Development and Production of Commercial Therapeutic Monoclonal Antibodies in Mammalian Cell Expression Systems: An Overview of the Current Upstream Technologies

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Abstract: This article provides an overview of the upstream technologies used in the industrial production of therapeutic monoclonal antibodies (mAbs) based on the cultivation of mammalian cells. More specifically, in a first section, after a short discussion of relevant biochemical characteristics of antibodies, we review the cell lines currently employed in commercial production and the methods of constructing and isolating production clones. This is followed with a review of the most current methods of commercial scale production and their associated technologies. Selected references and short discussions pertaining to emerging and relevant technologies have been embedded throughout the text in order to give a sense of the overall direction the field is taking.

INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) are as of today a well accepted class of therapeutics especially in the fields of oncology, immunology, and organ transplant, where the use of these targeted biologics has profoundly revolutionized treatments paradigms. This is especially true in the treatment of various cancers where mAbs have proven to carry fewer side effects than the traditional cytotoxic drugs and have resulted in improved patient quality of life [1-3]. As of today, there are twenty two therapeutic mAbs, or fragments, currently registered for marketing in the US (see table 1) with a market size over $ 17B in 2007 and an expected cumulative annual rate of growth of 14 % [4, 5]. This success is likely to carry for many years since it has recently been reported that the number of mAbs that entered clinical studies tripled over the past decade [4].

Commercial production of currently registered mAbs relies on the cultivation of mammalian cells that have been genetically engineered to over-produce the mAb of interest. One exception is the use of the bacterium *Escherichia coli* in the production of fragments. The production of mAbs at industrial scale is a multifaceted endeavor that encompasses many technically complex and lengthy steps. Briefly, a commercial mAb production process starts with the generation of a mAb via immunization of an animal or via molecular biology methods, the identification and optimization of the coding DNA sequence and the construction and identification of a highly producing and stable clone. These steps are followed with the development of a well designed cultivation process that encompasses the full control and scale up of associated operations that will support early clinical evaluations. Typical cycle times for these activities usually range between 16-24 months [6]. In this article, we will provide an overview of the state of the current technologies that lead to achieving high efficiency in these process steps and will briefly discuss potential technological improvements which are likely to benefit the current technology over the next few years.

1. OVERVIEW OF RELEVANT BIOCHEMICAL FEATURES AND PROPERTIES OF MONOCLONAL ANTIBODIES

This section will familiarize the reader with the basic concepts that are later discussed in the following sections.

1.1. The Concept of Therapeutic Antibody

*Via* interaction with specialized components of the immune system, the *in vivo* role of antibodies is to clear the host from invading pathogens and from any non-self molecules these micro-organisms may release (toxins for example). Antibodies present exquisite specificity for their target (antigens), with the ability to recognize and bind exclusively to a small region (epitope) of a given antigen. When binding to an antigen, the complex formed allows for rapid recognition and clearance by specialized components and cells of the immune system. Natural killer cells NK or NKC recognize antibody-target cell complexes and trigger the lysis and destruction of the invading cell in a process known as antibody dependent cell-mediated cytotoxicity (ADCC). Another facet of the immune response involves the complement, a multi-protein complex that sequentially bind to the antibody-target complex leading to either its recognition and engulfment by macrophages in a process known as opsonization, or to the lysis of the target cell in a process known as complement-dependent cell cytotoxicity (CDC). This very succinct and simplified overview of the immune response can be supplemented by consulting the following references for more extensive discussions [7, 8].

These properties have made antibodies a very attractive choice for novel therapeutic approaches to diseases where externally exposed membrane-bound or circulating proteins could be specifically targeted for a specific action. The over-
all expectation is that specific interactions between the mAb and its specific circulating or membrane-bound protein target will either result in the blockage of key signals of the disease pathology, will achieve the specific delivery of conjugated small molecules such as cytotoxins or radionucleides to target cells, or will lead to the destruction of the target cell via ADCC or CDC. Several therapeutic mAbs whose mode of action fits one or several of these desired traits are currently in clinical use. A few are highlighted here.

For example, the mAbs cetuximab (Erbitux®) and trastuzumab (Herceptin®) interfere with the over-expressed membrane-bound growth factor receptors EGFR and Her2, both known to be implicated in signaling for proliferation and survival of cancer cells. By preventing ligand binding, cetuximab prevents cancer cells from proliferating. Although the exact mode of action is still unclear, trastuzumab is believed to act via interactions with the effectors of the immune system. The association of the mAb with a membrane protein triggers various mechanisms that can lead to the destruction of the targeted cell by triggering apoptosis (a pre-programmed self destruction pathway) or cell lysis via CDC or ADCC. Both these two mAbs are two highly successful clinical examples of interference with a membrane-bound receptor. Based on a different approach, rather than directly interfering with a receptor, the mAbs bevacizumab (Avastin®) and adalimumab (Humira®) respectively block tumor angiogenesis and the signals leading to rheumatoid arthritis by respectively sequestering the circulating ligand, VEGF and TNFα. The concept of delivering a toxic payload to specific cells targeted for destruction is exemplified by three mAbs that are currently in use in the treatment of cancers. Mylotarg® (gemtuzumab) is conjugated to ozogamycin, a derivative of the antitumor-antibiotic calicheamycin and targets CD33, a protein often over-expressed on myeloid cells. Bexxar® (tositumomab) conjugated with the radionucleide 131I, and Zevalin® (ibritumomab) conjugated with either 111In or 90Y both target CD20, an antigen expressed on the surface of myeloid cells. Palivizumab (Synagis®) which is directed at the envelope transmembrane F (fusion) protein of the respiratory syncytial virus (RSV is a very serious pediatric infection) is the only mAb currently in use for the treatment of infectious disease.

 Fragments of mAbs that are made up of the domain of the mAb that confers affinity to its target but that do not include the domain of the molecule that interacts with the effectors of the immune system are also used. In addition to not having interactions with the effectors of the immune system, fragments are rapidly cleared from the body, a second feature that is useful when seeking short term therapeutic action. Three fragments are currently registered with the FDA. Reo-pro® (abciximab) is used to control platelet aggregation during percutaneous coronary intervention. Lucentis® (ranibizumab) which is directly delivered to the eye has been very successfully used in the management of age related wet macular degeneration. Very recently, the approval of Cimzia® (certolizumab pegol), a pegylated fragment directed at TNFα, marked the introduction of fragments designed for extended in vivo life time through conjugation with the polymer PEG. These few selected descriptions exemplify how mAbs have been successfully used in the treatment of complex and serious diseases. The list of the 22 currently US approved mAbs, their protein target and their clinical applications can be found in Table 1. Additional general information can be found in the following publications [9-16].

1.2. Biochemical Overview of Therapeutic Antibodies

As of today all therapeutic mAbs are of the Immunoglobulin G (IgG) sub-class. These molecules are made up of two heavy chains and two light chains that are held together and folded via intra and inter-chain disulfide bounds. The average molecular weight of each sub-chain is about 25Kd (or about 220 amino acids) for the light chain and 50 Kd (about 450 amino acids) for the heavy chain. The heavy chain is made up of 3 constant and one variable domains, while the light chain is comprised of one constant and one variable domains. Specificity of the antibody molecule is dictated by the amino acid sequence at specialized sites, called complementarity determining regions (CDR) present in the variable domains of both light and heavy chains. It is these loop-forming hyper-variable regions of the molecule that interact and form a tight association with the intended antigen target. All IgG sub-class antibodies are based on this general structure and mostly differ in the sequence of the constant regions of the heavy chains, the part of the molecule that interacts with other components of the immune system via ADCC and/or CDC. The IgG1 sub-class molecules present the strongest association with the receptors of the effector components of the immune system [17]. IgG antibodies have an additional layer of complexity through a conserved N-linked glycosylation site at ASN-297 on each of the heavy chains [18]. These short glycans are of bi-antennary structure and can be variable in many ways that include; the terminal sugar composition (presence or absence of galactose, capping with a sialic acid) and the presence or absence of bisecting fucose and N-acetyl glucose amides. This high degree of variability and relative abundance of each glycan species results in the high likelihood that glycans of a different structure are attached to each of the heavy chains of a given mAb molecule. The presence and composition of these glycans has been found to greatly affect the activity of the antibodies in terms of their interactions with the immune system effector functions [19]. For example, glycans devoid of the bisecting fucose will confer the mAb a stronger interaction between the antibody and the NKC, resulting in higher ADCC mediated immune response [20-26]. Similar increases in ADCC function have also been noticed when the bisecting N-acetyl glucose amide is present, although this effect appears to be more antibody dependent [27-29]. However while all ADCC activity is abolished by the complete removal of the glycans, partial removal of the terminal sialic acid and galactose have no gross apparent impact on ADCC [30]. Although they represent a small part of the antibody molecule, glycans, because they are assembled post translationally are more likely to exhibit higher diversity and to thereby influence the overall activity of the mAb. Fig. (1) presents a composite pictorial of an antibody with a composite of the glycan chain presented in detail.

