Current Status and Perspectives of Oral Therapeutic Protein and Peptide Formulations: A Review

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Authors’ contributions

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ABSTRACT

Medicinal formulations have evolved from the use of small molecules that act by blocking various receptors. On the contrary, therapeutic proteins are a class of medicines that have gained increased popularity owing to its low toxicity, high stability and exquisite specificity. Oral delivery of protein drugs is a very interesting but a highly challenging area of medicine that requires advancements in terms of bioavailability of oral drugs. The main objective of the present review is to provide a systematic overview of the various physiological barriers of delivery of therapeutic proteins and novel approaches available in this field in order to counter these physiological barriers. Advances in terms of inhibitors of proteases, permeation enhancers, mucoadhesives, short peptide conjugates, particulate delivery system including nanoparticles. Oral therapeutic proteins face

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challenges with regard to oral bioavailability, stability of the protein and reproducibility. Among the various strategies, a co-administration of permeation enhancers with protease inhibitors have proven most effective, while particulate delivery system is still under clinical studies in order to be establishes as a method. Overall, a thorough and focused research with sufficient knowledge on the structure-function relationship, substrate specificity and physiological parameters can deliver a potent therapeutic protein with high efficiency.

**Fig. 1. Graphical abstract**

**Keywords:** Oral therapeutic proteins; protein formulation; bioavailability; barriers; physicochemical properties; oral delivery.

1. **INTRODUCTION**

Virtually all the drugs marketed by the pharmaceutical industry comprises of small molecules with a size <900 D. These drugs are targeted to act on different protein molecules that are either enzymes or receptors involved in various pathways of metabolism and signalling resulting in the desired effect [1,2]. Yet, advances in protein chemistry have resulted in the use of recombinant DNA technology and solid-phase peptide synthesis for the production of protein-based therapeutic agents [3,4]. In this regard, insulin is regarded as the first commercially available protein with an U.S. FDA approval obtained from the recombinant DNA technology [5]. This identification of this protein-based therapeutic agent has revolutionized the field of therapeutic proteins with increasing focus on the identification of enumerable proteins for the treatment of various disorders [6]. Varying degree of success has been attained in the synthesis of proteins such as enzymes, physiological regulatory proteins, recombinant hormones, interferons, interleukins, hematopoietic growth factors, tumor necrosis factors, blood-clotting factors, thrombolytic drugs for the treatment of various disorders [7]. This also applies to bioactive peptides, which have been concerned as one of the most effective therapeutic agents alongside proteins [8]. Apart from animal and bacterial source, these can also be obtained by plant sources [9]. These therapeutic agents are better accepted primarily because they do not merely block the action of their target proteins but treat the condition itself [10]. In addition, these proteins achieve greater specificity over small molecular drugs primarily because of their structural similarity to the target proteins. Furthermore, these proteins mimic the naturally existing ones thus being less
immunogenic with lower adverse effects [6,7].

Unlike small molecular drugs, therapeutic proteins are highly specific to their targets as they primarily target the key molecules of the disease pathogenesis while ensuring to maintain the immune response exerted by the body [11,12]. In order to do so, these proteins interact with the cell surface receptors or extracellular components as they do not have the ability to cross the cell membranes [13]. In this regard, recent advances have exploited the possibility of directing therapeutic proteins to intracellular targets by crossing the membrane barriers [14]. Their primary action is exerted by either restoration of deficient components or degradation of specific extracellular molecules [15]. It also has the ability to inhibit the extracellular receptors thus inactivating further signal transduction [16]. Overall, based on their therapeutic efficiency, the proteins can be selected as drug molecules for delivery through different mode of administration [17]. In reality, any protein encoded by the human genome cannot be repurposed as a therapeutic component. The foremost requirement of any protein to be used as a drug is its safety. Proteins administered in the form of drugs can elicit immunogenic responses similar to that observed during the pathogen entry to the body. These responses not only reduce the efficacy of the administered drugs but also may be fatal resulting in severe conditions [18]. Hence, careful assessment of the immunogenicity of therapeutic proteins is essential. Furthermore, proteins as such lack physical and chemical stability that results in a short half-life in vivo thereby rendering it difficult to be used as therapeutic components. They are easily digestible in the intestine thus affecting its bioavailability [19].

With these complexities, the route of administration is critical for ensuring optimal delivery of therapeutic proteins as drug molecules. In this regard, intravenous, intraperitoneal and intramuscular administrations are commonly followed. Yet, oral administration is the most widely accepted method as it is less painful [20]. The production of such drugs is often less expensive and easy to store as it does not require specialized equipments for its storage. Despite its wide acceptance, their development is complicated because of their high molecular weight, low stability under physiological conditions, low permeability to the intracellular targets and shorter half-life [17,21].

On these lines, an ideal oral therapeutic protein should have efficacy along with protective ability against varying pH and proteolytic damage, and ensure optimal absorption from the intestine to release its action through the blood stream [22]. The stability and functionality of the therapeutic proteins can be improved by performing certain chemical modifications with a thorough assessment of their pharmacokinetic and pharmacodynamic properties for it to be represented as oral therapeutic proteins [23]. Some of the popular therapeutic proteins/peptides are listed in Table 1.

