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EXPERT REVIEW

Enhancing the Buccal Mucosal Delivery of Peptide and Protein Therapeutics

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ABSTRACT With continuing advances in biotechnology and genetic engineering, there has been a dramatic increase in the availability of new biomacromolecules, such as peptides and proteins that have the potential to ameliorate the symptoms of many poorly-treated diseases. Although most of these macromolecular therapeutics exhibit high potency, their large molecular mass, susceptibility to enzymatic degradation, immunogenicity and tendency to undergo aggregation, adsorption, and denaturation have limited their ability to be administered via the traditional oral route. As a result, alternative noninvasive routes have been investigated for the systemic delivery of these macromolecules, one of which is the buccal mucosa. The buccal mucosa offers a number of advantages over the oral route, making it attractive for the delivery of peptides and proteins. However, the buccal mucosa still exhibits some permeability-limiting properties, and therefore various methods have been explored to enhance the delivery of macromolecules via this route, including the use of chemical penetration enhancers, physical methods, particulate systems and mucoadhesive formulations. The incorporation of anti-aggregating agents in buccal formulations also appears to show promise in other mucosal delivery systems, but has not yet been considered for buccal mucosal drug delivery. This review provides an update on recent approaches that have shown promise in enhancing the buccal mucosal transport of macromolecules, with a major focus on proteins and peptides.

KEY WORDS buccal permeability · chemical penetration enhancers · formulation · peptides · proteins

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INTRODUCTION

For many years, the lack of industrial manufacturing processes for peptides and proteins had limited their use and availability as therapeutic agents [1]. However, with modern advances in the fields of biotechnology and genetic engineering, as well as the development of sophisticated delivery technologies, the potential for clinical use of various biomacromolecular therapeutic agents is becoming a reality [2–4]. Widely-used biomacromolecules include monoclonal antibodies for the treatment of cancer and autoimmune diseases, vaccines for immunization against hepatitis A and/or B, insulin for the treatment of diabetes, human growth hormone for supplementation in hormone deficiency, interferon α for the treatment of hepatitis B and/or C [5] and peginesatin for the treatment of anemia in dialysis patients with chronic kidney disease [6].

While oral administration of such peptides and proteins would be the most conventional and patient-friendly approach for their delivery, intestinal absorption of these macromolecules is generally limited by their hydrophilic characteristics and large molecular mass. Moreover, other common properties of these macromolecules such as susceptibility to proteolytic degradation, a short plasma half-life, immunogenicity, and their tendency to undergo aggregation, adsorption, and denaturation are also limiting factors preventing the oral delivery of peptides and proteins for treatment of various diseases [7, 8].

Given the limitations associated with oral delivery, parenteral administration (e.g. intravenous or subcutaneous delivery) has consequently been the traditional route for delivering these

macromolecules [5, 9]. However, disadvantages such as discomfort at the site of injection, inconvenience associated with storage and administration, and poor patient compliance have often limited the parenteral route for delivering such macromolecules [10]. Various alternative routes of delivery have therefore been explored for such molecules, including the nasal, vaginal, transdermal and pulmonary routes, but limitations associated with each route may prevent their acceptability for certain therapeutics [11]. While nasal delivery is an attractive route because of the ease of administration, adverse effects including rhinitis and rhinorrhea, often induced by excipients (such as absorption enhancers and surfactants), may render this route of administration unacceptable for certain molecules [12]. Moreover, long term nasal administration may compromise the integrity of the nasal mucosa, and there are reports suggesting untoward effects of penetration enhancers on mucociliary activity [13, 14]. While intravaginal delivery of peptides has been attempted, it is characterized by poor and variable bioavailability, which has been associated with formulation factors, vaginal physiology, age of the patient and alterations occurring through the menstrual cycle [15]. Moreover, formulations to be administered via the intravaginal route may exhibit low retention to the vaginal epithelium and induce local irritation, negatively affecting patient compliance [16]. While the transdermal route has the advantages of ease of access and the availability of a large surface area, irritation at the site of application and the visibility of the formulation (such as patches) can often limit patient acceptability of this route of delivery [17]. The pulmonary route offers a large and highly absorptive surface area (80–120 m^2), extensive vascularization and low thickness of the alveolar epithelium, which contribute to a rapid onset of pharmacological action [18]. However, the presence of drug-metabolizing enzymes and alveolar macrophages may limit the pulmonary absorption of peptides and proteins [5, 18]. Moreover, long-term application may cause irritation and irreversible lung damage [5], and many patients have difficulty using inhaler devices correctly, often resulting in suboptimal therapy [19]. Thus, while each of these routes can be potentially exploited for the delivery of macromolecular therapeutics, potential disadvantages associated with each of them can limit their usefulness for the systemic delivery of these agents.

Over the last two decades, considerable attention has been devoted to the buccal mucosa as an alternative route for drug administration because of its excellent accessibility and physical robustness, as well as the avoidance of intestinal and hepatic metabolism, which is particularly favorable for peptide and protein delivery [20, 21]. Unlike the skin, the human buccal mucosa consists of 40 to 50 layers of non-keratinized cells, which makes it a more permeable membrane, and is particularly important for the systemic delivery of hydrophilic biomacromolecules such as peptides and proteins [22]. The accessibility of the buccal mucosa favors the administration of formulations at this site, and, in the event of adverse reactions, formulations can be easily and quickly removed (an option not possible with oral or pulmonary delivery). In addition, buccal and sublingual regions have low enzymatic activity, with enzymes such as trypsin, chymotrypsin and pepsin (which are present in the gastric and intestinal fluids) being largely absent in the oral mucosa [23]. This specific property of the buccal mucosa is particularly favorable for peptide and protein delivery given their susceptibility to enzymatic degradation, which is a very common reason for their poor oral absorption [24]. Furthermore, the buccal mucosa is a well-vascularized tissue, from which blood vessels drain directly into the jugular vein. Therefore, molecules that are able to penetrate the buccal mucosal epithelium are likely to be delivered rapidly into the systemic circulation, avoiding the hepatic first pass effect [25]. It should also be noted that, particularly for sustained-release mucoadhesive devices, cellular turnover time in the buccal mucosa is slower (4–14 days) than in the gastrointestinal tract, allowing formulations to adhere to the buccal mucosa for relatively longer periods of time [26]. The relatively high permeability of the buccal mucosa translates into a lower required dose of macromolecule, thus reducing the cost of goods and limiting the potential for undesirable side effects associated with higher doses. Despite these advantages, the buccal mucosa has a low surface area available for molecular absorption (relative to the gastrointestinal mucosa), and the constant flow of saliva can reduce the transport of macromolecules across the buccal mucosa through clearing such molecules into the throat. In addition, molecules with a strong taste are not desirable for buccal delivery and therefore taste issues must be considered prior to application [22]. Although some of these limitations may not be controlled, the benefits associated with buccal mucosal administration outweigh the limitations, making it a promising route for the administration of macromolecular therapeutics, such as peptides and proteins.

Given that peptides and proteins are generally larger than most conventional drugs (with molecular masses ranging from 600 to 100,000 Da) [27], the inherent barrier nature of the buccal mucosa still represents a challenge, and various enhancement strategies are often required to ensure a suitable absorption profile is achieved. The first attempts to use the buccal mucosa for insulin delivery, for example, were made as early as 1925. Given the limited permeability of insulin across this membrane, several attempts have been made since then to improve buccal absorption by, for example, adding chemical enhancers or by modifying the physicochemical properties of the peptide. After multiple attempts at improving buccal delivery of this protein, a buccal system for this protein (Oral-lyn®) has been developed by Generex Biotech, for the treatment of both Type 1 and Type 2 diabetes mellitus [28–30]. This product consists of a liquid formulation of mixed micelles containing insulin and specific chemical

penetration enhancers, which encapsulate and protect this peptide, as well as assisting its permeability across the buccal membrane [31, 32]. This successful example highlights the importance of understanding the physiology of the membrane, the factors limiting permeability and the exploitation of various enhancement strategies in achieving successful buccal delivery.

The addition of chemical penetration enhancers such as surfactants, fatty acids and chitosan in formulations is the most common approach to enhance the delivery of peptides across the buccal mucosa, although minimal increases in absorption may be achieved and histological damage can be observed with these enhancers [30, 33]. Subsequently, new approaches to improve the buccal mucosal delivery of macromolecules have been exploited, including chemical modification, physical methods (such as iontophoresis), and the incorporation of particulate systems and anti-aggregating agents. The purpose of this review is to provide an update on recent approaches that have been successfully employed to improve the buccal delivery of macromolecules, particularly peptides and proteins. We also highlight some approaches that have shown promise for the delivery of macromolecules across other membranes, but are yet to be exploited for buccal mucosal delivery.

STRUCTURE AND PHYSIOLOGY OF THE BUCCAL MUCOSA

By understanding the structure and physiology of the buccal mucosa, rational approaches to optimize the permeability of macromolecules across this biological membrane will be possible. The oral cavity comprises the lips, cheek, tongue, gingiva, hard palate, soft palate and floor of the mouth [24]. While each of these regions has its own physiological role to support mastication, speaking and eating, the sublingual (under the tongue and on the floor of the mouth) and buccal (located on the inner lining of the cheek) mucosae are the most important for the purposes of drug and macromolecular drug delivery, as they are the most permeable of the oral mucosal membranes [34]. Both the sublingual and buccal mucosae are non-keratinized (Fig. 1A), providing flexibility and elasticity necessary for mastication and speech processes [35]. On the other hand, keratinized mucosa, which includes the hard palate and gingiva (Fig. 1B), is present in regions of the oral cavity subjected to physical or chemical stress and is therefore more resistant to abrasion [36]. Non-keratinized and keratinized mucosae not only occupy different sites within the oral cavity, but also differ in their intercellular lipid composition. The intercellular spaces of the keratinized mucosae contain neutral lipids such as ceramides and acylceramides, whereas the intercellular spaces of the non-keratinized mucosae lack acylceramides and only have small amounts of ceramides [37] and neutral but polar lipids, mainly cholesterol sulfate and glucosylceramides [38].

