A Study of the Practicality of Cell-Free Protein Synthesis for the Commercial Manufacture of Monoclonal Antibodies

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Title: A Study of the Practicality of Cell-Free Protein Synthesis for the Commercial Manufacture of Monoclonal Antibodies

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Declaration:

"I hereby declare that this project is entirely my own work and that it has not been submitted for any other academic award, or part thereof, at this or any other education establishment".

Signed:---------------------

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Abstract

Background: Monoclonal antibodies are tetrameric proteins with a complex folded structure, bound together by numerous disulfide bonds, and decorated with a functionally important oligosaccharide. Current production methods rely on recombinant cell cultures.
Aims: The aim of this project was to highlight the benefits of a transition to cell-free manufacturing of monoclonal antibodies, to identify the technological gaps which prevent this transition, to suggest possible solutions, and to critically assess the state of development of these new technologies with a view to suggesting areas for further research.
Method: A literature review was performed based on a list of pre-defined search terms. The information was grouped into themes aligned with the aims.
Results: Cell-free protein synthesis has been used in research for many years, and it is apparent that the technological foundations exist for large-scale cell-free protein manufacture, boosted by recent advances in understanding of folding mechanisms and disulfide bonding. Nevertheless, the complex glycosylation of monoclonal antibodies cannot yet be performed *in vitro*. In fact, even current cell culture techniques cannot produce a pure glycoprotein. Counterintuitively. this is a strong argument for developing cell-free production technologies as these are less complex, are easier to control, and are more amenable to alternative technologies for glycosylation. Three emerging technologies were identified as possible solutions, each of which may be capable of attaching a pre-formed, pure oligosaccharide to the non-glycosylated monoclonal antibodies that are currently produced in cell-free systems: gene code expansion, antibody-drug conjugation techniques, and glycoengineering.
Conclusion: The results are significant as they indicate that cell-free techniques may soon become the predominant platform for the production of mono-clonal antibodies, bringing financial and societal benefits, as well as faster development timelines. A shift to pure glycoforms could mean a much faster introduction of biosimilars, or even their replacement by identical biogenerics.

Keywords: monoclonal antibody, cell-free protein manufacture, gene code expansion, synthetic glycobiology, glycoengineering, antibody-drug conjugation

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List of Abbreviations & Acronyms

AaNGT	*Aggregatibacter aphrophilus N*-glycosyltansferase
aaRS	mRNA aminoacyl synthetases
ADC	Antibody-drug conjugates
ADCC	Antibody-dependent cellular cytotoxicity
ApNGT	*Actinobacillus pleuropneumoniae N*-glycosyltansferase
Asn	Asparagine
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
BiP	Binding protein
Cas9	CRISPR-associated protein 9
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-defining regions
CECF	Continuous exchange cell-free
CFCF	Continuous flow cell-free
CFPS	Cell-free protein synthesis
CH	Constant (heavy)
CHO	Chinese hamster ovary
CL	Constant (light)
CRISPR	Clustered regularly interspaced short palindromic repeat
Cys	Cysteine
DARPin	Designed ankyrin repeat protein
DHA	Dehydroascorbate
DMC	2-chloro-1,3-dimethylimidazolinium chloride
DNA	Deoxyribonucleic Acid
DSB	Disulfide bond
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
Endo-F3	Endoglycosidase F3
EndoM	N175Q mutant of *endo*-β-*N*-acetylglucosamidase
EndoS	ENGase mutant from *Streptococcus pyogenes*
ENGase	Endoglycosidase
ER	Endoplasmic reticulum
Fab	Fragment (antibody)
Fc	Fragment (crystallisable)
GalNAcα-Thr	α-N-acetylgalactosaminyl-L-threonine
GCE	Genetic code expansion
GH	Glycosyl hydrolases
GlcNAc	*N*-Acetylglucosamine
GlmAse	glucosamine-*N*-acetyltransferase
Gly	Glycine
GT	Glycosyltransferase
HC	Heavy chain

HILIC	Hydrophilic interaction chromatography
Hsp	Heat shock protein
ICH	International Conference on Harmonisation
Ig	Immunoglobulin
IgG	Immunoglobulin G
IRES	Internal ribosome entry sites
LC	Light chain
Lin	Linker
mAb	monoclonal antibody
mRNA	Messenger ribonucleic acid
mTGase	Microbial transglutaminase
NGT	*N*-glycosyltansferase
nnAA	Non-natural amino acid
NTP	Nucleoside triphosphates
o-aaRS	Orthogonal mRNA aminoacyl synthetases
OST	Oligosaccharide transferase
PDI	Protein disulfide isomerases
PEP	Phosphoenol pyruvate
ppGT	Polypeptide-modifying glycosyl transferases
PTM	Post-translational modification
PURE	Protein Synthesis Using Recombinant Elements
Pyl	Pyrrolysine
QbD	Quality by design
R&D	Research & development
RF1	Release factor 1
RNAP	Ribonucleic acid polymerase
RNAse	Ribonuclease
rRNA	Ribosomal RNA
scFV	Single-chain variable fragments
Sec	Selenocysteine
Ser	Serine
SGP	Sialoglycopeptide
SPAAC	Strain-promoted alkyne-azide cycloaddition
TALEN	Transcription activator-like effector nuclease
TGase	Transglutaminase
TIR	Translation initiation region
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
UCOE	Ubiquitous chromatin opening elements
UDP-Glc	Uridine diphosphate glucose
UDP-GlcNAc	Uridine diphosphate *N*-acetylglucosamin
UDP-GT	Uridine diphosphoglucosyltransferase
Und-PP	Undecaprenyl-pyrophosphate

VH	Variable (heavy)
VL	Variable (light)
ZFN	Zinc-finger nuclease

1. Introduction

Monoclonal antibodies make up a large proportion of current blockbuster drugs, and their exceptional selectivity has allowed them to provide life-saving treatments in areas such as oncology and autoimmune diseases. They are structurally complex protein molecules which must be manufactured by whole-cell cultures under stringently controlled conditions in order to achieve the required quality specifications. Manufacturing techniques have been optimised over recent decades to provide a reliable production platform, and the process is relatively mature.

However, this approach has disadvantages. The use of live mammalian cells means that the process must cater to all the metabolic needs of the cell, not just those associated with the production of the desired protein. A new cell needs to be genetically engineered for each new monoclonal antibody product. Also, living cells are extremely complex and sensitive systems, so the level of control over processing conditions must be very high, as even slight variations can lead to changes in the product quality. This is especially true for co- and post-translation modifications, which are chemical and/or structural changes to the amino acids in the protein. These are introduced by a range of enzymatic processes in the cell.

Thesis Statement: *A transition to the cell-free manufacture of monoclonal antibodies would deliver a simpler, better understood biosynthetic system with more control over quality, and although the lack of a practical technique to attach oligosaccharides selectively at the asparagine-297 position is a significant barrier, developments in genetic code expansion may offer solutions in the future, as well as recent developments in glycobiology.*

The most complex post-translation modification is the addition of an oligomeric sugar molecule, or oligosaccharide. This is critical to the biological function of many monoclonal antibodies; however, all cell-based production processes generate a mixture of so-called glycoforms. For each product, a particular mixture of glycoforms undergoes clinical trials, and then the same mixture is then commercialised.

Although this is an acceptable situation, it is not ideal as different glycoforms can have differing biological impacts. A better approach would be to develop a manufacturing process that delivered a single glycoform. This would have the additional societal benefit of allowing biosimilar manufacturers to enter the market more easily, as they would be also to use the same technology to make a single identical molecule, rather than trying to adjust to the complex mixture from the originator.

Cell-free systems for the synthesis of a range of proteins have existed for decades, though mostly on a small research scale. This system relies on the lysis of the desired production organism's cells, followed by some limited purification, to provide the essential biological machinery of protein production. Functionality is maintained and such lysates will produce proteins under the appropriate conditions despite the lack of a living cell. The advantage of this system is its simplicity. Only the required cellular components are retained, and the inputs and controls are solely dedicated to protein production. In principle, the same lysate can be used to manufacture any protein by just changing the DNA or mRNA input.

Although a transition to the cell-free manufacture of monoclonal antibodies would deliver a simpler, better understood biosynthetic system with more control over quality, this has not yet been achieved for any monoclonal antibody. The research problem which is addressed in this dissertation is to examine the reasons for this, to identify what areas of research might be taken forward to close any gaps, and to highlight what the benefits might be for the commercial production of monoclonal antibodies.

This dissertation is divided into the following sections. Firstly, the technical and societal context of the work has been described, as well as the value of this work. Secondly, a detailed description of antibody biosynthesis has been provided, with the purpose of identifying all structural and quality aspects which would need to be achieved by cell-free system. Thirdly, a detailed literature review of the area of cell-free protein synthesis has been presented, with a focus on how this relates to monoclonal antibodies. Finally, the current limitations and drawbacks of cell-free production of monoclonal antibodies have been identified, as well as the improvements which are required, together with suggestions for possible solutions and future areas of work. Gene code expansion was identified as a key technology in this regard.

The importance of this work lies in the fact that it draws together research findings from a range of disciplines in the areas of chemistry, biochemistry, molecular biology, genetics, and pharmaceutical manufacture to identify gaps and opportunities for future work, whilst highlighting the benefits of cell-free manufacturing for monoclonal antibodies.

2. Context: Importance of Biopharmaceuticals

The purpose of this section twofold. Firstly, it provides some background on the basic technologies and the societal importance of the biopharmaceutical industry, and monoclonal antibodies (mAbs) in particular. Secondly it identifies the main disadvantages of the current cell-based manufacturing platforms, thereby justifying efforts to identify alternative cell-free approaches and underscoring the importance of this work.

2.1. Technical Background

Antibodies represent an ever-growing sector of the pharmaceutical market, and continue to offer novel treatments for disease (Kaplon and Reichert, 2021). Newer, related biologics such as antibody fragments (Nelson, 2010) and DARPins (Stumpp *et al.*, 2008) are expanding the possibilities for treatments even further. These recent advances in disease treatment have been underpinned by dramatic increases in our understanding of cell and molecular biology. Headline-grabbing advances have included the mapping of the human genome, cloning, the harnessing of stem cells, transgenic organisms, and gene editing. However, a great number of other developments have been pivotal to the successful manufacture of therapeutic proteins, such as powerful display and selection platforms, exquisitely powerful analytical techniques, high-titre cultures, and efficient purification techniques.

Such research has allowed scientists to understand the function of cells, and together with the advancing areas of genomics and proteomics, to uncover in ever greater detail the molecular origins of disease. It also provides the theoretical and practical framework for the use of whole cells to provide therapeutics.

The harnessing of the living cell for protein synthesis forms the basis of our modern biopharmaceutical industry. The biopharmaceutical industry can now reliably add foreign DNA to the genome of a cell and coax it into producing a protein that has been identified by scientists as a therapeutic molecule. This involves the manipulation and control of extremely complex cellular processes, but we can now reliably manufacture biopharmaceuticals on a large scale.

2.2. Societal Benefit

The discovery, development and commercialisation of biopharmaceuticals have been a great success story in the treatment of human disease. This is particularly true for monoclonal antibodies, which are now routinely used to treat conditions for which there were very few options previously. The most common indications are cancer, inflammatory & autoimmune diseases, haemophilia and diabetes (Walsh, 2018). The future also looks bright with many of the top-selling drugs being antibodies (Table 1) and continued strong sales growth predicted (Walsh, 2018).

Table 1: World's Top-selling Antibodies in 2020 (Sagonowsky, 2021)

Brand Name	Generic Name	Therapeutic Area	Vendor	Global Sales/bn (2020)
Humira	Adalimumab	Autoimmune diseases	AbbVie	$20.4
Keytruda	Pembrolizumab	Oncology	Merck & Co.	$14.4
Stelara	Ustekinumab	Autoimmune diseases	Johnson & Johnson	$7.9
Opdivo	Nivolumab	Oncology	Bristol Myers Squibb	$7.9
Avastin	Bevacizumab	Oncology	Roche	$5.3
Ocrevus	Ocrelizumab	Multiple sclerosis	Roche	$4.6
Rituxan	Rituximab	Autoimmune diseases & Oncology	Roche	$4.5
Remicade	Infliximab	Autoimmune diseases	Johnson & Johnson	$4.2

2.3. Limitations of Whole-Cell Approach: Cell Line Development

Commercial biopharmaceutical processes use live whole-cell cultures to produce antibodies, with mammalian cell lines such as the Chinese hamster ovary (CHO) cell now predominating (Walsh, 2018). Culturing entails the suspension of suitably modified, genetically engineered (recombinant) cells in a nutrient-rich solution under strictly controlled conditions, allowing them to grow and multiply, all the while synthesising the desired biomolecule. After the maximum yield of the product is obtained, most cell

cultures are then terminated, and the product harvested using complex filtration and purification steps.

However, there are significant disadvantages associated with the use of whole cells to express biopharmaceuticals. Cell-line development and clone selection is a time-consuming process which is required to create, optimise and select a suitable production clone (Le *et al.*, 2015). The key process to enable cells to produce or express a protein of interest is transfection, whereby foreign genetic material is introduced into a cell (Chong *et al.*, 2021). Chemical, physical, or biological methods may be used, however there is a low success rate, and many clones need to be screened to find one with stable, efficient expression. Newer technologies, such as the ubiquitous chromatin opening elements (UCOE) expression system, are leading to improved success rates in the identification of high-producer clones (Nair *et al.*, 2011), however significant resource is still required.

This lack of precision and predictability means that cells must then be screened to find high-producing clones. This process is called 'amplification' and is used to select high-producing cells for further optimisation (Kingston *et al.*, 2002).

This process eventually delivers a selection of suitably stable, fast-growing, efficient production clones, from which a final production clone is selected based on their suitability under commercial manufacturing conditions. Many aspects of cell metabolism must then be optimised, such as growth profile, propensity for apoptosis, levels of antibody secretion, glycosylation pattern, and genetic stability (Zhu, 2012). This is generally done by directly measuring antibody production levels using an analytical method such as enzyme-linked immunosorbent assay (ELISA). The biological activity of the antibody is also confirmed at this stage.

These selection and optimisation steps are due to the unpredictable nature of the transfection process and require a lot of resource and effort. This adds to the cost and timelines in the development phase.

2.4. Limitations of Whole-Cell Approach: Manufacture

Over the last few decades, the stability and productivity of commercially relevant host cells has been improved. Directed evolution and genetic & metabolic engineering of producer clones have been used to decrease programmed cell death, reduce lactate accumulation, control glycosylation patterns, and manipulate cell growth (Li *et al.*, 2010, Zhu, 2012).

The biggest advantage of the current predominant use of murine cell lines such as CHO and non-secreting murine myeloma (NS0) cells for antibody expression is their ability to produce large complex proteins with post-translation modification (PTM) profiles which are generally well tolerated by humans. However, there can still be some immunogenicity since murine glycosylation patterns are not exactly the same as in humans. This has led to increased interest in newer cell lines such as human embryonic kidney cells (HEK293), and the retina-derived human cell-line PER.C6 (Zhang, 2010), which only produce human PTMs.

An alternative approach is to use targeted gene editing tools (Fischer *et al.*, 2015) such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) to genetically modify non-human host cells to express the desired human glycosylation patterns (Felberbaum, 2015, Aumiller *et al.*, 2003).

However, despite these improvements, cell culture conditions still need to be very tightly controlled, and even relatively small deviations can lead to an impact on product quality, especially modifications such as glycosylation patters, disulfide linkages and folding. All living cells are extremely complex, and their behaviour is highly sensitive to a wide range of parameters such as pH, osmolality, temperature, and the concentrations of nutrients, oxygen, carbon dioxide, and waste products.

Efficient, but low-shear mixing is a critical requirement for bioreactors, though perfusion reactors approach this issue somewhat differently. The impurity profile also depends on the health of the culture itself, especially cell density and viability.

There are also intra-cellular interactions, the greatest implication of which is the need to passage cell cultures into ever larger vessels, rather than using a single large vessel from the start of the culturing process.

Even with the use of high-throughput experimental techniques, the need to control all these interdependent factors means that a great deal of development work is required to explore and optimise the cell culture medium, feeding strategy and conditions. However, this is vital to understand the variability of product quality throughout the process design space, so-called quality by design (QbD). This allows comprehensive bioprocess modelling and the development of a robust process control strategy.

Even once the desired conditions are identified during development, it is a challenge to control all such factors in a bioreactor at the level required. Even low levels of variability or inhomogeneity in these parameters during manufacturing can influence production quality and reproducibility (Andersen *et al.*, 2000, Restelli *et al.*, 2006). There can be significant local inhomogeneity in hot spots, eddies, and infeed entry points where there may be variations in the temperature, pH, concentration of oxygen, nutrients, or waste products. Cells passing through such an area will be subject to a change in their local environment, and this is regarded as detrimental to cell performance (Guillardand and Trägårdh, 1999). Unfortunately increasing the agitation will subject the cells to higher shear, so the options for addressing this issue are limited.

After the product has been successfully produced, it must be isolated from the cell culture. Cell death and lysis during culturing and harvesting releases proteases and glycosidases which begin to degrade the product, making the procedure time-sensitive (Chee Furng Wong *et al.*, 2010). The cell harvest also contains a great number of different cellular proteins, metabolites, nucleic acids, various organelles, and other cellular structures. These contaminants can have significant implications for quality and therefore need to be removed at great expense using sophisticated downstream purification processes (Figure 1). A carefully controlled combination of filtration, centrifugation and chromatography is required to separate out the desired product at the required level of purity.

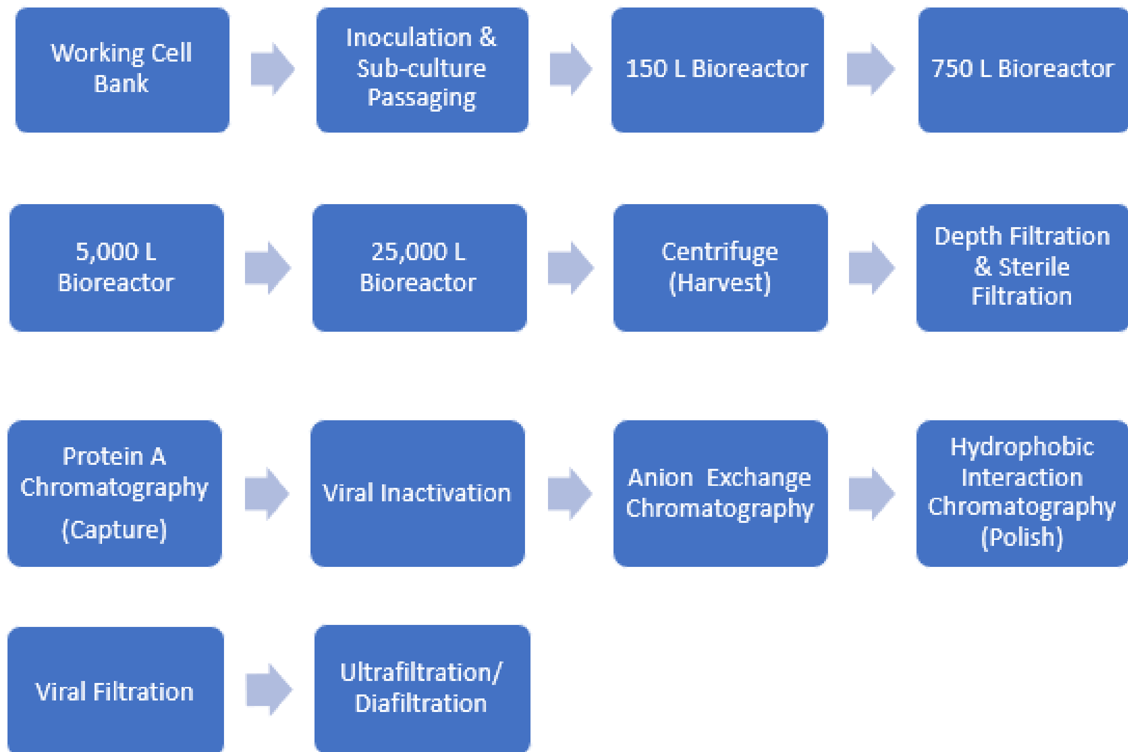

Figure 1: Typical Unit Operations for Drug Substance Production

In addition to the elevated production costs arising from the sensitivity of cell cultures, the cell-culture process itself is inherently wasteful. A significant proportion of the input materials, resource and time is wasted in producing the parts of the cell which are not directly involved in the production of the target biomolecule.

2.5. Financial Pressures

The whole-cell approach is still regarded as the only commercially viable manufacturing process for antibody production, however the need for such tight process control introduces significant costs due to the need for sophisticated equipment, control systems, administration, and oversight. Despite the huge societal and health benefits associated with the advances in the treatment of disease arising from biopharmaceutical innovation, patient access and affordability continues to be an issue for the majority of people in the world (Makurvet, 2021), and there is ever-growing concern of the impact of spiralling cost of biotherapeutics on health systems (Hampson *et al.*, 2019). The introduction of biosimilars was supposed to have a similar cost-reducing effect to small-molecule generics, but this has not been the case to date (Chen *et al.*, 2018).

2.6. Solution Overview

Having highlighted the disadvantages of the current whole-cell approach to biopharmaceutical production and recognising the continued rapid advance in our understanding of cellular biochemical processes, there is a need to look critically at the current orthodoxy in the biopharmaceutical industry to understand whether there are fundamentally simpler approaches to antibody synthesis, and what the implications of a genuine paradigm shift might be.

Therefore, there is a need to address the technical limitations and drawbacks associated with antibody production, particularly in order to reduce costs, while maintaining or improving quality. One way to address the weaknesses in the whole-cell approach is to use cell-free techniques, or even synthetic biology. The aim here is to strip out the myriad biochemical processes which are associated with the maintenance of the cell and life itself, and just to use the biochemical pathways and enzymes which are required to produce the target protein. This approach would remove a lot of variability and cellular complexity, reduce waste, and greatly simplify the development and execution of biopharmaceutical manufacturing.

2.7. Value of this Work

Many challenges remain before cell-free manufacture of monoclonal antibodies is routine at a commercial scale, however the idea itself is not new, and several new enabling technologies are currently converging to make this a real possibility. The importance of this work lies in the identification of technologies which can be applied to the cell-free production of biopharmaceuticals, reviewing their current state of development, and identifying gaps which will require further research to provide solutions to the outstanding gaps which currently prevent progress in this area.

2.8. Materials and Methods

Two systematic reviews of the literature were performed. Both used PubMed as the primary search engine and relied on a list of pre-defined search terms. The information was grouped into themes as indicated by the bulleted points below.

Literature Review #1: Background on mAbs

The aim of the first review was to provide an up-to-date technical background covering the detailed structure-function relationship for mAbs, the therapeutic importance of their structure, and the biosynthetic processes which ensure structural control *in vivo*. The review was not an exhaustive one of all literature in the area due to its great breadth, however it has identified and summarised key papers and reviews to provide the reader with the relevant scientific context.

Title of Review: Control of Antibody Structure in Cellular Environment
Focus Areas: Structure-function relationship, control of folding, control of glycosylation
General Search Terms (Google and PubMed, reviewed first 20 results):

- Antibody structure-function relationship
- Antibody structure function relationship
- Control of antibody folding
- Control of antibody glycosylation

Based on the results of the general search, the following more targeted terms were used
Targeted Search Terms (Google and PubMed, reviewed first 10 results):

- Antibody chaperones
- Formation of disulfide bonds in antibodies
- Proline isomerisation in antibodies
- Small directing helices in antibodies
- Enzymatic coupling of oligosaccharides to antibodies
- Enzymatic coupling of glycans to antibodies
- Glycosylation of Asparagine (Asn) 297 *in vitro*

Literature Review #2: Cell-free mAb Manufacture

The aim of the second review was narrower than the first; to review the literature thoroughly to identify the current state of development of cell-free manufacture, with a view to its applicability to monoclonal antibodies.

Title of Review: Cell-free Manufacture of Antibodies
Focus Areas: Basics of cell-free protein synthesis (CFPS), challenges of applying CFPS to mAbs, current systems for CFPS, technologies which may enable the manufacture of glycosylated mAbs
Search Terms (Google and PubMed, reviewed first 20 results):

- Cell-free Manufacture of proteins
- Cell-free Manufacture of Antibodies
- Bacterial glycoengineering
- Cell-free glycoengineering
- Genetic code expansion
- Click chemistry for derivatisation of mAbs
- Financial aspects of cell-free protein synthesis

The aims were to summarise the current state of development of cell-free technologies in general, and then to identify any papers which address their use in a commercial production setting.

The question to be addressed was what the gaps are between current commercial biopharmaceutical production techniques and expectations, and the current state of development of cell-free protein synthesis and its apparent future direction. The outcome of the review is discussed in chapter 4, with the implications for future research and manufacturing discussed in chapter 5.

3. Background: Antibody Structure & Synthesis

The background section is divided into three parts. The first general part summarises the therapeutically relevant aspects of the structure and function of antibodies, with a focus on the related critical quality requirements for manufacturing.

The second part focuses on the control of folding during production *in vivo*, describing the associated biological quality assurance systems. It then explores recent literature on how this may be achieved *in vitro*, a key challenge for any cell-free system.

The third part similarly focuses on the control of glycosylation during production *in vivo*, describing the imperfect biochemical cellular machinery. It then explores recent advances in chemical and enzymatic methodologies for the construction and conjugation of oligosaccharides to proteins, with a focus mAbs where applicable.

