An Introduction to Cell Culture: A Historical Perspective

Early Enabling Innovations in Cell Culture

Cultured mammalian cells are the workhorse for the manufacturing of the class of pharmaceuticals known as biologics, including viral vaccines and many protein medicines. What may not be known to many is the art-like nature of growing cells at the dawn of cell culture, a skill that required a great deal of creativity, inquisitiveness, passion, and perseverance, in addition to all the good traits of a scientist. Around the turn of the twentieth century, explants of animal tissues began to be cultured on glass surfaces that were submerged in an animal's tissue fluid. Cells grew from the tissue clumps and remained viable and observable for a few days.[1] From the outgrowth of tissue clumps, some cells could be isolated and eventually dissected out and expanded to new glass surfaces. The capability of passaging or expanding cell population was an important step towards genuine cell culture. Among the first cell lines established that could be continuously expanded in culture was the mouse L cell.[2] The early cell lines could be sustained in the lab only by continuous passaging; they could not be frozen, stored away, and later thawed to resume growth. In that era, the complex nutrient mixture could not be sterilized by heat. Rather, serum, ascetic fluids, or chicken embryo extract were carefully isolated from animals in order to maintain sterility for cell cultivation (Figure 1.1).[3] Imagine the amount of work involved in maintaining cultured cells!

The discovery of cryopreservation for freezing animal sperm and later other cells allowed cell growth to be paused and resumed in the lab.[4] Another important advance was the use of trypsin for cell passaging, instead of relying on dissection to dissociate cells from the surface. The first human cell line, HeLa, derived from human cervical cancer, fully took advantage of this.[3, 5]

Critical to cell culture advances was the arrival of membrane-filtration-based medium sterilization, first by ultrafiltration with a nitrocellulose membrane and later by microfiltration. While saline and media for microorganisms could be autoclaved for sterilization, the complex nutrients needed by animal cells are destroyed at high temperatures. Membrane sterilization accelerated the development of a chemical nutrient medium consisting of glucose, amino acids, vitamins, and balanced salts.[6] This not only advanced our knowledge of the nutritional needs of cells, but also greatly simplified the logistics of growing cells, leading to the establishment of many important cell lines and eventually to the industrialization of cell culture.

The early cell lines that could be continuously passaged, including mouse L and HeLa, were derived from cancerous tissues (Panel 1.1). Morphologically, they looked abnormal and were distinct from primary cells first grown from normal tissues. Later continuous cell lines were isolated from various animal tissues, including baby hamster kidney (BHK) from the Syrian hamster,[7] Vero from green monkey kidney,[8] and Chinese hamster ovary (CHO) from

Panel 1.1. Cell Substrate Example

Primary Cells
- Tissue explant, limited cell expansion

Cell Lines
- e.g., Mouse L, HeLa, BHK, CHO

Differentiated Cell Lines
- e.g., HepG2 (liver), PC12 (neuronal)

Stem Cells
- Multipotent, pluripotent
- Capable of directed differentiation *in vitro*

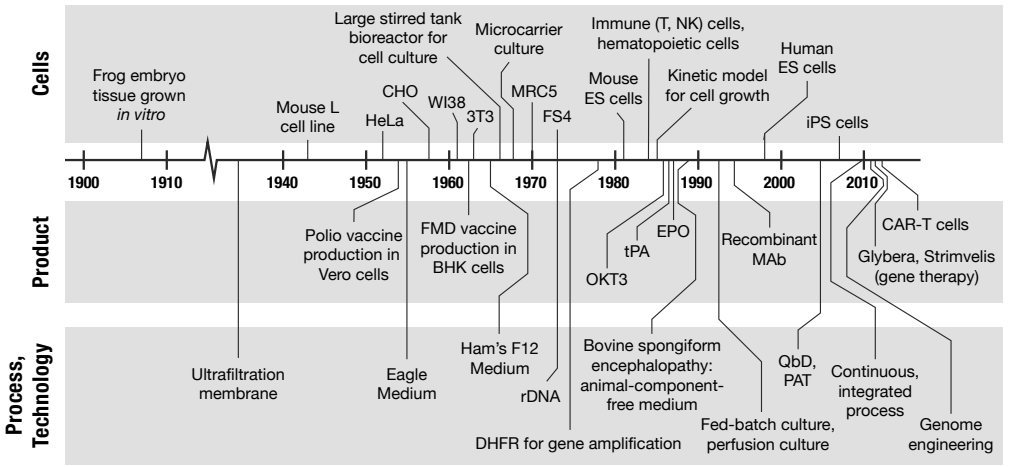

Figure 1.1. Milestones in cell culture technology.

Chinese hamster ovary.[9] These cell lines carried mutations that allowed them to bypass their cells' internal growth control mechanisms. These cells were not phenotypically normal. They often did not exhibit contact inhibition. With abundant nutrient supply, they grew into multiple layers of cells on a surface. Later, 3T3, a cell line which is adhesion dependent, exhibits contact inhibition, and does not undergo senescence, was established.[10] But the karyotype (or chromosome composition) of 3T3 cells, as well as other continuous cell lines, was aneuploid, not diploid. Around the same time, phenotypically normal human fibroblastic cell strains, such as WI-38 and later MRC-5 and FS-4, were isolated.[11, 12, 13] These cells were diploid and exhibited contact inhibition but were not continuous cell lines like 3T3. They senesced after repeated passaging in culture. For many decades, these cell lines and cell strains served important roles in biological science and medical research, and many were used in the industrial production of viral vaccines and other biologics.

Differentiated Cell Lines

The early cell lines were important tools for the *in vitro* study of cell physiology, biochemical mechanisms, and cancers. However, those cell lines didn't possess the differentiated functions that tissues do. Later, cells of various differentiated tissues would be isolated, including HepG2 from hepatocellular carcinoma,[14] Jurkat from human T cell leukemia,[15] and PC12 from rat pheochromocytoma.[16] Most of those differentiated cell lines were isolated from cancerous tissues instead of normal ones. Compared to their normal counterparts, they were easier to isolate due to their reduced dependence on various growth factors and cytokines needed for *in vitro* growth. Nevertheless, they carried the phenotypic characteristics of the tissue that they were derived from, and these differentiated cell lines became valuable tools for biomedical research.

Differentiated Cells in Culture

The arrival of rDNA technology in the 1970s made the production of various protein reagents a simpler task. It expanded the repertoire of biochemical reagents and greatly increased our ability to culture cells that require growth factors and cytokines. Primary cells, such as endothelial cells, keratinocytes, chondrocytes, and hepatocytes, began to be isolated from normal human tissues and cultured *in vitro*. Primary tissue cells isolated from tissues typically retain many tissue-specific activities, and thus may be exploited for tissue engineering applications such as repairing tissues or augmenting tissue functions. However, most differentiated cells have very limited proliferative potential and show phenotypic instability in culture.

Stem Cells

The quest for cells with differentiated properties soon extended from isolating terminally differentiated cells to stem cells that could be differentiated to specific lineages. Stem cells are classified by their differentiation potential: totipotent, pluripotent, and multipotent. Totipotent cells can become any type of cell, including extraembryonic tissues. Pluripotent cells (embryonic stem cells, induced pluripotent stem cells) can become any type of cell found in any of the three germ layers of ectoderm, mesoderm, and endoderm.[17] Multipotent cells can differentiate into different cell types of the same lineage. For example, hematopoietic stem cells (HSCs) can differentiate into any type of blood cell by exposure to different combinations of growth factors, such as stem cell factor (SCF), thrombopoietin (TPO), and granulocyte colony-stimulating factor (G-CSF). Multipotent HSCs have been isolated from bone marrow, peripheral blood, and umbilical cord blood. Mesenchymal stem cells (MSCs), also multipotent, were first isolated from adult bone marrow[18] but are now found in almost all tissues. They have the ability to differentiate mainly into osteogenic, chondrogenic, and adipogenic cells.[19] They also have the capacity to produce a large number of bioactive molecules and are being explored as signaling cells for possible immunomodulatory and trophic effects.

Pluripotent stem cells

Stable mouse embryonic stem cells (mESCs) were first derived from the inner cell mass (ICM) of early mouse embryos.[20] Nearly twenty years elapsed before two laboratories derived human embryonic stem cell lines (hESCs).[21, 22] These embryonic stem cells were pluripotent and capable of differentiating into cell types of all three germ layers. They were karyotypically normal when grown under the appropriate culture conditions. However, hESCs were derived from fertilized human eggs, making their use ethically controversial. In contrast, induced pluripotent stem cells (iPSCs) are derived by introducing four exogenous genes (OCT4, SOX2, KLF4, and c-Myc (OSKM)) into adult somatic cells. They can be readily derived from different individuals and are therefore free of ethical controversy. After the introduction of these genes, a small fraction of the transfected cells proliferated, bypassed apoptosis and cell senescence, lost somatic cell characteristics, and were reprogrammed into pluripotent cells.[23–25] Since then, the reprogramming of adult somatic cells to iPSCs has been accomplished with some modifications of gene combinations, or even with cocktails of small molecules that induce epigenetic changes.

