INTRODUCTION

Since the last few decades, proteins have emerged as the major class of pharmaceuticals with more than 200 protein-based products currently available in the market, of which 90% are used as therapeutics (1). The market for protein therapeutics is continuously growing and stands at $100 billion in 2012 (Figure 1A). The demand for protein therapeutics is estimated to increase and the pharmaceutical market is expected to grow at a Compounded Annual Growth Rate (CAGR) of 10.94% between 2012 to 2017 (Markets and Markets, 2013) and be worth $168 billion by 2017. The protein engineering market is bolstered by the need for drugs with improved efficiency, specificity, technological capabilities, rise of antibody based drugs and steady growth in the therapeutic market. Monoclonal antibodies (mAbs) is the fastest growing segment in the therapeutic market, though the other segments comprising non-mAb recombinant proteins like Insulin, Erythropoetin (EPO), Interferons (INF), Interleukins (ILs) and Somatotropin (hGH) are also in great demand for therapy (Figure 1B).

All drugs available for therapy maybe broadly classified into two major classes, i.e. protein therapeutics and small molecule drugs. Although, the latter is currently the more pre-dominant therapeutic agent in use, the impact of protein therapeutics is increasing, mainly due to the advances in recombinant DNA technology. Moreover, proteins exhibit high specificity, less immunogenicity and are widely used as therapeutic agents in the treatment of various disorders and diseases. In order to improve their already proven potential in the clinics, second generation recombinants are being developed, each exhibiting improved efficiency and potency. In contrast, small molecule drugs benefit from the ability to reach intracellular targets, oral bioavailability, ease of manufacture, stability over long periods of time and hence have a long shelf-life. However, these drugs (usually less than 1000 Da) have limited surface area available to contact a target protein and in most cases, need the presence of a deep hydrophobic pocket in the target protein for having a favourable interaction, thus limiting the number of potential druggable targets (2). In contrast, protein drugs are large and do not have this limitation, thus making them important tools in human disease therapy, and treatment or management of some crippling diseases such as chronic renal failure, infertility and dwarfism has been made possible (3). Moreover, the higher binding selectivity and specificity of protein therapeutics aids in targeting specific steps in disease pathology, thus revising the treatment paradigm of certain diseases (4, 5) and subsequent supplementation or replacement of small molecule drug therapies.
**Sources, targets and mode of action of Protein Therapeutics**

The human body has an effective and adaptive immune system that works round the clock, fighting off and preventing diseases. However, there are cases when some molecular components are produced either erroneously or in lower amounts leading to disorders or diseases of various types and gravity. Hence, an extrinsic modulation of the immune system by supplementation of natural human proteins or regulators is an important strategy adopted for curing of diseases. Before the discovery and development of recombinant DNA technology, important therapeutic proteins such as human growth hormone (hGH), follicle stimulating hormones (FSH) etc. were purified from the body fluids of healthy individuals and used in therapy of deficient individuals. Currently, many more therapeutic proteins such as recombinant antibodies and antibody fragments, hormones, cytokines, interferons and enzymes of human origin are produced industrially in bacteria, yeast or mammalian expression systems due to improvements made in these protein expression systems.

Any molecule that is connected directly or indirectly with the pathogenesis of a disease is a potential target for protein therapeutics. Protein therapeutic molecules almost always target cell surface receptors or extracellular molecules since they are not able to diffuse and traverse across cell membranes unlike small drug molecules, though more recently, researchers have explored the possibility of directing protein therapeutics to intracellular targets. Protein therapeutics have three different modes of action based on disease pathology. Firstly, if the disease is caused by unwanted extracellular molecules such as cell metabolites or cell lysate, enzyme therapeutics can be used to degrade these targets. Secondly, if the disease is caused by a deficiency in certain types of proteins, such as enzymes, protein therapeutics can be used to replace them and thus restore the individual’s good health. Thirdly, if the disease involves improper immune responses or wrongly regulated signalling pathways such as in the case of autoimmune disorders, chronic inflammatory diseases, infectious diseases or cancer; protein therapeutics act as inhibitors or activators of cell surface receptors.

**Engineering effective protein therapeutics**

Protein therapeutics are very important among modern pharmaceuticals, though they remain at an early stage of development and application, and substantial improvement must be made in almost all aspects, including drug target identification, protein engineering and design, protein expression and purification, drug delivery and marketing.

**a) Challenges for Protein therapeutics**

Proteins, by nature have not evolved for use in therapy and therefore lack optimal affinity, specificity, avidity, and/or stability; important characteristics for any therapeutic agent for successful disease treatment or management. Protein instability and immunogenicity are the most important challenges the success of protein therapeutics face. Proteins have limited physical and chemical stability, resulting in low half-life in the body, hence limited efficacy and increased frequency of drug dosage. Secondly, they are more difficult to produce, have shorter shelf lives, cannot be administered orally since they are quickly digested in the intestines and so always need to be administered as injections, thus affecting patient compliance, therapeutic outcomes and the need to comply to stricter FDA laws for production of parenteral drugs, all of which leads to increased cost of pharmaceutical commercialization.

