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Biopharmaceuticals: an overview

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Review article

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Abstract:

Biopharmaceuticals drugs structurally mimics compounds found within the body and are produced using biotechnologies. These have the potential to cure diseases rather than merely treat symptoms, and have fewer side effects because of their specificity, for example, cytokines, enzymes, hormones, clotting factors, vaccines, monoclonal antibodies, cell therapies, antisense drugs, and peptide therapeutics. Emerging technologies in the area of biopharmaceuticals include manufacture of monoclonal antibodies in protein free media, designing chemically defined cells, genome based technologies, improving vaccine manufacturing processes, a potential cancer treatment and non-ribosomal peptide synthesis. Biopharmaceuticals have changed the treatment ways of many diseases like diabetes, malignant disorders; since these can be tailored for specific medical problems in different individuals. With biotechnology, any drug can be genetically modified using cell fusion or deoxyribonucleic acid (DNA)-recombinant technologies to alter specificities for individual diseases. Some distinct advantages of biotechnological processes include fewer side effects and more potent effect on target cells. Biopharmaceuticals' greatest potential lies in gene therapy and genetic engineering.

Keywords: Biopharmaceuticals; Bioprocessing; Biotechnology; Transgenics

Introduction

Biopharmaceuticals make up about one-third of drugs currently in development and refer to pharmaceutical substances derived from biological sources. These are medical drugs produced using biotechnology especially genetic engineering or hybridoma technology or *via* biopharmaceutical techniques such as recombinant human technology, gene transfer and antibody production methods. Virtually all biotherapeutic agents in clinical use are biotech pharmaceuticals. Alternatively, any medically useful drug whose manufacture involves microorganisms or genetically modified organism or substances that living organisms produce (e.g. enzymes), or bioprocessing is termed a biopharmaceutical [1, 2]. Biopharmaceutical drugs are large, complex protein molecules derived from living cells. Manufacturing of pharmaceutical proteins including antibodies has been reported on a large scale. The production systems available include mammalian cells, yeast, insect cells and bacteria, and the schematic production work flows of important product groups are given in Figure 1. The choice of production systems depends on the nature of the protein being produced. However, there is no precise scientific definition of a biopharmaceutical.

Biopharmaceuticals, outcome of the exploitation of genetic information of all living material have already developed into an autonomous discipline. The biopharmaceutical industry is in a growth phase and is greatly changing the way that drugs are produced-from the use of chemical synthesis (traditional pharmaceuticals) to biomanufacturing (biologics). 'Quality by design' requires a thorough understanding of a biopharmaceutical product and its manufacturing processes, necessitating an investment in time and resources upfront in the discovery and development of a product [3]. The aims of development of a biopharmaceutical are: it should be clinically effective, approvable by regulatory authorities and commercially viable.

Development of new drugs and vaccines *via* biopharmaceutical research require concentrated efforts on many levels, as well as multiple skills and expertise. Various technologies such as manufacture of monoclonal antibodies in protein free media; designing chemically defined cells, genome based technologies, improving vaccine manufacturing processes, a potential cancer treatment and non-ribosomal peptide synthesis [4,5] developed in the last decade function similarly to unit operations for producing advanced biopharmaceuticals [6]. The biopharmaceutical industry is the most important sector in industrial biotechnology, and is one of the most rapidly growing high-tech industries [7, 8].

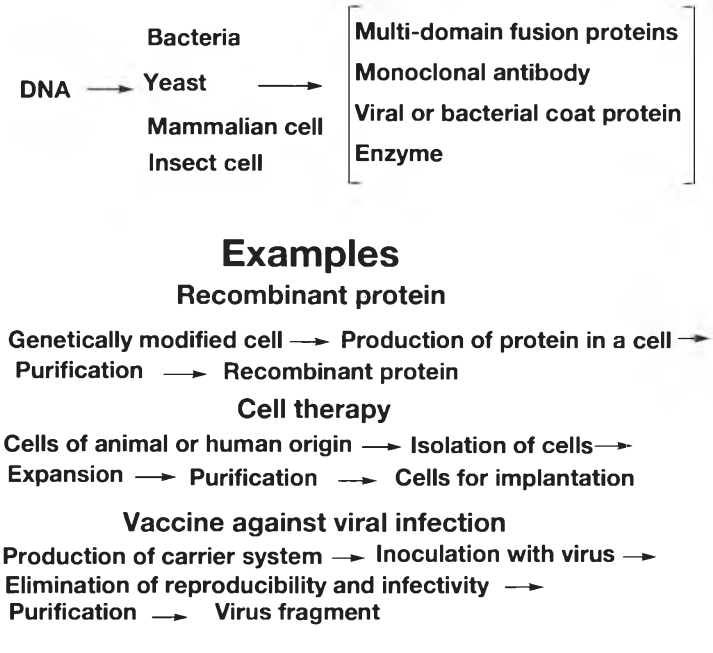

Figure 1 Schematic production work flows of important product groups (For example, recombinant protein, cells for implantation, virus fragment)

Biopharmaceuticals are proteins (including antibodies), nucleic acids (DNA, RNA or antisense oligonucleotides) used for therapeutic or *in vivo* diagnostic purposes, and are produced by means other than direct extraction from a native (non-engineered) biological source. The key areas of investigation in the field, covers drug production, plus the biochemical and molecular mechanisms of action together with the biotechnology of major biopharmaceutical types on the market or currently under development [9]. The first biopharmaceutical substance approved for therapeutic use was biosynthetic 'human' insulin made *via* recombinant DNA technology in 1982. In the late 1990s advances in manufacturing and processing revolutionized the production of biopharmaceuticals such as recombinant DNA technology and hybridoma technology. In other words, biopharmaceuticals have revolutionized the treatment of many diseases like diabetes, malignant disorders etc. More than 150 biotech drugs (human insulin, interferons, human growth hormones and monoclonal antibodies, as well as thirteen blockbuster drugs) are currently marketed around the world [10]. The biopharma market is growing at an annual rate of around 15%-far higher than pharmaceuticals (c.6-7% per annum). In future, the market is forecast to be significantly driven by a shift in usage from conventional drugs to biopharma products [11]. Majority of biopharmaceuticals products consist of glycoproteins and methods are now becoming available that allow the production of recombinant monoclonal antibodies bearing pre-selected oligosaccharides-glycoforms-to provide maximum efficacy for a given disease indication [12].

