Proteins and Peptides: Non-Invasive Delivery

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INTRODUCTION

Innumerous advances in biotechnology over the past few decades, in particular in the area of recombinant DNA technology (1), have produced a number of protein- and peptide-based (bio)pharmaceutical products approved for the treatment of a large number of human afflictions. As scientific areas such as genomics and proteomics continuously mature in development, additional macromolecules with potential therapeutic applications are likely to be discovered (2), reflecting the growing importance of biotechnology-derived (macro)molecules as therapeutic candidates (3,4). Biosynthetic advances, mainly based on recombinant DNA technology, are enabling the availability of increasingly complex protein molecules including those with multiple specific carbohydrate moieties (4–7). In the same way, solid-phase synthesis is now capable of producing high-purity peptides and macropeptides up to ca. 40 amino acid moieties (8). The ability to engineer specific functional properties of protein macromolecules, such as higher potency, further increases the likelihood of commercial success by reducing both dose requirements and the amount of active (bio)pharmaceutical ingredient.

Notwithstanding the (bio)technological advances leading to the discovery of growing numbers of new protein and peptide molecules, potential candidates for pharmacological applications, the challenges associated with both formulating and administering such types of molecules still remain high (9–11). An assessment of the commercially available protein- and peptide-based biopharmaceutical products shows that the major formulation strategy involves lyophilization, with delivery being effected via subcutaneous, intramuscular, or intravenous injection (12). Whereas the need to reconstitute a lyophilized pharmaceutical product prior to administration is essentially a convenience issue, the subsequent mode of delivery strongly limits product potential in the market because patients do not readily accept injections, especially in the treatment of chronic diseases. Only a few ready-to-use pharmaceutical formulations of proteins and peptides in solution are available in the market and minimize to some extent the inconvenience of dose preparation, but however do not address the invasive nature of administration.

Many carriers of protein and peptide moieties, such as nanoparticles, liposomes, micelles, nanoemulsions and microemulsions have been studied as delivery systems to obtain better therapeutical efficacy (13–16). A complete review on this subject was provided by Moutinho and colleagues (1).

Since it is generally recognized that injection-based delivery methods for biopharmaceuticals are a major impediment to the commercial success of therapeutic proteins and peptides, scientific research in both academia and industry continues to focus on ways to overcome this problem. Indeed, all possible routes of administration have been under scrutiny for the systemic delivery of protein- and peptide-based biopharmaceuticals by non-invasive routes, including nasal, buccal/sublingual, oral, trans-dermal, pulmonary, ocular, vaginal, and rectal. The large surface area associated with most of the aforementioned routes makes them attractive targets for drug delivery (Table 1).

Although biopharmaceutical administration by these routes (Table 1) is considered a more logical and achievable option for non-invasive local treatments, systemic delivery of protein and peptide molecules is significantly more challenging. However, scientific studies designed and conducted to demonstrate the feasibility of systemic delivery of such biopharmaceutical molecules are often carried out in animal models, leaving highly uncertain translation of the findings to human subjects. Some of the scientific research has evolved to evaluation in human patients, but many of the reported successes fail to address how the technology will be transformed into a commercial product. The only remarkable exceptions have been the successful commercialization of nasal formulations for systemic delivery of a limited number of therapeutic peptides, and the quite regulatory approvals of both pulmonary and buccal delivery systems for systemic delivery of insulin. Furthermore, an oral formulation of a small peptide analog (cyclosporin) has been commercialized, but this molecule is not entirely representative of typical proteins and peptides since it contains unnatural amino acid moieties.

Currently, there are more than 885 protein-based pharmaceuticals in clinical studies, with an extensive market potential for protein-based therapies. The impact of biotechnology-derived products on the pharmaceutical

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Table 1 Available options for the non-invasive delivery of both protein and peptide molecules

Administration route	Estimated surface area[a] (m^2)	Level of enzymatic activity (17)
Trans-dermal	1–2	+
Pulmonary	100	+
Buccal	0.01	++
Nasal	0.015	++
Vaginal	0.036	+
Ocular (Area corresponds to human conjunctiva. Pharmaceuticals delivered by this route are also absorbed through inferior nasal and gastrointestinal mucosa) (18)	0.0018	n.a.
Rectal	0.02–0.04	+++
Oral (19)		
Gastrointestinal tract (GIT)	200	
Stomach	0.1–0.2	
Small intestine		+++++++
As a whole	100	
Taking intestinal microvilli into account	4500	
Large intestine	0.5–1.0	

[a]For source details, please refer to references cited throughout the text.

industry can be demonstrated by the fact that ca. 30–40% of new drug approvals are in the "biological category", including proteins and peptides (20).

A demand for conversion of therapeutically active proteins and peptides into widely used medicines is increasing, consequently intensifying demands for improved formulations. Such new formulations need to more adequately address the pharmacological and therapeutic requirements for each particular protein and/or peptide molecule. This not only means to search for alternative routes to a parenteral administration but, crucially, through the development of new formulations to increase the range of therapeutic proteins that can be used clinically.

This chapter examines the current status of non-invasive delivery of protein and peptide biopharmaceuticals, with particular emphasis on technologies that appear to offer a viable solution to overcoming the challenges associated with delivering this class of macromolecules. Factors pertinent to the technological development and the issues influencing potential realization of commercial products based on a given technology are also considered and scattered throughout the text.

NON-INVASIVE (MUCOSAL-BASED) DELIVERY

Lungs, skin and GIT are all in direct contact with the environment. These organs are likely to be a first port of entry for nanomaterials encasing proteins and/or peptides into the body (21). The physicochemical properties of most peptide and/or protein moieties, such as their molecular size, hydrophilicity, susceptibility to proteolytic degradation and lack of absorption, exclude them from being delivered via other potential routes of administration. The notion of non-invasive protein delivery took an extraordinary impulsion with the approval and marketing of Exubera®, a pulmonary insulin formulation that was available from September 2006 to October 2007 in the United States of America (USA). Currently, there are very few marketed non-parenteral protein formulations, including nasal calcitonin spray (Fortical®) and a buccal formulation of insulin (Oralyn®), with a few peptide formulations for either oral or nasal administration (20).

In essence, there are extensive similarities in the barriers presented to non-invasive protein and peptide delivery via oral, pulmonary or intranasal routes, although, depending on the route, the intensities of these obstructions (enzymatic degradation, mucous layer, ciliated cell membrane, as well as the expression of surface receptors on epithelial cells) are dissimilar. The first physiological barrier, a mucociliary clearance, has evolved to effectively remove deposited material, and it is frequently controlled by the use of mucoadhesive materials such as chitosan, polyacrylic acid derivatives or thiolated polymers. These (bio)materials bind non-specifically to the mucus (through formation of ionic bonds, hydrogen bonds, van der Waals and/or hydrophobic interactions), and in this way extend the residence time of the delivery system (20). Second-generation approaches focus on bioadhesion, highly specific and direct interactions with receptors or receptor-like structures on the apical membrane of epithelial cells. These are based on plant lectins, which bind specifically to the sugar moieties of the glycocalyx expressed on the intestinal epithelial cells, which are generally resistant to digestion within the GIT (22).

Bioadhesion phenomena presents new perspectives to trans-mucosal delivery of peptides and proteins, as: **(*i*)** the attachment to cell surfaces is independent of mucus turnover; **(*ii*)** there is a potential for achieving site targeting, and also **(*iii*)** there is a possibility of triggering endocytotic uptake and trans-cytosis by epithelial cells. This approach is being particularly exploited in the design of vaccine formulations (22).

The fundamental step for transport to occur through the mucosal surface is to cross the epithelial cell lining. For large peptide and protein macromolecules, the transport can occur exploiting either paracellular (tight junctions) or trans-cellular routes. The mucosal epithelial cells are connected by tight junctions, with paracellular spaces in the order of 10 and 30–50 Å. This suggests that a "particle" with a radius greater than 15 Å (approximately 3.5 kDa) cannot be transported via this route. Accordingly, the delivery of large peptides and proteins across mucosal epithelia using the paracellular pathway is strictly limited. However, incorporation of the so-called permeation enhancers into protein-based formulations has been shown to increase the permeability of the mucosal epithelium. Permeability enhancement has been reported following utilization of excipients with diverse characteristics, which perform via different mechanisms, including rising membrane fluidity (*e.g.* surfactants), decreasing mucus viscosity (mucolytic agents), and disrupting tight junctions (chitosan, calcium chelators such as EDTA) (23).

Formulations for delivery of peptide and protein biopharmaceuticals across the mucosal surfaces are often based on designing nano-sized carriers (1). These are typically polymeric particles, complexes or liposomes, normally surface-modified/decorated by mucoadhesive moieties and permeability enhancing materials (24). The use of these systems eliminates the need for chemical conjugation and also protects incorporated proteins and peptides from the action of intestinal proteolytic enzymes. Stabilization of the protein and/or peptide moieties can also be correlated with a change in the thermodynamical conditions of the environment surrounding each particle, since the movements of solvent (water) molecules in their microneighbourhood are seriously restricted by the effect of encapsulation, which is in agreement with postulated stabilization mechanisms for protein residues by Ragoonanan and Aksan (25).

OVERVIEW ON GENERAL BARRIERS TO NON-INVASIVE DELIVERY

Although each potential site for non-invasive administration of biopharmaceutical molecules such as peptides and proteins has its own unique features, some general barriers to delivery do exist. Such barriers can be broadly categorized as physicochemical (structural), enzymatic, and cellular (physical). The physicochemical barrier relates specifically to the complex structural properties specific to proteins and peptides. The enzymatic barrier deals with the susceptibility to proteolytic enzymes associated with a given route of delivery, and the cellular barrier refers to the epithelial membrane that must be crossed by the biomolecules for absorption into systemic circulation. All these types of barriers will be detailed below.

Physicochemical Barrier

The unique physicochemical properties of protein and peptide (macro)molecules impart a level of structural complexity not usually encountered with traditional, low-molecular weight pharmaceuticals. To retain the therapeutical effect, the integrity of the primary (linear sequence domain), secondary (local folding domains), tertiary (global folding domain) and quaternary (assembly of multiple subunits, in the case of multimeric proteins) structural elements specific to a given protein or peptide molecule must be properly maintained. Any disruption of these properties can lead to diminished activity or impart serious toxicological, immunological, or pharmacological consequences (10,11,26). Therefore, much of the time and effort invested in developing protein and peptide (bio)pharmaceuticals relates to understanding which factors will negatively impact their native structure, and devising means to control or eliminate potentially deleterious effects.