3.1. General

The structure of antibodies, including the folding pattern of the individual domains, has been discussed in several reviews (Padlan, 1977, Amzel and Poljak, 1979, Gilliland *et al.*, 2012, Feige *et al.*, 2010).

As is generally true for all proteins, mAb structure can be divided into four levels (Rüker, 2021). This work focuses on the most therapeutically relevant immunoglobulin, IgG. All immunoglobulins are tetrameric, with two identical heavy chains (H) bound together in a Y-shape, and each heavy chain bound to one of two identical light chains (H_2L_2). The tetramer is stabilised by several inter-chain disulfide bonds (Figure 2).

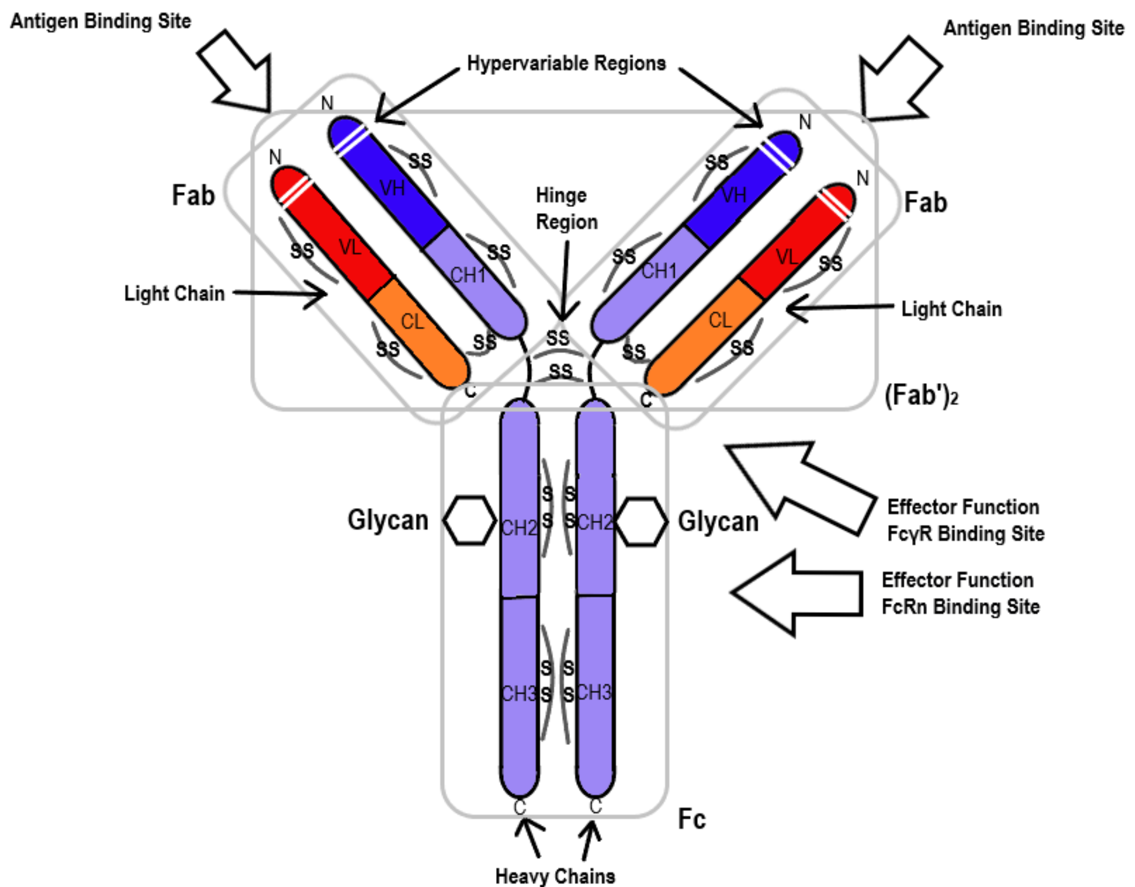

Figure 2: Schematic Representation of IgG Antibody

The primary structure consists of the sequence of amino acids. The secondary structure consists of alpha helices and/or beta pleated sheets, the latter being composed of parallel or antiparallel beta strands stabilised by hydrogen bonding. Individual beta strands also arrange into more complex structures such as barrels. Tertiary structure is maintained by intra-chain disulfide bonds, and quaternary structure is generally mediated by disulfide bonds as well, but between separate amino acid chains.

Both types of chain, heavy and light, are composed of repeating units of tertiary structure called immunoglobulin (Ig) domains, each with approximately 110 amino acid residues. The heavy chains are composed of four such domains, linked by a chain of amino acids. The domains are similar to one another, but not identical. Each H has one variable domain at the antigen binding end (V_H), which differs according to the target antigen, as well as three constant domains (C_H1, C_H2 and C_H3). Each light chain has two domains,

one variable domain (V_L) which forms the antigen binding surface along with V_H, as well as one constant domain (C_L).

The four domains at the antigen-binding amino termini of the antibody are the variable domains, two V_H and two V_L. The constant light domain (C_L) occurs in two variants, kappa (Cκ) and lambda (C_Λ).

Two of the three constant domains on the heavy chain (C_H2 and C_H3) form the stem of the antibody, the Fc fragment. Binding sites located in the Fc fragment allow the antibody to escape natural degradation pathways for a prolonged period, and also mediate the cellular effector functions of the antibody, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Goldberg and Ackerman, 2020). The heavy chain class or isotype (α, δ, ε, γ or μ) determines its effector functions, and defines its classification as either IgA, IgD, IgE, IgG or IgM.

For IgG subclasses IgG1, IgG2, IgG3 and IgG4 there are respectively two, four, eleven and two disulfide bonds between the two heavy chains in the hinge region. For all subclasses, there is one disulfide bond between each light chain and its paired heavy chain, though the location of the anchor point on the heavy chain varies with subclass. These inter-chain linkages constitute the quaternary level of structure (Figure 3).

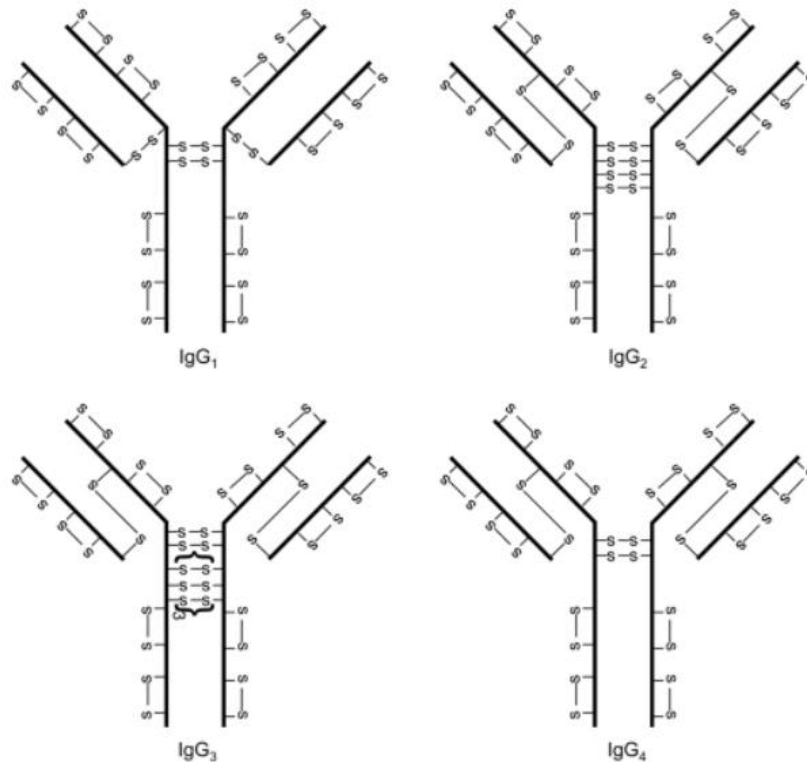

Figure 3: Classical IgG disulfide bond structures (Liu and May, 2012)

3.2. Folding

The core secondary structural feature of all twelve domains is termed the immunoglobulin fold and comprises a sandwich of two beta pleated sheets, rotated through a characteristic beta-sheet angle (Figure 4). For variable domains this consists of nine anti-parallel beta strands. Constant domains have only seven beta strands. Each domain is arranged in a 'Greek key barrel' topology (Rüker, 2021), which resembles a flattened barrel as not all 'staves' (beta strands) are fully hydrogen-bonded to their neighbours. There are either four beta strands in one sheet, ↓A ↑B ↓E ↑D, and five in the other ↓C" ↑C' ↓C ↑F ↓G for variable domains, or else three in the other ↓C↑F↓G for constant domains (Chiu *et al.*, 2019).

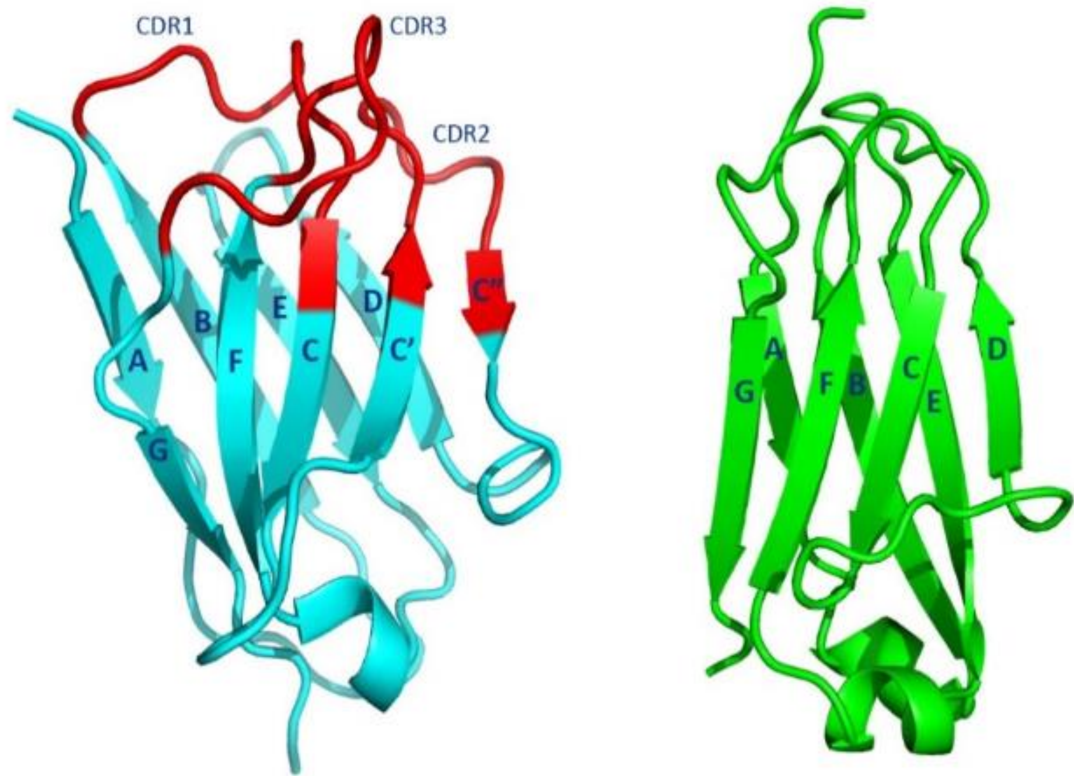

Figure 4: The immunoglobulin fold. VH domain on left with CDRs in red. CL on right (Chiu et al., 2019).

The geometry of these beta pleated sheets results in every second amino acid residue projecting from the opposite side of the sheet to the previous one. Because the interior of the beta pleated sandwich is hydrophobic, this requires every second amino acid to be lipophilic, and therefore gives a characteristic alternating pattern of hydrophobic and hydrophilic residues. This also limits the tolerance of the protein structure to variability, so it is outside this area that diversity in the amino acid sequence can be found, in the free loops which link the ends of each beta strand to the start of the next one. These project as loops from the end of the barrel and are longer in the variable domains to allow sufficient scope for paratope variability in the hypervariable complementarity-defining regions (CDRs).

In addition to the hydrophobic interactions which bind the pair of beta pleated sheets in each domain, there is also a single intra-domain disulfide bond. This stabilises an antibody's tertiary structure.

An antibody's quaternary structure comprises the linkage of the two heavy chains to each other by homodimerisation of the C_H2 and C_H3 domains to form the Fc stem of the

antibody Figure 5. Only the C_H3 domains pack tightly together using non-covalent forces; the C_H2 domains have no observable protein-protein contacts, with the space between them being occupied by the carbohydrate chain attached at Aspagagine-297 (Chiu *et al.*, 2019). The heavy chain dimer has additional stabilisation at the hinge region via interchain disulfide bonds.

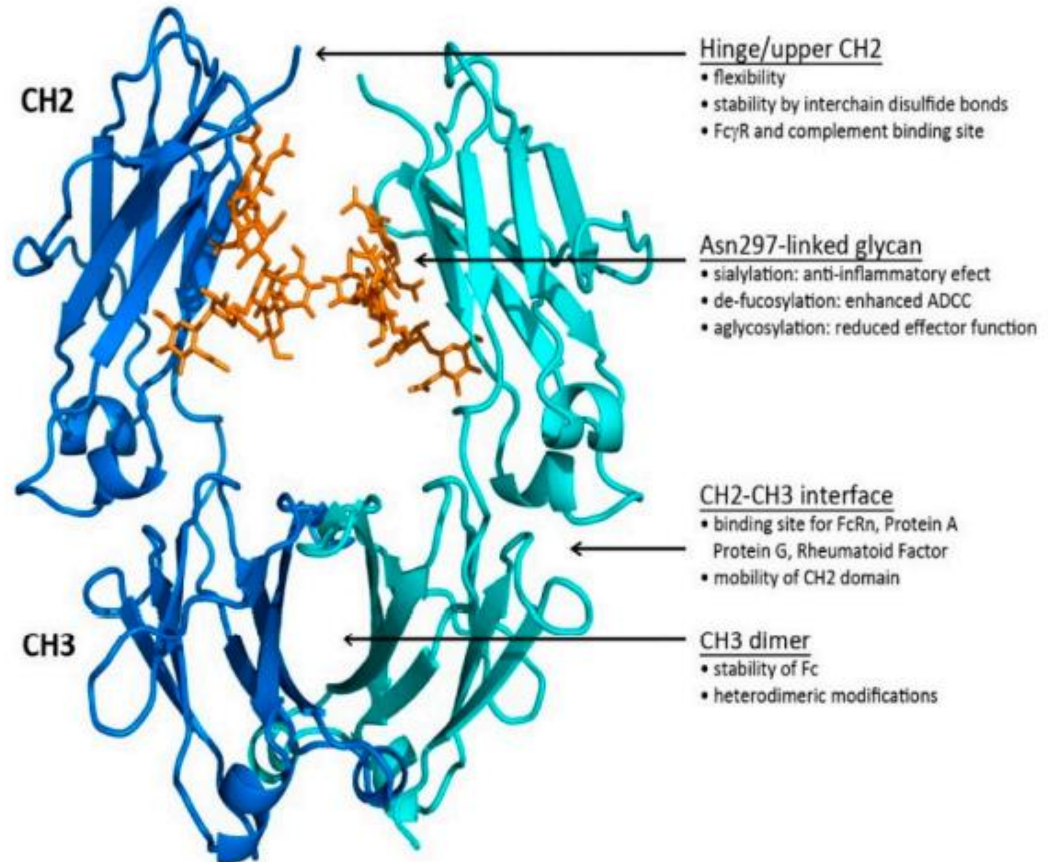

Figure 5: Structure and functionality of human IgG1 Fc (Chiu et al., 2019)

Further disulfide bonds link each light chain to its partner heavy chains. However paired domains (V_H with V_L, C_H1 with C_L, and the two identical C_H3 domains) are also stabilised by interchain interactions between hydrophobic patches a their interface (Wang *et al.*, 2007, Krapp *et al.*, 2003).

3.2.1. Impurities & Post-Translational Modifications

Biopharmaceuticals such as mAbs are not generally isolated pure, but as a complex mixture of similar molecules called isoforms. Point mutations and deletions in the sequence of amino acids occur as the fidelity of transcription and translation is not 100%. Post-translational changes cause heterogeneity in the product, including oxidation of

susceptible chemical groups such as methionine, incorrect glycosylation patterns, loss of terminal amino acids, deamidation, and fragmentation (Jenkins *et al.*, 2008). These transformations occur unavoidably to some extent during cell culturing, purification or storage. In addition, the complex three-dimensional structure of mAbs mean that a proportion of the molecules do not achieve the correct folding and assembly pattern and are prone to agglomeration (Roberts, 2014).

3.2.2. Structural Control *in Vivo*

The correct folding of proteins is a prerequisite for their correct function. Folding is spontaneous and does not require the input of energy because each folding intermediate represents a progression through decreasing energies towards the final stable native state, and this is indeed the case *in vitro* under the appropriate conditions (Feige *et al.*, 2010). In fact, folding processes already start in the exit tunnel of the ribosome (Kudva *et al.*, 2018). The conformational progression towards the native state is thought to follow a funnel-like energy landscape. One of the main driving forces is the association and burial of hydrophobic residues, however despite considerable progress, current models still cannot successfully predict protein folding pathways (Balchin *et al.*, 2020).

However, partially folded intermediates, whether they lie on the correct folding pathway, or are misfolded, can represent kinetic traps on the folding landscape, where slow-folding, hydrophobic intermediates have time to aggregate (Varela *et al.*, 2019). In addition, there is a high risk of unwanted intermolecular interactions with unrelated proteins in the crowded cellular environment which can also lead to aggregation, so molecular chaperones are used *in vivo* to promote the rapid efficient folding of newly synthesised amino acid chains (Dahiya and Buchner, 2019). One of the main mechanisms of chaperone action is to bind to the exposed hydrophobic regions of nascent proteins to prevent aggregation in these vulnerable areas until they are correctly folded into the hydrophobic core of the protein.

Antibodies are assembled in the endoplasmic reticulum (ER) before being secreted via the Golgi apparatus. Each level of structural control must be performed and assured *in vivo*, from the primary amino acid sequence, through formation of beta sheets, the stabilisation of the immunoglobulin fold with disulfide bonds, the formation of the L_2H_2

tetramer, and finally the completion of any post-translational modification, especially glycosylation.

Secondary structure is composed of beta-pleated sheets forming the core structural element, and to a lesser extent alpha-helices, which form early on during the folding of constant domains, thereby directing the subsequent folding stages towards the required native structure (Feige *et al.*, 2008). However, the secondary structure does not form in isolation from subsequent tertiary and quaternary processes; there are complex interdependencies between all these levels.

Tertiary and quaternary structure rely heavily on the formation of the correct disulfide bonds and are critical for protein structure, especially for secreted proteins (Liu and May, 2012). Intradomain disulfide bonds in immunoglobulins are buried within the hydrophobic interior of the immunoglobulin fold. These bonds are critical for the stability of Ig domains *in vitro* (Kikuchi *et al.*, 1986) and of complete antibodies *in vivo* (Bergman and Kuehl, 1979) as they hold the surfaces of paired domains together. This burial of the disulfide bond suggests that the oxidative linkage occurs before the domain is fully folded, to allow access of oxidative agents to the buried cysteine residues before the hydrophobic pocket is formed (Gross *et al.*, 2010).

3.2.3. Enzymes and Chaperones

Protein disulfide isomerases are a family of proteins (more than 20 in humans) which catalyse disulfide formation via thiol/disulfide exchange reactions, corresponding to the cysteine/disulfide interconversion. These are located in the endoplasmic reticulum where secretory and surface membrane proteins undergo co-translational and/or post-translational modifications such as disulfide bond formation (Okumura *et al.*, 2015).

A complex network of components in the endoplasmic reticulum acts to maintain protein equilibrium in the body (protein homeostasis or proteostasis), including the correct conformation during protein production. This also includes an ER quality control system (Hammond and Helenius, 1994) based on a complex of folding enyzmes, chaperones and co-chaperones. These aid the initial folding of secreted and membrane-bound proteins, as

well as identifying and then refolding misfolded proteins (Meunier *et al.*, 2002, Dahiya and Buchner, 2019, Clausen *et al.*, 2019).

Antibodies generally assemble first as heavy chain dimers, driven by the interactions between the two C_H3 domains, but also stabilised by disulfide bonds in the hinge region (Feige *et al.*, 2010). The C_H2 domains only interact via the *N*-linked glycans which are sandwiched between them, (Feige *et al.*, 2010, Huber *et al.*, 1976). This determines their orientation and spacing, which is crucial for the binding of downstream effectors (Feige *et al.*, 2009, Krapp *et al.*, 2003). However, although heavy chain glycosylation is a pre-requisite for the assembly and transport of IgM (Sitia *et al.*, 1984), the absence of glycans in IgG does not prevent maturation (Hickman and Kornfeld, 1978).

Due to the great variety of antibodies, many of which can be a novel response to an antigen, some individual variants are more prone to mis-folding or mis-assembly due to the particular combination of amino acid residues. In response, immunoglobulin domains have evolved diverse folding strategies ranging from spontaneous intra-domain disulfide formation upon entry into the cisterna of the ER during translation (Bergman and Kuehl, 1979), to enzyme-directed folding, to the presence of intrinsically disordered domains which only fold correctly once they are united with their partner domains. These mechanisms are discussed below.

3.2.4. Disulfide Bonds & Oxidative Folding *in Vivo*

The oxidative folding *in vivo* of a protein such as an immunoglobulin (Ig) proceeds from an unstructured linear peptide precursor through increasingly structured intermediates. As the various cysteine residues are oxidised and converted into disulfide bonds, the chain begins to be constrained into a more compact shape. The formation of the intradomain disulfide bond is generally a prerequisite for the efficient folding of Ig domains (Feige *et al.*, 2010), as evidenced by the observation that the absence of a disulfide bridge increases the tendency to aggregate for single-chain variable fragments (scFV) (Ramm *et al.*, 1999). Disulfide bonds are also required to link the two heavy chains together in the hinge region, and to bind the two light chains to their heavy chain partners (Baumal *et al.*, 1971). This is achieved by the post-translational enzymatic oxidation of a pair of sulfhydryl (thiol) groups located on cysteine residues. This can occur in the endoplasmic

reticulum, Golgi apparatus or mitochondrion (Fass and Thorpe, 2018). This process is termed the oxidative folding of proteins.

The required pair of cysteines does not necessarily connect immediately. A transitional phase of relatively random cysteine pair-swapping allows the molecule to achieve ever lower energy, eventually reaching an energy minimum on its conformational energy landscape, which is generally the native folded state of the protein with the desired disulfide bonds and biological activity (Arai and Iwaoka, 2021).

One of the main enzyme families performing this reaction *in vivo* is represented by the protein disulfide isomerases (PDI) a type of thiol-disulfide oxidoreductase which is found in the ER of eukaryotes. An analogous system is found in prokaryotes such as *E. coli* (Figure 6), where the disulfide bond (DSB) machinery is found in the bacterial periplasm (Gruber, 2006).

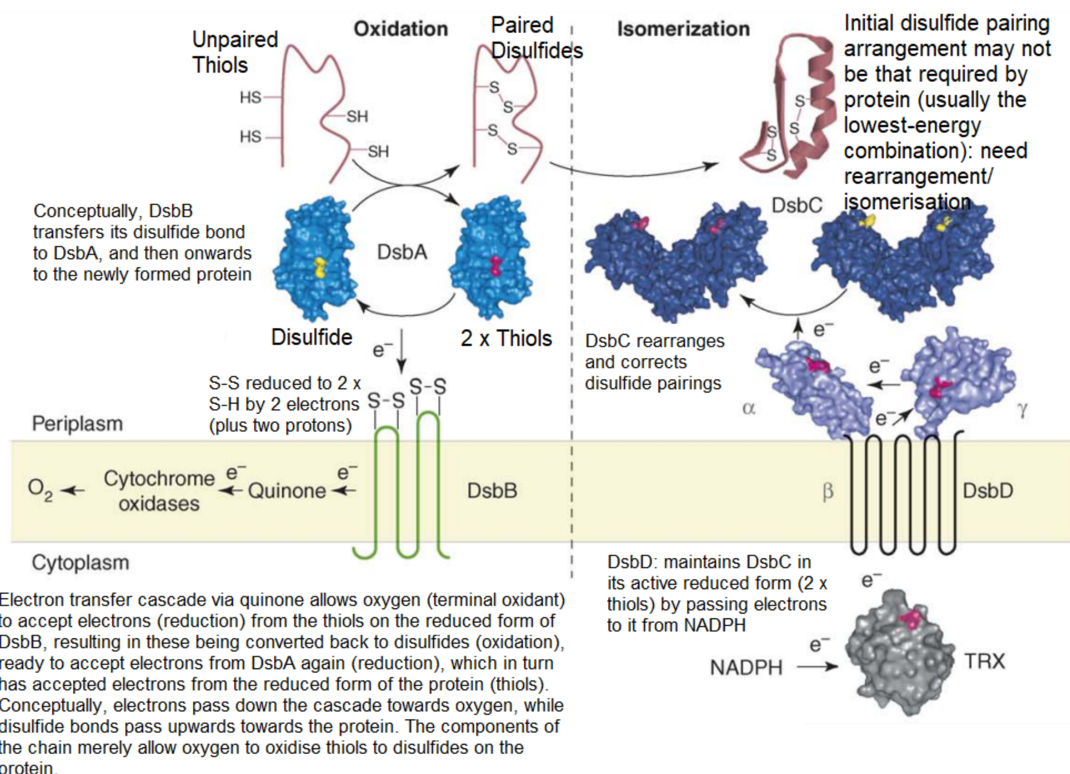

Figure 6: Summary of bacterial disulfide formation and isomerisation machinery, adapted from (Gruber, 2006)

Initial disulfide formation can result in incorrect, non-native disulfide pairings. However, because these are generally less stable, higher-energy forms, they can be corrected by enzymes which can catalyse thiol-disulfide exchange reactions. This is slow in the absence of catalysis. Under catalysis, it proceeds via the very rapid, reversible attack of a

cysteine thiolate anion on a disulfide bond, displacing one half of the target disulfide bond as a free cysteine thiolate. This free thiolate then goes on to attack another disulfide, forming a new disulfide linkage, while simultaneously displacing another thiolate, and so on (Figure 7). In effect, rapid equilibrium between various disulfide bonds in a protein allow the eventual formation of pairings which constitute the most stable constellation of disulfide bonds. This is driven by energy minimisation and achieves the most stable folding pattern of the protein (Gruber, 2006, Netto *et al.*, 2016).

