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Review Article

ADVANCES IN THERAPEUTIC PROTEIN PRODUCTION AND DELIVERY

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ABSTRACT

A budding interest in production and delivery of therapeutic proteins has generated a number of advancements throughout recent years. Therapeutic proteins offer great importance in the treatment of various diseases like cancers, heart attacks, strokes, cystic fibrosis, diabetes, anemia and hemophilia. They are produced by using microbial fermentation on cell cultures in transgenic plants and animals. Various therapeutic proteins are mainly administered by parenteral routes. Other routes of administration of therapeutic proteins are pulmonary, nasal, oral, buccal, transdermal, mucosal, rectal and vaginal. Many new approaches of delivery of therapeutic proteins have been investigated and designed to achieve maximal efficacy with minimal side effects. This review highlights current state and future of production and delivery of therapeutic proteins for the use in human health care.

Key words: Proteins; Therapeutic proteins; Protein production; Protein delivery.

INTRODUCTION

During past few decades, therapeutic proteins have emerging in prominence as potential drug candidates for the future to treat patients suffering from various diseases like cancers, heart attacks, strokes, cystic fibrosis, diabetes, anemia, hemophilia, Gaucher's disease etc. Proteins are large molecules composed of long chains of sub-units called amino acids. Since, proteins play critical roles in cell biology and they have many potential therapeutic uses in preventing and curing various diseases. The first protein used to treat disease was insulin, a small peptide that revolutionized the diabetic treatment.[1] In addition; the antigens used for vaccinations to induce immunogenic responses are often proteins. Now, a new series of therapeutic protein-based pharmaceuticals have made their presence felt with the discovery and development of methods to clone and express the cDNA encoding heterologous proteins. Proteins, which are engineered in the laboratories and in plants or animals for various pharmaceutical use are commonly known as biotechnological proteins.[2] These protein marketed to date are recombinant therapeutic proteins produced by living microorganisms, in plants and in animals to fight against various diseases. Currently, most of the commercially available therapeutic proteins are used parenterally, because oral administration is limited due to enzymatic degradation. They also suffer from short biological half-lives. So, daily multiple injections would be required to maintain the effective therapeutic levels of these drug candidates. To limit this drawback, development of controlled release parenterals would be beneficial. There is undoubtedly an urgent need to explore alternative non-parenteral routes of administrations like oral, buccal, pulmonary, nasal, transdermal, mucosal, rectal and vaginal. Alternatively, other approaches such as implants or self-regulatory devices can be equivocally be exploited.[3-4] Thus, challenges to develop a new protein-based formulation include not only cloning or synthesis of protein candidates, but also improving bioavailability with patient compliances. This review outlines the production of therapeutic proteins by various ways and related possible delivery techniques in detail.

PRODUCTION OF THERAPEUTIC PROTEINS

Unlike synthetic drug candidates, therapeutic proteins are usually produced through microbial fermentation, by cell cultures in transgenic animals and transgenic plants (Table 1). Short peptide chains (< 30 amino acids) are synthesized chemically, large proteins produced by living cells and in some cases proteins are secreted by cells have been used in therapeutics.[5] Producing biotechnological therapeutic proteins is a time-consuming and very complicated process. Many years can be spent for just identification of protein molecules determining its gene sequence. Production of these candidates is commonly done by growing host cells that have been transformed to contain the gene of interest in carefully controlled conditions of a balanced temperature, oxygen, acidity and other variables. These proteins are isolated from cultures.