Biopharmaceuticals versus conventional chemical drugs

Biopharmaceuticals are fundamentally different from the conventional small molecule chemical drugs [13]. There is a fundamental difference in the average size of the two types of drugs. The chemically synthesized products are known as "small molecules" drugs (e.g. aspirin, molecular weight 180 Da). In general, the biopharmaceuticals are complex macromolecules that are over 100 times larger (e.g. interferon beta, molecular weight 19,000 Da) with complex structural and appropriate biological activity requirements [14]. Biopharmaceuticals have more potential heterogeneity than small molecule drugs. The large majority of biopharmaceutical products are derived from life forms. Small molecule drugs are not typically regarded as biopharmaceutical in nature by the industry. The nature of the manufacturing process, and the safety and efficacy profile of biopharmaceutical products are also different.

The majority of first generation biopharmaceuticals are unengineered murine monoclonal antibodies or simple replacement proteins displaying an identical amino acid sequence to a native human protein. Modern biopharmaceuticals are engineered, second-generation products. Engineering can entail alteration of amino acid sequence, glycocomponent of a glycosylated protein, or the covalent attachment of chemical moieties such as polyethylene glycol. Engineering has been applied in order to alter immunological or pharmacokinetic profile of protein, or in order to generate novel fusion products [15].

Biopharmaceutical classification system

Biopharmaceutical classification system (BCS) is a drug development tool that deals with the contributions of three major factors, dissolution, solubility and intestinal permeability, affecting oral drug absorption from immediate release solid oral dosage forms. According to BCS, drug substances are classified into different classes [16]. Class I: high solubility-high permeability; Class II: low solubility-high permeability; Class III: high solubility-low permeability; Class IV: low solubility-low permeability.

Types of biopharmaceuticals

Biopharmaceuticals are being developed to fight cancer, viral infections, diabetes, hepatitis and multiple sclerosis and these can be grouped into various categories. i) cytokines ii) enzymes iii) hormones iv) clotting factors v) vaccines vi) monoclonal antibodies vii) cell therapies viii) antisense drugs, and ix) peptide therapeutics.

i) ***Cytokines:*** Cytokines are hormone-like molecules that can control reactions between cells. They activate

cells of the immune system such as lymphocytes and macrophages [17]. *Interferon* is potent glycoprotein cytokine that acts against viruses and uncontrolled cell proliferation [18]. *Interleukins* function as messengers for various steps in the immune process.

Interleukin-2 (IL-2): IL-2 stimulates T lymphocytes. The FDA has approved a recombinant variant of IL-2, aldesleukin (Proleukin®), for treating renal cell carcinoma [19]. The antitumor effect of IL-2 and its recombinant variant was found directly proportional to amount of the agent administered. Endogenous IL-2 is scarce; aldesleukin can be mass-produced but has adverse side effects at relatively low levels of administration [20]. The mechanism of action, methods of delivery, efficacy, and side effect profile of the cytokines IL-2 and interferon alfa were reported [21].

Interleukin-3 (IL-3): IL-3 is an interleukin, a type of biological signal (cytokine) that can improve the body's natural response to disease as part of the immune system. It acts by binding to the Interleukin-3 receptor. IL-3 stimulates bone marrow stem cells. Stimulation of hematopoietic IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) appears to be able to stimulate sympathetic nerve growth, *via* specific cytokine receptors on neurons, which lead to activation of the mitogen-activated protein (MAP) kinase pathway that then mediated the observed neurotrophic effects [22]. Researchers suggested that IL-3 neuroprotected neuronal cells against neurodegenerative agents like amyloid-β protein (Aβ) [23].

Interleukin-1 (IL-1): A protein produced by various cells, including macrophages. IL-1 raises body temperature, spurs the production of interferon, and stimulates growth of disease-fighting cells, among other functions. The IL-1 family of cytokines comprises 11 proteins (IL-1F1 to IL-1F11) encoded by 11 distinct genes (IL1A, IL1B, IL1RN, IL18, and IL1F5 to IL1F11 in man, Il1A to Ilf11 in mice) [24-26].

The main function of IL-1-type cytokines is to control proinflammatory reactions in response to tissue injury by pathogen-associated molecular pattern (such as bacterial or viral products) or damage-or danger-associated molecular patterns released from damaged cells (such as uric acid crystals or ATP) [27, 28]. Thus, they are considered major mediators of innate immune reactions and blockade of IL-1 by the interleukin-1 receptor antagonist (IL-1RA) has proven a central role of IL-1α or IL-1β in a number of human auto-inflammatory diseases [29-31].

The signaling of the founding members, IL-1α and IL-1β, share only 24% amino-acid sequence identity but have largely identical biological function [32]. Further, IL-1 pathway has been reported [33]. IL-1 is made mainly by one type of white blood cell, the macrophage, and helps another type of white blood cell, the lymphocyte, to fight infections. It also helps leukocytes pass through blood vessel walls to sites of infection and causes fever by affecting areas of the brain that controls body temperature.

Interleukin 1β (IL-1β): IL-1β is a potent proinflammatory factor during viral infection. Interleukin-1 made in the laboratory is used as a biological response modifier to boost the immune system in cancer therapy [34]. An IL-1 blocker, anakinra (Kineret®), has been approved for treatment of rheumatoid arthritis. Another, rilonacept (Arcalyst®, has been approved for cryopyrin-associated periodic syndromes [35].