Guiding Cell Differentiation in Vitro

Some cell lines can undergo differentiation *in vitro* when they are exposed to the appropriate signals. For example, a subclone of 3T3 cells,

designated 3T3-L1, can be induced to differentiate into adipocytes.[26] PC12 cells, upon exposure to nerve growth factor or dexamethasone, can terminally differentiate into neuron-like cells. The potential of using cell lines capable of differentiation for *in vitro* drug testing—even for therapy—has been long recognized.

Most primary cells isolated from tissues have a very limited proliferation capacity. Primary hepatocytes, chondrocytes, and neuronal cells all proliferate to a very limited extent in cultures and cannot be cultured for a long period. In some cases, it is possible to enrich a subpopulation of cells from a cell mixture isolated from tissues using chemical cues that preferentially stimulate their proliferation. For example, T lymphocytes and natural killer (NK) cells isolated from peripheral blood can be cultured and expanded into larger populations. The isolation and culture of those cells from the immune system two decades ago paved the way for cell therapy today.

Adult stem cells isolated from tissues, like HSC and MSC, can also be guided to differentiate into cells of related lineages (MSC to bone and muscle lineages and HSC to lymphoid lineage). These adult stem cells are multipotent and can only be directed to differentiate into their respective related lineage. With pluripotent stem cells, directed differentiation to lineages of all three germ layers is now possible, although most differentiation protocols still generate only tissue-like cells with an insufficient extent of maturation.

It is also possible to directly reprogram adult somatic cells from one lineage to another.[27] Instead of ascending to the ESC-like state, the cells are transduced with a combination of gene factors and reprogrammed directly into the target differentiation state upon culturing in a differentiation medium.[28] Beta cells, cardiac cells, neurons, and hepatocytes have all been derived directly from somatic cells.[29–33]

Cell Culture as Process Technology

Early cell culture explorations aspired to establish a biological platform for scientific research, but even then the potential applications of cell culture were never far from the minds of the scientists involved. Soon after primary cells became culturable, viruses were produced in primary cell culture. Cell culture quickly began to take the place of animals and embryonated chicken eggs as the production vehicle of viral vaccines. Notably, foot and mouth disease (FMD) viruses were produced in primary calf kidney cells[34] and polio vaccines were produced in primary monkey kidney cells in the 1950s.[35] Subsequently, continuous cell lines became the production vehicle of viruses, including BHK cells for FMD virus[36] and Vero cells for polio virus.[37] Human fibroblasts MRC-5

were used in human vaccine production, while interferon was produced by FS-4 cells.[13] However, as we have witnessed in the past two decades, it was recombinant DNA (rDNA) technology and the use of mammalian cells for the production of therapeutic proteins that propelled cell culture to its place as a major manufacturing workhorse. Recent advances in T cell therapy offer hope that therapeutic cells will become a new class of product of cell culture processes.

Viral Vaccines

The most effective way to fight viral diseases has been widespread immunization by vaccines. The majority of viral vaccines are now produced in cell culture. While animal tissues are no longer used, embryonated eggs are still part of the production of many viruses, including influenza virus. Because of their prophylactic nature and impact on public health, vaccines do not command the same price as therapeutic proteins. The combined commercial value of vaccines is substantially lower than the total of pharmaceuticals, with total annual sales worldwide in the order of ~30 billion US dollars in 2015. Influenza virus vaccines, among the few viral vaccines with an annual sales value of more than 1 billion US dollars, have only about 10% of their doses produced in cell cultures. The rest are still produced in chicken eggs. Table 1.1 lists a number of viral vaccines for human use. Although viral vaccines have made major strides in advancing health care, many viral diseases, such as HIV, are still in need of vaccines.

The majority of viral vaccines employ whole viruses, either live attenuated viruses or virus particles that have been inactivated by formalin or another chemical treatment to render the virus incapable of infection but still capable of eliciting the immune response. Live attenuated viruses used in immunization have been adapted, often by a long serial passaging in a nonhuman host, to reduce their virulence to humans. The attenuated virus is still capable of replication, but often at a slower rate. They continue to replicate after being administered, thus requiring a smaller amount for immunization than if an inactivated virus had been used.

Viral vaccines may also be subunit vaccines consisting of protein subunits produced in microbial or insect cell systems that retain antigenicity for immunization. Another type of vaccine, VLP,

Table 1.1. Viruses Produced in Cell Culture

Virus	*Cell substrate*
Poliomyelitis	Vero
Rabies	Vero, MRC-5
Measles	Chicken embryo fibroblasts
Mumps	Chicken embryo fibroblasts
Rubella	WI-38, MRC-5
Adenovirus (vaccine)	MRC-5
Adenovirus (gene therapy vector)	HEK293
Rotavirus	Vero
Smallpox (vaccinia)	Chicken embryo fibroblasts, BHK
Varicella	MRC-5, 2BS
Influenza	MDCK, Vero

consists of proteins that are produced by recombinant DNA technology in microbial or cell systems but assembled into viral particles without the virus genome. A prominent example is the VLP vaccine against human papillomavirus virus, which is produced in yeast or insect cells.

The dose of viral vaccine for each immunization is low. Cell culture facilities for viral vaccine manufacturing are therefore relatively small in comparison to those for protein biologics. Nevertheless, the cell culture technologies established for vaccine production in the 1960s and 1970s laid the foundation for subsequent large-scale bioprocessing for therapeutic protein production. The basic cell culture processes of cell banking, medium design, process control, suspension culture, and adherent growth on microcarriers in stirred tank bioreactors were all first established for the manufacturing of vaccines and later adopted for recombinant protein production.

Many viral diseases in the industrialized world have all but vanished in the past few decades because of the availability of vaccines. However, many basic vaccines that are routinely administered to the general population in industrialized countries are out of reach for a large fraction of the world's population. Reducing the cost of those vaccines and improving their distribution to make this basic human necessity available to everyone is still a major challenge. Nowadays, an outbreak of an infectious disease in any part of the world has the potential to quickly become a worldwide pandemic. Establishing manufacturing technology capable of quickly producing vaccines for new strains of viruses and providing a rapid response to affected regions during a pandemic remains a challenge.

Protein Molecules as Therapeutics

Proteins derived from blood, tissues, and cell cultures were used for therapeutic purposes long before the arrival of recombinant DNA technology. Examples include insulin for treating diabetes, urokinase for stroke, factor VIII for blood coagulation disorder hemophilia, and interferons for viral infection. The first wave of recombinant DNA therapeutic proteins, including human growth hormone and insulin, were produced in *Escherichia coli* (Table 1.2). The subsequent products were also of human origin but required complex post-translational modifications—such as complex disulfide-bond formation and glycosylation—that could not be carried out by microbial cells (Table 1.3). Hence, mammalian cells were employed.

The early development of cell-culture-based therapeutic proteins focused on monoclonal antibodies produced by hybridoma cells. The hybridoma cells were derived from the fusion of non-antibody-secreting, but continuously proliferating, myeloma cells and the specific antibody-secreting, but non-dividing, B lymphocytes. Those fused cells that could both grow and produce the desired antibody were isolated for production.

Since the B cells were obtained from immunized mice, the method produced mouse antibodies rather than human or humanized antibodies.

Hybridoma technology thus soon gave way to recombinant DNA-based methods using Chinese hamster ovary (CHO), mouse myeloma, and a few other cell lines. By introducing the transgene coding for the

Table 1.2. Therapeutic Protein Biologics Produced in Non-Mammalian Hosts

Product	*Activity / Use*
Granulocyte colony-stimulating factor (Neupogen)	White blood cell growth for neutropenia
Insulin (Humulin)	Diabetes
α-Interferon (Intron-A)	Anticancer, viral infections
Somatropin [human growth hormone] (Humatrope/Protropin/Nutropin)	Growth deficiencies
Interleukin-2 (Proleukin)	Kidney cancer
Human papillomavirus VLP	Vaccine
Hepatitis B virus surface antigen	Vaccine

Table 1.3. Non-Antibody Products Produced in Mammalian Cells*

Trade name	*Type*	*Therapeutic use*
Aldurazyme	Laronidase	Mucopolysaccharidosis I
Cerezyme	β-glucocerebrosidase	Gaucher's disease
Myozyme	α-galactosidase	Pompe disease
Fabrazyme	α-galactosidase	Fabry disease
Naglazyme	N-acetylgalactosamine 4-sulfatase	Mucopolysaccharidosis VI
Orencia	Ig-CTLA4 fusion	Rheumatoid arthritis
Luveris	Luteinizing hormone	Infertility
Activase	Tissue plasminogen activator	Acute myocardial infarction
Epogen/Procrit	EPO	Anemia
Aranesp	EPO (engineered)	Anemia
Pulmozyme	Deoxyribonuclease I	Cystic fibrosis
Avonex	Interferon-β	Relapsing multiple sclerosis
Rebif	Interferon-β	Relapsing multiple sclerosis
Follistim/Gonal-F	Follicle stimulating hormone	Infertility
Benefix	Factor IX	Hemophilia A
Enbrel	TNF receptor fusion	Rheumatoid arthritis
Tenecteplase	Tissue plasminogen activator (engineered)	Myocardial infarction
ReFacto	Factor VIII	Hemophilia A
Advate	Factor VIII (engineered)	Hemophilia A

*all listed produced in CHO cells

Panel 1.2.
Evolution of Therapeutic Proteins

- Native proteins
  - Factor VIII, tPA, EPO
- Engineered native proteins
  - B-domain deleted factor VIII, glycosylation-modified EPO, recombinant IgG
- Fusion proteins
  - TNFR-Fc fusion protein, IL-1R-Fc fusion protein
- Bispecific antibodies
- Drug-conjugated antibodies

product and amplifying its copy number, the transduced CHO cell can be made into a high producer of the transgene product. Furthermore, the transgene can be engineered to increase its binding affinity to the antigen or to humanize its protein sequence. After the introduction of tissue plasminogen activation (tPA) by Genentech in 1987, erythropoietin (EPO) and factor VIII soon followed to reach the market (Figure 1.1).