In addition to the differences inintercellular lipids between these two types of mucosae, the way in which the lipids are packed differs between non-keratinized and keratinized tissue. The intercellular lipids of the keratinized tissue are in an ordered, lamellar state, which hinders the ability of molecules diffusing through the intercellular (paracellular) space [39, 40]. In contrast, the intercellular lipids of the non-keratinized mucosa are in a more amorphous state, with occasional short stacks of lipid lamellae [39], which offer less hindrance to the diffusivity of molecules. This difference in the packing order of the intercellular lipids is important, as it suggests that most molecules permeate the buccal mucosa via the paracellular rather than the transcellular route (Fig. 2) [41]. While both routes may coexist for all drugs, it is the route with the least penetration resistance that is usually preferred [42]. It is suggested that hydrophilic compounds such as peptides and proteins permeate the buccal mucosa

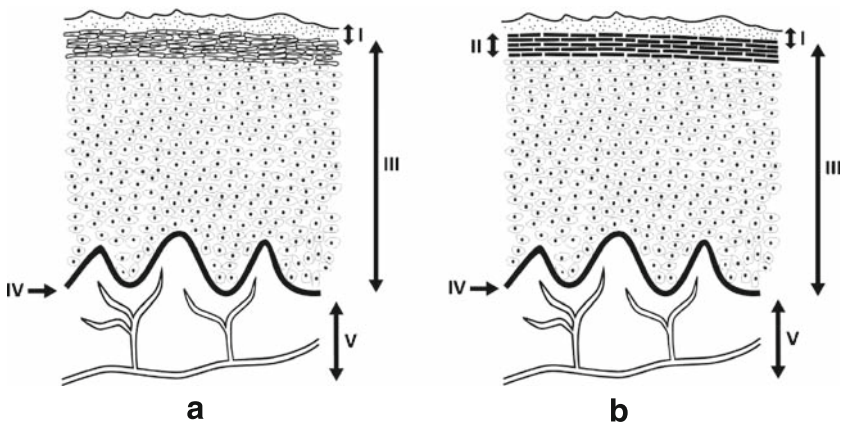

Fig. 1 Structure of the non-keratinized (a) and keratinized (b)stratified squamous epithelium lining different regions of the oral cavity. This structure includes mucus layer (I), stratum corneum (II), stratified squamous epithelium (III), basement membrane (IV) and submucosa (V), which contain blood vessels through which any absorbed compounds are delivered to the systemic circulation. Modified from [44] with permission.

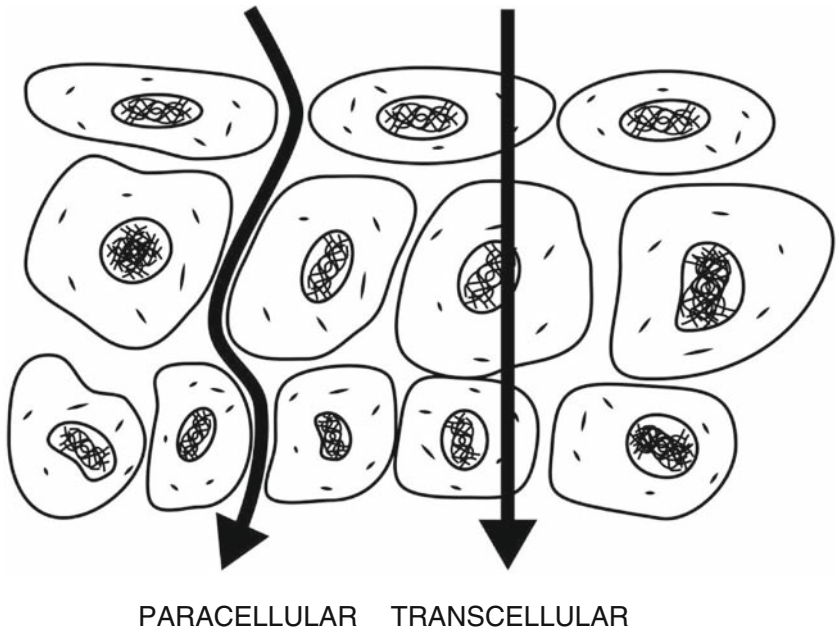

Fig. 2 The two potential transport pathways (*paracellular and transcellular*) that molecules could utilize when permeating the buccal mucosa.

via the paracellular route as they are more soluble in the aqueous fluids filling the intercellular spaces [43]. Given that the intercellular lipids forming the paracellular space of non-keratinized mucosa would provide less resistance than those of keratinized tissue, peptide and protein absorption is therefore likely to occur at a faster rate through non-keratinized tissue (i.e. buccal and sublingual mucosa) than keratinized tissue.

For the reasons mentioned above, the buccal and sublingual mucosae are the most attractive sites within the oral cavity to be exploited for systemic macromolecular delivery. While the sublingual mucosa is thinner, it is more commonly used for the systemic administration of small molecular weight drugs requiring an immediate pharmacological effect (e.g. glyceryl trinitrate) [34]. Given the thicker nature of the buccal mucosa, this membrane is more appropriately exploited for therapeutics where a slower onset, but longer duration of action is desired. As shown in Fig. 1a, the buccal mucosa is composed of a stratified squamous epithelium, a basement membrane, and underlying connective tissue. The epithelium consists of 40–50 cell layers (500–600 μm thickness), which migrate from the basal layer to the superficial layer, and protect the underlying tissue against fluid loss and entry of potentially harmful environmental agents. As indicated above, the barrier nature of the buccal mucosa is imparted by the intercellular lipids [44], which are released by membrane-coating granules of the superficial epithelial cells [35]. Once a molecule has permeated through the intercellular spaces of the buccal mucosa (the most likely route for peptides and proteins), it then enters into the connective tissue, which is separated from the epithelium by a lamina propria and contains a network of capillaries that deliver material into the systemic circulation [26]. While the intercellular lipids are considered the major barrier to buccal mucosal absorption [44, 45], additional barriers such as saliva and mucus, and to some extent enzymatic activity, have the potential to minimize the ability of a molecule to permeate the buccal mucosa.

Saliva and Mucus

Saliva is secreted by three major salivary glands (parotid, submaxillary and sublingual) and minor salivary or buccal glands [46]. The parotid and submaxillary glands produce non-viscous aqueous secretions, whereas the sublingual glands produce mainly viscous saliva with limited enzymatic activity [24]. The pH of the saliva is slightly acidic (6.6) during the rested state and increases to 7.4 when stimulated due to an increase in the bicarbonate ion concentration [47]. Saliva mainly consists of water (95–99% per weight), enzymes, inorganic salts, lipids, and glycoproteins (which are referred to as mucins). MG1, a high molecular weight mucin composed of disulfide-linked subunits, is able to adhere to the surface of the oral epithelium, representing another penetration barrier, the mucus layer [48] (Fig. 1). Saliva not only lubricates the oral cavity, assisting in processes such as swallowing and speaking, but it also helps to maintain the integrity of the hard tissues of the teeth. Saliva also allows carbohydrate digestion and regulates oral microbial flora by maintaining the oral pH and enzyme activity [49]. From a drug delivery perspective, saliva may facilitate the removal of drugs from the site of absorption, thereby limiting the absorption of a desired therapeutic agent unless an administered formulation can be retained [50, 51]. This limitation may be overcome by using bioadhesive formulations that are able to prolong adhesion to the mucosal

surface and maximize the drug concentration gradient, leading to increased bioavailability [52]. While the presence of saliva can potentially increase the clearance of molecules from the oral cavity, it also provides a water rich environment that can be favourable for dissolution of molecules released from delivery systems, benefiting drug absorption across the buccal mucosa [24].

Enzymatic Activity

The membranes of the oral cavity exhibit less enzymatic activity than the gastrointestinal tract [24, 26],making the oral cavity more suitable for the delivery of peptides and proteins, which are typicallysusceptible to enzymatic degradation. However, the metabolic capacity of the oral cavity cannot be ignored as a number of peptides have been shown to be degraded in the presence of buccal tissue homogenates, including insulin and proinsulin [53], enkephalin analogues [54], thyrotropin releasing hormone [55], calcitonin [56] and substance P [57]. Given that these studies were undertaken with buccal tissue homogenates, it is not clear whether the enzymes responsible for the peptide and protein degradation were intracellular or extracellular. Although the incubation of peptides in mucosal tissue homogenates has been used to characterize proteolytic activity [58], this is not able to discriminate cytosolic, membrane-bound and intercellular proteolytic activities, leading to inconclusive results [59]. If the enzymes are present intracellularly, and given that peptides and proteins are likely to traverse the buccal mucosa via the paracellular route, it is possible that intracellular enzymes do not affect the overall absorption process of peptides and proteins. For example, it has been reported that a small percentage of oligopeptides escape hydrolytic processes in the buccal mucosa, resulting in appreciable absorption into the blood, as the oligopeptide is transported via the paracellular route and has limited interaction with intracellular hydrolytic enzymes [60]. Furthermore, insulin and enkephalin, which are predominantly transported via the paracellular pathway, may escape extensive metabolism because the proteolytic activity against these substrates is primarily cytosolic [27]. In contrast, there are various studies suggesting that enzymes such as aminopeptidases, carboxypeptidases, phosphatases, carbohydrases, esterases and endopeptidases within the oral cavity can limit the buccal bioavailability of macromolecules because these molecules may be converted into inactive forms [53, 54, 61, 62]. As a result, specific enzyme inhibitors may be considered to overcome this potential barrier when attempting to optimize the delivery of certain peptides and proteins across the buccal mucosa.

The activities of aminopeptidase and esterase in buccal mucosal tissues have also been evaluated in different animal models (rat, rabbit, guinea pig, and dog), using L-leucine-β-naphthylamide as a substrate [63]. Although most buccal permeability studies have been carried out in porcine buccal mucosa, this membrane model was not considered in this analysis. For aminopeptidase activity, the relative activity ranking was dog < rat < guinea-pig < rabbit, whereas for the esterase activity the ranking was guinea-pig < rat < rabbit. In view of these differences, animal models should be carefully selected when predicting the buccal transport of peptides and proteins.