Inflammasome: The inflammasome is a multiprotein complex that mediates the activation of caspase-1, which promotes secretion of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18, as well as 'pyroptosis', a form of cell death induced by bacterial pathogens. Members of the Nod-like receptor family, including NLRP1, NLRP3 and NLRC4, and the adaptor apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) are critical components of the inflammasome that links microbial and endogenous 'danger' signals to caspase-1 activation. Several diseases are associated with dysregulated activation of caspase-1 and secretion of IL-1β. In view of above, understanding inflammasome pathways may provide insight into disease pathogenesis that might identify potential targets for therapeutic intervention [36]. Inflammasomes and IL-1 are involved in the pathogenesis of several inflammatory disorders. The remarkable progress in this field has offered new hope for many patients with these disorders

and also highlighted the role IL-1 might have in other inflammatory disorders, such as systemic juvenile idiopathic arthritis (sJIA), adult-onset Still's disease (AOSD), and rheumatoid arthritis [37].

Granulocyte-colony stimulating factor (G-CSF) stimulates the bone marrow to produce neutrophils (antibacterial leukocytes), and is used for cancer treatments that are immunodepressants [38].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the bone marrow to produce neutrophils and macrophages, and is used for chemo and radio therapy that suppresses bone marrow function [38].

ii) ***Enzymes:*** These are complex proteins that cause a specific chemical change in other substances without being changed themselves. For example, alteplase (Activase®, TPA) (dissolves blood clots); dornase alfa (Pulmozyme®) (a recombinant DNAse I that digests DNA in the mucous secretions in lungs); imiglucerase (Cerezyme®)-a recombinant glucocereborsidase for Gaucher's disease, bone destruction and enlargement of the liver and spleen [38]. Factor IX (Alphanine® SD, Benefix®, Bebulin® VH, Profilnine® SD, Proplex® T) belonging to peptidase family S1, is one of the serine proteases of the coagulation system. Deficiency of this protein causes hemophilia B [39]

iii) ***Hormones:*** These chemicals transfer information and instructions between cells in animals and plants. Examples include insulin (Insugen®, Humulin®, Novolin®), human growth hormone (Ascellacrin®, Crescormon®), glucagon, growth hormone, gonadotrophins (Ovidrel®)

iv) ***Clotting factors:*** These include any factor in the blood that is essential for the blood to coagulate [40].

v) ***Vaccines:*** These are microorganisms or subunit of microorganisms that can be used to stimulate resistance in a human to specific diseases as well as to stimulate immune response. Examples include hepatitis B virus [Baraclude (Entecavir®), Adefovir dipivoxil (Hepsera®), Lamivudine (Epivir®-HBV, 3TC), Alfa Interferon (Intron® A, Infergen®, Roferon®)], Ebola virus (No commercially available Ebola vaccines are available). Researchers have identified a protein in infected liver cells that is essential for hepatitis C virus replication. Inhibiting this protein is highly efficient in blocking virus replication [41].

vi) ***Monoclonal antibodies:*** Monoclonal antibodies are produced from immortal cells with an antibody producing spleen cells. Examples include Infliximab (Remicade®), adalimumab (Humira®), rituximab (Rituxan® , MabThera®). Monoclonal antibodies now account for approximately one third of all new treatments. Their applications include the treatment of breast cancers, leukemia, asthma, rheumatoid arthritis, psoriasis, chronic gastrointestinal inflammatory disease and transplant rejection. First fully human monoclonal antibody was launched in 2003 (Humira®) in UK-removing potential for immunogenic reactions. New indications and therapies are emerging all the time. The development of human antiviral monoclonal antibody therapies regarding antigenic variability of circulating viral strains and the ability of viruses to undergo neutralization escape was reported [42]

vii) ***Cell therapies:*** Cell therapy describes the process of introducing new cells into tissues in order to treat a disease. Several stem cell therapies are routinely used to treat disease today. Adult stem cell transplant e.g. bone marrow stem cells, adult stem cell transplant e.g. peripheral blood stem cells and umbilical cord blood stem cell transplant. Umbilical cord blood stem cell transplants are less prone to rejection than either bone marrow or peripheral blood stem cells. The best-known stem cell therapy to date is the bone marrow transplant, which is used to treat leukemia and other types of cancer, as well as various blood disorders [43].

Regenerative medicine using stem-cell research, tissue engineering and gene therapy is cutting-edge research and it focuses on the repair, replacement and regeneration of cells, tissues or organs to restore damaged function resulting from diseases and ailments. Stem cell-based therapies, tools and targets are our future. The big challenge for the stem cell community is therefore to facilitate the best possible interaction with the population at large i.e. one stem cell world [44].

Stem cell treatments are a type of genetic medicine that introduces new cells into damaged tissue in order to treat a disease or injury. Many medical researchers

believe that stem cell treatments have the potential to change the face of human disease and alleviate suffering. The ability of stem cells to self-renew and give rise to subsequent generations that can differentiate offered a large potential to culture tissues that can replace diseased and damaged tissues in the body, without the risk of rejection and side effects [45]. A number of stem cell treatments exist, although most are still experimental and/or costly, with the notable exception of bone marrow transplantation. Medical researchers anticipate one day being able to use technologies derived from adult and embryonic stem cell research to treat cancer, Type 1 diabetes mellitus, Parkinson's disease, Huntington's disease, Celiac Disease, cardiac failure, muscle damage and neurological disorders, along with many others [46].

