their tendency to have sharp signals thus different formulations can be compared based on these molecules to report on the translational diffusion and relaxation rate.
6.5 Discussion

The key aim of this Chapter was to see the effect of Arg·Glu on LLPS and explore whether NMR can provide additional information on events associated with opalescence and LLPS for mAb5 formulations. There is less available literature concerning LLPS of mAb solutions compared to aggregation and reversible self-association, though several studies are published (Nishi et al. 2010, Wang et al. 2011, Ying Wang et al. 2014). LLPS and aggregation occur under different conditions; with LLPS the protein retains its native structure however in aggregation this is associated with both native and/or non-native protein species that are either reversible or irreversible (Raut and Kalonia 2016a). However, fewer than 20 proteins appear to be mentioned in LLPS studies and not all (such as lysozyme) are relevant to mAb-related investigations; this is probably because LLPS is a metastable transition which is not commonly observed or reproduced (Asherie 2004, Wentzel and Gunton 2007). The ability of Arg·Glu to reduce aggregation and increase stability has already been determined in Chapter 3, so in this part of the study it was of interest to see if Arg·Glu could reduce or prevent LLPS and associated opalescence, and how its efficiency would compare with Arg·HCl. To gather more systematic and comprehensive experimental data on the nature of the protein-lean and -rich phases (e.g. using Rₑ and Rₚ parameters) and the underlying molecular mechanism leading to LLPS, and the influence of Arg salts, NMR spectroscopy was employed. This would be the first report to use NMR as a sensitive analytical tool in this context of mAb phase separation.

The colloidal stability of mAbs is a vital consideration in formulation as this looks at the self-association of the native protein which can arise through attractive electrostatics or short range attractive forces or via complementary surface effects (He et al. 2011, Kheddo et al. 2014). The folded, globular proteins can be thought of as soft-sphere colloidal molecules which may undergo ‘condensation’ leading to aggregation or LLPS (Ying Wang et al. 2014). The likelihood of condensation is determined by mainly attractive short range PPIs (e.g. van der Waal’s forces) which are attributed to the metastable nature of LLPS (Mason et al. 2010). A link between LLPS and aggregation has been suggested as LLPS can arise from protein self-association and/or irreversible aggregation with both of these phenomena being concentration dependent (although LLPS does not necessarily result in aggregation). During phase separation the protein retains its native conformation in solution (Ahamed et al. 2007, Nishi et al. 2010, Mason et al. 2011, Raut and Kalonia 2016b); other authors have shown that the protein self-association in the lean phase is negligible with mostly monomeric species however in the rich phase attractive electrostatic intermolecular interactions predominate that are responsible for causing LLPS (Nishi et al. 2010). The thermodynamics of these phenomena are different, with LLPS manifesting predominantly at lower temperatures, while aggregation can occur both at lower temperature following cold denaturation (Lazar et al. 2010) or at elevated temperatures (Raut and Kalonia 2016a).
The results shown in the visual inspections done for the mAb5 formulations (Figure 6.1A) were in agreement with the historical data at MedImmune, showing increased opalescence with increasing salt concentrations (0-30 mM) and after several freeze-thaw cycles phase separated in the highest ionic strength sample (30 mM). It is known from the literature that the addition of additives such as NaCl and PEG can induce LLPS (Annunziata et al. 2003, Asherie 2004, Roosen-Runge et al. 2014) with 40 mM NaCl shown to cause LLPS at lower temperatures in other experimental studies (Nishi et al. 2010). Previous work by Salinas et al suggested the increase in opalescence at high ionic strength was attributed to charge shielding of long-range electrostatic repulsive forces, such that at high concentrations attractive interactions become predominant, also leading to LLPS upon lowering of the temperature (Salinas et al. 2010). From the visual inspections the addition of 50 mM Arg·Glu to the control sample set clearly reduced the increasing opalescence of the solutions with increasing NaCl concentrations, most importantly preventing LLPS in the solution with the highest ionic strength. The increase in Arg-Glu up to 200 mM completely eliminated this opalescence and LLPS. In comparison to Arg-HCl the opalescence seen in the control sample set was reduced with 50 mM however there was no further impact upon increasing the concentration of Arg-HCl up to 200 mM. The results indicate that the opalescence observed in these samples is not solely from Rayleigh scattering (proteins are typical Rayleigh scatterers of visible light (Salinas et al. 2010)) but some degree of protein self-association is occurring. Arg-Glu attenuated self-association at 200 mM, hence preventing LLPS, in contrast to Arg-HCl which partially reduced self-association (at 50 mM) with no further effect up to 200 mM (Figure 6.1). Moreover, after several freeze-thaw cycles, Arg-HCl still did not mitigate the phase separation as there was some degree of opalescence still remaining in the bottom of the vial (Figure 6.2).