**b) Strategies for designing Effective protein therapeutics**

To address these issues in protein therapeutics, various protein engineering and design strategies have been developed, which maybe classified based upon the technology used for the improvement of the efficacy of the therapeutic protein, i.e. Protein fusions (Albumin, Fc or Transferrin), Glycosylation, Sequence modification and humanization, PEGylation, use of display technologies (Phage, Bacterial or Yeast) and Genetic engineering.

Pharmacokinetics involves the determination of the fate of substances administered externally to a living organism and the efficacy of a therapeutic protein in the human body can be improved by a number of protein design strategies including fusions, glycosylation and chemical modification. **Fusion** of the protein of interest to an endogenous human protein was first demonstrated by CD4-Fc. Since then, several fusions have been tried and the most commonly used fusions are Human serum albumin (HSA), Fc fragment of antibodies and transferrin. Protein fusions increase the effective size of the protein, reduces renal clearance and in the case of fusion to Fc, protects the protein from degradation by the “recycling” system in the lysosome, by allowing its release into the plasma (due to the interaction between Fc and FeRn). Glycosylation involves the addition of carbohydrates to the protein surface, thus increasing protein size and reducing renal clearance, but in addition also plays a role in enhancement of protein solubility, protection of the protein from damage by heat, free radicals, proteolysis and immune surveillance; ultimately resulting in increased serum half-life. Finally, chemical modification by methods such as PEGylation, which involves the conjugation of polyethylene glycol (PEG) to the protein to increase the hydrodynamic volume and hence retention in serum, can endow the protein with brand new capabilities and increase its clinical potential. The most clinically and commercially successful protein conjugates so far have been with PEG with at least 8 PEGylated proteins already approved for human therapy since it is possible to tune the half life of proteins by varying of the number of PEG molecules, their size and extent of branching. Also, PEGylation is also known to reduce the immunogenicity of some proteins. A major drawback of this system though, is the impairment of protein function, especially in the case of low molecular weight proteins due to site-specific PEGylation and/or tailoring of the PEG. Secondly, PEG is not found in nature, is apparently non-biodegradable, can induce vacuolation in animals and hence, its impact on long-term safety is still unknown.
Reduction of Immunogenicity

In general, reduction of immunogenicity involves alteration of protein therapeutics to avoid immune surveillance. PEGylation as discussed previously, is non-toxic and reduces immunogenicity by shielding the immunoreactive sites on the protein from recognition by antibodies or surface receptors (13), enhances solubility thus preventing aggregate formation, which are highly immunogenic and also deters proteolysis of the PEG-protein conjugate, thus avoiding cleavage of protein into small peptides and subsequent display on MHC class II molecules. Glycosylation interferes with antibody binding (14), but should be of a human pattern for greatest effectiveness (15). mAbs are commercially produced in animal cell lines such as CHO or NS0, have requisite specificity for their therapeutic targets, but do not always trigger the appropriate human effectors systems of complement and Fc receptors in vivo, and the ones that work are acted upon by the patient’s immune system due to the human anti-murine-antibody response (HAMA), thus cutting short the therapeutic window, an exception however is the mAb OKT3, used in prevention of graft rejection. Antibody engineering has been used to generate antibodies with altered Fc regions and hence altered activity, by “Antibody Humanization”, which involves the removal of as much non-human content from the constant and variable regions of the murine and mouse-human chimeric antibodies as possible, thus reducing the immunogenicity.

Genetic Engineering

Genetic engineering strategies are broadly classified into rational design, directed evolution and semi-rational design. These have been extensively utilized for engineering of proteins with altered physical and chemical properties or with novel functions. Rational or computation design methods have been used for improvement of stability and solubility, or to predict and reduce immunogenicity of protein therapeutics (16-18). The primary drawback of this method however, is the need of knowledge regarding protein structure, mechanisms and protein structure – function relationships to a certain extent. In contrast, using Directed evolution, molecular diversity is created at the DNA level in a stochastic manner and so does not have these limitations, but suffers from other key challenges such as: How to find the variant with a desired property in a library of up to a billion variants? which necessitates the requirement of high-throughput selection or screening methods, some of which have been developed for different applications (19). Among the library selection and screening approaches, display technologies are being increasingly used, especially proficient in the engineering of protein therapeutics with improved affinity and specificity. These methods create physical linkage between the genotype and the protein displayed on the platform, so that the library of target protein variants is directly accessible to binding analysis, thus selectable and recoverable for further engineering. Using surface display, in vitro affinity maturation of an antibody generated variants with the highest affinity reported (femtomolar range) (20). Ever since, a number of display platforms have been developed including phage display, cell surface display and cell-free display. Phage display is the earliest and most widely utilized display platform for the affinity maturation of proteins. Recent advances have enabled the selection of phages on more complex biological systems such as cultured cells and in vivo. Peptides and proteins like antibodies have been successfully displayed on phages and epitope mapping carried out, though the display of complex human membrane proteins such as MHC and T cell receptors has had limited success, mainly due to the limitation in protein folding and post translational modifications (which are important for mammalian protein functions) in the bacterial host used for phage propagation.