viii) ***Antisense drugs:*** Antisense drug is a medication containing part of the non-coding strand of messenger RNA (mRNA). Antisense drugs work at the genetic level to interrupt the process by which disease-causing proteins are produced. Instead of attacking the bacteria or viruses that cause diseases, antisense drugs will literally throw a wrench into the portion of a cell's genetic machinery that produces disease-related proteins. Among much new molecular therapeutics being explored for cancer therapy, antisense oligonucleotides are emerging as a novel approach to cancer therapy, and used alone or in combination with conventional treatments such as chemotherapy and radiation, with numerous antisense agents being evaluated in preclinical studies and several anticancer antisense drugs in clinical trials [47]. One of the treatments for genetic disorder or infections is antisense therapy. When the genetic sequence of a particular gene is known to be causative of a particular disease, antisense drugs hybridize with and inactivate mRNA, thereby, restricting a particular gene from producing the protein for which it holds the recipe [48]. Although specificity and selectivity are the key features of antisense oligonucleotides, the need to target the right tissues and reach the nucleus remains a challenge to overcome [49, 50].

ix) ***Peptide therapeutics:*** Peptide therapeutics represents a novel class of therapeutic agents. Currently, only selected cationic antimicrobial peptides have been licensed, and only for topical applications [51]. It is now possible to produce very large quantities of therapeutic peptides with tight specifications by using the wide possibilities offered by liquid phase and solid phase technologies, alone or in combination depending on the specific features of a given project. Moreover, many peptides currently in the preclinical or clinical stages contain non-natural amino acids (β-amino acids or amino acids having D configuration) to make them more active or more stable [52]. Selection methodologies addressing protease resistance have been developed and when combined with methods such as pegylation antibody Fc attachment and binding to serum albumin look likely to finally turn therapeutic peptides into a widely accepted drug class [53].

Technologies

PEGylation

It is the process of covalent attachment of poly (ethylene glycol) or PEG polymer chains to another molecule, normally a drug or therapeutic protein. PEGylation has been proven as a powerful new drug delivery technology. Therapeutic proteins have been modified chemically by the covalent addition of PEG, or dextrans or other sugars, or by cross-linking to other proteins with the main aims being to extend the circulation time and/or the avoidance of immunogenicity or toxicity of those protein drugs. The recent achievements in PEGylation processes with an emphasis on novel PEG-drugs constructs, the unrealized potential of PEGylation for non-injected routes of delivery has been reported. [54]. Five PEGylated biopharmaceuticals: pegademase bovine (Adagen®) [PEG- bovine adenosine deaminase, used to treat cross-linked severe combined immunogenicity syndrome, as an alternative to bone marrow transplantation and enzyme replacement by gene therapy], pegaspargase (Oncaspar®) (PEGylated L-asparaginase for the treatment of acute lymphoblastic leukemia in children who are hypersensitive to the native unmodified form of L-asparaginase), pegylated interferon alfa-2a (Pegasys®) or pegylated interferon alfa-2b (PegIntron®) [PEGylated interferon alpha for use in the treatment of chronic hepatitis C and hepatitis B],

pegfilgrastim (Neulasta®) (PEGylated recombinant methionyl human granulocyte colony-stimulating factor for severe cancer chemotherapy induced neutropenia), and doxorubicin HCl (Doxil®) (PEGylated liposome containing doxorubicin for the treatment of cancer) were commercialized.

Releasable PEGylation employs customized linkers that reversibly bind a therapeutic moiety with polyethylene glycol polymers. Based on the bioconjugates of cytokines, peptide hormones, immunotoxins, enzymes, and reporter proteins, researchers have described both aromatic and aliphatic based customized linkers that release the unaltered original drug under physiological conditions and at therapeutically useful release rates [55]. PEGylation has become the biopharmaceutical delivery technology of choice for intravenously administered therapeutic proteins [56]. The benefits of PEGylation to a known protein-based biopharmaceutical versus a non-PEGylated version include: i) improved pharmacokinetics i.e enhanced solubility, improved stability, sustained absorption, continuous biopharmaceutical action, ii) increased circulation time i.e. decreased amount of protein required for therapeutic efficacy, decreased dosing frequency due to optimized biodistribution, reduced renal clearance, increased circulation time, iii) decreased toxicity, improved safety profile, reduced immunogenicity, reduced proteolysis. Structural properties of PEGylated proteins could play an increasingly important role in developing optimal therapeutic protein drugs [57]

Biologicals are produced under controlled conditions and newly generated proteins undergo complex post-translational modifications. These are very sensitive to production conditions and minor changes can have major impacts on biological activity [58-60]. Post-translational modifications add to complexity, for example, degree of glycoslyation can affect receptor binding, and pegylation can affect receptor binding and metabolic removal [61].

The separation and purification of biopharmaceuticals represents one of the most time and cost intense downstream operations in the manufacture of commercial biopharmaceutical products. Separation and purification of protein systems are usually achieved chromatographically with all of their disadvantages including high buffer requirements, large footprint, reuse and storage of resin studies as well as costs.

Bio-crystallization

The crystallization of biopharmaceuticals is poorly understood and is a rarely used commercial process for the primary separation and purification of proteins. The development of the technology base for an essentially new biopharmaceuticals unit process operation-biocrystallization is desirable. The benefits of protein crystallization in biopharmaceutical processing include i) isolation and purification: streamlining the manufacturing process and making biopharmaceuticals less expensive [62-64], ii) dosage levels-bioavailability: crystals are the most concentrated form and is beneficial for drugs such as antibodies, which require high doses at the delivery site [65], iii) protein crystallization may significantly improve some aspects of protein handling, and change the way biopharmaceuticals are produced, formulated, and delivered [66], iv) sustained release-stability :reduced chemical degradation and as such may enhance drug efficacy over prolong period [67], v) handling/processing/delivery-formulation: ability to achieve high concentration, low viscosity formulation and controlled release protein delivery [68], vi) engineering to suit purpose: the ability to control crystal shape or habit and polymorphism as in the case of urate oxidase [69].