As a next step, solubility testing of mAb5 was carried out by using PEG, a macromolecular crowding agent. PEG is frequently used for liquid-liquid partitioning, bio macromolecules precipitation (Annunziata et al. 2002) and in solubility studies as a function of solution formulations since PEG increases the phase separation temperature (Raut and Kalonia 2015b). Other commonly used synthetic crowding agents include Ficoll, dextran and polyvinyl alcohol. These primarily work via excluded volume effects by reducing the solvent accessibility region for proteins thus resulting in PPIs; additionally as these polymers are neutral and relatively hydrophilic, thus PEG does not interact with the protein (Ellis 2001, Kuznetsova et al. 2014). The findings from the PEG solubility assay conducted here showed a decrease in the relative solubility of mAb5 in the presence of increasing salt concentrations. This can be explained by the protein solution undergoing changes in its chemical potential or due to the increased intermolecular interactions (Raut and Kalonia 2016a). Remarkable increases in the relative solubility was observed for mAb5 formulated with 30 mM NaCl with the addition of Arg-Glu in comparison to salt alone. It is important to note that the determination of solubility using the PEGs assay is a relative solubility measurement. Protein solubility is a consequence of the relationship between two opposed effects: polarity and hydrophobicity. Well-folded protein indicates hydrophobic patches are hidden within the protein core but due to the
flexibility of the protein structure some of these hydrophobic patches become solvent accessible; thus to minimise the free energy of the system the protein will refold and interact with other molecules to minimise hydrophobic exposure. Increased hydrophobic exposure can ultimately reduce protein solubility and increase chances of PPIs. These effects described are intrinsic protein factors however extrinsic factors can also influence the solubility; such as the addition of salt. The addition of high salt concentrations totally screens the charges on the protein surface hence minimising charge repulsion resulting in closer protein interactions encouraging molecular interactions between hydrophobic patches thus reducing solubility. If in a particular condition hydrophobic interactions are stronger than electrostatics, the solubility limit will be achieved even at low salt concentrations as these intrinsic and extrinsic parameters impact molecular interactions and self-association (Pindrus et al. 2015). The findings from the PEG assay showed its potential use as a predictive tool to determine mAb formulations which may be susceptible to LLPS as these results correlated well with the visual inspection as well as the findings from the NMR studies. There is scarce published literature on the effect of low salt concentrations on protein solubility; although recent studies by Zhang et al investigated the effect of the monovalent cation Na⁺ in the form of a number of different salts, one being NaCl where the authors found low salt concentrations decreased solubility due to the presence of counterions (i.e. presence of NaCl) which neutralised the net charge of the protein thus decreasing electrostatic repulsive interactions resulting in reduced solubility; this could explain the decrease in the relative solubility of mAb5 with the increasing NaCl added (Zhang and Zhang 2012).

LLPS is a thermodynamically driven effect disrupting the homogeneity of protein solutions resulting in two distinct phases which differ in concentrations (Chow et al. 2016). The different states proteins can exist in is highly dependent on a number of variables such as temperature and protein concentration all impacting the protein phase behaviour. Characteristic features of a genuine LLPS sample would follow as a prominent increase in the cloudiness of the protein solution on decreasing of the temperature followed by a clearing of this as the solution separates into two distinct clear phases (concentrated and dilute phases in equilibrium with each other) separated by a sharp meniscus caused by the change in protein and solution density between the two phases. Taking into account these LLPS explanations, the type of phase separation observed in the studies conducted in this Thesis was indeed LLPS with the presence of two distinct phases but because the protein-rich phase in particular was not completely clear some degree of solid-liquid phase separation was probably superimposed into this region. Furthermore, the missing milligrams of protein could be attributed to these solid aggregates which were not detected during the protein concentration measurements. The phenomenon observed indicates more complex events are occurring.