Since enzymes are selective for their substrates and can be differentially distributed in regions of the buccal mucosa (mucus, epithelium, connective tissue), studies should be performed to identify where a peptide or protein of interest is most sensitive to degradation. In this way, more targeted formulation approaches can be efficiently applied to minimize the possibility of degradation of the peptide or protein. For example, it is suggested that insulin is mainly degraded by intracellular cytosolic-bound proteases, given that aprotinin (a serine protease inhibitor) did not affect the overall permeability of insulin (in line with the assumption that insulin and other peptides and proteins would traverse the buccal mucosa via the paracellular route) [64, 65]. Furthermore, no proteolysis of insulin was observed when it was applied to the surface of porcine buccal mucosa, suggesting that enzymes responsible for insulin degradation are not present on the buccal mucosal surface, and are therefore likely to beintracellular [65]. Such knowledge can guide the type of buccal formulation used in insulin delivery, and the lack of surface enzymes suggests that a bioadhesive buccal formulation would be suitable to deliver insulin. Indeed, there are various examples demonstrating appreciable bioavailability/permeability of insulin when administered in a mucoadhesive formulation [66–68]. In another study, the location of dipeptidyl peptidase IV activity was determined by assessing endomorphin-1 (ENI) stability in full-thickness buccal epithelium and partial-thicknessbuccal epithelium [69]. Superficial layers of the epithelium had been scraped off and removed by surgical blades for preparing the partial-thicknessbuccal epithelium. ENI was more unstable in the presence of full-thicknessbuccal mucosal epithelium (14 *vs.* 58% intact drug detected after 24 h in experiments with full- and partial-thicknessbuccal epithelium, respectively), which suggests that the enzyme responsible for the metabolism of ENI is located in the superficial layers of the buccal epithelium layer. In the presence of diprotin-A, a potent inhibitor of dipeptidyl peptidase IV [70], the buccal stability of ENI in full thickness buccal mucosal epithelium was significantly enhanced (23 *vs.* 71% of the peptide remaining intact after 6 h) [69]. Therefore, dipeptidyl peptidase IV was found to be responsible for ENI degradation.

APPROACHES FOR ENHANCING PEPTIDE AND PROTEIN BUCCAL TRANSPORT

With an understanding of the physiological factors affecting the buccal mucosal transport of peptides and proteins, it has been possible to design various approaches to overcome the barriers and enhance the buccal mucosal delivery of these macromolecules. These include the use of chemical modification, chemical penetration enhancers, pH modulation, iontophoresis and various particulate and mucoadhesive formulations, which will be highlighted in the following sections.

Chemical Modification

Because of their polarity and size, peptides are very poorly transported across biological membranes, which are generally lipophilic in nature. As a result, various chemical modifications have been explored to improve the biopharmaceutical properties of peptides and proteins, to assist in enhancing their inherent buccal permeability, as well as increasing their resistance towards enzymatic degradation [71]. Overall, the chemically modified derivatives are suggested to be cleaved in the systemic circulation and/or target tissue, resulting in the delivery of the free active peptide at its site of action following absorption [72].

One commonly used chemical modification approach for general enhancement of peptide absorption is the introduction of acyl chains to the N- terminal positions of peptides and proteins, which has led to increased lipophilicity and improved stability [73, 74]. With respect to buccal absorption, acylation of a dipeptide (Trp-Leu) has been achieved by covalent attachment of myristic acid to the terminal amino group (Myr-Trp-Leu) [71]. Using an *in vitro* modified Franz diffusion model, the acylated peptide displayed greater affinity to porcine buccal epithelium and greater accumulation in the tissue (with 80% of the modified peptide detected in the buccal mucosa), although surprisingly this modification reduced the overall permeability of the peptide in the receptor chamber. In contrast, the non-acylated peptide had lower retention in the buccal mucosa (between 3 and 4%) with 55% of the dipeptide being detected in the receptor chamber. These findings suggest that the N-terminal acylation is not effective in enhancing the buccal absorption of this peptide, at least *in vitro* and over this experimental period of 24 h, and thatthe enhanced tissue retention could be attributed to either increased interaction with the lipophilic domains of the buccal mucosa, enhanced stability within the buccal mucosa, or a combination of both processes. Whether this modification leads to increased buccal absorption over a prolonged period of time *in vivo* remains to be assessed; however, the enhanced tissue retention may result in a slower release of this peptide into the plasma over time. Therefore, without further studies on this approach, N-terminal acylation should not be discounted as a potential mechanism to enhance buccal absorption of peptides *in vivo*.

Thyrotropin-releasing hormone (TRH) has been investigated as a model peptide for buccal absorption in various delivery systems [75, 76]. TRH has been modified by N-acylation of the imidazole group of its histidine residue with chloroformates to improve its lipophilicity and thereby protect it against rapid enzymatic inactivation in the systemic circulation [77]. TRH is rapidly hydrolyzed in human plasma by a TRH-specific pyroglutamyl aminopeptidase, although N-alkoxycarbonyl derivatives have been shown to be more resistant to cleavage, suggesting that increased plasma levels are probably due to its higher lipophilicity and therefore permeability through the tissue [78]. Thus, modifications aimed at increasing the lipophilicity of macromolecules may be considered as relevant approaches to enhance buccal mucosal absorption of these peptides.

Chemical Penetration Enhancers

Given that absorption through biological barriers is often limited by the structural elements that provide a physiological barrier, it is often necessary to employ chemical penetration enhancers to overcome these barriers to result in appreciable absorption. There have been multiple studies assessing the impact of chemical penetration enhancers on buccal mucosal absorption of both small and large molecules, and the possible mechanisms for their enhancing effects include (i) increasing the partitioning of molecules into the buccal mucosal epithelium, (ii) extracting the buccal mucosal intercellular lipids, (iii) interacting with buccal mucosal epithelial protein domains, and/or (iv) increasing the retention of molecules at the buccal mucosal surface [27, 44]. Hassan *et al.* (2010) [79] have also proposed that penetration enhancers are able to alter mucus rheology by reducing its viscosity, and to increase the solubility of the permeants, but limited research has been performed in this area to confirm such mechanisms. The commonly used and evaluated penetration enhancers for increasing peptide and protein buccal delivery include surfactants and bile salts, fatty acids and polymers such as chitosan, which will be described in the following sections [80]. Particular attention has been devoted to polymers since they may be considered safer and are generally assumed not to be absorbed into the systemic circulation from the buccal mucosa. Furthermore, they may not only improve the permeability profile of molecules but they may exhibit mucoadhesive properties, inhibit the activity of peptidases, or exhibit antimicrobial action [81], and, as such, have been referred to as "multifunctional polymers". Chitosan is one of the more extensively studied of these materials [81].

One of the major concerns in applying chemical penetration enhancers is their potential toxicity to the buccal mucosa, although the buccal mucosa has been suggested to rapidly

recover after removal of certain enhancers [82, 83]. It remains unclear as to whether the beneficial effects of enhancers (and potential irritation) are transient, and so it is essential to select the appropriate type and concentration of enhancer in order to minimize irritation of the mucosal membranes, but still induce an enhancing effect [84]. It is also recommended that the effects of chemical enhancers on epithelial damage, local irritation, long term toxicity (recovery kinetics) and enhanced permeability of pathogenic microorganisms be carefully considered prior to their selection for buccal transport studies [85]. If the buccal mucosa is significantly damaged by an enhancer, recovery of barrier function may result from tissue turnover or other self-repairing mechanisms, and this could be a slow process. Conversely, the recovery process may be much faster if the enhancer only transiently alters tissue structure [82]. Therefore, an in-depth morphological and/or toxicological profiling of enhancers should be undertaken prior to advancing such enhancers into the clinic for potential use in enhancing buccal mucosal delivery of peptides and proteins.

Surfactants and Bile Salts

There are a number of studies demonstrating the beneficial effects of surfactants and bile salts on the buccal mucosal absorption of macromolecules, including peptide and protein therapeutics (Table I). Although some authors have suggested that surfactants cause removal of the superficial cell layers [86–88], thereby affecting the barrier properties of the buccal mucosa, a greater body of evidence suggests that intercellular lipid extraction is the main mechanism by which these agents enhance buccal permeability [44, 89, 90]. This lipid-solubilizing effect generally modifies paracellular transport of agents, although transcellular transport has been suggested to also be increased (when lipids from the cell membranes are extracted) if high concentrations of surfactant are present [91, 92]. For example, Hoogstraate (1996) [93] observed in *in vitro* experiments using porcine buccal mucosa that 10 mM sodium glycodeoxycholate (SDGC) was able to enhance the flux of fluorescein isothiocyanate–dextran (a hydrophilic macromolecule having a molecular mass comparable to that of therapeutic peptides) only through the paracellular route. However, higher concentrations (100 mM SDGC) enhanced permeability of this macromolecule through both the paracellular and transcellular pathways.

However, it should be noted that higher concentrations of surfactant may not always necessarily correlate with improved buccal absorption. Physico-chemical features such as the log P and charge of the macromolecule should also be taken into account when considering surfactants or bile salts for buccal absorption studies. For example, the impact of different concentrations of SDGC at 1, 2, and 5% on the buccal mucosal absorption of calcitonin has been assessed, with 1% being found to be the optimal concentration [33]. It was suggested that, because calcitonin is transported via the paracellular pathway (due to an effect of its strongly positive charge), it is unlikely that high surfactant concentration (and therefore involvement of transcellular permeation) would have an impact on such a strongly charged entity. Although Nicolazzo *et al.* (2004) [86] have considered small molecules in their studies, they also suggest that the effect of surfactants on buccal absorption is governed not only by surfactant concentration, but also the physicochemical properties of the permeant. For example, while sodium dodecyl sulfate (SDS) increased the porcine buccal penetration of the hydrophilic marker caffeine (probably through increased paracellular diffusion), SDS either had no impact on the absorption of the lipophilic steroid estradiol, or in fact reduced estradiol absorption at concentrations of SDS above its critical micellar concentration. This result was associated with micellar solubilization of the lipophilic estradiol, which would decrease the concentration of free estradiol available for absorption – an effect that would not have occurred for the hydrophilic caffeine. As peptides are generally hydrophilic, it is expected that such micellar solubilization would not occur with increasing concentrations of surfactant. However, for those with limited aqueous solubility, a reduction in buccal permeability might be expected with higher concentrations of surfactant and bile salt, and therefore, when assessing the impact of such agents on macromolecular transport, various concentrations of these enhancers should be assessed.

In addition to the micellar solubilization and lipid-solubilizing effects of bile salts and surfactants, bile salts may also cause inhibitory effects on buccal membrane peptidases, which would contribute indirectly to improving buccal absorption. Dihydroxy bile salts (sodium deoxychocolate, sodium glycodeoxycholate, sodium taurodeoxycholate, sodium chenodeoxycholate, sodium glycochenodeoxycholate, sodium taurochenodeoxycholate) have been shown to reduce degradation rates of calcitonin in rat oral mucosa homogenate (from 14 h^{-1}/mg protein to 2 h^{-1}/mg protein) [56]. In addition, sodium glycocholate (SGC) is reported to inhibit the proteolytic degradation of insulin 5-fold in homogenates of rabbit buccal mucosa [53]. The selection of new absorption enhancer candidates for macromolecules should consider this aspect, as enhancing the buccal delivery and preventing degradation are both crucial, particularly for peptides and proteins.