In addition, it is also important to note that not only are antibody molecules highly complex in their primary structure and heterogeneity; they also have complex secondary and tertiary structures that greatly influence their activity. This
short overview of the biochemistry of IgG antibodies highlights the fact that synthesis of biologically active mAbs is a highly complex and well orchestrated biochemical process that encompasses folding, trafficking, post-translational modifications, and effective secretion. The choices of a producing platform (host cell) and of the physico-chemical conditions that will support mAb synthesis have been shown to greatly affect the abundance and quality of the N-linked glycans, molecule folding, and the yield of the mAb of interest.

Since the advent of mAb production in the late 1970s, production platforms have relied on mammalian cells. As discussed above, monoclonal antibodies are highly complex molecules that require post translational modifications such as inter and intra-chain disulfide bond formations, and the addition of N-linked glycans at specific sites. Until recent metabolic engineering advances, only mammalian cells which possess the intrinsic required machinery could produce mAbs with the desired folding and post-translational modifications, and were therefore the de facto production platforms for mAbs. Although considerable progress has recently been (and continues to be) made in the use of other biological production platforms [31-33], to our knowledge, as of today only mammalian cells are used for the production of commercial and late stage clinical mAb supplies. Judging by the pace and the significance of the discoveries made in the use of yeasts, it is however very likely that during the next decade, these non-mammalian expression systems will be in use for the production of clinical or commercial mAbs.

2. GENERATION AND MOLECULAR BIOLOGY BASED MANIPULATIONS OF MABS.

2.1. General Principles

Traditionally, the generation of most therapeutic antibodies relies on the immunization of mice or other mammals with the desired antigen target. Upon repeated injections, the animals develop a strong response to the antigen and will present a large number of cells secreting antibodies against the injected antigen. It is important to understand that multiple antibodies directed at different epitopes of the antigen are secreted by a mixed population of B cells with each cell only secreting one specific antibody [34]. Therefore if one secreting B cell was to be isolated and expanded (cloned), only one type of antibody molecule (monoclonal or mAb) would be secreted. Unfortunately, secreting B cells can only replicate a limited number of times, therefore rendering production of mAbs by their cultivation all but impossible. In vitro production of mAbs was only a concept until the groundbreaking hybridoma technology was developed in the 1970s by Kohler and Milstein [35-37]. Antibody secreting cells originating from the spleen of an animal immunized with the antigen target of choice are fused with immortalized (i.e. cells that will divide forever when cultivated in permissive conditions) non-antibody secreting cells. To facilitate the isolation of the desired fused clones, the immortalized non-secreting cells are engineered to be deficient in a key metabolic pathway. For example, deficiencies in the hypoexan-
thine guanidine phosphorybosyl transferase (HGPRT), an enzyme that holds a key role in the synthesis of guanidine tri-phosphate (GTP) in the “nucleotide synthesis salvage pathway” have been extensively used. These cells are still able to synthesize their nucleotides via the main (or de novo) pathway, however, when cultivated in the presence of aminopterin, an inhibitor of dehydrofolate reductase (DHFR) an enzyme that produces tetrahydrofolate, a key co-factor in the synthesis of thymidine tri-phosphate (TTP) from dUMP in the main nucleotide synthesis pathway, these cells can not grow since each of the pathways leading to the production of nucleotides are non-functional under these conditions. Since they have a functioning HPRT enzyme, B cells originating from the spleen are able to survive in the presence of aminopterin. However as somatic cells, they only can replicate a few times before dying. Only fusion clones that have inherited the ability to replicate indefinitely from the non-secreting cells and the functional HPRT activity from the B-cells will grow for extensive number of generations in the presence of aminopterin. When coupled with the isolation of colonies originating from one fused cell, this technology rendered possible the production of mAbs. Clones that produce the mAbs with affinity to the desired target can be identified using classical affinity biochemistry methods. The development of this technology in the late 1970s was hailed as a major breakthrough and opened the door to generation of therapeutic monoclonal antibodies and is now a well established protocol [38-40]. Fig. (2) represents an overview of the process of mAb production by generating hybridomas.

Although the first recombinant mAbs were produced using this technology, the mAb hybridoma production platform presents some serious drawbacks. Since the coding region of the mAb directly originates from the immunized animal without any subsequent genetic manipulations, the mAb produced is of mouse origin in its amino acid sequence, and upon repeated injections rapidly triggers an immune response from the human recipient (referred to as Human Anti Mouse Antibody or HAMA). Upon these repeated infusions, the therapeutic mAb is rapidly cleared from the circulation and therefore rendered therapeutically ineffective. About 80% of the patients develop an immune response to the first commercial therapeutic mAb, OKT3, a murine antibody used in the treatment of organ transplant rejection [41]. Further improvements were obviously needed in order to make monoclonal antibodies a therapeutic reality, and consequently there are only two murine therapeutic monoclonal antibodies approved at this time (Table 1).

2.2. Sequence Humanization

Following these first clinical attempts to use mAbs of murine structure, molecular biology manipulations greatly improved the immunogenic profiles of the subsequent mAbs tested in humans by reducing the amount of amino acid sequences of mouse origin while retaining the appropriate affinity for the intended target. The most common approach starts with the generation of mouse hybridomas as described above. Once clones are generated, they are screened for their ability to produce a mAb with the desired affinity for the target antigen, employing biochemical screening methods [42-47]. The selected clone is further cultivated and the mAb genetic coding sequences are isolated and modified to yield antibodies that are more and more human-like in their structure. Briefly these technologies have evolved over the past decade, from first the construction of chimeric antibodies, made up of the constant regions of human antibodies attached to the variable region of mouse origin (responsible for the binding to the target), to techniques of generating "humanized" antibodies by "grafting" and "veneering" only the essential mouse amino acid residues needed for affinity
Table 1. Therapeutic Monoclonal Antibodies and Antibody Fragments on the US Market