While conducting these studies, there was no obvious literature describing the characterisation of LLPS of biopharmaceuticals using liquid-state NMR spectroscopy. Commonly used techniques employed to study phase separation include DLS, SEC and turbidity
measurements (Nishi et al. 2010). DLS is the primary tool in measuring PPIs which reports on the interaction parameter ($k_D$) with a good predictive measure of protein aggregation, viscosity and solubility (Lehermayr et al. 2011, Connolly et al. 2012, Raut and Kalonia 2015a). Recently, experimental work was conducted to investigate if numerous light scattering analysis ($B_{22}$, $k_D$, weight-average molecular weight and hydrodynamic diameters) at low mAb concentrations could be predictive of phase separation at high mAb concentrations; amongst these techniques the author found $k_D$ measurements at low mAb concentrations correlated well with phase separation at higher concentrations whereas this agreement was not seen with $B_{22}$ (Chow et al. 2016). Although these techniques exist, samples require diluting to obtain meaningful data and these do not provide information on the molecular mechanisms of LLPS, thus, as a complementary tool NMR can provide new information with the phase separated samples being analysed in situ. A selection of mAb5 formulations were chosen here for NMR studies that represented different degrees of opalescence and LLPS alongside formulations with Arg-Glu or Arg-HCl in the phase separated sample. By measuring the normalised and viscosity-corrected signal intensities for these formulations at decreasing temperatures, it was informative to see that mAb5 formulated in the presence of 30 mM NaCl (phase separated sample) displayed the lowest peak intensity at the start of the experiments (20 °C) which was well above the phase separating temperature, meaning that this sample had initially high-level of self-associated protein. Addition of 100 mM Arg-Glu to this sample dramatically increased the peak intensities with sharper peaks, almost twice higher than those achieved in the presence of Arg-HCl, indicating Arg-Glu was preventing self-association and increasing monomeric species in solution, and more efficiently than Arg-HCl. This correlated with Arg-Glu preventing LLPS for this salt-containing sample. A recent study has investigated the effect of excipients on LLPS to see whether a number of already established excipients (PEG 400, Tween 80, sucrose and hydroxypropyl β-cyclodextrin) could prevent LLPS. They found only sucrose and hydroxypropyl β-cyclodextrin was able to reduce LLPS (Raut and Kalonia 2016a). Thus, the ability of Arg-Glu to also prevent opalescence and LLPS is attractive due to the scarce information on excipients which can prevent LLPS. Monitoring changes in the signal intensities can be used as an informative indicator for formulations which may be prone to phase separation particularly useful in early drug development when there is sample limitation and a large amount of potential lead molecules (Chow et al. 2016). Another measureable parameter explored in this Chapter was the translational diffusion coefficients; however it was not possible to measure mAb signals due to the decay of the signals during the experiments, thus $D$ of the buffer constituents only was measured at decreasing temperatures. It is expected that $D$ for these small molecules in the buffer would not be affected by protein self-association as if aggregation were to happen there would be more space for the buffer molecules to diffuse. mAb5 formulated with 30 mM NaCl in the presence of Arg-Glu or Arg-HCl displayed superimposable trends with the diffusion being much faster in comparison to the sample which went on to phase separate showing the slowest diffusion. This clearly indicates that these excipients are preventing protein self-association thus making $D$ faster. From the Stokes-
Einstein equation it was possible to calculate the solution viscosity using $D$, the results showed there was no change in microscopic viscosities in comparison to the expected changes in water viscosity at each temperature.