Table 1: Important therapeutic proteins with their potential applications and expression cells

Therapeutic proteins	Potential applications	Expression cells
Insulin	Diabetes mellitus	*E. coli*
Erythropoietin	Anemia	Mammalian cell line
Factor VIII	Hemophilia	Chinese Hamster ovary cell
Human growth factor	Treating hypopituitary dwarfism in children	*E. coli*
Hepatitis-B vaccine	Hepatitis-B	*S. cerevisiae*
Alpha interferon	Leukemia, hepatitis-B, cancers	*E. coli*
Beta interferon	Sclerosis	Chinese Hamster ovary cells, *E. coli*
Gamma interferon	Chronic granulomatus disease	*E. coli*
Streptokinase	Acute myocardial infarction	*E. coli*
Interleukin-2	Renal cell carcinoma	*E. coli*

Production of therapeutic proteins by microbial cell culture: Some human proteins can be produced by microbial host. To produce therapeutic proteins by bacteria, yeast and fungi, hybridoma technology has provided as a major molecular tool. The best protein – expressing hosts are *Eschrichia coli, Staphylococcus cerevisae, Aspergillus niger* and *Pseudomonas pastoris*. Scalability and well characterized genetics of microbial host systems made it as the best option for protein production at relatively low cost.[6] Combinatorial biosynthesis of metabolites by microorganisms is a powerful method for producing non-ribosomal peptides with desirable drug functions.[7-8] The major problem of production of therapeutic proteins by microbial cell culture is post production recovery of proteins. The improved bioprocess strategies include the secreting of signal peptide fused to N-terminal of target proteins and addition of charged acid residues or mutation of amino acids of protein for improving purification.[9]

Production of therapeutic proteins by animal cell culture: Animal cell lines such as mammalian cell lines and Chinese cell lines etc. have been widely utilized as host for therapeutic protein production like factor VIII, erythropoietin, β-interferon and tissue plasminogen activator etc. Many of them used for large production of therapeutic proteins undergo apoptic death due to deprivation of nutrients like amino acids, glucose, serum, oxygen etc, in the bioreactor environment.[10] These are required to be monitored carefully to reduce to avoid unintentional transmission of viral contaminants that could infect and to increase yield.[11]

Production of therapeutic proteins by transgenic animals: Transgenic animals are used to produce large quantities of complex human proteins. Cow, sheep, goat, pig, rabbit are used as transgenic animal species due to several advantages like higher expression, level, low initial investment and reproduction facility also.[12] These animals are genetically engineered by introduction of gene designed to direct the synthesis of proteins without affecting the health of the animal. This process involves the microinjection of the DNA molecules into the pronucleus of embryo using fine glass needle. The infected zygote is then transferred into a hormonally prepared recipient and brought to term. Finally, positive transgenic animals are matured and the level of expression of transgene is determined. The rate transgenesis is 5-25 % of line birth, but the unit cost per protein should be significantly less if animals are used as bioreactor.[13] Some common applications of these proteins include the treatment of hemophilia and sepsis or trauma from accidents and surgery.

Production of therapeutic proteins by transgenic plants: Recently, transgenic (i.e., plants engineered to produce specific proteins) plant expression systems were developed as alternative sources for the production of biologics, known as plant-made pharmaceuticals.[14] The types of plants and the types of plant tissues used for the production of therapeutic proteins include the followings:[15]

1. Leaf and stem tissues of various species and varieties of tobaccos, *Arabidopsis thaliana*, alfalfa, spinach and potatoes,
2. Seeds of rice, beans, maize and tobacco,
3. Root vegetables like carrots,
4. Fruits like tomatoes and strawberries,
5. Aquatic weeds like *Lemna sp*.,
6. Hairy root cultures derived from various plants, via, *Agrobacterium rhizogenes* transformation,
7. Single cell cultures of the algae *Chlorella* and *Clamydomonas*,
8. Suspension cell cultures of tobacco,
9. Transformed chloroplasts of a variety of plant species.

Transgenic single-cell cultures offer the advantages over whole plant systems of a high level of contaminant and possibility of producing proteins in bioreactors under GMP (-) conditions, as in currently the case with conventional fermentation or cell culture techniques. In general, the use of plants for production of therapeutic proteins means a lower cost of production and easier expansion for large volume production than cell culture systems. Plant expression systems can potentially produce hundreds of kilograms per year of a purified protein whereas the cost of a similar production capacity using mammalian cell cultures may be prohibitive. To achieve specific protein molecules in plants, the DNA that encodes the desired proteins must be inserted into the plant cell. This can be done as a stable transformation when foreign DNA is incorporated into the genome of the plant that dictates to the plant to produce the desired protein molecules. Transgenic plants are suitable for large volume production of proteins.[16] The first therapeutic protein produced in plants was human growth hormone, which was expressed in transgenic tobacco in 1986.[17] Other proteins that have potential therapeutic applications after expression and purification from plants[18] are listed in Table 2.

