Chapter 8

Noninvasive Strategies for Systemic Delivery of Therapeutic Proteins — Prospects and Challenges

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Additional information is available at the end of the chapter

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Abstract

It is well established that proteins have great physiological importance, thus possessing great potential for therapeutic use. There is increased interest in protein/peptide pharmaceuticals delivery due to recent improvements in analytical methods, advancements in molecular biology and genetic engineering, and a better understanding of regulatory roles of proteins and peptides. There are however major challenges that need to be overcome for systemic delivery of these biological molecules. The major hurdles that contribute to low biological activity are low stability, immunogenicity, and toxicity. A combination of strategies can be used to overcome these challenges and improve the bioavailability of protein drugs. Alternative delivery routes (e.g., nasal and pulmonary) and the development of new methods for overcoming delivery challenges (e.g., nanomedicine, and PEGylation), along with the development of innovative formulation strategies (e.g., spray-freeze drying, supercritical fluid methods, fluidized-bed spray coating, lyophilization, jet milling and spray drying), have resulted in improved pharmacokinetics of protein drugs and in some cases increased patient compliance.

Keywords: Protein drugs, peptides, protein stability, bioavailability, systemic delivery

1. Introduction

Proteins and peptides are polymers of amino acids that differ in chain size. These compounds are an assorted class of biological macromolecules with amino acid sequence characterized by a unique three-dimensional structure [1]. The three-dimensional structure is not only responsible for the biochemical reactions but also useful in feedback mechanism, transport, and solubility in physiological solutions. Proteins have a variety of important physiological roles, which are due to their ability to specifically bind to respective biological counterparts.

Enzymes, hormones, antibodies, globulins, hemoglobin, myoglobin, and numerous lipoproteins are all proteins and peptides involved in catalysis and transport of substances within the body [2].

In 2010, it was reported that around 20 antibody products and 150 protein-based products were approved for use in the US market alone [3]. In that same year, it was reported that >100 approved protein drugs were in use, and ~800 were being developed to treat numerous conditions, including cancer, Alzheimer's, Huntington's, and Parkinson's diseases [4], with worldwide sales figures estimated to be around $70 billion [5]. It is clear that the protein drug class is continuing its strong economic growth, with the global market for bioengineered protein drugs being valued at $151.9 billion in 2013 and expectations that the market could reach $222.7 billion by 2019 (http://www.bccresearch.com/pressroom/bio/global-market-for-bioengineered-protein-drugs-to-reach-$222.7-billion-in-2019). Most of these protein and peptide-based products are still administered by daily or weekly injections [5]. Besides administration route, shelf life imposes a major challenge to the pharmaceutical industry as there are no general rules on how to stabilize protein products and guarantee its safety and activity during the time it is supposed to be marketable [6].

Protein drugs are biopharmaceuticals, which include other biological drugs such as nucleic acid-based drugs, monoclonal antibodies, and recombinant proteins. Although recent advancements in genomics and proteomics have created a large number of protein drug candidates, most fail to be biologically promising *in vivo* [5]. Proteins by nature are prone to denaturation and structural modifications by heat and agitation once in aqueous environments or organic solvent [5].

Major obstacles that contribute to low biological activity are low stability, immunogenicity, and toxicity [7]. A combination of these factors results in low plasma half-life, ranging from minutes to several hours, which necessitates repeated administrations, which in turn leads to higher costs and lower patient compliance [7]. To overcome the challenges that protein drugs present, the development of strategies that focus on improving the bioavailability of the drugs by alternative routes of delivery (parenteral, oral, nasal, pulmonary, etc.) and innovative formulation strategies (spray-freeze drying, supercritical fluid methods, fluidized-bed spray coating, lyophilization, jet milling and spray drying) are trending topics in the protein delivery sphere. The aim of these strategies is to improve protein stability during manufacturing, storage and *in vivo*, following drug administration, allowing the drug to reach the intended biological target. Furthermore, the development of new strategies for overcoming drug delivery challenges such as the use of Nanomedicine and PEGylation can eventually lead to improved pharmacokinetics of some protein drug candidates. In this chapter, we will examine the major challenges facing noninvasive protein drug delivery and strategies for overcoming these problems.

2. Major protein delivery challenges

The use of proteins as therapeutic agents is hampered by their chemical/physical instabilities, low oral bioavailability due to enzymatic degradation in the gastrointestinal tract, low

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permeability across the epithelial cells lining of the small intestine, and rapid elimination from the circulation. In this section, major obstacles to be overcome in order to successfully deliver protein drugs are examined.