Therapeutic applications of proteins include treatments for acute conditions, such as cancer, cardiovascular disease and viral disease, and chronic conditions, such as diabetes, growth hormone deficiency, haemophilia, arthritis, psoriasis and Crohn's disease. Protein crystals have shown significant benefits in the delivery of biopharmaceuticals to achieve high concentration, low viscosity formulation and controlled release protein delivery. The utilization of protein crystals in biopharmaceutical applications has been reviewed [70].

The regulatory and commercial pressures to accurately characterize complex biopharmaceutical molecules have lead to analysts looking beyond the traditional tried and tested methods. Analytical techniques include proteomic methods, mass spectrometry, 2D-PAGE,

CE-SDS gel method and sensitive micro/nano chromatography [71, 72].

Biopharmaceutical manufacturing

Biopharmaceutical manufacturing is complex and variable. These may be produced from microbial cells (e.g. recombinant *E. coli* or yeast cultures), mammalian cell lines, plant cell cultures and moss plants in bioreactors of various configurations, including photobioreactors [73, 74]. The process of manufacturing a biopharmaceutical product entails two major steps that are referred to as upstream and downstream processing. Upstream processing skills include those associated with the culture and maintenance of cells and downstream processing skills included those associated with the chemical and physical separations necessary for the isolation and purification of the product itself, from the complex culture mixture [75, 76].

For proteins that require glycosylation, mammalian cells, fungi or the baculovirus system are chosen. The two most utilized yeasts are *Saccharomyces cerevisiae* and *Pichia pastoris*. Yeasts can produce high yields of proteins at low cost, proteins larger than 50 kD can be produced, signal sequences can be removed, and glycosylation can be carried out. The most popular system for producing recombinant mammalian glycosylated proteins is that of mammalian cells. Genetically modified animals secrete recombinant proteins in their milk, blood or urine. Similarly, transgenic plants such as *Arabidopsis thaliana* and others can generate many recombinant proteins [77]. The possible mechanisms by which glycosylation improves the molecular stability of protein pharmaceuticals have been reported [78]. Insulin Aspart (NovoLog®/NovoRapid®) and insulin Glargine (Lantus®) are manufactured from recombinant DNA technology using the yeast, *Pichia pastoris*. Erythropoietin-alpha is a 165 amino acid glycoprotein manufactured by recombinant DNA technology and its biological effects are the same as naturally occurring erythropoietin. Streptokinase (Kabikinase®, Streptase®) is manufactured by recombinant DNA technology from *E. coli* as a non-glycosylated polypeptide chain. Various aspects of biopharmaceuticals development and clinical manufacturing have been reported and an outline of drug substance production involving upstream and downstream process is shown in Figure 2. [79, 80]

The production technologies and operations that occur in the manufacturing facility were reported [81]. Scientists described practices applicable to the large-scale processing of biotechnological products [82]. Protein therapeutics-therapy using protein-based drugs especially monoclonal antibodies and recombinant proteins, have emerged as the hottest approach in targeting and treating a number of diseases. The demand for new and effective biotherapeutic production has resulted in new technologies associated with protein expression and purification. In biopharmaceutical manufacturing, process development accounts for 30% of costs, upstream

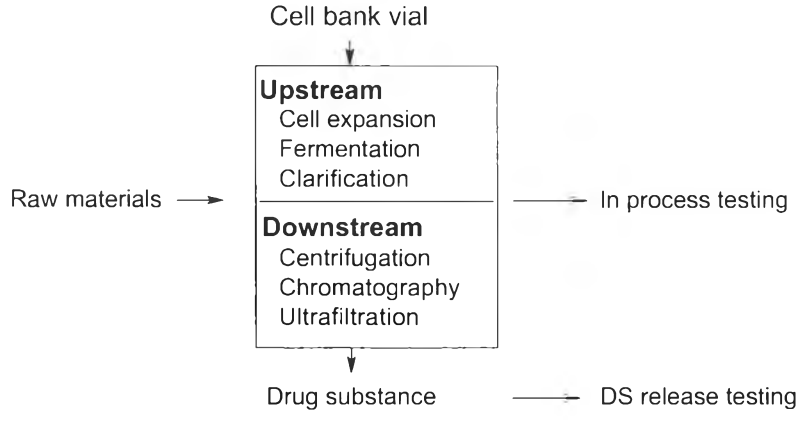

Figure 2 An outline of biopharmaceutical manufacturing involving upstream and downstream process for drug substance (DS) production

processing for 20%. However, the highest outlay in biopharmaceutical manufacturing is attributed to downstream processing, which is responsible for a massive 40% of the total costs incurred [83]. There has been considerable pressure to reduce the cost of downstream processing and cut the bottlenecks in the biopharmaceutical production process. Monoclonal antibody manufacture presents substantial current and upcoming challenges to the biopharmaceutical industry [84]. In the future, experts are of opinion that successful companies will have fewer ton-scale proteins and will look for economical ways to produce low-scale products.

Characterization of biopharmaceuticals

Most commonly used spectrophotometric, chromatographic, and electrophoretic methods are used to characterize biopharmaceutical product. Mass spectrometry can be used, in conjunction with protein and carbohydrate chemistry, to solve a variety of structural problems ranging from de-novo protein sequencing, to the characterization of recombinant proteins, including vaccine and antibody products. Particular emphasis is positioned on the identification of post-translational modifications, such as glycosylation. Glycosylation is important for the biological activity of proteins. The functions of the glycocomponent include protein folding, protein trafficking, protein targeting, ligand recognition, ligand binding, biological activity, stability, pharmacokinetics and immunogenicity. Scientists have applied the MS-Mapping technique routinely to the characterization of recombinant protein and glycoprotein products [85]. Scientists highlighted the role that mass spectrometry can and should play in the biopharmaceutical industry beyond the presently assigned task of primary structure analysis [86]. Methods and genetically engineered cells useful for producing an altered N-glycosylation form of a target molecule was described [87]. Analytical ultracentrifugation and field flow fractionation are two important biophysical methods for measuring in characterization of therapeutic proteins in the biopharmaceutical industry [88].