Protein and peptide molecules can be chemically or physically denaturated by a variety of mechanisms (27). Specific individual aminoacids, or sequences of aminoacids, are subject to chemical transformations such as deamidation, oxidation, covalent cross-linking, disulfide scrambling, and fragmentation, resulting from exposure to extremes of pH and temperature, hydrophobic air interfaces, metal ions, or chemical agents. Physical denaturation pathways involve disruption of higher-order structural domains (e.g., folding - in the case of monomeric proteins) and assembly (in the case of multimeric proteins)) induced by mechanical (agitation or shear) stress, air-liquid (hydrophobic-hydrophilic) interfaces, solvents, pH and/or temperature extremes. Aggregation, precipitation, and surface adsorption, are undesirable consequences of physical denaturation of proteins. The factors involved in manufacturing operations, distribution, storage, and handling, of injection-based protein and peptide therapeutical formulations, in general, can all potentially expose them to conditions that may result in disruption of the native structure of said proteins and peptides. Each of these aspects must be carefully examined during product development, to ensure that the structural integrity of the molecules is maintained. Extensive studies are also being performed to demonstrate that the final biopharmaceutical products have sufficient stability over their shelf-life (typically 1.5–2 years, for commercial products), because time-dependent degradation of the protein or peptide molecules can negatively influence potency, pharmacology, immunogenicity, or toxicology profiles (10,11,26).

The general requirements related to maintenance of both structural integrity and stability will apply to non-invasive delivery systems for proteins and peptides. Formulation strategies, manufacturing processes, and delivery mechanisms, associated with non-invasive systems can include exposure to pH extremes, organic solvents, higher temperature, high shear, severe agitation, air-liquid interfaces, formulation additives (e.g., permeation enhancers) or other unfavorable conditions, all capable of causing chemical or physical denaturation. Many of these situations are not typically encountered (or are completely avoided) with injection-based (invasive) systems for proteins and peptides. Since structural complexity and inherent fragility are common traits of protein and peptide molecules, there is no simple way to overcome the physicochemical barrier to non-invasive delivery. Devising appropriate formulation and processing strategies that both minimize or eliminate any impact to the (fragile) structural properties of said biomolecules is the only way to manage this problem. If a device is required to deliver a formulation, then additional testing and controls are needed to ensure full integrity of the active protein or peptide molecules.

Enzymatic Barrier

Protein and peptide moieties are accessible to enzymatic (proteolytic) action due to the susceptibility of specific amino acid sequences, and such proteolysis is an *in vivo* naturally occurring metabolic process. Degradation pathways generally involve hydrolysis of peptide bonds by a variety of exopeptidases and endopeptidases, and the specific proteolytic enzymes associated with non-invasive routes of administration have been identified in some detail by several researchers (17,28). Enzymatic activity varies largely depending on the delivery route (Table 1). Since a significant portion of the protein intake consumed by humans is assimilated by means of a highly evolved and effective digestive system, it is not surprising that the highest enzyme levels are associated with the oral route. The pulmonary and trans-dermal routes are considered to be relatively low in enzymatic activity, whereas the levels of enzymatic activity in all the other sites are intermediate. It is important to note that proteolytic enzymes are ubiquitous and are present, for example, in the blood, liver, kidneys, and vascular endothelia. Thus, in addition to the site of absorption, protein and peptide molecules may experience the enzymatic barrier en-route to (e.g., hepatic first-pass metabolism following oral absorption) and in systemic circulation.

An obvious strategy to overcome the enzymatic barrier involves co-administration of inhibitors in formulations for non-invasive delivery systems. Some examples described in the scientific literature include (but are not limited to): aprotonin, boroleucine, puromycin, trypsin inhibitor, chymotrypsin inhibitor, ethylenediaminetetraacetic acid (EDTA), phosphinic acid analog, and bestatin (a potent inhibitor of some aminopeptidases) (17,29). Since multiple enzymes are implicated in degradation pathways, combinations of inhibitors are typically required. Furthermore, use of these agents alone will not overcome the cellular barrier. Potential physiological and toxicological consequences associated with the use of these agents must be considered when employing this strategy for the design of non-invasive delivery systems.

Structural modifications and formulation are two additional approaches proposed for overcoming the enzymatic barrier (28). Using recombinant DNA technology or synthetic techniques, selective modifications to the protein or peptide sequence can be introduced, thus effectively reducing proteolytic susceptibility, but these changes must not have a significant impact on the pharmacological properties of the biomolecule (e.g., reduced potency or altered selectivity). Moreover, modifications to address a specific enzymatic action will not eliminate vulnerability to others. The formulation approach essentially involves encapsulation systems to protect the protein or peptide molecules from reactions with enzymes, and selected examples include emulsions, multiple emulsions, liposomes, nanoparticles, or enteric-coated capsules (1,28).

Cellular Barrier

The cellular architecture associated with each non-invasive route of administration represents a formidable physical barrier to the effective absorption of both protein and peptide moieties into systemic circulation, and the large molecular weights and relatively hydrophilic nature of these macromolecules does not favor effective permeation. The mechanism of protein and peptide absorption has been extensively studied by many researchers but, however, the exact details about such process(es) remains poorly understood. A general, but somewhat simplified, representation of the cellular barrier (Fig. 1), is often used to describe pathways for absorption. Potential mechanisms of transport across cells include: passive paracellular, passive transcellular, facilitated transcellular, active carrier-mediated, and trans-cytosis. Although the cellular barrier for each non-invasive delivery route is more complex than what is depicted in Fig. 1, paracellular and trans-cellular transports are typically proposed as likely mechanisms for absorption of protein and peptide molecules.

Much is known about the specific anatomical, histological, and physiological features of each absorption site, and the design of effective delivery systems must take these aspects into consideration so that maximum absorptive potential can be achieved. In most cases, the epithelium is composed of multiple layers of cells, each having specific structural properties (viz. dimensional characteristics, keratin, or cilia). Adding to this complexity is the local physiology connected with the target route. Cellular secretions such as mucus, saliva, or other fluids lining epithelial surfaces, together with the mechanical effects resulting from ciliary action, can add up to minimize residence times of protein and peptide therapeutical molecules at the site of absorption. Other clearance mechanisms may also be present (e.g., macrophages in the lungs), that can effectively eliminate protein or peptide molecules prior to their absorption. Finally, each potential delivery route is part of a dynamic system subject to changes in cellular and molecular organization brought about by normal processes (e.g., menstrual cycle or aging process), or resulting from other factors such as allergies, infections, illness, disease, disorders, and other lifestyle habits (e.g., smoking). A general strategy for overcoming the cellular barrier consists in incorporating permeation enhancers into formulations intended for non-invasive routes of administration (28,29). Table 2 lists some representative permeation enhancers screened for potential use in non-invasive delivery systems of proteins and peptides. Commonly accepted mechanisms of action of said permeation enhancers are also included in Table 2, which typically involve disruption of the cellular architecture and/or molecular interaction with the protein and peptide molecules to facilitate transport. Cell damage and non-specific absorption are key concerns associated with the use of permeation enhancers, together with the impact of these agents on the structural properties of protein and peptide biopharmaceuticals (30).

PHARMACOKINETIC AND PHARMACODYNAMIC CONSIDERATIONS

A threshold level of systemic drug concentration and an optimal duration of exposure (pharmacokinetics, PK) following administration of a protein or peptide biopharmaceutical are

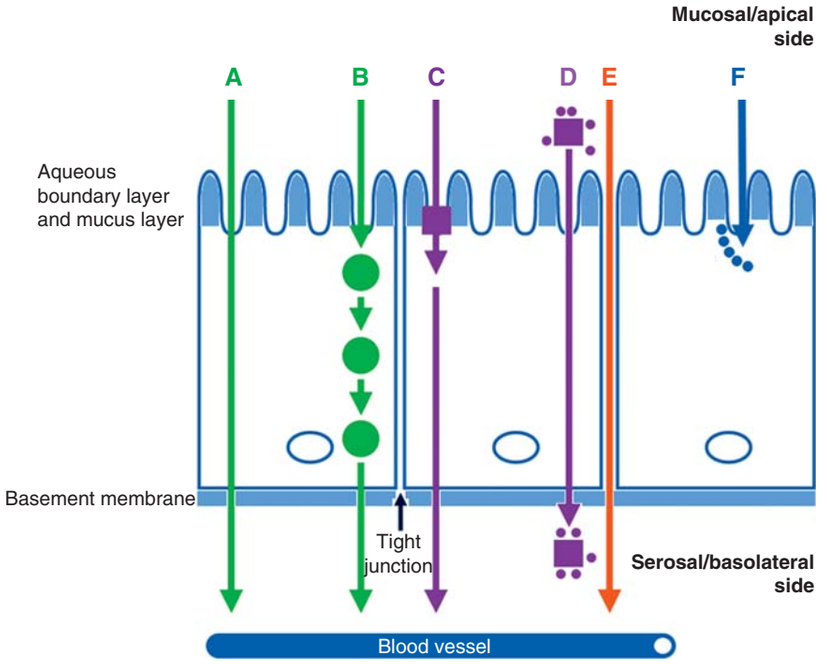

Figure 1 Schematic illustration of potential mechanisms of transport across the cellular barrier. (**A**) passive transcellular, (**B**) via trans-cytotic vesicles; (**C**) active carrier-mediated, (**D**) facilitated transcellular, (**E**) passive paracellular via tight junctions, and (**F**) trans-cytosis.

Table 2 Types of permeation enhancers used in non-invasive delivery systems for protein and peptide molecules (28,29)

Class	Examples	Postulated mechanism of action
Bile salts	Sodium deoxycholate Sodium glycocholate Sodium taurocholate	-Formation of reverse micelles, solubilization of proteins/peptides -Removal of epithelial cells and formation of transient pores -Inhibition of proteolytic activity -Reduction of mucus viscosity
Dihydrofusidates (DHF)	Sodium tauro-DHF Sodium glyco-DHF Sodium phospho-DHF	-Formation of micelles -Inhibition of proteolytic activity -Perturbation of membrane layers
Complexing and chelating agents	Cyclodextrins EDTA Salicylates Citric acid	-Formation of inclusion complexes -Increase in stability -Increase of paracellular transport by (i) removing calcium ions, and (ii) widening of tight junctions
Surfactants	Sodium lauryl sulfate Polyoxyethylene oxide-9 ethers	-Solubilization of proteins/peptides -Perturbation of membrane layers -Extraction of membrane proteins and lipids
Fatty acids and derivatives	Sodium caprylate Sodium caprate Sodium laurate Oleic acid Monoolein	-Increase membrane fluidity by (i) creating disorder in phospholipid domain of the membrane, and (ii) facilitating the leaching of proteins from the membrane

both necessary to produce the desired therapeutical effect (pharmacodynamics, PD). PK and PD parameters must be fully understood in both animal models and human subjects to demonstrate the therapeutical utility of the molecules and therefore enable selection of both dose levels and suitable dosing regimens. In addition, the required exposure must be appropriately balanced against toxicological safety margins and control of any undesired side effects. Thus, both the pharmacology and toxicology of a molecule determine the therapeutical window, which is the ratio between the maximally

tolerated dose and the minimally effective dose, dictating the degree of PK control that needs to be achieved. Depending on the medical condition, either a bolus (rapid onset and elimination), basal (sustained level), or a pulsatile exposure profile of the pharmaceutical agent in systemic circulation may be required. However, achieving a specific profile is often difficult because many factors can influence the PK profile following administration of a protein or peptide biopharmaceutical, including (but not limited to): properties of the biomolecule, formulation conditions, route, and/or site of administration, and natural clearance mechanisms. Evidence also indicates that gender and race differences can further influence the pharmacological response to therapeutical agents (31,32). One additional complicating factor associated with non-invasive delivery is the limited availability of validated *in vitro* and animal models that allow the accurate prediction of PK/PD responses in human subjects.