2.1. Chemical and physical instabilities

Peptide and protein drugs although generally formulated in solid state still undergo multiple degradative reactions [6]. Chemical degradation involves covalent modification of the primary protein structure via bond cleavage or formation, while physical degradation refers to changes in higher-order structure by denaturation and noncovalent aggregation or precipitation. Physical and chemical types of reactions do not necessarily occur in isolation, but rather one may cause or facilitate the other [7]. Prominent mechanisms of chemical degradation in the solid state include deamidation, oxidation, and the Maillard reaction. Chemical instability due to deamidation has been observed for a number of important proteins, including human growth hormone, recombinant human interleukin-1 receptor antagonists, and recombinant bovine somatotropin [8]. A common deamidation reaction in peptides and proteins in drug formulations is the nonenzymatic intramolecular deamidation reaction of Asn residues. The use of peptide model VYPNGA has led to a better understanding of the Asn deamidation degradative process [8]. By examining the amino acid sequence of a particular peptide or protein with therapeutic potential, residues, which may be susceptible to deamidation, can be identified and formulation decisions made to minimize this type of degradation. The potential for degradative oxidation reactions can be found at various stages of production, packaging, and storage. For instance, peroxide contamination has been found in formulation excipients such as polytethylene glycols and surfactants leading to oxidation of these products [9]. The activation of molecular oxygen to more reactive species requires light or presence of a reducing agent and trace levels of transition metal ions, which can then convert molecular oxygen into more reactive oxidizing species such as superoxide radicals (O2-*), hydroxyl radical (*OH), or hydrogen peroxide (H_2O_2) [9]. Transition metal ions are often present in excipients, and processing in stainless steel equipment can lead to significant iron contamination [9] and thus represent potential sources, which may contribute to degradative oxidation of peptide or protein drugs. After formulation, the final packaging decision can also have an effect on drug stability. It was demonstrated that even low levels of oxygen (1%) in the vial headspace can result in complete oxidation of a particular product [9]. By understanding the potential sources of contaminants leading to oxidation at all stages of drug production, and the mechanisms by which oxidation reactions take place, formulation strategies can be adopted to minimize these events. For instance, in the process of developing the PTH (1-34) microprojection patch for transdermal delivery, oxidation was identified as the major chemical degradation pathway [10]. It was determined that the addition of 0.03% EDTA to the drug formulation could effectively delay oxidation, which was measured using RP-HPLC. Further, stability testing in the presence and absence of antioxidizing agents and other excipients was performed to assess the compatibility of the patch with packaging components. Indeed, several of the patch components were shown to contribute to volatile compounds, which were chemically and/or physically incompatible with the coated PTH (1-34) formulation. Overall, the selection of appropriate packaging materials and the use of a desiccant sachet in the package appeared to

make it possible to achieve the target of 95% PTH (1-34) purity at the end of a two-year shelf life at ambient storage temperature [11]. In some protein formulations, reducing sugars (e.g., fructose, maltose, lactose, glucose, and xylose) react with basic protein residues such as lysine, arginine, asparagine, and glutamine [9]. In a stability study of recombinant human relaxin, it was discovered that the use of glucose as an excipient in a liophilized formulation resulted in covalent adducts of glucose with amino groups on the side chains of the protein formed by Maillard reaction [12]. In this case, switching to the nonreducing sugar (trehalose), or a polyhydric alcohol (mannitol), resulted in greater relaxin stability.

Protein drugs very often undergo physical changes that may result in change of pharmacological effect and potency. Physical instability involves changes in the three-dimensional conformational integrity of the protein and does not necessarily involve covalent modification. These physical processes include denaturation, aggregation, precipitation, and adsorption to surfaces [8]. Protein drugs may undergo these changes during manufacturing, shipping, storage, and administration. Earlier concerns focused on denaturation (unfolding), oxidation, and deamination of protein drugs. Recently, aggregation has emerged as the main problem with protein therapeutics [11]. Protein aggregation is a multistage process that involves unfolding or misfolding of monomeric units of protein along with one or more assembly steps of monomeric protein to form soluble or insoluble oligomers or higher-molecular-weight aggregates [13]. Protein aggregates are often considered ordered if they occur as long, rigid fibrils or filaments. The most characterized aggregation state is the amyloid fibril, associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [14, 15]. Protein aggregation can be problematic during drug manufacturing, especially if it is insoluble and tends to precipitate, and typically reduces drug stability and shelf life [15]. In worst-case scenarios, aggregation might have an undesirable impact on drug potency, pharmacokinetics, and immunogenicity [16]. Protein aggregation can be triggered by many factors such as shear stress, high temperatures, changes in pH, and high protein concentration [16]. Unfortunately, it is very difficult to predict if the protein drug will undergo aggregation because of the complexity of the mechanism involved in the aggregate formation.