Table 2: Various therapeutic proteins produced by transgenic plants

Therapeutic proteins	Potential applications	Transgenic plant host
Protein C	Anticoagulant	Tobacco
Hirudin	Thrombin inhibitor	Canola
Granulocyte macrophase colony stimulating factor	Neutropenia	Tobacco
Somatotropin, Chloroplast	Growth hormone	Tobacco
Erythropoietin	Anemia	Tobacco
Enkephalins	Antihyper analgesic	Arabidopsis
Epidermal growth	Wound repair, control of cell proliferation	Tobacco
Alpha interferon, Beta interferon	Hepatitis B & C	Rice, turnip and tobacco
Serum albumin	Liver cirrhosis, burns, surgery	Tobacco
Hemoglobin-∞, β	Blood constitute	Tobacco
∞-1 antitrypsin	Cystic fibrosis, liver diseases	Rice
Aprotinin	Transplant surgery	Maize
Lactoferrin	Antimicrobial	Potato
Angitensin converting enzyme	Hypertension	Tobacco and tomato
∞- Tricsanthin	HIV Therapies	Tobacco
Glucocerebrosidase	Gaucher's disease	Tobacco

The conventional commercial therapeutic production from microbial fermentation and mammalian cell lines has limitations of cost, scalability, product safety, authenticity, quality and potential contamination with animal pathogens. Producing recombinant animal and human proteins in plant systems has several advantages. However most of the plant-based systems currently available lead to generation to plant-like complex glycans on human and animal proteins that are potentially immunogenic. This greatly diminishes their value as therapeutics. A comparison of production of therapeutic protein from different sources is listed in Table 3.

Table 3: Comparison of production systems for therapeutic proteins from different sources

Production systems	Overall cost	Production time	Scale--up capacity	Product quality	Contamination risk	Storage cost
Bacteria	Low	Short	High	Low	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Low	Moderate
Mammalian cell culture	High	Long	Very low	Very high	Viruses. Prions, Oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very high	Viruses, Prions, Oncogenic DNA	Expensive
Plant cell culture	Medium	Medium	Medium	High	Low risk	Moderate
Transgenic plants	Very low	Long	Very high	High	Low risk	In--expensive

PURIFICATION OF THERAPEUTIC PROTEINS

Therapeutic proteins which are obtained from different sources are needed to purify to remove proteinaceous and non-proteinaceous contaminants without affecting their biological activities. Nearly all therapeutic proteins have to be isolated from proteinaceous substances. Thus, most common impurities in therapeutic proteins are various proteinaceous substances. Protein impurities can cause various allergic reactions or make the therapeutic effects different from the desired effects. A slight difference between a recombinant protein and its endogenous counterpart can elicit an adverse immune response, which may be harmful. There are several methods for purification of these proteins. They are extraction, precipitation and differential solubilization, ultracentrifugation and chromatographic methods viz, assize exclusion chromatography, ion exchange chromatography, affinity, metal binding, immunoaffinity chromatography, hydrophobic interaction chromatography, HPLC and buffer exchanges. Purified proteins are concentrated by lyophilization that simply removes volatile components leaving the protein behind or by ultrafiltration which concentrates a protein solution using selective permeable membranes.[19] Then they are formulated into products. All these procedures are in strict compliance with FDA regulations.[19]