<table>
<thead>
<tr>
<th>Name</th>
<th>Generic name</th>
<th>Target</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituxan®</td>
<td>Rituximab</td>
<td>CD20, a protein expressed on B cells but not on stem cell and differentiated antibody secreted cells. Can trigger cell death via ADCC, CDC and by initiating apoptosis.</td>
<td>Non Hodgkin Lymphoma of B-Cells</td>
</tr>
<tr>
<td>Zevalin®</td>
<td>Ibritumomab</td>
<td>CD20. Same target as rituximab, often used in conjunction with rituximab</td>
<td>Non Hodgkin Lymphoma of B-Cells</td>
</tr>
<tr>
<td></td>
<td>Conjugated with either Indium ¹¹¹ or yttrium ⁹⁰°. A murine antibody precursor to rituximab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bexxar®</td>
<td>Tositumomab, a conjugate with I¹³¹</td>
<td>CD20. Specifically delivers a radionucleide to target cells. ADCC, CDC, and induction of apoptosis via ionizing radiation.</td>
<td>Non Hodgkin Lymphoma of B-Cells</td>
</tr>
<tr>
<td>Herceptin®</td>
<td>Trastuzumab</td>
<td>Her2, a tyrosine kinase receptor over-expressed on the surface of cancer cells. By blocking the most upstream signal, it reduces cell proliferation and survival. Can also trigger cell death via ADCC and CDC.</td>
<td>Cancer (breast)</td>
</tr>
<tr>
<td>Mylotarg®</td>
<td>Gemtuzumab ozogamicin A conjugate with the cytotoxic ozogamycin,</td>
<td>CD33, a protein often over-expressed on cancerous myeloid cells but not on pluripotent haematopoetic stem cells. Specifically delivers a cytotoxic drug.</td>
<td>Cancer (Acute myeloid leukemia)</td>
</tr>
<tr>
<td>Campath®</td>
<td>Alemtuzumab</td>
<td>CD52, a protein expressed on the surface of differentiating lymphocytes. Elicits cells death via ADCC, CDC, and the triggering of apoptosis.</td>
<td>Cancer (Chronic myeloid leukemia)</td>
</tr>
<tr>
<td>Erbitux®</td>
<td>Cetuximab</td>
<td>EGFr, a tyrosine kinase receptor over-expressed on the surface of cancer cells. By blocking the most upstream signal, it reduces cell proliferation and survival. Can also trigger cell death via ADCC and CDC.</td>
<td>Cancer (Colon)</td>
</tr>
<tr>
<td>Vectibix®</td>
<td>Panitumumab</td>
<td>VEGF, a circulating protein that triggers vascularization, a needed step for tumor development</td>
<td>Cancer (colon)</td>
</tr>
<tr>
<td>Avastin®</td>
<td>Bevacizumab</td>
<td>VEGF, a circulating protein that triggers vascularization, a needed step for tumor development</td>
<td>Cancer (Colon)</td>
</tr>
<tr>
<td>Orthoclone®</td>
<td>Murumob</td>
<td>CD3a protein associated with T cell receptors. Steric inhibition by OKT3 induces pan-T cell depletion</td>
<td>Kidney transplant rejection prevention</td>
</tr>
<tr>
<td>OKT 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zenapax®</td>
<td>Daclizumab</td>
<td>CD25, an IL2 receptor present on the surface of activated but not resting T-lymphocytes, thereby blocking activation of cytotoxic lymphocytes involved in transplant rejection</td>
<td>Kidney transplant rejection prevention</td>
</tr>
<tr>
<td>Simulect®</td>
<td>Basiliximab</td>
<td>CD25</td>
<td>Kidney transplant rejection prevention</td>
</tr>
<tr>
<td>Remicade®</td>
<td>Infliximab</td>
<td>TNFα, a proinflammatory cytokine involved in various inflammatory chronic diseases</td>
<td>Crohn’s disease, RA</td>
</tr>
<tr>
<td>Humira®</td>
<td>Adalimumab</td>
<td>TNFα</td>
<td>RA</td>
</tr>
<tr>
<td>Xolair®</td>
<td>Omalizumab</td>
<td>IgE, prevent the binding of this proinflammatory signaling immunoglobuling with its receptor</td>
<td>Allergy related asthma</td>
</tr>
<tr>
<td>Raptiva®</td>
<td>Efalizumab</td>
<td>CD11a, a subunit of the T cell β integrin LFA-1, a ICAM receptor involved in T-cell activation and migration, a hallmark of psoriasis.</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>Tysabri®</td>
<td>Natalizumab</td>
<td>4 subunit of VLA-4 integrin of activated T cells, preventing interaction with its brain endothelium counter receptor VCAM-1, thereby preventing T-cells from crossing the blood brain barrier.</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Synagis®</td>
<td>Palivizumab</td>
<td>F protein (fusion transmembrane protein) of the Respiratory Synsytial Virus. Binding to the F protein elicits recruitment of the immune system effector components and triggers virus clearance.</td>
<td>RSV infections</td>
</tr>
<tr>
<td>Soliris®</td>
<td>Eculizumab</td>
<td>Terminal complement protein C5. Prevents complement mediated lysis of red blood cells deficient in the regulatory proteins CD55 and CD59, which makes the cells sensitive to complement dependent destruction.</td>
<td>Paroxysmal Nocturnal Haemoglo- binuria</td>
</tr>
</tbody>
</table>
to the antigen target to a human antibody framework. Finally, the production of fully "human" antibodies through the use of transgenic mice, which expresses human genes for IgGs, has been implemented to recently yield its first commercialized mAb, Vectibix® (panitumumab), an IgG2 molecule [48].

All these approaches which rely on the immunization of animals require the construction of hybridomas and present limitations in some situations such as when generating a mAb directed against a toxin or a highly conserved antigen across species. To overcome these limitations, the generation of mAbs by recombinant technology was developed. Post isolation and amplification of the variable HC and LC genetic coding sequences from B lymphocytes by PCR, it relies on the construction of highly diverse libraries that have the potential to generate up to $10^{10}$ different mAbs via recombination. Most screening protocols use phage display technology, an approach that by fusing the Fab region coding genes with the gene of a phage coat protein results in linking genotype to phenotype. Briefly, the phage which displays the resulting Fab on its coat can be isolated by affinity technologies. After amplification in a bacterium, the genome of the isolated phage contains the matching DNA sequence which can be isolated and further manipulated as desired such as increasing affinity for the antigen target [42-47, 49]. Other methods such as yeast and bacterial displays have also been developed and may present a simpler and faster approach to the development of synthetic mAbs [42-47]. As of today, the use of phage display technology has been successfully implemented in the generation of fully human antibodies, with Humira® an antibody directed at the human protein TNFα, being the first to reach commercialization.

Development of the production of chimeric mAbs followed by the refinements described here were the needed boosts that by greatly reducing or eliminating patient immune response to the therapeutic mAb allowed repeated infusions and propelled therapeutic mAbs to widespread and successful clinical uses.

### 2.3. Construction of Genetic Vectors

The construction of producing clones follows the overall paradigm of classical molecular biology developed for microbial systems. After selection of a hybridoma clone secreting the desired antibody (selection based on affinity for its target), the DNA sequence coding for the IgG is obtained and engineered to reduce immunogenicity as described above. Further refinements aimed at increasing the affinity of the mAb to its target antigen using phage display technology can take place at that point during the development cycle of a mAb [49]. This is sometimes a required step since affinity of the original murine mAb for its target typically decreases during the humanization process. The finalized mAb coding sequences are then inserted on classical genetic vectors (plasmids) that contain a selectable genetic marker and that are mass produced using microbial technology. The entire coding mAb sequence can be enclosed into a single plasmid, or two plasmids each containing the genes for the heavy chain (HC) and light chain (LC) respectively can be constructed [50, 51]. The purified plasmid(s) is/are then transfected into non-antibody secreting immortalized mammalian cells, where upon insertion of one or multiple copies into one or several chromosomes, the foreign gene is transcribed to eventually yield secretion of a fully functional mAb. Various transfection approaches that all rely on the temporal destabilization of the membrane integrity, thus allowing the plasmid to gain access to the cytoplasm, have been used with success [52]. Vector design uses the same promoters for both heavy and light chains and in theory can support similar production of HC and LC, although more LC may be required since HC lower transcription efficiency and lower transcript stability has also been observed. Studies seem to indicate that excess LC synthesis may lead to better stabilization of the HC and to higher overall mAb secretion [53]. To the best of our knowledge, tunable expression systems that favor the transcription and translation of LC or that offer the possibility of controlling the onset of synthesis via induction have not yet been commercially implemented although the technology does exist [54].

Since transfection and integration of genetic material into the chromosome of mammalian cells are low efficiency processes, in order to facilitate selection of cells that are likely to have integrated the foreign DNA and to secrete the desired mAb, selection protocols are routinely used. These rely on the principle that when the genes of the mAb and of a resistanceSelectable marker are co-localized on the vector they will co-integrate and co-express with a high probability. Usually resistance to an antibiotic or the ability to grow in a

<table>
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<th>Name</th>
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<th>Target</th>
<th>Indication</th>
</tr>
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<tbody>
<tr>
<td>Reo-Pro®</td>
<td>Abciximab (a Fab fragment)</td>
<td>GPIIb/IIIa receptor complex of platelets. Binding to these integrins inhibits platelet aggregation and prevent blood clots formation.</td>
<td>Adjunct in coronary interventions</td>
</tr>
<tr>
<td>Lucentis®</td>
<td>Ranibizumab (a Fab fragment)</td>
<td>VEGF. Binding with the VEGF ligand prevents interaction with its receptors VEGFR1 and VEGFR2 on the surface of endothelial cells. When locally injected, it reduces endothelial cell proliferation, vascular leakage and the formation of new blood vessels on the central part of the retina.</td>
<td>Wet macular degenerescence</td>
</tr>
<tr>
<td>Cinzia®</td>
<td>Certolizumab pegol (A Fab fragment conjugated with poly-ethylene-glycol)</td>
<td>TNF-α.</td>
<td>Crohn's disease</td>
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It has been established that the site of integration of the foreign DNA clearly influences the level of expression. Targeting highly transcriptionally active regions of the genome for the integration of the foreign genes has been attempted and has shown some positive but limited signals [55]. One of these approaches relies on first developing and isolating high producing clones where the inserted transgene is flanked with bacteriophage-derived short DNA strands (lox P) that are substrate to a specific recombinase (Cre) thereby creating pre-characterized sites for the future exchange and insertion of other transgenes. Assuming that integration of any foreign trans gene to this specific site will yield high producing clones, targeted integration can be achieved via reciprocal site specific integration [56]. Other approaches have, rather than targeting transcriptionally active regions, developed the use of short nucleotide sequences that help the foreign DNA to be more transcriptionally active regardless of its site of integration. When linked to the trans genes these genetic elements are believed to influence the accessibility of the chromatin environment in the vicinity of the integration site and to thereby increase transcription efficiency. Several technologies have been developed and although different in their respective mode of action, they all rely on this general philosophy.