2.2. Biopharmaceutical challenges

Therapeutic activity of proteins is highly dependent on their conformational structure, which is flexible and sensitive to external conditions such as pH, temperature, and impurities in the excipients [15]. The detection of structural changes in protein is complex in pharmaceutical formulations and is a major challenge for the development and quality of protein drugs [16]. In addition, protein-based drugs have been shown to be immunogenic and in some cases the production of neutralizing antibodies have led to the inhibition of the therapeutic effect [16].

Until recently, injections (i.e., intravenous, intramuscular, or subcutaneous routes) remained the most common means for administering protein drugs. However, the bioavailability of protein drugs using these routes are low due to their rapid elimination from the circulation through renal filtration, enzymatic degradation and uptake by the reticuloendothelial system [17]. Oral dosage is the most attractive route of administration because of the accompanying

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decreased medical costs and improved patient compliance. However, oral administration of protein drugs results in even lower bioavailability [2]. The main reasons for the low oral bioavailability are the instability of these drugs in the low pH environment of the gastrointestinal tract as well as inactivation and digestion by proteolytic enzymes in the intestinal lumen. In addition, protein drugs have high molecular masses and are hydrophilic, with logP values <0. This means that these drugs are more difficult to transport via the paracellular route across the epithelial cells lining of the small intestine [2, 16].

3. Strategies for mitigating delivery challenges

3.1. Processing strategies for improving protein stability

Despite their many attractive features, protein drugs come with many complications with regards to formulation. Unlike their small molecule drug counterparts, they are often complex molecules with secondary, tertiary, and quaternary structures and generally contain side chains with various chemical properties. The disruption of this complexity can lead to loss of function of the drug. Thus, the formulation of these biological compounds for therapeutic use must maintain their shape and form thus maintaining their activity [2].

3.1.1. Spray-freeze drying

Spray-freeze drying was first introduced in 1994, and it was at first classified as a variant of dry milling [18]. Since pharmacological therapeutics have limited stability in liquid solutions, several methods, such as spray-freeze drying and spray drying, have been developed for proper removal of liquids such as water. Spray-freezing involves spraying a solution containing the macromolecule of interest into a vessel containing cryogenic liquid such as oxygen, nitrogen, or argon, which results in freezing of the droplets due to the low boiling point of the cryogenic fluids used [19]. Spray-freeze drying is the sublimation of solid water (ice) following freezing of the solution, and despite its disadvantages, it has become quite an established method in the pharmaceutical industry [20]. The reason for this is quite clear, spray-freeze drying significantly increases the shelf life of pharmaceuticals, it is an extremely sterile process (compared to spray drying), and the product can be readily reconstituted at the time of use. Spray-freeze drying entails three major steps: (1) freezing, the crystallization of water; (2) primary drying, the removal of ice via sublimation; and finally (3) secondary drying, the desorption of residual water from product [19].

Even though this method is used for obtaining protein particles, stresses involved with freezing and drying have been shown to cause irreversible damage to the protein due to degradation, aggregation, and eventual loss of biological activity [19]. Similar to spray drying the majority of the damage occurs in the atomization and freezing of the droplets in the cold vapor phase of the lyophilization process [21].

3.1.2. Supercritical fluid methods

Supercritical fluids (SCF) are used in variety of extraction and analytical methods. They can also be used for the production of pharmaceutical powders, mainly those intended for inhalation such as inhalation steroids dexamethasone, flunisolide, and triamcinolone acetonide [22]. SCFs have properties of gases and liquids at temperatures and pressures above their critical point. Advantages of SCFs include density values resulting in high dissolving power, lower viscosity levels compared to other liquids, and higher diffusivity allowing high mass transfer [19]. Among present SCFs, CO_2 is the most commonly used due to its low critical temperature (31.2°C) and pressure (7.4 MPa); other advantages include nonflammability, nontoxicity, and cost-effectiveness [19].

There are two major principles for particle precipitation when using SCFs. SCFs can be used as (1) solvents (rapid expansion of supercritical solution (RESS) and particles from gas saturated solutions (PGSS)) and (2) antisolvents (gas antisolvent (GAS), aerosol solvent extraction system (ASES), supercritical fluid antisolvent (SAS), precipitation with compressed antisolvent (PCA), and solution enhanced dispersion by supercritical fluids (SEDS)). Since the early 2000s, solutions consisting of proteins along with a cosolvent, such as ethanol, have been precipitated using CO_2 [23]. The integrity of such proteins depends on the operating conditions such as temperature, pressure, flow rates, and the concentration of the ingredients used, and by properly optimizing such conditions, the precipitation of proteins using the SCFs, such as CO_2, has become a promising method for particle precipitation [24].