The early recombinant cell culture products were native human proteins administered to patients who suffered from deficiency of the protein due to congenic disorders or some disease conditions (Panel 1.2). The second wave of therapeutic proteins was antibodies. Antibody products make up the bulk of protein drugs in clinical use today. Early antibody products, called chimeric antibodies, retained the sequence of the immunized species (mostly mouse, since mouse hybridoma was the primary source of antibody molecules) in the variable region of the molecule while using a human sequence in the constant region. Later generations of recombinant antibody molecules were humanized (retaining only the hypervariable region of the mouse sequence) or completely human. Table 1.4 lists some examples of antibody-based therapeutic proteins. Trastuzumab (trade name Herceptin) binds to an overexpressed protein, HER2, which is so named because of its structural similarity to the human epithelial growth factor receptor 1 (HER1), on the surface of breast cancer cells. About 20% of breast cancer cells overexpress the HER2 protein and can be treated with the antibody. Its success in clinics paved the way for subsequent growth in antibody drugs and antibody Fc-region-based drugs. More recently, antibodies against PD-L1 (programmed death ligand 1) that has become overexpressed in many cancer cells have become an important class of medicines. PD-L1 is present in many normal cells, where it binds to PD-1 on T cells to suppress the T cell mediated immune response. However, some cancer cells also express PD-L1 to evade the immune response by T cells. The binding of an antibody to PD-L1 or PD-1 prevents the suppression of T cell activation by cancer cells, thus allowing the cancer cells to be killed by T cells.

The success of antibodies as a class of therapeutics was facilitated by decades of vast investment in biomedical research by the federal government and a better understanding of disease mechanisms. Once a binding target in a disease pathway is identified and the antigen isolated, an antibody against the antigen can be obtained and its affinity toward the antigen optimized. The antibody-based drug discovery is thus mechanism-based and design-oriented. This is in contrast to the discovery of traditional biochemical drugs such as antibiotics, immunosuppressors,

and anticancer drugs, which rely largely on screening using binding assays or bioassays. The success rate of a candidate antibody biologic is much higher than that of a biochemical drug. Nevertheless, it is sobering to recall that some had taken the failure of the 1992 clinical trial of Centoxin, an antibody against the *Staphylococcus aureus* toxin for treating septic shock, as the obituary of antibody therapeutics. The real success of antibody therapeutics and the rapid increase in the number of FDA-approved biologics did not truly occur until the second half of 1990s, nearly twenty years after the arrival of recombinant DNA technology and the first wave of venture capital-funded biotech companies.

Antibodies are among the most abundant proteins in blood circulation. They are highly soluble and can be secreted by B cells at high levels.

Table 1.4. Therapeutic Antibody Products

Trade name	*mAb type*	*Therapeutic use*	*Host cell*
Orthoclone OKT3	Muromomab CD3	Reversal of acute kidney transplant rejection	Hybridoma
ReoPro	Anti-Abciximab	Prevention of blood clots	SP2/0
Rituxan	Anti-CD20 mAb	Non-Hodgkin's lymphoma	CHO
Zenapax (Daclizumab)	Humanized, anti-α-subunit T cell IL-2 receptor	Prevention of acute kidney transplant rejection	NS0
Simulect (Basiliximab)	Chimeric, anti-α-chain T cell IL-2 receptor	Prophylaxis of acute organ rejection in allogeneic renal transplantation	SP2/0
Synagis (Palivizumab)	Humanized, anti-A antigen of RSV	Prophylaxis of lower-respiratory-tract disease	CHO
Remicade	Anti-TNF-α mAb	Active Crohn's disease	SP2/0
Herceptin	Anti-HER2 mAb	Metastatic breast cancer	CHO
Mylotarg	Anti-CD33	Acute myeloid leukemia	CHO
Campath	Anti-CD52 mAb	Chronic lymphocytic leukemia	CHO
Zevalin	Anti-CD20 murine mAb	Non-Hodgkin's lymphoma	CHO
Humira	Anti-TNF-α mAb	Rheumatoid arthritis	CHO
Xolair	Humanized, Anti-IgE mAb	Moderate/severe asthma	CHO
Bexxar	Anti-CD20 mAb	Follicular non-Hodgkin's lymphoma	CHO
Raptiva	Anti-CD11a mAb	Chronic psoriasis	CHO
Erbitux	Chimeric antibody raised against human EGF receptor	EGF receptor–expressing metastatic colorectal cancer	CHO
Avastin	Anti-VEGF	Metastatic colorectal cancer and lung cancer	CHO
Soliris	Antibody binding to C5	Paroxysmal nocturnal hemoglobinuria	NS0
Vectibix	Anti-EGFR mAb	Metastatic colorectal cancer	CHO

Portions of the antibody molecule, namely the Fc region, are major components of many therapeutic fusion proteins. In those fusion proteins, the functional domain (or fragment) of a protein is joined to the carrier domain through a linker segment (Figure 1.2). The Fc fragment provides many of the properties of an antibody. A prominent example is the fusion molecule of the Fc fragment of IgG and the tumor necrosis factor α (TNFα) binding fragment of the TNFα receptor (TNFRα). The molecule binds to TNFα and suppresses its inflammatory effect. Such non-natural proteins are increasingly being explored as medicine. Bispecific antibodies (BsAbs), as the name implies, use antigen-binding sites from two different antibody molecules to simultaneously target two components of the cellular pathways and thereby improve clinical efficacy. Bispecific T-cell engagers (BiTEs) are a special class of bispecific antibodies which engage the T cells of the host's immune system in order to treat cancers. Increasingly, antibodies are being derivatized so as to contain drugs. These antibody-drug conjugates (ADCs) deliver cytotoxic agents specifically to diseased cells through their recognition of a particular antigen on the cell surface. Many biologics in the pipeline include antibody-drug conjugates, bispecific antibodies, and bi- or tri-specific immune cell engagers.

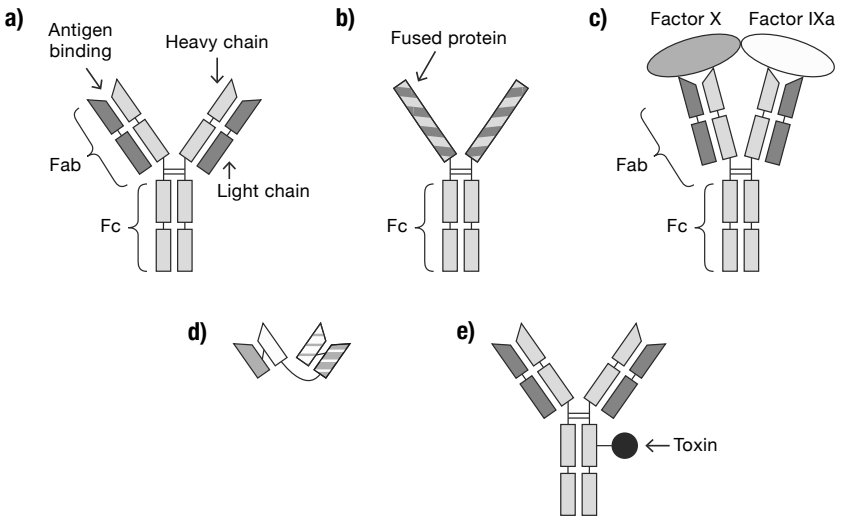

Figure 1.2. Conventional and new generation antibodies. (a) A traditional monoclonal antibody (mAb), a tetramer of two heavy and two light chains. (b) A fusion protein where the Fc region of the antibody is fused to different proteins/receptors. (c) A bispecific antibody which combines two antigen-recognizing elements into a single construct, enabling it to bind to two or more targets. In this example, one of the antigen-recognizing elements recognizes blood coagulation factor Factor X, and the other binds to Factor IXa, mimicking the function of Factor VIII to enable blood coagulation. (d) A bispecific antibody which exclusively recruits the T cell activity is called a Bispecific T-cell engager. BiTEs do not have a Fc region. (e) An antibody covalently linked to a chemical toxin, or an antibody-drug conjugate (ADC).