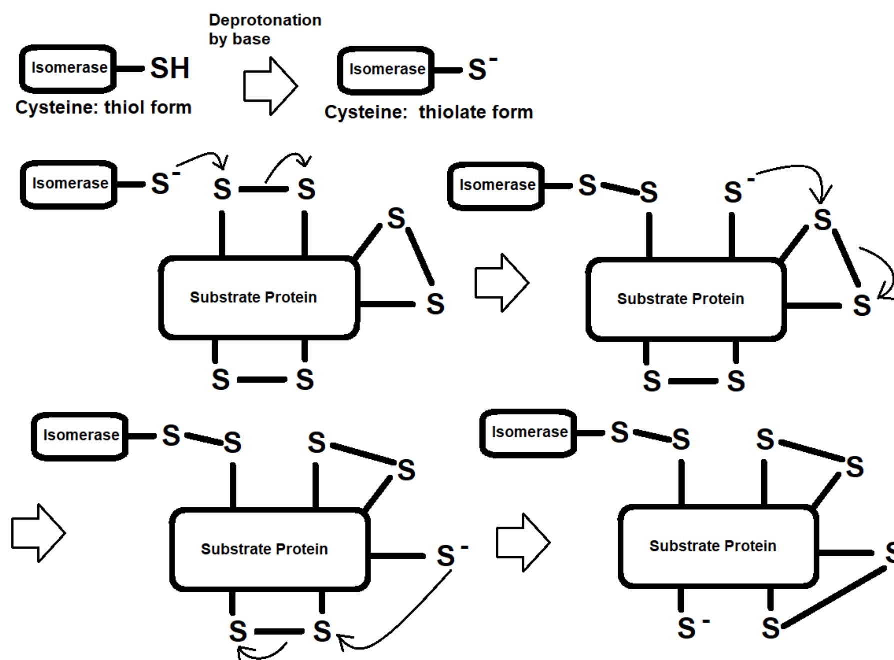

Figure 7: Disulfide isomerisation process

For the isomerisation process there is no net oxidation or reduction, just swapping of disulfide partners. The PDI needs to be in the reduced dithiol state to perform this function. Conversely, for the net formation of disulfide bonds, the PDI enzyme needs to be in the oxidised disulfide form and is attacked by free cysteine thiols in the target protein. There needs to be an oxidant such as dehydroascorbate (DHA) present to reoxidise the PDI back at the end of each catalytic cycle (Fass and Thorpe, 2018).

The thiol-disulfide exchange reaction is accelerated significantly in hydrophobic environments, and such pockets commonly surround the active site in thiol-disulfide oxidoreductases (Netto *et al.*, 2016). These hydrophobic regions are also thought to be important for targeting by chaperones of unfolded or mis-folded regions of proteins,

which are often composed of exposed hydrophobic residues which should be inside the native protein structure (Byrne *et al.*, 2009, Denisov *et al.*, 2009).

3.2.5. Disulfide Control Non-Antibody Proteins

Some progress has been made in this area with disulfide bond control in non-antibody proteins (Pardee *et al.*, 2016, Goerke and Swartz, 2008). Elucidation of the oxidative folding pathways *in vivo* (Qiao *et al.*, 2001, Qiao *et al.*, 2003) has enabled an alternative efficient chemical synthesis of human insulin by using oxidative native chain assembly whereby unprotected native A and B chains assemble spontaneously under thermodynamic control (Arai *et al.*, 2018).

However the oxidative folding pathway of a given amino acid chain is not only determined by its sequence, but also by the conditions under which disulfide bond and folding is performed, such as temperature, pH and the presence of metal ions, thereby allowing scope for optimisation (Arai and Iwaoka, 2021).

Small-molecule catalysts, such as the inexpensive reduced tripeptide Cys-Gly-Cys, have been used with some success to mimic the activity of PDI and thioredoxin in generating and isomerising native disulfide bonds, however the rate of catalysis was several orders of magnitude lower than *in vivo* (Woycechowsky and Raines, 2003).

More sophisticated diethylenetriamine-derived organocatalysts (Figure 8) achieved more rapid isomerisation of non-native disulfide bonds to native ones in a model ribonuclease (RNase) A enzyme with 8 cysteine residues. They work in a very similar manner isomerases, as described in Figure 7. The small molecule catalyst contains two thiol groups, each of which can be deprotonated at biological pH to reveal a nucleophilic thiolate, which then triggers a cascade of disulfide cleavage events, each time releasing another thiolate, leading to further rearrangement events (Figure 9).

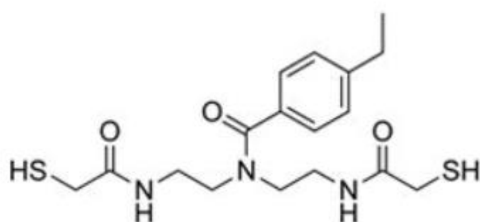

Figure 8: Example of small-molecule PDI mimic (Lukesh et al., 2014)

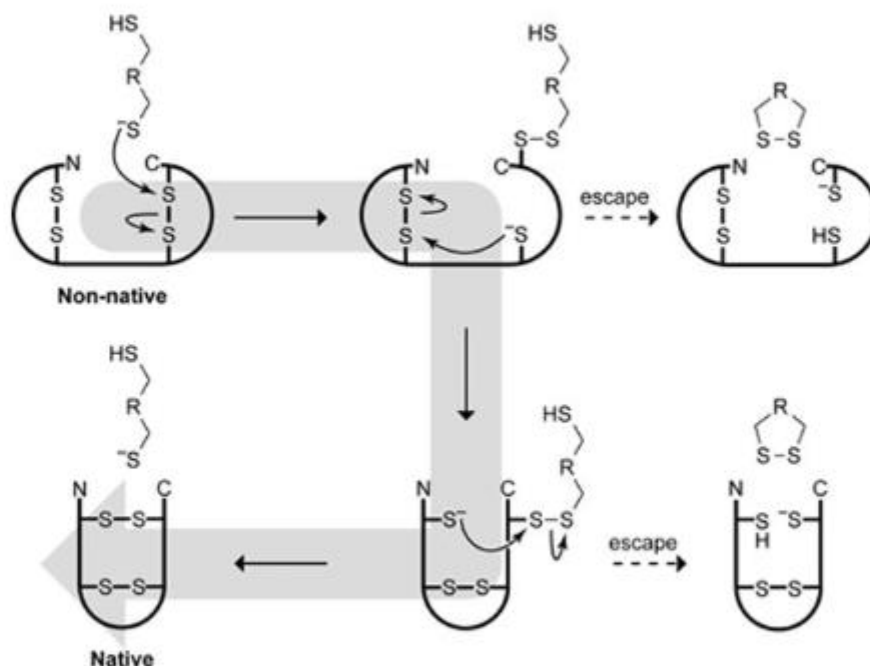

Figure 9: Putative mechanism of catalysis of disulfide isomerization (Lukesh et al., 2014)

These catalysts were attached to a lipophilic moiety designed to bind to the hydrophobic unfolded portion of the proteins resulting in 47% higher yield of native protein compared with the uncatalysed system (Lukesh *et al.*, 2014). There are advantages in small-molecule-based systems compared to enzyme-based systems. Small molecules can easily be removed during purification of their protein substrate.

Work on small-molecule diselenide catalysts has demonstrated effective disulfide isomerisation and significant suppression of protein aggregation, which often occurs due to intermolecular attraction between exposed hydrophobic residues in misfolded proteins (Mikami *et al.*, 2021). In fact, replacement of two cysteine residues in a protein with two selenocysteine residues results in the rapid selective formation of a diselenide bond, which is more stable than a disulfide bond (Pegoraro *et al.*, 1999, Musiol and Moroder, 2012). This work showed that there was little impact on biological activity in the model system. The purpose of the work was to investigate folding mechanisms, but it has the potential to allow protein folding to be directed more quickly and efficiently towards the desired native state, albeit with a non-native diselenide bond embedded in the structure. Similar results were obtained in the case of selenoinsulin, a biologically active, natively folded analogue of insulin where the A and B chains are linked by a diselenide (Arai *et al.*, 2017).

N-(thioethyl)urea and *N*-(thioethyl)guanidine can also accelerate folding and lead to higher yields of native proteins (Okada *et al.*, 2019). Insulin is a small heterodimeric globular protein (5.8 kDa) with two peptide chains A and B containing one intrachain and two intrachain disulfide bonds. Insulin is produced as a single proinsulin chain *in vivo*, where the two chains are connected by an extra 35-residue peptide linker, which is excised by proteolysis after correct folding has been achieved (Steiner, 1998). Biomimetic synthesis *in vitro* using enzymatic processing of proinsulin with either native or novel linkers has been performed on a commercial scale (Mayer *et al.*, 2007).

Minimal proinsulin surrogates have also been chemically synthesised which contain alternative linkers, such as a hydrolysable ester linkage between Thr-Glu residues that are naturally adjacent to each other in the final folded insulin molecule (Sohma, 2010). Tethering the two chains allows the entropically more challenging association of the two chains to be pre-ordained, thereby allowing the subsequent native folding and disulfide formation to proceed with high efficiency under redox conditions with dithiothreitol.

3.2.6. Disulfide Control for mAb Manufacture

Mismatched or missing disulfide bonds are a source of inhomogeneity in monoclonal antibodies. Enzymes are available commercially to assist with disulfide bond formation, and even to correct mis-oxidised substrates (New England Biolabs, 2021).

This concept has also been successfully implemented as a rescue strategy on a manufacturing scale to re-oxidise reduced disulfide bonds using optimised redox conditions (cysteine/cystine ratio, pH, temperature, time) while an antibody was immobilised on a protein A resin during purification. A mixture of H, L, HL, HH, HHL and H_2L_2 (5%) was converted to the desired H_2L_2 with a product purity >95% over 24 h, thought to be facilitated by continued non-covalent association between the chains after reduction of the disulfide chains (Tang *et al.*, 2020, Tan *et al.*, 2020).

It has been shown that an incorrect folding in the tumour necrosis factor TNFα receptor-IgG1 Fc fusion protein Etanercept/Enbrel®, was reversible *in vitro* under serum-like

redox conditions using physiological cysteine concentrations, restoring potency to normal levels (Lamanna *et al.*, 2017).

Protein folding and mis-folding is an active area of research due to ongoing efforts to develop treatments for diseases which are thought to be caused by protein misfolding, such as Alzheimer's and Parkinson's diseases, areas of unmet medical need.

Limited work has been performed in the area of *in-vitro* folding/misfolding for antibodies, where the structure is more complex than that of model systems like insulin. The suitability of many so these systems is not known for antibodies. Also, the difficulty of achieving the correct folding varies from mAb to mAb. There are examples of development molecules which are inherently difficult to express which need changes to their primary structure to improve manufacturability by enabling correct folding during cell culture (Mathias *et al.*, 2020).

3.2.7. Proline Isomerisation

Proline is the only natural amino acid which can exist in two isomeric forms, *cis* and *trans*. *Cis*-proline is required to achieve the desired shape of the backbone. A *trans-cis* isomerisation of proline residues is required to allow the formation of the tight hairpin turns which connect the beta strands in the Ig folds. This is catalysed by a member of the cyclophilin family of peptidyl-prolyl isomerases (PPIases) *in vivo* (Feige *et al.*, 2004, Fischer *et al.*, 1998).

However this represents a slow stage in the folding of the Ig domains (Goto and Hamaguchi, 1982), increasing the probability that the unfolded protein might undergo misfolding or aggregation. Therefore replacement of such prolines with optimised, flexible loops might be a useful strategy to accelerate protein folding (Jakob *et al.*, 2010). In addition it is possible to influence the cis/trans equilibrium of the proline residue (Meng *et al.*, 2006) and/or increase the activity of PPIases (Zoldak *et al.*, 2009) by optimising the sequence of amino acids adjacent to the proline.

3.2.8. Folding Mechanisms

Although all IgG domains are very similar, and all progress through intermediate partially folded states (Figure 10), they can be grouped into three different folding categories based on *in vitro* investigations of the folding of the denatured protein. Both folding and the formation of disulfide bonds (Bergman and Kuehl, 1979, Bergman and Kuehl, 1978) occur during transcription. Protein folding has been shown to progress through partially folded intermediate states or folding intermediates along the pathway to the final native state (Feige *et al.*, 2008).

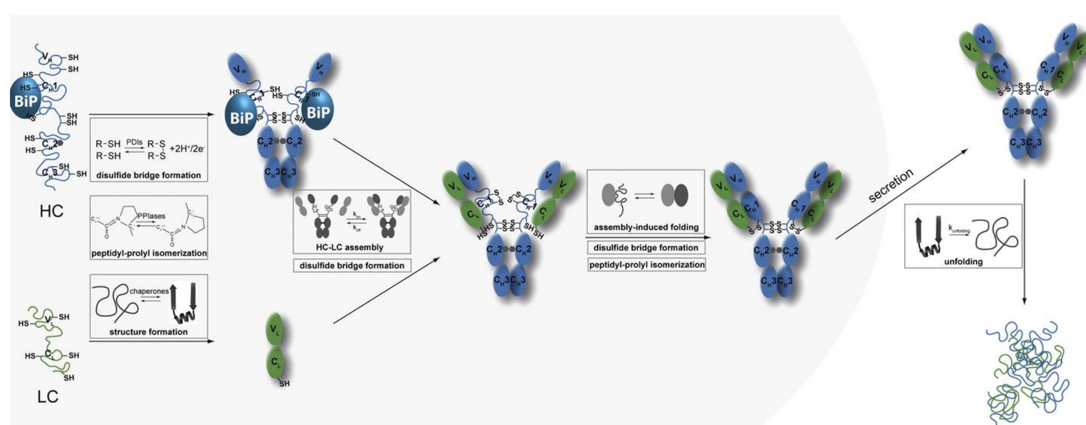

Figure 10: Key stages of antibody folding (Feige and Buchner, 2014)

3.2.8.1. *Category I: Autonomous Folding*

In the first category are domains which undergo autonomous folding without the pre-formation of the embedded intra-chain disulfide bond (Figure 11). It is known that C_L will fold autonomously *in vitro* (Isenman *et al.*, 1979, Goto and Hamaguchi, 1982). Furthermore there is no significant disruption to the structure of the beta-sandwich if the embedded disulfide bond is reduced in the native protein, though it is less stable (Goto and Hamaguchi, 1979).

However it is also clear that the presence of an intra-domain disulfide bond limits the degrees of freedom of a denatured immunoglobulin domain, forcing it to follow a more highly structured sequence of intermediates, thereby increasing the chance of formation of a properly folded final protein (Feige *et al.*, 2007). The presence of a covalent bound between two otherwise distant points on the primary structure can act as an initiation point for a cascade of interactions along the amino acid chain which lead to the formation

of the desired three-dimensional structure, not only after deliberate experimental denaturation of the protein, but also during de novo folding in the endoplasmic reticulum (Feige *et al.*, 2007).

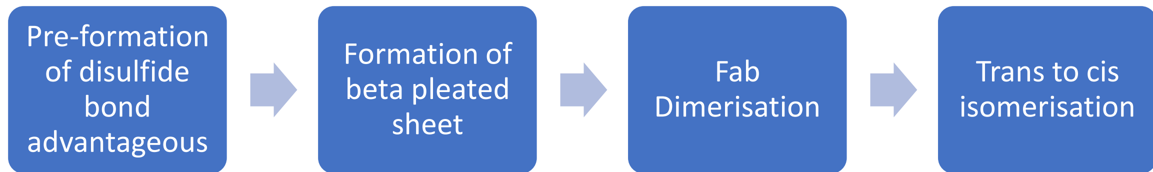

Figure 11: Assembly and dimerisation of C_L fragment

3.2.8.2. Category II: Dependence on Proline Isomerisation

The second category of immunoglobulin domains is represented by C_H3 (Figure 12). Experiments have shown that unfolding is completely reversible (Thies *et al.*, 1999). Prolyl trans-to-cis isomerisation is the rate limiting process here (Isenman *et al.*, 1979), however in contrast to the first category, beta sheet formation does not occur until proline isomerisation is complete. However, if this is sufficiently accelerated by catalysis, the assembly of the beta sheet becomes rate limiting. This is significantly slower than for other domains, but once the sheet is formed, the folded immunoglobulins dimerise rapidly to form a non-covalent homodimer (Thies *et al.*, 1999).

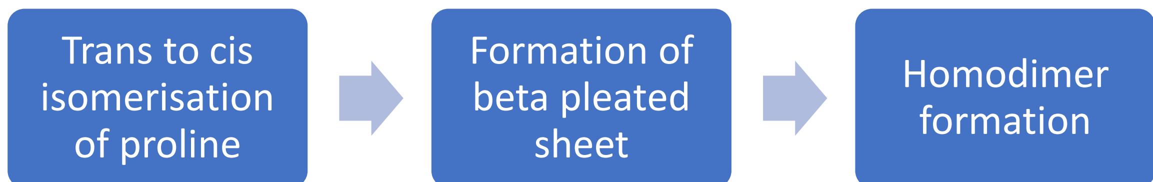

Figure 12: Assembly and dimerisation of C_H3 domain

3.2.8.3. Category III: Folding as a Quality Control

The third category of folding behaviour is represented by the template-assisted folding of the C_H1 domain (Figure 13). This domain cannot undergo folding until it is partnered with the folded C_L domain on the light chain. This is reflected in the kinetics of Fab folding, where C_H1 folding was observed to be the slowest step, only occurring after association of HC and LC (Lilie *et al.*, 1995).

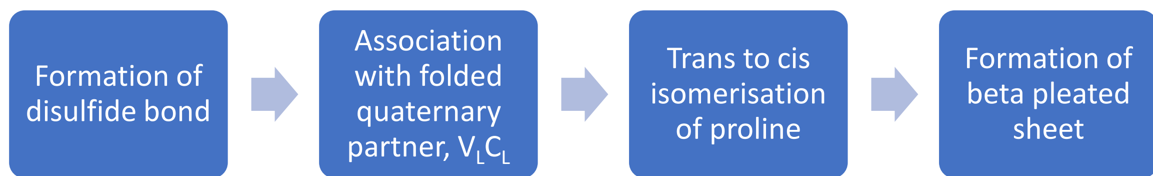

Figure 13: Assembly of the C_H1 domain

In-vivo studies have revealed that this is part of the quality control mechanism in the endoplasmic reticulum (ER). An immunoglobulin heavy chain binding protein (BiP), which is the ER equivalent of Hsp 70 chaperones, has been shown to associate with unassembled heavy chains pending association with the LC, thereby preventing their premature secretion from the ER (Meunier *et al.*, 2002). BiP associates specifically with the unfolded hydrophobic region, usually as a complex with other chaperone molecules such as GRP94, the oxidoreductases PDI (Meunier *et al.*, 2002), ERp57, and pERp1, which assists in the oxidative folding of Ig (Shimizu *et al.*, 2009).

Once the C_H1 domain has paired with the folded C_L domain, the BiP protein dissociates (Bole *et al.*, 1986). This quality control gate prevents the secretion of incomplete antibodies. It has however been shown *in vitro* that deliberate removal of BiP results in the rapid autonomous folding and intradomain disulfide bond formation in the C_H1 domain. Therefore it does not need BiP nor association with the C_L domain in order to enable folding to occur; in fact the BiP chaperone acts to inhibit C_H1 folding until it is dislodged by C_L (Lee *et al.*, 1999).

A further ER chaperone system uses uridine diphosphoglucosyltransferase (UDP-GT) to *N*-glycosylate unfolded regions of nascent proteins, which provides the binding site for the ER chaperones calnexin and calreticulin. Cleavage of the glucose is the performed by glycosidase II, removing the calnexin/calreticulin binding site. The unfolded protein remains trapped in this cycle of glycosylation and deglycosylation, held in the ER by calnexin/calreticulin until folding is complete and the UDP-GT cannot rebind, releasing the folded protein from the ER (Meunier *et al.*, 2002).

3.2.9. Small Directing Helices

A further aspect of the structure of constant immunoglobulin domains which appears to be critical for self-directed folding is the presence of two small helices between strands a

& b, and between strands e & f (Huber *et al.*, 1976). They appear to achieve their native structure very quickly as part of one of the folding intermediates which lies along the pathway to the native state. It then acts as an organising centre for the correct formation of the beta fold, directing the hydrophobic residues towards the core of the beta fold. The optimisation of these helices has been shown to increase folding efficiency and decrease the tendency to misfold in other members of the Ig superfamily (Feige *et al.*, 2008). These small helices are however missing in variable domains, limiting the wider applicability of this approach.

3.2.10. Aggregation

It is however thought that certain V_L or V_H domains may be inherently difficult to fold due to the particular constellation of amino acids in the CDRs, thereby requiring pairing with the partner on the other chain in order to facilitate folding, which is consistent with assembly-assisted category 3 (Feige *et al.*, 2010).

The agglomeration of antibodies due to misfolding is one of the main reasons for the presence of chaperones in the ER. In fact, even fully assembled antibodies tend to self-associate and aggregate at higher concentrations. This tendency varies depending on the balance of hydrophobic and hydrophilic residues, and it is now possible to improve solubility by engineering antibody frameworks, domain interfaces and antigen binding loops to prevent aggregation of natively and non-natively folded antibody fragments (Perchiacca and Tessier, 2012).

The unfolding and refolding of antibodies are equilibrium processes for reversible non-covalent association, such as ionic attraction, hydrogen bonding, hydrophobicity, and Van der Waals forces. The position of the equilibrium is affected by environmental conditions such as pH, ionic strength, temperature, and protein concentration. Once unfolding of antibodies has occurred, the tendency to agglomerate depends on the solubility of the Ig domains, with higher solubility resulting in slower aggregation. This could in principle lead to the engineering of highly stable, non-aggregating Ig domains which could resist environmental stress and re-fold back to their native structure, as exemplified in particular by camelid VH domains (Gil and Schrum, 2013).

The body uses so-called stabilising osmolytes to favour the folded state of proteins, especially when cell osmolality changes (Khan, 2010). They consist of small molecules such as glycine, alanine, *N*-methylglycine (glycine betaine), trimethylamine-*N*-oxide, polyols, sorbitol or sucrose (Rabbani and Choi, 2018). These are the opposite of protein denaturants and work by destabilising the unfolded state of proteins. The equilibrium between native and unfolded states is shifted depending on the relative energy of intramolecular hydrogen bonding between residues on the amino acid backbone (native), and intermolecular hydrogen bonds between the amino acid residues and the solvent (unfolded) (Bolen and Rose, 2008). If the protein backbone is repelled from interacting with its surroundings and focuses on various intramolecular interactions, such as beta-sheets and alpha-helices, this causes the protein to contract to its native state, minimising the contact area with the solvent, thereby hindering opening and unfolding of the protein structure when under osmotic stress (Street *et al.*, 2006).

These are used instead of K^+ or Na^+ because these can perturb protein function at high concentrations, whereas organic osmolytes do not (Yancey *et al.*, 1982). Osmolytes such as trehalose and sucrose are already used in the biopharmaceutical industry to protect protein products from aggregation, but also as osmoprotective agents for cell cultures (Øyaas *et al.*, 1994). There is now considerable interest in using osmolytes to treat so-called amyloidogenic disorders such as Parkinson's and Alzheimer's diseases, which are caused by protein misfolding and aggregation (fibrillation) (Powers *et al.*, 2009). Osmolytes have also been used to reverse unwanted dimerisation of Fab fragments (Nelson *et al.*, 2012).

In summary, most immunoglobulin domains will fold autonomously into the correct three-dimensional shape. Once their intradomain disulfide bond has formed, completion of folding is dependent the kinetically limiting peptidyl-prolyl isomerisation, after which the final beta immunoglobulin fold is achieved, and inter-chain association and disulfide bonding can occur, leading to dimerisation of the heavy chain and association of the two light chains.

3.3. Glycosylation

A further structural feature of antibodies is that they are glycoproteins. This structural aspect is a key determinant of antibody structure and function (Cymer *et al.*, 2018, Chiu

et al., 2019, Wang and Ravetch, 2019, Wang *et al.*, 2020b). Glycosylation is a post-translational modification, mediated by glycosyltransferases once the heavy chains reach a certain size during translation; in other cases, glycosylation can occur after completion of translation of the heavy chain (Bergman and Kuehl, 1978).