3.1.3. Fluidized-bed spray coating

Fluidized-bed spray coating is a commonly used method in the pharmaceutical industry for coating of small particles [25]. Coating of particles has several advantages, such as providing acid resistance [24], modifying the release of the particles [26], protecting the therapeutic agent from light, and moisture and masking the taste of a substance [27]. In this process, particles to be coated come in contact with droplets of coating solution after being fluidized via air currents and are then dried using heated air; thus, after several loops through the spraying and drying zones, a uniform coat is achieved [28]. Further, the uniformity and success of the coating is dependent on the spreading of the droplet on the surface of the particle [29].

As a result of recent advancements in fluidized-bed spray coating systems, particles as small as 50 μm can be coated. For instance, fluidized-bed spray coating was applied to recombinant human deoxyribonuclease (rhDNase), and the coating was examined via scanning electron microscopy [30]. The coating demonstrated strong integrity when introduced to mechanical force; however, the process resulted in aggregation of the proteins,, which may be mainly due to the thermal stress involved with the process [29]. Aggregation was significantly reduced however, when rhDNase was formulated with calcium ions, thus suggesting in presence of proper stabilizers fluidized-bed spray coating is a feasible method for coating and preparing dried pharmaceutical proteins [31].

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3.1.4. Lyophilization

Protein instability is one of the major reasons protein drugs are still administered via injections and not orally. Therefore, in order to overcome this hurdle, proteins must be made into solid forms to achieve an acceptable shelf life [32]. Lyophilization has become one of the most commonly used methods for generating solid protein drugs [33]. Lyophilization consists of three steps beginning with (1) freezing, where the solution that is to be dried is frozen at a controlled rate thus removing water from the protein as ice crystals, at this point ~20% of water remains in a unfrozen phase known as maximal freeze concentrate [31]. Freezing is followed by (2) primary drying, where frozen water is removed via sublimation resulting in significant desorption of water (~10%). The remaining water is removed at higher temperatures in the final stage, (3) secondary drying, via desorption which lowers the fluid moisture content to few tenth of a percent [34].

A variety of stresses caused by lyophilization, such as low temperatures, formation of dendritic ice crystals, pH changes, phase separations, increase in ionic strengths, and removal of protein hydration shell, can result in reversible [33] and irreversible [34] structural changes in the protein [33]. Thus, for proteins that are sensitive to the listed stresses, specific cryo-/lyo-protectants can be used, such as commonly used sugars/polyols, nonaqueous solvents, polymers, protein itself, surfactants, and amino acids [33]. Further, extra effort is made to fully customize the lyophilization cycles in order to avoid lyophilization cycle-related stresses such as freezing rate and temperature, thermal treatment conditions, drying rate and temperature, and final moisture content [33].

3.1.5. Jet milling

Jet milling is a method used for particle size reduction using interparticle collisions and abrasion to produce particles ranging between 1 and 20 μm [19]. Although Jet milling is a great method for particles size reduction (1–20 μm), it does come with some drawbacks, such as lack of control over size, shape, surface properties, and morphology. Furthermore, the high energy input can be detrimental to proteins as it can lead to protein degradation [19]. In a study where horseradish peroxidase was coprecipitated with carbomer and jet milled for two different time points, it was demonstrated that the longer the jet milling process, the more significant the reduction in protein activity [35]. In fact, grinding the power for 10 min in a mortar almost completely eliminated the activity of peroxidase [36] However, pulmonary activity of salmon calcitonin along with a variety of absorption enhancers (oleic acid, lecithin, citric acid, taurocholic acid, dimethyl-beta-cyclodextrin, and octyl-beta-D-glucoside) micronized after freeze drying with lactose showed significant blood levels of calcitonin in rats [37]. Further, it has been suggested that microparticle preparation is possible via melting, pregrinding, and a final jet milling step for particle size reduction [36]. Lastly, as peptides fed into the machine have to be coarse enough to allow for free flow and fine enough not to block the hopper and pipe work, they are at times required to be lyophilized which can result in protein degradation if no lyoprotectant is used [36].

3.1.6. Spray drying

Spray drying is a method used for forming protein particles in lower molecular range [38]. The solution is automatically fed into droplets that rapidly dry due to high surface area and large amount of air–water interfacial area. During the drying process, which can range from 100 milliseconds to seconds, the critical increase in temperature is prevented by the evaporation of the solvent, which results in the temperature of particles remaining significantly lower than the temperature of the gas and drying powder is quickly removed from the drying zone to prevent overheating [25]. Spray drying allows control over a variety of particle design features, including particle size and distribution, surface energy and rugoses, particle density, surface area, porosity, and microviscosity [39]. Since the average radius of particles obtained ranges between 2 and 6 μm, spray drying is generally used for pulmonary particle delivery [40] such as the protein insulin [41].