The STAR elements (Stabilizing and Antirepressor elements) reduce the extent of histone deacetylation pattern and the spread of methylation in the vicinity of the inserted trans genes. Correlatively, the region is kept acetylated and transcriptionally more active [57]. The S/MAR elements (Scaffold/Matrix Associated Regions) interact with the nuclear matrix and create loops where gene expression is coordinated and insulated from repression [58, 59], while the UCOE elements (Ubiquitous Chromatin Opening Elements) which are derived from housekeeping genes, a group of genes that present high histone acetylation, create a highly transcriptionally active environment [60].

One very apparent impact of these new technologies is the potential to reduce the need for the evaluation of a large number of transfectants which could significantly shorten development timelines [55]. As of now, although several publications outline the benefits of these elements, their introduction is too recent to know when they eventually will be incorporated into the design of a commercially used clone.

### Table 2. Commercialized mAbs Production Cell Platforms

<table>
<thead>
<tr>
<th>CHO</th>
<th>NS0</th>
<th>Sp2/0</th>
<th>Murine Hybridomas</th>
</tr>
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<tbody>
<tr>
<td>Avastin, Campath, Herceptin, Humira, Raptiva, Rituxan, Vectivix, Xolair, Zevalin</td>
<td>Mylotarg, Soliris, Synagis, Tysabri, Zenapax</td>
<td>Erbitux, Remicade, Reopro, Simulect,</td>
<td>Bexaar, Othoclone</td>
</tr>
</tbody>
</table>

3. CURRENT CELL LINES IN USE

Although many immortalized mammalian cells lines can be potentially used, since they were in use at the time the first mAbs were developed for clinical and commercial applications, as of today, with the exception of the two mAbs produced by hybridoma technology, the production of all currently commercialized therapeutic mAbs is achieved in mammalian cells of mouse (NS0 and SP2/0 cell lines) or Chinese hamster ovary (CHO cell line) origin. For references, Table 2 lists a few selected commercialized mAbs and their respective production platform.

3.1. NS0 and SP2/0 Cell Lines

These cell lines originate from mouse plasmacytoma cells that have undergone several steps of cloning and selection to yield immortalized non-IgG secreting B cells [36, 37, 61-65]. Both NS0 and SP2/0 cell lines have been extensively used for the production of mAbs employing fusion technology. More recently, molecular biology techniques have allowed NS0 and SP2/0 cells to be used as the production platform for several therapeutic mAbs currently on the market. These cell lines can be cultivated in serum and non-serum containing cultivation media and are reasonably amenable to scale up in large cultivation vessels (up 20,000-L scale). However, NS0 cells present some drawbacks that complicate their cultivation. Unlike most mammalian cell lines, NS0 cells seldom grow in the absence of exogenous cholesterol, and are therefore routinely cultivated in the presence of cholesterol, which is usually delivered via the use of serum [66-70]. Since the presence of ingredients of animal origin as well as any proteins in cultivation media is undesired, it complicates the use of NS0 cells due to the difficulty of supplying cholesterol in a protein free medium as it requires the use of carriers such as cyclodextrins to enhance cholesterol "solubility" [71, 72]. It has been demonstrated that cholesterol independent NS0 cells can be developed, although with a certain unpredictability, a factor that has limited their use [73-75]. Recent advances into the mechanism of cholesterol requirement has assigned the epigenetic gene silencing caused by methylation upstream of the region coding for the 3β-hydroxysteroid dehydrogenase type 7 that catalyzes the conversion of lanosterol to lathosterol as the cause of this deficiency [76-78]. Although industrial groups have developed cholesterol independent NS0 lineage [79], to the best of our knowledge, no commercial production of mAbs using NS0 cholesterol independent cells is currently on-going. It is quite possible, since they offer greater simplicity of use, that these biochemically and genetically understood cholesterol independent lines will eventually be used for commercial production.
Although reasonably similar, the glycosylation pattern distribution ratio (G0F, G1F, and G2F) of the IgGs produced by NS0 and SP2/0 is not that of circulating human IgGs. In addition, these cells produce small amounts of murine-like glycans such as the addition of an extra galactose (α-Gal) to the terminal galactose and the insertion of N-glycolyneuraminic acid (NGNA) in place of NANA, [80], which have the potential to trigger an immune response. Clinical adverse events, anaphylactic shock, have been reported for cetuximab which is produced by cultivation of SP2/0 cells [81]. Nevertheless, and because the consistency of the glycoforms can be controlled and characterized, NS0 and SP2/0 cells have demonstrated reliability and have proven their worth in the production of several well marketed mAbs.

Several selection strategies for selecting clones that have integrated the desired mAb trans genes have been developed and successfully applied. The overall philosophy is to use cells with a deficient metabolic background, while the genetic element containing the mAb coding region carries a gene that once expressed will complement the metabolic deficiency. Upon transfection, one or several copies of the genes carried on the plasmid will integrate into one or several chromosomes of the recipient cells where they will be transcribed and translated. Post transfection and during cloning of the cells, a selective pressure aimed at favoring those cells that have incorporated the gene coding for the correction of the metabolic deficiency is applied in order to only favor the growth of those cells that have integrated the foreign DNA. Assuming that both foreign genes have stayed linked during their integration into the genome of the recipient cells, the majority of the cells surviving the selective environment will be mAb producers. Several variations on this strategic theme are described below.

A popular NS0 cell lineage is the GS-NS0 commercialized by Lonza biologics. NS0 cells have very low levels of the endogenous enzyme glutamine synthetase (GS), and require exogenous glutamine in order to grow. It is only when transfected with a copy of the GS gene that the cells can grow in the absence of glutamine [61, 82]. A vector, designed to carry both the genes coding for the mAb to be expressed and for GS, once integrated into the genome will allow the expression of both genes and the selection of producing clones. In order to impose selective pressure against rejection of the foreign DNA and to select for clones having integrated the genes in a region of high expression, the cells are cultivated in a medium devoid of glutamine and in the presence of methionine sulfoximide (MSX), an inhibitor of GS. Only those clones that produce large amounts of GS will survive. Correlatively, these clones are likely to also produce large amounts of the mAb. Interestingly, this approach seems to yield high producing clones that have integrated low copy number (less than five) of the trans gene [61, 82].

Another method of selection used relies on the fact that SP2/0 and NS0 cells are deficient in HGPRT a negative genetic trait can be exploited by inserting a copy of a microbial gene (from E. coli) coding for the synthesis of XGPRT, an enzyme that can substitute for HGPRT. Only the cells that have integrated the XGPRT gene will be able to use the nucleotide synthesis salvage pathway. When cultivating the transfectants in a selective medium containing aminopterin which inhibits the de novo nucleotide synthesis pathway, only those cells that have incorporated the XGPRT gene will grow. Since the gene coding the IgG of interest is co-located with the XGPRT gene on the plasmid, the probability of a co-integration is high, and transfectants expressing high levels of IgG are likely to be selected by using this method [79, 83].

3.2. CHO Cells

These cells originally isolated from a Chinese Hamster Ovary in 1957, are proline auxotrophs and have been spontaneously immortalized during their successive transfers. [84-87].

In the mid-1980s CHO cells were modified to be deficient in the dehydrotetrafolate enzyme (DHFDR). This enzyme is required for the synthesis of reduced tetrahydrofolate, a cofactor required in the synthesis of DNA precursors. Commercial production cell lines are derived from two major CHO dhfr minus lineages, DG44 and DUKX-B11. Both lines were developed at different times by the same group at Columbia University [88-90]. This metabolic deficiency allows for a selection strategy similar in its philosophy as described previously. Here, the use of metotrexate (MTX), an inhibitor of DHFR is implemented. Since the plasmid contains a copy of the DHFR gene along with the mAb of interest, only the cells that have stably integrated both genes carried on the plasmid will be able to grow and produce mAb in the presence of MTX [91]. Using increasing concentrations of MTX will lead to the selection of high producing clones which are likely to have incorporated high copy numbers of the trans genes [51, 92]. The DHFR selection strategy can be enhanced with the use of resistance to aminoglycoside antibiotics such as neomycin and kanamycin conferred by an added resistance gene on the plasmid [93, 94]. It is believed that the incorporation of high copy numbers of the trans gene may lead to genetic instability.

More recently, a selection based on the fact that the parent CHO-K1 cells have a low glutamine synthetase (GS) expression level has been developed. Transforming the cells with a plasmid co-expressing GS and an IgG of interest and cultivating the transfected cells in the absence of glutamine and in the presence of MSX allows for only those cells that have stably incorporated the foreign genes to grow. Since it only relies on a weak expression rather than a metabolic deficiency, this system requires that the presence of MSX be maintained during cell expansion in order to keep a sufficient genetic pressure to prevent deletion of the foreign DNA [82].