In humans IgGs glycosylation consists of biantennary (forked) structures which are *N*-linked via an asparagine at position 297 in the C_H2 domain of the Fc region. The structure of the oligosaccharide is critical for therapeutic efficacy, having an important impact on the pharmacokinetics, pharmacodynamics, stability, immunogenicity, and activation of cell-mediated complement functionality such as CDC and ADCC, as well as prolonging antibody half-life in the blood (Liu, 2015, Higel *et al.*, 2016, Jefferis, 2012, Boune *et al.*, 2020, Liu *et al.*, 2020). Oligosaccharides have been shown to be involved in the correct folding of proteins in general (Shirakawa *et al.*, 2021) and contribute to the correct structure of mAbs (Liu, 2017).

Structural patterns vary significantly from species to species, with non-native structures being capable of triggering a strong immunological response, making this aspect of antibody structure a critical quality attribute (Reusch, 2015). An immunological response in the patient can result in deactivation of the therapeutic antibody via antibody-drug conjugates (i.e. patient-generated antibodies against the therapeutic antibody). It can also result in anaphylactic shock in some cases.

The oligosaccharides in human antibodies are based on a core bi-antennary structure Asn-GlcNAc-GlcNAc-Man-$GlnAc_2$, which is referred to as a G0 glycoform as it has no terminal galactose residues (Figure 14). By adding galactose to each of the two core GlnAc residues, the G1 and G2 glycoforms are obtained. Human IgGs are usually fucosylated on the core GlcNAc residue, leading to an F notation (G0F, G1F, G2F).

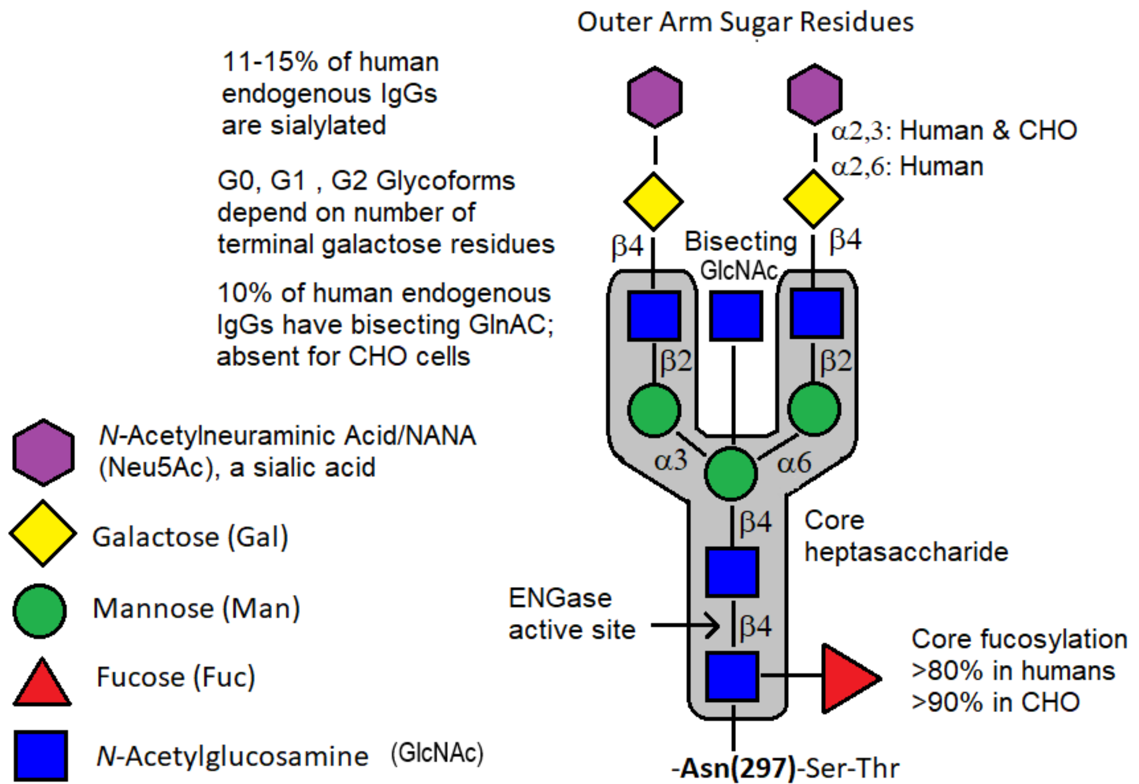

Figure 14: Typical human glycosylation patterns (Boune et al., 2020)

Each glycosidic bond can have an α or β stereochemistry, and most glycosides contain 5 different alcohol groups for bond formation, with the carbonyl group at position 1 used to form one side of the glycosidic bond (the so-called reducing end of the sugar). The relevant glycosyltransferase ensures fidelity with respect to the stereochemistry and regioselectivity *in vivo*.

A great deal of work is ongoing to understand the impact of varying the glycoforms of mAbs and there are still many areas of uncertainty regarding their therapeutic impact (Shirakawa *et al.*, 2021), however it is clear that non-human patterns represent an immunogenic risk. There is an impact on the effector functions, with fucosylation and galactosylation strongly impacting ADCC and CDC respectively (Loebrich *et al.*, 2019). The absence of fucose enhances ADCC activity markedly (Boune *et al.*, 2020).

3.3.1. Glycoprotein Biosynthesis

N-linked glycoproteins, such as mAbs, are formed initially in the ER lumen by the transfer of a preformed glucosylated high-mannose glycan substrate ($Glc_3Man_9GlcNAc_2$) by an oligosaccharyltransferase (OST) enzyme to the amide nitrogen of an asparagine

residue in the Asn-X-Ser/Thr recognition sequence (sequon) of the nascent polypeptide. This is a co-translational activity, i.e. the initial glycosylation occurs during formation of the polypeptide chain by the ribosome. This is followed by the sequential removal by glycosyl hydrolases (GHs) of the three-unit chain of glucose units from its attachment point on one of the three mannose antennae (Shirakawa *et al.*, 2021) (Figure 15).

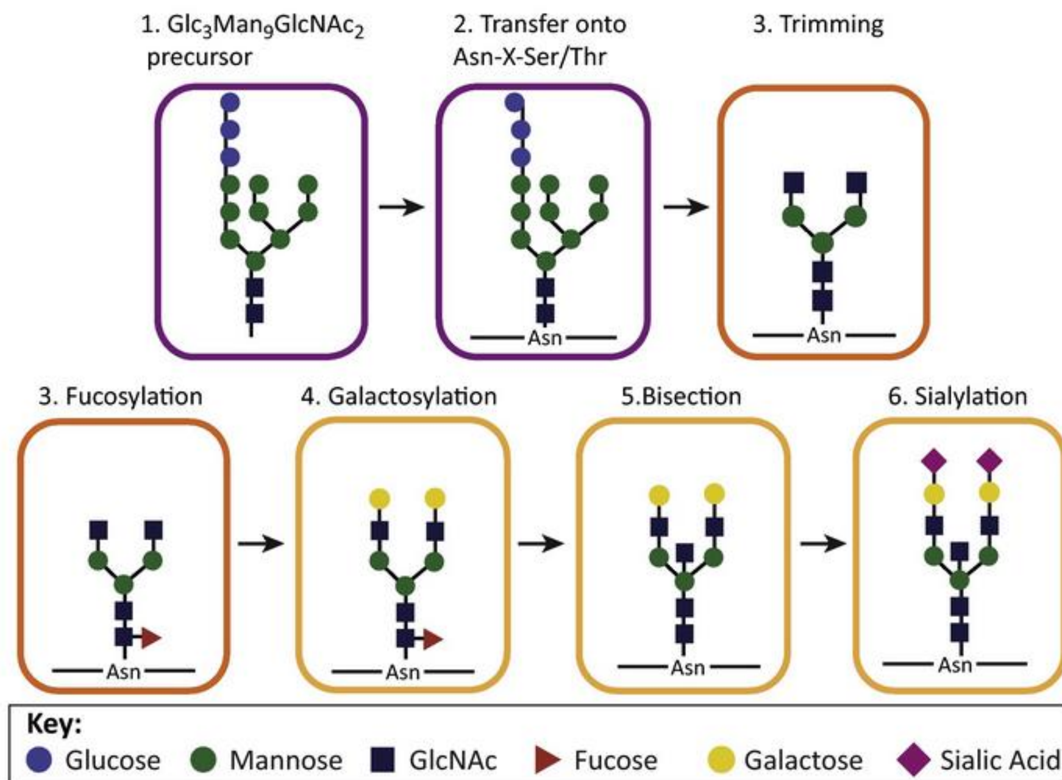

Figure 15: Construction of Human mAb Glycan (Jennewein and Alter, 2017)

The resulting high-mannose glycoform is subjected to further enzymatic modification by glycosyl hydrolases (GHs) to remove more mannoses, going from a trifurcated structure to a bifurcated one, revealing the core for the typical biantennary ‘complex’ glycan found on human antibodies.

Then the structure starts to get built back up again. The two terminal mannose units are *N*-acetylglucosylated and the core *N*-acetylglucose is fucosylated. This is followed by galactosylation of the two terminal *N*-acetylglucose units. This is optionally followed by *N*-acetylglucosylation of the mannose at the bifurcation point. Then the terminal galactose units are optionally sialylated. The ER quality control system ensures that only the properly folded protein with the required glycan structure is then exported to the

Golgi apparatus (Arigoni-Affolter *et al.*, 2019), though this still results in a mixture of glycoforms.

After export to the Golgi apparatus, the core is elaborated to the species-specific glycosylation pattern, which for humans usually means adding two *N*-acetyl glucosamines, followed by zero, one or two molecules each of galactose and sialic acid. The sequential modification of the glycan is achieved by the spatial separation of the various glycosylation enzymes along the pathway which the protein follows through the Golgi (Arigoni-Affolter *et al.*, 2019).

A small proportion is bisected by a third *N*-acetylglucosamine, and the majority carries a core fucosylation (Unverzagt and Kajihara, 2013). The key to the sequential modification of the initially attached glycan is the localisation of different enzymes and substrates in the ER and Golgi, and there is now a quite detailed understanding of the reaction pathways and kinetics for mAb glycosylation (Arigoni-Affolter *et al.*, 2019)

Unprocessed high-mannose forms can occur as unwanted glycoform impurities. Partially processed 'hybrid' structures also result due to incomplete processing of the high-mannose glycan during mAb production, especially under stressed conditions. This contributes to the considerable heterogeneity seen in biological systems.

3.3.2. Natural Variability of Glycans

Antibodies in nature occur with a variety of glycosylation patterns dependent on species. This has clear implications for the use of non-human host cells for the production of mAbs. Human glycosylation patterns require a biantennary heptasaccharide core consisting of *N*-acetylglucosamine and mannose (Figure 14), essential for activation of the complement system and Fcγ receptors. Host cells are therefore selected for manufacturing which can provide a human-like glycan isoform.

However, humans (and other species) do not display just one isoform. There is significant variability, the level of which can change depending on environmental conditions and age. Unlike the DNA-driven biosynthesis of proteins, glycan biosynthesis is controlled by the availability, concentration, and specificity of enzymes such as glycosyl transferases

(Lairson *et al.*, 2008). Because of the complexity of this process, products of natural protein glycosylation pathways are typically heterogeneous mixtures of glycoforms.

There is considerable heterogeneity of the outer residues involving galactose and bisecting *N*-acetylglucosamine, as well as optional core fucosylation. Sialylation with *N*-acetylneuraminic acid is not frequent in humans but it is observed. It is also possible to have different glycosylation patterns on each of the two heavy chains (Jefferis, 2012).

There is an ever increasing level of understanding of the importance of the structure-function relationship of glycan structures, both desired and undesired, occurring naturally in humans as well as produced during cell culturing (Cymer *et al.*, 2018). The importance of glycans as regulators and mediators of biological processes has driven an increasing interest in the synthesis of pure oligosaccharides in order to attach them to proteins of interest to investigate their structure-function relationship (Edgar, 2021). However, their *in-vivo* heterogeneity defeats most attempts at purification or quantitation for study.

The structure of mAbs is heterogenous during cell culturing due to the inherent complexity of their biosynthesis and their sensitivity to low levels of environmental variation. Generation of homogenous glycosylation patterns during commercial production of monoclonal antibodies is therefore a great challenge, and generally the aim is to produce the same mixture in each batch, rather than aiming for a pure glycoprotein. Maintaining and monitoring the stability of the glycoform profile is a quality-critical task during the production of monoclonal antibodies.

The fucosylated core pattern predominates in mammalian cell cultures such as CHO, with lower levels of galactose than in humans, however deviant patterns can be generated under stressed conditions for pH, temperature, nutrient levels (Jefferis, 2012). The production of a desired glycoforms may be enhanced by careful selection of a suitably stable and robust cell line, or genetic-level glycoengineering of host cells to make their glycosylation patterns more homogenous, or to knock out undesired glycyltransferases. However, variability in the cell culture conditions (Pacis et al., 2011), nutrient levels (Fan *et al.*, 2014) and the concentrations of trace media supplements (Radhakrishnan et al., 2017) can still lead to altered glycosylation patterns.

In general however, high levels of control of the cell culture conditions are required for stable production of the desired glycoform profile (Jimenez del Val *et al.*, 2011). It is possible to use this sensitivity as a control lever by deliberately using additives and bioreactor conditions to modify an undesired glycosylation pattern. This is especially interesting for manufacturers of biosimilars, who need to match the quality profile of the originator product, despite not having access to the originator cell line (Ehret *et al.*, 2019, Loebrich *et al.*, 2019).

Inevitably with better understanding comes a desire for better control and much work has been performed to improve the homogeneity of glycoforms for commercial production of antibodies, though cell-based processes still generate a mixture of glycoforms (Hossler *et al.*, 2009, Chang *et al.*, 2019). *In-vitro* approaches may however offer solutions to glycoform inhomogeneity.

3.3.3. Glycosylation of mAbs *In Vitro*

Given the interest in reducing glycoform heterogeneity, there have been great efforts in understanding the biological glycosylation machinery with a view to optimising it. By understanding each of the glycosylation enzymes and searching for similar more convenient variants in other organisms, as well as deliberately mutating them, glycobiologists are now able to mimic aspects of the glycosylation of mAbs *in vitro*.

The construction of oligosaccharides *in vivo* is achieved using various glycosyltransferase (GT) enzymes which transfer specific activated glycosyl donors (nucleotide sugars) such as uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) to the desired nucleophilic acceptor such as a hydroxyl group on a glycoside acceptor, or the amide group on Asn, in order to form glycosidic linkages with the correct stereochemistry. Oligosaccharide transferases (OSTs) therefore act as the key controller of *N*-glycosylation due to the combination of their preference for a particular protein, their specificity for a particular consensus sequence on the protein chain, and finally their specificity towards a particular glycosyl donor which will be attached to the protein *en bloc*.

3.3.4. mAb Remodelling using Glycosidases & Glycosyl Transferase

In-vitro glycoengineering has been made possible by using enzymes to remodel non-optimal glycosylation patterns using chemo-enzymatic manipulation of monoclonal antibodies (Ma *et al.*, 2020, Cymer *et al.*, 2018, Malik *et al.*, 2021, Li and Ye, 2020).This chemoenzymatic approach uses native glycoside hydrolases (glycosidases for short), or more specifically an endoglycosidase (ENGase), to release the native oligonucleotide from a glycoprotein by hydrolysing the glycosidic bond between two *N*-acetylglucosamine residues near the link to the protein, hence the 'endo' terminology. They can therefore be used to trim back a heterogenous mixture of glycoforms arising from a cell culture, leaving just the innermost saccharide unit bound to the antibody.

The oligosaccharide can then be re-built into the desired glycoform using individual glycosyl transferases. There are now even one-pot multienzyme approaches which leverage the highly specific nature of glycosyl transferases to increase the efficiency of this process significantly (Tong *et al.*, 2018).

Depending on the starting glycoform mixture, the residual *N*-acetylglucosamine (GlcNAc) at Asn-297 may be present with or without core fucosylation after treatment with endo-β-*N*-acetylglucosaminidase. This GlcNAc can then be used to provide an attachment point to deliberately build up a new homogenous glycoprotein in a stepwise manner using the appropriate sequence of glycosyl transferases.

3.3.5. En-Bloc Transfer Using Oligosaccharide Transferase

As well as building up oligosaccharides residue by residue, there are also OSTs which transfer pre-formed oligosaccharides to an acceptor *en bloc*. This is the case for *N*-glycosylation of mAbs *in vivo*. Individual GTs build up an oligosaccharide separately on an activated lipid carrier (dolichol phosphate) anchored in the membrane of the ER. The entire oligosaccharide is only then transferred to asparagine 297, which sits within a consensus Asn-X-Ser/Tyr or NX(S/T) sequon which is used to direct OST in nascent human antibodies. This initial oligosaccharide is subsequently trimmed in the ER by GHs and then re-glycosylated by GTs after transfer to the Golgi apparatus to give the final oligosaccharide structure.

However, there are significant hurdles to replicating this approach *in vitro* using glycoengineering. The human OST enzyme is unstable, membrane-bound, and has a very complex structure with up to nine protein subunits (Bai *et al.*, 2018, Wild *et al.*, 2018, Ramírez *et al.*, 2019). It is unable to glycosylate folded proteins, and the natural substrate not only has a long lipid chain for anchoring to the ER membrane, it is also not the final desired oligosaccharide and therefore needs further processing (Wang and Amin, 2014). To date, only limited success has been achieved with eukaryotic OSTs, and only on short synthetic peptide acceptors rather than fully folded proteins (Arigoni-Affolter *et al.*, 2019).

However, certain proteobacteria from the *Campylobacter* and *Helicobacter* species also use *en-bloc* transfer of glycans from undecaprenyl-pyrophosphate (Und-PP) glycolipids onto conserved amino acid sequons. This finding led to the transfer of the *N*-glycosylation machinery from *Campylobacter jejuni* into *E. coli*, which gave rise to the area of bacterial glycoengineering. The OST from *Campylobacter jejuni* requires a more extended glycosylation sequon that the mammalian equivalent; the so-called 'minus-two rule' requires an acidic amino acid at the -2 position with respect to the glycosylation site (Kowarik *et al.*, 2006b).

However, other bacterial OSTs have been identified which are not subject to this constraint (Ollis *et al.*, 2015). These enzymatic tools therefore allow the specific *N*-glycosylation of proteins after the insertion of the required sequon using genetic engineering and may be used in cell-based or cell-free systems (Kowarik *et al.*, 2006a, Ollis *et al.*, 2015). Despite their complexity and transmembrane nature, bacterial OSTs can be readily overexpressed in a recombinant bacterial host such as *E. coli*, and large-scale purification procedures have been established (Jaffee and Imperiali, 2013, Jaroentomeechai *et al.*, 2017), making *in-vitro* bacterial glycoengineering a standard tool.

3.3.6. Attachment to *N*-Acetylglucosamine Core using Glycosidases

Given that the *in-vitro* use of OST poses difficulties, and that GTs require relatively expensive nucleotide sugar donors, researchers also looked at reversing the reactivity of GHs or glycosidases to force them to form glycosidic bonds, rather than hydrolysing them. One example of the successful application of this approach this is the so-called

endoglycosidase or ENGase family of enzymes, which are present in many species, including fungi, bacteria and mammals. In nature these enzymes hydrolyse the chitobiose core of *N*-linked glycans, i.e. the dimer of *N*-acetylglucosamine which is linked to the asparagine at position 297 in mAbs, and therefore remove the oligosaccharide substrate, except for a single *N*-acetylglucosamine still attached to the peptide chain (Fairbanks, 2017).

The N175Q mutant of *endo*-β-*N*-acetylglucosamidase M ($EndoM^{N175Q}$) has been developed with this aim and can be used in reverse to attach an activated glycosyl donor (a pre-formed oxazoline derivative of an oligosaccharide) to mono-glycosylated mAbs (Asn-GlcNAc) as well as core fucosylated mAbs (Asn-GlcNAc-Fuc) (Fairbanks, 2017, Lomino *et al.*, 2013, Giddens *et al.*, 2016). This reverse reactivity, forming glycosidic linkages instead of hydrolysing them (Figure 16), has led to these mutant enzymes being categorised as endoglycosynthases.

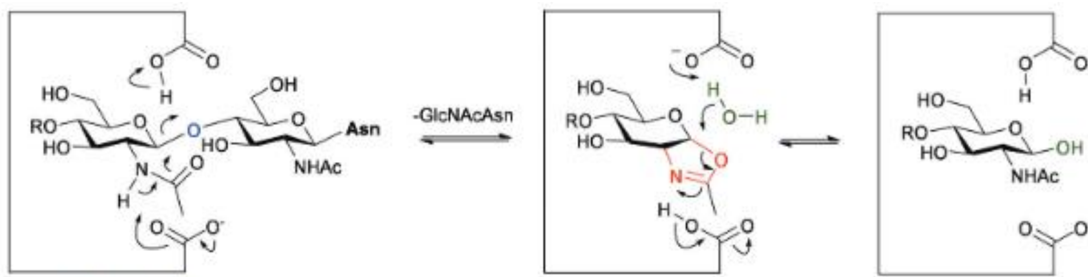

Figure 16: Endoglycosynthetase Mechanism (Fairbanks, 2017)

This technology has given rise to a two-step glycan remodelling procedure where the wild type ENGase is used hydrolytically to trim back the various glycoforms in a glycoprotein mixture, and then the mutant ENGase is used to attach a pre-synthesised homogenous oxazoline-activated oligosaccharide to the remaining *N*-acetylglucosamine (Tang *et al.*, 2017, Ou *et al.*, 2021). This enzymatic approach replicates the *N*-glycosylation process *in vitro* but using different, more convenient enzymes and glycosyl donors to avoid the issues associated with OST.

This system has the advantage of easy access to the enzymes, the use of readily available glycosyl donors, and promiscuous specificity for acceptors (Li and Wang, 2018). A range of ENGase mutants has been developed showing differing substrate specificities and

limitations (Fairbanks, 2017, Manabe *et al.*, 2019) some of which are suitable for the glycosylation of mono-glycosyl mAbs such as rituximab (Huang *et al.*, 2012, Li *et al.*, 2016). This approach can be used to attach any of the typical biantennary glycoforms commonly seen in antibodies, getting around the difficulties of using the very complex and sensitive OST.

However, the target protein must already be mono-glycosylated for this approach to work. Fortunately however, approaches have recently developed to achieve this, using *in-vitro* enzymatic protein glycosylation based on the recently discovered bacterial biochemistry.

3.3.7. Installing the Attachment Point using N-Glycosyltransferase

The use of endoglycosidase has limited use for cell-free synthesis of antibodies as they are not glycosylated by typical *E. coli* cultures, so there is no glycoside residue present in the first place on which to build the desired glycan. Fortunately, the single glycoside attachment point which is required for ENGases may be installed on the protein using a cytoplasmic *N*-glycosyltansferase (NGT) from *Actinobacillus pleuropneumoniae* (ApNGT). These recently discovered bacterial enzymes are unrelated to OST but can glycosylate the eukaryotic NX(S/T) amino acid sequence selectively and therefore provide the correct attachment point for the rest of the oligosaccharide (Schwarz *et al.*, 2011). The ApNGT has also been functionally transferred into *E. coli* for wider application (Kightlinger *et al.*, 2019, Cuccui *et al.*, 2017), however, the originally discovered system only adds a single glucose residue from the activated sugar donor UDP-glucose (UDP-Glc), rather than the usual *N*-acetylglucosamine residue (Naegeli and Reymond, 2014).

Fortunately, further work on mutants of *N*-glucosyltransferase ApNGT has revealed that it is possible to transfer glucosamine (GlcN) from UDP-GlcN to a peptide using a deliberately engineered mutant of from *Actinobacillus pleuropneumoniae* ($ApNGT^{Q469A}$) (Song *et al.*, 2017) (Figure 17). Once the initial glucosamine unit is attached, glucosamine-*N*-acetyltransferase from *Clostridium acetobutylicum* (GlmAse) can be used to acetylate the glucosamine and provide the required *N*-acetylglucosamine attachment

point for human antibodies (Reith and Mayer, 2011) as this system recognises the consensus sequon (N-(X≠P)-T/S).

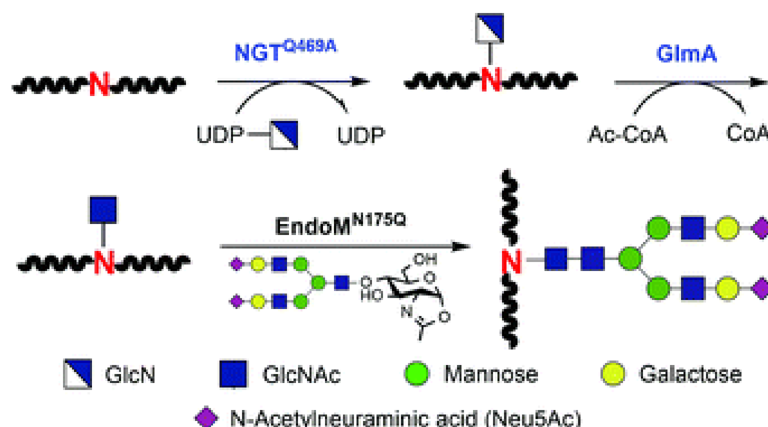

Figure 17: Enzymatic synthesis of glycopeptides carrying natural eukaryotic N-glycans (Xu et al., 2017)

The resultant *N*-acetylglucosamine attachment point allows the use of the oxazoline derivative of the desired pre-formed human oligonucleotide to construct glycans by ENGase-catalysed transglycosylation directly onto a range of short peptides, though not onto actual proteins (Xu *et al.*, 2017) (Figure 18).