Although the air-drying process of spray drying prevents thermal degradation of proteins, the atomization process may present a different obstacle. The high shear rate required for the atomization process can lead to degradation of macromolecules. For instance, a study demonstrated degradation of human growth hormone (hGH) during the air–liquid interface following atomization, while the tissue-type plasminogen activator (t-PA) remained intact [42]. There have been several proteins that have been successfully air-dried. Niven et al. spray dried formulations of recombinant human granulocyte colony-stimulating factor (rhG-CSF) [43], and Dalby et al. produced fine protein particles in a process where they combined nebulization, air drying, and electrostatic collection [44].

4. Formulation strategies for overcoming protein delivery challenges

4.1. Nanotechnology approaches

Another promising approach to enhance the stability of therapeutic peptides and proteins is encapsulation into a micro- or nanoparticle, with the aim of protecting the drug from the hostile environments in the body [45]. At their target location, biodrugs typically are released from the particle by diffusion, swelling, erosion, or degradation [46]. Biological systems are usually protected by nanometer-sized barriers, which are extremely specific with regards to transport of biological molecules. Permeation through such barriers and their access to specific biological compartments is dictated by chemical properties, size, and shape of biological molecules [47]. Nanotechnology, defined as development and application of materials, structures, devices, and systems by modeling and manufacturing of the matter in the nanoscale range (1–100 nm), can be used to provide protection against the degreadation of biological agents [48]. As nanoparticles are similar in scale to biological molecules, nanoparticles can have many medical applications and be engineered to have various functions. As their properties allow them to cross biological barriers, nanoparticles can be used to transport therapeutic molecules to sites of interest, providing access to molecules of interest and thus modulation of molecular interactions [48, 49].

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One of the main issues for lack of efficacy for some current therapeutics is the inability to be fully delivered to the required sites, which can be due to their low solubility. Low efficacy may result in increasing dosage, thus correlating with increased side effects [50]. The use of nanocarriers can aid in overcoming some of these obstacles. The surface of nanocarriers usually consists of polymers or biodegradable molecules that are customized to ensure biocompatibility and selective targeting [48]. In fact, nanocarriers may offer numerous advantages over free drugs, such as protecting the drug from premature interaction with unwanted biological entities and degradation, enhancing the absorption of the drug into a specific tissue of interest (cancers or tumors), increasing the pharmacokinetics of the drug, and improving the intracellular penetration of the drug [51].

Nanoparticles and nanocarriers can be used for delivery of peptide drugs to specific sites of interest, avoiding degradation in the GIT and first-pass metabolism via the hepatic route. In fact, peptides have been transported using nanocarriers. Hyaluronic acid–Fe_2O_3 hybrid magnetic nanoparticles were designed to deliver peptides to HEK293 and A529 cells at a 100% level [52]. Further, functionalized gold nanoparticles consisting of a drug peptide ligand and a targeting peptide were shown to be both effective and enhance the activity and selectivity of such peptide multifunctionalized conjugates [53]. Furthermore, carrier molecules have been shown to increase membrane permeability of protein therapeutics such as insulin, interferon α2b, and human growth hormone through epithelial membrane of small intestine thus increasing the bioavailability of listed protein therapeutics [54].

4.2. PEGylation

Proposed in 1970s [55], PEGylation is the attachment of polyethylene glycol to drug molecules as a method of transforming proteins, peptides, small molecules, and oligonucleotides into more potent drugs [56]. PEGylated molecules tend to be more clinically useful compared to their unmodified counterparts as they tend to have higher stability and solubility, longer half-life in the systemic circulation, reduced renal clearance, reduced immunogenicity and antigenicity [57], and higher potency [58].

Protein drugs, such as enzymes, cytokines, and antibodies, have been shown to be significantly improved as a result of PEGylation [59]. Although it is common to see improvements in retention within circulation and reduction of immune response and degradation, the loss of biological activity due to PEGylation is quiet common [60]. The loss of biological activity is however compensated with improved pharmacokinetics as seen in α-interferon Pegasys® [61]. Currently, there are several PEGylated drugs available for public use, including PEG–adenosine deaminase (Adagen®) [62], PEG–asparaginase (Oncaspar®) [63], PEG–interferon α2b (PEG–Intron®) [64], PEG–interferon α2a (Pegasys®) [63], and PEG–growth hormone receptor antagonist (Pegvisomant, Somavert®) [65]. Before PEGylated drugs are made available to public use, they must undergo biological tests to ensure that the advantage of PEGylation does not result in increased toxicity [62].