Normally, the strategies devised to modulate the PK profile achieved with injection-based therapy of insulin may not produce the same pharmacological outcome in a non-invasive delivery system. A formulation approach may not be directly transferable to a non-invasive delivery system because additives needed to effect absorption may be incompatible with an existing composition, or the specific requirements to reach the target site may require a totally different design. Moreover, use of an absorption enhancer to overcome the cellular barrier could also facilitate a very rapid transport of the active agent into systemic circulation, resulting in an undesirable burst effect. In the case of insulin analogs, the designed molecular properties may not produce the same PK profile when administered by a non-invasive route due to environmental differences at the site and characteristics associated with absorption. The PK and PD considerations described above are applicable to all protein and peptide moieties with biopharmaceutical applications, and therefore must be carefully evaluated to determine the suitability of a given non-invasive route of administration.

FACTORS INFLUENCING COMMERCIAL FEASIBILITY OF NON-INVASIVE DELIVERY METHODS

The availability of technologies that overcome the general barriers to non-invasive delivery of protein and peptide molecules and address the pharmacological needs associated with a particular therapy, will not necessarily guarantee commercial feasibility. Although advances in biotechnology have enabled production of large amounts of highly purified (recombinant or native) proteins and/or peptides for use as biopharmaceutical entities, there are additional constraints when considering non-invasive delivery systems. Typical values reported for bioavailabilities in most human proof-of-concept studies evaluating non-invasive protein or peptide delivery technologies range from 1% to 10%, suggesting that tremendous increases in annual production may be required to supply a commercial market compared to treatment using a conventional injection-based approach. The absolute amounts of protein/peptide moieties required will further depend on the target disease, size of the patient population, and whether treatment is chronic *vs*. acute. Thus, in addition to the cost of manufacturing, the mere feasibility of producing such large-scale quantities must be thoroughly evaluated. If the technology requires the use of delivery agents, enzyme inhibitors and/or absorption enhancers as part of the formulation, the cost of production associated with these molecular entities must also be considered. In the same way, a technology requiring a specific device to administer a formulation will result in additional manufacturing costs.

With the exception of a few approved products for the nasal administration of peptides, and the very regulatory approvals of delivery systems for both pulmonary and buccal delivery of insulin, there is relatively little precedence with the worldwide regulatory approval process for non-invasive delivery systems incorporating protein or peptide biopharmaceuticals. It is reasonable to expect that delivery systems utilizing chemical agents to affect absorption will require rather comprehensive and lengthy toxicological studies to demonstrate acceptable safety profiles in human subjects, particularly in chronic use situations. Specific details concerning requirements for chemistry, manufacturing, and control data are also lacking at the present time. It can also be anticipated that data covering a broad range of experimental factors, including information acquired under extreme stress (e.g., temperature, relative humidity, and/or mechanical agitation) conditions, will be necessary to demonstrate the overall performance and reliability of the delivery system, especially if a device is involved. Data demonstrating structural integrity of the protein or peptide molecule in the formulation throughout shelf-life and during the in-use period, will likewise be required. Collection of all the necessary data to support a regulatory submission involving a non-invasive delivery system will ultimately add both time and cost to the overall development process.

Assuming that a particular non-invasive protein or peptide delivery system can be developed and can ultimately gain regulatory approval, there are additional factors related to patient and/or healthcare professionals that could ultimately influence whether the technology is successful in the commercial market. Simplicity of design is a key attribute, since physicians or patients will not readily accept highly complex delivery systems that are cumbersome and difficult to use particularly when self-administration is involved in the treatment regimen. There are also inherent disadvantages associated with some of the routes available for non-invasive delivery. For example, patient acceptance of ocular, rectal, or vaginal administration is not anticipated to be very high. Although exploratory studies evaluating the feasibility of ocular, rectal, and vaginal delivery have been reported by several researchers (18,33–35), numerous complicating factors shed some doubt on the commercial viability of delivery systems intended for administration by these routes, except perhaps for local treatment. There are obvious concerns over the potential impact on vision resulting from ocular delivery, particularly in chronic treatment situations. Rectal administration faces significant psychological barriers, hampering patient acceptance, and interruptions resulting from defecation can

diminish effective dose absorption. The vaginal route is gender-specific and sexual activity, menstruation, or pregnancy, can further complicate administration and impact absorption of the biopharmaceutical dose. The buccal/sublingual, nasal, trans-dermal, pulmonary, and oral routes of administration are receiving most of the attention by the scientific community, with some technologies showing promise as potentially feasible commercial products. In the following sections, each of these non-invasive delivery routes will be examined in greater detail.

Buccal/Sublingual/Palatal Administration

The human oral cavity is a readily accessible site for the potential non-invasive delivery of protein and peptide biopharmaceuticals, providing a total internal surface area covering 0.01 m^2, and a highly vascularized mucosa with relatively low enzymatic activity (36–39). Administration via this region also avoids first-pass hepatic metabolism. However, despite these advantages, a multilayered epithelium that is coated with mucus and a constant flow of saliva are major impediments to achieve effective absorption. Permeability of the mucosa in the oral cavity varies, with the non-keratinized buccal (cheeks) and sublingual regions being most permeable and serving as the primary targets for non-invasive delivery systems.

A variety of protein and peptide biopharmaceuticals has been evaluated for buccal/sublingual administration using both animal models and human subjects (37,38). In the research effort by Trapani and colleagues (40), nanoparticles consisting of chitosan and selected cyclodextrins have been prepared and characterized *in vitro* as potential formulations for oral glutathione (GSH) delivery. Their results confirmed that by selecting the most suitable cyclodextrin, it was possible to modulate the physicochemical characteristics of the nanoparticles and their ability to load GSH.

Among the various trans-mucosal sites available, the soft-palatal mucosa was also found to be a most convenient and easily accessible novel site for the delivery of therapeutical agents and achieving systemic delivery as retentive dosage forms, because it has abundant vascularization and a rapid cellular recovery time after exposure to *stress*. The palatal mucosa offers several additional advantages for controlled drug delivery over extended periods of time. The mucosa is well supplied with both vascular and lymphatic drainage and both first-pass metabolism in the liver and pre-systemic elimination in the GIT are avoided. The area is well suited for a retentive device and appears to be acceptable to the patient. With the right dosage form, design, and formulation, the permeability and the local environment of the mucosa can be controlled and manipulated to accommodate drug permeation. Palatal drug delivery is a promising area for continued research aiming at systemic delivery of orally inefficient drugs as well as a feasible and attractive alternative for non-invasive delivery of potent peptide and protein drug molecules (41).

Typical delivery systems evaluated for buccal/sublingual administration of proteins and peptides involve conventional solutions or tablets. Strategies to improve absorption include the use of permeation enhancers and enzyme inhibitors in the formulations, or specific chemical modifications of the biomolecules to increase lipophilicity (37). As stated, a common problem encountered with conventional solutions or tablet dosage forms is the lack of retention at the absorption site due to continuous dilution by salivary flow. Therefore, immobilized bioadhesive delivery systems (tablets or patches) have been developed to extend the residence time at the site of absorption, thus allowing to achieve therapeutical drug levels. Bioadhesion is defined as the attachment of natural or synthetic biocompatible polymers to a biological substrate, and examples of bioadhesive agents include (but are not limited to): hyaluronic acid, chitosan, cellulose derivatives, polyacrylates, gelatin, and agarose (37,38). Conventional solutions or tablet delivery systems rarely result in measurable absorption, with the bioavailabilities being typically much lower than ca. 10%. In general, bioadhesive systems tend to yield higher bioavailability. Gutniak and co-workers (42) evaluated a bioadhesive, biodegradable buccal tablet formulation of glucagon-like peptide amide in human subjects with Type-II diabetes. Although potentially therapeutic levels were demonstrated via such delivery system, the aforementioned authors concluded that further development was required since an extended duration PK profile would be necessary for optimal therapy with this pharmaceutical peptide. The research work developed by Gutniak and co-workers (42) clearly demonstrates that simply achieving effective absorption of a therapeutic agent may not be sufficient to ensure commercial feasibility of a non-invasive delivery system. Departing from typical buccal/sublingual delivery system design strategies, Modi and co-workers (43) described a solution formulation containing insulin and absorption enhancers (Oralin™), administered as a fine mist into the oral cavity using a metered dose inhaler. The results of clinical studies evaluating this buccal delivery system in healthy subjects as well as in patients with Type-I or Type-II diabetes have been reported by several researchers (43,44). In healthy subjects and Type-I diabetes patients, the PK profile obtained with Oralin™ was similar to that observed for subcutaneously administered, rapid-acting, insulin analogs. Late-stage clinical testing of this delivery system still appears to be in progress, and a regulatory approval has been obtained in Ecuador.

A number of questions still remain about the safety and commercial viability of buccal/sublingual delivery systems for protein and peptide biopharmaceuticals. Low bioavailability will be a major impediment to commercialization. The use of permeation enhancers and enzyme inhibitors to address this issue raises concerns about the effects on the oral mucosa, particularly when used in combination with bioadhesive systems, and long-term toxicology data are currently scarce (45). The alternative strategy involving chemical modifications to improve lipophilicity is not generally applicable to all peptides and proteins, and remains largely unproven. Finally, the limited PK data obtained in human studies suggest that buccal/sublingual delivery systems produce a bolus effect that may not be an appropriate pharmacological response for all protein and peptide biopharmaceuticals.

Nasal and Pulmonary Administration

Pulmonary and nasal routes are the other mucosal pathways that are attracting considerable attention as alternative routes for peptide and protein non-invasive systemic delivery since they involve very large surface areas and less intracellular and extracellular enzymatic degradation (46), and thus have demonstrated to be a commercially viable alternative to injection administration as evidenced by the number of products currently on the market, including (but not limited to): calcitonin (3.4 kDa), nafarelin (1.3 kDa), desmopressin (1.18 kDa), oxytocin (1.0 kDa) and buserelin (1.3 kDa).