Biosimilars and the Expanded Reach of Protein Therapeutics

The patent that gives the market exclusivity of a mammalian cell-based therapeutic protein has a limited life span of 20 years after it is granted. After the expiration of a patent for a particular protein therapeutic, the generic version of the product, called the biosimilar, can be marketed by other manufacturers without the permission of the innovator of the original medicine. The availability of the generic version drives down the price of the drug and makes it more accessible to patients outside of industrialized countries. The regulation of biosimilars differs from that of generic drugs. The clinical effectiveness of chemical drugs (such as cholesterol-controlling statins and pathogen-killing antibiotics), whose molecular weight is relatively small compared to proteins, is usually assured once their chemical structure and purity are ascertained. The generic version of these small molecule drugs, upon verifying its chemical identity, purity, and drug product quality, can enter the market after the patent of the original drug expires (Panel 1.3). In contrast, both the structure and manufacturing process of protein biologics are complex. The biological activity and clinical efficacy of a biologic medicine is not readily predictable when a therapeutic protein is produced using a different cell line and a different process by a different manufacturer. The status of molecular folding, glycan composition, and even the composition of minute impurities may affect activity and immunogenicity. Biosimilars thus require regulatory approval and must undergo clinical trials, although on a smaller scale than the original (innovator) medicine.

A biosimilar must match the same product quality criteria as the original drug—it must have the same protein sequence, similar post-translational modification patterns (such as the profile of glycans, phosphate, or sulfate), and other quality indexes. However, such information is not available from the manufacturer of the original innovative medicine. Biosimilar makers thus are obliged to acquire the product from the market (often at different times and locations to cover a wide range of variabilities) and perform extensive product characterization to develop a product quality profile. They must also establish a producing cell line and

Panel 1.3. Biologics, Drugs, and Patents

Original		Off-Patent Version
• Drugs (small molecules) - Require clinical trials	→	• Generic drugs - No clinical trials needed prior to market entry
• Biologics (proteins) - Require clinical trials	→	• Biosimilars - Limited clinical trials

develop a process that generates a product that meets the same quality indexes as its counterpart from the innovator. It is possible that a biosimilar may have improved clinical performance compared to the innovator molecule; these are called "biobetters."

Producing a biosimilar with an acceptable post-translational glycosylation pattern is a challenging task, but one essential for receiving regulatory approval. The pathway for glycosylation is very complex. Many factors can affect the glycosylation pattern of a product, including the expression of glycosylation genes, the metabolic state of the cell, and the supply of nucleotide sugars. Controlling the flux, or even just modulating the flux in the glycosylation pathway, is not an easy task. Coupling the power of '–omics'-based analyses with a systems approach could possibly create a predictive tool to help steer the glycosylation pattern of the biosimilar to better match that of the innovator molecule.

Gene Therapy

Gene therapy aims to treat congenic diseases by delivering a correct copy of the gene to the cell of the patient via the introduction of a DNA molecule with the correct genetic sequence. The affected tissue can then be "corrected" to perform its proper function, or, in the case of the affected gene product being a secreted protein, the correct protein can be produced in the patient's body at a functional level. Due to the large number of genetic diseases that may benefit from gene therapy, there have been numerous ongoing studies and clinical trials. Most efforts have focused on diseases caused by a single gene mutation. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) gene treats cystic fibrosis, and the factor VIII gene treats hemophilia. Some approaches seek to "permanently" incorporate a corrected copy of the gene into the cells of the patient, while others aim to transiently express the correct gene to rectify the disease conditions for a limited period of time. With the advances in genome engineering technology, the exploration of gene therapy has been extended from giving a correct copy to the repair or silencing of the incorrect copy in the host cell.

Panel 1.4. Gene Therapy

- Viral vectors
  - Adenovirus, adeno-associated virus, retrovirus, lentivirus
- Non-viral vectors
  - Nanoparticles
- Treatment
  - *Ex vivo* and *in vivo*

Gene therapy may be achieved by isolating a patient's target cells and performing gene transduction *ex vivo* before the "corrected" cells are returned to the patient (Panel 1.4). It may also be accomplished by suspending the replacement gene (or other DNA elements) in nanoparticles or a plasmid or viral vector, and then injecting them into the patient and allowing the DNA element to enter a fraction of the cells in the body and perform its function. Most clinical trials that have been conducted with viral vectors used adenovirus, lentivirus, or adeno-associated virus.

Producing viruses for gene delivery is similar to producing viruses for vaccines. However, there are also important differences. While the number of viruses per dose for vaccines is rather small, that for gene therapy in most applications is very high (100 to 1000 times higher). For one dose, the volume of culture fluid needed is thus much larger for gene therapy than for vaccine applications. This also means that the number of impurities carried in culture fluid that must be removed from the final product is much larger in a gene therapy product than a vaccine. In the manufacturing of live viruses, at the end of production the culture fluid cannot undergo the same type of extensive purification carried out in the production of therapeutic proteins. Thus, the purification and concentration of viruses for gene therapy face greater challenges. In a typical virus production process, a very large fraction of the virus particles produced do not contain complete viral genomes and are not infectious. While empty virus particles are still immunogenic, and can be effective for vaccination, these non-infectious particles do not deliver the DNA cargo needed for gene therapy. Increasing the infectious virus particle content is thus important in gene therapy virus production.

Recent approval of gene therapy products by regulatory agencies has been very encouraging for cell culture bioprocessing. However, the cell culture bioprocess profession still faces major challenges in the manufacturing technology required for virus production for gene therapy.

Cell Therapy

Bone marrow transplant, which has been practiced for more than thirty years, is one of the oldest forms of cell therapy (Panel 1.5). Following the discovery of HSC, its expansion and differentiation into separate lineages for transplantation was long envisioned. Some also predicted the enrichment and expansion of a particular lineage of cells, such as platelets, T cells, or NK cells. As the capability of scientists to isolate and culture various differentiated cells increased, many also attempted to culture and expand hepatocytes or beta cells for cell transplantation to functionally rectify liver failure or diabetes. Functional differentiated cells were also incorporated into scaffolds to construct tissue mimicries for implantation. The research field of employing functional cells for the construction of tissue analogues is more frequently referred to as tissue engineering.

Panel 1.5. Cell Therapy

- Cell transplant (autologous cryopreserved cell)
  - e.g., bone marrow transplant
- Donor cell transplant (heterologous)
  - e.g., liver cell transplant, bone marrow transplant
- *Ex vivo* expanded cell transplant
  - e.g., stem cell or stem-cell-derived differentiated cell transplant
- *Ex vivo* genetically altered cell transplant
  - e.g., Car-T cell therapy

The emergence of various multipotent and pluripotent stem cells gives hope to regenerative medicine. The use of stem cells as the source of differentiated cells for cell

therapy can potentially alleviate the problem of donor cell procurement in those applications. PSCs can provide an almost unlimited supply of liver and NK cells for regenerative applications. Many such applications would involve the infusion of differentiated cells into regenerated tissues or organs. Cell therapy thus entails overlapping applications with tissue engineering, regenerative medicine, etc.

An area of cell therapy that has shown much promise is the treatment of cancers using cells from immune systems, either the native cells or genetically engineered ones. Cells of immune systems may be cultured *ex vivo* and genetically modified to express cytokines, receptors, and chimeric tumor-antigen receptors for cancer immune therapy. T cells that have been engineered to express a chimeric T cell receptor with a single-chain variable fragment (scFv) and a high affinity for tumor-associated antigen (TAA), called chimeric antigen receptor T (CAR-T) cells, have been used successfully to recognize and kill tumor cells.[38]

Cell therapy, with its initial clinical success and great promise in cancer treatment, still faces major challenges. These are not only in producing a large number of cells and engrafting them at the target site, but also in cell isolation, expansion, and final preparation as a drug product. The challenge is especially acute in the autologous (from patient) applications that require a large number of cells. Without the economy of scale of production, manufacturing a large number of doses runs the cost of goods for those autologous treatments exceedingly high. Even for allogeneic (from external donor) applications, many challenges await cell culture bioprocess technologists in bringing the field forward. The manufacturing process must generate cells of high viability and therapeutic activity. This is in contrast to the production of protein therapeutics, in which the cell viability at the end of a fed-batch process is sometimes only somewhat higher than 50%. The recovery of those highly active cells for cell therapy poses another challenge. Unlike the production of protein biologics, in which the product can be subjected to various salts, pHs, and osmolality conditions that are considered abusive to cells, cells for cell therapy can only be subjected to a minimal number of steps while achieving the necessary degree of purification. Therefore, many challenges in process technology are yet to be overcome in the wide applications of cell therapy.