With this background, the present review aims to provide a insight on the various factors responsible for the formulation of proteins for their therapeutic potential for its usage as an oral therapeutic protein.

| Name of the drug               | Application                                      |
|-------------------------------|-------------------------------------------------|
| Angiotensin-2 (ACE-2) inhibitor | Reduction of blood pressure, cardiovascular diseases |
| Bradykinin                    | Vasodilation                                    |
| Cholecystokinin               | Suppress appetite                               |
| B-endorphin                   | Relieves pain                                   |
| Bursin                        | Selective B cell differentiating hormone         |
| Interferons                   | Enhance activity of killer cell                  |
| Gastrin inhibitor             | Reduce secretion of gastric acid                 |
| Pancreatitin                  | Digestion enhancer                              |
| Human Growth Stimulant        | Treats dwarfism                                 |
| Insulin                       | Treats diabetes mellitus                        |
| Vasopressin                   | Treats diabetes insipidus                       |
2. METHODOLOGY

A thorough literature survey was conducted using electronic databases including Google Scholar, PubMed, SpringerLink, Wiley-Blackwell, and Web of Science. An extensive number of studies published in peer reviewed journals like Progress in Biomaterials, International Journal of Pharmaceutics, Scientific Reports, Nature and many others were collected. Authors performed the search using keywords including “Oral therapeutic proteins”, “hurdles in oral therapeutic proteins”, “characterization of oral therapeutic proteins” and “methods for the production of oral therapeutic proteins”, which resulted in the gathering of much literature. From around 5780 identified studies, a total of 140 studies were retained on completion of the different levels of screening. Among them, based on repetitive results some of the studies were eliminated.

3. EMPHASIS ON THE IMPORTANCE OF PHYSICOCHEMICAL PROPERTIES

Formulation of any biological molecules is essential for their optimal delivery to the target site. As such, this formulation is a crucial step for the pharmaceutical companies but formulation of protein-based drugs is more complex owing to their chemical nature [24]. Therefore, a thorough understanding of the critical points regarding its physicochemical properties is essential in order to optimize its efficacy. Among them, the most important factor that is responsible for a protein activity is its 3D conformation that ensures the physiological property of the protein. Proteins remain stable as long as the physiological parameters such as pH, temperature and salt concentrations are maintained. Thus, the formulation should ensure to protect the protein through minute changes that occur during the processing of oral drugs [25]. Any changes in these conditions result in physical instabilities resulting in the making or breaking of the interactions between different amino acids to maintain structural integrity causing a denaturation [22].

Furthermore, proteins demonstrate aqueous solubility depending of various factors such as pH, temperature, isoelectric point, presence of metallic ions and hydrophobicity that are measured using modern analytical methods such as X-ray diffraction, dynamic light scattering, size exclusion chromatography, mass spectroscopy (MS), circular dichroism spectroscopy, nuclear magnetic resonance spectroscopy, and Fourier transform infrared (FTIR) spectroscopy [26]. A formulation of therapeutic proteins should consider the fact that phenomena such as association, aggregation and hydrogen bonding between amino acids occur that likely change the properties of that protein [27]. In addition, covalent interactions such as glycosylation and non-covalent interactions such as hydrophobic and ionic interactions, hydrogen bonding and others contribute to the stability of the protein and therefore should be carefully reviewed during the formulation [28]. Biological factors such as its interaction with the substrate or receptor for the formation of a complex should be considered in order to ensure optimal bioavailability of the therapeutic drug. These parameters are assessed using the pharmacokinetic profiles of the protein in terms of absorption, distribution, metabolism and excretion (ADME) studies carried out using Mass spectroscopy-based methods [23]. Apart from ADME parameters, structure-activity relationship also is crucial for the formulation. In order to address the challenges towards ensuring stability of proteins, the ideal way could be its use in a native form [29]. However, use of native proteins is often associated with minute contaminants caused from degraded or misfolded components occurring primarily due to stringent purification process. It is highly challenging to maintain structural integrity of the native protein as it is passed through various steps of pharmacological processing in order to render it as a druggable molecule [30]. As per USFDA guidelines, the stability for biological products is defined as “the capacity of a drug product to remain within the established product specifications, to assure its identity, potency, quality and purity”. Therefore, an effective formulation should consider the various aspects of protein degradation that can hinder in the optimal activity of the drug [31].

4. PHYSICOCHEMICAL PROPERTIES ASSESSMENT

Various stress exerted on therapeutic proteins should be well understood in order to determine methods for its stabilization during the pre-formulation stage. They should assess the structure and conformational stability, molecular weight, aggregation and solubility of the protein that provides an insight on the various hurdles that may arise during the drug delivery, optimal route of administration, protective groups that can be added in order to prevent degradation and denaturation [32,33]. One of the critical issues of engineering therapeutic proteins is the
labile amino acids that hamper the stability of the protein. These residues can align the binding site pocketsuch that the native protein attains its active 3D structure [34]. Excipients and covalent modifications that are possible in order to enhance the stability of the protein should be assessed by various analytical and bioinformatic methods. Instrumentations such as UV–visible spectroscopy, fluorescence measurements, circular dichroism (CD), FTIR spectroscopy are used for the assessment of influence of excipients on the protein stability whereas differential scanning calorimetry is used for thermal stability assessment [26]. The microenvironment of the protein is determined by the UV absorption range between 250-320 nm that primarily originate because of the presence of aromatic amino acids. Further, a shift in this absorbance represents a change in the 3D conformation of the protein thus determining a perturbation in its active structure. Furthermore, absorbance at 330 nm is indicative of a fully unfolded protein that suggests a possibility of protein aggregation [35]. Therefore, various aspects of the stability of protein can be well explained by mere spectroscopic analysis of the protein under UV range.