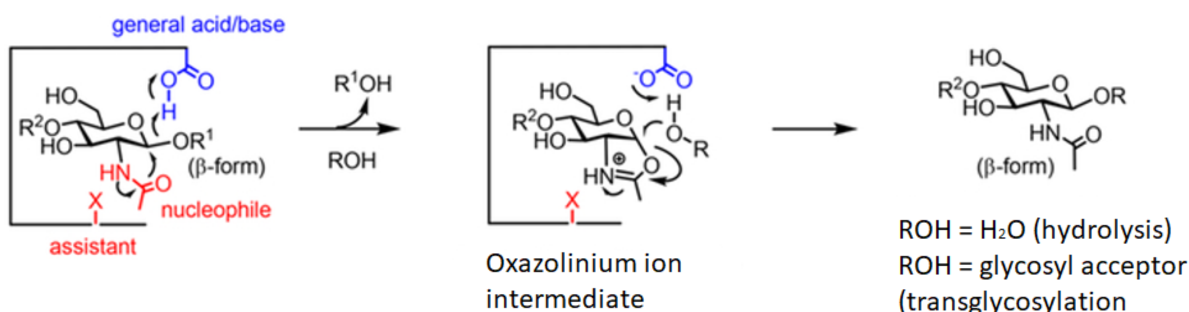

Figure 18: Glycosidase mechanism showing N-acetylglucosamine as glycosyl donor (Li and Wang, 2018)

Furthermore, a naturally occurring NGT has been identified from *Aggregatibacter aphrophilus* (AaNGT) (Kong *et al.*, 2018) which will add a range of glycosyl donors, including glucose (UDP-Glc) and glucosamine (UDP-GlcN) to short peptides with the NX(S/T) sequon *in vitro*. More specifically, both ApNGT and AaNGT have been shown to glycosylate a short peptide mimic of the glycosylation sequon of human IgG1 (Kong *et al.*, 2018), though not yet a full mAb.

A complex array of glycosylation enzymes is coming to the fore, which holds out the promise of efficient engineering of glycoproteins (Kightlinger *et al.*, 2020, Lin *et al.*, 2020). This complexity needs to be better understood, however. The GlycoSCORES high-throughput system uses CFPS and mass spectrometry to rapidly screen polypeptide-modifying glycosyltransferases (ppGTs) to discover their substrate specificities (*i.e.,* where they glycosylate amino acid chains, and what they use as glycosyl donors). These substrate amino acid sequences were then inserted into heterologous proteins and successfully glycosylated in living *E. coli* cytoplasm. This included a human monoclonal antibody (Kightlinger *et al.*, 2018). GlycoSCORES therefore facilitates fundamental understanding of glycosyltransferases specificity in terms of glycosyl donor and polypeptide acceptor, and therefore greatly facilitates the engineering of synthetic glycoproteins

3.3.8. Chemical Attachment of Glycans

This alternative approach takes its inspiration from current progress and research directions in the manufacture of antibody-drug conjugates, combined with opportunities arising from gene code expansion and the use of non-natural amino acids, though in this case non-glycosylated ones. Significant developments have occurred in this area in recent years (Degruyter *et al.*, 2017, Bird *et al.*, 2021).

By reviewing the range of cysteine conjugation methods which are available for ADCs (Kang *et al.*, 2021), it is clear that there are many options. These include more novel solutions, such as reactions with alkynes and their isomeric allenes, as well as the better established maleimide Michael addition (Figure 19).

However, it is also clear that none of them will not deliver a linker to the oligosaccharide which is similar to the native asparagine structure. The degree of similarity which is required is not known, but the ideal situation would involve very small changes, or none.

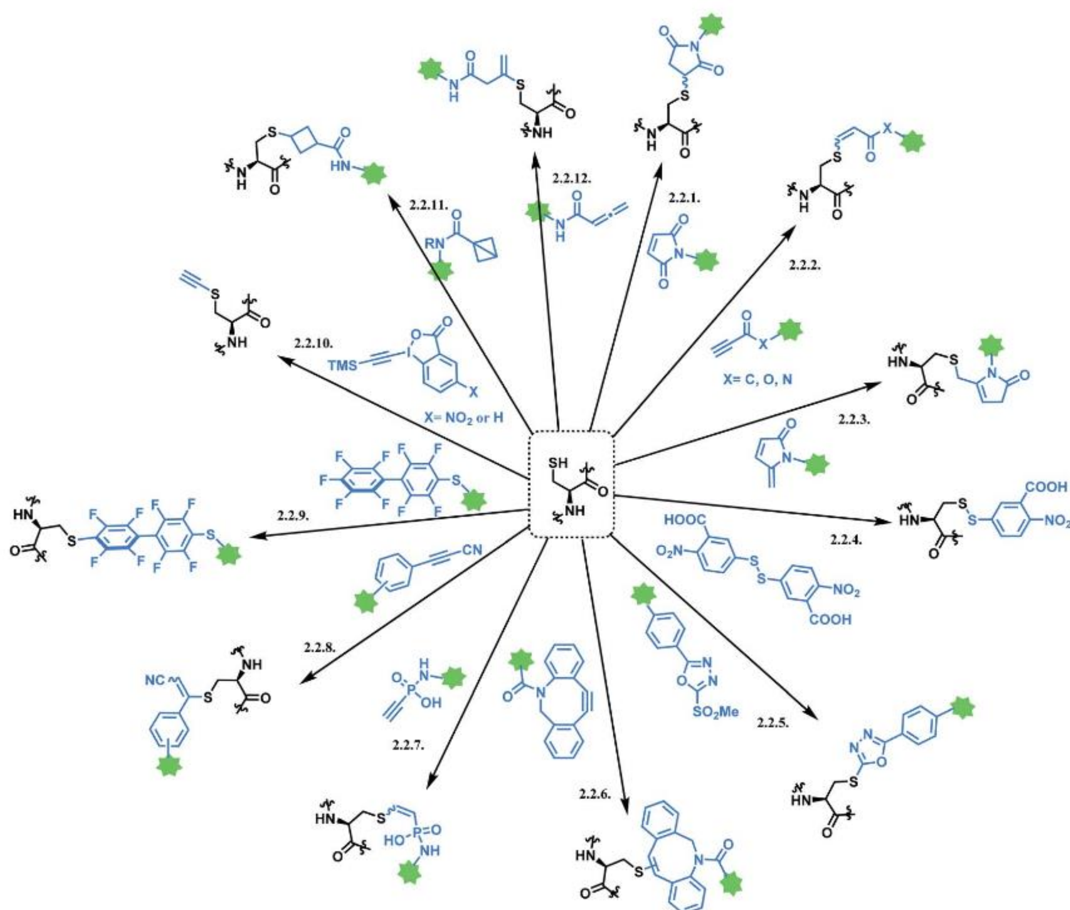

Figure 19: Cysteine Conjugation Methods (Kang et al., 2021)

Macmillan's substitution of a cysteine residue in erythropoietin followed by reaction with glycosyl-β-*N*-iodoacetamide is simple, and although no reaction with the pre-existing native disulfide bonds was observed, success would be dependent on the complete pairing of all native disulfide bonds, leaving the attachment points at Asn 297 free (Macmillan *et al.*, 2001). It is a promising technique, which does not appear to have been applied to the glycosylation of mAbs, possibly because of their greater complexity compared to the erythropoietin used in the original work. It would be interesting to try this chemistry with a selenocysteine-substituted mAb to assess the selectivity, as well as the functional impact. An iodoacetamidoglycan would have an extra -$SeCH_2$- bridging component (Figure 22), but would be relatively similar to the native linkage.

Further work from the Department of Chemistry at Edinburgh University has developed the alkylation concept further. The Hulme Group has used chloromethyl-triazole alkylating agents to selectively alkylate cysteine residues on proteins, to yield a lysine

analogue in place of the cysteine (Brewster and Hulme, 2021) (Figure 20). The purpose of this work was to allow the selective derivatisation of proteins with lysine-specific PTMs using structural mimics. The work still relies on the low abundance of cysteine residues in proteins and may still give rise to problems in a molecule as complex as a mAb.

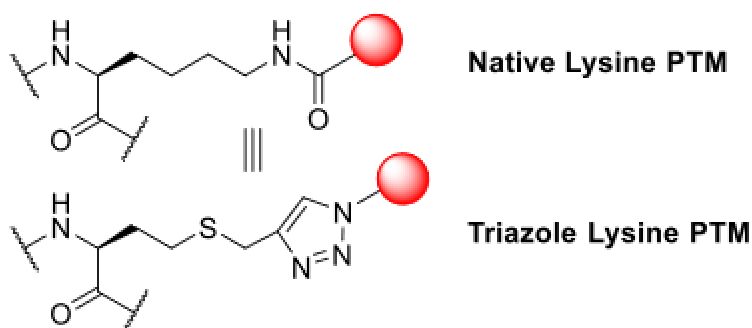

Figure 20: Structural Analogue of Lysine Derived from Cysteine (Brewster and Hulme, 2021)

However, as with Macmillan's work, the selective biosynthetic replacement of Asn-297 with selenocysteine (Sec), by using Sec-specific tRNA (Liu *et al.*, 2017a), might yield interesting results. Selenocysteine is a more reactive nucleophile than cysteine and allows the reaction with alkylating agents at the desired location. It may therefore be more selective to react selenocysteine with a chloromethyl-triazole alkylating agent. This would of course result in a lysine analogue at the 297 position, rather than the native asparagine, and it is unknown how this difference would impact the biological function of the glycosylated mAb.

Another approach uses allenes, rather than alkylating agents. This has been used for the site-specific conjugation of ADCs. Selenocysteine-modified trastuzumab scFv-Fc-Sec has been shown to react with a high yield specifically at selenium with allenes, such as the fluorescent probe below, with no other site on the protein reacting (Pedzisa *et al.*, 2016) (Figure 21). Although the aim of this work did not include conjugation to glycans, the reaction may be an attractive option for selective conjugation to glycans (Figure 22).

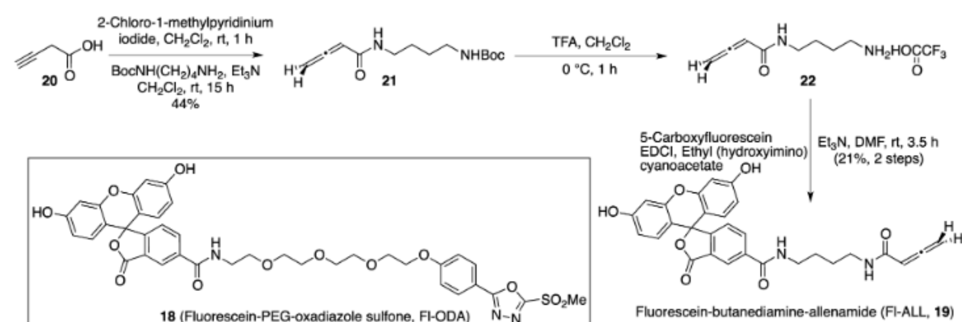

Figure 21: Allenes as Conjugation Agents for Selenoproteins (Pedzisa et al., 2016)

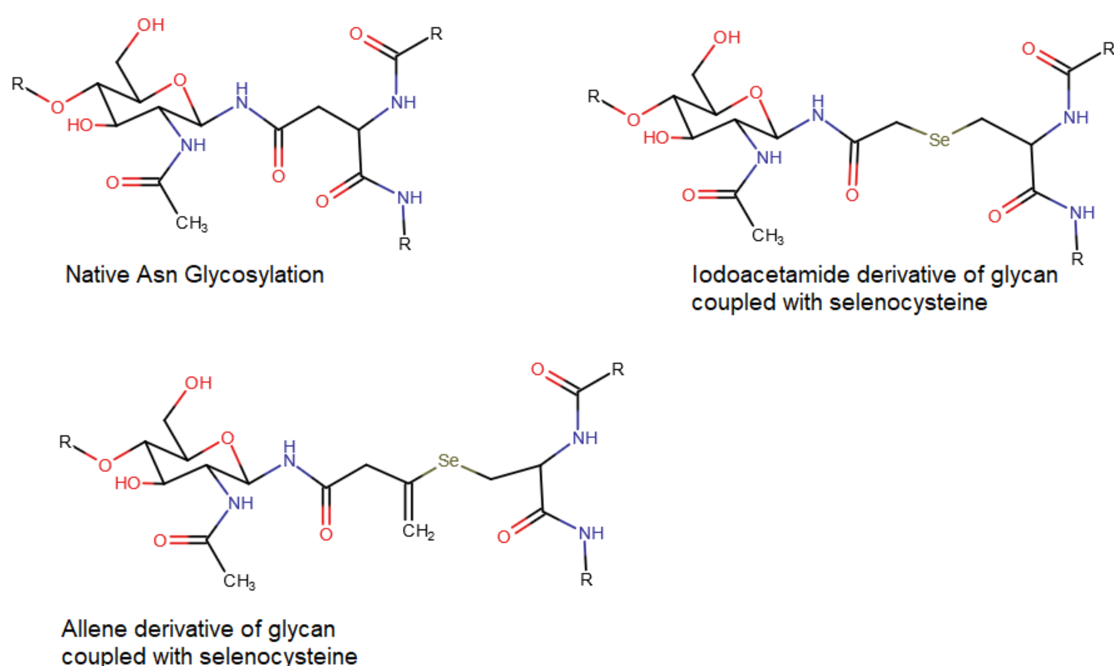

Figure 22: Options for Coupling with Selenocysteine

In order to synthesise the required glycosyl alkynes, a highly stereoselective *C*-glycosylation reaction has been developed whereby glycosyl acetates can be coupled with terminal alkynes via $ZnBr_2$-activation at room temperature (He and Qin, 2018). This procedure has the potential to provide glycosyl alkynes for conversion to allenes (an isomer of alkynes), but without the amide linker in the Pedzisa work, thereby making the length of the linker more similar to the natural linker. The limitation here is that the isomerisation of the alkyne to the allene may scramble the stereochemistry at the anomeric centre of the saccharide.

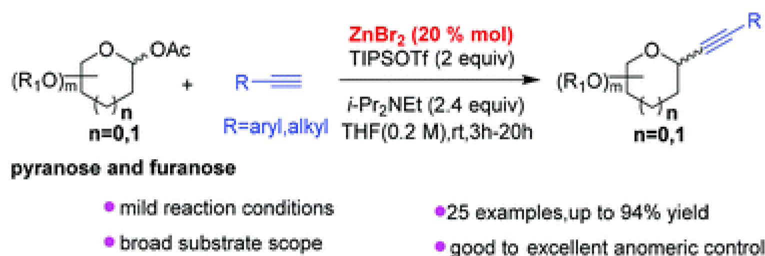

Figure 23: Synthesis of Alkyne-Derivatives of Saccharides

A different angle on this would be to make the mAb electrophilic rather than nucleophilic. This can be also done by replacing the Asn-297 with selenocysteine, which can then be selectively deselenised to give the highly reactive electrophile, dehydroalanine, without desulfurisation of cysteine (Liu *et al.*, 2017a). This is very reactive towards nucleophiles but would likely react with other nucleophilic groups on the mAb, such as cysteine, so a very nucleophilic derivative of a glycan would need to be present *in situ* to react preferentially. For this reason opting for a highly electrophilic attachment point on the mAb in an environment full of nucleophiles may be the wrong direction conceptually. The previously described use of selenocysteine as a nucleophile is likely to be more successful.

Traceless Staudinger reactions are a promising coupling strategy which does not rely on selenocysteine. This chemistry forms an amide bond, but between two chemical groups which have a strong affinity for each other but are not found in proteins. However, no remnant of the original chemical groups remains after the reaction however, just an amide (or peptide) bond. The amine side of the intended amide bond is formed from an azide group, which loses N_2 during the reaction. The carboxyl partner is represented by a diphenylphosphine substituted thioester.

One can imagine the insertion of an azide-containing non-natural amino acid at mAb position 297, and its subsequent reaction with a suitably functionalised saccharide. There is precedent for the incorporation of azide-functionalised the non-natural aromatic amino-acids *para*-azido-*L*-phenylalanine and *para*-azidomethyl-*L*-phenylalanine into proteins for the purpose of strain-promoted azide-alkyne cycloaddition (Zimmerman *et al.*, 2014). This technology is widely used for bio-orthogonal conjugation, however the linker

residue which remains is very large compared with the glycosylated Asn and is therefore unlikely to work directly for glycan coupling. However, the use of azido amino acids in the traceless Staudinger might be more fruitful. Azido-homoalanine has been incorporated into proteins using an engineered orthogonal tRNA/tRNA synthetase pair for genetic code expansion (Saleh *et al.*, 2019). This would then need to be coupled to a glycan with the required phosphinomethylated thioester at the anomeric position. This clearly has significant structural differences from the native molecule, however the impact of such changes on complement functionality is not currently known. Reversing the direction of coupling would be better, by placing the azide function on the sugar, with the coupling partner comprising a thioester-containing non-natural amino acid, as this would maintain the amino group at the anomeric position. However, no such non-natural amino acid has yet been devised, and the diphenyphosphino variant is likely to be too large to easily accommodate in current genetic code expansion techniques. The dimethylphosphino variant might be more successful, however this may not be stable enough with regard to oxidation.

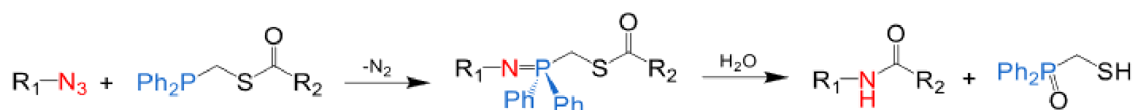

Figure 24: Traceless Staudinger Reaction

Overall, the area of chemical coupling of glycans is looking more and more promising based on advances which are being made in the area of specific ADC coupling reactions. Driven by the therapeutic need for more homogenous ADCs to assure predictable clinical outcomes, this area is likely to deliver interesting options to conjugate appropriately derivatised glycans to non-natural amino acids as position 297. Selenocysteine appears to be a leading candidate due to its analogous but superior reactivity compared to cysteine, as well as the fact that it is a naturally occurring amino acid.

3.3.9. Modification of Alternative Natural Glycopeptides

A great number of steps is required for a total chemical synthesis of the biantennary oligosaccharides required by antibodies. This effort can be greatly reduced by starting the synthesis with a readily available core oligonucleotide which can be modified into the desired mAb structure using specific enzymes.

Hen's egg yolks provide a natural source of a sialoglycopeptide (SGP), which may be isolated on a gram scale in very homogenous form from egg yolk powder by extraction with aqueous acetone and hydrophilic interaction chromatography (HILIC) at a recovery of 1 g/kg of cheap, commercially available yolk powder (Makimura *et al.*, 2006, Sun *et al.*, 2014, Liu *et al.*, 2017b). It has a human-identical, *N*-linked, complex-type, disialyl biantennary oligosaccharide with the same structure as a defucosylated glycan in Figure 14: PeptideAsn-GlcNAc-GlcNAc-Man-(Man-GlcNAc-Gal-Neu5Ac)2.

This was hydrolysed in buffer with native ENGase (EndoM from Mucor hiemalis) between the two *N*-acetalglucosamine residues to release the free complex-type glycan with a yield of 88%. The acetyl glucosamine residue was then be converted without protection to its oxazole derivative using 2-chloro-1,3-dimethzlimidazolium chloride in 15 min with 90% isolated yield. This was then subjected to transglycosylation onto a pentapeptide model using EndoM mutant-N175A giving 88% yield (Huang *et al.*, 2010). It is now possible to directly glycosylate immunoglobulins using a further type of ENGase mutant from *Streptococcus pyogenes* (EndoS) and significant work is continuing in this area (Lin *et al.*, 2015, Thomann *et al.*, 2015, Wang *et al.*, 2019, Parsons *et al.*, 2016).

Alternatively, enzymatic glyco-trimming may be used to remove unwanted terminal saccharide residues from such a naturally available oligosaccharide, followed by enzymatic glycosylation using a panel of mammalian glycosyltransferases to build up a wide range of bi-, tri- and tetra-antennary oligosaccharides (Liu *et al.*, 2019).

3.3.10.Core Fucosylation Using Glycosidase

A further elaboration of the oligosaccharide will be required for some human-like structures: core fucosylation. However, it is becoming clear that the presence of fucose suppresses ADCC activity significantly, so omitting it may actually be beneficial for mAbs whose mode of action depends on ADCC (Suzuki *et al.*, 2007, Imai-Nishiya *et al.*, 2007).

ENGases are however available for the glycosylation of α1,6-fucosylated GlcNAc-peptides using a synthetic sugar oxazoline as a donor (Huang *et al.*, 2011). The oxazoline derivative of a 2-acetamido oligosaccharides is easily made in a single chemical step using 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (Noguchi *et al.*, 2009).

Core fucosylation still allows for the attachment of a bi- and tri-antennary oligosaccharides: Endoglycosidase F3 (Endo-F3) mutants from *Elizabethkingia meningoseptica* were found to be devoid of hydrolytic activity but were able to accept glycan oxazolines as a glycosyl donor in transglycosylation, and one mutant in particular showed a high specificity towards α1,6-fucosyl-GlcNAc-Asn, proving to be very effective for the glycosyl remodelling of the Rituximab mAb (Giddens *et al.*, 2016).

3.3.11.Chemical Synthesis of Oligosaccharides

In contrast to the biosynthesis of glycans, their chemical synthesis *in vitro* is more controllable and predictable, giving relatively pure glycans (Krasnova and Wong, 2019, Nomura *et al.*, 2021). However, glycans are extremely complex branching structures with a huge level of possible structural diversity. Their synthesis involves the stepwise formation of glycosidic linkages from the single anomeric centre of a glycosyl donor to one of the hydroxyl groups on a glycosyl acceptor, thereby building up the desired branched structure. However, because both reaction partners are saccharides, and both contain multiple hydroxyl groups of similar reactivity, it is quite challenging to direct the formation of the bond to the desired hydroxyl *in vitro*. This means that a significant number of extra 'protection' and 'deprotection' steps are required.

Nevertheless, the chemical synthesis of entire glycosylated proteins such as erythropoietin is now possible (Wilson *et al.*, 2013), however for the purposes of mAbs, the chemical approach is only practicable for the oligosaccharide part due to the greater complexity of the antibody protein. Although sugar chemistry is notoriously complex, great advances have been made in the chemical synthesis of oligosaccharides (Li and Ye, 2020), with the successful synthesis of various multi-antennary structures (Wang *et al.*, 2009a, Walczak *et al.*, 2013, Shirakawa *et al.*, 2021, Edgar, 2021, Luber *et al.*, 2018). There is even a programmable system for the one-pot synthesis of oligosaccharide libraries (Cheng *et al.*, 2018).

These advances present two future directions. Firstly, they allow researchers to investigate differences in the biological function of different glycoforms using pure glycoproteins, and even to label them for tracking *in vivo*. Secondly, the resultant increase in understanding of the function of each of the possible glycan structures in an antibody is likely to lead to an increased desire to produce a single isomer reliably on a commercial scale, which would require the development of scalable solutions for industrialisation.

3.3.12.Chemical Coupling of Oligosaccharides to mAbs

Once an oligosaccharide donor has been synthesised, it must be coupled to the monoclonal antibody, which is challenging. There are research-scale methods (native chemical ligation) which synthesise fragments of the final target protein using solid-state synthesis, attaching the oligosaccharide to the fragment, before coupling the fragments together chemically (Unverzagt and Kajihara, 2013). This would not be suitable for large proteins like mAbs however due to yield losses and microheterogeneity in the amino acid chain composition due to the cumulative effect of even low levels of infidelity during the stepwise build-up of such a long peptide chain.

As an alternative, chemoselective ligation uses a tagged protein that has been obtained using site-specific mutagenesis (‘tag and modify’ method). This typically involves the insertion of a free cysteine residue, replacing Asn-297 in a mAb for example. The free cysteine residue has particular properties (soft nucleophile) which allow it to be coupled chemoselectively to a tagged sugar with a cysteine-reactive functional group (Chalker *et al.*, 2011). The selective *in vitro* chemical glycosylation of erythropoietin provides a precedent for the use of site-directed mutagenesis (Asn → Cys) to provide non-native cysteine residues as an attachment point for glycosylation with a glycosyl-β-*N*-iodoacetamide. No reaction with the pre-existing native disulfide bonds was observed (Macmillan *et al.*, 2001).

Glutaminases are a class of enzyme whose function is to cross-link an ε-amino of a lysine residue and a γ-carboxamide of a glutamine residue via an isopeptide bond and have found use in the selective functionalisation of ADCs (Schneider *et al.*, 2020). Work has

also been carried out on the selective conjugation of photosensitiser-antibody conjugates for use in photodynamic oncology therapy. This approach used site-specific chemo-enzymatic modification of a heavy-chain glutamine residue (Gln 295) by microbial transglutaminase (mTGase E.C 2.3.2.13) to introduce a 'clickable handle' (Figure 25), a specific functional group (stained alkyne) which reacts specifically with the desired conjugation via a highly efficient, biologically orthogonal chemistry (azide cycloaddition) (Sadiki *et al.*, 2020) (Figure 26 and Figure 27). The commercially available mTGase catalyses a transamidation reaction in which the unsubstituted amide in the glutaminyl residue is converted into a substituted amide using an amine substrate, which contains the click-chemistry moiety of interest, an alkyne. The alkyne may be attached to any molecular structure of interest.