Industrial Cell Lines

Cell lines have been used for the industrial production of human and veterinary vaccines for more than half a century. For human viral vaccine production, human diploid cell strains (MRC-5 in particular) are the preferred cell substrate. Traditional viral vaccines consist of whole virus particles where the viral genome is packaged in the virus particle

after the replication of its genetic materials in the production cell. The entire viral particle is then injected into the patient to elicit an immune response. There is a low-level risk that the virus genome might recombine with the genetic elements of the production cell and bring the genetic element of the host cell into the virus particle. This poses a potential risk of transmitting an activated oncogenic or other adventitious genetic element to the patient. To minimize such a risk, the vast majority of human virus vaccines are produced in normal diploid human cells. Vero and MDCK cells (along with chick embryos) are notable exceptions of non-human continuous cell lines used for human vaccine production (Table 1.5). In the case that a continuous cell line is used for human vaccine production, its passage number is carefully monitored and controlled to alleviate concerns raised by the report that prolonged passage of Vero cells poses an increased risk of tumor formation.[39] However, advances in molecular analytics in recent years are rapidly extending our capability to characterize cells as well as products. There is an increasing interest in exploring the use of continuous or even tumorigenic cell lines for the production of viral biologics. For veterinary vaccines, the repertoire of host cells is much larger. Both cell lines and tissue-derived cell strains with limited life spans are widely used (Table 1.6).

Table 1.5. Major Cell Strains and Lines for Human Biologics Production

Human Vaccines	
Primary cells	Green monkey kidney cells (no longer used), chicken embryo cells
Cell strains	MRC-5 (human lung fibroblast)
Cell lines	Vero (monkey kidney epithelial cells) MDCK (dog kidney epithelial cells)
Recombinant Proteins	
Cell line origin	
Human	HEK293
Mouse	C-127, NS0, hybridoma cells, SP2/0
Chinese hamster	CHO
Syrian hamster	BHK
Gene Therapy	
Human	HEK293, PER C6
Insect	Sf9

Most cell lines used for the production of recombinant therapeutic proteins are derived from rodents, including the mouse, Chinese hamster, and Syrian hamster. Human cells are only used for the production of a handful of products. The vast majority are produced using Chinese hamster ovary (CHO) cells lines (Panel 1.6). CHO cells were a favorite for cytogenetic research even before they were commonly used for therapeutic protein production. A large number of CHO cell mutants were isolated in cytogenetic research. This was in contrast to other mammalian cell lines; due to their diploidity, mutants are usually difficult to obtain since only dominant phenotypes are observable. However, the rate of obtaining mutants is relatively high for CHO cells. They were sometimes described as a functional haploid.

Using a non-human cell line to produce a therapeutic protein carries the risk of having contaminants from the host cell (nucleic acids, proteins)

Table 1.6. Cell Lines Used in the Production of Veterinarian Vaccines

Vaccines	*Cell line*
Bovine viral diarrhea virus	MDBK
Bovine parainfluenza virus type 3	MDBK
Bovine rhinotracheitis virus	MDBK
Bovine respiratory syncytial virus	MDBK
Feline leukemia virus	FL72
Feline panleukopenia virus	CRFK
Feline chlamydia	CRFK
Canine parvovirus	CRFK
Canine distemper	Vero
Canine adenovirus type 2	Vero
Ehrlichia canis	DH82
Rabies	BHK21
Eastern equine encephalitis virus	Vero
Western equine encephalitis virus	Vero
Equine rotavirus	MA104
Equine rhinopneumonitis virus types 1 and 4	Equine Dermal
Equine influenza virus	MDCK
Foot and mouth disease virus	BHK21
Swine parvovirus	ST, PK
Swine influenza virus	MDCK

Panel 1.6. CHO Cells

- Aneuploid, heterogeneous population in karyotype, chromosome number
- Many mutants were isolated, sometimes called functional haploid
- Easy for cell adaptation (to suspension growth, varying nutritional requirements, etc.)
- Glycans on proteins are similar to human
  - Does not express α2,6-sialyltransferase as in human glycoproteins
  - Has only α2,3-sialyltransferase

in the product even after extensive purification. This necessitates the reduction of contaminating host cell proteins and DNA in the product to an acceptable level to minimize the possibility of their causing any immunogenic response or other adverse effect. The required level is based on per dose, rather than per unit mass or volume of the therapeutic protein to be administered. Thus, the higher the protein dose is, the harder it is to reduce the host cell contaminants to an acceptable level. Many therapeutic antibodies are administered at gram level per dose, in contrast to tens of milligrams for growth factors and cytokines (Table 1.7). For such high dosage products, it may be advantageous to use a human cell line for production. However, the advantages of easy genetic manipulation and the high probability of obtaining a hyper-producing cell line thus far outweigh the requirement of removing residual host cell proteins.

Many viruses, including lentivirus, adenovirus, and adeno-associated virus, are used as carriers to deliver the corrective genetic material to the host tissue *in vivo* or host cell *in vitro* for gene therapy applications. The production of these viruses is often carried out in HEK293 cells. The HEK293 cell line was derived from human embryonic kidney cells immortalized by the insertion of a segment of adenovirus type 5 DNA into its genome.[40] In some cases, insect cells are also used for virus vector production.

No particular cell line of embryonic stem cells, iPSCs, or MSCs has yet become a commonly used or "standard" cell line in clinical trials involving allogeneic stem cells or other tissue cells. In the industrial production of biologics, cells for production are characterized by their

cytogenetic characteristics, scrutinized for their history in terms of exposure to animal serum, viruses, or other adventitious agents, etc., and then expanded for banking as master and working cell stock. Such meticulous cell characterization and banking may not be possible for autologous cell therapy applications. For allogeneic cell therapy applications, even though no standard cell lines for cell therapy have emerged yet, it is expected that the cell line stock for such applications will follow a similarly rigorous procedure.

Table 1.7. Dose of Some Antibody Products

Product	*Disease indication*	*Approximate dose*
Amevive	Psoriasis	7.5mg / 0.5ml; 15mg / 0.5ml
Enbrel	Rheumatoid arthritis	25mg
Herceptin	Breast cancer	440mg / 30mL
Humira	Rheumatoid arthritis	40mg (1ml prefilled syringe)
Remicade	Crohn's disease, rheumatoid arthritis	100 mg / 20mL
Rituxan	Non-Hodgkin's lymphoma	100mg / 10mL; 500mg / 50mL
Synagis	Respiratory syncytial virus	100mg
Xolair	Allergic asthma	150mg / 5mL

Other Production Systems

Mammalian cells are the workhorse for the production of protein therapeutics that require post-translational modifications (e.g., glycosylation) or complex protein folding. Many proteins that do not require extensive post-translational modifications are produced in *E. coli* or in yeast *Saccharomyces cerevisiae*, including insulin, human growth hormone, and some cytokines. Even some proteins that requires glycosylation and other post-translational modifications are produced in other host cell systems that are capable of carrying out those functions. Some of those systems have been explored as the production vehicles of therapeutic proteins (Table 1.8, Panel 1.7).

Insect Cell Culture

The use of insect cells for protein expression dates back over three decades ago, to around the time that yeast and mammalian cells were being explored as a production vehicle for therapeutic proteins.[41] The expression usually involves the cloning of a transgene (or transgenes) into a viral vector such as Baculovirus. The vector is then packaged in insect viruses by transfecting the insect cells with the vector. The virus produced is then used to infect the host cell in the production. Virus replication in the host cell results in a large number of transgene copies. By using a strong promoter to drive the expression of the transgene, a high level of protein expression can be achieved.

A popular cell line for heterologous protein expression, Sf9, was derived from the insect *Spodoptera frugiperda*. Insect cells are capable of

Table 1.8. Recombinant Proteins Produced in Yeast

Product	*Use*
Medway (recombinant human serum albumin)	Blood expander
Hepatitis B vaccine	Hepatitis B
Interferon-alpha	Hepatitis C / cancer
DX-88	Hereditary angioedema (HAE), a debilitating condition characterized by acute attacks of inflammation
Recombinant human insulin	Diabetes
Recombinant collagen	Medical research reagents and dermal filler
Botulism vaccine	Botulism vaccine product

Panel 1.7. Other Therapeutic Protein Production Technologies

- *E. coli* for aglycosylated products
- Yeasts
  - *Saccharomyces cerevisiae*
  - *Pichia pastoris*
- Insect cells
- Transgenic animal
  - Transgenic goat
- Transgenic plant
  - Transgenic tobacco cell culture

glycosylation; however, the glycoforms of the proteins produced in insect systems are somewhat different than those produced in mammals. For example, the N-glycans synthesized in insect cells have high mannose content and fucose with different glycosidic bonds than those seen in mammals. Insect cell lines are thus not used in the production of therapeutic proteins, though they are used in the production of proteins for research use or toxicity studies. They are also frequently used in the production of recombinant proteins for use as antigen proteins or virus-like-particles in vaccine applications. Additionally, they are used in the production of human and veterinary viruses as vaccines or as transgene vector production vehicles in gene therapy. The insect cell culture remains attractive because the cultivation is relatively straightforward and the process development time can be comparatively short.