Similarly, fluorescence spectroscopy reveals the presence of tryptophan and tyrosine. The information on the environment of these amino acids in the tertiary structure of a protein and changes in these environments when the structure is perturbed are also provided by the intrinsic fluorescence. The indole side chain of tryptophan is highly sensitive to polarity of the solvent and the resulting microenvironment. Any changes in this microenvironment results in a corresponding change in its fluorescence that contributes a great deal of information about the kinetics of protein refolding. This refolding that is essentially carried out using guanidine hydrochloride solution can be followed using fluorescence spectroscopy [36]. Furthermore, circular dichorism (CD) spectroscopy provides information on the secondary structure of the protein and its related changes. For samples that do not show absorbance in UV spectroscopy can be clearly assessed using CD as this method assesses the electric and magnetic dipole moments of the sample. While peptide bonds itself show measurable CD signals, secondary structures such as α-helices, β-sheets, turns, and disordered structures produce different and measurable magnetic dipole moments that are detectable using CD spectroscopy. The secondary structures have specific absorption, for instance, α-helices show two distinct minima at 208 and 222 nm and maxima at 192 nm whereas β-sheets show a broad minimum at 217 nm and a maximum at 193 nm. Likewise, CD at a range of 200-240 nm is known to provide a remarkable amount of information about the conformational changes in the protein structure. During the step of formulation, CD is expected to provide important information about the quantification and content of the secondary structure of the protein. For example, the microenvironment created by phenylalanine, tyrosine, and tryptophan residues is indicated by the ellipticity of the bands in the ranges 250–260, 270–280, and 290–300 nm and disappear or reduce once the protein unfolds. Similarly, a weak or no CD bands are represented by molten globule or a partially folded protein [37].

IR spectra are also known to provide more accurate information over that of UV and CD spectroscopy. The amide I-IR is clearly shown by an absorption at 1600-1700 cm-1 that estimate β-sheets and turns whereas information on the α-helix content of a protein still relies on UV-CD spectroscopy over IR [38]. Formulation of therapeutic proteins also requires its assessment using FTIR spectroscopy that provides information of the content of secondary structures and protein folding. It takes an edge over all the other methods mainly because of its ability to monitor samples present in different state such as sample in solution, dried or lyophilized samples [39]. This method also provides information of the thermal aggregation of proteins in D2O solution that has pharmaceutical significance. A strong band at 1618 cm-1 and a weak band at 1685 cm-1 represents this aggregation and assigned to the intermolecular formation of β-sheets [40]. Further information of aggregation is provided by the use of methods such as size exclusion chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and analytical ultracentrifugation [41].

5. TECHNOLOGICAL ADVANCES IN PROTEIN FORMULATION

One of the key factors responsible for an optimal activity of the therapeutic agents is the route of delivery. Although the most commonly employed administration is through subcutaneous, intramuscular and intravenous systems, oral drugs are the most widely accepted as they are less painful during administration [19,20,22]. Advancement in technology has led to the association of certain microparticles with proteins
Therapeutic proteins are delivered via subcutaneous and intramuscular injections, whereas oral delivery systems are often least preferred. In this section, a careful review of the various strategies for formulation of therapeutic proteins for its delivery through oral system is discussed.

An overview of the criteria for formulation development is depicted in the Fig. 2. The various resources that are the prerequisite for formulation and development are discussed herein. The foremost step in the protein formulation is the purification step from the bulk extract with information on the impurity and degradation from component mixtures. Ingredients such as chelating agents, buggers and pH modifiers should be carefully chosen to ensure safety and compatibility with the protein and be approved by the FDA [51,52]. Certification for good manufacturing practices is essential for the manufacturing unit, which

**6. STRATEGIES FOR ORAL PROTEIN FORMULATIONS**

Oral protein delivery system concentrates on strategic enhancement of its bioavailability by enteric coating to eliminate its digestion through the digestive tract enzymes. This has a disadvantage of rendering the protein inactive owing to varying pH through the tract, which is also associated with increased risk of polymerization of the enteric coating material used that leads to inability of the protein to be released at its target site [50]. Protease inhibitors and absorption enhancer molecules are sometimes co administered with the oral therapeutic proteins in order to overcome these constraints. Attempts to alter the membrane permeability, designing particulate delivery system comprising of microparticles, liposomes or nanoparticles and use of mucoadhesive polymers with the goal of increasing time spent at the target site are few of the other strategies to improve the delivery of oral therapeutic proteins [19,20].
Fig. 2. Criteria followed for protein formulation development

| Purified protein | Representation of manufacturing process; Sufficient production to meet up the dose bracket; formulation variables; stress conditions; complication by impurity |
|------------------|--------------------------------------------------------------------------------------------------|
| Qualified excipients | Pharmaceutically acceptable quality; manufacturing with certified protocols at sufficient scale; specifications on critical impurities; clinical study and commercialization quality achievement |
| Access to finish facility | Capability to sterilize container components; Filling under aseptic environment; head-space purge system; drying equipment |
| Analytical instruments | Structural analyses; concentration determination; chromatographic analyses; electrophoretic assays; other microcharacterizations |
| Stability achievement | Controlled temperature; controlled light exposure; control of relative humidity, devices to provide controlled agitation |

should essentially be equipped with suitable units for keeping dust, fumes and particulate matters at bay [53]. Advanced instrumentation facility comprising of all the high-end instruments such as UV, CD, HPLC (high-performance liquid chromatography), fluorescence spectroscopy, mass spectrometry, and SDS-PAGE for protein analysis should be present in the manufacturing unit [54]. The efficacy of protein formulation is determined by the assessment of its stability conducted using ambient conditions such as refrigerators, agitators, stability chambers and alike. Once the protein is formulated, one of the priority components is the packaging, storage and delivery of the final product that should be carefully planned [55].

7. PHYSICAL BARRIERS OF DRUG DELIVERY SYSTEM

7.1 Gastrointestinal Tract

Oral protein delivery encounters a major hurdle in the gastrointestinal tract because of the release of various GI enzymes. Drugs from the oral route are primarily absorbed through the jejunum. The large surface area of the jejunum facilitates active absorption of the drug molecules [56]. Unless specified, proteins administered through the oral route are absorbed through the same route and come across various hindrances. The systemic entry of the drug molecules is prevented right at the mucosal surface of the GI tract because of the presence of a monolayer of tight junctions attached by tight junctions [57]. A strategically planned approach can achieve great success in the oral delivery system by employing permeation enhancers, modification of physicochemical properties of the proteins, and using particulate systems.