5. Approaches for decreasing protein aggregation and enhancing bioavailability

Numerous strategies have been developed and applied to control or prevent protein aggregation. Protein aggregation can be reduced by introducing disulphide bonds, salt bridges, and metal ions to stabilize and rigidify regions involved in local unfolding. However, extensive clinical trials will be required to confirm if there are any adverse effects associated with these modifications [15, 16]. A more direct approach is to alter the formulation of the protein. One approach to reduce aggregation is to work with protein solutions at lower concentrations and correspondingly larger volumes. A number of excipients have been used with varying success to reduce protein aggregation; however, each has its own limitations [14]. Nonionic detergents can be used to reduce aggregation induced by shear and heat. Cyclodextrins can also be used to reduce aggregation. For example, cyclodextrins suppress the aggregation of insulin. Another approach to reduce aggregation is to use lyophilized dosage forms [16]. Lyophilization is generally regarded as an effective means to stabilize proteins [13, 14]. However, proteins can undergo reversible conformational changes in the lyophilized state, which makes them more susceptible to undesirable side reactions. For example, the aggregation of lyophilized insulin can be ameliorated by the presence of trace moisture [15, 16].

To improve the oral bioavailability of protein drugs, many strategies have been developed. One strategy involves the modulation of the physiochemical properties of the gastrointestinal tract. This could be achieved by the use of protease inhibitors as an additive to reduce the rate of enzymatic degradation [18]. For example, the enzymatic degradation of insulin in the intestine is known to be mediated by the serine proteases trypsin, α-chymotrypsin, and thiol metalloproteinase insulin degrading enzymes. The use of additives that inhibit these enzymes was found to increase the intestinal absorption of insulin by 10% in rats [66]. Also, the modulation of the tight-junction permeability to increase paracellular transport of protein molecules has been studied. However, this approach requires further investigations before it can be applied [18].

Another strategy to improve protein drug bioavailability involves chemical modification of the protein. This includes the synthesis of a protein analog with an improved enzymatic stability and/or membrane penetration. For example, insulin tends to self-associate to form hexamers. The absorption of hexameric insulin is lower than that of the monomeric insulin analog [2]. Mutation of the amino acids that are involved in self-association results in the formation of the monomeric insulin analog known as insulin lispro (Humalog®, Eli Lilly), which is characterized by rapid onset of action following subcutaneous injection [16]. Chemical modification could also be used to produce a prodrug, which could be useful to protect the drugs against enzymatic degradation [18]. The introduction of novel functional groups to protein drugs that are recognized by transporters can also aid in their absorption. For example, adding a dipeptide that is recognized by peptide-influx transporters in the gastrointestinal tract to a protein drug results in significant improvement in its oral bioavailability [16, 18]. Another way to improve protein stability is acylation of the protein drug, which involves the attachment of fatty acids to the exposed residues on the protein surface. This kind of modifi-

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cation increases the affinity of the protein to the serum albumin resulting in an increase in its half-life. Acetylation of insulin led to the development of insulin detemir (Levemir®, Nova Nordisk), which is a long-acting insulin analog [16]. The conjugation of protein drugs with polymers is one of the approaches used to improve the bioavailability of protein drugs. Currently, poly (ethylene glycol) (PEG) is the most widely used polymer. Protein conjugation with PEG reduces the plasma clearance rate by reducing the metabolic degradation [67].

It is also possible to increase the bioavailability of protein drugs by using pharmaceutical technologies. For example, the use of the mucoadhesive delivery system was found to prolong the residence time of protein drug at the local site of absorption and to increase the concentration gradient between delivery system and intestinal membrane, which ultimately results in a higher rate of drug absorption [18]. In addition, protein delivery systems represent an effective method to effectively deliver protein drugs. The most often used delivery systems include liposomes, micelles, microspheres, and hydrogels. The use of carriers provides a higher degree of protection against enzymatic degradation and other destructive factors because the carrier wall completely isolates drug molecules from the environment [18]. Liposomes possess the most suitable characteristics for protein encapsulation. Encapsulating insulin in liposomes results in enhanced oral absorption of insulin [16], [18]. A combination of these approaches can also be used for the development of a successful [16], [18] approach for the delivery of protein drugs, for example, the multifunctional smart polymer that is equipped with a pH-dependent drug release, Ca^{+2} deprivation ability, and mucoadhesive characteristic. The use of this system to deliver insulin by the oral route results in 10% bioavailability [18].