THERAPEUTIC PROTEIN DELIVERY SYSTEMS

Delivery of therapeutic proteins has found an important position in therapeutics. Recent advances in pharmaceutical biotechnology have led to an increase in the number of protein products in the market. As these therapeutic proteins are made available, it will be essential to formulate these drugs to ensure safety, consistency, potency and effectiveness of delivery systems. Despite the attractive features that protein offers, a large majority of them have some serious limitations. The chemical and structural complexities involved demand an effective delivery system in which the physicochemical and biologic properties including molecular size, solubility, stability, light sensitivity, moisture, temperature, biological half-life, immunogenicity, dose requirements, and complex feedback control mechanisms are duly main considerations.[3] Physical instabilities like denaturation, aggregation, precipitation and adsorption onto surfaces etc., and chemical instabilities like oxidation, hydrolysis, deamidation, β-elimination, racemization and disulfide exchange etc., may occur for a given protein, due to the presence of multiple susceptible sites. The most important challenge to formulations of therapeutic proteins into effective dosage forms is to ensure their stability over their shelf-lives. In the gastrointestinal tract (GIT), digestive enzymes normally break down proteins. Even injection may not ensure effective delivery to the target cells. To be effective, many injected drugs need to survive transport through liver and encounters with enzymes. Therefore, compared to the formulation of other drugs, formulation of therapeutic proteins is very critical and, regardless of the route of administration, product development should start with preformulation studies including physicochemical characterization, solubility determination, stability determination under various conditions, isoelectic point determination, pH determination etc. Also, the choice of buffer system, pH of vehicle, solvent selection and preservation of the formulation as well as selection of suitable pharmaceutical excipients, are among the factors that should be considered in the formulation of therapeutic proteins, in order to prevent or minimize the various physical and chemical degradation pathways. Pharmaceutical formulations of therapeutic proteins comprise the preservation of their biological activities with an acceptable shelf-life, effective and safe transport at the site of action. But unfortunately, there is no single strategy to follow in formulating such a product and proteins have to be evaluated on a case-by-case basis.[20]

Invasive protein delivery routes: The most protein pharmaceuticals are usually formulated as solution or suspension and delivered by invasive routes such as intravenous (i. v.), intramuscular (i. m.) and subcutaneous (s. c.) administration, which are not well tolerated by patients usually. Although, the clearance after this particular type of administration of therapeutic proteins may range from a few minutes to several days, most protein has short half-lives in the blood stream. After administration, unwanted deposition may occur, resulting in the need of frequent administration of high doses to obtain therapeutic efficacy.[21-22] Both unwanted distribution and repeated dose administration of therapeutic proteins can lead toxic side effects. Upon subcutaneous injection, protein bioavailability may be as 100%, but also may be much lower, the fate depending on molecular weight, site of injection, muscular activity and pathological conditions.[23] While proteins over 16,000 Daltons can diffuse through the blood endothelial wall entering blood capillaries at the injected site, or enter the lymphatic system and the systemic circulation mainly via, thoracic duct, lower molecular proteins are predominantly absorbed in the systemic circulation via, local blood capillaries.[24] Injectable continuous release systems deliver drugs in controlled predetermined pattern and are particularly appropriate when it is important to avoid large fluctuation in the plasma-drug concentrations.

Non-invasive protein delivery routes: Alternative non-invasive protein delivery routes are currently emerging of greater importance. Among them, mucosal absorption has been rather neglected in advanced drug delivery market, perhaps because of the limitations that will have to be solve in order for these routes to become commercially viable alternatives for the delivery of a large number of biomolecules.[22-23] The mucous surfaces of the body (mouth, eye, nose, rectum and vagina) offer less barrier than the skin or the gastrointestinal tract to the systemic absorption of drug molecules and the advantage of by passing the hepato-gastrointestinal first-pass elimination associated with the oral route. They are ideal for rapid absorption but practical difficulties include the fact that most mucosal sites are no suitable for a long period.[25] For the efficient drug delivery of therapeutic proteins by non-invasive routes, in particular via, gastrointestinal tract (GIT), novel concepts are needed are needed to overcome significant enzymatic and diffusion barriers. In general, the difficulties associated with development of effective oral therapeutic protein delivery are normally ascribed to poor intrinsic permeability across gastric mucosal membrane due to the hydrophobic nature and large molecular size, susceptibility to enzymatic attack, rapid post-absorption clearance and chemical instability. To overcome this problem, a variety of permeation enhancers including mixed bile salts fatty acids micelles, chealators, surfactants, medium chain glycerides etc., have received considerable attention in an attempt to increase the absorption of proteins. Nevertheless, nasal, ophthalmic, buccal, transdermal, pulmonary, rectal and vaginal routes have been extensively studied for therapeutic protein delivery.[24, 26-33] Delivery routes and novel technologies for therapeutic protein formulations are listed in Table 4.