Figure 25: Schematic of mAb with glutamine 295 highlighted (Sadiki et al., 2020)

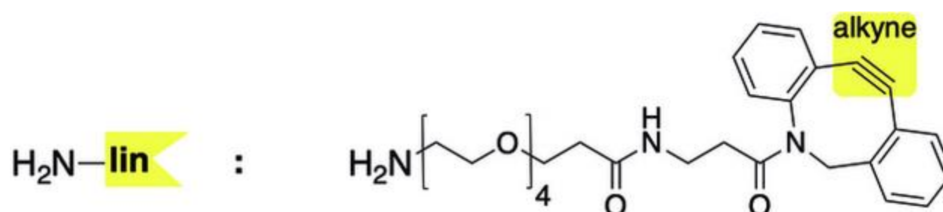

Figure 26: Example of an alkyne-containing linker which can be specifically attached to Gln-295 using transglutaminase (Sadiki et al., 2020)

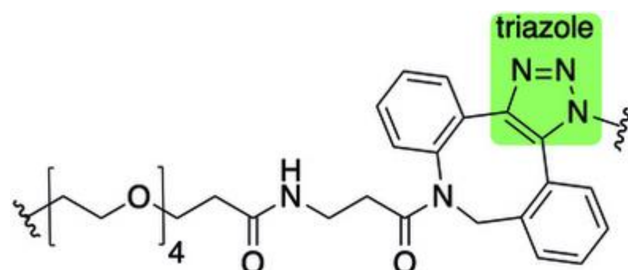

Figure 27: Triazole product of reaction between strained non-linear alkyne and alkyl azide (Sadiki et al., 2020)

4. Literature Review: Towards Cell-Free mAb Manufacture

The background section has provided relatively detailed information on the structure and biosynthesis of mAbs in preparation for an exploration of how this can be achieved *in vitro*. This work now reviews the current state of technologies which could be used in cell-free manufacturing systems for the production of monoclonal antibodies. It examines each of the main technical impediments and challenges which prevent the adoption of such technologies for the commercial production of monoclonal antibodies. Finally, recent developments are presented which have the potential to overcome these issues. It also includes any literature discussions of the advantages and disadvantages of a cell-free approach for commercial production.

4.1. Overview of Cell-Free Protein Synthesis (CFPS)

4.1.1. Basics of CFPS

In recent years, the biopharmaceutical industry has become ever more important, and the complexity, effort and expense associated with the use of live cells in the manufacture of therapeutic proteins has driven a significant amount of work towards cell-free expression systems (Chiba et al., 2021).

CFPS systems rely on a combination of biological and chemical agents to replicate the required cellular functionality to produce a protein. They present significant advantages over cell-based systems (Endo and Sawasaki, 2006). There is no requirement to support non-productive biochemical processes associated with maintaining cell viability and growth, allowing the system to focus resources on the biosynthesis of a single product. The openness of the system ensures that synthesis is not concealed behind a cell wall, which facilitates real-time monitoring, and ensures that any adjustments to the processing conditions are transmitted throughout the medium more rapidly, subject only to stirring and solution dispersion. This also facilitates control over the concentration and identity of enzymes, chaperones and feed components which are present. It is easy to add non-native substrates, purified functional proteins, RNA or recombinant DNA (Silverman et al., 2020). It also allows the use of non-native additives as they do not have to pass the cell wall. This ability to manipulate and experiment with the system has made it very useful for small-scale fundamental research into the key principles of biological systems. The open nature of the system also facilitates analysis during experiments as well as

production, as the mixture is relatively homogenous with all components accessible to sampling.

However, historical constraints such as low and variable protein yields, small reaction scale, short reaction durations, high reagent costs, limited ability to fold complex proteins, and lack of PTMs have limited the application of this technology. Nevertheless, recent developments are addressing each of the issues above, and the number of applications in areas like synthetic biology are increasing (Silverman et al., 2020). Most interestingly, the technology behind cell-free protein CFPS is advancing at a rapid pace and is moving beyond its historical role as a purely small-scale research tool (Carlson et al., 2012). Far cheaper inputs are now able and can deliver titres of more than 1 g/L, with reaction times greater than 10 h and reaction scales of 100 L (Sun et al., 2013).

Conceptually CFPS can be regarded as the separation of the biosynthesis of the required transcription and translation enzymes and co-factors (cell growth) from their later use in protein production (Kwon and Jewett, 2015). A standard CFPS requires a cell extract to provide the biosynthetic transcription and translation machinery. This is usually derived from *E. coli* (Stiege and Erdmann, 1995). The *E. coli* system has the advantages of being cheap, well understood, high-yielding, and scalable (Chiba et al., 2021).

In practice this means that a culture is subjected to lysis to liberate the required biochemical machinery. The culture can be bacterial, particularly *E. coli*, yeast, mammalian (CHO), or plant (wheat germ). This is followed by purification to remove cell debris and chromosomal DNA, leaving the all the required components for transcription and translation, such as aminoacyl-tRNA synthetase and ribosomes (Dondapati et al., 2020). The nucleic acid of interest is added, either as mRNA, DNA from plasmid culture, or linear DNA from PCR. Transfection procedures are not required, and although these are quite reliable, they are very time-consuming, difficult to optimise and sensitive to cell viability, cell passage number, purity and type of nucleic acid. Additionally, there are no requirements regarding the maintenance of cell viability, nor is there a requirement for passaging, and the system is inherently scalable because there is no sensitivity to cell density.

For a ‘coupled’ system with both transcription and translation a DNA template is used along with RNA polymerase, whereas in an uncoupled system mRNA alone is used to code the desired amino acid sequence. After adding amino acids, salts, nucleotides, RNA polymerase (RNAP), an energy source, and any required co-factors, the bacterial extract produces the desired therapeutic protein. Disulfide isomerase chaperones may be added to aid the correct linking of cysteine residues, with added glutathione redox buffer. In this way, all the energies of the biochemical machinery are devoted to the production of the required biotherapeutic, allowing the generation of high antibody titres in a bioreactor within hours rather than weeks.

4.1.2. History of CFPS

From a historical perspective, the dissection of cells to isolate and study their constituent parts originally led to the discovery of organelles such as the cell nucleus, the mitochondrion, the Golgi apparatus, and the endoplasmic reticulum. More sophisticated studies used staining and labelling to study the function of such organelles, and it was discovered that much of the biochemical activity of cells is maintained even after disrupting the plasma membrane.

Studies of CFPS were initiated as far back as the 1950s (Nirenberg and Matthaei, 1961, Nirenberg and Leder, 1964). Tissue homogenates and extracts, or cell-free systems, were used to study cellular biochemistry and have elucidated processes such as the incorporation of amino acids into proteins (Winnick, 1950, Borsook, 1950), the role of adenosine triphosphate (ATP) in protein synthesis (Hoagland *et al.*, 1956), and the discovery of the role of the codon in the translation of mRNA into proteins (Matthaei and Nirenberg, 1961, Matthaei, 1961, Söll *et al.*, 1965, Holley *et al.*, 1965). The latter area of research led to the awarding of the Nobel Prize for Physiology or Medicine to Nirenberg, Holley, and Khorana in 1968.

Separating the intact cellular components based on their size and density using differential centrifugation led to a more precise understanding of the organelles involved in protein synthesis in the context of the structural and functional organisation of the cell. This work led to the awarding of the Nobel Prize in Physiology or Medicine to Albert Claude, Christian de Duve and George E. Palade in 1974.

In a significant step towards applying this understanding to CFPS, Ledermann and Zubay (1967) achieved coupled transcription-translation using a cell-free system using a bacterial extract which allowed DNA to be used as a template. Although these efforts were not directed at the commercial extra-cellular production of therapeutic proteins, they did act as a proof of concept: specific cellular functionality is feasible without the support of a fully functioning living cell.

4.1.3. Cell Extracts

Current technologies rely on large volumes of cell extracts, particularly from bacterial cultures such as *E. coli* (Gregorio et al., 2019). This well understood host offers several advantages, such as the use of inexpensive growth medium, rapid accumulation of biomass, high cell density and ease of scale up (Baneyx and Mujacic, 2004).

After lysis, the cell extract is purified by centrifugation and dialysis (Hiering *et al.*, 2022), as well as treatment with nucleases to remove endogenous DNA and RNA. Ensuring repeatable high activity can be challenging, and cell lysis can be hard to standardize, however more reliable methodologies are becoming available on scales up to 10 L of broth (Kwon and Jewett, 2015).

It is also possible to produce mAbs directly in bacterial cells (Simmons *et al.*, 2002, Frey *et al.*, 2008), however these will not be glycosylated with mammalian structures in their natural state. Also, there are additional problems with the aggregation of misfolded and partially folded intermediates into so-called inclusion bodies in the crowded environment of the bacterial cytoplasm (Baneyx and Mujacic, 2004). However, in recent years the emerging area of synthetic glycobiology has allowed the transfer of non-native glycosylation pathways into *E. coli* from other organisms enabling the production of complex glycoproteins in the intact cell (Valderrama-Rincon *et al.*, 2012, Keys *et al.*, 2017, Tytgat *et al.*, 2019).

Although cell lysates are the most feasible approach for commercial production, it has also been possible to reconstitute all the required components of the *E. coli* translation

mechanism from more than 100 individually purified components to deliver high translation efficiency and an easily purified product (Shimizu *et al.*, 2001).

4.1.4. Advantages of CFPS

CFPS offers several advantages (Table 2). Production times are in the order of hours to reach harvest titres, rather than weeks. Current cell-based systems require many weeks from the inoculation to the isolation of the final purified bulk drug substance, with one month being a typical cycle time for the upstream cell culturing process alone (Lacki *et al.*, 2018). Protein yields are now in the grams per litre range, with reaction volumes above 100 L, and costs have been reduced by orders of magnitude (Carlson *et al.*, 2012).

Table 2: Comparison of cell-free and cell-based protein synthesis

Parameter	Cell-Free	Cell-Based
Synthesis time	Fast (hours)	Slow (weeks)
DNA template	Simply add mRNA/DNA from PCR or DNA from plasmid	Requires DNA to be transfected via plasmid vector
Versatility	Easy to change nucleic acid input	Fixed host cell system
Commercial Applicability	Mainly limited to R&D currently, but progressing upscale	Proven, reliable and well understood for R&D and commercial production
Regulatory	Not recognised for commercial production	Comprehensive regulatory guidance and precedent in place
Protein yields	Yields up to several mg/mL	Yields above 10 mg/mL.
Purification	No lysis required prior to product harvest. No issue with liberation of nucleases and proteases as these can be removed from the lysate prior to use. However bacterial lysates present extra risk of contamination with endotoxins	Need to additionally lyse the cells for product harvest, releasing myriad unwanted cellular components as well as proteases and nucleases. There is also a risk of endotoxin contamination, though exogenous and lower for mammalian systems.

Parameter	Cell-Free	Cell-Based
Post- and co-translational modifications	Limited PTMs in prokaryotic and eukaryotic lysates. *O*-Glycosylation not possible	All PTMs are possible including glycosylation
Non-standard amino acids	Suited to the incorporation of non-canonical amino acids	Difficult due to cell membrane barrier and cytotoxic effects
Scale of reaction volume	Ranging from few µL (chip-based) to 100 L reaction (bioreactor)	Typical minimum of 5 mL up to tens of thousands of litres
Control	Open system, no cell membrane, easy control of reaction conditions	Completely closed system with constrained ability to manipulate

There is also the great advantage that the instruction for desired protein is a free-floating ribonucleic acid, which means that the laborious cell cloning step is not required. During clone development, first a suitable plasmid needs to be generated, whose genetic makeup will require the insertion of a promoter sequence, an antibiotic resistance gene, and a selectable marker using restriction enzymes. Then the DNA of the plasmid is cleaved by a specific restriction enzyme at one of its restriction sites, leaving 'sticky' ends. The same restriction enzyme is used to isolate the gene of interest from the donor organism, leaving complementary sticky ends. A proportion of the plasmids will then incorporate the target DNA in a process called transformation, however many plasmids will just re-close, or else they can insert with the wrong orientation, making the process rather hit-or-miss. It may then be necessary to sequence the insert as a check step, before the transfection process can start, which is also rather hit or miss, requiring further work to select a successfully transfected host cell which is expressing the gene of interest stably and efficiently. Cell-free systems skip all these steps.

A further advantage of CFPS is that the adjustment of conditions to optimise quality and to improve protein yield and folding is straightforward, without the complication of the cell membrane barrier, or of complex, poorly understood biological interdependencies, inhibition pathways, and feedback loops.

CFPS can now be implemented on a wide range of scales for the production of full-length, correctly folded, aglycosylated versions of IgG1 antibodies such as trastuzumab (Herceptin ®), as well as single-chain Fv (scFv) and Fab antibody fragments (Yin *et al.*, 2012).

It is also possible to produce cytokines, complex disulfide-bonded proteins, on a commercially relevant scale above 100 L (Zawada *et al.*, 2011), achieving protein titres above 1 g/L, with batch reactions continuing for up to 10 hours. This long-lived expression was achieved using a maltose metabolism with efficient phosphate recycling and represents a significant achievement in terms of obtaining a useful titre and yield from each batch of cell extract (Caschera and Noireaux, 2014). It compares very favourably with the multiple weeks which are required for cell passaging and growth in conventional CHO systems (Zawada *et al.*, 2011).

4.1.5. PURE System

Instead of using a cell lysate or extract, it is now possible to use a CFPS system referred to as Protein Synthesis Using Recombinant Elements (PURE) to rapidly express proteins from their genes (Shimizu *et al.*, 2005, Shimizu *et al.*, 2001). This system consists of individually purified components, which are obtained from cells. These consist of amino acids, nucleoside triphosphates (NTPs), a pH buffer, salts (potassium glutamate and magnesium acetate), spermidine, creatine phosphate, a redox buffer, as well as the essential elements of the *E. coli* translation system (ribosomes, tRNA mix, peptidyl-prolyl isomerase, kinases for energy cascade), a range of required recombinant protein factors for initiation, elongation, release, and ribosome recycling, 20 aminoacyl tRNA synthetases, and methionyl tRNA formyltransferase (New England Biolabs, 2022, Kuruma and Ueda, 2015).

The PURE system is a useful technique for functional genomics, which integrates molecular biology and cell biology to relate the genotype to the phenotype by fully understanding the dynamic interplay of transcription, translation, protein-protein interactions, and epigenetic regulation of gene expression. It is also a useful tool for proteomics, the larger-scale study of the expression and interaction of proteins in an organism. It is not used for commercial-scale production of biopharmaceuticals.

It does however have several features which would be advantageous for commercial production of antibodies. This system does not contain any proteases which can degrade the protein product, which can lead to impaired quality and reduced yield. It is also free of nucleases which can degrade the RNA components required for successful protein synthesis. It allows the components of the CFPS system to be chosen based on their function and the needs of the protein to be synthesised. This includes the addition of bacterial OSTs for the targeted glycosylation of proteins (Guarino and Delisa, 2012). It also features polyhistidine tags on all protein factors, which enables their targeted removal during product purification by using a nickel nitrilotriacetic acid (Ni-NTA) resin.

However there are significant limitations associated with the PURE system, which become apparent once it is compared with the more traditional *E. coli* cell lysate system (Li *et al.*, 2017). Yields of over 100 µg/mL are possible with PURE (New England Biolabs, 2021), which compares with up to 400 µg/mL with a cell lysate (Prime, 2009), and up to 10 mg/mL with a modern commercial mAb process (Li *et al.*, 2010). Cell lysates contain ancillary components, such as recycling enzymes, metabolic enzymes, chaperones, and foldases, and may explain the greater productivity of extract-based systems (Hammerling *et al.*, 2020). Although acceptable for small-scale research, the cost of PURE is also prohibitive, with an estimate of a factor of 1000 more for the milligram-scale production of a protein (Hong *et al.*, 2014b).

4.2. CFPS for mAbs

4.2.1. Advances in CFPS

There have been several obstacles associated with cell-free protein synthesis in general, (Katzen and Chang, 2005), however recent advances have addressed many of the issues, all of which are relevant to the cell-free production of mAbs.

The period of protein production can be short due to degradation of required components, especially for batch reactions. By switching to a fed-batch format with replenishments of the energy source, such as phosphoenol pyruvate (PEP), as well as additional arginine, cysteine, tryptophan and magnesium, the final concentration of a cell-free synthesised

protein (chloramphenicol acetyl transferase) increased more than 4-fold compared to a batch reaction (Kim and Swartz, 2000).

The rapid degradation of template mRNA in the presence of ribonuclease under cell-free conditions can also lead to the unwanted termination of the protein production process. The complete removal of ribonuclease is very difficult so the issue has been addressed by coupling translation with continuous transcription of source DNA into the required mRNA using nucleoside triphosphate as the energy source in an *E. coli* lysate (Kitaoka *et al.*, 1996).

The rate of protein production can be low and there is also a challenge to get the large amount of nutrients and energy to the biochemical machinery fast enough, and to remove the by-products rapidly to prevent them causing deleterious changes to the chemical environment of the biosynthesis. Therefore, to address these issues and produce meaningful amounts of protein, the batch-type approach can be replaced with dialysis reactors, which allows a longer run time Figure 28. The initial design of this semi-continuous approach was the continuous flow cell-free (CFCF) translation system (Spirin *et al.*, 1988). Feeding buffer is actively pumped into a reaction vessel, with a balancing flow of liquid then being pumped out of the vessel through an ultrafiltration membrane, which retains the large-molecule components of the system.

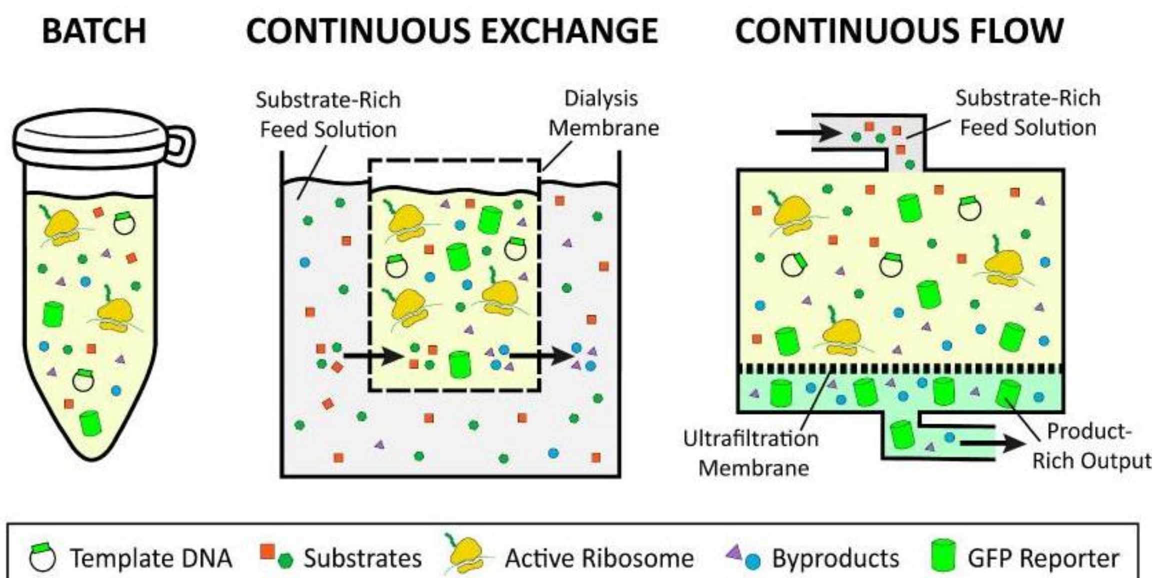

Figure 28: Continuous Exchange vs. Continuous Flow Modalities (Gregorio et al., 2019)

This system was later improved to the continuous exchange cell-free (CECF) system, which relies on passive exchange of substrates. Diffusion of reaction components occurs across a membrane between a feeding reservoir which contains the required small-molecule nutrients and energy sources, and a reaction chamber where protein production occurs. A continuous flow of feeding solution, coupled with the removal of by-products keeps the production chamber at a relatively steady concentration with respect to inputs and by-products, while the concentration of product increases.

The constant supply of fresh nutrients and energy molecules addresses the degradation issue, and by continuously removing inhibitory by-products, the chemical environment is maintained in a productive state. This system can extend protein synthesis periods to tens of hours, with a hundredfold increase in yield. It is even possible to reactivate the biochemical machinery to restart production after it has been terminated for an initial harvest (Lim *et al.*, 2018).

However, the efficiency and economics of the CECF system has been an issue due to the large excess of small-molecule feed substrates required, with the excess of the energy sources (phosphoenol pyruvate and creatine phosphate) being of particular concern. The use of glucose, or glycolytic intermediates such as fructose-1,6-biphosphate as an energy source in *E. coli* lysates has however addressed this issue, with up to 10 mg/mL of protein being produced at greatly reduced input costs (Kim *et al.*, 2008, Shrestha *et al.*, 2014).

4.2.2. Mammalian Lysates

As well as using bacterial lysates, it is also possible to use mammalian systems such as rabbit reticulocyte lysate (i.e., immature red blood cells). The advantage of mammalian lysates is that they may retain the ability to form disulfide bonds and perform complex PTMs. This is particularly relevant to mAbs as they contain complex oligosaccharides.

Progress has also been made on the use of CHO cell lysates containing microsomes, the still-functional, vesicle-like remnants of the ER after lysis. By attaching an ER-targeting signal sequence to the DNA encoding the antibody, the antibody polypeptide is translocated into the microsomes, allowing antibody assembly with the expected

functionality and post-translational modification (Stech *et al.*, 2017, Dondapati *et al.*, 2020, Martin *et al.*, 2017).

This cell-free approach combines the advantages of an open system, with the functionality of the usual eukaryotic biochemical machinery to ensure proper protein folding, assembly and disulfide bond formation (chaperones and peptidyl-prolyl isomerase), as well as successful glycosylation. However, given that this system relies on the slow growth and high sensitivity of CHO cells anyway, it is unlikely to progress beyond the early-phase development purposes outlined in the paper, especially as the yields were low.

Recently cell-free systems have been developed which can perform post-translational modifications (Goerke and Swartz, 2008). Cell-free systems can achieve glycosylation by adding CHO microsomes to the lysate (Gurramkonda *et al.*, 2018), however these systems still rely on the culture of mammalian cells. An alternative approach relies on the insertion of heterologous membrane-bound oligosaccharyltransferases into bacterial cell-membrane-derived vesicles in cell-free lysates (Hershewe *et al.*, 2021).

4.2.3. Cell-Free Bacterial Glycoengineering

Bacteria have the advantage of much more rapid growth than mammalian cells, and the ability to use a standard extract for all mAbs as there is no requirement for transfection. However, a significant drawback is that bacterial cells are normally unable to perform complex PTMs such as glycosylation, and therefore cannot produce glycoproteins like antibodies.

However, recent developments are changing this due to the emergence of the field of cell-free synthetic glycobiology (Czlapinski and Bertozzi, 2006), which is enabling the construction of glycoproteins outside of living cells by synthesising glycosylation enzymes *in vitro* and allowing them to perform the required glycosylation (Jaroentomeechai *et al.*, 2020). Genetic manipulation has equipped *E. coli* cells with glycosylation enzymes which allow them to produce glycoproteins bearing the therapeutically relevant core *N*-glycan $Man_3GlcNAc_2$ (Valderrama-Rincon *et al.*, 2012, Glasscock *et al.*, 2018)

This field has allowed the uncovering of the underlying mechanisms of glycosylation, as well as the preparation of structurally pure glycoproteins using a range of cell-free platforms based on purified enzymes and cell lysates (Dudley *et al.*, 2015, Perez *et al.*, 2016, Silverman *et al.*, 2020). There have been significant improvements in yield (Des Soye *et al.*, 2019, Caschera and Noireaux, 2014) and operational volume (Zawada *et al.*, 2011, Yin *et al.*, 2012)

A cell-free platform has been developed for the rapid synthesis of *N*-glycosylating oligosaccharyltransferases. These are transmembrane proteins, rather than free-floating, so lipid nanodiscs were used as cell membrane substrate mimics, and titres of up to 0.4 mg/mL were obtained (Schoborg *et al.*, 2018). The enzymes were shown to successfully *N*-glycosylate based on their natural sequons.

In a further development in bacterial glycoengineering, a one-pot *E. coli*-based CFPS system has been developed which seamlessly incorporates asparagine glycosylation (Guarino and Delisa, 2012). The lysate may also enriched in free, cytosolic oligosaccharyltransferases and lipid-linked oligosaccharides (Jaroentomeechai *et al.*, 2018). Further work has characterised and increased the concentration of native cell membrane vesicles in bacterial cell lysates, allowing their enrichment in oliogosaccharyltransferases and lipid-linked oligosaccharyltransferases (Hershewe *et al.*, 2021).

Glycosylation on a chip has also been explored and allows the continuous flow of a substrate solution through a sequence of microfluidic cells, each containing a different immobilised oligosaccharyltransferase (Aquino *et al.*, 2021). This temporal and spatial separation of each of the enzymes means that there is better control over the final structure of the glycan.

Improvements have also been made to the efficiency of folding in yeasts by engineering a non-native oxidizing environment in the cytoplasm, and by expanding the ER to increase production capacity, combined with the over-expression of the peptidyl-prolyl isomerase CPR5, leading to a tenfold increase in output (De Ruijter *et al.*, 2016). *N*-linked glycosylation machinery of the consensus sequence (Asn-Xaa-Ser/Thr) has been

discovered in the bacterium *Campylobacter jejuni* and has been transferred into *E. coli*, though the glycosylation pattern was not eukaryotic, the insertion of an *N*-glycosylation cassette into the workhorse *E. coli* system opened up the possibility of further development of recombinant *N*-glycosylating systems for wider application (Wacker *et al.*, 2002).