Furthermore, a layer of watery fluid comprising of mucus and glycocalyx runs through the entire surface of the intestine. This is a barrier that restricts the entry of proteins into the epithelial cells. Mucin, a gel-like layer constituting of glycoproteins stabilizes and alters the diffusive nature of the cellular membranes. The ability of the protein to diffuse is dependent on its molecular weight, charge and lipophilicity [58,59].

7.2 Epithelial Cells

Epithelial cells present lining the intestinal lining constitute as the important surface for absorption of drug molecules that occur primarily by active or passive transport and endocytosis. While active transport is an energy-driven process that occurs against the concentration gradient, passive transport occurs along the concentration gradient through simple diffusion and dependent on certain physiological factors such as the degree of ionization, the pH of the lumen and its lipophilicity [60]. Similarly, another mode of transport of molecules across the cell membrane is endocytosis that requires adhesion of molecules to the cell membrane, which is then internalized by the formation of an extended membrane. Passive diffusion and active transport are the major modes of uptake of drug
molecules. Several carrier molecules also facilitate the transport of proteins and peptides across the membrane barriers. In addition, there exists an intestinal proton-driven peptide transport system that is highly specific to the type of peptides. While amino acids are directed transported using passive and active transport mechanisms, short peptide fragments are transported through specific transporters driven by electrochemical gradient [61]. The designed therapeutic proteins should also show properties of lipophilicity because they have to pass through the membrane barriers that are lipophilic in nature. Overall, uptake of these drugs via passive mode is preferable whereas active transport through the paracellular pathway is challenging because programming cells towards this uptake involves a great of engineering [17,61].

7.3. Physiological Barriers to Drug Delivery

Oral proteins encounter several hurdles upon entry to the GI tract. The low pH of the stomach and various degrading enzymes present in the gastric secretions hinder the bioavailability of the oral therapeutic proteins. They may undergo denaturation owing to the low pH of the gastric fluids or be degraded because of the action of proteolytic enzymes of the GI secretion [60]. The extreme pH conditions can lead to the addition or elimination of charges on the 3D structure resulting in a distortion of the structure. Furthermore, enzymes such as trypsin, chymotrypsin, pepsin and various other proteolytic enzymes constitute a major part of the luminal enzymes that are responsible for protein degradation. Jejunum being the major site of absorption for the oral drugs, the presence of these enzymes act as a major barrier for optimal bioavailability of the drugs [62]. Fig. 3 describes an overview of the various barriers in efficient absorption of oral proteins.

8. STRATEGIES TO COUNTER THE HURDLES FOR ORAL DELIVERY OF THERAPEUTIC PROTEINS

Oral therapeutic protein delivery has been a challenging area of research. With technological advances, several modifying aspects have been understood in order to ensure better bioavailability of the drug and increased absorption. They primarily involve the use of physicochemical modifications, enzyme inhibitors, carriers for drug delivery and permeation enhancers [19]. Proteins can be protected from degrading enzymes by the use of coating polymers that facilitate its delivery to the target site. However, care should be taken to choose polymers that do not undergo unrestrained polymerization in course of its shelf-life formulation. The formulations discussed in the subsequent sections should be used individually or in combination to enhance intestinal absorption of the drug [63].

8.1 Enzyme Inhibitors

Peptidase inhibitors are chemical compounds that inhibit the action of these proteolytic enzymes reversibly or irreversibly by the formation of an enzyme inhibitor complex thus blocking the enzyme action. The inhibitors are chosen based on their specificity towards the
target enzyme [64]. While this is one of the crucial steps in the formulation, the selection also depends on factors such as its capacity to reach target site and its ability to compartmentalize at the subcellular level. Several commercially available enzyme inhibitors are in use such as aprotinin, soybean trypsin inhibitor, FK448 and chicken egg white inhibitor (inhibits chymotrypsin and trypsin), bestatin, puromycin, amastatin, boroleucine, and borvaline [31]. Use of pH modifiers has also gained popularity because they are known to modify the active site of the enzyme thereby rendering it inactive [65]. Studies have proven the successful co-administration of insulin with a protease inhibitor in enhancing the bioavailability of insulin. In one of the studies carried out by Yamamoto et al., co-administration of insulin with aprotinin, Na-glycocholate, bacitracin in combination or camostatmesylate individually demonstrated a profound increase in the insulin bioavailability [66]. Yet, there are certain setbacks in the use of enzyme inhibitors such as its requirement in a larger quantity for better activity and higher cost of production in combination. Also, regular use of these compounds leads to toxicity and eventually lowers the intestinal absorption of the therapeutic agent itself [67]. As these inhibitors are required in a large amount, they also hinder with the enzymatic degradation for the normal digestion of food. To address this issue, studies have proven the efficacy of the use of protease inhibitors with permeation enhancers as a unique combination to achieve optimal delivery of the therapeutic proteins to the target site [68,69]. Table 3 details few of the popular enzyme inhibitor drugs along with their targets.