Controlled release formulations show numerous advantages, including protecting the protein molecules over an extended period from both degradation of molecules and elimination, improved patient compliance and also delivering capacity of protein molecules at the site of injection, thereby minimizing systemic exposure to reduce adverse effects. Techniques used for controlled/pulsed release of intact molecules of various therapeutic proteins includes-the use of a pump with a catheter and fixed needle to administer drugs for local or systemic delivery, liposomal dispersions, formulation in amorphous form or as crystals (e.g., insulin) to ensure release over a short period of up to 2 days and hydrogel-based techniques.[21] Some examples of these formulation in pipeline are given in Table 5.

COMMERCIAL SUCCESS

For commercial success of therapeutic protein delivery by various routes like oral, nasal, pulmonary, ocular, rectal and vaginal etc, have been more opportunistic rather than application of platform technologies applicable to every proteins has been employed the use of various polymeric carriers.[34] More success has been achieved by the use of chemical modifications to improve delivery system remain limited.[35] Major hurdles remain in order to overcome the combined natural barriers of drug permeability, drug stability, pharmacodynamics and pharmacokinetics of protein therapeutics.[36]

Table 4: Various delivery routes and novel technologies for therapeutic protein formulations

Therapeutic protein delivery routes		Formulation and devices of therapeutic proteins
Invasive	Direct injections: Intravenous (i. v.), Subcutaneous (s. c.), Intracerebral (i. c.)	Liquids or reconstituted solids, i. v. Injectable liposomes
	Depot systems (s. c. or i. m.)	Biodegradable polymers, liposomes, microspheres, implants
Non--invasive	Oral	Solids, emulsions, microparticles, nanoparticles (with or without absorption enhancers)
	Pulmonary	Liquids or powder formulations, nebulizers, metered dose inhalers, dry powder inhalers
	Nasal	Liquids (usually require permeation enhancers)
	Transdermal	Iontophoresis, electroporation, sonophoresis, transferosomes with permeation enhancers
	Ocular, buccal, vaginal, rectal	Gels, suppositories, bioadhesives, microparticles, nanoparticles

Table 5: Various therapeutic protein delivery systems in pipeline

Therapeutic protein delivery systems	Therapeutic proteins	Potential applications
Intra-nasal delivery	Insulin and other proteins	Diabetes mellitus and other diseases
Inhaled delivery	Insulin	Diabetes mellitus
Powder jet	Prophylactic and therapeutic DNA vaccines	Viral diseases and cancers
Intra jet	Conventional vaccines, insulin and other proteins	Diabetes mellitus and other diseases
Liposomal delivery	Ambisome	Modify pharmacokinetics and reduce toxicity
Targeted delivery	Monoclonal antibodies, tumorotropic ligands for DNA and antisense	Cancers
Gene delivery approaches	Viral vectors	Cancers and gene defective diseases

CONCLUSION

The biotechnological and pharmaceutical scientific community has reached a new stage in the understanding of the production and properties of therapeutic proteins. Production of proteins for therapeutic purposes shows great promise, with some therapeutic proteins in clinical trials and many others under investigation. The delivering various therapeutic proteins through various routs are extremely challenging. Research on production of new therapeutic proteins and also delivery of these new therapeutic proteins with realistic capacity is to be encouraged, and in future they will play a pivotal role in the treatment of various diseases. As we look to what is on the horizon for new protein therapeutic proteins with important therapeutic application will emerge. We expect that with continued support, the medical and pharmaceutical field will be an important beneficiary of therapeutic proteins for years to come

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