Yeasts

The yeast *Saccharomyces cerevisiae* has been used to produce serum albumin, cytokines, and some virus-like particles for use as vaccines (Table 1.8).[42] However, the glycans on the recombinant proteins synthesized in yeasts bear significant differences to those seen in mammals. Thus, *Saccharomyces cerevisiae* is not used for the production of therapeutic proteins that require extensive glycosylation. The yeast in the genus *Pichia* is capable of synthesizing N-glycans that are not the mannose-rich types produced in *Saccharomyces*. Advances have been made in 'humanizing' the glycosylation characteristics in the yeast *Pichia pastoris* for the production of therapeutic proteins. The development of a multistep genetic engineering process that first eliminated non-human glycosylation

enzymes from *Pichia pastoris* and then introduced human glycosylation enzymes led to the synthesis of proteins with humanized glycans. The secretion rate of the synthesized recombinant proteins does not match that seen in recombinant mammalian cells; however, this might be compensated for by the high cell concentration achievable in the bioreactor. With further improved secretion capacities and glycosylation patterns, these engineered yeast strains may be capable of producing proteins with consistent glycosylation patterns, or even with uniform glycans.

Transgenic Animals

The use of transgenic animals, including goats, pigs, and rabbits, for the production of biotherapeutics has been in development for three decades. Most frequently, the product protein is tissue-specifically expressed in the mammary glands for secretion into milk.[43] These production systems need a low initial capital investment compared to the traditional manufacturing plant necessary for biologics. The downstream purification process is relatively simple, as the proteins are fully glycosylated. The product ATryn (antithrombin III), which was produced in transgenic goat's milk by GTC Biotherapeutics, has been approved by the regulatory agencies in the USA and Europe.

An advantage of transgenic animal production is its high titer in milk, on the order of 2–10 g/L. However, over the years the titer in cell culture processes has increased to a range of 5–10 g/L, thereby diminishing this particular advantage of transgenic animal production.

Manufacturing

Upstream Process

The basic process flowsheet for cell culture manufacturing is almost identical to that for microbial fermentation (Figure 1.3). Although laboratory-style culture devices, including roller bottles and multiple plates, are still used in some vaccine and cell therapy processes, the vast majority of vaccine, gene vector, and protein production processes use stirred bioreactors. Compared to microbial processes, the metabolic load of cell culture, in terms of nutrient and oxygen consumption rate per unit reactor volume, is at least one order of magnitude lower, as is the power input for mechanical agitation.

Viral vaccines, cytokines, and growth factors are administered to patients in relatively small quantities. The manufacturing scale for those low-dose products is therefore also relatively small. In contrast, antibodies are often administered in high doses, as shown in Table 1.7, to elicit longer-lasting effects. Thus, in general, the size of manufacturing plants

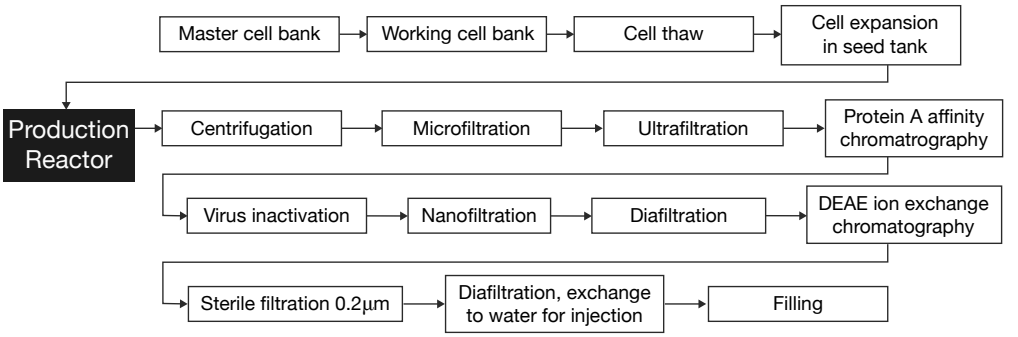

Figure 1.3. A typical cell culture manufacturing process for recombinant antibodies.

for antibody products tends to be larger than those for viral vaccine production, etc., except in the case of some orphan drugs that are used only for small patient populations.

A typical process uses a few small reactors for cell expansion before switching to the production reactor. The process cycle tends to be longer than in microbial fermentation. A fed-batch culture may last 10–14 days in the production scale and 30–40 days in total (including seed cultures). Perfusion cultures are operated as continuous processes and last from two to six months.

The fundamental principles of process engineering are virtually identical for cell culture and microbial fermentation. In both processes, the stoichiometric relationship is used as the guide for medium design, the kinetics of growth and production as the foundation for process optimization, and transport phenomena as the key for reactor design. Even the reactor technology for the production of cell culture biologics was mostly adopted from microbial fermentation. However, cell culture engineering, with its great contribution to the bioeconomy, has outshone microbial fermentation in process innovation. The technological advances in the past two decades in product separation and analysis and manufacturing facility design, the adoption of platform concepts in cell line and process development, the wide application of single-use reactors, and the recent exploration of continuous processes have been all driven by cell-culture-based production. Cell culture processes are still expanding to meet the increasing number of new, innovative drugs and biosimilars. With cell and gene therapy on the horizon, cell culture engineering will continue to be a key driver in bioprocess technology.

Single-Use Systems and Continuous Process

The increasing number of licensed products in the past decade has heightened the demand for production capacity. This increased demand has not seen a proportional increase in the volumetric capacity of the manufacturing plant, but has instead been met by increased productivity

and improved efficiency in facility utilization. The manufacturing process for cell culture products has undergone some important changes in the past decade (Panel 1.8). To shorten the time spent in cell expansion before reaching the production bioreactor, some have employed a large quantity of cell stock in a working cell bank to initiate the seed train in larger bioreactors. In some cases, the production reactor is inoculated at a much higher cell concentration by employing some form of cell perfusion in the preceding seed culture (often called N-1) stage in order to shorten the time it takes to reach the peak of productivity.

The past decade has seen an increase in the use of disposable or single-use reactors. Used only in inoculum preparation and in small-quantity production a decade ago, single-use stirred reactors, at a volume of up to two thousand liters, are becoming common for manufacturing. This is feasible because of the relatively low power requirement for agitation in cell culture process, allowing the plastic vessel to sustain the mechanical stress of agitation. A plant based on disposable bioreactors offers the advantage of lower capital investment and faster plant construction in setting up the manufacturing facility, plus increased flexibility and modularity in process implementation.

Single-use bioreactors do have some shortcomings. Most of them cannot be easily pressurized to transfer the fluid between vessels using fixed pipes. Many have limited choices of impeller design. For some cells, especially those grown on microcarriers, the agitation mechanism may not be optimal. Nevertheless, the wide application of single-use bioreactors has changed the landscape of biomanufacturing. Due to the size limitation, to increase the production volume beyond the maximum size of the vessel one must resort to using multiple reactors. The alternative is to increase the concentration or the throughput of the product. This is achieved by increasing the cell density and prolonging the duration of fed-batch culture processes, or by running the process in a continuous fashion.

Panel 1.8. Disposable Single-Use Systems

- Reduced capital investment for manufacturing plants
- Reduced lead time for plant construction
- Transfered many manufacturing logistics to single-use equipment suppliers
- Reduced reactor size, with more operational constraints
- Compensates for smaller reactor volume with increased cell density and product throughput if a large product volume is needed, by:
  - High cell density perfusion
  - Hybrid process of perfused fed-batch

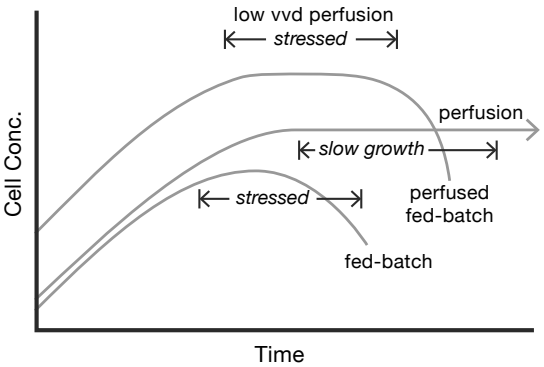

Figure 1.4. Cell culture manufacturing processes: fed-batch, perfusion, and hybrid (perfused fed-batch).

Most current cell culture biomanufacturing processes are operated in a fed-batch mode (Figure 1.4). After a few days of rapid cell growth, the culture is fed with medium containing high concentrations of nutrients a few times over the production period to increase the culture volume step-wisely to its maximum. The addition of medium increases the osmolality of the culture broth. This, along with increased metabolite accumulation, slows down cell growth. During the slow growth and stationary phase period, the product accumulates to a high level.

Cell culture processes are rarely operated as a simple continuous culture because the cell concentration at a steady state is too low to achieve a high productivity. A continuous cell culture process is performed as a perfusion culture, in which a cell separator is used to recycle a concentrated cell stream separated from the effluent back into the bioreactor to increase the cell concentration. However, only a small number of current processes employ perfusion culture. Most of the perfusion processes have been those involving labile proteins that need a short residence time in the reactor or proteins that are expressed only at very low levels. The lack of an effective and robust in-line device that can concentrate cells and return them to the bioreactor has contributed to the slow adoption of the perfusion process.