The phase separated sample was further studied to independently characterise the association state of the protein in each phase. The expected mAb concentrations were found in both phases with the lean phase being much less concentrated (8.6 mg/mL) than the rich (114.1 mg/mL); these phases displayed different volumes, with 3:1 lean:rich volume ratio. Raut et al also reported a similar split of protein concentrations of ~4 mg/mL in the lean and ~130 mg/mL in the rich phase; moreover, they showed the protein concentrations in the two phases remained the same regardless of the initial concentrations as these phases are in equilibrium with each other (Raut and Kalonia 2015a). Here, 1D $^1$H NMR spectra were acquired from the two phase independently. The lean phase displayed greater signal intensities in comparison to the rich phase despite being less concentrated; this can be explained by the rich phase experiencing much more self-association (and hence having broader peak signals) in comparison to the lean phase. To explore the effects of Arg-Glu in each phase, 100 mM Arg-Glu was added to these phases; this resulted in increased signal intensities (peak at 1 ppm) in the rich phase with the intensities in the lean phase remaining the same. There was not any additional effect from Arg-Glu in the lean phase most probably due to the absence of measurable self-association of mAb5 at this low concentration present in this phase. On the other hand, addition of 100 mM Arg-Glu resulted in a 4-fold increase in the signal intensity in the rich phase, likely due to the reduction in self-association of mAb5 in this concentrated phase. The translational diffusion measurements were informative for the lean phase as differences between the presence and absence of Arg-Glu were seen however in the rich phase it was not possible to discriminate between the presence and absence of Arg-Glu. This can be explained by the increased protein concentration in the rich phase causing crowding effects in addition to the presence of Arg-Glu molecules also adding to this which reduces the ability of the protein molecules to diffuse, these similar observations with $D$ was observed in Chapter 5. It was more useful to measure $D$ for the buffer ion, acetate as these small ions (and other small molecular probes) display strong signal intensities which are easily trackable and generally do not distort the characteristic protein signals in the aliphatic region of the spectra. This ties up well with the findings in Chapter 5 as $D$ was also not a very informative parameter in those studies especially in concentrated solution although monitoring $D$ for the buffer ion, citrate was more informative. The transverse relaxation rate, $R_2$, also strengthened this observation that monitoring acetate ion rather than protein signals (here the buffer ion caused spectral distortion in the protein signals) was more useful. A faster $R_2$ for acetate ion was observed in the lean phase which was expected as the solution is less concentrated hence molecular tumbling is faster in comparison to a slower tumbling in the rich phase. For future experiments, the use of a small molecular probe such as the buffer ion would be favourable in reporting $D$ and $R_2$ of different mAb formulations as these ions usually show strong intensities.
In this Chapter, problems were encountered in measuring $D$ for acetate ion as the signal overlapped with Arg-Glu signals which was difficult to separate through DOSY. An important consideration before selecting the ion would be to ensure there are no overlapping signals from the buffer solution with the ion of interest; this information can be readily found through published literature to check their chemical shift value. Commonly used NMR reference probes are tetramethylsilane (TMS) and 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) as the protons in these compounds are in the same environment thus displaying one single peak in the spectra which is easily recognisable and can be assigned with chemical shift at 0 ppm. The nature of these probes also needs to be considered as these are very sensitive to pH, salt and temperature as well as these may have the tendency to interact with the solvent or possibly stick to the protein (Becker 1959, Wishart et al. 1995).

In conclusion, the incorporation of Arg-Glu in a mAb formulation which otherwise phase separated mitigated any opalescence and LLPS probably due to the reduction in protein self-association; moreover, Arg-HCl was not as effective in reducing opalescence. This Chapter further emphasised the high sensitivity of NMR not only in observing molecular changes in self-association (see Chapter 5) but also it was possible to link events associated with opalescence and LLPS seen in the visual inspections to NMR observable parameters such as signal intensity changes. Thus, the information gathered from NMR could be used as a predictive tool to hypothesis if a particular mAb formulation in early drug development could potentially phase separate. NMR was also able to monitor subtle changes in less stable mAbs as mAb5 used in these studies was more prone to physical instabilities than the mAb (mAb2) used in Chapter 5 which was a highly stable and tolerant mAb. Finally, it was interesting to see the usefulness of monitoring a small molecular probe in the mAb formulations (buffer ion) rather than the mAb signals as these small ions/compounds have sharper and more intense signals; thus observing changes based on probe molecules can reliably report on differences in selecting the 'best' mAb formulations.
Chapter Seven: Discussion and Future direction

The main aim of the Thesis was to assess the ability of Arg-Glu, which previously showed a potential to increase protein solubility (Golovanov et al. 2004), to prevent aggregation and to increase stability and solubility in industrially-relevant mAbs. This was investigated using a number of biophysical techniques, and the potential issues with toxicity of these excipients were investigated in cell culture models.