4.2.4. Folding & Disulfide Bonds

In addition to glycosylation, one of the main challenges for cell-free systems is to ensure that their protein products are correctly folded, and that the right intra- and interchain disulfide bonds in place. Previous studies of the folding pathways in the human endoplasmic reticulum have led to a screen of chaperone proteins which identified the disulfide isomerase DsbC and the peptidyl-prolyl isomerase FkpA as key folding chaperones for *in-vitro* antibody synthesis, such as trastuzumab (Groff *et al.*, 2014).

A disadvantage of bacterial cell lysates is that the usual collection of mammalian chaperones and proline isomerases is not present. There is also no endoplasmic reticulum or Golgi apparatus, the key mammalian organelles which allow for compartmentalisation of protein production between unfinished molecules, and finished ones, which are ready for their final destination, such as secretion.

On the other hand, the open nature of the system means that any externally added folding catalysts or chaperones has immediate access to the protein synthesis without needing to cross the cell membrane. Purified chaperones such as DnaK, DnaJ, GroEL and GroES have demonstrated some benefit for the synthesis of single chain and Fab antibodies (Jiang *et al.*, 2002, Ryabova *et al.*, 1997).

Achieving high levels of native folding of proteins *in vitro* using such an approach requires significant optimisation, however there are significant advantages compared with the environment *in vivo* where the nascent or new peptide chain must fold onto itself despite the many competing interactions with myriad other cellular components in a cell. It must also contend with changes in the osmotic state of the cell, which is not a static environment. This results in ca. 30% misfolded protein output, which is then degraded by proteosomes instead of being released from the ER (Schubert, 2000). In contrast, the concentration of proteins can be controlled in a cell-free system, and the number of

interfering proteins is greatly reduced, especially in purified lysates (Rosenblum and Cooperman, 2014).

Studies have shown that the formation of disulfide bonds between the light and heavy chains is one of the main limitations of Fab biosynthesis (Feige and Buchner, 2014). Intradomain bonds need to be in place to generate the initial folding intermediate. CFPS lacks an endoplasmic reticulum, the oxidising environment in eukaryote cells where disulfide bond formation occurs. However this can be overcome, and cell-free systems have been shown to correctly form disulfide bonds (Goerke and Swartz, 2008). By eliminating the reducing dithiothreitol from the cell-free extract, using a suitable glutathione redox buffer, and adding disulfide isomerase, the formation of disulfide bonds can be optimised (Kawasaki *et al.*, 2003). The cell-free production of single-chain variable fragment (scFv) was possible on a laboratory scale with the use of protein disulfide isomerase as part of a glutathione redox system in combination with molecular chaperones (Ryabova *et al.*, 1997). In fact, the open nature of the cell-free system in amenable to the optimisation of the redox environment to maximise correct disulfide formation.

Strains of *E. coli* are available commercially which contain DsbC disulfide isomerase and have been shown to be effective for the correct formation of disulfide bonds in proteins with multiple linkages (Dopp and Reuel, 2020). The supplementation of chaperone proteins and rational design of processing conditions have also been shown to be successful (Focke *et al.*, 2016, Henrich *et al.*, 2015, Goerke and Swartz, 2008).

Another key feature of eukaryotic cells which makes them more amenable to the production of antibodies is the presence of peptidyl-prolyl isomerase, which also facilitates folding. A poor-yielding yeast system, whose ERs are not suited to the production of complex human antibodies, displayed a ten-fold increase in antibody production by using a strain with an enlarged ER and overexpression of CPR5 peptidyl-prolyl isomerase (De Ruijter *et al.*, 2016).

4.2.5. Application to mAbs

CFPS has relatively recently been successfully applied to the production of aglycosylated mAbs from a eukaryotic system for the first time based on commercially available CHO extract, however only on a laboratory scale for research purposes. The reaction conditions were optimised to ensure disulfide bond formation and to increase protein yields to >100 mg/mL, with a key factor being the separate addition of the heavy and light chain plasmids to ensure intact mAb production (Martin *et al.*, 2017). Shortly afterward, a microsome-containing translationally active CHO lysate was developed where some limited glycosylation was achieved by fusing the antibody gene to an ER-specific signal sequence, thereby ensuring that the antibody underwent the required folding and assembly in the ER lumen (Stech *et al.*, 2017). Again, this was on a laboratory scale for research purposes. Although these represent useful developments, the use of CHO lysates negates many of the advantages of cell-free systems.

In fact, several advances have been made in the introduction of glycosylation biomachinery into the more practical *E. coli* extract, which is bringing the commercial production of fully glycosylated mAbs closer. It is now possible to synthesise active bacterial OSTs from a range of species such as *Campylobacter jejuni*, despite their large size and transmembrane nature, by using an *E. coli* cell-free extract containing lipid nanodiscs as cellular membrane mimics (Schoborg *et al.*, 2018). The primary aim of this work was to allow the routine synthesis and characterisation of OSTs to identify possible uses in biotechnology. This is particularly important given that only 1,862 glycosyl transferases have been characterised from the nearly 850,000 which are listed in the CAZy database of carbohydrate-active enzymes (Drula *et al.*, 2022). In addition, this work included the demonstration of *in-vitro* glycosylation of a single-chain Fv antibody fragment which was deliberately engineered to have the appropriate *C. jejuni* glycosylation sequon DQNAT.

The rate-limiting process in CFPS is translation initiation, not elongation, however modifications in the translation initiation region (TIR) of the DNA template have enabled significant improvements; the introduction of the light chain DNA first ensured that the mAb heavy chain was subsequently able to fold correctly using the known LC-dependent templating effect, and delivered a functional Fab LC-HC heterodimer (Yin *et al.*, 2012).

In fact, this work showed that it is possible to produce fully assembled aglycosylated IgG with titres of several hundreds of mg/mL over many hours of production, followed by a three-step purification using protein A affinity chromatography, hydrophobic interaction chromatography (HIC), and size-exclusion chromatography, achieving standard specifications for host cell proteins and endotoxins. It was noted however that the heterotetramer IgGs consisted of a mixture of covalently and non-covalently bound tetramers.

4.3. Expanding the Genetic Code

4.3.1. Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) are one of the fastest growing types of oncology drugs (Baah *et al.*, 2021), and some aspects of their production may be usefully applied to the cell-free synthesis of mAbs. ADCs combine the specificity of antibodies towards a chosen target on cancer cells with a cytotoxic chemical payload, which is attached via a chemical linker (Khongorzul *et al.*, 2020). Commercial products use rather random attachment of amine-reactive or thiol-reactive payloads to lysine or cysteine residues respectively. The large number of such residues leads to great variability in the number, site, and distribution pattern of conjugation on the antibody.

4.3.2. Non-natural Amino Acids

An alternative is the use of clearly defined bioconjugation sites, which can be selectively derivatised despite the myriad reactive sites present on mAbs (Walsh *et al.*, 2021, Huang and Liu, 2018). This approach can greatly improve stability, pharmacokinetics, efficacy and the safety profile (Shastri *et al.*, 2020). This relies on the repurposing of one of the three naturally occurring stop codons by genetically incorporating a specially developed tRNA/aminoacyl tRNA synthetase pair, so that it mis-recognises the stop codon and incorporates a non-natural amino acid (nnAA), instead of terminating the translation (Chin, 2017, Wang *et al.*, 2001, Wang *et al.*, 2003, Hong *et al.*, 2014a, Wang *et al.*, 2020a).

This genetic code expansion (GCE), an important tool of synthetic biology, is commonly based on the promiscuous nature of the aminoacyl-tRNA synthetase corresponding to the 22nd proteinogenic amino acid, pyrrolysine (Pyl) with respect to its amino acid and the

anticodon of tRNA(Pyl). This is actually one of the three stop codons (the so-called amber stop codon TAG/UAG in DNA and RNA respectively), but has been repurposed for pyrrolysine in the methane producing archeon, *Methanosarcina barkeri*, where it was first discovered (Srinivasan *et al.*, 2002). This amino acid is not present in humans. The promiscuity of the pyrrolysine system and its engineered derivatives has allowed the amber stop codon to be repurposed in other organisms to allow the genetic incorporation of more than 150 non-natural amino acids using bacterial, mammalian or yeast cells (Wan *et al.*, 2014, Dunkelmann *et al.*, 2020, Nguyen *et al.*, 2009).

Challenges with the incorporation of nnAAs have included the low catalytic efficiency of the non-natural or orthogonal mRNA aminoacyl synthetases (o-aaRSs), as well as competition from the natural release factor RF1, which responds to the amber stop codon by terminating the protein chain (Hong *et al.*, 2014a, Hammerling *et al.*, 2020). One such approach is to entirely genomically modify an RF1-deficient strain of *E. coli* to replace all of its amber stop codons (TAG) with the alternative TAA codon, thus allowing the removal of the corresponding RF1 (Ko *et al.*, 2013, Hong *et al.*, 2014b, Martin *et al.*, 2018).

A further innovation is a ribozyme-catalysed *in-vitro* aminoacylation system called Flexizyme. This relies on a ribozyme to catalyse acylation reaction between a tRNA and a chemically derived, activated ester derivative of a nnAA (a cyanomethyl ester, a dinitrobenzyl ester or a [2-aminoethyl]amidocarboxybenzylthioester) (Lee *et al.*, 2019). Flexizymes aminoacylate any tRNA with a wide range of nnAAs regardless of anticodon, though this has not been achieved with glycosylated nnAAs. This could however be a significant challenge not only due to the steric bulk of the sugar, but also the possible reaction of the sugar hydroxyls with the activated esters.

4.3.3. Commercial Systems

Many technical limitations have now been addressed and a commercial system is now available from Ambrx (EuCODE™) and has been implemented for the culturing of mAbs containing conjugatable nnAAs in mammalian cells in 2000L bioreactors, with titres greater than 1.0 g/L. The defined location of the nnAA allows the precision derivation to form ADCs for oncologic targets (Kang *et al.*, 2018, Shastri *et al.*, 2020, Ambrx, 2021,

Jackson *et al.*, 2014, Axup *et al.*, 2012). Homogeneity levels greater than 90% are claimed after forming a stable oxime linked to the non-natural *para*-acetyl phenylalanine residue. However, this system is reliant on mammalian cell culture.

A cell-free expression platform (XpressCF+ ™) from Sutro Biopharma (California, US) is based on a repurposed amber stop codon and uses an aaRS-tRNA pair which is orthogonal to the host system, i.e., the nnAA-specific synthetase does not recognise any of the host tRNAs or cognate natural amino acids, and the orthogonal tRNA is not aminoacylated by any host aaRS. It claims to deliver g/L yields of proteins in 8-10 h at any scale (Sutro Biopharma, 2021). It uses the continuous fermentation of biomass to provide lysate from a mutant *E. coli* strain (Zimmerman *et al.*, 2014) and is able to produce proteins, including mAbs, with non-canonical amino acids such as *para*-azidophenylalanine or *para*-(azidomethyl)phenylalanine by repurposing the amber codon and suppressing the natural release factor termination (Yin *et al.*, 2017).

These ncAAs are then used as conjugation sites for ADCs to attach a toxin or drug (‘warhead’) to the antibody to form an antibody-drug conjugate (ADC), using strain-promoted alkyne-azide cycloaddition (SPAAC) chemistry. This is very a very efficient and specific ‘click’ chemistry which attaches the warhead to the desired nnAA, rather than a variable selection of the natural cysteines (Oliveira *et al.*, 2017, Lang and Chin, 2014, Sletten and Bertozzi, 2009). However, this system can only produce aglycosylated versions of mAbs (Yin *et al.*, 2012).

There are several drugs at the clinical stage, including a Bristol Myers Squibb candidate for multiple myeloma, manufactured under GMP conditions at Sutro Biopharma. The process is based on the continuous culture in a bioreactor of a proprietary *E. coli* strain. This strain has been optimised by incorporating IgG-folding chaperones DsbC and FkpA, and orthogonal tRNA for nonnatural amino acid production. Enzyme knockouts have been used to stabilise the system, and chaperones were re-optimised (Groff *et al.*, 2022). The lysate is clarified and may be freeze dried for transport. It is then reconstituted and combined with a plasmid containing the DNA of interest, RNA polymerase, synthetase and tRNAs. Standard chromatography is used to purify the antibody, giving a crude pre-harvest yield of 233 g from an 800 L reaction (0.3 g/L) after a 24 h culture cycle. The

yield after purification was 147 g. The subsequent azide-based click chemistry is reported as having >85% yield to attach the payload (Anik, 2020).

4.3.4. Application to mAb Glycosylation

A further possibility for non-natural amino acids is to insert a glycosylated version of asparagine at the 297 position of a mAb using genetic code expansion, and then use this handle for the attachment of the desired pre-formed glycan using a glycosyl transferase enzyme. Some work has been performed on the incorporation of various glycosylated amino acids as an initial handle for the subsequent construction of a more elaborate glycan.

In the area of *O*-glycosylation, so called suppressor tRNA technology (Sisido and Hohsaka, 2001, Wang *et al.*, 2009b) has been used to evolve a new tRNA aminoacyl synthetase – tRNA pair which incorporated α-*N*-acetylgalactosaminyl-L-threonine (GalNAcα-Thr) into a model protein (streptavadin) at the *N*-terminus via ribosomal translation in a cell-free system (Matsubara *et al.*, 2013), by chemoenzymatically attaching GalNAcα-Thr to an amber suppressor tRNA. It was not possible to incorporate the nnAA internally in the peptide however.

Although *N*-glycosylation is required for mAbs, this work indicates that GalNAcα-Thr peptides can pass through the peptide tunnel of ribosomes, and showcases the power and continued development of suppressor tRNA technology in the area of protein *O*-glycosylation, which has now been expanded to include *O*-linked *N*-acetylglucosamine (Ge and Woo, 2021).

However molecular modelling calculations for the application of suppressor tRNA technology to glycosylated amino acids have shown that the model aminoacyl-tRNA-synthetase for *p*-benzoyl-*L*-phenylalanine is limited in its ability to selectively accommodate larger non-natural amino acids, such as *N*-acetyl glucosamine-β-asparagine, even if mutations are introduced *in silico* (Armen *et al.*, 2010), so there may be limitations to this area of work. In addition, failed attempts to recreate work which claimed to show that non-natural glycosylated amino acids could be incorporated into

proteins by *E. coli*, led to the retraction of two papers (Check Hayden, 2009, Antonczak *et al.*, 2009).

4.4. Cost and Challenges of Cell-Free Manufacturing

Although the scale up of the cell-free production of cytokines and aglycosylated mAbs has been shown to be straightforward and linear up to 100 L scale (Zawada *et al.*, 2011, Yin *et al.*, 2012), there is still little practical experience or data regarding the economics of commercial-scale cell-free antibody production, especially for glycosylated antibodies, however a basic financial modelling exercise has shown that the cell-free system is likely to be financially viable for high-value pharmaceuticals such as ADCs, though the economics improve substantially if phosphoenol pyruvate is replaced by glucose as an energy source (Silverman *et al.*, 2020, Shrestha *et al.*, 2014). A reassessment of the necessity of each of the small-molecule inputs typically used in cell-free systems has indicated a 95% reduction in the reagent costs for the production of the IgG trastuzumab (Cai *et al.*, 2015).

However, a recent financial modelling exercise has shown that the in-house manufacture of the bacterial extract required for cell-free manufacturing would lead to an approximate increase of 80% in the cost of goods compared with conventional cell-based systems (Stamatis and Farid, 2021). It also identified the need for improvements in the titre.

In summary, although the costs of cell-free systems have reduced, especially the inputs, there are further requirements for cost reductions before the technology becomes competitive with the current cell-free technologies. However, there is little real-life commercial experience of cell-free systems, and all technologies become cheaper as they are more widely adapted, more resource is focused on optimisation, and economies of scale are realised.

5. Overall Conclusions & Recommendations

This section is divided into three. The results section summarises the literature review, critiquing the state of development of key technological hurdles in the context of the requirements of the cell-free manufacture of mAbs. It presents work which has advanced the control of folding and disulfide bond formation and shows that these are now at a moderately advanced state of development. However, it also highlights the gaps than remain in the area of glycosylation and brings together the latest results which have the potential to provide breakthroughs in this area.

The discussion section builds on the current state of research to propose possible near-term technical advances and solutions in key technologies. It concedes that cell-free mAb manufacture is currently not feasible, but makes recommendations to industry based on the likelihood of near-term progress

The final conclusions section reiterates the benefits of cell-free manufacture from the point of view of regulatory requirements, quality expectations, cost, and process understanding. It summarises the future research directions which need to be prioritised in order to make cell-free manufacture of mAbs a reality.

5.1. Results: Summary of Current Status of CFPS for mAbs

As a result of this literature review, the state of progress towards cell-free manufacture of mAbs became apparent. Firstly, there were areas of technical complexity which are relatively well understood, or have been overcome to various degrees, particularly folding chaperones and disulfide bond formation. These areas have been described individually below.

Secondly, technological gaps were identified which have been a challenge historically and are not fully resolved to date. This primarily concerned glycosylation and its current status has been described below.

Thirdly, recommendations have been made regarding the composition of a putative cell-free system, with the cost implications of some of the choices highlighted. Finally, a future evolution of regulatory requirements and quality expectations and have been

proposed driven by increases in process capability, understanding and control. Cell-free mAb manufacture has been proposed below as a means to meet these requirements.

5.1.1. Current Progress and Challenges

The complex structural features of mAbs which have been explored in this work, have highlighted that the production of a single molecular product, rather than a mixture of isoforms, is a challenge for current cell-based technologies. In fact, inherent weaknesses in the biological glycosylation apparatus were highlighted. The most obvious incongruity is the unnecessary extra steps in the formation of the oligosaccharide, which is firstly significantly deconstructed, before being built up again, with variability in each step, leading to different glycoforms, which can be viewed as impurities.

Any future commercial cell-free production system will need to address this challenge. Indeed, the fact that state-of-the-art systems such as CHO are still not capable of producing a pure molecular entity may indicate that the various cell-based platforms have reached the limit of what they can achieve, and that a new approach is required. The demands of maintaining a living cell result in compromises which negatively impact the homogeneity and therefore the quality of the product, as well as production efficiency. A newer technology such as cell-free manufacture may be able to perform better given the significant differences in its operational principles.

This is not to say that all the challenges inherent to cell-free systems have been overcome. Three areas stand out as particularly complex in the context of mAb production: protein folding, disulfide bond formation, and glycosylation. The outlines of a new cell-free approach are becoming clearer, and this review has identified several key technologies, each of which addresses a particular opportunity for the commercial cell-free production of mAbs. Their applicability to CFPS is discussed below in the context of their current state of development, and likely future direction. However, it is clear that cell-free manufacture of glycosylated mAbs is not yet possible, and current cell-based systems are required.

5.1.2. Folding Chaperones in Cell-Free Systems

A review of the current knowledge regarding mAb folding has shown that it is spontaneous in energetic terms, and that each domain will pass through a sequence of partially folded intermediates to achieve a final energy minimum. No external energy input is required as the process is thermodynamically favourable. However, it is also clear that even in the cellular environment, a significant level of misfolding occurs, especially at higher protein concentrations. For this reason, a biological system exists to identify and degrade misfolded proteins in a lysosome.

Furthermore, changes to the amino acid sequence, especially in structurally critical regions such as the β-pleated sheet and the small directing helices, can alter the relative energies of the folding intermediates, and can disrupt the smooth progression towards the desired low-energy folded state. This is important for novel mAbs, as the impact of possible structural destabilisation would need to be considered during the rational design process. As computing power increases however, and it becomes more feasible to predict the impact of changing amino acids on the folding (Lai *et al.*, 2022, Bitran *et al.*, 2020), it will become easier to design mAbs which have smoother folding pathways, with fewer kinetic mis-folding traps.

As well as the impact of the amino acid sequence in the substrate a range of physical variables will influence folding, such as temperature, pH, redox potential and the presence of metal ions. The advantage of an open cell-free system is that these parameters can easily be varied as part of a statistically designed experiment to identify optimal conditions.

There are also additives and techniques which can aid the folding of unfolded proteins, or even allow misfolded proteins to be correctly folded. Refolding is already applied to pharmaceutical grade antibody fragments by treating them with detergents, denaturants, redox agents, aggregation suppressors and folding assisting agents, giving the antibodies a second chance to achieve the correct folding pattern (Arakawa and Ejima, 2014). In a commercial setting however, this would add extra manufacturing steps, cost, and yield losses. It also clashes with the right-first-time approach to quality and is inelegant in terms of fundamental process understanding and control, amounting to a reprocessing

step. However, it would be perfectly reasonable to incorporate some of the additives, into a cell-free system to use their properties to improve the initial mAb folding.

An initial examination of cell-free systems would suggest that an appropriate chaperone is needed for the correct folding of mAbs. However, a major reason why chaperones are needed *in vivo* is the high concentration and great variety of proteins which can interfere with the correct folding of mAbs. A compositionally simpler cell-free system with a lower overall concentration of functional cellular proteins would suffer less from this problem, especially if the lysate was purified enough to remove the bulk of unnecessary components. An optimal dilution would also need to be defined to allow acceptable volume productivity, while still minimising protein aggregation.

Nevertheless, it is still likely that a protein chaperone will be necessary or desirable to improve the success rate of mAb folding. In fact, it is likely that several chaperones will be required, with the recent discovery that different categories of chaperone deal with different categories of misfolding; the Hsp70 system refolds stably misfolded proteins (i.e. those in a kinetic trap), while GroEL/ES merely accelerates the progression of conformationally dynamic intermediates towards the native state (Balchin *et al.*, 2020).

There is wide range of chaperones (e.g., Hsp 70 and Hsp 90 families), co-chaperones (e.g., Hsp 40 family), and chaperonins (e.g., Hsp 60 family) involved in the control of protein folding (Bascos and Landry, 2019). These can be structurally very complex, with eukaryotic chaperones consisting of 8 or 9 subunits (e.g., the GroEL/GroES system). Ideally, the optimal composition and concentration of these components would need to be defined for each new mAb based on its folding difficulty, however in a commercial setting it may be more efficient to define a platform with a composition which is appropriate to most mAbs, and then genetically modify the organism which generates the lysate to produce the required chaperone mixture.

There is however a dependency on the presence and structure of the oligosaccharide at Asn-297. It is well known that this can have an influence on folding, particularly as it is located in a pocket between the two CH_2 domains in the Fc fragment. The attachment of the oligosaccharide is discussed later.

In general terms, the state of knowledge, techniques, and examples of applications around disulfide bond formation indicate that control over this aspect of antibody formation should be achievable *in vivo* but will require the presence of complex chaperones derived from the lysate.

5.1.3. Disulfide Isomerisation in Cell-Free Systems

The rapid formation of the correct constellation of disulfide bonds would also be advantageous to achieve correct folding as quickly as possible. Fortunately, however, it seems that such isomerisation is achievable with relatively simple molecules, pH control, and the maintenance of a suitable redox potential with a buffer. The inexpensive tripeptide Cys-Gly-Cys or diethylenetriamine-derived organocatalysts, have been shown to be efficient in mimicking the activity of PDI and thioredoxin for the generation and isomerisation of native disulfide bonds.

However, given that the rate of catalysis was several orders of magnitude lower than PDI, it may be more efficient to rely on the bacterial PDIs from the lysate. The level of the enzyme could be optimised by genetically engineering the bacterium (or other lysate generator) to provide the desired activity.

The possible incorporation of selenocysteine into rationally designed mAbs is another interesting future direction. The replacement of key cysteines with their selenium analogue could selectively and rapidly form conformationally critical intra-chain linkages, driving the folding progression rapidly towards initial intermediates which favour domain folding, such as the bond which is buried in the hydrophobic core of the mAb β-barrel folds. This selective nudging towards conformationally constrained intermediates would be expected encourage the progression through the desired folding intermediates.

This control of disulfide formation determines the success of the folding of each of the individual domains, which is critical in turn to the inter-chain association of domain pairings to form the antibody tetramer.

In general terms, the state of knowledge, techniques, and examples of applications in the area of disulfide bond formation indicate that control over this aspect of antibody formation should be achievable *in vitro*, either with lysate components, or quite likely with small molecule additives and the optimisation of physical parameters.

5.1.4. Glycosylation in Cell-Free Systems

Although glycosylation is possible in CFPS, it relies on so lipid nanodiscs as cell membrane substrate mimics, and crucially these must be derived from mammalian cells in order to contain the required glycosylation enzymes, which are missing from bacteria. If the highly structured glycosylation machinery and compartmentalisation in an actual cell cannot generate a high-purity glycosylation pattern, then a lysate is unlikely to perform any better. Considering this, it is unlikely that the genetic engineering of bacteria to contain glycosylation apparatus will lead to a better outcome that the current CHO system.

A number of unorthodox alternatives to cellular glycosylation are emerging, which have the potential to allow access to purer glycosylated mAbs. The challenge is that once the mAb and glycan are combined, the physical separation of different glycoforms is practically impossible. The overall properties of the molecule are overwhelmingly determined by the larger protein component, and any relatively small differences in the glycan portion are swamped by the behaviour of the protein. This is exacerbated by the buried position of the glycan between the two CH_2 domains of the heavy chain dimer. Although it may be possible to use biological recognition systems to differentiate between different glycoforms, possibly using recognition sites on proteins from the complement system in a similar way to protein A chromatography, this is likely to be expensive.