8.2 Advantages of Permeation Enhancers

These molecules are conferred with unique properties like low molecular weight and amphiphilic nature that facilitate the permeation of molecules across the epithelial membrane barriers. The modes of action of these compounds vary depending on the compound used such as membrane disruption, opening of tight junctions, lowering viscosity of the mucosa and enhancing membrane fluidity. These compounds overall facilitate in increasing the intestinal absorption of therapeutic proteins [70]. Permeation enhancers are selected based on their compatibility with the therapeutic proteins and other components of the formulation, nature of the delivery system and knowledge on the dissimilarities in absorption of different therapeutic proteins at different target sites. With these factors in mind, and ideal agent as a permeation enhancer should qualify criteria such as chemically inert, safe for the body, non-toxic and non-allergic [71]. Chelating agents (EDTA [ethylenediaminetetraacetic acid], citric acid, and salicylates), cationic polymers (chitosan and derivatives), bile salts (sodium deoxycholate, sodium taurodeoxycholate, sodium taurocholate, and sodium glycodeoxycholate), anionic polymers (polycrylic acid derivatives and carbapol), Surfactants (ionicsodium lauryl sulfate; nonionic- tween 80, and polysorbite), acyl carnitines(lauroyl-l-carnitine chloride and palmitoyl carnitine chloride), fatty acids (linoleic acid, oleic acid, caprylic acids, mono- and diglycerides), and miscellaneous enhancers (zonula occludens toxin and, 5-(p-chlorophenyl)-l-cysteine (PCP)-cysteine belong to the class of permeation enhancers [31,72]). Surfactants disrupt the intestinal membranes by interfering with the membrane lipids thereby increasing the transcellular permeation. Likewise, bile acids enable increased absorption of the proteins by lowering the intestinal mucus viscosity and blocking the peptidase activity. They also possess the ability to form micelles thereby disrupting the acyl chain in the phospholipids and in turn facilitating protein absorption [73]. Similarly, chelators assist in opening the tight junctions lining between adjacent cells by interacting with the Ca$^{2+}$ ions in the epithelial cells. This modifies the cell structure leading to intracellular space between the cells causing paracellular transport [74]. Use of polymers on the other hand facilitate in adhesion, opening of junctions via ionic interactions or increase permeation [75]. Conversely, fatty acids interact with the Ca$^{2+}$ ions and lead to contraction of actin filaments resulting in paracellular transport [76]. Furthermore, carnitine exerts its action by opening of tight junctions causing a disruption in the membrane integrity and leading to an increased permeation. Several other enhancers are available such as zonula occludens toxin that binds to specific receptors on the cell surface to bring about opening of tight junctions between the enterocytes [77].

Like most other synthetic agents, permeation enhancers also are associated with certain long term effects. Regular use of permeation enhancers leads to toxicity to the epithelium and intestinal membrane erosions and ulcers of the GI tract [70]. In addition, opening of tight junctions for an extended period leads to disrupted permeability causing the entry of various unwanted and harmful molecules into the
cell, which likely leads to further toxicity. Therefore, studies on their chronic toxicity, dosage and frequency of dosing should be formulated before its formulation with the therapeutic proteins [78].

8.3 Physicochemical Modifications

Some of the physicochemical properties of the can be modified to facilitate increased absorption of the therapeutic protein. Such modifications include PEGylation, altering of the amino acids, hydrophobization and a few others.

8.3.1 PEGylation for enhanced bioavailability

PEGylation is the attachment of polyethylene glycol, a biopolymer inert in nature that does not elicit immunogenicity, toxicity and antigenicity. In addition, they possess increased solubility in water and organic solvents, demonstrate better mobility in solution, better clearance from the body and possess GRAS (generally recognized as safe for internal administration) status [79]. Proteins associated with PEG possess several advantages over the native ones including elevated solubility, reduced toxicity, enhanced shelf-life, increased stability and protection against proteolytic degradation. Several PEGylated therapeutic proteins are being marketed [80]. Cimzia®, for the treatment of Crohn’s disease, Macugen®, for the treatment of age-related macular degeneration, Mircera®, for treating anemia due to chronic kidney diseases, and Somavert®, for the treatment of acromegaly are some of the widely used PEGylated proteins. Even for the oral delivery of insulin, PEGylation has been used. Furthermore, PEGylation has been reported to enhance pH and thermal stability of therapeutic proteins [81,82].

8.3.2 Amino acid alterations enhance stability

In nature, d-amino acids are more stable against enzymatic degradation in comparison with that of L-amino acids. Substitution such d-amino acids present greater stability to the therapeutic proteins and are less likely to be degraded during their decent through the GI tract. Luteinizing hormone releasing hormone is one such protein that contains 6, 10 or both positions modified with d-amino acids [83]. Cetrorelix, a decapeptide and an antagonist is prepared after modifying this protein sequence containing d-amino acids. This peptide can survive for up to 50 h when maintained at 37°C while the native peptide can survive for only 2 h [84].

8.3.3 Hydrophobization to alter surface charge

This is an approach that coats the protein with a polymer or surfactant leading to an alteration in the hydrophobicity by changing the surface charge and mucoadhesive properties of the protein. This modification is carried out by incorporating hydrophobic residues to the protein backbone or conjugation of the therapeutic protein with a lipophilic agent in order to elevate its cellular uptake [85]. Conjugation of proteins and peptides with fatty acids such as lauric acid, butyric acid, palmitic acid, esters of carboxylic acid and cholylsarcosine have demonstrated promising improvement in the intestinal permeability of these proteins through the membrane barriers [86].

8.4 Use of Drug Delivery System with Particulate Formulations

Formulations comprising of liposomes, microemulsions, solid-lipid core, nanoparticles, nanosuspensions and microspheres have demonstrated beneficial applications in improvising delivery of the drug to its target site by protecting the active conformation of the protein.