6. Exploration of various delivery routes

Currently, the most accepted method of delivery of protein/peptide compounds is the parenteral administration of liquid formulations [68]. This method has become widely accepted by the pharmaceutical companies as it is the fastest way toward achieving commercialization [69]. However, as the therapeutic range of protein/peptide compounds increases, so does the demand for improved formulations. Even though designing novel drug delivery systems is not essential for the success of such compounds, they are important for increased efficacy, patient compliance, and reduced errors in drug administration [20]. Thus, along with parenteral administration of protein/peptide compounds, other routes of delivery such as oral, nasal, pulmonary, ocular, and transdermal delivery have been explored [69].

6.1. Parenteral delivery

Parenterally administered protein drugs are most likely to be commercially successful since most animal studies and early clinical trials are performed via direct injections, thus making parenteral drug delivery one of the most popular forms of delivery assuming that injections meet the desired safety and efficacy targets [17]. Drugs delivered parenterally, whether intravenously or intramuscularly, gain full access to the systemic circulation due to rapid drug

absorption. The short half-life of peptide drugs within the bloodstream results in repeat doses which correlate with oscillating drug concentrations within the blood [69].

There are numerous injection devices available for patients. For patients with daily injection requirements such as insulin, there are small diameter needle and syringes available. In fact, to improve patients' quality of life, the patient is now provided with prefilled syringes, syringe injectors, injectors, and pen devices, which may be preloaded with the drug of interest, and autoinjectors are regularly utilized as patients require more flexible and convenient injection devices moving away from the traditional syringe and vial [70]. For instance, insulin is now available in a variety of syringe injectable forms, infusion pumps, jet injectors (a needleless system that transports insulin transcutaneously), and reusable and prefilled pens [70]. All injectable devices must undergo stringent testing to ensure that the patient receives the proper dose of the drug in its expected form. For instance, in needleless systems that depend on high velocity liquid injection, which is dependent on compressed gas, the sheer stress can compromise the configuration of the protein [71, 72].

The rapid absorption of parenterally delivered drug is quickly followed by a rapid decline in the drug levels in the systemic circulation, which can be problematic in cases of chronic conditions where daily or weekly injections may be required for years. The requirement for numerous injections can result in decreased patient compliance, thus resulting in the development of prolonged release parenteral drug delivery systems [71]. These drug delivery systems include use of implants, which are biodegradable drug delivery systems mainly composed of either polylactic acid (PLA) or polyglycolic acid (PLGA) to control the release of the therapeutic drug [73]. However, since implants often require surgery and have been shown to have poor content uniformity in lower drug doses, in situ microparticle systems have been shown to be more advantageous due to the presence of an external oil phase [74]. ISM showed comparable drug release profile to drug release of microparticles prepared by solvent evaporation method, thus potentially becoming an alternative to more complicated microencapsulation methods [75].

6.2. Oral delivery

One of the major challenges for protein drug delivery via the oral route is the susceptibility of the protein drug to proteolytic degradation in the gastrointestinal tract (GIT), which is at its highest in the stomach and the duodenum of the small intestines and is significantly lower in the mouth, pharynx, esophagus, ileum, and the colon. Another challenge is the bioavailability of the drug, which is dependent on two major factors, the molecular weight (MW) of the drug and its solubility. Bioavailability is essentially independent of MW for drugs less than 700 Da; however, with increasing MW passed this threshold, there is a decrease in the bioavailability of the drug. Drug compounds also need to meet a certain level of hydrophobicity criteria as they are required cross biological membranes. However, most biological drug compounds considered for therapeutic use are frequently greater than 700 Da and are hydrophilic [76]. Thus, degradation and poor absorption are the main bioavailability barriers. There are however suggested methods for increasing the survival of the peptides as it moves through the GIT [75].

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There are current methods for increasing the bioavailability of peptidic drugs such as (1) modifying the N- or C-terminus to increase half-life (encephalin, conversion of C-terminal methionine to methioniol group [77]); (2) altering the terminal amino acids from L-amino acid to a D-amino acid (arginine-vasopressin analog 1-deamino-8-D-arginine [77, 78]); (3) converting the linear peptide into a cyclic analog to avoid degradation by carboxy- and amino-peptidases (successfully performed in a model hexapeptide [77]); (4) use of peptidomimetics, which are molecules that mimic the action of peptides but are no longer peptidic in nature and prodrugs, which can be metabolized in the body releasing the therapeutic agent in return; and (5) coadministration of the peptide drug with digestive enzyme inhibitors [79]. The GIT presents both physical and chemical challenges for oral delivery of protein drugs; however, recent developments have made progress in facing each of those challenges and obstacles. Even though the development of new formulation methods, which have shown to improve bioavailability of protein drugs, are still costly, the progress in this field is still quite promising [20].