Nasal Delivery

Since the very beginning, the nasal route has received considerable attention as an ideal site for delivery of both low molecular weight compounds and macromolecules, due to several characteristics, namely a rapid drug absorption, ease of administration, and both patient acceptance and compliance. Advances in administration device technologies together with a greater understanding of formulation and dosage form aspects that enable enhanced systemic absorption, have provided an enhanced driving force for considering the nasal route as an ideal one to deliver biopharmaceuticals (47,48). The nasal mucosa has therefore emerged as a highly therapeutically viable route for systemic drug delivery. In general, among the primary targets for intranasal administration are pharmacologically active compounds with poor stability in gastrointestinal fluids, poor intestinal absorption and/or extensive hepatic first-pass elimination, such as peptides, proteins and polar drugs.

The nasal cavity possesses a rather porous endothelium and a highly vascularized mucosal membrane that provides a large surface area of approximately 0.015 m^2 for absorption. As mentioned previously, nasal administration has the added advantage of avoiding hepatic first-pass metabolism. However, and despite all these advantages, several barriers do exist that need to be successfully overcome to achieve sufficient bioavailability for protein and peptide therapeuticals. Such barriers include: **(i)** a low membrane permeability of the nasal epithelium, that has been shown to display a molecular weight cut off ca. 1 kDa, hence excluding many (bio)pharmaceutical molecules with molecular size greater than 1 kDa (49); **(ii)** a mucociliary clearance mechanism that quickly and effectively renews the mucus layer every 15 - 30 minutes, having therefore a high impact on the contact time between the formulation and the nasal epithelium; and **(iii)** the presence of various peptidases, which cause proteolytic degradation of the protein and peptide molecules. Consequently, the bioavailabilities for nasally administered protein and/or peptide biopharmaceuticals in human subjects are typically quite low, in the range of 1–3% for calcitonin and insulin, for example (50). Nevertheless, the intranasal route for brain targeting is gaining much attention in the scientific community due to the particular anatomical and physiological functions of the nasal cavity. A number of advantages are particularly attractive such as non-invasive rapid systemic absorption, fast onset of action, avoidance of first-pass metabolism in the liver, increasing drug bioavailability, and less systemic side effects (51). The nasal delivery appears therefore to be a favourable way to circumvent the obstacles for blood brain barrier (BBB), allowing the direct delivery of active compounds in the biophase of central nervous system (CNS) (52). Intranasal delivery has been used to target a wide variety of therapeuticals to the CNS, namely neurotrophins (NGF), insulin-like growth factor (IGF-1), neuropeptides (hypocretin-1 and exendin), cytokines (interferon β-1b) and erythropoietin (52–54). Intranasal administration of hexarelin, a growth hormone-releasing neuropeptide for nose-to-brain targeting, was also enhanced by N-tridecyl-beta-D-maltoside as a permeation enhancer. Greater hexarelin concentrations in olfactory bulb and olfactory tract on the treated side of brain tissues were observed in this manner (55). Basic fibroblast growth factor (bFGF) can also be directly delivered into the brain following intranasal administration, imparting protection against brain ischemia/reperfusion in adult rats (56).

When a drug is nasally administered to induce systemic effects or to act into CNS, it needs to pass through the mucus layer and epithelial membrane before reaching the blood stream or pass directly to the CNS. The passage across the epithelium may occur by trans-cellular or paracellular mechanisms, as described before. The first mechanism includes passive diffusion through the interior of the cell and it is especially involved in the transport of lipophilic pharmaceuticals. However, it seems that compounds with a molecular weight higher than 1 kDa, such as peptides and proteins, are trans-cellularly transported by endocytic processes (57). Since bioavailability of nasally administered drugs is particularly restricted by a low drug solubility, rapid enzymatic degradation in nasal cavity, poor membrane penetration and rapid mucociliar clearance (MCC), several approaches have been under study to overcome such limitations, including the use of prodrugs, enzymatic inhibitors, absorption enhancers, development of mucoadhesive delivery systems and new pharmaceutical forms. Common problems associated with low nasal bioavailability of drugs, challenges and possible solutions, can be found in Table 3.

Chitosan is used in several intranasal pharmaceutical forms, including powders, liquids, gels, microparticles and microspheres. For some pharmaceuticals, it is well documented that the addition of chitosan to nasal formulation increases drug bioavailability. One of the most studied pharmaceuticals for nasal non-invasive delivery is insulin. Poly (ethylene glycol)-grafted chitosan (PEG-*g*-chitosan) nanoparticles obtained by ionotropic gelation, administered intranasally to rabbits, enhanced the absorption of insulin by the nasal mucosa to a greater extent than a suspension of insulin-PEG-*g*-chitosan and a control insulin solution (58). Chitosan also enhanced the brain bioavailability of intranasally administered nerve growth factor by a 14-fold increase, when comparing with a preparation without chitosan (59).

Cyclodextrins are used as complexing agents to improve nasal absorption of pharmaceuticals by increasing both drug solubility and stability. Hybrid chitosan-cyclodextrin

Table 3 Common problems associated with low nasal bioavailability of drugs, challenges and possible solutions[a]

Problem	Challenge	Solution
Poor physicochemical properties of drug and/or formulation	Improve physicochemical properties of drug and/or formulation	Pro-drugs Co-solvents Cyclodextrins Pharmaceutical excipients Novel drug formulations
Enzymatic degradation	Reduce drug affinity to nasal enzymes Inhibit nasal enzymes Protect drugs from nasal enzymes	Pro-drugs Enzymatic inhibitors Co-solvents
Low permeability through nasal membrane	Increase drug permeability and dissolution Modify nasal membrane Enhance drug residence time in nasal cavity	Pro-drugs Co-solvents Absorption enhancers Mucoadhesive systems Gelling/Viscosifying agents

[a]For source details, please refer to references cited throughout the text.

nanoparticles have also demonstrated their potential for enhancing the transport of complex molecules across the nasal barrier (60). Hyaluronan is another example of a mucoadhesive polymer used in nasal formulations. It has demonstrated its ability to improve the brain penetration of a hydrophilic peptide via the nasal route (61).

Peptide and/or protein carriers, with average sizes in the nanometer range, such as liposomes and nanoparticles, exhibit some well-defined and delicate characteristics, which have created an attractive and efficient approach for pulmonary delivery of pharmaceuticals. Solid lipid nanoparticles (SLNs), introduced in 1991, represent an alternative carrier system to traditional colloidal carriers (62). Wang and colleagues (63) described that insulin-loaded thiolated chitosan nanoparticles considerably improved absorption of insulin across the nasal mucosa as compared to non-thiolated chitosan nanoparticles. Thiolated chitosan nanoparticles showed a faster swelling and release as compared to plain chitosan nanoparticles, which might facilitate diffusion of the encapsulated drug. *In vivo* evaluations showed that after intranasal administration of the insulin-loaded thiolated nanoparticles to rats, the plasmatic glucose levels of the animals quickly decreased. The glucose levels of these animals were similar to those that received insulin subcutaneously. Novakovic and co-authors (64) published some interesting results, indicating that intranasal delivery to a mouse of [D-Leu-4]OB3, a synthetic peptide amide with leptin-like activity, with Intravail™, was a more effective method of peptide administration than injection methods, and suggested that it may have potential as a novel, non-invasive approach to the treatment of obesity and its associated metabolic dysfunctions in humans. These results were subsequently confirmed by Lee et al. (65).

Matsuo and co-authors (66) investigated the potential of poly(γ-glutamic acid) nanoparticles (γ-PGA NPs) with entrapped antigenic proteins as cancer vaccine carriers, to be intranasally administered, and determined the antitumor effects and associated immune responses in a mouse tumor model. The findings obtained revealed that intranasal administration of tumor-associated antigens (TAA) entrapping γ-PGA NPs elicited a potent tumor immunity based on TAA-specific and long-term cellular immune responses. Intranasal vaccination of antigen-entrapping γ-PGA NPs was actually considered a non-invasive and effective vaccine delivery system.

The primary strategies to improve systemic uptake of protein and peptide biopharmaceuticals via nasal non-invasive delivery include: (*i*) the use of permeation enhancers (50,67) to change the absorptive membrane to increase permeability, (*ii*) the use of excipients/additives to increase retention time of the formulation in the nasal cavity and/or protect the active agent against enzymatic degradation, and (*iii*) modification of protein and/or peptide chemical structure to increase resistance to enzymatic degradation and thus improve absorption. Typical classes of permeation enhancers evaluated by researchers include surface-active agents such as bile salts, fusidic acid, fatty acids, and bile salt-fatty acid mixed micelles that are believed to work by the mechanisms described in Table 2. In the case of insulin, incorporation of permeation enhancers increased bioavailability by 8–15% (68,69). However, potential barriers for clinical use of enhancers include toxicity concerns such as nasal irritation and membrane damaging effects (70). Classical enzyme inhibitors such as bacitracin and bestatin have been evaluated as agents to improve nasal absorption of peptides, but they have the same issue as permeation enhancers in terms of their safety for clinical use.