Chapter 1 provided an Introduction and overview of the field and research techniques used in the study. The work presented in Chapter 3 investigated the effect of Arg-Glu on the conformational and colloidal stability of mAbs as a function of different solution conditions. The conformational stability refers to the propensity of the protein to fold and unfold providing information about the protein conformation whereas the colloidal stability refers to the reversible self-association of the protein molecules. These two stabilities hold a dynamic relationship as the thermodynamic pathways driving unfolding and aggregation can occur simultaneously, thus it is imperative to study these independently to ensure neither of these stabilities are compromised (Blake et al. 2015). The results showed that Arg-Glu markedly increased the colloidal stability of all four chosen mAbs in a concentration-dependent manner, which was particularly noticeable at neutral pH where the mAbs were inherently more colloidal unstable (but more conformationally stable) in the absence of Arg-Glu. The conformational stability studies of these same mAb conditions revealed Arg-Glu did not show any obvious effects on the conformational stability, however, this was not the case with mAb1 where with increasing Arg-Glu concentrations there was a decrease in $T_m$ particularly at pH 7. An explanation for this could be that mAb1 was the least inherently stable which showed noticeable increases in $T_m$. Nevertheless, by simply increasing the pH closer to the pI, this was enough to cause a noticeable increase in $T_m$ for these mAbs. A speculative explanation for this is by increasing or decreasing the pH away from the pI this increases the protein charges causing increased charge repulsion and destabilising the protein. On the other hand, increasing the solution pH towards the pI induces compact aggregate formation due to the reduced protein charges which can be explained by the decrease in the electrostatic barrier (Nicoud et al. 2016). Thus, these combined results indicate that the conformational and colloidal stability are intricately linked and an optimum pH probably exists which minimises monomer depletion but increases colloidal stability. However, the increased conformational stability of the least stable mAb3 could be attributed to this mAb having more chances of facing perturbations in the structure. This Chapter revealed that Arg-Glu can minimise aggregation at a pharmaceutically-acceptable concentration and osmolality range without significantly compromising the conformational stability; thus it would be of interest to understand why Arg-Glu caused conformational destabilisation of mAb1 at pH 7 but colloidal stabilisation. It was initially speculated this may due to differences in the mAbs PTMs; however all the mAbs have the same glycosylation at Asn297. It is well-known that Arg-HCl inhibits protein self-association with little influence on the conformational stability; thus the findings with mAb1...
were somewhat unexpected. Arg-HCl is expected to stabilise the least stable mAbs via its association at the interface of poorly hydrated regions (specifically hydrophobic regions that are transiently exposed to the solvent). The ability of Arg-HCl in stabilising mAbs without causing further unfolding can be partly explained by the guanidine moiety experiencing a larger volume of exclusion (Baynes et al. 2005, Schneider et al. 2011). It is important to note that the ability of Arg-HCl to reduce self-association has been reported generally at concentrations much greater than 0.1 M; for example, one study showed reduced protein aggregation using 0.5 M Arg-HCl (Baynes et al. 2005) whereas another study showed this at 0.15 M (Schneider et al. 2011). Schneider et al. stated Arg-HCl at concentrations below 0.5 M is not strongly bound nor excluded from the protein surface however beyond 0.5 M it is increasingly excluded from the protein surface. Thus, the differences in mAb conformational stability in this Chapter could be due to these varying effects from Arg-HCl (Schneider and Trout 2009). Furthermore, the selected mAb conditions taken forward for accelerated stability studies at concentrations more relevant to biologics emphasised the ability of Arg-Glu to increase the stability of less stable mAbs more than the existing excipient Arg-HCl. Additionally, the stability of the mAbs at pH 7 showed comparable results to mAb conditions at pH 5.5. Thus, the data supports the earlier published work that Arg-Glu can increase the stability of aggregation-prone proteins (Golovanov et al. 2004) and additionally demonstrate that it can be effective for pharmaceutically relevant mAbs. As all the mAb conditions selected for the accelerated stability studies included either Arg-Glu or Arg-HCl, supporting experimental work would include comparing the stability of these exact mAb conditions in the absence of any excipient to make direct comparisons of the degree in the percent monomer retained in the absence and presence of Arg-Glu. Moreover, as mAb3 is expected to have a greater inherent $T_{m1}$ at pH 7 with Arg-Glu than at pH 5.5 as shown in Figure 3.6, it would be beneficial to address this by assessing the long-term stability of mAb3 at pH 7 in comparison to Arg-HCl. A key conclusion from these studies is that Arg-Glu enhances the colloidal stability of mAbs at pH values closer to their pI; additionally, simply increasing the pH towards neutral increases the conformational stability of mAbs. Typically, mAbs are formulated at mildly acidic conditions, however, the findings from this Chapter provides an opportunity to formulate mAbs at pH’s shifted to neutral where the addition of Arg-Glu could potentially provide greater conformational and colloidal stability.