6.3. Nasal delivery

The use of the nasal cavity as a site for systemic peptide drug delivery has several benefits, such as (1) rapid absorption rate, which in some cases have been shown to be as effective as intravenous injections and require lower doses; (2) high permeability due to the nasal epithelium (up to 1 kDa) [80]; along with (3) high total blood flow; (4) avoidance of first-pass metabolism; and (5) accessibility. The combination of such characteristics allows for a faster onset of pharmacological activity and lower side effects [81]. The olfactory nerves also allow for the direct transport of drug to the brain as the blood–brain barrier provides a challenging obstacle for numerous drugs such as antibiotics, antineoplastic agents, and other drugs that are active in the central nervous system [82].

There are a variety of formulation factors that can affect the absorption of drugs within the nasal cavity. Such factors include dose and volume, pH, osmolarity of the solution which the drug is dissolved in, viscosity, excipients used (such as absorption enhancers), dosage form (spray, powder, or drops), administration techniques (inhalation or mechanically assisted), and devices used to administer the drug [83]. Further, despite the fact that nasally administered drugs are able to avoid first-metabolism, bioavailability of peptide drugs is still limited due to the presence of broad range of metabolic enzymes that reside in the nasal mucosal cavity and the epithelial cell lining [84].

Currently, drugs ranging from small to large macromolecules such as protein drugs, hormones, and vaccines are delivered through the nasal cavity [85]. For instance, in the presence of absorption enhancers, which aid in modulating the nasal epithelium permeability, insulin can be effectively administered through the nasal cavity [84].

6.4. Pulmonary delivery

The large surface area of the lung along with its well vascularized thin epithelial lining provides a noninvasive method for drug delivery, direct access to systemic circulation, and

allows for avoidance of first pass metabolism and GIT degradation [86]. It has been suggested that to achieve successful pulmonary delivery of drug, the drug must reach the alveoli or deep lung; thus, the particle size must range within 1–2 μm for optimum absorption [87]. The inhalation of therapeutics is an effective means for providing therapies for respiratory [88] and a wide range of other disorders [89].

Even though animal studies have shown the bioavailability of drugs delivered via the pulmonary route ranging from ~10% to 50% depending on the type of protein, similar success rates have not been seen in human studies [70]. For instance, pulmonary insulin delivery demonstrated just below 50% bioavailability in animals [90]; bioavailability in humans is merely 10–15% [70]. These findings suggest that the bioavailability of a drug in animals is not necessarily predictive of that of a human. Another obstacle associated with pulmonary delivery is the clinical toxicology of the lungs, particularly when dealing with cytokines and growth factors that may have a local effect on the tissue [70]. The particle size of the drug also plays a critical role in its bioavailability in the lungs [91]. As previously stated, the smaller the particle size, the deeper it will penetrate the lung thus the higher the bioavailability. However, for larger molecular drugs, patients may need to use different dosages and multiple administrations to achieve the desired therapeutic effect [70]. Another challenge for pulmonary delivery of protein drugs is the rapid increase in levels of the drug in the serum, which may not be problematic in cases where the drug has a large margin of safety [89]. Although, this may be desirable for instances where fast delivery of a drug is required, such as insulin preceding a meal, it can result in unwanted side effects and will require more doses to achieve and maintain the required serum levels. However, new technologies, such as rapid-acting, slow-release analogs of drugs, such as insulin, have been developed to allow for an improved pulmonary peptide drug delivery [91].

7. Concluding remarks

In order to develop an analog of a protein drug, which can be deemed, therapeutically viable there are a variety of factors that need to be considered. A logical first step would be to gain a full understanding of physiological and chemical barriers, which the protein drug will encounter. Although this field is only a few decades old, significant progress has been made in many areas. There has been an increase in understanding of the advantages and disadvantages of different routes of protein drug delivery. Thus, with proper modifications, such as modifications of the N- or C- terminus, alteration of terminal amino acids, converting the conformation of protein from linear to a circular form, use of peptidomimetics, enzyme inhibitors, or PEGylation, protein drugs can be engineered based on the existing knowledge of proteolytic enzymes, which the protein drug will face once within the systemic circulation. Furthermore, recent advancements in the field of nanomedicine has allowed for the encapsulation of proteins resulting in increased bioavailability. Modifications and new developments in formulation technologies have also allowed for the potential production of protein drugs, which were originally thought to be uneconomical for large-scale production. Proper formulation of the drug product is dependent on choosing the most stable form of the protein drug

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and fully understanding the chemical and physical properties and the stability of the compound in varying conditions in short and long-term studies.

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