A cell surface display library (e.g. using bacteria, yeast, insect or mammalian cells) is generated by transforming the cells with DNA variants and screening for mutants with a desired phenotype by fluorescence activated cell sorting (FACS) or magnetic cell sorting (MACS). High-throughput enrichment of positive binders is achieved in a quantitative manner, but cannot be applied to phage display libraries due to the small size of the phage particles (21, 22). Out of the different cells types tested, yeast display and bacterial display have attracted the most attention. While, yeast display has the advantage of possessing eukaryotic post-translational processing pathways (23), enabling the folding and glycosylation of complex human proteins, generation of large libraries is limited due to the low transformation efficiencies found in yeast. On the other hand, gram negative bacteria such as E. coli, have relatively higher transformation effi-

Figure 1. The Global Therapeutic Market. (A) 5 year growth in revenue in the global therapeutic market between 2001 and 2015 (in Billion USD) (B) Segment-wise break up of the global therapeutic market revenues (in %). The monoclonal antibodies is the largest segment (based on the revenue earned)
ciencies and large libraries of about 109 – 1011 are easily obtained, moreover, variants that express well in bacteria can be identified, amplified and the engineered proteins produced in large scale. Bacteria, however, lack eukaryotic post-translational processing pathways, and so their use has been limited to the display of simpler proteins and antibody fragments. Thus, depending upon the complexity of the protein to be displayed, any one of these two systems are used. Cell-free display (also known as “in vitro display”) represents an emerging technology that has proven useful in the discovery and engineering of therapeutic proteins with high affinity (24). Ribosome display, the first cell-free system developed for complete in vitro protein engineering, generated antibodies with higher affinities (picomolar range) than those obtained with phage display (nanomolar range) (25). However, the biggest advantage of cell-free display methods is that the transcription and translation steps take place in vitro, abolishing the need of introduction of DNA into the host cells (which often limits the library size accessible to other display approaches) and hence library sizes usually several orders of magnitude higher than that obtained using other display methods (upto 10^{14} - 10^{15}) are achieved (24).

The cell-free feature of these methods make them more amenable to automation, potentially allowing ultra high throughput identification of new drug targets on a genomic level.

**Examples of Protein Therapeutics**

The protein engineering and design strategies described previously have been applied for engineering the activity, stability, specificity and increased productivity of a wide variety of protein therapeutics.

a) **Monoclonal antibodies (mAbs) and Next generation antibody Therapeutics**

The exquisite specificity, ease of engineering fragments by display technologies and chimerization or humanization of antibodies to enhance stability has made antibodies the most swiftly expanding class of therapeutics for treatment of various human diseases. Monoclonal antibodies have been reviewed extensively and hence will not be discussed here.

b) **Enzyme Therapeutics**

In addition to binding soluble molecule targeting proteins and membrane-bound receptors, enzyme therapeutics also target other molecules in the extracellular environment; a primary example of which is the use of amino acid-degrading enzymes as an anticancer strategy. Unlike healthy cells, rapidly growing tumor cells may be auxotrophic for certain metabolites, whose depletion in the plasma can selectively inhibit tumor growth e.g. lymphoid tumors are auxotrophic for asparagine because they lack asparagine synthetase activity, and recombinant, PEGylated L-asparaginase (Oncaspar®, Enzon) is an FDA-approved drug for treatment of Leukamia (26). A second enzyme, PEG-arginine deiminase (ADI-PEG 20, Phenixi Pharmalogics), is currently undergoing clinical trials for the treatment of the arginine-auxotrophic tumors melanoma (clinical trial phase I/II completed) and hepatocellular carcinoma (clinical trial phase II/III in progress) (27).