Transgenics

A potentially controversial method of producing biopharmaceuticals involves transgenic organisms, particularly plants and animals that have been genetically modified to produce drugs. Transgenic plants are an attractive platform for the production of biopharmaceuticals since they offer bio-safety, lower cost of goods and flexibility/scalability. One potential approach to this technology is the creation of a transgenic mammal that can produce the biopharmaceutical in its milk (or blood or urine). The first such drug manufactured from the milk of a genetically-modified goat was antithrombin (ATryn®). Scientists created, moss strains with non-immunogenic humanized glycan patterns and an overview of the relevant aspects for establishing moss as a production system for recombinant biopharmaceuticals was reported [89]. Other technology trends include development of efficient mammalian expression systems and cloning technology, though there is still some way to go before cloning becomes part of normal treatment procedure [90].

The expiry for patent protections for biologics has created a new marketplace to be exploited by generic competition [91]. Biosimilars, or follow-on proteins, are new versions of existing biopharmaceuticals whose patents have expired [92]. They are produced using the same core genetic material and are approved on the basis that they are equal to the reference product in terms of both safety and efficacy. Biosimilars are large, complex molecules produced by living organisms, which are sensitive to manufacturing changes. Biosimilars is an official term used by the European medical authorities; the US terminology is follow-on protein products. The promise of profits from biosimilars grows greater, but only when a number of significant market, regulatory and clinical hurdles can be overcome [93].

Bioprocess membrane technology

Bioprocessing is a crucial part of the biotechnology/ biopharmaceutical sector and it is anticipated that within the next five to ten years, up to 50% of all drugs in

development will be biopharmaceuticals; a large proportion of these will be recombinant protein therapeutics. Bioprocessing encompasses a wide range of techniques used in the development and manufacturing of bioscience based medicines, (known as biopharmaceuticals or biologics). Applications include tissue engineering (manufacturing approaches for tissue products); biopharmaceutical formulation and delivery (novel mechanisms for formulating and delivering biopharmaceuticals); bioscience underpinning bioprocessing (understanding the cellular and molecular processes which are predictive of process performance and which can inform strategies for process design and metabolic engineering); improved tools for bioprocessing (tools to accelerate bioprocess development including high throughput bioprocess research); process modeling, improved analytics and ultra scale-down systems

Researchers provided an overview of recent developments in membrane technology, focusing on the special characteristics of the membrane systems that are now used for the commercial production and purification of recombinant protein products [94-98]. The large-scale production of recombinant human monoclonal antibodies demands economical purification processes with high throughputs. Membrane chromatography has already proven to be a powerful alternative to traditional packed-bed chromatography in flow-through operations, such as polishing for the removal of viruses and contaminants in biologics manufacturing. The benefit of using membrane chromatography is receiving increased recognition and is now becoming a routine process step in large-scale biopharmaceutical manufacturing processes, as it opens the opportunity for interesting new application areas, such as removal of impurities and viruses. Some of the upcoming technologies that can help to optimize time and cost of biopharmaceutical manufacturing have been recently reported [99]. These technologies include i) tangential flow filtration microfiltration capsule for cell clarification and harvest, ii) hydrophobic interaction membrane chromatography and iii) solutions for high parvovirus retention. The disadvantages and advantages of using Q membrane chromatography as a purification unit in large-scale production were reported [100]. The technology used in biopharmaceutical filtration and separation (the harvesting of mammalian cells to produce new drugs and medicines) has been reported [101].

Disposable systems in biopharmaceutical manufacturing

Disposable technologies are available for almost every aspect of biopharmaceutical drug processing. In the case of a recombinant biotech product, completely disposable manufacturing process means that each unit process operation, from fermentation through purification, to final fill-and-finish, ideally should be redesigned and retooled to enable the economical single use. The benefits of disposable manufacturing are many; their relative significance depends on the type of drug to be manufactured and the focus, size and resources of the individual biopharmaceutical company. These benefits include economy, speed to market, capacity and drug process security [102].

Single-use technology in biopharmaceutical manufacturing

Single-use systems are designed with partnerships between end-users and suppliers and users must evaluate supplier emerging technologies, sometimes with only minimal in-house. In biopharmaceutical manufacturing, single-use components and systems can offer distinct advantages over reusable, cleanable systems and will continue to grow in importance and demand as these have potential to reduce cross contamination, and elimination of many of the cleaning procedures [103]. One challenge in using single-use technology in bioreactors is that not all cell lines are compatible with disposable bioreactors [104-107].

As most biopharmaceutical drugs are manufactured in small batches, the advantages offered by the use of disposables would seem especially applicable to this market segment. The use of disposables in biopharmaceutical manufacturing can significantly impact manufacturing process efficiency by reducing capital costs, improving plant flexibility, reducing startup time and costs, eliminating some or all non-value added process steps, eliminating cross-contamination, significantly reducing process waste, reducing labor costs and

reducing on-site quality and validation requirements. However, the development of fully disposable manufacturing platforms to supply all scales of biopharmaceutical manufacturing remains in the future and is dependent on the development of new technologies, specifically in the areas of disposable chromatography, tangential flow filtration (TFF) and centrifugation by the developers and producers of these technologies. However, the long-term future of disposables looks bright as applications for disposables are expanding. They will be incorporated into the toolbox of technologies available to the end user with the development of other disposable processes [108-112].

Miscellaneous aspects

New and emerging large-molecule bioactive agents delivered from stent surfaces in drug-eluting stents (DESs) to inhibit vascular restenosis in the context of interventional cardiology has been reported. New therapeutic agents representing proteins, nucleic acids (small interfering RNAs and large DNA plasmids), viral delivery vectors, and even engineered cell therapies require specific delivery designs distinct from traditional smaller-molecule approaches on DESs. Many of the larger-molecule and biopharmaceutical approaches have been reported recently for stent-based delivery with the challenges associated with formulating and delivering these drug classes compared to the current small-molecule drugs [113].