In the past decade, in-line cell separation devices have advanced markedly. This has made continuous perfusion culture easier to implement and allowed for a new hybrid mode of perfusion/fed-batch to be carried out. A hybrid perfusion/fed-batch culture often starts with a higher cell concentration than a typical fed-batch culture. This is accomplished by using a cell separation device to concentrate the seed culture stream and inoculate more cells into the reactor. The operation is similar to a fed-batch culture, though the high cell concentration results in a high accumulation of metabolites, requiring the continuous removal of culture medium to reduce their levels. Continuously flushing out salts, metabolites, etc., through medium withdrawal alleviates the growth inhibition and prolongs the production phase.

Product Recovery

The recovery process of cell culture products is simpler than that for bacterial-based recombinant proteins. The majority of cell culture processes now use media with a low concentration of proteins. At a high concentration in the range of 5–10 g/L, the product is the predominant protein in the spent medium at the end of the cell culture process. The product isolation and purification process is thus substantially simpler, since only relatively small quantities of contaminating cellular proteins and other medium components must be removed.

An example of the recovery of antibody IgG is shown in Figure 1.3. After the cell culture process, the cell suspension from the reactor is chilled in a holding tank to prevent cell lysis. Centrifugation or microfiltration

is used to remove the cells. To prepare for subsequent chromatographic steps and membrane processes, the particulate matters in the supernatant are removed by microfiltration. The process stream is then concentrated using ultrafiltration. For some very high-productivity processes, this step may not be necessary, as the product concentration is already high. The retentate from ultrafiltration is then fed into a protein A affinity chromatography column. This chromatography step captures the product at a very high yield and at a high degree of purification. After washing, the adsorbed IgG is eluted with a low-pH buffer for several hours. This inactivates endogenous retroviruses from within the host cell that may form virus particles. Sometimes, another means of virus inactivation is used. After virus inactivation, a nanofiltration step is used to remove the virus particles. Because protein aggregation sometimes occurs, a gel-permeation chromatography step may also be employed to remove aggregated IgG (not shown). Non-human host cells invariably shed cellular proteins and DNA fragments into the medium and contaminate the product. To reduce the level of those contaminants, anion exchange chromatography is used. After purification, the product solution is filter sterilized for final preparation in a formulation solution.

With the renewed interest in continuous perfusion culture, there have been studies conducted concerning the possibility of making the product recovery process continuous. As can be seen in the typical recovery process, virtually all unit operations are continuous-flow open systems but operated in a periodic batch mode. The strategy is to connect those unit operations and adapt them to a continuous feed stream from the bioreactor by using multiple smaller pieces of equipment for each unit operation. They would cycle through the different stages of the operations such that there would always be a piece of equipment receiving the feed from the preceding operation. Some have envisioned using rather low-capacity equipment which cycles through at a short period so that the equipment completes its life cycle quickly. In such a way, the equipment can be disposable and be replaced after a short duration of continuous operation. Whether such a vision will be implemented, and how much operational and cost benefit it may bring, has yet to be seen.

Product Quality in Cell Culture Processing

Quality of the Product

The quality of a cell culture product is judged by its capability to deliver its target biological activities while minimizing the risk of causing any biological harm. In most cases, a direct measurement of the biological activity, in the form of a bioassay or even an animal assay, is not readily assessable. Instead, one examines some physical features and

Panel 1.9. Protein Product Quality Characteristics

- Identity
- Functional activities
- Purity/contaminants
- Chemical structural variations

chemical structural features that are readily measurable to assess the product quality. For example, peptide finger printing is used to establish the identity of a protein product after production (Panel 1.9).

The quality of the product is not static and is liable to change over time. Such quality variability may occur at different stages of the cell cultivation process through changes in product synthesis. It may also occur during product recovery or even after the protein has been purified and stored in the formulation solution. Depending on the nature of the chemical or physical change, product quality variation may occur at fast or slow kinetics. Therefore, quality is not only measured on the drug substance and the product after it is formulated. For variables that change in slow kinetics, quality is also assessed in inventory conditions. For example, after a long storage of a purified and formulated protein, color may develop or the solution may become cloudy due to aggregation. Such quality changes can only be assessed over time.

In general, the product must be void of some known contaminating species or be capable of keeping them below certain levels. For example, protein therapeutics produced in rodent cells may carry some host cell constituents even after product purification. The host's proteins, DNA contaminants, and endogenous viruses that are not removed in the product purification process and are present in the final product must be kept below an acceptable level in order to minimize the risk to the patient incurred by the presence of those contaminants.

Structural variants of a protein (e.g., an amino acid in the sequence of a protein molecule which differs from the one specified by the coding sequence) are frequently seen in proteins produced by cell culture. Even if no adverse effect of the structural variant is known, the variant must be kept below a certain level that is accepted as low-risk.

The glycan structures of the proteins invariably exhibit heterogeneity. In the case that the glycan structure affects the protein's biological activity, the content of the glycan with the desired or undesired structure must be kept within a bound. Even in the case that the glycan structure does not directly influence the biological activities of the protein, the extent of the heterogeneity of the glycan must be specified, and different lots of product produced in different times or at different manufacturing sites must all meet the same specifications.

Structural features and biological activities

The products of cell culture processes—proteins, viruses, and cells—are all structurally complex. The assessment of their quality is not easy. Ultimately, the quality of a virus intended for use as a vaccine is determined by its ability to elicit an immunogenic response. For gene therapy,

the quality of the product virus is dictated by its capability to infect the target cells and express the cargo gene. The mechanism by which different therapeutic proteins deliver their biological effect is very diverse. Some bind and neutralize or remove the target molecules, while others bind to the target cell and elicit the killing by killer cells. Direct evaluation of such biological functions in a process setting is not feasible. Instead, one uses the mechanistic knowledge of the mode of action to develop metrics of product quality based on the structural characteristics that are critical to the biological functions. For example, for influenza virus, in addition to the virus particle count, the hemagglutinin level can be used as a quality index. In gene therapy, the ratio of transgene copy to the virus particle may be used as a measure of quality. In the production of therapeutic IgG for applications involving antibody-dependent cell-mediated cytotoxicity (ADCC, also referred to as antibody-dependent cellular cytotoxicity) activity, the fraction of fucosylated glycan is used as a quality measure because the presence of fucose on the glycan significantly reduces the ADCC activity of the IgG. Understanding the mode of action of the product involved is thus important in using the structural attributes of the product as quality metrics.

Glycosylation Profile

The glycans attached to a glycoprotein are structurally heterogeneous. This is seen in glycoproteins in blood circulation as well as in therapeutic proteins produced in cell culture. The glycan structure of some proteins plays a critical role in the protein's therapeutic efficacy (Panel 1.10). For example, the presence of mannose 6-phosphate on the glycan of a number of lysosomal enzymes is critical in mediating the protein binding to the mannose 6-phosphate receptor on cell plasma membrane for uptake, a step necessary for targeting the enzymes to lysosomes. The abundance level of mannose 6-phosphate on the glycan of those enzymes is thus critical in treating lysosomal storage diseases. In some cases, the glycan structure does not directly influence the protein's biological activities but affects its pharmacokinetic behavior. A higher content of sialic acid on erythropoietin and many other proteins, for example, increases the circulation half-life of those proteins. Without the sialic acid, the exposed galactose would bind to the asialoglycoprotein receptor on the surface of liver cells, leading to the internalization and degradation of the protein.

Panel 1.10. Glycosylation Profile

- Glycans are not uniform, but heterogeneous *in vivo* and in recombinant proteins
- Glycan distribution must be within a prescribed range of the product
- Roles of glycans
  - Affect solubility
  - Affect pharmacokinetics, e.g., sialic acid content
  - Affect biological activities, e.g., fucose in Asn292 glycan
  - Critical for biological activities, e.g., mannose 6-phosphate for lysosomal enzymes

A protein may have multiple sites in its sequence for glycosylation. The glycan structure

on a given site is not uniform. Rather, it consists of a number of somewhat different structures. This heterogeneity is the result of its biosynthesis reactions in the Golgi apparatus. The profile of glycans may also vary somewhat in different production runs, or under different production conditions or cell lines. The impact of such variation on its clinical efficacy is dependent on the mode of action of the protein. As discussed above, for some it is very important; for others, it may affect the blood circulation half-life, but not the biological activities. However, from a regulatory perspective, the glycan distribution of the product must be within a specified bound. Thus, confining glycan distribution to an acceptable range is important for the quality control of the product. A better understanding of the mode of action of the product can help define the acceptable range of heterogeneity for the product.

Protein Structural Variants

Prior to the selection of the production cell line of a given product, the DNA sequence of all the copies of the product gene integrated into the genome should be verified to ensure the transgene integrity. One thus expects that the primary sequence and the secondary, tertiary, and even quaternary structures of the protein will be uniform. However, structural variants of the protein do occur, both in natural and in recombinant proteins. They are caused by translational errors, incomplete post-translational enzyme processing, post-secretion extracellular enzymatic or chemical reactions, and even post-process events (Panel 1.11). At a higher order structural level, protein molecules may form dimers or oligomers, and may scramble disulfide bonds or even form large aggregates and precipitate. At a primary sequence level, sequence variants include incorrect amino acid incorporation and chemical modifications of amino acids in a protein.