A diverse group of solid tumor types including lung, prostate, and bladder tumors are methionine-dependent and PEGylated recombinant methioninase has been studied as a cancer therapy in animal models. Many of these enzymes are immunogenic, likely due to their production in bacterial sources, and as a result PEGylation was needed to reduce immunogenicity and prolong serum half life (26). Finally, PEGylated recombinant human arginase has been proposed as a treatment of arginine-dependent hepatocellular carcinoma resistant to PEG-arginine deiminase and is currently undergoing preclinical study. Enzyme therapeutics have also found use in the treatment of cystic fibrosis, in which patients affected by frequent bacterial infections lead to accumulation and eventual lysis of neutrophils in the lungs, releasing extracellular DNA to form abnormally viscous mucus. Dornase alfa, or recombinant human DNase I (Pulmozyme®, Genentech), is delivered to the lungs as an aerosol and degrades extracellular DNA to improve lung function, quality of life, and prevent aggravation of the disease. Dornase alfa is produced by CHO cell culture, resulting in a glycosylated protein with minimized immunogenicity, but this also contributes to the high cost of the drug.

c) **Protein Hormones**

Some medical conditions characterized by deficiency or complete loss of an endogenous protein can be treated by replacement therapy. The most widely recognized protein therapeutics in this area are the protein hormones, which include insulin and human growth hormone. Millions of individuals worldwide suffer from Type I and Type II diabetes, and many are dependent on injection of insulin, a peptide consisting of one 21-amino-acid chain (A chain) and a separate 31-amino-acid chain (B chain) linked by two disulfide bonds. Extensive molecular engineering of this simple molecule has resulted in numerous short- and long-acting insulin analogs that are used to control meal time glucose spikes and meet the body’s basal insulin need, respectively. These analogs can differ in terms of primary amino-acid sequence or may be chemically modified.

The second well-known protein hormone, recombinant human growth hormone (rhGH), has been available for several decades for the treatment of growth hormone deficiency in children and adults. rhGH also finds use in treating growth failure in children caused by other disorders (28), as well as in treatment of HIV-associated wasting and lipodystrophy. Current manufacturing processes generate two pharmacologically equivalent products: a 191-aminoacid protein identical to the natural human growth hormone or a 192–amino-acid protein possessing an additional N-terminal methionine. The short plasma half-life of rhGH forces daily injection to maximize therapeutic benefit, making compliance an issue, particularly for pediatric patients. Recently, conjugation of human growth hormone to human serum albumin and PEG (29) have been studied as a means to create more stable, longer-acting formulations for less frequent injections. To lower the potential for immunogenicity, analysis was focused on the core of the protein rather than its surface, and resulted in mutants with improved van der Waals interaction, hydrophobic substitutions at polar serine and
higher thermostability may improve the stability of rhGH in the body, thereby increasing its half-life.

d) Coagulation Factors
Management of acquired or genetic loss of coagulation factors is another long-standing field of protein replacement therapy, and transfusion of human plasma–derived coagulation factors is the traditional treatment of bleeding disorders. The sequencing of genes encoding human coagulation factors, as well as advances in molecular biology techniques and mammalian cell culture, has made recombinant production a reality (30). The availability of safe, pathogen-free coagulation factors has also created the opportunity to explore these therapies in “off-label” uses in non-hemophilic patients. For example, while plasma-derived protein C is used to treat inherited protein C deficiency, recombinant activated protein C has been investigated in treatment of sepsis, an often fatal condition that leads to a rapid depletion of protein C and limited protein C activation (31). Reasoning that the ability of activated protein C to reduce mortality in septic patients is related to its anti-inflammatory and anti-apoptotic activity but unrelated to its anticoagulant activity, researchers used site-directed mutagenesis to engineer a mutant protein C with reduced coagulant activity but normal anti-apoptotic activity (32). In the future, this mutant may prove effective at treating activated protein C depletion during sepsis without leading to severe bleeding complications (33).

e) Enzyme Replacement Therapy
Enzyme replacement therapy (ERT), is used to treat the acquired or hereditary loss of an enzyme and several rare genetic diseases, including lysosomal storage disorders have been treated in this manner. In this case, successful therapy is dependent upon delivery of exogenously supplied replacement enzyme to the intracellular lysosome and targeting specific cell types for maximum effectiveness. Gaucher disease ERT (Cerezyme®, Genzyme Corp.) is accomplished by post-translational modification of the replacement enzyme β-Glucocerebrosidase (GCase). Glycosylated GCase is produced in CHO cells, and sugar residues are subsequently removed from the carbohydrate chains of the glycosylated protein to generate a terminal mannose (34). These mannoseterminated glycosylation residues interact with the mannose receptor, which is restricted to the macrophage plasma membrane, to deliver GCase specifically to macrophage lysosomes (35).

CONCLUSION
Medicine is approaching a new era in which approaches to manage diseases are being made at the genetic and proteomic levels. As of today, recombinant human proteins make up the majority of FDA approved biotechnology medicines; and the future potential for these therapies is huge, given the thousands of proteins produced by the body. Protein engineering has been used extensively to improve any issues of immunogenicity and pharmacokinetics that may be present and represents a powerful method for improvement of therapeutics. Given all these developments, it can be safely concluded that protein therapeutics are the future of human therapy.
REFERENCES