It is often necessary to utilise high concentration of synthetic surfactant or bile salts to provide appreciable enhancement in macromolecular buccal absorption, but such concentrations may result in mucosal irritation. For this reason, some researchers have turned to natural surfactants to overcome this limitation. Lysalbinic acid, a non-ionic surfactant obtained from alkaline hydrolysis of albumin, has successfully enhanced the buccal absorption of α-interferon and insulin

Table I Effects of Surfactants and Bile Salts on the Buccal Mucosal Permeability of Macromolecules

Macromolecule	Surfactant or bile salt	Model	Outcome	Reference
Buserelin	Sodium glycodeoxycholate (SGDC)	Buccal delivery devices were administered to pigs *in vivo*	Co-administration of SGDC (0.45%) increased the absolute buccal bioavailability from 1.0% to 5.3%	[171]
Calcitonin (CT)	Sodium deoxyglycocholate (SDGC)	*In vitro* permeation with excised porcine buccal mucosa	Flux of CT increased 18-fold in the presence of SDGC	[33]
Endomorphin-1 (ENI)	Sodium glycocholate (GC) and sodium taurocholate (TC)	*In vitro* permeation with excised porcine buccal mucosa	GC and TC were not effective in enhancing the permeation of ENI	[69]
Fluorescein isothiocyanate (FITC)-labeled dextrans (4,10 and 20 kDa)	Sodium glycodeoxycholate (SGDC), sodium taurodeoxycholate (STDC), sodium glycocholate (SGC) and sodium taurocholate (STC)	*In vitro* permeation with excised porcine buccal mucosa	A maximal enhancement of approximately 2,000-fold was obtained for lower molecular weight dextran (4 kDa) after treatment with SGDC. No significant differences in the degree of permeation enhancement was observed between these four bile salts	[93]
Fluorescein isothiocyanate (FITC)-labeled dextran (4 kDa)	Sodium glycodeoxycholate (SGDC)	Buccal delivery devices were administered to pigs *in vivo*	Co-administration of 10 mM SGDC increased the absolute bioavailability of 4 kDa dextran from 1.8% to 12.7%	[172]
Insulin	Laureth-9 (L9)	Insulin solution was administered to rats *in vivo*	The hypoglycemic response of insulin increased from 3.6 to 27.2% in the presence of L9	[64]
	Laureth-9 (L9), polysorbate 20 (Tween 20), PEG laurate, propylene glycol laurate, sorbitan laurate, glyceryl monolaurate, octoxynol-9, sodium dodecyl sulfate (SDS), sodium glycocholate (SGC), sodium deoxycholate (SDC), sodium laurate (SL), sodium lauryl sulfate (SLS)	Administration of solutions to rats *in vivo*	In the absence of an absorption enhancer, buccal insulin was less than 4% as effective as i.m. insulin. Bile salts (SGC and SDC), SL, SLS and L9 were the most effective (efficacy relative to i.m. insulin > 20%)	[173]
	Sodium cholate (SC), sodium aurocholate (STC), lysophosphatidylcholine (LPC)	Buccal administration of insulin in anesthetized dogs *in vivo*	Glucose back permeation flux remained unchanged for SC and it decreased by 80% 5–8 h after exposure to STC and LPC (>hypoglycemic effect)	[82]
	Brij-35, sodium taurocholate (STC), sodium lauryl sulfate (SLS), sodium deoxycholate (SDC)	Application of solution formulation to rabbits *in vivo*	Brij 35 resulted in the greatest hypoglycemic effect of insulin, followed by STC, SLC, and SDC	[174]
	Octylglucoside and dodecylmaltoside	Buccal administration of solution formulation to rats *in vivo*	The hypoglycemic effect increased from 1% (control) to 20 and 30% in the presence of octylglucoside and dodecylmaltoside, respectively	[175]
	Soybean lecithin	Application to rabbits and rats *in vivo*	Blood glucose levels decreased significantly in diabetic rabbits (54%) and rats (60%) after buccal administration with soybean lecithin	[67]
	Lysalbinic acid	*In vitro* permeation with excised hamster cheek pouch	Lysalbinic acid increased buccal permeability of FITC–insulin 5-fold	[94]
α-interferon	Sodium taurocholate (STC), Tween 80 and sodium dodecyl sulfate (SDS)	Application of buccal preparation to rats *in vivo*	Buccal bioavailability increased 18-, 6- and 8-fold with the	[176]

Table I (continued)

Macromolecule	Surfactant or bile salt	Model	Outcome	Reference
			addition of STC, Tween 80 and SDS, respectively	
	Lysalbinic acid	*In vitro* permeation with excised hamster cheek pouch	Lysalbinic acid increased α-interferon transport 6–9 fold	[94]
Luteinizing hormone releasing hormone (LHRH)	Sodium taurodeoxycholate (STDC), sodium deoxycholate (SDC) and sodium cholate (SC)	Bilayer mucoadhesive devices were administered to beagle dogs *in vivo*	The following rank order was observed for relative buccal bioavailability: SDC > SC > STDC (237, 151 and 84%, respectively)	[120]
Pituitary adenylate cyclase-activating polypeptide (PACAP)	Sodium deoxycholate (SDC), cetrimide	*In vitro* permeation with excised porcine buccal mucosa	Buccal permeation enhancement of PACAP was 18.6-and 46.5-fold in the presence of SDC and cetrimide, respectively	[177]
Recombinant human basic fibroblast growth factor (rhbFGF)	Sodium glycocholate (SGC)	*In vitro* permeation with excised rabbit buccal mucosa	Flux of rhbFGF increased 2.3-fold with the addition of SGC	[178]
Phosphorothioate antisense oligonucleotide (ISIS 3082)	Sodium glycocholate (SGC)	*In vitro* permeation with excised porcine buccal mucosa	Co-administration of 10 mM SGC increased the buccal permeability of ISIS 3082 17-fold	[179]

*Studies in which macromolecule has been tested alone (no enhancer) were not considered in this analysis.

through hamster cheek mucosa. Co-administration of 1 and 5% lysalbinic acid resulted in 5- and 9-fold increases, respectively, in the buccal permeability of α-interferon [94]. Furthermore, 0.3% of this surfactant was able to increase the buccal permeability of insulin 5-fold through hamster cheek pouch [94]. While promising, these results should be extrapolated to human buccal mucosal absorption with caution due to the fact that the hamster mucosa is keratinized [95], whereas that of humans is non-keratinized [96]. Furthermore, potential buccal mucosal-irritating effects of lysalbinic acid have not been investigated, and the mechanism by which this natural surfactant increases macromolecular transport has not been fully characterized, albeit intercellular lipid solubilization has been proposed as one mechanism [94]. Therefore, while this natural surfactant may offer some potential benefit in enhancing macromolecular buccal absorption, further studies on its impact on the absorption of peptides and proteins and irritation on a non-keratinized buccal mucosa (e.g. porcine buccal mucosa, or preferably, human buccal mucosa) would be required.

Fatty Acids

Fatty acids have been shown to increase the buccal absorption of insulin [97], [D-Ala^2, D-Leu^5]enkephalin [98] and ergotamine tartrate [99]. Although the mechanism of penetration enhancement of fatty acids on buccal absorption has not been fully elucidated, it is suggested that fatty acids decrease the lipid packing in buccal epithelial cells [100]. Factors such as fatty acid chain length of the triglyceride, the degree of saturation and the volume of lipid administered have been shown to influence oral absorption [101] and could also potentially affect buccal delivery, and should be considered during development of buccal formulations.

Oleic acid, one of the most widely investigated fatty acids, has been incorporated into the cubic phase of glyceryl monooleate (GMO) to improve the *in vitro* buccal permeability of [D-Ala^2, D-Leu^5]enkephalin, an opioid peptide with analgesic properties [98]. When incorporated in the cubic phase of GMO alone, oleic acid had limited permeability enhancing effects, whereas a significant enhancing effect was observed with the addition of PEG 200 (enhancement ratios ranging from 1.15 to 3.99 depending on the PEG concentration). The addition of PEG 200 also increased the *in vitro* release of oleic acid from the formulation up to 7-fold. In another study, the effect of oleic acid on the buccal permeability of ergotamine tartrate (molecular mass 1,313 Da), also known as peptide-type ergot alkaloid, was investigated and compared with that of cod-liver oil extract (CLOE) using a keratinized epithelial-free membrane of hamster cheek pouch mucosa [99]. Pretreatment with CLOE provided 8- and 2-fold higher permeability flux and solubility, respectively. Although pretreatment with oleic acid increased the permeation of ET approximately 2-fold, this enhancing effect was approximately 3-fold lower than that of CLOE. Given that the effect of each fatty acid (eicosapentaenoic acid, docosahexaenoic acid, palmitic acid, oleic acid) on the permeation flux of ET was significantly lower than that of CLOE, a synergistic action of the major fatty acids was proposed by the authors.

While fluorescence polarization studies have suggested that oleic acid strongly reduces the lipid packing in both the hydrophobic and the polar head-group region of the non-keratinized buccal epithelial cell bilayer [100], Coutel-Egros *et al.* [102] have suggested that it may improve the permeability of compounds by increasing their partitioning into the tissue, although there is little evidence to support this hypothesis. Several experiments have been performed to explain the mechanism of the effect of oleic acid on the transdermal permeation [103], but the results from these studies should not be extrapolated to the buccal mucosa given that the lipid composition and packing are quite different between human skin and buccal mucosa.