Biologicals as a group are highly effective in the treatment of rheumatoid arthritis. Biologicals were efficacious both in treatment naïve and methotrexate-refractory patients [114]. A recent advance in the management of rheumatoid arthritis is the use of biological agents which block certain key molecules involved in the pathogenesis of the illness. They include tumour necrosis factor (TNF)-blocking agents such as infliximab (Remicade®), etanercept ((Enbrel®) and adalimumab (Humira®), the anti-CD 20 agent rituximab (Rituxan® and MabThera®) and CTLA-4 Ig abatacept (Orencia®). Other agents which are in development include anti-IL6 tocilizumab (Actemra®, RoActemra®), anti-CD22 (epratuzumab) and monoclonal *anti*-BLyS antibody (anti-lymphostat B). Scientists have discussed the efficacy and side effects of these agents, their impact on current clinical practice and future trends. [115]. The status on the use of biopharmaceuticals in the treatment of rheumatoid arthritis indicated that blocking of TNF-alpha, co-stimulation of CD28+ T-cells and depletion of CD20+ B-cells were effective ways to diminish inflammation and joint damage [116].

Viral safety is a key issue for biological and biotechnological medicinal products. Scientists discussed the most up-to-date scientific knowledge and regulatory aspects in the areas of virus safety of recombinant proteins, monoclonal antibodies, plasma-derived medicinal products and advanced technology medicinal products [117, 118].

Fusion proteins are successful biopharmaceuticals. Fusion proteins can be categorized into several groups according to their features. In the first group, effectors molecules are fused to Fc domains, albumin or transferrin to extend the plasma half-life of the fusion product. In the second group, toxicity is conveyed by fusion proteins to toxins, enzymes or cytokines. The third application, which is not yet in clinical trials, utilizes fusion partners to enable novel delivery and targeting routes. Besides some specific disadvantages, many examples of fusion proteins suffer from the challenge of immunogenicity; however, future applications with novel fusion partners will reach beyond cancer, immunology and inflammation [119]. The potential applications of erythrocytes in drug delivery have been reviewed with a particular stress on successful erythrocyte loading and characterization of the different classes of biopharmaceuticals [120]. In recent years, recombinant therapeutic proteins produced in mammalian cells demand has increased dramatically and is now driving the development of a variety of improvements to maximize their expression in mammalian cells [121]. Glycoengineered yeast can be used to produce functional full-length monoclonal antibodies at commercially viable productivities [122]. DNA has the potential to meet the demands of emerging and existing diseases and of particular interest for therapeutic use is plasmid DNA that makes use of cellular machinery to express proteins or antigens. The production

stages of fermentation and downstream purification using DNA as a drug was reported [123]. Scientists have described application of using RNAi technology to increase cellular productivity and the quality of recombinant proteins that are produced in Chinese hamster ovary (CHO) cells, the most important mammalian cell line used in producing licensed biopharmaceuticals [124]. Researchers focused on the use of aqueous two-phase liquid-liquid extraction technique as an option for the downstream processing of biopharmaceuticals therapeutic proteins for the purification of monoclonal antibodies, growth factors and hormones [125]. Monoclonal antibodies have become major therapeutic drugs for the treatment of a number of diseases, thanks to a remarkable molecular engineering. The success of the first generation of monoclonal antibodies opens the way to new challenges such as antibody functional optimization, better control of unwanted side effects, or low cost production at an industrial scale. A new generation of antibodies is now emerging and one can already foresee the future: oligoclonal approaches based on the use of specific antibodies cocktails, selection of eligible patients, and antibody production at low costs [126].

Twenty five percent of women who have breast cancer express a growth receptor called HER2. Breast cancer with this receptor expressed grow exceptionally fast compared to other types. A genetically engineered monoclonal antibody trastuzumab (Herceptin®) by Genentech, Inc, is a drug designed specifically to block the activity of the HER2 receptor. It does not work in patients who do not express the HER2 receptor, but it works exceptionally in those patients who do express it. Scientists consider it as the ideal example of a truly targeted biopharmaceutical [127].

New antibody discovery tools have increased the speed and precision with which potent neutralizing human antiviral monoclonal antibodies (mAbs) can be identified. As longstanding barriers to antiviral mAb development, such as antigenic variability of circulating viral strains and the ability of viruses to undergo neutralization escape, are being overcome, deeper insight into the mechanisms of mAb action and engineering of effector functions are also improving the efficacy of antiviral mAbs [128].

Regulatory authorities and the biopharmaceutical industry are continuously seeking to improve methods for the detection, identification, inactivation and removal of potentially contaminating pathogens in biotherapeutics. The biopharmaceutical industry has employed a multifaceted approach in pathogen detection, including the rigorous screening of blood/plasma donations; documented sourcing and screening of raw materials; thorough testing of production cell substrates and cell culture harvest material during processing, and at the stage of a final purified drug substance; and the evaluation of microbe clearance during purification operations [129]. High-tech bioreactor-derived bioactive phytomolecules and biopharmaceuticals hold the prospect of providing permanent remedies for improving human well-being [130].

Some specific uses of biopharmaceuticals

Erythropoietin: Erythropoietin is the hormone responsible for inducing red blood cell production by the body's bone marrow. The most common use is in people with anemia (low blood count) related to kidney dysfunction. Drugs such as epoietin alfa (Epogen®, Procrit®) and darbepoetin alfa (Aranesp®) increased the production of red blood cells. They are used to treat anaemia associated with chronic kidney failure, cancer chemotherapy, and antiretroviral HIV therapy [131]. Erythropoietin plays an important role in the brain's response to neuronal injury and is also involved in the wound healing process [132-134].