8.4.1 Liposomes

They are the bilayered vesicles which are constituted by aqueous core and a phospholipid surface with a size ranging between 10 and 400 nm. They have the ability to deliver small molecules and extensively used for the delivery of over 13 liposomal formulations approved by the USFDA [87]. Even for larger biomolecules such as proteins and nucleotides liposomal delivery system is beneficial. It is the property of liposomes to form a micelle-like structure in association with the protein that is targeted. The proteins accommodate themselves in the core of the liposomes by aligning the hydrophilic residues in the inner aqueous core that is protected by further by the multiple amphiphilic layers constituted by phospholipids. However, they are entailed with certain limitations such as size of the vesicles vary causing a varying concentration of proteins encapsulated and possible leakage of hydrophilic drugs within the GI passage [88]. In order to overcome these limitations, researchers have exploited the properties of novel liposomal formulations obtained from archeosomal membranes. They are made up of ether lipids and exhibit stability for a gradient pH and against GI fluids. In
practise, use of liposomes with insulin when administered orally showed superior results in terms of stability and efficacy [89,90]. Furthermore, silica nanoparticles coated liposomes have is yet another method that has demonstrated protective ability against coalescence of droplets. They also enhance the stability of oral delivery system [91].

8.4.2 Microemulsions

They are stable formulations comprising of water, oil along with surfactants. They are constituted of oil in water or water in oil forming emulsions, where the oil phase comprises of medium chain fatty acids. They are beneficial for extending good permeability and increase stability of the proteins thereby protecting them against enzymatic degradation [92,93]. In practise, solid-water-oil emulsion system for the delivery of insulin has been successful, which is obtained by coating insulin with surfactants. This formulation was found to enhance insulin absorption through the intestine. A microemulsion-based technology was prepared for proteins and peptides using oil droplets. They have proven efficacy in the delivery of macromolecules, yet they are often associated with some hindrances. They are not useful for long term storage that leads to short shelf-life of drugs associated with microemulsions [94,95]. More recently, self emulsifying technologies have been developed that obviates the use of organic solvents that benefit in withstanding high shear conditions. Cyclosporine is a class of cyclic peptides among which Neoral is a commercially marketed type of self-emulsifying technology [96].

8.4.3 Solid-lipid core particles

This class of protein modifiers are constituted of either nanoparticles or microparticles. They are prepared using lipids that are solids at physiological temperature with an average diameter of 50-100 nm. They are synthesized by double emulsification technique using equipments such as high-pressure homogenizers [97]. Commercially, tripalmitin and PEG stearate are the commonly used solid-lipid nanoparticles particles used as a formulation that benefit in the dissolution of proteins and peptides in aqueous medium [98]. This is followed by encapsulation of the proteins using oil or solubilisation in melted lipids resulting in the formation of hydrophobic complexes. These particles made up of cetyl palmitate have been evaluated for the administration of insulin. On the contrary, lipid microparticles are larger in size obtained from glyceryl monostearate or trimyristin [99]. Insulin conjugated with these microparticles also have shown promising delivery of insulin with an added advantage of slow release of the protein up to 25 h after an initial burst of about 20% [100].

8.4.4 Nanoparticles

Recent technological advances have moved the focus on the use of nanoparticles for drug delivery system. Oral delivery of therapeutic proteins has also exploited the use of nanoparticles for obtaining stable formulations that are capable of targeted drug delivery. Engineering of nanoparticles enable drug delivery to specific sites and guided release of the encapsulated molecule by mere alteration in some of the parameters. Nanoparticles are taken up by the cells via endocytosis including phagocytosis, pinocytosis and receptor-mediated mechanisms [101]. Nanoparticles can be modified on the basis on physicochemical properties of the protein, charge carried on the surface of the target site and others. Nanoparticles are developed by the use of anionic or cationic polymers that elevate protein stability, mucoadhesive properties, survival time in the GI and intestinal permeability [102]. Insulin complexing agent such as diethylene triaminepentaacetic acid was used to conjugate insulin with the nanoparticle. This proved beneficial by opening the tight junctions and preventing proteolytic degradation of the therapeutic proteins [103]. Further, in a study synthesis of nanoparticles using chitosan, γ-glutamic acid, and DTPA resulted in increased insulin absorption through the intestine. Blood glucose levels were effectively maintained for a 24h period with the administration of folate-PEG-PLGA nanoparticles once a day that improved the bioavailability of insulin by two-folds. Use of PLA (poly lactic acid)-PEG nanoparticles for tetanus toxoid, PLGA–polyanhydrides and dextran–alginate–chitosan for insulin, PLGA–chitosan for calcitonin, PLA–cyclodextrin for BSA are also documented for their beneficiary potential [104]. Yet, some of the drawbacks include its scalability, cytotoxicity, long term stability and reproducibility. The most promising advantage of nanoparticles is its ability to be targeted to specific cell organelles such as mitochondria, Golgi's apparatus, cell membrane, lysosomes and others [105].
8.4.5 Microspheres

They are the particles synthesized by double emulsion, spay-drying or double emulsion solvent extraction methods. They are 1-100 μm in size and effective in protecting therapeutic proteins from enzymatic degradation, increase absorption and facilitate targeted delivery [106]. Synthesis of PLGA microspheres associated with cholera toxin has proven beneficial in increasing its absorption. They were assessed for its immunogenicity in specific antibody-secreting cells of mice that demonstrated the production of T cell response from cytotoxic T cells causing immunization in mice [107]. Microspheres are developed using polymeric matrices of chondrotin sulphate/chitosan, which is further developed into ovalbumin-associated microparticles. This enabled the release of 30% ovalbumin in the first 24 h while it further led to a slow release of the protein. Polymeric coacervation method was effective in the development of microsphere-loaded insulin containing mucin and sodium alginate, which revealed promising efficiency in lowering blood glucose levels for over 5 h [108]. Although they appear to be effective, modifications in terms of drug loading efficiency, release pattern and kinetic study will enable a remarkable improvement in the microsphere-mediated protein delivery. Decapeptyl® and Pamorelin® are the commercially available microspheres obtained from Debiopharm useful for the delivery of triptorelin for a period of 3–6 months [109]. Table 2 lists few of the well-known microsphere drug products.