Other fatty acids have also been employed to enhance the buccal delivery of peptides. Unsaturated fatty acids (oleic, eicosapentaenoic and docosahexaenoic acid) were added individually into Pluronic F-127 (PF-127) gels in order to maximize the absorption of insulin following buccal administration to Wistar rats [97]. Despite the presence of unsaturated fatty acid actually decreasing insulin release from the gel, a higher buccal bioavailability of insulin was observed in the presence of these fatty acids (with oleic acid increasing the bioavailability to 15.9%). The enhanced absorption of insulin in the presence of these fatty acids was therefore probably not due to an increase in the free fraction of insulin available for absorption, and more likely due to an effect of the fatty acids on the mucosal tissue. Given that the buccal administration of PF-127 gels without unsaturated fatty acids resulted in a negligible bioavailability for insulin (1%), the enhancer effect observed in the presence of these fatty acids is probably also not associated with mucoadhesive properties of the PF-127 gel or increased residence time at the site of absorption. Therefore, while the exact mechanism by which fatty acids increase buccal absorption is not fully characterized, examples of their enhancing effects on peptide and protein buccal absorption have been reported, suggesting they could play an important role in assisting the buccal delivery of otherwise impermeable macromolecules.

Chitosan

Chitosan is a biodegradable and biocompatible polysaccharide which has been widely used in pharmaceutical formulations [104]. Recently, it has also been employed in transmucosal drug delivery devices due to its bioadhesive properties, which result from the cationic charge of the primary amino groups interacting with the negative functional groups of mucus or epithelial cells [105]. It is more likely that, in the buccal mucosa, the mucoadhesive properties of chitosan enhance the residence time of permeants at the surface of the membrane, allowing for enhanced bioavailability. To further enhance the mucoadhesive properties of this polymer, additional chemical modifications have also been undertaken at its two hydroxyl groups (primary or secondary) and one primary amine group [104]. Moreover, chitosan has been combined with 5-methyl-pyrrolididone, a well-known skin permeation enhancer, improving the permeation enhancing properties of this polymer as well as drug release [106].

Several types of chitosan are commercially available, differing in the molecular mass, degree of deacetylation and presence of free or substituted amino groups (chitosan base or chitosan salt). Chitosan hydrochloride and chitosan glutamate are the two most widely used salts [81]. The effect of different concentrations of chitosan glutamate (ranging from 1 to 100 $\mu g\ mL^{-1}$) on the buccal permeability of fluorescein isothiocyanate labeled dextrans (FD) of different molecular mass (4, 10 and 20 kDa) has been evaluated in TR146 cells, an *in vitro* model of the human buccal epithelium [107]. The most effective concentration of chitosan glutamate observed to increase FD buccal absorption was 20 $\mu g\ mL^{-1}$ (which increased the permeability coefficient values 2.9-, 1.8- and 1.7-fold for FD4, FD10 and FD20, respectively). For chitosan concentrations higher than 20 $\mu g\ mL^{-1}$, the solutions become more viscous, and diffusion of the macromolecules was hindered. This again demonstrates the importance of utilizing multiple concentrations of particular enhancers when attempting to improve the buccal mucosal absorption of macromolecules, as higher concentrations do not always correlate with enhanced permeability.

Since chitosan is only soluble in acidic solutions (pH below 6.5), which is required to ensure protonation of the primary amine, there are limitations to its use for delivery in physiological systems. An elegant way to impart new properties to chitosan is through chemical modification of the chain, generally by grafting of functional groups, without modification of the initial skeleton [104]. Sandri *et al.* [108] subjected two different chitosans (molecular mass of 1,460 and 580 kDa) to methylation reactions obtaining two series of three trimethyl chitosan (TMC) derivatives and evaluated the influence of different quaternization degree (4, 35 and 90%) on buccal permeability of FD4. FD4 was selected as a model because it has a molecular weight comparable with that of peptides. The TMC derived from the lower MW chitosan and characterized by the highest degree of quaternization presented the best mucoadhesive and penetration enhancement properties (with the amount of FD4 permeating porcine buccal mucosa being approximately 8-fold higher compared to the control after 6 h at pH 6.4). This experiment was conducted at pH 6.4 because the trimethylation of chitosan did not present any change in its penetration enhancement properties when an aqueous medium was selected. Therefore, these TMCs are likely to be the most appropriate chitosans for improving the buccal bioavailability of hydrophilic molecules and biomacromolecules such as peptides and proteins. In another study, the same researchers investigated the mechanism responsible for the

penetration enhancing effects of TMC hydrochloride and suggested that it involved a repackaging of the epithelial cells up to the basal membrane and a partial disarrangement of desmosomes [109].

Thiolated chitosans have also been developed to improve buccal permeability via immobilization of thiol groups on the primary amino groups of chitosan [110]. These thiolated chitosans have numerous advantages over unmodified chitosan, such as significantly improved mucoadhesive and permeation-enhancing properties since thiol groups are able to form disulfide bonds with cysteine-rich subdomains of mucus glycoproteins [111, 112]. For example, the mucoadhesive properties to porcine buccal mucosa of a chitosan-4-thio-butylamidine conjugate were shown to be 140-fold greater than unmodified chitosan [113]. Moreover, because of the ability of thiomers to bind divalent metal ions such as zinc ions, these polymers may inhibit zinc-dependent proteases such as carboxypeptidases A and B, as well as most membrane-bound peptidases [114]. The strong adhesive properties of thiolated chitosans render them highly appropriate excipients in controlled drug release dosage forms [113]. With this in mind, Langoth *et al.* exploited thiolated chitosans to enhance the bioavailability of pituitary adenylate cyclase-activating polypeptide (PACAP) [115]. A buccal bioavailability of approximately 1% was obtained in pigs following buccal application of PACAP delivery systems consisting of thiolated chitosan, whereas no PACAP was detected in the plasma of pigs in the absence of thiolated chitosan. In addition, the authors suggest that cationic therapeutic peptides should be embedded in a cationic or nonionic mucoadhesive polymer such as thiolated chitosans because incorporation in anionic polymers may retard peptide release due to strong ionic interactions between the peptide and the polymeric network [115]. Therefore, formulation scientists should also consider the matrix in which peptides and proteins are formulated, so as to not only enhance permeability but ensure that release of the peptide is not hindered.

pH Modulation

Modulation of pH can affect the buccal delivery of peptides and proteins via two potential mechanisms: through ensuring the peptide is in an unionized form to maximize permeability or through enhancing stability. The control of pH is critical for the successful buccal delivery of ionizable compounds [116, 117]; as with all other biological membranes, maximal permeability occurs at the pH at which macromolecules are predominantly in the unionized form [118]. pH modifiers can be included in formulations to temporarily modulate the microenvironment at the application site, improving not only buccal absorption [50] but also stability against enzymatic degradation given that various enzyme-catalyzed degradation processes are pH-dependent [119]. Acidic and basic pH modifiers have been added into luteinizing hormone releasing hormone (LHRH)-loaded mucoadhesive formulations to evaluate the effect of pH on buccal absorption in Beagle dogs [120]. It was demonstrated that the protonation of the peptide's histidine in the acid pH formulation resulted in a higher plasma profile and greater bioavailability (C_{max} and bioavailability were, respectively, 3.1 and 2.6 higher than those of formulation without pH modifier), although higher mucosal irritation response was also observed. Similarly, at a higher pH where the histidine was in a non-protonated form, the buccal absorption of this peptide was dramatically reduced. These simple studies demonstrate that the pH-partition hypothesis is also relevant for macromolecular drug delivery, although the issues of tissue irritation with pH modification should always be considered.

In addition to modifying the extent of ionization of permeating molecule, alterations to pH can also impact on the beneficial effects of excipients. Polymers are often used to prepare mucoadhesive formulations for buccal delivery and they can be ionized to different extents depending on the pH, which may affect the strength of mucoadhesion and subsequently the buccal permeability of the formulated macromolecule. Polycarbophil®, a polyacrylic acid cross linked with divinyl glycol, is one such polymer that has been used to prepare mucoadhesive formulations for improvement of buccal transport of low molecular weight drugs. Studies have demonstrated that at a pH higher than the pKa of polyacrylic acid, polycarbophil tends to absorb water up to 100–800 times its weight, which compromises the adhesive interactions between the buccal mucosa and the mucoadhesive formulations prepared by polyacrylic acid [121]. Therefore, pH control strategies may affect not only the ionization state of the macromolecule and its inherent permeability across the buccal mucosa, but also the state of the polymers used to prepare the matrix. Such factors should be considered by the formulation scientist so as to not jeopardize mucoadhesion, and therefore residence time of the macromolecule on the mucosa.

Another aspect to be considered with respect to pH changes is the ionization state of epithelial or mucus proteins, which may also play an important role in the buccal transport of peptides and proteins. At physiological pH or at a pH above the isoelectric point (pI), epithelial structures (due to their sialic acid and sulfate residues) are negatively charged and can interact with positively-charged solutes [27]. This could therefore assist in the absorption of positively-charged peptides and proteins, but minimize the absorption of negatively-charged peptides and proteins. Modifying the pH could therefore affect these interactions, potentially compromising the

overall buccal absorption of positively-charged peptides and proteins.

Iontophoresis

Recently, iontophoresis has been used to improve delivery of molecules across biological barriers, particularly the skin [122, 123]. Iontophoresis is a non-invasive and patient-friendly method that enables hydrophilic charged molecules to penetrate through biological barriers to achieve both local and systemic effects [123]. Iontophoresis enhances the rate of movement of ionic compounds across membranes by an externally applied electric potential [124]. The iontophoretic system consists of a donor solution containing the drug in its ionized form and a receptor solution separated by a limiting membrane, in this case buccal mucosa, where a voltage is generated by connecting an anode and cathode to a voltage source that supplies direct constant electric current. The circuit is completed as the ions carry the current through the tissue barrier. Similar to the skin, the buccal membrane has a net negative charge, facilitating the delivery of positively-charged compounds [125]. This method therefore has potential to be applied to buccal delivery systems of drugs with poor penetration properties, particularly proteins, peptides and oligonucleotides [126].

Molecular transport during iontophoresis can be attributed to three component mechanisms: (enhanced) passive diffusion, electromigration and convective solvent flow, also called electroosmosis [123]. Epithelial proteins from buccal mucosa are negatively charged at physiological pH and may act as a cation-selective ion-exchange membrane [127]. As a consequence, under the influence of an electric field, a convective solvent flow is generated in the anode-to-cathode direction. Assuming that each phenomenon is independent, the total flux of a molecule during iontophoresis can be described as the sum of the fluxes resulting from those three processes described above [123].