These two genetic strategies rapidly allowed the use of CHO cells since it conferred a rapid method to select for producing clones and to identify high mAb producers. CHO cells can be cultivated in suspension in serum-free chemically defined cultivation media in large scale conventional bioreactors. They display a high resilience to cultivation conditions, do not require cholesterol, and tend to remain viable for a longer period of time when compared to NS0 cells. This ease of genetic selection coupled with their very amiable character to large scale industrial cultivation have resulted in the CHO cell line being used in more than half of the registered mAb production processes.
However, because of their rodent origin, the glycosylation pattern distribution ratio (G0F, G1F, G2F) of mAbs produced by CHO, NS0, and SP2/0 cells do not completely match that of circulating human IgG1. In addition, CHO cells produce small amounts of non-human like glycan patterns, such as α2-3 linked sialic acid residues that have the potential to be immunogenic [80]. On the other hand, these non-human glycans are present in very low proportions (a few %) and mAbs produced by cultivation of CHO cells have remarkably safe profiles in the clinic [41].

3.3. Emerging Mammalian Cell Lines

Other cell lines of animal and non-animal origin have been considered for expression of mAbs. Since this discussion is limited to mammalian cell lines in its scope, and although some significant advances have been made in using microbial expression systems, this topic will not be reviewed here. Among emerging mammalian cell lines, the Per.C6® cell line appears to be most advanced in its usage and acceptance. Per.C6® cells are human embryonic retina cells that were immortalized by the use of the early gene E1 of Adenovirus [95]. Per.C6® cells offer the potential for human like glycosylation pattern with the added advantage of a lack of undesirable murine glycans. Several recent communications have disclosed that Per.C6® cells can be cultivated to very high densities at large scale and that they are capable of supporting elevated recombinant protein yields of up to 10 g/L of a test mAb [96, 97]. These promising data will certainly help the Per.C6® cell line to gain appeal in the next few years as a potential platform for the production of recombinant mAbs. However, to the best of our knowledge, no mAb production platform based on the cultivation of Per.C6® has yet reached the regulatory registration stage.

A few additional cell lines are believed to be in development such as the avian EBx and the rat myeloma YB2/0 cell lines. Their potential advantage is significantly reduced fucosylation, and therefore the potential to increase the level of ADCC of the manufactured antibody. Their development in the production of mAbs is less advanced than that of PerC6 cell. Unlike well established cell lines such as CHO, NS0, and SP2/0 that have been used for the production of several commercial mAbs, new and previously unregistered cell lines are likely to face high regulatory scrutiny. This is especially true on the topic of associated viruses, and in the clearance of residual genetic material. We are however positive on the prospect of the most advanced of these additional cell lines, Per.C6® cells, to soon join NS0, SP2/0 and CHO cells as platforms for the commercial production of mAbs.

4. SELECTION AND DEVELOPMENT OF HIGH PRODUCING AND INDUSTRIALLY AMENABLE CLONES

4.1. Clonality and Selection of High Producing Clones

Once cultivated under selective conditions, the cells are rapidly subjected to cloning by the use of various methods. Cloning is required for ensuring that the produced mAb molecules will have high homogeneity and that cultivation of the selected clone will be consistent and predictable. In addition to being a process need, clonality is also a regulatory requirement. The most popular and simpler method is the use of limiting dilution where the cells are diluted to concentrations that will likely dispense one or less than one cell per culture well. The cells are allowed to grow under the desired selective conditions until confluence is observed. This is achieved by classical dilutions and estimations of cell population based on viable cell counts. An improvement to this method is the use of automated cell sorting equipment (flow cytometer). Although these methods are simple and predictable, they require extensive amounts of labor and time and improvements or alternative methods have been sought. Adding to the advantage of simple cell sorting, several technologies have evolved with the aim to directly identify potential high producing cells at this stage. One approach has been to estimate the secretion potential of a cell by entrapping it in a gel. After an incubation period, the secreted entrapped mAb is detected using a fluorescent-labeled antibody. The entire gel drop, containing cells and their respective captured secreted product, are sorted by the flow cytometer according to their surrogate level of mAb production, thereby potentially achieving a rapid selection [98-100]. Other approaches have relied on detecting the intra-cellular co-expression of DHFR [101], when employing CHO cells and this specific popular selection system, or on measuring an easily detectable mAb co-expressed protein directed to the external surface of the membrane [102]. However, these methods only reflect the secretory potential of a cell for a short amount of time under conditions that may not be highly reflective of the intended production environment.

In an attempt to potentially better capture the production potential, other methods rely on the cultivation of the cells on a soft agar surface, which retains the antibody secreted in the vicinity of the cells, and on detecting extended production potential using a labeled antibody [103, 104]. The clones can then be directly picked and cultivated for further evaluation. The potential advantage of this technique is that unlike the FACS based methods, which submit the cells to high shear stress, it offers a more cell-friendly environment and captures antibody secretion over a longer period of time, thereby increasing accuracy of detection.

In order to verify clonality, microscopic observations of each culture well are performed during the incubation period which can last several weeks. The cells are further expanded in larger cultivation vessels where their potential to produce large amounts of mAb is evaluated [105-107]. Upon confirmation of their mAb production level and quality (i.e., glycan distribution, correct amino acid sequence, ..) , the best producing clones are selected for laboratory scale cultivation under process conditions [108]. Since several hundred clones are evaluated during a typical screen, as mentioned in the previous section, the use of genetic elements that can target the foreign genes to highly transcriptionally active regions has the potential to tremendously reduce both the amount of work and the development timelines. Of these clones that maintain high productivity and that produce mAbs with the desired quality, a few are selected for further re-cloning. This is achieved by either a second round of limiting dilution or by sorting the cells using a flow cytometer or any other method described above.
Post second round of cloning, an expansion protocol similar to the one described above is put in place and the best producing clones (usually 3-5) are selected for stability evaluation. Since mammalian cells have a very plastic genome and tend to reject, modify or relocate integrated foreign DNA, it is important to ensure that the clone selected will exhibit genetic stability over the desired length of cultivation. Stability is not only measured in terms of production levels but also on the biochemical attributes of the mAb such as sequence integrity and fidelity in the glycosylation pattern [109]. This is achieved by cultivating the cells for extended numbers of generations under conditions that mimic process conditions as closely as possible, including an evaluation beyond the number of generations that are targeted in the manufacturing process. It is not uncommon to observe a reduction in the amount of mAb produced over these successive transfers as well as to detect changes in some of the biochemical characteristics of the molecule [107, 110-114].

Most important to the selection of a clone is maintaining biochemical profile of the molecule as quality attributes are essential to the safety and potency of the product. Once these steps are completed, the selected clone is preserved in a set of cell banks that upon qualification (purity, lack of adventitious agents, etc.) will be used for commercial production of the therapeutic mAb.

These procedures are lengthy, require a highly specialized and competent workforce, expensive equipment and therefore weigh heavily on the overall product development cycle time, since these activities are performed on the critical path. This is where the advantage of integrating novel technologies can be of great impact since they have a high probability of reducing the extent and duration of the screening activities. Fig. (2) presents a pictorial overview of the steps associated with clone selection.

4.2. Cell Line Engineering

In addition to selecting suitable and stable clones for commercial production of mAbs, many research groups have attempted to modify the producing host cells in order to either improve or control the quality and quantity of the mAbs produced. Rather than presenting an exhaustive list, a few key examples pertaining to each area of investigation will be discussed.

Improving or influencing the quality of the monoclonal produced is of importance since it can translate into better therapeutic efficiency. As mentioned earlier, of significance is the importance of the glycosylation pattern. For example, mAbs with non-fucosylated glycans have been shown to have greater interaction with the effector cells of the immune system, translating into greater cell target lysis by ADCC. CHO cell lines that are devoid of fucosyl transferase have been constructed by several groups, with the resulting mAbs exhibiting up to 100 fold increase in ADCC activity [20-26]. Other approaches have targeted the modification of the Fc region of the protein sequence in order to increase affinity for the receptor of the effector cells or to improve mAb stability [45, 115, 116]. Since high ADCC activity seems to correlate with the efficacy and response rate of several mAbs used in the treatment of cancer especially in the case of Rituxan® [117], there is a reasonable likelihood that one or several engineered mAbs with increased effector function achieved via either glyco or protein engineering will reach the clinics and the market in the next decade.

In addition to improving the end-product, engineering of the production host may provide a means to improve mAb production yields. Investigated areas cover a wide range, and the following selected examples will briefly present an overview of the current research.