In Chapter 4, toxicity studies were conducted to assess if any toxic or adjuvant-like effects would manifest from the combination of Arg-Glu at increasing osmolalities compared to the reference salt NaCl (positive control), L-Arg-HCl and NaGlu using in vitro cell culture systems. The isotonicity of the parenteral formulation with the human plasma is very important so as to avoid tissue damage during the route of administration (Mehmood and Farooq 2015). Adherent and nonadherent cell lines showed very similar toxicity profiles for Arg-Glu with decreases in cell viability that were comparable with NaCl. The greatest loss in cell viability was observed with Arg-HCl; it was hypothesised this was due to NO release however this effect could not be reconciled experimentally to this; nevertheless, the equimolar mixture with
L-Glu clearly ameliorated the toxicity. Arginine is commonly used as a therapeutic oral supplement due to its crucial role in the synthesis of NO, urea and creatine thus useful in treating insufficiencies in these products. L-Arg is usually given in supplements as the Cl\(^-\) salt or other salt forms including pyroglutamate; studies have reported the use of Cl\(^-\) may itself be deleterious as Arg-HCl in excess can provoke a hyperchloremic acidosis. Another speculative reason for Arg-HCl causing greater loss in cell viability could be attributed to the presence of the Cl\(^-\) salt (Grimble 2007). Interestingly, it has been reported glutamine and glutamate are closely associated with intestinal arginine metabolism which may explain why the addition of Glu restores the loss in viability (Reeds et al. 2000). Moreover, the effect of NaCl at hypertonic concentrations showed a very similar cell death mechanism to Arg-Glu which was via apoptosis. This was an important finding as this indicated there would not be a pro-inflammatory response which was further confirmed by lack of effects of Arg-Glu on markers for cellular stress or activation. This is a very promising result as Arg-Glu did not display any signs of adjuvant-like effects which may stimulate an inflammatory response (Schijns 2000, Allison and Byars 1991). Therefore, it was possible to conclude Arg-Glu would most likely not have any further detrimental effects on cell viability beyond what was seen with the already established excipient NaCl; thus it was found that the loss in cell viability is solely due to changes in osmolality. For future work, the in vivo cellular responses would need to be investigated, as immediately after administration the drug formulation is dispersed and exchanged in to the bodies’ physiological medium which may impart other biological consequences. The FDA requires nonclinical (animal) and clinical (human) studies for any novel excipients even if the excipient has been used before in an alternative route of administration. In vivo challenges have been previously reported for example with sorbitol and PEG as excipients in oral drugs where PEG 400 lowered drug bioavailability (Mei-Ling Chen et al. 2013).

It was discussed in Chapter 4, that Arg-Glu itself is already an approved drug (Glutargin) in human use for liver therapy (Degtyaryova et al. 2012). Glutargin has been stated to have distinct hepatoprotective, detoxifying, antioxidant and antihypoxic effects by binding to ammonia in the blood converting it into a non-toxic compound – glutamine (HealthTips 2016). Based on the already approved human use of Glutargin, the incorporation of Arg-Glu as an excipient in mAb formulations has some precedence.

Another aspect which was addressed in these toxicity studies was the ability of Arg-Glu to reduce aggregation in upstream processes of protein production rather than only in the downstream areas which was the main focus in this Thesis. This part of the work was a focussed narrow study. Protein aggregation is commonly experienced during protein expression and purification thus we wanted to assess if Arg-Glu could be beneficial in preventing aggregation in upstream processes. HEK cells were used as a model system for their ability to secrete proteins into the cell culture medium similarly to how mAbs are generally produced. Arg-Glu was incorporated at two different aggregation-prone stages; during the
entire protein expression and purification stages or only at the latter stage. These specific stages have been clearly displayed in Figure 2.1 from the protein expression to the elution stage.

Although the presence of Arg-Glu from the protein expression stage caused some disruptions in the cell density observed from cell morphology studies, the light scattering data generated from MALS clearly showed a decrease in the presence of high molecular weight species with the addition of Arg-Glu during the entire protein expression and purification stages and the addition only at the purification stage with a more pronounced decrease with Arg-Glu added from the protein expression stage. Moreover, the percentage mass of protein retained in each condition was markedly increased in the presence of only 50 mM Arg-Glu. On a larger scale, this excipient concentration may not be economically viable in these upstream processes thus lower Arg-Glu concentrations could also be investigated. More in depth experimental work would be required with the HEK cells by carrying out cell viability assessment (similarly to the two cell lines used in the initial part of Chapter 4) in parallel with measurements of protein yield and aggregation in order to optimise conditions. The data obtained in this Chapter represents a step forward in gathering the toxicity data required for novel additives to be included in the GRAS category (Pifferi and Restani 2003, Ogaji et al. 2011).