Results from *in vitro* transbuccal experiments using iontophoresis suggest that the total drug flux is related to the current density applied and the initial donor concentration under the influence of competitive ions [22]. Previous transdermal studies have considered that a level of electrical current of approximately 0.5 mA is physiologically acceptable, although Guy suggests a reduction in these levels for buccal mucosa given its lower barrier properties compared to skin [128]. While iontophoresis has been effective in delivering macromolecules through the skin [126], iontophoretic parameters should be considered carefully as this technique can negatively affect physicochemical stability of macromolecules [129]. For example, 65% of thyrotropin-releasing hormone has been reported to degrade when a current value of 0.5 mA was applied [130]. Therefore, studies monitoring peptide stability should be carried out until appropriate iontophoretic parameters are identified, including the orientation of the electrode. Depending on the charge of the macromolecule, the electrode orientation for successful iontophoretic delivery may be cathode-to-anode (anodal) or anode-to-cathode (cathodal) [131]. The selection of appropriate electrodes is also highly relevant in order to avoid tissue irritation, reduction in drug stability and variations in release of the drug. Ag/AgCl active electrodes are commonly selected because inactive electrodes such as carbon or platinum induce proton production, which may lead to the above-mentioned issues [132].

The enhancing effect of iontophoresis has been investigated for the buccal mucosal delivery of four model macromolecular compounds (dextrans – 3 and 10 kDa, bovine serum albumin – 64 kDa and parvalbumin – 12 kDa) [133]. The effect of parameters such as electrode polarity, pH of the donor solution and different levels of electric current on buccal absorption was also evaluated. Dextrans and parvalbumin were successfully delivered across porcine buccal mucosa after an anodal pulsed electrical stimulation (cathode to anode orientation). The enhancement in flux following application of iontophoresis ranged from 32 to 38-fold for dextrans and 36-fold for parvalbumin. It was reported that iontophoretic delivery was approximately 37 times faster than passive diffusion. These results clearly support the use of a physical technique to efficiently deliver peptides and proteins through the buccal mucosa. However, the iontophoretic delivery of peptides with a pI between 4 and 7 has been challenging. As the peptide becomes uncharged, the iontophoretic force will no longer apply; alternatively, as the peptide cations permeate the tissue, their concentration will decrease and at some point will be neutralized by the higher pH values, leading to the precipitation of peptide [134, 135]. There also might be a molecular size cut off for the beneficial effects of iontophoresis. For example, iontophoresis was shown to have no effect on bovine serum albumin (molecular mass 66 kDa), whereas it had a 36-fold enhancing effect on parvalbumin (molecular mass 12 kDa) [133].

Given that macromolecules in solution are not readily amenable to incorporation into iontophoretic devices, semi-solid formulations such as hydrogels may be required. These dosage forms should present optimal mechanical properties, appropriate electro-conductivity, bioadhesion and viscoelastic properties. In this context, three macromolecular model compounds (3 and 10 kDa dextrans and 12 kDa parvalbumin) were incorporated into polymeric hydrogel delivery systems based on poly(vinyl alcohol) (PVA), hydroxypropyl methylcellulose (HPMC) and PVA/HPMC binary systems, which were subjected to iontophoresis [136]. The iontophoresis was included with the aim of enhancing the release of these permeants from hydrogels and their subsequent transfer through the buccal mucosa. Enhancement ratio (ER) values for each of the three sandwich systems (hydrogel and buccal

mucosa) were significantly higher than for the buccal mucosa alone. ERs for parvalbumin were lower than those obtained for the two dextrans (47.0–54.6 *vs.* 32.7–39.5), which may be related to its higher molecular weight, while ERs for 10 kDa dextran were lower than those obtained for 3 kDa dextran. The three hydrogel systems enhanced iontophoretic delivery for each of the three permeants, suggesting that hydrogels have potential as delivery vehicles for exploiting iontophoretic enhancement.

The combination of chemical penetration enhancers and iontophoresis has also been reported recently [33]. In this study, the *in vitro* delivery of salmon calcitonin through porcine buccal mucosa was evaluated. In the presence of iontophoresis alone, the buccal absorption of calcitonin was 66-fold higher than in the absence of iontophoresis. The combination of iontophoresis and chemical enhancers (5% sodium deoxyglycocholate in 10% ethanol) further enhanced the transbuccal delivery of calcitonin 165-fold, demonstrating that the combination of iontophoresis and chemical penetration enhancers is a viable approach for enhancing peptide and protein buccal delivery. The synergistic effect has been attributed to enhancement of the interaction with polar intercellular lipids from bile salts, which then improves the effectiveness of iontophoresis because it enhances drug delivery mainly through the paracellular pathway, and calcitonin is primarily absorbed by this route [33]. More importantly, the combination of chemical penetration enhancers and iontophoresis would not be more complicated than the processes required to set up iontophoresis alone (with the latter likely to be the limiting factor in the clinical utility of this enhancement approach) [137].

In future studies exploring the potential of this technique, it is suggested that different parameters should be evaluated simultaneously in order to optimize the effect of iontophoresis on the buccal absorption of macromolecules; these include the composition of formulation (drug concentration, pH of donor solution, presence of co-ion, ionic strength), physicochemical properties of permeant (molecular weight, lipophilicity, charge) and experimental conditions (current density and profile, duration of treatment, electrode material and polarity of electrodes) [138].

Novel Formulation Approaches

Particulate Systems/Delivery Vectors

Particulate systems such as emulsions, liposomes, micro- and nanoparticles have been widely used and remain a promising approach for buccal delivery of macromolecules. The design and development of colloidal systems, which are very fine solid particles (typically ranging from 10 nm to 10 μm) suspended in a fluid phase, can help to overcome limitations of macromolecular buccal transport such as poor stability, low bioavailability and the associated short half-lives of peptides and proteins [139].

Among the hydrophilic polymers studied, chitosan appears to be the most suitable polymer given that it is able to retain the peptide in the buccal mucosa, as described above. For example, insulin-loaded chitosan-ethylenediaminetetraacetic acid hydrogel films exhibited a mucoadhesive force over 17,000 N/m^2 which remained for 4 h in a simulated oral cavity. This approach resulted in a pronounced hypoglycemic effect following buccal administration to healthy rats, leading to a 17% pharmacological availability (which is obtained by comparing areas above the glucose reduction–time curve between oral and subcutaneous administration) when compared to a subcutaneous insulin injection [140].

Alternatively, the use of alginates and poloxamers (both hydrophilic) as encapsulation materials also represents a promising approach to overcome those restrictions (poor stability, low bioavailability and the associated short half-lives of peptides and proteins). Such materials are able to swell substantially and retain significant fractions of water in their structure without dissolving, which can lead to the formation of hydrogels [141]. Due to their high biocompatible and hydrophilic nature, hydrogels offer a preferable environment for peptide and protein formulation and have been shown to stabilize the complex structure of proteins [142, 143].

Although more hydrophobic polymers have been preferentially selected to increase the affinity to lipid bilayers, this could potentially lead to precipitation of proteins during storage or under specific physiological conditions due to differences in the polarity of proteins and carriers and physiological medium [144]. Thus, the addition of surfactants to increase the affinity between protein and polymer or the incorporation of peptides and proteins in hydrophilic polymers such as polyethylene glycol, chitosan, alginates and poloxamers may be alternatively used [141]. With the recognition of the role of surfactants in increasing the affinity between proteins and polymers, lecithin and propanediol encapsulated in nanostructured systems have been utilized in an attempt to improve the buccal delivery of insulin in rabbits [145]. The average bioavailability of insulin-loaded nanoparticle systems was 18.3% (buccal delivery *versus* subcutaneous injection), indicating that this system may be promising for the buccal delivery of insulin and other proteins.

Diblock copolymers composed of a poly(D,L-lactide) (PLA) core with a hydrophilic chain of poly(ethylene glycol) (PEG) have also been considered for the delivery of peptides and proteins through the buccal mucosa. Nanoparticle systems coated with PEG have been designed to increase the stability of insulin when in contact with physiological fluids and provide a controlled release [139]. Moreover, it is well established that PEG has a mucoadhesion-promoting effect [146]. Although Giovino *et al.* have suggested that nanoparticles coated with PEG can provide controlled buccal delivery of proteins based on *in vitro* release experiments, buccal transport studies from optimized systems were not carried out [139]. In another

study, biodegradable and redox-responsive complex systems have been prepared for the transmucosal delivery of proteins and peptides. Insulin-loaded chitosan/poly (L-aspartic acid) submicron capsules prepared using the layer-by-layer technique were shown to release the peptide from these systems when exposed to different levels of glutathione [147]. This system has shown acceptable biocompatibility (cell viability was above 90%) and mucoadhesive properties (adsorbed mucin up to 48.1 μg/mg submicron capsules), extending the residence time after mucosal administration. Despite these *in vitro* release studies, the buccal absorption of insulin from these novel particulate systems has not been investigated, but remains an interesting field to explore.

The combination of particulate systems and bile salts has been assessed to determine whether this leads to improved buccal delivery of macromolecules. Phospholipid deformable vesicles (transfersomes) with and without sodium deoxycholate have been prepared by reverse phase evaporation methods and their impact on the delivery of encapsulated insulin across the buccal mucosa assessed [148]. While differences in the *in vivo* buccal permeability of insulin were not observed between vesicles with and without surfactant, the relative pharmacological bioavailability and the relative buccal bioavailability after delivery of the insulin-deformable vesicles to rabbits were 15.6 and 19.8%, respectively (compared to subcutaneous administration of insulin solution). This effect was significantly higher than with conventional insulin vesicles, blank deformable vesicles and insulin mixture groups. It has been suggested that transfersomes may respond to external stresses by rapid shape transformations requiring low energy, allowing them to deliver macromolecules across barriers. Given that these systems allow for significant interaction with the buccal mucosa, their use for buccal delivery of other peptides and proteins might be considered in the future [148].

Immobilized Drug Delivery Systems

In view of the fact that aqueous suspensions and solutions do not provide high retention of drugs at the buccal mucosa due to continuous dilution by salivary flow, particles have been immobilized in buccal films or other solid systems. The buccal mucosa is a very suitable region for bioadhesive systems because of its smooth and relatively immobile surface as well as its easy accessibility [42].

Mucoadhesive systems are essential to maintain an intimate and prolonged contact of the formulation at the absorption site, resulting in continual absorption of therapeutic into the systemic circulation [24]. The process of mucoadhesion consists of two main steps: the contact step (wetting) followed by the consolidation step (the establishment of the adhesive interactions). While the first step of this process is characterized by an intimate contact between the mucoadhesive and mucous membrane, various physicochemical interactions may occur in the second step to consolidate and strengthen the adhesive joint, leading to prolonged adhesion [149, 150]. The relative importance of each step will depend on the individual application. If the formulation is exposed to significant dislodging stresses such as mouth movements and strong or prolonged adhesion is required, a second consolidation step should be ensured [151]. Given that peptide and protein therapeutics generally result in low bioavailability, both steps need to be optimized so that they may lead to enhanced mucoadhesion. It is also desirable that the polymers employed are able to inhibit key proteolytic enzymes.