Improving cell longevity should result in increased mAb production since the specific rate of mAb secretion (mass per cell per unit of time) tends to be fairly constant during the cultivation stage. Although cells are adapted to grow in suspension the combination of stressful shear bioreactor environment, the potential nutrient limitations, toxic by-product accumulation, and the burden of producing large amounts of a foreign protein all have the potential to trigger the onset of apoptosis, a highly regulated pre-programmed self destruction process that cells will initiate when facing highly stressful conditions [118, 119]. Since the apoptotic pathway is highly complex and involves many activator as well as repressor proteins, multiple targets with the aim to reduce cell death have been evaluated. Mutants that contain additional copies of genes coding for resistance to apoptosis have been constructed for both NS0 and CHO cell lines, while other approaches have relied on the use of inhibitors of apoptosis [120-127]. Overall increases in longevity have been observed and some correlations with increased mAb secretion have been observed. These published works, however, have translated to limited improvements from low production levels to modest production levels at best and remain to be translated to industrial cell lines. Possible explanations include the fact that since industrial cell lines undergo an extensive, lengthy, and stressful selection process, only the most resilient cells survive and end up selected, and limited benefit is gained from these additional alterations. It is highly possible that in the coming years the use of naïve host cells engineered to exhibit high resistance to apoptosis will be used in commercial mAb production. When coupled with the use of genetic elements and of rapid identification methods for high producers, the introduction of engineered cells may help in speeding up the time required for the identification of clones that are amenable to cultivation conditions.

Another aspect of cell line engineering currently investigated is the modification of the protein assembly and secretion machinery of the cells. Once foreign genetic material is integrated into a highly transcriptionally active region, supported by retrospective investigations employing proteomic and genomic analyses, it is now believed that the assembly and secretion step may in itself be more rate limiting than the abundance of mRNA [128, 129]. This hypothesis is supported by the fact that maximum secretion rates for mAbs have been relatively flat over the past 6-8 years, with most specific production rates of industrial cell lines comprised in the 25-40 pg/cell/day range. It is speculated that mis-folded proteins that are degraded in situ can account for substantial mAb losses, and it is believed that these yield losses could be reduced through achieving a better control of the oxidative/reductive enzymatic cell machinery and of the interactions with the chaperone proteins involved in cellular trafficking and secretion of the mAb [130-132]. These very
novel approaches have yet to reach a technological level where they can be implemented in the design of improved industrial cell lines. However, they present long term potential and merit to be closely monitored for their eventual implementation.

5. CULTIVATION PROCESS

5.1. Cultivation Medium

Media used for the cultivation of mammalian cells are highly complex and contain several dozens of ingredients that range from amino acids, vitamins, trace elements, and nucleosides to name a few groups [133-136]. Development and manufacturing of these finely balanced media are complex and time consuming and require considerable expertise. Until recently, the use of serum was standard and provided the cells with many necessary nutrients and growth supporting molecules. However, recent events linked to abnormal prions which cause bovine spongiform encephalitis (BSE) have generated an incentive for the avoidance of animal-sourced ingredients in the manufacture of biopharmaceuticals such as therapeutic mAbs [137]. Early serum-free cultivation media used the addition of complex plant or yeast hydrolysates to their formulation in order to supply the nutrients and growth supporting molecules present in the serum [138-143]. While these components did replace animal-sourced materials, hydrolysates are still relatively undefined raw materials that can vary significantly between lots and vendors. Recently, the development of protein-free chemically defined media has been successfully achieved for both CHO and NS0 cell lines, with the formulation of media supporting extremely high cell densities [144]. Particularly, the development of non-animal sourced cholesterol carriers such as methyl-β-cyclodextrin (mβCD) complexed with synthetic cholesterol have met with success in supporting the cultivation of NS0 cells in serum-free media [71, 73].

Some companies choose to develop their own proprietary medium for many reasons including cost, control of the medium production process, ability to design medium to fit the specific clones and to avoid reliance on a single vendor since the formulations of commercially distributed cultivation media are not in the public domain. In house medium development is a significant investment in time and resources and often includes not only the design of a basal medium, but also the formulation of the seed expansion and production medium as well as the related feed solutions if a fed-batch strategy is to be implemented. One must also consider if liquid formulations are sufficient or if powder formulations should be developed. Given the anticipated scale of manufacturing batches for therapeutic mAbs, the storage of several hundreds of thousands of liters of cultivation medium under refrigerated conditions is a logistically complex and expensive proposition. Although not discussed in the public domain, the development of powder media is highly complex in itself, with the addition sequence of the ingredients and the type of milling equipment used believed to be keys to a successful formulation. When considering the number of high purity ingredients, the complexity of the manufacturing, and the required tests implemented for quality and consistency, it should not be surprising that the cost of mammalian cell cultivation media can reach costs in excess of $20 per liter, even at industrial scale. This is one of several costs of goods (development time, cultivation medium, purification resins, and facilities) that significantly contribute to the overall pricing of therapeutic mAbs.

5.2. Cell Banks and Seed Train Processes

Cells destined for cryopreservation are usually cultivated in small vessels, harvested during exponential growth phase, concentrated, and mixed with a cryo-protectant such as DMSO. Cell banks are traditionally stored using glass or plastic cryovials that range from 1-5 mL in volume. Very often, two tiered cell banks are created (Master Cell Bank and Working Cell Bank) in order to ensure long term supply for marketed products. The Master Cell Banks are made large enough to ensure supplies for the entire expected lifetime of the commercial process. Storage logistics and protection of the Master Cell Banks are keys to ensuring consistency of operations and should ensure legacy of the product. Recent publications have discussed larger volume cell banks using cryobags (up to 100 mL) [145-147]. Since these start from a larger volume, such an approach reduces the number of expansion steps needed in the seed train, which also results in shorter timelines. However, this is still a relatively new proposal for cell bank generation. A few hurdles remain regarding industrial acceptance of larger volume cell banks - the increased storage space requirements and more importantly maintaining integrity of the bag-containers under cryo-conditions.

The development of the seed or expansion train is often overlooked or minimized in its importance. While the seed train has a simple goal – generation of enough cell mass to inoculate the final production reactor – there are several important decisions that need to be made during seed train development. These decisions include the following: will the seed train be regenerated with each production batch, how many expansion steps will be used, will disposable vessels be used, what type and size of cell bank will be generated?

There are two common approaches for seed train processes – the rolling seed train and a de novo seed train for each production batch. Of importance is that mammalian cells are slow to divide with an average doubling time of about one day, and must not be excessively diluted when transferred to a new cultivation vessel. It is usual to consider a 10 fold dilution factor as maximum and safe for these transfers, and this will minimize the number of vessel transfers. When taken together, these limitations render the expansion of mammalian cells a very lengthy and labor intensive exercise. Some processes are designed at lower dilution factors, which allow for more flexibility in inoculation volumes and may shorten the seed train process. A de novo seed train consists of the thawing of a vial (or several vials) of a cell bank for each production batch. It is not unusual for de novo expansion protocols to span over more than one month when targeting the inoculation of very large bioreactors (10,000-L or greater). In addition to their length, as their name indicates, de novo expansion protocols must be implemented for each batch, consume a large number of banked cell vials over time, can be logistically difficult to manage, and consume a large amount of resources.
A "rolling seed train" is generated from cells contained in a vial (or several vials) that are thawed and sequentially expanded to yield a desired volume that is used to inoculate a bioreactor. Instead of being operated as a batch, the reactor is maintained in operation for many months. Every few days, a substantial volume is drained from the reactor and used to seed the production bioreactor. An equal volume of fresh medium is added to the seed reactor, thus allowing the remaining cells to multiply again. This drain-fill protocol can be repeated every few days, and constitute the basis of the "rolling seed train". The advantage of a rolling seed train is that smaller cell banks can be generated since each vial can support multiple production batches over periods of several months. In addition, less time is required to generate enough cell mass for production batches once the rolling seed train is established. However, in order to use a rolling seed train successfully, cell lines must be stable over much longer time periods – i.e. 3-6 months rather than 1-2 months. As discussed previously, the selection of a suitable clone for industrial production of mAbs must take into account the potential implementation of a rolling seed train. This is a very important point to consider since the quality of the mAb generated using cells produced by a rolling seed train must be consistent. Additional considerations for implementing a rolling seed train require that the facility be equipped with additional reactors dedicated to the seed train. On the other hand, a de novo seed train can be implemented with more flexibility as a facility can use disposable reactors for the seed train.

The number of expansion steps is a function of cell bank vial size, volume of available bioreactors, cell growth rates, and desired split ratios. Traditional expansion vessels include spinner flasks, shake flasks, and stirred tank reactors. Recently, many processes have implemented the incorporation of disposable vessels, such as Wave bioreactors©, in order to build in more flexibility into the expansion process and to control production costs (disposable vessels will be addressed in Section 5.3.3). A schematic overview of cell bank preparation protocols and of seed train expansion strategies is presented in Fig. (3).