8.4.6 Colon-targeted delivery systems

GI tract facilitates the absorption of molecules from the food, which occurs and an uneven level through the entire surface. The upper GI tract has an elevated degradation of the administered proteins thereby resulting in a restricted absorption. Therefore, to counter this hindrance colon-specific delivery has been developed as an alternative because it bypasses the route through the GI tract. This offers an edge over the conventional drug delivery system because it reduces the exposure to degradation enzymes, shows improved response to permeation enhancers and extends duration in the colon [110,111]. Also, delivery to the colon is beneficial mainly because it promotes absorption by providing a feasible environment unlike the highly acidic pH of the gastric secretions. It can be achieved by the activation of pH- and temperature-dependent microbial flora of the colon [112,113]. It has been successfully established for the delivery of proteins and peptides such as interferons, insulin, vasopressin, glucagon and many others. Materials like Eudragit L100, Eudragit S100, and methacrylic acid copolymers can be used to attach the proteins that help in better drug delivery by maintaining the ambient pH of the colon. This delivery system however demonstrates lower bioavailability of the drug mainly because of the reduced opening of tight junctions that facilitate in absorption. Therefore, release of the protein from the formulation is likely hindered [114].

8.4.7 Mucoadhesive polymer and stimuli responsive hydrogels

They have been in focus for their efficacy in interacting with the mucosal membrane of the intestine that facilitates in extended stay of the drug and in turn improve its absorption. Chitosans, thiolated polymers and carbopol are used to develop these polymers that benefit by increasing the residence period by the drug at the absorption site as well as increase permeation [115]. Insulin associated with chitosan nanoparticles demonstrated improved permeation and regulated blood glucose levels for an extended period of time. In order to protect the peptide, a new technology has been developed that involves the production of stimuli-responsive hydrogels made up of absorptive substances that respond to even minute changes in the pH [104]. They possess high sensitivity to changes in the pH and recognize intestinal pH resulting in the release of the therapeutic protein in the intestine. Optimization of specific polymers to exert sensitivity towards pH, temperature, electrical charge variations and biochemical changes are being exploited to optimize this method of drug delivery as it offers highly promising system [116].

8.4.8 Cell penetrating peptides

They are the amphiphilic peptides that exert its action by an energy-independent translocation of the plasma membrane of eukaryotes. They act as vehicles that transport molecules with the ability of direct penetration through the lipid bilayer [117]. Insulin associated with the cell-penetrating peptides was evaluated in the Caco-2 cell monolayer. This hybrid was capable of transporting the molecule across the membrane
Table 2. Commercially available microsphere drug products [51]

| Name of the drug | Commercial name          | Name of the company       |
|------------------|--------------------------|---------------------------|
| Risperidone      | Risperdal® Consta®       | Alkermes                  |
| Naltrexone       | Vivitril®                | Alkermes                  |
| Leuprolide       | Lupron Depot®            | TAP                       |
| Somatropin       | Nutropin® Depot          | Genentech/Alkermes        |
| Triptorelin      | Trelstar™ Depot          | Pfizer                    |
| Buserelin        | Suprecur® MP             | Sanofi-Aventis            |
| Lanreotide       | Somatuline® LA           | Ipsen-Beafour             |
| Bromocriptine    | Parodel LAR™             | Novartis                  |

Table 3. Enzyme inhibitor drugs [51]

| Enzyme specific inhibitors                                                                 | Enzymes                      |
|-------------------------------------------------------------------------------------------|------------------------------|
| Diazoacetyl DL-norleucine methyl ester; 1,2-epoxy-3(Q-nitrophenoxy) propane; Pestatine     | Acid Protease                |
| Bestain; Baccitracin                                                                       | Aminopeptidases              |
| Chymostatin; N-Tosyl-L-phenylalanine chloromethyl ketone                                   | Chymotrisin                  |
| α2-Macroglobulin                                                                           | Endopeptidase                |
| Phosphoramidon                                                                            | Metaloproteases              |
| Ethylenediamine tetra-acetic acid (EDTA)                                                   | Metaloproteases              |

through transcytosis with a efficiency of eight-fold in terms of intestinal permeability. HIV-1 Tat, oligoarginine, and penetratin are few of such peptides that have been used for the delivery of proteins, peptides and DNA. However, they show toxic effects on the membranes and organelles of the epithelium preventing them from being widely used. Further formulation of these peptides in order to render them less toxic is essential [118].

Yet another advanced drug delivery system involves the use of prodrugs that are pharmacologically inert type of the actual drug produced by certain chemical modifications. The advantage of its use is their ability to transform itself into an active drug after guided enzymatic or non-enzymatic processing inside the biological system. This is one of the most ideal and effective mode of drug delivery as it protects the active molecule from various types of adverse conditions. They also possess the properties of increased permeability, greater shelf life and enhanced stability unlike any other protein conjugates discussed so far [119]. N-hydroxymethyl derivatives of N-acetyl-L-phenylalanine amide are the prodrugs that are synthesized to protect the phenylalanine group from proteolytic cleavage, cyclization of (Leu5)-enkephaline, using phenylpropionic acid is yet another strategy to increase the permeability of drug by over 1680 folds upon evaluation using Caco-2 cell monolayer [120]. Overall, this is the most promising technology for optimized drug delivery. Yet, its use is limited to only a few proteins and peptides. Research should thus be focused on designing such prodrugs for a wide range of therapeutic molecules including proteins and peptides [118].

9. PHARMACOKINETICS OF ORAL THERAPEUTIC PROTEINS

9.1 Absorption

The most widely accepted mode of delivery for oral therapeutic proteins are parental routes namely, intravenous, intramuscular and subcutaneous that is entirely dependent on parameters such as molecular mass, hydrophilicity and gastric degradation [121]. More recently, pulmonary delivery system obtained as aerosol formulations are also widely accepted [48]. For instance, oral mode of administration is considered relatively slower when compared with that of the small molecular drugs [122]. The slower action in this case is because of parameters such as intrinsic factors such as age, sex, body weight, physical activity. Other parameters such as dose concentration also play an important role for optimal absorption [123]. However, there are limited studies that describe the mechanism and pathways for drug absorption through the subcutaneous mode and a lot still needs to be elucidated.