In order to address the rapid removal of pharmaceuticals due to the mucociliary clearance mechanism, mucoadhesive systems that promote bioadhesive interactions with a mucous membrane and therefore increase retention time by prolonging the contact between the formulation and absorption site have been evaluated as a strategy to enhance systemic absorption following nasal administration (71). Chitosan, a cationic polysaccharide, has been extensively evaluated as a bioadhesive agent to improve nasal absorption of peptides and proteins including calcitonin, insulin, and human growth hormone. In a research effort conducted using normal subjects, a bioavailability of 9–15% was

reported when insulin was administered in a chitosan solution (50). In the case of peptides such as leuprolide and salmon calcitonin, nasal bioavailabilities of approximately 20% have been reported in clinical trials (72). The absorption-enhancing mechanism of chitosan is believed to be due to a combination of mucoadhesive properties and opening of the tight junctions. Chitosan is biocompatible, an important attribute it shares with compounds such as cyclodextrins and phospholipids, and from a practical perspective, it is likely to be safer for human use compared to other permeation enhancers. Formulation and dosage form aspects that can affect the site of deposition within the nasal cavity are a key consideration for nasal delivery. The site of deposition can affect absorption due to the differences in the permeability and residence time of the anterior and posterior portions of the nose. A wide variety of dosage forms such as nasal drops, sprays, gels, powders, or microspheres, are available for administering (bio)pharmaceuticals by the nasal route, and these options need to be carefully evaluated because the type of dosage form can affect where the formulation is deposited in the nasal cavity and, in turn, affect the systemic absorption. For example, the bioavailability of nasally administered desmopressin was significantly increased in a spray formulation compared to drops (73), most likely because the spray formulation delivered the peptide molecules to the posterior region of the nose (as compared to the drops), which is highly rich in ciliated cells and has a larger surface area, thereby contributing to increased absorption. Modulation of viscosity properties via addition of viscosity-enhancing agents has also been shown by several researchers to increase systemic absorption (74). Powder dosage forms can also offer several advantages over solutions for protein and peptide nasal non-invasive delivery. For example, a bioavailability of ca. 10% calcitonin was observed in human subjects following administration as a nasal powder containing microcrystalline cellulose (a cellulosic polymer) (75). The use of cyclodextrins has been also investigated in clinical studies for the nasal delivery of several peptide and protein (bio)pharmaceuticals such as calcitonin, glucagon, recombinant human granulocyte colony stimulating factor (rh-GCSF) and insulin. With an intranasal insulin/dimethyl-β-cyclodextrin powder formulation, average absolute bioavailabilities of ca. 3.4% and ca. 5.1% were obtained in healthy volunteers and patients with diabetes respectively (76). The nasal route can therefore be a viable option for the non-invasive delivery of proteins and peptides. However, the collective knowledge gained indicates that although it may be feasible to deliver smaller peptides with acceptable bioavailability levels, for larger peptide molecules (with more than 20 aminoacid moieties) acceptable systemic absorption typically can only be achieved with the use of permeation enhancers (77). A number of protein and peptide molecules such as insulin, human growth hormone, and human β-interferon are currently in early clinical development for delivery via the nasal route, and the experience gained from these studies and other ongoing research works in this field should eventually help to establish in a near future, whether the nasal route can be successfully used to deliver a broad range of proteins and peptides with acceptable safety and efficacy.

Pulmonary Delivery

Pulmonary drug delivery is attractive for both local and systemic drug delivery as a non-invasive route that provides a large surface area (78), thin epithelial barrier, high blood-flow and avoidance of first-pass metabolism in the liver (79). Murata et al. (80) investigated the feasibility of surface-modified liposomes for the pulmonary delivery of the peptide elcatonin (eCT) (a calcitonin derivative used as an anti-parathyroid agent). In the study by these researchers, chitosan oligosaccharide (oligoCS) and polyvinyl alcohol with a hydrophobic anchor (PVA-R) were used as surface modifiers. The effect of liposomal surface modification on the behavior of the liposomes for pulmonary administration and potential toxicity were both evaluated *in vitro* and *in vivo*. The therapeutical efficacy of eCT after pulmonary administration to rats was significantly enhanced and prolonged for 48 h after separate administration with oligoCS- or PVA-R-modified liposomes. Liposomes modified with oligoCS adhered to lung tissues and caused opening of tight junctions, which in turn enhanced eCT absorption. On the other hand, PVA-R-modified liposomes induced long-term retention of eCT in the lung fluid, leading to sustained absorption. These researchers have concluded that surface modification of liposomes with oligoCS or PVA-R has high potential for the effective peptide drug delivery through pulmonary administration. Poly (lactic-co-glycolic acid) (PLGA) nanospheres coated with chitosan for the pulmonary delivery of eCT are also an example of the advantages of physical modifications to nanocarriers (81). Powder formulations of protein-loaded chitosan nanoparticles suitable for pulmonary non-invasive delivery were prepared by spray drying (82). Amidi et al. (83) prepared N-trimethyl chitosan (TMC) powder formulations using a supercritical CO2 (SC-CO2) drying process for the pulmonary delivery of insulin. The particles had an average aerodynamic diameter of 4 μm, suitable for peripheral pulmonary deposition. After one-year storage at 4 °C, the particle characteristics were maintained and the insulin structure was largely preserved. In a follow-up study, the potential of TMC and dextran powder formulations for the pulmonary delivery of insulin was evaluated in diabetic rats. Pulmonary administration of TMC-insulin microparticles, as compared to dextran-insulin microparticles, significantly enhanced the systemic absorption of insulin, with a bioavailability of ca. 95% relative to subcutaneously administered insulin (84).

Lately, much attention has been centered on the pulmonary non-invasive delivery of GLP-1 (Glucagon-like peptide-1) mimetic peptide, in the treatment of type II diabetes. Qian et al. (85) assessed the feasibility of delivering BMS-686117 (an 11-merGLP-1 receptor peptide agonist) to the lung in rats via intratracheal administration. The PK profiles of three spray-dried, prototype inhaled powder formulations, 80/20 BMS-686117/trehalose (I), 100% BMS-686117 (II), and 20/80 BMS-686117/mannitol (III), as well as a lyophilized BMS-686117 powder, were compared to intravenously and subcutaneously administered peptide. The spray-dried formulations were mostly spherical particles with a narrow particle size distribution

between 2–10 μm, which are better suited for inhalation delivery than the lyophilized, irregular shape powder, with a wide particle size distribution between 2–100 μm. Prototype III exhibited the best physical characteristics and *in vivo* performance, with a bioavailability of ca. 45% relative to subcutaneous administration. The study described by Qian et al. (85) clearly demonstrated that pulmonary delivery is a promising, non-invasive route, for the administration of BMS-686117.

Patton (86) reviewed in detail the specific anatomical, histological, and physiological features associated with the human respiratory tract and the suitability of the lungs as an organ for the systemic delivery of pharmaceuticals. In particular, the lungs are an attractive target for the systemic non-invasive delivery of protein and peptide molecules due to (*i*) avoidance of hepatic first-pass metabolism, (*ii*) a large absorptive surface area of the alveolar region (ca. 100 m^2), (*iii*) a relatively low enzymatic activity, (*iv*) a highly vascularized mucosa and (*v*) a thin epithelial (0.1–0.2 mm) barrier that is not ciliated and is free of mucus. Notwithstanding these advantages, a significant impediment to the pulmonary administration is the physical delivery of the active biopharmaceutical to the site of absorption (deep lung, alveolar region), which involves passing through many bifurcations of the lung airways. Simply achieving the required deposition will not guarantee effective absorption, since the transport process into the systemic circulation involves passage through a series of additional barriers including (but not limited to) (*i*) surfactant layer, (*ii*) epithelial surface lining fluid, (*iii*) epithelial monolayer, (*iv*) interstitium and basement membrane, and (*v*) capillary endothelium. The absorption efficiency can also be significantly reduced by natural phagocytotic (via macrophage) clearance mechanisms designed to eliminate inhaled foreign particles from the lungs. The exact mechanism of absorption is not fully understood, although paracellular and trans-cellular mechanisms have been postulated depending upon molecular weight of the protein or peptide moieties (86).

A pulmonary delivery system for the local administration of the enzyme rhDNase used in the treatment of cystic fibrosis (87) is now commercially available. However, until now, no products for systemic delivery of protein or peptide biopharmaceuticals via the pulmonary route had received regulatory approval. This is partly due to limitations in currently available nebulizers, pressurized metered-dose and dry powder inhaler systems (88), that are either not suitable due to poor formulation stability and inefficiency and/or cannot reproducibly deliver such molecules to the site of absorption to achieve sufficient drug levels in the systemic circulation. Nevertheless, intense research efforts in the area of inhalation administration have produced a greater understanding of the aerodynamic properties of particles that are important for their deep lung deposition (89), resulting in significant developments in protein and peptide pulmonary delivery systems that appear to have overcome some of the deficiencies in current inhaler designs. To achieve effective deposition into the alveolar region, particles containing protein or peptide molecules must possess an appropriate aerodynamic diameter in the range of 1–3 mm (90,91). The aerodynamic diameter can be expressed in simple mathematical terms as the product of a particle geometric diameter multiplied by the square root of its density. Particles with aerodynamic diameters that are too large will become lodged in the nasopharynx region, whereas those that are too small are exhaled. Most developments in inhalation technology are based on optimized formulation and device combinations that are capable of efficiently and reproducibly deliver particles with the correct size to the deep lung region.

Technologies currently under development for delivering protein and peptide biopharmaceuticals involve either liquid formulation or dry powder inhaler systems (92,93). Liquid-based systems atomize solution formulations into fine inhalable droplets via mechanical means through a jet, ultrasonic atomization, or electrospray (94,95). Each delivery system (92) employs particle engineering approaches using various excipients (e.g., aminoacids, carbohydrates, and/or phospholipids) included in the formulation and specific spray drying process conditions to achieve the desired properties of the finished powder (96–98). Processing conditions are also controlled to produce particles having shape and surface morphologies that minimize cohesive and adhesive forces enabling efficient dispersion. The ability to escape clearance by alveolar macrophages is an additional claimed advantage of the large particle design strategy (98–100). Alternative design strategies for protein and peptide pulmonary delivery systems have been described in the literature. Studies have been reported evaluating the inclusion of permeation enhancers and enzyme inhibitors in formulations as a means to improve absorptive potential (30,101). However, the safety implications with any chemical agent that potentially modifies normal lung physiology must be considered especially for chronic treatment situations. A strategy involving a carrier-based formulation utilizing self-assembling diketopiperazine derivatives that can encapsulate protein and/or peptide moieties into microspheres (Technosphere™) has also been proposed by several researchers (102,103). The resulting particles are harvested and dried to produce a powder that can be administered with a passive inhaler device. A delivery system for insulin based on this design has been evaluated in human proof-of-concept studies (102,103). However, some concerns with this methodology include the long-term safety of the diketopiperazine derivatives that appear to act as an absorption enhancer, and the additional costs associated with including such compounds in the formulation. Another approach involves a formulation containing micronized crystalline insulin produced by jet milling, to achieve the appropriate geometric size range for inhalation, blended with a lactose monohydrate carrier. Dispersion of the dry powder is achieved with an electromechanical, breath-actuated device. This insulin delivery system, referred to as Spiros™, has also been evaluated in human clinical trials (104).

The feasibility of pulmonary administration for a wide variety of proteins and peptides has been evaluated in both

animal models and in some human proof-of-concept studies (88,105,106). A wide range of bioavailabilities (from less than 1% up to 95%) has been reported. An insulin pulmonary delivery system (Exubera™) consisting of a dry powder formulation composed of small geometric diameter particles produced by spray drying and utilizing a mechanical device for dispersion has won regulatory approval in both the USA and Europe. Data evaluating this system in numerous clinical trials, including large-scale studies involving both patients with Type-I or Type-II diabetes over extended periods of use, have been published (107–111). The other dry powder technology utilizing formulations composed of geometrically large, but low-density, particles that are dispersed with a passive device is being developed for pulmonary administration of insulin (93). Both safety and efficacy results evaluating this delivery system in patients with Type-I diabetes have also been reported (112). A liquid-based, microprocessor-controlled insulin delivery system identified as AERx™ is advancing in clinical trials involving both patients with Type-I or Type-II diabetes, and the results of these studies have also been published (113–116). Another liquid-based, breath-actuated, insulin delivery system (Aerodose™) is also progressing in development, and evaluations conducted in patients with Type-II diabetes have been reported (95,117). Based on all the published information pertaining to clinical trials, some generalizations about pulmonary insulin administration can be made (118,119). The PK profile of inhaled insulin is characterized by a faster onset when compared to the subcutaneous administration of insulin solution formulations, and is at least as fast as rapid-acting insulin analogs. The duration of action is intermediate between that of subcutaneously administered rapid-acting insulin analogs and regular insulin. Estimated bioavailabilities are typically in the range of ca. 10–20% relative to insulin administered via subcutaneous injection, although higher values have been reported in studies involving the Technosphere™ technology.