5.3. Production Process

5.3.1. Environment Conditions

Bioreactor controls are one of the most well-defined areas of mammalian cell culture, encompassing temperature, pH, O₂, CO₂, and agitation controls. Initial work with bioreactor control approaches was performed in hybridoma platforms, but these approaches have also been implemented with success in CHO, NS0 and Sp2/0 platforms. Mammalian cells are typically cultivated at 37°C, pH 7.15 while maintaining dissolved O₂ levels at 30-60% dissolved oxygen (DO). The accumulation of byproducts can be detrimental to a culture, particularly when the goal is to maintain the culture over multiple weeks. Typical manufacturing processes are 12-14 days in length with no design for byproduct removal, so the accumulation of byproducts such as lactate and ammonium can lead to stressful conditions and higher probability of apoptotic cultures. Therefore, many different approaches have been investigated to reduce byproduct accumulation. One of the benefits of the GS system (see section 3.1) is the elimination of ammonium accumulation by eliminating the need for exogenous addition of glutamine in the culture medium. Both pH shifts and temperature shifts reduce cellular metabolism which leads to lower lactate production and sometimes even a shift in the lactate accumulation profile. Alternative medium components can also help in

![Fig. (3). Cell expansion logistics.](image-url)

Top part, production of Master and Working cell banks (MCB and WCB).

Bottom part, classical cell expansion protocol and rolling seed strategy.
controlling byproduct accumulation as many processes have demonstrated through the substitution of galactose for glucose (see section 5.3.2). Many studies have been performed to examine the effects of ammonium and lactate on CHO and NS0 cultures. Some studies show minimal negative effects on cell growth, but productivity may actually benefit from increasing lactate [148, 149]. Dissolved CO₂ (measured as partial pressure; pCO₂) levels are closely monitored due to a desire to mimic physiological levels (pCO₂ = 31-54 mmHg), however, levels can reach >150 mmHg in high cell density cultures [150, 151].

In order to maximize mAb production, many bioreactor processes have been designed with a two-phase approach where conditions are optimized first for growth and then altered to optimize mAb production. Unlike microbial cultures, mammalian cells do not typically carry inducible promoters; therefore, these processes cannot be induced in the traditional sense. On the other hand, bioreactor controls such as temperature and pH can be used indirectly to control mAb production either by increasing specific productivity or by shifting cell resources away from cell growth and towards mAb production. By designing for a two-phase process, after the desired cell mass has been accumulated, cell mass is maintained or incrementally increased, but at a very substantially reduced growth rate. This allows the cells to redirect the cellular metabolism toward mAb production. Low temperature and low pH are particularly effective in CHO and hybridoma systems, where temperatures as low as 30-35°C and pH as low as 6.7-7.0 can reduce cell growth and sometimes even increase specific productivity. Studies have found that growing CHO cells at lower temperatures may keep cells in the G1 stage of the cell cycle, which allows for more protein production, less nutrient consumption, less waste production (e.g. lactate), and decreased cell death [152-157]. The control of pH is also a critical parameter in controlling cell metabolism. Through lowering the pH of the cultivation medium, it is possible to decrease nutrient (especially glucose) consumption rates which in turn greatly reduce waste (lactate and ammonia) production. Lower pH limits are cell line and clone dependent where too low a pH will result in lack of cell growth or even cell death. Typical lower pH ranges where CHO cells can still proliferate and produce mAb is 6.7-7.0. The final effect is maintenance of higher viability cultures over longer periods of time. Some clones even show increased specific productivity when cultivated at lower temperatures and lower pH. Since clones vary in their sensitivity to pH and temperature variations, experiments must be conducted to balance cell growth with specific productivity as a function of these parameters. In addition, as discussed in the clone selection section, the identification of clones fully amenable to industrial cultivation conditions should ideally include the potential implementation of pH and/or temperature shifts.

CHO cultures do not show much sensitivity to DO levels – in most cases, CHO cells can be cultivated at DO levels ranging from as low as 5% to >60% DO without negative effects. Generally, O₂ levels are maintained through the sparging of a combination of air and O₂ supply and by increasing flow rate and agitation, as oxygen demand increases during exponential growth. Oxygen uptake rates (OUR) can be a useful tool for culture monitoring as the OUR values are very sensitive to culture conditions and closely reflect cellular metabolism. In fact, control automation programs can be designed to trigger events based on OUR values.

High cell density cultures can reach high pCO₂ levels (>150 mmHg), and these levels will alter cell growth, protein production, and protein glycosylation [151, 158]. Some of these effects are believed to be due to the increase in osmolality resulting from typical pH control strategies. When employing the most commonly used buffer system, bicarbonate-buffered medium, increasing pCO₂ resulting from high respiratory cellular activity will result in acidification of the medium. In order to keep the pH value close to the desired neutral range, base addition is required and will result in higher osmolality. To avoid this undesired cycle, the use of bicarbonate-free buffers were successfully implemented in a perfusion process resulting in 70% reduction in pCO₂ levels and subsequent positive effects on cell growth and specific productivity [159]. However, the negative effects of elevated pCO₂ still appear to be significant even when decoupled from osmolality effects. How each variable affects the growth phase and the production phase can be different depending upon the cell line, clone, and process. For example in CHO cultures, increasing pCO₂ only affected cell growth rates minimally whereas, the impact of osmolality was quite significant on cell growth rates. On the other hand, both factors had an impact on the production phase in terms of mAb titers [160]. Some work has been published on the effect of pCO₂ and osmolality on NS0 cultures. These papers describe different effects regarding the impact and conclude that there are differences due to clonal variation and process design [161-163].

As mentioned above, agitation is one of the variables used to control DO and pCO₂ levels. Historically, animal cells were considered highly shear sensitive, but more recent research confirms that animal cells are quite robust with respect to pure hydrodynamic forces [164-167]. Shear sensitivities are greatest at the air-bubble interface; therefore, the presence of protective polymers in the culture medium is a critical component. Typical additives are Pluronic F68 (most common), albumin, and dextran [168], that help in protecting the cells from high shear generated during bubble break up.

### 5.3.2. Nutrient Feeding

The simplest mammalian culture process is a batch process where all nutrients are added into the medium prior to addition of the cells. The culture is maintained until cell death and no further nutrient additions are made, on the assumption that sufficient nutrients are available in the basal medium to achieve desired cell growth. While a batch process is simple and easy to implement, it is difficult to provide sufficient nutrients in the basal medium without reaching toxic levels of certain components or without generating excessive levels of waste byproducts. A typical cell growth profile in a batch cultivation shows a phase of exponential growth which translates into a peak cell density that rapidly decreases as cells quickly loose their viability and lyse. Researchers found that cell viability could be maintained over an extended period of time, and even that a low level of cell growth could be generated, if some nutrients were added at different times during the cultivation. Initially, only simple nutrients, such as glucose and glutamine were added as feeds
during the culture. Fed-batch cultures became more sophisticated as feed solutions were developed into more complex mixtures [169]. One approach is to concentrate the basal medium and feed the concentrated mixture at specified times during the cultivation. Another approach is to analyze spent medium to determine the consumption of medium components and design a feed solution based on this information [144, 170-172]. While less empirical, the later approach can be lengthy and may not yield a complete set of information since cultivation media contain several dozens of ingredients, with some of them at difficult to measure concentrations. However, since some components may increase cell mass while other components may increase specific productivity, a better understanding of the effect of individual components will allow different feed solutions to be designed for different stages of the culture process and will support a more economical cultivation process. Feed solutions can also be designed to minimize undesirable byproduct generation [173-178]. An obvious example is the substitution of galactose, a more slowly metabolized carbon source, for glucose, which will support lower lactate production [173, 174, 177]. The majority of industrial mammalian culture processes use fed-batch technology since with expertise, the feed solutions and feeding regimens are reasonably simple to design and to implement. Given the popularity of this feed approach, the design of the production facility is simple and process development and validation are easy to perform. In addition, production phase process cycle times are reasonably short and reduce process exposure to adverse events such as equipment failure, and each fed-batch is easily associated with a product lot.