In Chapter 5, solution NMR spectroscopy studies were conducted to assess if this technique could be a suitable method for monitoring mAbs self-association and if so whether this approach could be used as an orthogonal technique in mAb formulation screening. Several key observations were drawn from Chapter 5. First, the Chapter revealed the utility of NMR as an orthogonal method in mAb formulation screening, particularly at high concentrations. The acquisition of good quality NMR spectra depends on samples that are stable and show minimal aggregation, self-association and viscosity. This ideally complements the requirements in mAb formulations, where an optimal formulation for clinical use depends on these parameters. From 1D $^1$H spectra, it was possible to monitor changes in signal intensities of characteristic protein signals in the methyl region to assess the stability and aggregation state of mAbs in different formulations. Literature have reported the use of NMR as a biophysical technique to study self-assembly by observing changes in the signal intensities; the signal intensities of monomers decreased due to self-assembly to NMR-invisible complexes (extremely broad signal beyond detection) (Sparks et al. 2012). A useful observable parameter was the increase in the signal intensities with the addition of Arg-Glu corresponding to an increase in the monomeric fraction of protein in solution; this simple observation can be used as a criterion in the development of optimal mAb formulations. Furthermore, the temperature-dependent experiments showed that the derived parameter, the relative signal intensity $L_{Ni}^N$ (normalized signal integral corrected by the viscosity change due to Arg-Glu addition) can report on both the conformational and colloidal stability of mAbs.

The diffusion coefficient $D$ was measured using DOSY reporting on the translational motion of the mAb in solution; this parameter measured in different formulations turned out not to be
very informative and not sensitive to formulation conditions. It is well-known from the literature that protein-self diffusion is hampered in crowded environments in concentrated solutions or crowding buffer components (Price et al. 1999, Price 2009, Balbo et al. 2013). However, the molecular tumbling rate, linked with the transverse relaxation rate $R_2$, was a useful reporter of the rotational dynamics of mAbs in solution which did not suffer from this ‘crowding’ effect (Lee et al. 2006). These parameters successfully paralleled the reduction in aggregation caused by Arg-Glu in a range of mAb conditions; particularly the signal intensities markedly increased indicating more monomer species remaining in solution (broader peaks would be observed for aggregation). For future work it would be interesting to measure $D$ of these same mAb conditions using DLS as a complementary tool. DLS is the frequently used technique in formulation to determine $R_h$. These measurements can be compared to the data generated via NMR. Additionally, it would be of interest to characterise the protein interactions by utilising dilute solution properties to measure intermolecular parameters, namely $B_{22}$ and $k_d$ measured by SLS and DLS respectively. $B_{22}$ is the best measure of weak interactions and is the purest mathematically derived parameter, albeit, it is also the most difficult and time consuming parameter to measure; a negative $B_{22}$ indicates attractive PPI interactions whereas a positive $B_{22}$ relate to repulsive interactions. A number of studies have used $B_{22}$ as a measure of colloidal stability and a potential predictor of protein aggregation (Shi et al. 2013, Quigley and Williams 2015). $k_d$ is derived from the slope of the linear relationship between the diffusion coefficient vs concentration and is another parameter found to be useful in understanding weak PPIs with some studies reporting that $k_d$ may replace $B_{22}$ in formulation screening due to the ability of measuring this parameter on a DLS plate reader in a high-throughput manner (Shi et al. 2013, Blake et al. 2015). Moreover, $k_d$ has been used to predict the solution viscosity and stability for highly concentrated protein samples (Connolly et al. 2012). It would be interesting to observe if the NMR derived $D$ correlate with DLS derived measurements; moreover as $k_d$ is determined from $D$ this additional parameter can be determined reporting on the type of intermolecular interactions (i.e. colloidal stability). This can complement the findings not only in this Chapter but also from Chapter 3. However, it is important to note that these measurements are usually made in dilute protein solutions which may not be directly predictive of what will happen at high concentrations (Blanco et al. 2014, Roberts et al. 2015).