Two theories may be considered to explain the consolidation stage, the diffusion theory and dehydration theory, which are not applicable for highly hydrated dosage forms. According to the diffusion theory, the mucoadhesive molecules and the glycoproteins of the mucus mutually interact by means of interpenetration of their chains and the formation of secondary interactions [151]. For this to occur, the mucoadhesive device should present features favoring both chemical and mechanical interactions. In general, bioadhesive strength increases with the molecular weight, mucoadhesive polymer concentration, presence of hydrogen bond-forming groups (hydroxyl, carboxyl, amines and amides), chain flexibility, positively or negatively charged groups and reduced cross-linking density [152]. Physiological factors such as saliva secretion, food intake, local pH, and turnover of the mucus layer also strongly affect bioadhesion [42]. According to the dehydration theory, materials that are able to readily form gels in an aqueous medium when placed in contact with the mucus can lead to dehydration due to an osmotic pressure difference [151, 153]. Therefore, this concentration gradient draws water into the formulation until an osmotic balance is achieved, leading to the mixture of formulation and mucus. It is the water motion that consolidates the adhesive bond, and not the interpenetration of macromolecular chains [151]. Once this stage is understood, it is possible to select suitable polymers or other materials for preparing new formulations mucoadhesive according to its application.

Solid bioadhesive dosage forms are generally more convenient for the patient than gels and ointments [154], and solid systems typically offer greater drug stability and improved residence time at the buccal mucosa, potentially offering improved buccal mucosal absorption [155]. The design of buccal dosage forms, particularly films, patches and tablets may include (i) single-layer devices from which the drug is released multidirectionally, (ii) devices presenting an upper impermeable layer that minimizes the loss of drug into the oral cavity and prevents degradation of drug by salivary enzymes, or (iii) unidirectional release devices, from which drug loss is minimal since the drug is released only from the side adjacent to the buccal mucosa [42]. Buccal tablets have been the most commercially available dosage form for buccal

mucosal delivery, but the lack of physical flexibility has led to poor patient compliance for long-term and repeated dose, and other alternatives have been studied. Mucoadhesive buccal films share some of these disadvantages, but because of their small size and thickness, and their flexibility, they result in improved patient compliance [156].

In addition to traditional solid mucoadhesive forms, solid systems such as resorbable polymeric wafers and sponges have been proposed recently to deliver macromolecules through the buccal mucosa [157, 158]. However, there are limited examples of their use for the delivery of peptides and proteins across the buccal mucosa. Unlike semi-solid polymer gels, wafers can maintain their swollen gel structure for a longer period and therefore exhibit a longer residence time [159]. Because of their porous nature and higher surface area, they are able to contain higher amounts of drug compared to the thin and continuous solvent cast equivalent [160]. Polymeric wafers are usually prepared by freeze-drying techniques, and such wafers may provide an extended shelf life of the active permeant at room temperature offering more stable products that are suitable for storage at room temperature [158]. Given their advantages, research into the applicability of these systems for peptide and protein delivery should be considered.

Portero *et al.* [157] have also suggested the use of chitosan sponges as carriers of macromolecules in buccal systems. As insulin-loaded bilayered tablets presented poor release behavior (5% of the peptide being released over 10 h), which was attributed to the limited diffusivity of the insulin through compact matrixes, this group produced flexible sponges by the casting/freeze-drying technique, which presented a very porous structure and resulted in significantly faster *in vitro* release profiles. As would have become apparent from the previous paragraphs, there have been many suggestions of novel delivery devices for buccal mucosal delivery of peptides such as wafers and sponges, but these have not yet translated into *in vitro* or *in vivo* studies justifying their potential. Significant research into their applicability for actually delivering

Table II Advantages and Disadvantages of the Approaches to Enhance the Buccal Permeability of Macromolecules

Approach	Advantages	Limitations
Chemical modification	- Improved lipophilicity to increase permeability - Improved lipophilicity to minimize enzymatic degradation - Increased uptake into buccal mucosa potentially leading to controlled release into the systemic circulation	- Modified peptides and proteins may be chemically unstable – Problems with storage or shelf-life - Modified peptides and proteins may not be pharmacologically active - If conversion of the modified peptide or protein is enzyme dependent, high inter-patient variability may be observed
Chemical enhancers	- The possibility to combine different chemical enhancers reducing their concentration and enhancing the buccal absorption by different mechanisms - Potential reduction in enzymatic degradation	- May increase the absorption of xenobiotics. - May lead to membrane damage
pH modulation	- Potential to modulate the buccal delivery of ionizable peptides and proteins. - Potential to reduce enzymatic degradation - Potential to positively impact on mucoadhesive activity of excipients	- The difficulty in finding an optimal pH for both peptide and protein stability without mucosal irritation - Potential to negatively impact on mucoadhesive activity of excipients
Iontophoresis	- Potential to control the release kinetics by adjusting the electrical parameters. - High degree of programmability - therapy can be tailored to match the patient's requirements (blood levels of peptide or protein)	- Potential for irritation and pain on application of the electric current. - Accessibility and costs - May be restricted to compounds that can be formulated in their ionic form - Charged species may also be affected by an ionic competition
Particulates systems/ delivery vectors	- May provide a sustained release - May minimize chemical and enzymatic stability - The potential to add components with mucoadhesive properties	- Stability problems, particularly with liposomes - Incompatibility between hydrophobic polymers and peptides
Immobilized drug delivery systems	- Potential to maintain an intimate and prolonged contact of the formulation at the absorption site - Devices can present a unidirectional or multidirectional release according to intended use. - Systems (eg. polymeric wafers) can be prepared by freeze-drying technique, overcoming limitations associated with peptide and protein formulation	- Complex formulation approaches may be expensive - Rigid solid systems may result in low patient compliance
Anti-aggregating agents	- Prevention of *in vivo* protein aggregation and therefore inactive protein state - Potential combination chemical enhancers, maximizing enhancement in buccal absorption	- Low *in vitro-in vivo* correlation regarding this phenomenon (complex to predict this phenomenon *in vivo*)

macromolecular therapeutics should therefore be the focus of pharmaceutical and formulation scientists focusing on buccal mucosal delivery, if the potential of the buccal mucosa as an alternative site of delivery is to be recognized.

Addition of Anti-Aggregating Agents

Although the addition of anti-aggregating agents in buccal formulations seems promising for improving the buccal absorption of macromolecules, its effectiveness has not yet been assessed. The addition of anti-aggregating agents would be an indirect approach to reduce or avoid physico-chemical instability of macromolecules (self-aggregation phenomena, a common process for some peptides [161]), which could lead to precipitation and thus a reduction in buccal absorption. Many soluble proteins have been converted into insoluble fibrils due to self-aggregation phenomena occurring at the intermolecular level under particular solvents, temperature and pH conditions [161]. Nonspecific forces, such as hydrogen bonding could also contribute to this protein self-assembly [162]. Moreover, the lipid bilayer has been suggested to provide an environment in which the aggregated state of polypeptide chains appear to be more thermodynamically favorable than their monomeric forms [163]. In fact, protein aggregation has been recognized as a major reason for instability that can then impact on protein function *in vitro* and *in vivo* and result in toxic adverse events [164]. Overlooking the experimental and physiological conditions that accelerate the self-association of these macromolecules may lead to a reduction of their absorption in different biological membranes such as the buccal mucosa. Indeed, this self-association behavior has been reported for the two peptides most commonly tested in buccal transport experiments – calcitonin and insulin. Calcitonin has shown a tendency to aggregate in aqueous solutions and to form long, thin fibrillar aggregates, resulting in viscous and turbid dispersions easily detected by microscopic techniques [165]. *In vitro* insulin fibrillation occurs quite rapidly, particularly at low pH, high temperature, high ionic strength and on hydrophobic surfaces [164]. Thus, additives or co-solvents that prevent protein aggregation could be employed in order to stabilize macromolecules such as insulin and calcitonin in buccal permeability studies. Compounds that can either prevent unfolding of the native protein or sequester partially folded aggregation-competent intermediates have been effective in increasing the stability of native insulin [166, 167]. Carbohydrates and glycerols as well as low molecular weight compounds such as ectoine, trehalose, and citrulline have been shown to enhance the stability of insulin [166] through preferential exclusion of these co-solutes from the protein surface and subsequent enhancement of hydrophobic interactions within the native structure [168, 169]. Lecithins, cyclodextrins, and polymeric surfactants reduce insulin aggregation by binding to hydrophobic interfaces or hydrophobic insulin domains [167, 170]. Therefore, applying these compounds has the potential to prevent protein aggregation, potentially optimizing the macromolecular buccal delivery.

SUMMARY

Physical, chemical and formulation techniques have been applied to improve the buccal transport of peptides and proteins through the paracellular route, each with its particular advantages and limitations (as shown in Table II). When attempting novel approaches to enhance the buccal mucosal permeability of peptides and proteins, the particular physical and chemical characteristics of each macromolecule should be considered, as well as the impact of the enhancement process on the macromolecule itself and the potential for toxicity or tissue damage. To achieve optimal buccal penetration of peptides and proteins, it appears that a combination of various approaches that are able to enhance the stability, solubility and permeability of peptides and proteins is most suitable. Given the desirable characteristics of the buccal mucosa relative to the gastrointestinal tract and other biological membranes, exploiting this route with appropriate enhancement strategies has the potential to result in therapeutic systemic concentrations of otherwise poorly permeable macromolecules, overcoming the current limitations associated with peptide and protein delivery.

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REFERENCES

1. Adrio JL, Demain AL. Recombinant organisms for production of industrial products. Bioeng Bugs. 2010;1:116–31.
2. Swaan PW. Recent advances in intestinal macromolecular drug delivery via receptor-mediated transport pathways. Pharm Res. 1998;15:826–34.
3. Brown LR. Commercial challenges of protein drug delivery. Expert Opin Drug Deliv. 2005;2:29–42.
4. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug Discov Today. 2010;15:40–56.
5. Antosova Z, Mackova M, Kral V, Macek T. Therapeutic application of peptides and proteins: parenteral forever? Trends Biotechnol. 2009;27:628–35.