Interferon-α: Imatinib (Gleevec®) is a 2-phenylamino-pyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinase (TK) enzymes. It occupies the TK active site, leading to a decrease in activity. IMATINIB is used in chronic myelogenous leukemia, gastrointestinal stromal tumors and a number of other malignancies [135].

Interferon-β : This is used for treatment of relapsing multiple sclerosis [136].

Monoclonal antibody: The newest monoclonal antibody approved for the treatment of rheumatoid arthritis is rituximab (Rituxan®). Like infliximab (Remicade®), it is a chimeric mouse/human monoclonal antibody that is given

by intravenous infusion. Unlike Remicade®, it attacks the B cells as opposed to TNF. Several new monoclonal antibodies are in the development stage to treat rheumatoid arthritis and other conditions. Trends in benchmarks for various types of monoclonal antibodies, with an emphasis on that study as anticancer and immunological therapeutics were reported [137]. Researchers provided an overview of the upstream technologies used in the industrial production of therapeutic monoclonal antibodies based on the cultivation of mammalian cells. They reviewed the cell lines currently employed in commercial production and the methods of constructing and isolating production clones, followed with a review of the most current methods of commercial scale production and their associated technologies [138].

The recent knowledge on the use, effectiveness and safety of monoclonal antibodies in multiple sclerosis treatment has been reported [139]. Natalizumab (Tysabri®, a humanized monoclonal antibody) is a prescription medication approved for patients with relapsing forms of multiple sclerosis, and is now indicated for appropriate patients with Crohn's disease. Tysabri® binds to the "alpha4beta1" integrins of leukocytes, and blocks attachment to cerebral endothelial cells, thus reducing inflammation at the blood-brain barrier. The mandatory guidelines for natalizumab use in the treatment of multiple sclerosis were also reported. Natalizumab recently joined glatiramer acetate (Copaxone®, or copolymer 1) and interferon beta-1b (Betaseron®) as an approved therapy for controlling relapsing multiple sclerosis [140].

Colony stimulating factors; Colony-stimulating factors are medications used to increase the number of WBCs. Recombinant human granulocyte colony-stimulating factor (G-CSF) has been used for treatment of febrile neutropenia in systemic lupus erythematosus (SLE) and other systemic rheumatic diseases. However, scientists suggested that G-CSF therapy should be used with considerable caution in patients with SLE [141]. AVI-014 is an egg white-derived G-CSF and has safety, pharmacokinetic, and pharmacodynamic properties comparable to Neupogen (Filgrastim®) at an equal dose in healthy volunteers [142].

Glucocerebrosidase: This is used for treatment of Gaucher's disease which is the most common glycolipid storage disorder, characterized by storage of the glycolipid, glucocerebroside in the liver, spleen, and marrow. It is caused by a hereditary deficiency of the enzyme glucocerebrosidase (also known as acid β-glucosidase). Enzyme replacement therapy, with glucocerebrosidase purified from human placentae, was introduced in 1991. Recombinant human glucocerebrosidase, produced by Chinese hamster ovary cells in tissue culture, became available in 1994 and has replaced the placenta-derived product. These therapies have revolutionized the care of patients with type 1 Gaucher's disease, reversing many of the pathological consequences of this disease, and preventing further progression. Furthermore, they have served as a model for the treatment of other lysosomal storage diseases and inborn errors of metabolism [143, 144]. The relatively high prevalence of this disease within an ethnic group is believed to reflect a selective advantage. Treatment with enzyme replacement therapy is safe and effective in ameliorating the primary symptoms of the disease, yet there have been reports that some patients on enzyme replacement therapy have developed type 2 diabetes or metabolic syndrome, malignancies and central nervous system disorders [145].

Conclusion and perspectives

Biopharmaceuticals are medical drugs derived from biological sources and especially one produced by biotechnology. They are therapeutic proteins, cellular products, gene therapy products, vaccines and plasma blood product derivatives. Leading biopharmaceutical products include erythropoietins, insulins, and monoclonal antibodies, and biopharmaceutical companies are turning their focus on long-term conditions such as cardiovascular disease, diabetes and asthma. In light of limitations of cost and lack of long-term safety and efficacy data, newer agents for the time being are recommended for use as second- or third-line agents in patients with active rheumatoid arthritis. One alternative approach may be to limit the use in patients who can afford it, and

who are at high risk of radiographic progression and disability [146].

Biopharmaceutical products are considerably more expensive than traditional ones, largely due to the high-cost technology required for production. Cost-reduction technologies must be developed to bring the cost of manufacture down; it becomes more viable to manufacture biopharmaceuticals with large market size and high market growth. Although inherent simplicity of operation of small molecule therapies has given them an edge over newer ones such as stem cell therapies, the latter's ability to eliminate concerns over viral or prion contamination during organ replacement has gained a lot of popularity. Other advantages of biopharmaceuticals include fewer side effects and more potent effect on target cells. Pharmaceutical biotechnology is being further developed to fight cancer, viral infections, diabetes and hepatitis as well as for developing safer and more effective antibiotics, insulins, interferons, estrogens and human growth hormone [147].

Portable clean room technology, the use of cell culture perfusion for production of antibodies, sequential multicolumn chromatography, and extensive use of in-line dilution of buffer concentrates may allow for significantly less expensive and more modular facilities of sufficient capacity to match production needs just in time.

Biopharmaceuticals' greatest potential lies in gene therapy and genetic engineering. Currently product developers should develop products according to the medicinal products legislation, which also applies to biopharmaceuticals. Verification of the similarity of biosimilars to innovator biopharmaceuticals remains a key challenge. Critical safety issue and the immunogenicity of biopharmaceuticals, has been highlighted in recent years, confirming a need for comprehensive immunogenicity testing prior to approval and extended post-marketing surveillance.

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