The long-term stability studies conducted using NMR and the NMR-derived relative solution viscosities agreed well with the parallel comparison to the gold standard technique SE-HPLC and viscometer. Moreover, NMR was able to report on deuterium exchange rates from the proteins aromatic region which can be affected by solution viscosity. This was indeed the case as the increase in solution viscosity during the stability studies did account for the faster decay seen from the NMR data, compared with SE-HPLC. Apart from not taking into account the increase of formulation viscosity with time, analysis of the samples by SE-HPLC also required dilution, which may have led to overestimation of the fraction of monomers present in solution after storage. Thus, NMR provided a valuable additional criterion reporting on the monomeric state of the protein in situ, at high concentration, as well as the solution viscosity. The mAb
(mAb2) used in these studies was inherently very stable, for future work it would be ideal to use a more challenging mAb (i.e. unstable and aggregation-prone) to observe if this technique will perform better or worse at pulling out differences in formulation conditions. Hence, future work could explore a greater array of mAb formulations in a higher throughput manner. The number of mAb formulations susceptible to instabilities and aggregation is on a rise with an abundant amount of literature expressing this issue (Goswami 2013, Obrezanova et al. 2015, Pindrus et al. 2015). In light of this, the work conducted in this Thesis has great importance as further knowledge in this area is crucial to tackle these problems.

In Chapter 5, the work presented focussed on investigating the effect of Arg·Glu on LLPS and opalescence and exploring further the use of NMR in providing new information on these phenomena. Opalescence is another common problem associated with high mAb concentrations as this may initiate (not always) phase separation or indicates the presence of aggregates in solution (Raut and Kalonia 2015b). In LLPS, mAb formulations may separate into two concomitant phases of different concentrations resulting in an inhomogeneous solution which is also an unwanted attribute in the final product (Wang et al. 2011). We chose to analyse the sparsely-reported phenomenon of LLPS (Nishi et al. 2010, Wang et al. 2011, Ying Wang et al. 2014). The visual inspections clearly showed an increase in opalescence leading to phase separation in the control mAb5 samples with increasing ionic strength. Addition of increasing Arg·Glu concentrations reduced opalescence and LLPS, with noticeable reductions at concentrations as low as 50 mM which was completely mitigated at 200 mM. This was further supported by the increase in the relative solubility determined by the PEG-induced precipitation assay of mAb5 samples containing Arg-Glu. NMR was employed to understand these findings by determining the molecular tumbling rate changes upon self-association. The mAb5 sample in the formulation which showed LLPS displayed the lowest signal intensities at temperatures where LLPS was not yet expected, indicating the inherent tendency to self-associate in this formulation. The sample additionally containing Arg-Glu showed greatest signal intensities, followed by the sample containing Arg-HCl which was not as remarkable as Arg-Glu. Collectively, these results suggest Arg-Glu reduced protein self-association more effectively than Arg-HCl. This difference between Arg-Glu and Arg-HCl can somewhat be explained by molecular dynamic studies by Shukla and Trout (2011) (Shukla and Trout 2011). The authors proposed the molecular mechanism of Arg-Glu was via hydrogen bonding between Arg and Glu promotes the increase of these ions on the protein surface, enhancing the crowding effects hence preventing self-association (Shukla and Trout 2011). In these same studies Arg alone was shown to preferentially bind to the protein surface at a much higher degree than Glu alone, however, in the equimolar mixture this increase is probably due to the synergistic enhancement of the local concentration of these ions (Shukla and Trout 2011). These promising findings revealed by NMR have shown the potential for this technique to be used as a predictive tool in making decisions if a particularly mAb or formulation may be prone to opalescence or LLPS. Moreover, the molecular differences in the protein-lean and -rich phases were studied as a function of signal intensity change, which
showed that the addition of Arg·Glu dramatically increased the signal intensity by 4-fold in the rich phase, reducing protein self-association in this phase. By monitoring diffusion rates of small molecules in the buffer vs temperature, the NMR experiments also showed that the LLPS transition was not accompanied by any increase in the microscopic solution viscosity. This is an interesting observation as although the correlation between phase separation and viscosity is still not clearly understood, both these phenomena are caused by PPIs (Chow et al. 2016).

In conclusion, the findings in this Thesis support the initial aims. A complementary panel of analytical techniques provided robust evidence that the addition of Arg·Glu increases mAb stability, solubility and minimise aggregation and self-association under the conditions studied. Furthermore, demonstrating in vitro the non-toxic effects of Arg·Glu is a step towards the accumulation of data that could support a justification (in a regulatory context) for its use as a stabilising excipient in an injectable mAb product. Arg·Glu is therefore an excellent example of using alternative salt forms of GRAS-listed excipients to broaden the ‘molecular toolbox’ available to the formulation scientist. These emerging excipients may well meet the formulation needs associated with demands for increasing solution concentrations and non-mAb protein formats in the industry.
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