Panel 1.11. Protein Variants

- Amino acid misincorporation
  - Mutations, transcript editing
  - Amino acid limitations
- Charge variants
  - Acidic or basic variants may be present in a detectable proportion
  - Asn deamidation, glycation of lysine, C-terminal lysine clipping, N-terminal pyroglutamic acid, C-terminal proline amidation
- Higher order structural changes
  - Cystein-linking, aggregation

Many production cell lines have multiple copies of the product gene. With high-throughput DNA sequencing technology, the DNA sequences of the many copies of transgenes in the genome of a candidate production cell line are nowadays verified to ensure their integrity. Once a cell line is selected, the probability is very low that a mutation will subsequently occur to a copy of the transgene. Nevertheless, if under a rare occasion a missense mutation (i.e., a mutation causing a change of an amino acid in the protein) occurs in one of those copies, it will inevitably result in the presence of a fraction of mutated protein molecules.

A sequence variant may also arise from amino acid depletion in the medium, as reported for the substitution of asparagine by serine in a small fraction of IgG. In rare cases, incomplete leader peptide cleavage from the protein may be seen.

A number of commonly seen structural variants of recombinant proteins are listed in Panel 1.11. Many proteins have a lysine residue at their C-terminus. These lysine residues are cleaved intracellularly by a carboxypeptidase. The cleavage, however, is often incomplete. Uncleaved lysine at the C-terminus is frequently seen in naturally occurring proteins in circulation as well as in the production of recombinant proteins.

Industrial fed-batch cultures often employ high glucose concentrations. The secreted product protein molecules have therefore had a long exposure to a high glucose concentration. Glycation, the attachment of a sugar to an amino group in the side chain of an amino acid, is commonly seen. Glycation is also seen in proteins in circulation in the human body.

Some of the amino acid modifications result in changes in the net charge of the protein. These charged variants may be acids that elute in an anion exchange chromatographic column faster than the "normal" or reference product protein, or be basics that elute faster in a cation exchange column. Acidic charged variants may also arise from changes in the glycosylation pattern, for example by increased sialic acid or sulfate content.

Process Control and Product Quality

The heterogeneity of the glycan profile in a protein is mostly the outcome of intracellular biosynthetic events. Degradation of glycans (especially removal of sialic acid by sialidases) by enzymes released from cells after the protein was secreted into culture broth may occur when the cell viability is low. Protein structural variants mostly occur after the protein has been translated, either due to intracellular events or in culture broth after the protein has been secreted into the medium. Protein-processing enzymes may be released by cells via secretion or due to cell lysis. Extracellular proteolytic cleavage has been shown to lead to degradation of factor VIII and cause alterations to the ratio of single chain/double chain molecules of tPA and protein C. Some higher order protein structural changes, such as aggregation, oxidation of methionine, and deamidation of asparagine, may even take place in the product recovery process.

The increasing emphasis on product quality calls for a better understanding of how intracellular events affect glycan profiles and how the extracellular culture environment influences the occurrence of protein variants. Ideally, this should advance our ability to control the process conditions and deliver consistent productivity and product quality. The emphasis on the development of process technology is increasingly focused on process analytical technology (PAT) for on-line monitoring and control. Going hand-in-hand with PAT is Quality by Design (QbD).

The aim of QbD is to develop process understanding and identify the process variables that contribute most to product quality. This may lead to the development of strategies to control those variables and confine product quality attributes to an acceptable range.

In order to implement QbD, one needs knowledge of the attributes that define product quality and an understanding of the relationships between the process variables and the quality attributes. Initially, one may rely on empirical data that correlates process variables and quality attributes. Over time, a mechanistic process model becomes critical to developing a predictive control strategy. The quality and the productivity are not separable in such an effort. The mechanistic understanding of the culture environment in regards to productivity and quality is incorporated into a process model that translates the input of process perturbations into the necessary control actions to sustain productivity and product quality. The advances in genomics-, proteomics-, and metabolomics-based global analytical methods, in conjunction with a systems approach, will facilitate the implementation of PAT and QbD in cell culture bioprocessing.

From Discovery to Clinical Products

The cost of developing new medicine is staggeringly high. A typical drug discovery scheme begins with the initial disease treatment conception and the production of a discovery quantity of product for biological and toxicity testing and pharmacokinetic evaluation. Then comes the decision to proceed with clinical trials, the construction of production cell lines and initial process development for upstream, downstream, and formulation, and the development of chemical and bioassays for the product. Simultaneously proceeding are phase I trials, phase II trials, commercial manufacturing planning and implementation, documentation preparation, and finally submission to a regulatory agency. The traditional flowsheet staggers different teams and different stages of drug development roughly in sequence (Figure 1.5a).

A key to reducing the cost of developing medicines is to condense the timeline, compressing the typical 6–8 years of development time into 2–3 years, as the regulatory agency now permits for drugs with breakthrough drug status. Such accelerated development has changed the way cell culture technologists, discovery chemists, pharmacists, and protein engineers work together. The time duration for most tasks in drug development cannot be shrunk proportionally to accommodate the shortened timeline; for instance, cells' doubling time cannot be made shorter to allow for a shorter cell line development time. As a result, the compression of the overall development timeline is not achieved by proportionally

shortening the completion time of each task, but by overlapping them in time. Before the cell line construction is completed, the process development and the product assay development all have to start. The project flow thus changes from one task team handing over their completed object to the next team to different task teams simultaneously working together in a cross-communicative way (Figure 1.5b).

An important consequence of this change is that the scientists and engineers from different segments of development are increasingly working in concert. Each person has to gain the vocabulary and knowledge of the other segments of the drug development process. Two decades ago, at the dawn of biotechnology, the call was for engineers to learn biology and for scientists to understand some vocabulary of process engineering.

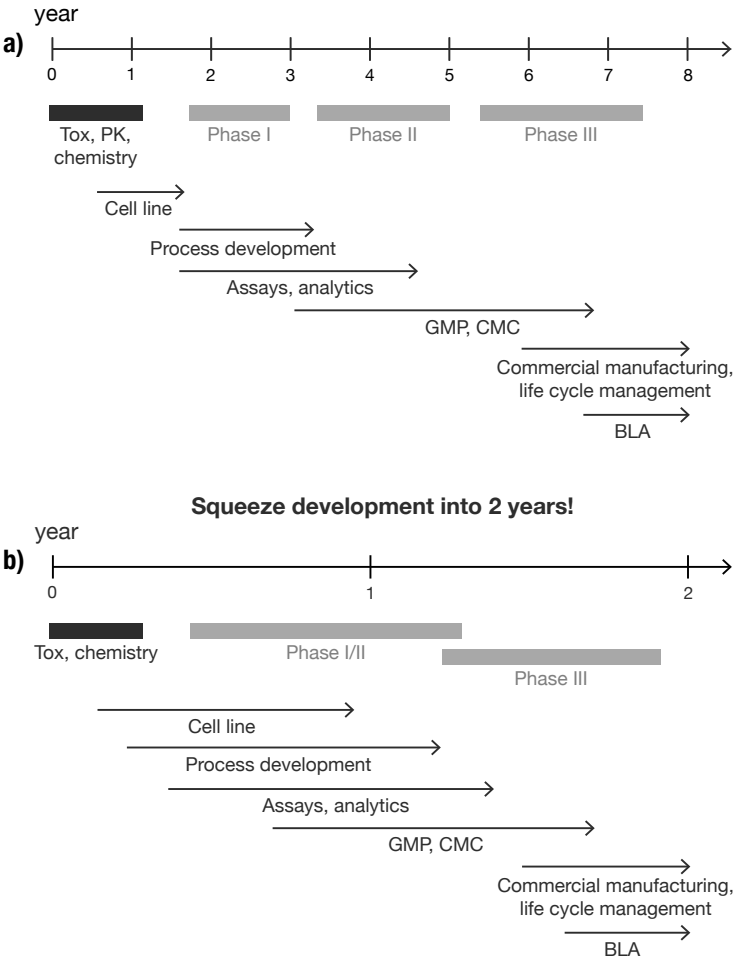

Figure 1.5. (a) Traditional biologics development. (b) Accelerated biologics development. Tox: toxicology; PK: pharmacokinetics; GMP: good manufacturing practice; CMC: chemistry, manufacturing, and controls; BLA: biologics license application.

Nowadays, engineers and scientists work hand-in-hand in the same segment of industry. This new cross-communication is far more than cross-disciplinary; it is cross-communication across segments of product development. Both upstream and downstream process technologists will need to understand the jargon of the regulatory specialists and that of the pharmacists developing bioassays, and vice versa. In the coming years, a new breed of cross-fertilized scientists and engineers will emerge to lead the advances in our field.