The overall bioavailability of a delivery system is influenced by various potential losses of the formulation in, for example, the device, packaging walls, mouth, oropharynx, or by exhalation. Intrapatient variability in PK and PD responses is low and similar to subcutaneously administered insulin. In special patient populations such as smokers, absorption of insulin is significantly greater, and different smoking patterns can theoretically influence dose requirements of inhaled insulin (115). This phenomenon, likely the result of cellular and/or physiological alterations in the lungs, could have important pharmacological implications for delivery of protein and peptide molecules in general. Other studies conducted in elderly subjects, asthmatics, or individuals with acute respiratory tract infections, also showed changes in PK/PD responses (119). The successful regulatory approval for pulmonary administration of insulin will likely open the door for other biomolecules to be considered for delivery via this route. Although the PK profile reported for inhaled insulin seems appropriate to meet prandial insulin requirements, it will not address basal insulin needs. In certain treatment regimens, an injection of a long-acting (basal) insulin preparation will be required unless a pulmonary delivery system capable of producing a sustained release profile is developed. One report describes the preparation of large porous particles containing insulin and protamine as a potential sustained release pulmonary formulation (120). The pulmonary administration of this formulation to rats produced sustained plasma insulin levels similar to those obtained following subcutaneous injection.

Trans-dermal Administration

Trans-dermal delivery is a prospective route of administration for peptide and protein biopharmaceuticals. However, physical and/or chemical enhancement technologies are necessary to make possible the successful delivery of these hydrophilic molecules (121). Microporation technologies offer an attractive approach to deliver both peptide and protein molecules. Electrically assisted technologies such as iontophoresis and electroporation have also been useful for the delivery of these (macro)molecules. Low-frequency sonophoresis has also enabled the trans-dermal delivery of both peptides and proteins. Formulation approaches have also been used for the trans-dermal delivery of smaller peptides. Trans-dermal delivery has the advantages that skin has a large and accessible surface area (1–2 m^2) with relatively low enzyme activity; it avoids first-pass hepatic metabolism and has the potential to provide sustained delivery of pharmaceuticals (122). However, skin also serves as an extremely effective permeability barrier, a property conferred by the thin (0.015 m) outermost layer of epidermis known as the stratum corneum. As a result, trans-dermal transport by passive diffusion is limited to low molecular weight (less than 0.5 kDa) and highly lipophilic pharmaceuticals and, indeed, all the marketed trans-dermal products to date fall into this category (123). The trans-dermal route for non-invasive systemic delivery of protein and peptide molecules has been hindered by the extremely low permeability of skin to these high molecular weight and typically hydrophilic molecules, and to achieve therapeutically useful systemic drug levels, permeation needs to be enhanced. Several approaches have been explored that provide additional driving force in the form of electrical (iontophoresis) (124,125) or ultrasound (sonophoresis) (126) energies, structural perturbation of stratum corneum (e.g., electroporation, thermal microporation, microneedles) (127,128), penetration enhancers (129,130), or a combination of these strategies. A summary of enhancement technologies for the trans-dermal delivery of peptides and proteins can be found in Table 4.

A variety of chemical penetration enhancers with or without protease inhibitors or colloidal vehicles (liposomes) have been investigated for their potential to enhance the skin permeability to peptides and proteins (129,130). One notable case is the use of the so-called Transferosomes™, ultraflexible liposomes containing a mixture of soybean phosphatidylcholine and sodium cholate, which are thought to reversibly create intercellular hydrophilic pathways through the skin to facilitate transport (151,152). Insulin-loaded Transferosomes™ applied epicutaneously to human volunteers have been shown to result in a glucodynamic response comparable

Table 4 Trans-dermal enhancement technologies for non-invasive delivery of peptides and proteins

	Microporation technology				**Microporation technology Electrically assisted techniques**			
Technology	**Microneedles**	**Thermal microporation**	**Radio frequency ablation**	**Laser Ablation**	**Iontophoresis**	**Electroporation**	**Sonophoresis**	**Formulation approaches**
Principle	Micron-sized needles which breach the stratum corneum to create microchannels through skin.	Creation of microchannels in skin can also be achieved when an array of electrically resistive filaments is applied.	Electric current in the range of radio frequency (100–500 kHz) is used to create microchannels in skin.	Microporation of skin can also be achieved with the help of medical lasers.	Application of small amounts of physiologically acceptable current to drive drug molecules into and across skin.	Use of high voltage (100–1000 V) electric pulses for a very short duration of time (several microseconds to milliseconds) to permeabilize skin.	The use of ultrasound to drive molecules into and across skin.	Used in conjunction with physical enhancement techniques for assisting trans-dermal delivery of proteins, such as nanotechnology- based carriers (liposomes and nanoparticles).
The pros	Offers elegant, effective and painless delivery of peptides and proteins.				Non-invasive technique, offers modulated and controlled delivery.	Used for electrochemotherapy and gene delivery.	Low frequency sonophoresis can deliver macromolecules into and across skin.	Simple approach without need of equipment.
The cons	Pore closure dynamics must be taken into account for planning effective drug delivery regimen and avoiding any potential infection risks.				Delivery limited to molecular weight of ≈ 13 kDa.	Safety of using this enhancement technique for drug delivery should be accessed.	Convenient hand held technologies should be developed.	Delivery limited to low molecular weight peptides or proteins.
References	(131–142)				(137,143,144)	(145–147)	(148,149)	(129,130,150)

to subcutaneously administered Ultralente preparation Ultratard™, albeit at approximately twice the insulin dose. With a single application of Transferosomes™ to patients with Type-I diabetes, normoglycemia has been maintained for 16 hours (153). This approach has also been successfully demonstrated with other polypeptides such as interferons α, β, and γ, calcitonin and superoxide dismutase in preclinical experiments (152).

The skin acts not only as a physical barrier but also as an immunological barrier, being enriched with various immunocompetent cells such as Langerhans cells (LCs), keratinocytes, dermal dendritic cells, and mast cells (153). In particular, LCs play a critical role as potent antigen-presenting cells against external antigens. Direct delivery of antigens to the LCs is expected to induce an effective immune response. Antigenic proteins applied to the bare skin, however, cannot access LCs in the epidermal layer, because the stratum corneum acts as a physical barrier to substance penetration (154). Huang et al. (155) established that the interaction between metal-based nanoparticles (gold-NPs) and the skin barrier leads to enhancement of skin permeability and effectively prompts percutaneous absorption of the co-administered proteins. The benefit of this co-delivery method is that it does not require "loading" of the bioactive molecules into the nanoparticulate system, but simply in a physical mixture instead. Consequently, a compromise in activity can be achieved for both protein drugs and nanoparticles due to the exclusion of complicated drug-loading processes. Such co-delivery effect highlights a new strategy for percutaneous protein delivery with obvious advantages in terms of simplicity and cost-effectiveness. It provides a promise in achieving self-administered

trans-cutaneous immunization, and may especially benefit those areas underserved for medical care.

The safe and effective delivery of peptides has also been successfully demonstrated in human subjects using iontophoresis, a technique that uses mild electric current to facilitate transport of molecules across the skin (125). Iontophoresis works primarily by a combination of two forces, electrorepulsion of charged drug molecules away from the electrode and into the skin, and electroosmosis, a convective solvent flow in the direction of the counter-ion transport. In general, cationic proteins and peptides are delivered more efficiently than anionic molecules, because electroosmosis works in the same direction as electromigration for cationic species, whereas it works against it for anionic species. Iontophoretic delivery of the luteinizing hormone-releasing hormone (LHRH) analog leuprolide has been demonstrated in clinical studies in human subjects (125,156,157) and showed an increase in luteinizing hormone levels comparable to those following a subcutaneous injection. Although this proof-of-concept demonstration occurred several years ago, it is not evident if this approach is anywhere close to commercialization for peptide drugs. The trans-dermal delivery of insulin remains a significant challenge due to low permeation rates at therapeutically useful rates. The delivery of insulin achieved in animal models appears to be far below the daily basal insulin needs for humans to be practical, and commercial viability remains questionable (118,158). Chen and colleagues (137) report unilamellar nanovesicles with membrane thickness of ca. 3–5 nm and an entrapment efficiency of ca. 89.05±0.91%, which can be driven by iontophoresis for enhancing transdermal delivery of insulin through microneedle-induced skin microchannels. The permeation rates of insulin from positive nanovesicles driven by iontophoresis through skin with microneedle-induced microchannels were 713.3 times higher than that of its passive diffusion. This approach offers a new strategy for the non-invasive delivery of peptides with large molecular weights using nanovesicles. Su et al. (159) reported a novel multilayer film in which a hydrolytically degradable loaded protein and oligonucleotide was incorporated for trans-cutaneous delivery. By applying a solid-in-oil (S/O) nanodispersion, Tahara et al. (160) successfully achieved trans-cutaneous immunization (TCI) without the use of any skin pre-treatment or adjuvant. The oil-based nanodispersion of hydrophilic drugs has effectively enhanced the permeation of proteins into the skin. TCI targeting the LCs of the epidermal layer is a promising needle-free, easy-to-use, and non-invasive vaccination method. Matsuo et al. (161) developed a hydrogel patch formulation to promote the penetration of antigenic proteins into the stratum corneum. These researchers investigated the characteristics of the immune responses induced by this vaccination method and the vaccine efficacy of TCI using a hydrogel patch containing tetanus and diphtheria toxoids.

Nanoparticles were shown to be promising carriers for trans-cutaneous vaccines. Due to the nano-bio interaction with skin lipids and the consequent induction of transient and reversible opening of the stratum corneum, encapsulation of antigens in nanoparticles is effective to enhance the trans-cutaneous delivery (162). Mattheolabakis et al. (163) examined the potential of poly(D,L-lactic acid) (PLA) nanoparticles as vectors for antigen delivery via the trans-cutaneous route. Using a double emulsification process, ovalbumin (OVA)-loaded PLA nanoparticles induced efficient cytokine responses. *In vitro* re-stimulation of cultured splenocytes with OVA elicited a little higher levels of IFN-γ and significantly higher levels of IL-2 in mice, compared to those immunized with OVA in solution. Nanoscopic therapeutical systems that incorporate biocompounds, such as protein and peptides, are emerging as the next generation of nanomedicines, aiming at developing therapeutical efficacy of pharmaceuticals. The delivery of peptides and proteins intended for therapeutical purposes poses unique difficulties in their encapsulation, especially because most of them possess a significant hydrophobic component and thus display a low tendency to adsorb onto surfaces (150,162). Such adsorptive behaviour can lead to distinct losses in the amount of macromolecule available for delivery.