A better approach conceptually would be to ensure that the glycan component is pure before attachment to the mAb. A given structural difference or inhomogeneity has a much greater contribution to the physical behaviour of a relatively small glycan, than it does to a very large glycoprotein, which facilitates purification. This would suggest that the glycan should be synthesised separately using chemical, enzymatic or cellular processes, and then attached selectively to the aglycosylated mAb at Asn-297. Although

the advantages of glycosylating a mAb selectively at Asn-197 with a fully pre-formed glycan have been discussed, currently no practical method exists to achieve this. However, there are two main approaches which may be successful in the future.

5.1.5. Synthetic Glycobiology & Glycoengineering

The emerging area of synthetic glycobiology is advancing rapidly and is beginning to uncover a complex array of glycosylation enzymes which hold out the promise of engineering. With these tools in hand, and promising initial results, it may be anticipated that this technology may be applicable to larger scales, and eventually may form a critical step in the cell-free manufacture of mAbs; the selective glycosylation of the protein chain. Two possible technical solutions are presented below: the use of a non-natural glycosyl amino acid, and the chemical attachment of oligosaccharides.

5.1.6. Glycan-containing Non-natural Amino Acids

An ideal solution to the problem of attaching an oligosaccharide specifically at the Asn-297 position would be to incorporate a non-natural amino acid bearing the required *N*-acetylglucosamine, preferably attached via an amide bond to an asparagine residue, as found in human mAbs. The challenge is that although ever more non-natural amino acids are being incorporated into proteins using gene code expansion, there is necessarily a limit to the size of substrate which can be accommodated by the ribosome complex.

To date, a wide range of nnAAs has been incorporated into proteins using genetic code expansion and codon reassignment in CFPS systems, including relatively large examples (Quast *et al.*, 2015, Dumas *et al.*, 2015). Although the incorporation of glycosylated versions of both serine and tyrosine (mannosyl, glucosyl and galactosyl derivatives) have been claimed in the literature (Fahmi *et al.*, 2007), there are indications this is was very much an outlier, especially given the state of development of the technology at the time.

The more the nnAA resembles the natural pyrrolysine substrate of the pyrrolysyl-tRNA synthetase system, or the tyrosine substrate of the tyrosyl-tRNA synthetase system, the greater the chance of success, as this increases the tolerance of the corresponding aminoacylated tRNA by the ribosome and associated factors. GlcNAcylated amino acids have not yet been added to the expanded genetic code, presumably due to their high

polarity. However phosphoserine acts as a precedent for incorporating such polar nnAA, where some components of the *E. coli* translation machinery were successfully re-engineered (Rogerson *et al.*, 2015).

A further potential problem arises with the use of glycyl amino acids is their stability *in vivo*. This is exemplified by Ser-O-GlcNAc which is metabolised as a carbon source by the *E. coli* host system which is used for evolving PylRS . However other glycyl amino acids, such as Cys-*S*-GlcNAc, are metabolically stable (Gorelik and Van Aalten, 2020). There is however little to be found in the literature regarding the metabolic stability of glycosylated asparagine.

Low yields are a key barrier to the wider adoption of nnAA incorporation, however a number of options are being pursued to increase the yield of nnAA-containing proteins, including high-yielding cell-based systems for antibody production (Roy *et al.*, 2020), the use of viral internal ribosome entry sites (IRES) to boost expression (Wang *et al.*, 2021), and reducing competition for the reassigned codon (Wu *et al.*, 2020). These developments would be very useful to boost the yield and efficiency of any future commercial process.

Although there is no convincing evidence yet of the successful use of glycosylated nnAAs in an evolved orthogonal aminoacyl-tRNA synthetase pair, it is clear that translation machinery can accommodate a range of structures. There is a great focus on this area currently, both on a research scale to investigate the impact of various PTMs on protein function and structure, but also on a manufacturing scale to develop new specific conjugation points for ADCs. This ongoing effort can be expected to continue the expansion of the nnAAs which are available, and to increase their yield, including in cell-free systems, which are relevant in this area to prevent negative effects on cell viability.

The lack of research, or publishable results for glycosylated asparagine is however an indication of the lack of development of this particular area of nnAA research. Alternatively, given the great commercial importance of mAbs, this lack of literature may indicate that although work has been performed in the area, the challenges have proven intractable. However, it is to be hoped that methodologies will be identified to allow the

site-specific insertion of the required mAb glycan attachment site as a nnAA, however this has not yet been achieved by gene code expansion,

5.1.7. Chemical Attachment of Glycans

Recent developments in the manufacture of antibody-drug conjugates have made a range of chemical conjugation techniques available. Combining this with the ability to use gene code expansion to insert non-natural amino acids with desirable chemical properties at the 297 position would allow the targeted attachment of linkers, which could then be attached to oligosaccharides. It seems likely that this area of research will be driven forward by the increase in the number of ADCs on the market, and its applicability to mAbs.

5.1.8. Components of CF Systems

A key question for the future optimisation and standardisation of cell-free systems for mAb synthesis is what component should be present in the lysate? What might a future commercial cell-free platform look like. This is particularly important when one considers that a precisely defined PURE system would likely be too costly, but also that a lysate may not be sufficiently well defined or controlled to provide an optimal translational environment. Therefore, a compromise or hybrid approach is likely to be necessary. This should minimise cost and complexity, but still deliver the required amount and quality of mAb.

The main components of CFPS systems are listed in Table 3, with a discussion of their current usage and recent developments below, in the context of what would need to be considered for an efficient large-scale cell-free commercial system.

Table 3: Key Components of Cell-Free System

Component	Function	Comment
Salts	Standard requirement.	Standard component of cell culture media.
Amino acids	Building blocks for protein	Standard component of cell culture media.
DNA	Primary genetic code for amino acid sequence	Easily available from plasmid culture or PCR

Component	Function	Comment
mRNA	Added directly to uncoupled systems as sole genetic code	Defined based on what protein product is required
Energy substrate	Required input to regenerate ATP, which is required by powered enzymes such as aminoacyl-tRNA synthetase	Technologies being developed which are moving towards cheaper energy inputs by using ATP recycling
Nucleotides & nucleosides	Small molecule building blocks for nucleic acids and for energy production	Expensive to synthesise chemically, better to recycle using cheap energy source such as sugars
RNA polymerase and associated co-factors	Needed for coupled systems where DNA is the primary instruction code	
Ribosomes	Helps loaded aminoacyl tRNA synthetase to bind to the next codon, and then catalyses formation of next peptide bond	Due to complexity, best to derive from lysate
Initiation, elongation, and termination co-factors, ribosome recycling factors	Escort amino acid-tRNA pair to the ribosome. Promote or down-regulate the initiation, elongation or termination of protein synthesis, as well as ribosome recycling	May need supplementation in CFPS
Aminoacyl-tRNA synthetase	Binds specific amino acid to 3’ end of cognate tRNA	Due to complexity, best to derive from lysate, unless nnAAs are required.
tRNAs	Binds to specific amino acid at one end, with the corresponding DNA anti-codon at the other end. Acts as amino acid donor during protein synthesis. Decodes mRNA codons into amino acid sequence.	Work on in-vitro production of tRNAs is ongoing, but lysate source would appear to be sufficient.
Disulfide isomerase chaperones	Required to speed up shuffling of disulfide bonds to reach optimal constellation	There are small-molecule options available.
Glutathione redox buffer	Maintains redox potential within range	External additive

Component	Function	Comment
	required for disulfide bond formation	
PPIase	*Cis-trans* peptidyl-prolyl ismerisation is crucial to β-sheet folding	

Amino acids are readily available from protein sources and are a standard component of culture media. Salts are also a standard component. RNA or DNA acts as the instruction for the desired protein in coupled and uncoupled systems respectively. Recent advances have been made in the efficiency of production of mRNA due to the covid-19 pandemic (Rosa *et al.*, 2021, Whitley *et al.*, 2022), which have improved the techniques and technologies for systems where mRNA is added directly. In the case of coupled systems, it will be important to ensure that the rates of transcription and translation are synchronised to ensure that mRNA does not form inhibitory secondary structures, such as stable hairpin loops, which can impact protein production efficiency (Hansen *et al.*, 2016, Webster and Weixlbaumer, 2021). Given recent advances, it may be advantageous to avoid the extra complication of transcription, and just add fresh mRNA as required to replace losses due to degradation.

The provision of energy to the lysate is one of the more complex requirements and is related to the requirement for the addition of nucleosides and/or nucleotides. Of course, it is possible to simply add adenosine triphosphate (ATP), but this is expensive and wasteful. *In-vitro* enzymatic ATP regeneration systems are more efficient, and several systems are available based on single enzymes, or cascades of adenosine kinases and polyphosphate kinases, using adenosine as substrate with polyphosphate as the inorganic input and (Sun *et al.*, 2021). It is clear from recent work that significant progress has been made in the area of low-cost, efficient, robust and long-lifetime ATP regeneration systems (Wei *et al.*, 2018), however some challenges remain, which may be addressed in the future using a bottom-up, *in-vitro* synthetic biology strategy (Chen and Zhang, 2020). So, although the current level of technological development is sufficient for high-value protein production, it can be expected further developments will bring significant improvements. In particular, the cost pressures associated with the biosynthetic production of small molecules are leading to ever more efficient systems using low-cost

sugar and starch inputs (Strohmeier *et al.*, 2019, Wei *et al.*, 2021), and any future commercial CFPS would need to use sugar or starch.

The ribosome forms the heart of the CFPS as it is where all the inputs come together to form the amino acid chain. It is a very complex biological machine, where a large proportion of the structure and function is defined by non-translated ribosomal RNA (rRNA) in conjunction with associated protein elements. Given this complexity, it is the keystone of the translation process, and its relative concentration against most of the other components is critical. Batch-to-batch variations in its lysate concentration could throw the entire biosynthesis out of balance, so it is likely to need control at a target concentration, with all other components needing to be adjusted to within an experimentally determined range, which ensures quality and titre.

The initiation, elongation, and termination co-factors interact with the ribosome, mRNA and tRNA to regulate the synthesis of proteins. The PURE system provides these co-factors as purified ingredients, and they may also be added to cell lysates to supplement the natural concentrations, leading to improved protein expression levels in some cases (Xu *et al.*, 2020). This indicates that maintaining the appropriate level of co-factors is an important consideration for commercial CFPS.

Although the Flexizyme system may allow the coupling of nnAAs to tRNA, enabling the incorporation of novel amino acid residues into proteins, the bulk of the protein will continue to be derived from aminoacyl-tRNA which has been produced by aminoacyl-tRNA synthetase. Like the ribosomes and co-factors, this input is likely to come from the lysate.

It is currently required to obtain tRNAs from the lysate, with additional exogenous tRNAs often being added (Xu *et al.*, 2020). A first glance, these chains of nucleotides could easily be synthesised *in vitro*, however they actually contain a number of substantially modified nucleotides which are functionally and structurally critical (Motorin and Helm, 2010). In the area of *in-vitro* production of tRNAs, a reduced set of tRNAs was identified, which did not contain nucleotide modifications, but still covered all the amino acids, due to the redundancy of the natural tRNA system where 64 codons encode only 20 amino acids (Hibi *et al.*, 2020).

This simplified system may be useful for genetic code engineering and specific areas of cell-free synthetic biology; however, it appears that the *E. coli* system is acceptable as a source of these components. Nevertheless, it might be useful to synthesise tRNA *in vitro* in order to add supplementary tRNA to a cell-free system, for example in cases where this component becomes rate-limiting due to tRNA degradation. This would have the advantage of prolonging commercial production runs.

Protein disulfide isomerase chaperones play a key role as a redox chaperone in the reshuffling of the initial disulfide bond arrangement and are likely to be derived from the lysate. However, it can be envisaged that even if the correct redox potential is maintained by titrating in glutathione redox buffer, the amount of active PDI may reduce over time. As well as the option of adding more PDI from purified lysate, it may be possible to add small molecule analogues such as the diethylenetriamine-derived organocatalysts discussed previously. These would be cheap and easy to produce using standard organic chemistry and may help to reduce costs in a commercial application.

PPIase is required to catalyse the cis-trans peptidyl-prolyl isomerisation, to allow the timely formation of the beta sheet, a pre-requisite for the formation of each of the domains. A slowing of the isomerisation of the proline residues is likely to lead to longer periods when unstable partially folded intermediates persist, which can lead to the mAb finding a local energy minimum on the folding pathway, rather than proceeding along the desired folding path towards the global energy minimum. It also increases the likelihood of protein agglomeration between hydrophobic patches and strands in partially folded mAbs. These proteins are relatively small, with the human peptidyl-prolyl isomerise A (cyclophilin A) consisting of only 165 amino acids. This might indicate that bulk production is possible to provide purified enzyme to add as a supplement to cell-free systems.

In summary, the use of a hybrid CFPS system may be beneficial, where the bulk of the components comes from a semi-purified cell lysate, but additional components are added or fortified to reach and maintain optimal concentrations. This represents a step in the direction of the high levels of control which are possible with the PURE platform, without the high cost of such a well-defined system.

5.1.9. Quality & Regulatory

The quality impact of the complexity of cellular biosynthesis of glycoproteins means that impurities can arise from incomplete hydrolysis or glycosylation. This is the reason why mAbs generally consist of a mixture of glycoforms. The best that mAb manufacturers can do is to ensure that batch-to-batch variability is minimal in order to ensure that patients receive the same product as was investigated during clinical trials. As discussed, this can be a challenge given the complexities and sensitivities of cell-based cultures.

However, as more and more is learnt about the influence of glycosylation on mAb function, particularly with respect to the complement system, the clearer it is becoming that different glycosylation patterns imply different biological effects. Some will be less active, and some may trigger undesirable side effects. Unfortunately, because clinical trials are carried out on a mixture, these structural implications are generally never fully understood.

For this reason, the approval of biosimilars relies on proving that its mixture of glycoproteins is equivalent to the reference product, and that the corresponding production system can reliably produce this mixture (Vulto and Jaquez, 2017). This adds to the cost of developing biosimilars, and partly explains why there have not been the large price decreases with their introduction as there are with generic small-molecule pharmaceuticals, whose structure is clear, and whose purity is usually very high.

It could even be the case that biosimilar producers are being forced to include inactive or even detrimental glycoforms in their product, just to match the profile of the reference product. In the past when analytical techniques and pharmacological understanding were less well developed, this situation may have been unavoidable. However, nowadays we should be striving provide as pure a drug as possible.

In a similar manner to the transition from serum-containing media to defined-composition, serum-free media, a transition to a fully characterised production platform would be a step forward. From a process and quality control perspective, cell-free systems are much simpler than whole-cell systems, with a finite, well understood

composition, and the ability to run experiments on an open system in order to define a robust operational design space.

This aligns well with cGMP and the latest regulatory guidance. ICH Q9 (Quality Risk Management) is based on the principle that all evaluations of quality risk throughout the product lifecycle should be based on scientific knowledge, whether that be during the definition of critical quality parameters during development (ICH Q11) or assessing the quality impact of a deviation or a proposed change (ICH Q12). Cell-free systems have fewer components, and each one of them has a well-defined purpose. This makes the system easier to characterise and understand and should lead to higher levels of robustness than sensitive cell-based systems.

However, this is still a very complex system, and it will require complex mathematical modelling to develop a control strategy. A battery of real-time process analytical tools will be needed to monitor the progress of the protein synthesis, and also to allow the timely charging of ingredient which are consumed or degraded during the production time.

5.2. Discussion: Likely Features of Cell-Free Manufacture of mAbs

The technological prerequisites for viable commercial cell-free manufacture of mAbs have been reviewed, and many of these are already in place. In terms of the input materials, these are already clear from the PURE system, and a hybrid between this and a more economical lysate system is proposed.

A two-stage approach will be required, where a fast-growing organism such as *E. coli* will be used to provide the lysate, and this will then be purified, with particular attention on the removal of higher-risk impurities such as nucleases and endotoxins. The advantage of this approach is that lysate production can be localised to a small number of specialised high-volume producers, reducing costs and improving product consistency, with the proviso that a significant number of mAb producers adopt the same platform approach for lysate. This would leave the option open to add supplements based on individual process needs.

It will be vital to avoid the use of expensive nucleotides as an energy source to make the process economical, but fortunately options are now available for recycling of ATP using simple sugars as the primary energy source. There are many examples of protein synthesis using cell-free systems, the main challenge would appear to be scaling up to a robust manufacturing platform which can compete with current cell-based systems.

The great number of components makes the optimisation of the relative concentrations a challenge, but is it worth examining this area, rather than blindly accepting the composition provided by cell lysates. Work in the area of synthetic biology based on the PURE system has identified the key parameters which have an impact on protein production by applying mathematical models and concepts of enzymatic kinetics (Matsuura *et al.*, 2018, Marshall and Noireaux, 2019), providing information on robustness and optimisation opportunities by indicating which components to modify and control, as well as which components are not critical.

Synthetic biology is able to identify and characterise the fundamental factors which control protein synthesis. This provides more meaningful critical process parameters during protein production as the concentration can be directly measured and adjusted, and approaches for optimising cell-free systems are available (Batista *et al.*, 2021). This is not directly possible in cell-based systems due to interference from cellular feedback, parallel cellular processes, or the sheer physical barrier of the cell membrane. For this reason, cell-based systems are harder to control because secondary control levers must be used, such as adjusting concentrations in the extra-cellular culture solution and waiting for the cell's protein production machinery to respond.

The available technologies for CFPS were critically assessed in relation to the three most challenging structural requirements of mAbs. The critical role of PPIase in the formation of the β-pleated sheet was discussed, and it is clear that this isomerase would be a required component of any commercial system, with such a complex component needing to be derived from the lysate.

The role of protein disulfide isomerase was examined, and the opportunity to replace this with small molecule analogues was critically assessed. Additional supplementation may be advantageous given their demonstrated activity, low complexity, and ease of synthesis,

but their low catalytic activity compared with native enzymes will mean that these will have only a supplemental role at their current state of development.

The practicality of the cell-free system was therefore clear for most proteins without PTMs, with examples provided which were relevant to a commercial scale, however the main technological gap was regarding glycosylation, and understanding whether there is likely to be a future development which overcome this challenge, and what this might be, became a key theme of this work.

Two areas of research were identified which may provide a way forward for glycosylation. The core technology for both is likely to be gene code expansion, which has the potential to allow the selective insertion of a nnAA instead of asparagine 297, thereby differentiating this position for oligosaccharide conjugation.

In the first approach, the nnAA would be a highly reactive moiety, which would then be coupled to a fully formed nucleotide. Several suggestions were made based on systems which have been demonstrated for ADC coupling. The usual balance of reactivity, specificity and stability would need to be met, but systems involving azide and alkynes meet these criteria. The main drawback here is that not only is the resultant coupling structure non-identical to the native glycosylated asparagine, but it is also very much larger and is unlikely to lead to recognition by the complement system, certainly for the strain-driven variant (SPAAC). This constraint is however not relevant to ADCs.

Other smaller possible linkers were proposed, again based on ADC technologies, and the selective reaction of glycosyl-β-N-iodoacetamide with cysteine at the 297 position was identified as a key piece of research which merits further development. The selectivity which was demonstrated in this work could be enhanced with the use of a selenocysteine at position 297, thereby furnishing an efficient glycan coupling reaction with a structure which is relatively similar to the natural asparagine linkage. However, it is likely that some reaction will occur at unwanted sites, such as cysteine residues. One possible solution would be to develop a chromatography system similar to protein A, but where receptor components from the complementary system are used to retain correctly glycosylated mAbs, whereas molecules with glycosylation at the wrong position would be expected to pass through the column.

Although it is not currently practical or feasible to manufacture monoclonal antibodies in cell-free systems, research into the main technological hurdles are already delivering useful results, and can be expected to make cell-free manufacture a viable option in the future. This indicates that the generally conservative biopharmaceutical industry should keep abreast of these developments, understand the potential benefits, and plan their future development, manufacturing, and product lifecycle strategy accordingly. It should be remembered however that predictions for such a fast-moving area are difficult, though this underlines the need to stay informed and adapt corporate strategies accordingly.

5.3. Conclusions & Future Direction

The findings of this research are that there are clear limitations regarding robustness, quality, fundamental process understanding, and control for cell-based production systems for mAbs. This is due to the inherent complexity of living cellular systems, and it seems that current platform production systems are unlikely to be able to deliver single glycoform products in the future. The current approach to mAb production takes this into account by having scrupulous control over production conditions, with the mantra that the process is the product. While the processing conditions will always define the product, the modern QbD approach to product quality and risk management would suggest that a more nuanced and sophisticated approach is required, rather than locking the process down. The ideal scenario would be that in a robust manufacturing process, the nucleic acid sequence defines the product, in addition to the quality of the oligosaccharide.

A conclusion of this work is that the direction of travel of global regulatory authorities, and the expectations in terms of quality, are likely to push manufacturers of mAbs towards better understanding and control of manufacturing processes, as well as towards single glycoforms, or at least the ability to determine the therapeutic benefit of each major glycoform separately. A further financial driver might be that the promise of lower cost biosimilar drugs has not been realised, and it would be much easier to achieve and demonstrate equivalence for single-glycoform products, helping to reduce the cost of biosimilars.!

Therefore, there is a driver to understanding mAb production processes better, and cell-free production offers a greatly simplified system, which is more amenable to a fundamental understanding of the entire system and its interdependencies, without the extra complications of cellular viability.

A review of the state of readiness of cell-free manufacturing techniques has highlighted that it is certainly possible to manufacture proteins on a moderate scale, and even those with disulfide bonds and complex structures. This includes aglycosylated mAbs, though not yet on a commercial scale. However, the cell-free production of glycosylated mAbs can only be realised using mammalian lysates containing lipid nanodiscs, which is unlikely to perform any better than the cells themselves.

In fact, glycosylation was revealed as the main barrier to cell-free mAb production. Although there are methods to produce pure oligosaccharides themselves, either from natural sources, or chemically, the specific attachment of these to the required site on the mAb is problematic. For this reason, at the moment is it easier to obtain the required glycosylation pattern using cell-based systems, even though a mixture results.

To address this limitation and unlock the potential of cell-free mAb manufacture, two areas of research need to be further developed, both based on genetic code expansion. Most simply, if the range of nnAAs were expanded to include *N*-acetylglucosamine functionalised asparagine, this could act as an attachment point for fully formed oligosaccharides using an oligosaccharyl transferase. An examination of the literature does not reveal very much progress in this area, though some work has been performed on *O*-glycosylated serine and tyrosine. Given the obvious relevance to the multi-billion-dollar mAb industry, this either indicates that there is a missed opportunity for useful discoveries, or else that this is a challenging area and has not provided any reportable successes to date.

An alternative approach involves the insertion of known nnAAs at position 297 and using a chemical approach to selectively attach a suitably functionalised oligosaccharide. This is very likely to give a non-native linker, rather than asparagine. An examination of the research performed for ADCs revealed that there is a wide range of possibilities, many of which have been successfully used to attach cytotoxic payloads to antibodies. However, a

comparison of their structures showed that only a handful might be similar enough to the native structure to have a chance of exhibiting near natural therapeutic behaviour. Again however, there was a lack of reported results in this area for mAb glycosylation, though in this case it may have been due to the great focus placed on ADCs at the moment due to their great therapeutic promise.

Looking forward, it is likely that continuing developments in genetic code expansion will have fundamental importance and significant practical applications for cell-free mAb production. The ability to target oligosaccharide attachment to mAbs may lead to a shift towards better understood manufacturing processes, better quality risk management, and reduced costs if cell-free production technology is widely adopted. However, a significant amount of work remains to be completed to allow this technology to be adopted commercially.

In addition, the continued advance of the relatively new areas of glycobiology and glycoengineering are likely to offer options for the crucial attachment of the first sugar residue on the amino acid chain. Combining this with the great number of glycosylation enzymes which are being discovered in both bacterial and mammalian sources, indicates that reliable *in-vitro* glycosylation of mAbs is on the horizon.

The implications of cell-free technology for monoclonal antibody production are significant. A fully characterised cell-free system should enable simpler, more transparent control over production conditions. The amount of each input should be reduced for the cell-free system itself, however it must be remembered that this will be offset by the energy, nutrients and media used to generate the lysate. The volume yield per unit time will be reduced significantly given that the proposed bacterial lysate is much faster growing than a mammalian cell culture, and the cell-free synthesis will likely take a day or two, rather than several weeks for mammalian cells.

This has important implications for the efficiency of the process, and the cost of production. Given the high prices of biologic medicines, this would represent a clear societal benefit. Lower costs are also likely to be generated from the ability to have a true platform technology where there is no need to engineer a new host cell for every product.

It may be an over-simplification, however it should be possible to add a new DNA or mRNA sequence to the same cell-free system in order to produce a new mAb.

Although this has implications for lowering cost, it also has implications for the profitability of mAb therapies. Once a patent has expired, it should be possible for biosimilars manufacturers to immediately produce an identical product, particularly as a future, well-developed cell-free system should be able to produce a single glycoform. In fact, it will no longer be a biosimilar, it will be an identical generic, similar to the situation for small-molecule drugs.

In summary, it is not currently possible to manufacture glycosylated monoclonal antibodies using cell-free technology. It is clear however that a transition to the cell-free manufacture has the potential to deliver a simpler, better understood biosynthetic system with more control over quality and costs. Although the current lack of a practical technique to attach oligosaccharides selectively at the asparagine-297 position is a significant barrier, developments in genetic code expansion may offer solutions in the future, as well as recent developments in glycobiology.

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