At the extreme, a continuous feed can be provided to the culture at a very low rate, where fresh medium is added to the culture and spent medium (containing the product of interest) is removed from the culture. Both rates need to be equal in order to maintain a constant volume. The advantage of this continuous culture approach is that the cultivation conditions are maintained constant over time and that waste products are constantly removed. However, this approach also results in the removal of perfectly viable cells from the bioreactor. To improve on continuous cultivation methods, the partial recycling of cells has been implemented and is known as perfusion culture [179, 180]. Under these conditions, a pre-set cell concentration is maintained by returning the desired amount of cells to the bioreactor. Perfusion processes are often maintained for many months, making it difficult to address regulatory issues on a per batch basis, and requires purification of the product from very dilute harvest streams. In addition, perfusion processes result in exposure to adverse events over a long period of time, require large amounts of expensive cultivation medium, and necessitate the design, maintenance and operation of very complex and difficult to scale up cell separation devices [181]. Also of importance is the need to use a production clone that exhibits excellent stability over several months of cultivation. For this reason, this technology is seldom used. However, the benefit of a perfusion process over a fed-batch process is that, for the production of a similar mass of mAb, it generally requires smaller cultivation vessels and smaller factory footprints [181]. In addition, perfusion processes minimize product exposure to adverse culture conditions, and it is for this reason that the one notable industrial exception is the production of Factor VIII where the product is highly unstable [182]. However, since mAbs are notoriously stable once secreted in the medium, the implementation of the highly complex and sensitive perfusion technology may not be the most efficient process. Fig. (4) pictures schematics and typical growth, cell viability, and mAb production in batch, fed-batch and perfusion systems.

5.3.3. Cultivation Equipment

Stainless steel bioreactors remain the workhorse of the industry. Stirred tank bioreactors used for production processes range from 1,000L to 25,000L in volume. These reactors are designed with baffles and a variety of impellers that result in the desired mixing profile [183, 184]. Airlift bioreactors, ranging from 2,000L to 5,000L, are also used for production processes. These reactors are similar to a bubble column where high air flow rates circulate upwards through an inner tube and bubbles are released at the top of the tube. The degassed liquid then flows downwards from the top of the tube; thereby, creating circulation of the medium and cells within the reactor [185, 186]. More recently, pilot-scale disposable reactors have become available in volumes ranging from 50L to 2,000L. The use of disposable equipment offers a wealth of advantages including the reduction of preparation time, the elimination of cleaning and sterilization steps, and a greater ease of use [187]. When factoring these benefits, the cost savings in terms of time and capital are likely to be significant. The manufacture of hundred of milligrams to gram quantities of recombinant proteins, produced via animal cell cultivation, is often required in order to support animal and clinical evaluations. Generally, these support activities rely on the cultivation of the animal cells in stirred laboratory- and pilot-scale bioreactors. While highly reliable and flexible, the preparation, operation, and cleaning of the reactors are time consuming activities. However, the recent commercialization of disposable and easy-to-use animal cell cultivation devices, such as the Wave Bioreactor™ (WBR), Hyclone S.U.B., and Xcellerex XDR™, offer the prospect of reducing the use of laboratory- and pilot-scale stirred bioreactors. In addition to their simplicity of use, the costs of disposable bioreactors and their ancillary accessories may be lower when compared to that of a sterilizable-in-place bioreactor.

Briefly, the WBR consists of a sterile disposable plastic bag that is half filled with cultivation medium with the head space filled with the desired gas mixture. The bags are placed on a rocking platform that delivers a wave-like motion to the liquid thereby delivering adequate mixing and gas transfer to the culture while avoiding the formation of damaging gas bubbles [188]. WBRs offer the possibility for continuous gassing and are available in nominal volumes ranging from 2-L to 1,000-L (www.wavebiotech.com). Since their market introduction, WBRs have been used for the cultivation of suspension [188, 189] and anchorage dependant mammalian cells [190, 191], as well as insect cells [192]. Most recently, Hami et al. report the use of WBRs for the clinical production of activated autologous T cells used in the treatment of various forms of cancers [193]. WBRs have also been fitted with a floating filter and successfully used as perfusion reactors, supporting cell concentrations of up to
3x10^7 cells/ml, a six-fold increase over the cell concentrations routinely achieved in batch cultures [194].

The Hyclone S.U.B. and Xcellerex XDR™ are more closely modeled after the stirred tank bioreactor. Both are contained within a stainless steel outer shell that is not disposable and provides the temperature control. Disposable sterile plastic containers are supplied from the vendor with ports for typical pH, DO, and temperature probes. A sterile, disposable impeller is built into each plastic container. The agitation is bottom driven in the Xcellerex XDR and top driven in the Hyclone S.U.B. The XDR™ is available from 200L to 2,000L (www.xcellerex.com), and the Hyclone S.U.B. (www.hyclone.com) is available from 50L to 1,000L. Minimum and maximum working volumes are similar to stirred tank reactors where impeller location and mixing profiles determine these values. Both models have been used to culture CHO and hybridoma cell lines, specifically for internal development projects at the respective companies.

Stainless steel stirred tank reactors still remain the industry standard, but disposable systems are gaining popularity for specific uses, such as seed train expansion or material generation. Since disposable systems do not require large capital investment, smaller organizations may find them useful to create short term capacity. Fig. (5) compares the most popular bioreactors currently in use for the cultivation of mammalian cells.

6. SCALE DOWN CULTIVATION SYSTEMS

Traditionally, experiments were sequentially performed in laboratory-scale bioreactors (i.e. 2L – 15L), scaled up to pilot-scale bioreactors (i.e. 300L – 2,000L), and confirmed at production-scale bioreactors (i.e. 5,000L – 25,000L). Today, shortened project timelines, the vast possibility of therapeutic molecules, and the cost-conscious evolution of the industry have forced process development groups to consider alternative scale down systems that have higher throughput, lower costs, and better predictability of production-scale bioreactors. Many candidate systems are available, but the industry continues to evaluate the options. Bioprocessors' SimCell system (www.bioprocessors.com) is one of the more extensive and complex systems available. The SimCell system consists of 700 μL reactors, 6 reactors per tray, where up to 210 trays (1260 reactors) can be handled by the integrated robotics and data analysis system. This is a standalone system that is integrated with a control system, incubators, sampling systems, and some analytical systems. At the other end of the complexity spectrum are well plate systems and disposable tube systems (www.sartorius.com, Culti Flask 50). These systems can be as simple as a shaker incubator and disposable well plates or 50 mL tubes that are manually inoculated, sampled, fed, and maintained with little to no on-line monitoring. Feeding, sampling, and harvesting can be automated by setting up a liquid handling system with an automation control system that can be programmed by the individual users. Applikon micro reactors (www.applikon-bio.com) are similar to well plate systems but with additional sensors and control systems included. The micro reactors look similar to 24-well, deep-well plates, but each plate contains sensors, which the automation system uses to control pH, temperature, and DO levels; although, currently, the system only has one-sided pH control for mammalian cultures (CO2-based control). The micro reactors were originally designed for microbial cultures using CO2 and ammonia gas for pH control, but these systems do show potential for mammalian cultures [195]. A common theme that scale down systems share is the use of alternative pH and DO probes – particularly fluorescent (optical) probes – instead of traditional electrochemical probes [196]. As a direct result of fluorescent technology, smaller, less invasive, disposable...
Fig. (5). Typical vessels used for the cultivation of mammalian cells.

Fig. (6). High level overview of the different activities leading to the implementation of a mAb production process at industrial scale. The timelines indicated are averages of what has been reported in Industry and can vary with the approach taken by each company and on the individuality of each mAb expressed.

probes that require little to no calibration (probes are delivered pre-calibrated) are now available in the form of optical probes. Optical probes are patches that are <1 mm in thickness and can be adhered to the inside of a clear reactor surface, and the optical instrumentation is external to the reactor, which results in a non-invasive system. The fluorophores
Development and Production of Commercial Therapeutic Monoclonal Antibodies

are immobilized on the patches, and in the case of DO probes, the emission of the fluorescent dye is quenched at a rate directly related to oxygen concentration. pH probes, on the other hand, use a fluorescent dye with an absorbance spectrum that changes as a function of pH.

Many scale down systems, ranging from highly complex to simple, are available for evaluation. As the field matures, we will see which systems are best for prediction of production-scale performance.

CONCLUSION

In this review of therapeutic mAb production by cultivation of mammalian cells, we have tried to provide the readers with an encompassing overview of the field. From the fundamentals of mAb biochemistry that drives many decision made during development to the current state of large scale cultivation of the engineered cells. References to the ongoing introduction of novel technologies, from advances in cell line engineering to the introduction of disposable bioreactors were provided to give the readers a sense of the direction the field is moving to.

In addition, we hope that we communicated that the discovery, development, and commercial production of therapeutically mAbs is a lengthy, scientifically and technically complex, and resource consuming process. Fig. (6) presents an overview of these steps and their associated time lines.

At this time, the cost of therapeutic mAbs reflects these economic burdens encountered during development and production, and has been challenged in many public forums [197]. Having taken root in the late 1980s, this industry is still very young and hopes are that continued development of more efficient technologies such as these described in this review will help in controlling cost of goods and will make mAbs available to an ever increasing number of patients.

REFERENCES


Development and Production of Commercial Therapeutic Monoclonal Antibodies


Development and Production of Commercial Therapeutic Monoclonal Antibodies