The design of new formulations, particularly lipid-based nanoscale carriers, appears promising for the disruption of stratum corneum through the nano-bio interaction of colloidal carriers with skin lipids, protection of proteins and peptides from the external milieu, and maintenance of long-term activity. The lipid-based vesicles of liposomes, especially elastic liposomes, may change the bioactive permeation kinetics due to an impaired barrier function of the stratum corneum, which is helpful for skin penetration. Results obtained by Li and colleagues (150,162) highlight the nanoscale formulation, flexible liposome, as a promising carrier for the trans-cutaneous delivery of antigen proteins. New studies have demonstrated that non-invasive trans-dermal iontophoresis can be used to deliver significant amounts of a structurally intact, functional protein, across the skin. In fact, results gathered by Dubey and Kalia (144) demonstrated the feasibility of using trans-dermal iontophoresis to non-invasively deliver an intact functional protein (such as ribonuclease A) across the skin.

Ultrasounds of low-frequency have also been used to deliver insulin, γ-interferon, and erythropoietin in *in vitro* studies (164), although validation of this approach in humans has not yet occurred. Other trans-dermal approaches that have been explored are the so-called minimally invasive technologies that physically circumvent the stratum corneum either by creating transient micropores by heat (thermal microporation) or the use of microneedles (128), or ablation of stratum corneum with a laser (165,166). Eppstein and colleagues (167) reported the use of a thermal microporation process to deliver insulin Lispro to human test subjects. The MicroPors™ technology employed in their research work uses electrical current to heat an array of small diameter wires pressed against the skin, resulting in the creation of pores in the stratum corneum. Basal serum levels of insulin Lispro were reached with this delivery system, but the sampling period only covered three hours. Similar to the minimally invasive approaches is electroporation, which involves application of high-voltage electrical pulses to create new low-resistance pathways through the stratum corneum (127,146). Despite all the efforts in this area, there are currently no

commercially available trans-dermal delivery systems for protein or peptide biopharmaceuticals. Among active transdermal technologies, iontophoretic delivery devices are in late-stage clinical trials with small non-peptide therapeutics (125). Although much effort has been spent on insulin, in general small and potent peptides are the best candidates for exploring the iontophoretic delivery option. Iontophoretic delivery has the advantage of achieving sustained and controllable delivery of peptides that makes it particularly attractive for molecules with a narrow therapeutical index, but in practice overcoming low bioavailability using a device at a reasonable cost is a key challenge. Although some of the minimally invasive technologies appear to be promising, the safety of these approaches, especially under chronic use conditions, remains largely unknown. Skin irritation is a concern even for passive trans-dermal patches (168), and permeation enhancers further increase the risk of skin toxicity (130). The potential effect of common permeation enhancers such as alcohols and surfactants on the higher order structure and physical stability is an additional challenge for protein and peptide therapeuticals.

Oral Administration

The oral route is the most frequently used way for administering drugs, although it may have serious inconveniences for peptide and protein biopharmaceuticals. The primary reasons for this are that during the transit through the GIT, these molecules are exposed to enzymatic degradation, and they also have to overcome the mucus layer and epithelial absorption barriers. The use of nanoparticles for encapsulating peptide and protein molecules is a promising approach (1) for protecting these biological molecules from enzymatic degradation. In addition, it has been stated that particles smaller than 500 nm can pass across the intestinal mucus layer by endocytocis. Formulations including chitosan, a classical permeability enhancer that demonstrates mucoadhesive and tight junctions opening functionalities, has been most often investigated (169,170). Several experimental approaches have been related to identify tight junction targets that are most appropriate for pharmacological manipulation in order to enhance paracellular delivery. Tight junctions are composed of three major types of integral membrane proteins, viz. occludin, claudins and junctional adhesion molecules, and the claudin family currently appears to be a confirmed potential target (23). Alginate-chitosan nanoparticles administered orally to diabetic rats were found to be effective for oral insulin delivery (171).

Protein and peptide (bio)pharmaceuticals are gathering increasing interest within the pharmaceutical industry (14), not only due to a better understanding of their role in physiopathology, but also because recombinant DNA technology is nowadays a well-established technology that allows large scale production of therapeutically important (recombinant) proteins (172). However, if oral administration is sought (14,173), two major drawbacks of such macromolecular (bio)pharmaceuticals lies in the fact that (*i*) they are easily hydrolyzed by proteolytic enzymes ubiquitous in the GIT, thereby leading to low bioavailability, and (*ii*) they possess an intrinsically low permeability across biological barriers and short half-life in the blood stream (14). The development of new drug delivery systems suitable for protein/peptide drugs, based on nanoencapsulation, has become therefore a major area of research, since it has been observed that a greater number of nanoparticles cross the GIT epithelium than do microparticles (14).

Several researchers reported that water-soluble chitosan nanoparticles enhance and prolong the intestinal absorption of bovine serum albumin, which makes them a potential protein delivery system (174). Chitosan nanoparticles containing an anionic cyclodextrin (SBE7μ–β-CD) appear to be extremely interesting as possible oral GSH carriers, since they combine improved GSH loading with the capacity to promote GSH transport through the intestine, as observed in a frog intestinal sac model. Another tight junction peptide (TJP) was shown to provide a dramatic improvement in drug permeation across epithelial tissue of salmon calcitonin, parathyroid hormone 1-34 (PTH1-34), and peptide YY 3-36 (PYY3-36). In addition, when the formulation containing this TJP was administered intranasally in rabbits, a dramatic increase in bioavailability was observed (175). Oral delivery technology based on hydrogels that exhibit reversible, pH-dependent swelling behavior appears to protect insulin from enzymatic degradation in a gastric milieu and releases it following an exposure to intestinal environment. The swelling of the hydrogel in the intestinal fluid leads to bioadhesion onto the intestinal wall. This technology demonstrated relatively high (ca. 8%) bioavailability in rats (176,177).

Nano-sized particles appear to be a highly promising solution for (non-invasive) peptide and/or protein administration, due to several characteristics namely **(***i***)** their intrinsic versatility for drug formulations, **(***ii***)** sustained-release properties, **(***iii***)** subcellular size and **(***iv***)** tissue and cell biocompatibility, and have therefore consistently been receiving more attention than do liposomes because of their therapeutic potential and greater stability in biologic fluids as well as during storage (14,178,179). To target the nanoparticles encasing protein and/or peptide moieties to a specific cell and/or site, advances in nanotechnology involve the decoration of nanoparticle surface with targeting moieties such as monoclonal antibodies or other compounds such as transferrin, lectin, or avidin (180,181).

Delivery through the oral route is, undoubtedly, the most attractive way for administration of any (bio)pharmaceuticals, but the development of commercially feasible systems for protein and peptide (bio)pharmaceuticals is a formidable challenge, mainly due to the fact that the GIT has evolved to efficiently degrade and assimilate nearly all of the dietary protein. Therefore, adding to the fact that proteins and peptides exhibit intrinsically low permeabilities, the fundamental problem lies in delivering the intact, (bio)active molecule to the site of absorption and protecting it from the onslaught of enzymatic degradation. A protein or peptide moiety that has been ingested has to resist not only the (highly) acidic pH environment and presence of proteolytic enzymes in the stomach, as gastric emptying occurs, but also the pancreatic enzymes launched into the small intestine (duodene) that continue the hydrolysis process with high efficiency. Besides constituting a significant

barrier to permeability, enterocytes present in the epithelium of the small intestine also present another enzymatic barrier both in the form of various enzymes associated with the brush border membrane as well as those contained in the mucus and the cytoplasm (182). Absorbed protein or peptide moieties are subject to further degradation via hepatic metabolism. In spite of these challenges, formidable in nature, the attractiveness of the oral route has prompted exploration of an incredibly diverse set of strategies aiming at delivering proteins and peptides (183–191). Such strategies include (*i*) use of permeation enhancers, (*ii*) addition of enzyme inhibitors, (*iii*) addition of mucoadhesives, (*iv*) use of multifunctional matrices that simultaneously incorporate strategies (i), (ii), and (iii), (*v*) addition of enteric coatings that confer protection against the acidic environment of the stomach, (*vi*) micro- and nanoencapsulation (within liposomes, microspheres, and nanoparticles), (*vii*) addition of pH-sensitive polymers, (*viii*) use of microemulsions (both simple and multiple ones), (*ix*) use of specific carriers (delivery agents), and (*x*) protein modification either to simply enhance permeability or to exploit specific transporters.

The use of liposomes to vehiculate protein drugs through the GIT was considered promising, because of their ability to protect the encapsulated drugs from enzymatic and pH degradation, and also to improve absorption. Nevertheless, poor success has been achieved, due to poor stability under the diverse physiological conditions typically found in the GIT. However, mucoadhesive liposomal systems prepared by coating lipid suspensions with mucoadhesive polymer solutions, such as mucin, showed some success in intestinal absorption of protein drugs, for example, insulin (192). This approach can be taken further by the use of a protease inhibitor as a coater: Werle and co-workers have investigated the efficacy of liposomes coated with the polymer-protease inhibitor conjugate chitosan-aprotinin for oral peptide delivery (193).