9.2 Distribution

Contrary to the small molecules, efficacy of therapeutic proteins has several limitations owing
to their size causing hindrance with their penetration ability. Furthermore, factors such as shape, charge, binding properties such as its uptake through specific receptors, route of administration and post-translational modifications that contribute to the structural integrity of the protein pose challenges for its distribution. A few studies in the past carried out modeling analysis to assess the binding affinity in terms of molecular size that was established for

Table 4. List of oral proteins that are under consideration

| Name of the protein/peptide | Molecular weight | Description | status | Reference |
|-----------------------------|------------------|-------------|--------|-----------|
| Desmopressin, analogue of vasopressin | 1,069 | Prolonged antidiuretic protein, resistant to degradation by vasopressinase | Clinically approved for patients with central diabetes insipidus and blood clotting disorders | Twarog et al., [128] |
| Insulin | 5,808 | with permeation enhancers like SNAC | Insulin tregopil, an analogue produced by Biocon is available. | Khedar et al., [129] |
| | | with permeation enhancer, soybean trypsin inhibitor and chelator | ORMD-0801 thrice a day reduced blood glucose AUC by 17%. | Eldor et al., [130] |
| Exenatide and semaglutide, glucagon like peptide receptors | 4,114 | Microsphere formulation | Developed by Novo Nordisk is under phase III trial. This is an ongoing study with the title Pioneer. | Suzuki et al., [131] |
| Salmon calcitonin | 3,432 | Enteric coating resistant to acid degradation with citric acid resistant to protease digestion | Reached phase III clinical trials | Binkley et al., [132] |
| Parathyroid hormone | 9,500 | With permeation enhancer 5-CNAC along with citric acid | PTH (1-34) from EnteraBio has passed phase I trials. | Hammerle et al., [133] |
| Somatostatin | 1,019 | Octreotide encapsulated with medium chain fatty acids and sodium caprate as permeation enhancer | Chiasma developed formulation has reached phase III trial | Tuvia et al., [134], Tuvia et al., [135] |
tumor targeting as well as human IL-2 delivery [124]. Oral proteins also encounter barriers by the gastric secretions and therefore several studies have carried out structural modifications of the proteins that act as blockers of degradation. Because of the nature of these molecules, they are likely to elicit immune responses, which are also countered by the use of blockers. In this regard, body perfusion studies to assess the bio-distribution of the oral proteins are carried out using rodent models to evaluate the distribution efficiency [125].

9.3. Metabolism

Oral therapeutic proteins, like any other molecule within the circulation, are eliminated through pathways such as proteolysis, receptor-mediated elimination, non-specific endocytosis and complement fixation through the immune system. After its uptake within the circulation and upon delivery into the cells, these proteins are broken down into peptides and amino acids. They are also capable of undergoing phagocytosis once they are presented on the surface of those cells that uptake such proteins [126]. Overall, metabolism occurs similar to most proteins present in the circulation. Yet, there is inter-patient variations based on the sex, age, weight and physical activity of the patients along with clinical factors such as renal and hepatic state that decides its efficiency to eliminate the proteins.

9.4. Excretion

Protein degradation products are eliminated through the renal route. Low molecular weight proteins can easily be excreted through the glomerular filtration whereas the others are infiltrated based on their molecular size. Those proteins that do not pass through the glomeruli are more often reabsorbed. Several studies carried out using radiolabelled proteins assessed in rodent models demonstrated that the protein degradation products are eliminated in the urine. Furthermore, studies also report the biliary excretion of therapeutic proteins such as insulin and epidermal growth factors [127].

10. PERSPECTIVES AND FUTURE PLAN

Advancements in protein engineering and regulation have led to a corresponding evolution of therapeutic proteins. Table 4 describes a comprehensive detail of various commercial products of oral therapeutic proteins and their status. Existing therapies are being optimized in order to achieve better drug delivery, stability and increased absorption by ensuring enhanced bioavailability of the drug molecule. This can only be achieved by a thorough knowledge in the structure-function relationship, mechanism of action, stability parameters and optimal physicochemical properties. This review has carefully stated the various emerging trends in improving the delivery of therapeutic proteins. Pharmacogenomics study has paved newer avenues in not only understanding the various parameters of a protein drug, it also establishes methods for the synthesis of these proteins using engineering. It is a reasonable anticipation that extensive use of proteins as therapeutic agents will become apparent in the near future. While protein engineering is no longer restricted to only altering the amino acid sequences, care should be taken to ensure that the designed therapy does not cause immunogenicity. There is a great deal of shift from mere synthesizing the protein drug to engineering them for various benefits. Proteins can be encapsulated in a micelle for easy transport across the membranes, conjugated with specific molecules that act as receptors in transport systems, constituted into nanoparticles for a guided delivery or conjugated with mucoadhesives for specific target sites. Overall, each method presents with specific benefit and the choice of upgradation therefore should be careful in order to achieve the required therapeutic effect with minimal adverse effects.

11. CONCLUSION

Technology has evolved a great deal in enhancing the bioavailability of oral therapeutic drugs without being degraded or denatured on its way to the target site. It therefore becomes apparent that use of high-throughput protein engineering has ushered in unprecedented avenues for the development of safer and effective drugs. Yet, these platforms are associated with certain risks, which can be understood in order to address the issues to achieve optimal targeted delivery of oral proteins. The present article provides comprehensive details of the various hurdles encountered in the path of oral therapeutic protein delivery system and discusses strategies to counter these hurdles.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our
area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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