In particular, one delivery system that uses a "carrier" (or "delivery agent") to facilitate macromolecular absorption, and another delivery system that uses covalent modification of the therapeutical protein moiety, appear most promising and are currently receiving most attention from the (bio)pharmaceutical industry. In the first strategy, a delivery agent (an aminoacid derivative or a small peptide-like compound) is selected from a library of molecules and used in combination with the therapeutical protein or peptide moiety (194). Although the exact mechanism by which delivery agents facilitate the macromolecular transport remains essentially unknown, it has been proposed by several researchers that these (mainly hydrophobic) agents bind non-covalently and reversibly to protein and peptide moieties and thus increase their lipophilicity (191). Also, it has been proposed that such interaction induces subtle perturbations in the higher order structure of a protein thus increasing flexibility and lipophilicity by exposing buried hydrophobic residues, thus resulting in a more transport-competent state (191,195,196). *In vitro* studies using intestinal tissue have demonstrated that these agents facilitate drug absorption via trans-cellular diffusion without compromising the integrity of both the cell membrane or tight junctions (191). The safety of these agents in both animal and human subjects has been established, at least in acute dosing situations (191). The first strategy, encompassing the delivery agent technology mentioned above, has been evaluated in human subjects for salmon calcitonin (197,198), insulin (191,199), and the non-peptide macromolecule drug heparin (200,201). In a Phase 1 clinical study, the oral absorption of salmon calcitonin was demonstrated in healthy male volunteers using a tablet formulation containing an unidentified caprylic acid derivative as the delivery agent (198), but the bioavailability attained was only ca. 1%. The formulation administered orally was well tolerated and produced all biological effects of calcitonin. In another Phase 1 clinical study, absorption of orally administered insulin was clearly demonstrated by a reduction in fasting plasma glucose levels (191). Although the exact details concerning bioavailability following oral administration were not reported by these authors, based on PD response the bioavailability relative to subcutaneous injection is likely to be ca. 10%. The potential of this technology to deliver the parathyroid hormone has also been reported in a primate study (202), resulting in a bioavailability of ca. 2% relative to subcutaneous administration via injection. The second strategy mentioned involves chemical modification of the protein or peptide moiety by attaching amphiphilic residues composed of both lipophilic alkyl groups and hydrophilic polyethylene glycol units to specific sites on the surface of the protein or peptide molecule (203). Besides improving stability against enzymatic degradation, this modification improves drug solubility in formulations that enhance absorption through the intestinal mucosa. An added advantage of this technology lies in the potential to increase circulation half-life by simply designing the covalent modification in a way that specific hydrolysis *in vivo* results in a pegylated therapeutical. An insulin derivative, hexyl-insulin monoconjugate 2 (HIM2), based on this technology has been evaluated by several researchers in clinical studies in human patients with Type-I diabetes (203,204), and a dose-dependent decrease in blood glucose has been clearly observed. Although no bioavailability studies have been conducted, given the limited relevance of traditional PK studies due to both hepatic uptake of orally administered insulin and the resulting effects on glucose homeostasis, an ''apparent'' bioavailability of approximately 5% has been suggested (203). The results obtained with this delivery system are apparently encouraging enough to warrant further development and clinical investigations in Type-II diabetic patients (205), at the same time that the utility of this technology to orally deliver a modified form of calcitonin (CT-025) has also been demonstrated in healthy volunteers (206).

Ocular Administration

The main problems of conventional ocular therapy are short residence time, drug drainage and frequent instillation. Nanoparticles seem to be promising vehicles for designing new controlled delivery systems to improve the ocular bioavailability of drugs for ophthalmic diseases (207). A review by Paolicelli and co-workers (208) provides an

updated overview of the advances made in ocular delivery of bioactive (hydrophilic) molecules, such as peptides and proteins, by means of chitosan-based nanosystems, and their potential relevance in clinical use. De la Fuente and co-authors (209) reported that their research group has designed and developed a delivery platform based on chitosan nanoparticles, which suits the requirements for the topical ocular route. These nanosystems have been specifically adapted for the delivery of peptide/protein drugs onto the eye surface.

Therapeutical molecules for treating diseases at the posterior segment of the eye are also emerging. Among them, protein drugs represent an important class of therapeutical entities (210). Topical administration of eye drops is the easiest way to deliver drugs. However, only less than 1/108 of protein drug may reach the back of the eye. Systemic delivery is another way to deliver drugs to the posterior segment of the eye, but a large amount of drug is needed to attain the required therapeutic level since the blood-retinal barrier (BRB) restricts the influx of drugs to the retina (211). The trans-scleral route has attracted much interest as a potential path for delivering protein and/or peptide therapeutical moieties into the posterior segment of the eye. There are several advantages: first, diffusion of macromolecules through the sclera is feasible, as demonstrated in *ex vivo* diffusion experiments using rabbit sclera; and second, the distance for drug molecules to penetrate into the posterior segment of the eye is shorter via the trans-scleral than via the trans-corneal route. After diffusing through the sclera, protein and/or peptide macromolecules are closer to the vicinity of the eye where most posterior diseases occur: the choroid and the retina. Cheung et al. (210) have explored the use of ultrasounds to non-invasively deliver protein therapeuticals into the sclera, so as to take advantage of the trans-scleral route. However, there is currently no investigation regarding the effects of ultrasounds in delivering macromolecules through the sclera. In their studies, these authors performed *ex vivo* experiments using rabbit eyes and tracked albumin penetration using a fluorescence microscope following a short ultrasound exposure. Cheung and colleagues (210) aimed at measuring the ultrasound enhancement of protein transport, proposed a potential mechanism for such transport and provided an initial assessment of the feasibility of such method.

Other Routes of Administration

As stated throughout the text, the commercial production of a variety of therapeutical molecules has intensified the study of drug delivery systems to improve bioavailability, therapeutical efficacy, and patient comfort. Efficient drug delivery systems aim at delivering proteins and/or peptides to the systemic circulation or local tissues via non-invasive routes, while maintaining at the same time a sufficient bioavailability. Many non-invasive drug delivery methods involve overcoming membrane and enzymatic barriers, where the underlying principle consists in enhancing the permeability of the membrane to the target (bio)pharmaceutical. The bioavailability is improved by both avoiding first-pass hepatic metabolism and achieving targeted delivery. In addition, such methods improve patient comfort by reducing the number of doctor visits, avoiding injections and facilitating administration. Membrane-based barriers such as the skin and mucosa have been extensively studied as potential drug delivery routes over the past decade. A mucosal barrier that has shown a great level of interest for both local and systemic delivery of peptides and proteins is the vaginal epithelium (212).

Vaginal non-invasive drug delivery, or the delivery of therapeutical molecules across the vaginal mucosa, both offer numerous advantages such as ease of access, prolonged retention, access to a highly rich vasculature, possibility of auto-administration, and a relatively low enzymatic activity. Intra-vaginal, non-invasive, drug delivery systems aim at enhancing permeability of the vaginal mucosa to the target peptide and/or protein moiety, and therefore provide an alternative administration route for peptide/protein-based therapies.

A genetically reconstructed fusion peptide of human epidermal growth factor (hEGF) with an undecapeptide YGRKKRRQRRR (P11), which have been shown to play important roles in ameliorating malfunctions in neurodegenerative diseases, was used by Zhao et al. (213) to investigate the permeability between cell membrane and the BBB via rectal administration. The results gathered by these researchers clearly suggest that the rectal non-invasive delivery of the P11 polypeptide-conjugated growth factor is an efficient way for BBB transduction, thus raising the hope of real therapeutical progress against neurodegenerative diseases via non-invasive rectal delivery.

CONCLUSIONS

Overcoming the need for (invasive) injection-based delivery of protein and/or peptide (bio)pharmaceuticals has been the driving force for intense research efforts exploring the feasibility of techniques allowing non-invasive administration of such macromolecules. Empowered with a much greater understanding of the intricate structural, physiological and biochemical characteristics of each non-invasive route, researchers have begun to devise promising delivery technologies to overcome the significant barriers posed to effective absorption of such biopharmaceutical macromolecules into the systemic circulation. Most successful among the various non-invasive routes are several nasal delivery systems incorporating highly potent (commercially available) peptide moieties that have already gained regulatory approval. Advances in inhalation research have resulted in the development of at least three pulmonary delivery systems centered in insulin, one of which has received regulatory approval in both the USA and Europe. This first successful approval may therefore open the way for additional pulmonary products to appear in the market, since the technology seems to be adaptable to other protein and peptide (bio)pharmaceuticals. Despite significant developments in the design of inhalation delivery systems for the nasal route, similar success has not yet been achieved for the buccal/sublingual, trans-dermal, vaginal, ocular, rectal or oral routes of administration. Some of the technologies under development have produced encouraging clinical

data in human proof-of-concept studies, but however with low bioavailability, thus making it difficult to achieve a specific pharmacological profile, associated to both uncertain toxicological impact (especially for chronic administration situations) and doubts about patient acceptance, which globally still represent significant hurdles to the successful commercialization of buccal/sublingual, trans-dermal, vaginal, ocular, rectal and oral delivery systems. Although tremendous progress has been made in the field of non-invasive drug delivery, significant drawbacks of current technologies limit their applicability to a broad range of protein and peptide biopharmaceuticals. Survival of these macromolecules in the body environment is therefore difficult, unless a suitable (protective) carrier is used. The selected carrier must, on one hand, guarantee proper protection to the protein/peptide moiety and, on the other, be able to escape uptake by macrophages. Several approaches have been under scrutiny to overcome the delivery problems of peptides and proteins by means of micelles, micro- and nanoemulsions, liposomes and solid lipid nanoparticles. Micelles and microemulsions (o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides. Hydrophilic drugs of this kind can be effectively associated to these systems, affording some protection against enzymatic degradation. During recent years, it has been recognized that nanoemulsions might also be used as drug carriers for lipophilic drugs through different routes. Liposomes exhibit a number of advantages in terms of amphiphilic character, such as biocompatibility and ease of surface modification, providing it a suitable delivery system candidate for peptide and protein molecules. Forthcoming research may eventually result in a breakthrough technology that effectively overcomes the multiple (diverse) barriers associated with the non-invasive delivery of both proteins and peptides, at the same time that simultaneously enables commercialization of cost-effective and patient-friendly biopharmaceutical products.

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ARTICLES OF FURTHER INTEREST

Absorption Enhancers, p.
Bioadhesive-Based Targeted Drug Delivery Systems, p.
Bioavailability Enhancement, p.
Biopharmaceutics, p.
Biotechnology and Biological Preparations, p.
Biotechnology-Derived Drug Products: Formulation Development, p.
Biotechnology-Derived Drug Products: Stability Testing, Filling, and Packaging, p.
Biotransformation of Drugs, p.
Clinical Pharmacokinetics and Pharmacodynamics, p.
Colloids and Colloid Drug Delivery System, p.
Dosage Form Design: A Physicochemical Approach, p.
Dosing of Drugs: Dosage Regimens and Dose-Response, p.
Drug Delivery: Buccal Route, p.
Drug Delivery: Controlled Release, p.
Drug Delivery: Gastro-Retentive Systems, p.
Drug Delivery: Nasal Route, p.
Drug Delivery: Needle-Free Systems, p.
Drug Delivery: Ophthalmic Route, p.
Drug Delivery: Oral Colon-Specific, p.
Drug Delivery: Oral Route, p.
Drug Delivery: Parenteral Route, p.
Drug Delivery: Pulmonary Delivery, p.
Drug Delivery: Pulsatile Systems, p.
Drug Delivery: Rectal Route, p.
Drug Delivery: Topical and Transdermal Routes, p.
Drug Delivery: Vaginal Route, p.
Drug Design: Basic Principles and Applications, p.
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Ultrasonic Nebulizers, p.

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