6. Osborne R. Fresh from the biotech pipeline-2012. Nat Biotechnol. 2013;31:100–3.
7. Yamamoto A, Iseki T, Ochi-Sugiyama M, Okada N, Fujita T, Muranishi S. Absorption of water-soluble compounds with different molecular weights and [Asu^{1,7}]-eel calcitonin from various mucosal administration sites. J Control Release. 2001;76:363–74.
8. Mahato RI, Narang AS, Thoma L, Miller DD. Emerging trends in oral delivery of peptide and protein drugs. Crit Rev Ther Drug Carrier Syst. 2003;20:153–214.
9. Motlekar NA, Youan BB. The quest for non-invasive delivery of bioactive macromolecules: a focus on heparins. J Control Release. 2006;113:91–101.
10. Sharma S, Kulkarni J, Pawar AP. Permeation enhancers in the transmucosal delivery of macromolecules. Die Pharm. 2006;61: 495–504.
11. Jitendra PK, Sharma S, Bansal AB. Noninvasive routes of proteins and peptides drug delivery. Indian J Pharm Sci. 2011;73:367–75.
12. Merkus FW, Verhoef JC, Schipper NG, Marttin E. Nasal mucociliary clearance as a factor in nasal drug delivery. Adv Drug Deliv Rev. 1998;29:13–38.
13. Merkus FWHM, Schipper NGM, Hermens WAJJ, Romeijn SG, Verhoef JC. Absorption enhancers in nasal drug delivery - efficacy and safety. J Control Release. 1993;24:201–8.
14. Morimoto K, Uehara Y, Iwanaga K, Kakemi M, Ohashi Y, Tanaka A, *et al.* Influence of absorption enhancers (bile salts) and the preservative (benzalkonium chloride) on mucociliary function and permeation barrier function in rabbit tracheas. Eur J Pharm Sci. 1998;6:225–30.
15. Hussain A, Ahsan F. The vagina as a route for systemic drug delivery. J Control Release. 2005;103:301–13.
16. Chatterjee A, Kumar L, Bhowmik BB, Gupta A. Microparticulated anti-HIV vaginal gel: *in vitro-in vivo* drug release and vaginal irritation study. Pharm Dev Technol. 2011;16:466–73.
17. Sozio P, Cerasa LS, Marinelli L, Di Stefano A. Transdermal donepezil on the treatment of Alzheimer's disease. Neuropsychiatr Dis Treat. 2012;8:361–8.
18. Swaminathan J, Ehrhardt C. Liposomal delivery of proteins and peptides. Expert Opin Drug Deliv. 2012;9:1489–503.
19. Newman S. Improving inhaler technique, adherence to therapy and the precision of dosing: major challenges for pulmonary drug delivery. Expert Opin Drug Deliv. 2014;11:365–78.
20. Rathbone MJ, Drummond BK, Tucker IG. The oral cavity as a site for systemic drug-delivery. Adv Drug Deliv Rev. 1994;13:1–22.
21. Devries ME, Bodde HE, Verhoef JC, Junginger HE. Developments in buccal drug delivery. Crit Rev Ther Drug Carrier Syst. 1991;8: 271–303.
22. Ciach T, Moscicka-Studzinska A. Buccal iontophoresis: an opportunity for drug delivery and metabolite monitoring. Drug Discov Today. 2011;16:361–6.
23. Stratford RE, Lee VHL. Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit - implications in peptide delivery. Int J Pharm. 1986;30:73–82.
24. Patel VF, Liu F, Brown MB. Advances in oral transmucosal drug delivery. J Control Release. 2011;153:106–16.
25. Sudhakar Y, Kuotsu K, Bandyopadhyay AK. Buccal bioadhesive drug delivery–a promising option for orally less efficient drugs. J Control Release. 2006;114:15–40.
26. Pather SI, Rathbone MJ, Senel S. Current status and the future of buccal drug delivery systems. Expert Opin Drug Deliv. 2008;5:531–42.
27. Veuillez F, Kalia YN, Jacques Y, Deshusses J, Buri P. Factors and strategies for improving buccal absorption of peptides. Eur J Pharm Biopharm. 2001;51:93–109.
28. Annabestani Z, Sharghi S, Shahbazi S, Monfared SSMS, Karimi F, Taheri E, Heshmat R, Larijani B. Insulin buccal spray (Oral-Lyn) efficacy in type 1 diabetes. Iranian J Diabetes and Lipid Dis. 2010;9.
29. Pozzilli P, Manfrini S, Costanza F, Coppolino G, Cavallo MG, Fioriti E, *et al.* Biokinetics of buccal spray insulin in patients with type 1 diabetes. Metab Clin Exp. 2005;54:930–4.
30. Heinemann L, Jacques Y. Oral insulin and buccal insulin: a critical reappraisal. J Diabetes Sci Technol. 2009;3:568–84.
31. Senel S, Rathbone MJ, Cansiz M, Pather I. Recent developments in buccal and sublingual delivery systems. Expert Opin Drug Deliv. 2012;9:615–28.
32. Generex. Generex Oral-lyn. http://www.generex.com/index.php/id/270 (accessed 05/05/2014).
33. Oh DH, Chun KH, Jeon SO, Kang JW, Lee S. Enhanced transbuccal salmon calcitonin (sCT) delivery: effect of chemical enhancers and electrical assistance on *in vitro* sCT buccal permeation. Eur J Pharm Biopharm. 2011;79:357–63.
34. Harris D, Robinson JR. Drug delivery via the mucous membranes of the oral cavity. J Pharm Sci. 1992;81:1–10.
35. Wertz PW, Squier CA. Cellular and molecular basis of barrier function in oral epithelium. Crit Rev Ther Drug Carrier Syst. 1991;8:237–69.
36. Teubl BJ, Absenger M, Frohlich E, Leitinger G, Zimmer A, Roblegg E. The oral cavity as a biological barrier system: design of an advanced buccal *in vitro* permeability model. Eur J Pharm Biopharm. 2013;84:386–93.
37. Squier CA, Wertz PW. Structure and function of the oral mucosa and implications for drug delivery. In: Rathbone MJ and Swarbrick J, editors. Oral mucosal drug delivery. New York: Marcel Dekker, Inc.; 1996. p. 1–26.
38. Diaz-Del Consuelo I, Jacques Y, Pizzolato GP, Guy RH, Falson F. Comparison of the lipid composition of porcine buccal and esophageal permeability barriers. Arch Oral Biol. 2005;50:981–7.
39. Law S, Wertz PW, Swartzendruber DC, Squier CA. Regional variation in content, composition and organization of porcine epithelial barrier lipids revealed by thin-layer chromatography and transmission electron microscopy. Arch Oral Biol. 1995;40:1085–91.
40. Wertz PW, Swartzendruber DC, Squier CA. Regional variation in the structure and permeability of oral mucosa and skin. Adv Drug Deliv Rev. 1993;12:1–12.
41. Zhang H, Robinson JR. Routes of drug transport across oral mucosa. In: Rathboneand MJ, Swarbrick J, editors. Oral mucosal drug delivery. New York: Marcel Dekker, Inc.; 1996. p. 51–64.
42. Hao J, Heng PW. Buccal delivery systems. Drug Dev Ind Pharm. 2003;29:821–32.
43. Senel S, Kremer M, Nagy K, Squier C. Delivery of bioactive peptides and proteins across oral (buccal) mucosa. Curr Pharm Biotechnol. 2001;2:175–86.
44. Nicolazzo JA, Reed BL, Finnin BC. Buccal penetration enhancers–how do they really work? J Control Release. 2005;105:1–15.
45. Senel S, Hincal AA. Drug permeation enhancement via buccal route: possibilities and limitations. J Control Release. 2001;72: 133–44.
46. Veerman ECI, van den Keybus PAM, Vissink A, Amerongen AVN. Human glandular salivas: their separate collection and analysis. Eur J Oral Sci. 1996;104:346–52.
47. Bardow A, Madsen J, Nauntofte B. The bicarbonate concentration in human saliva does not exceed the plasma level under normal physiological conditions. Clin Oral Investig. 2000;4:245–53.
48. Bykov VL. The tissue and cell defense mechanisms of the oral mucosa. Morfologia. 1996;110:14–24.
49. Jankowska AK, Waszkiel D, Kowalczyk A. Saliva as a main component of oral cavity ecosystem Part I. Secretion and function. Wiad Lek. 2007;60:148–54.
50. Salamat-Miller N, Chittchang M, Johnston TP. The use of mucoadhesive polymers in buccal drug delivery. Adv Drug Deliv Rev. 2005;57:1666–91.

51. Edsman K, Hagerstrom H. Pharmaceutical applications of mucoadhesion for the non-oral routes. J Pharm Pharmacol. 2005;57:3–22.
52. Jones DS, Woolfson AD, Djokic J, Coulter WA. Development and mechanical characterization of bioadhesive semi-solid, polymeric systems containing tetracycline for the treatment of periodontal diseases. Pharm Res. 1996;13:1734–8.
53. Yamamoto A, Hayakawa E, Lee VH. Insulin and proinsulin proteolysis in mucosal homogenates of the albino rabbit: implications in peptide delivery from nonoral routes. Life Sci. 1990;47:2465–74.
54. Kashi SD, Lee VH. Enkephalin hydrolysis in homogenates of various absorptive mucosae of the albino rabbit: similarities in rates and involvement of aminopeptidases. Life Sci. 1986;38:2019–28.
55. Dowty ME, Knuth KE, Irons BK, Robinson JR. Transport of thyrotropin releasing hormone in rabbit buccal mucosa *in vitro*. Pharm Res. 1992;9:1113–22.
56. Nakada Y, Awata N, Ikuta Y, Goto S. The effect of bile salts on the oral mucosal absorption of human calcitonin in rats. J Pharmacobiodyn. 1989;12:736–43.
57. Lee VH, Yamamoto A. Penetration and enzymatic barriers to peptide and protein absorption. Adv Drug Deliv Rev. 1990;4: 171–207.
58. Lee VH. Enzymatic barriers to peptide and protein absorption. Crit Rev Ther Drug Carrier Syst. 1988;5:69–97.
59. Lee VH. Peptidase activities in absorptive mucosae. Biochem Soc Trans. 1989;17:937–40.
60. Verhoef JC, Bodde HE, de Boer AG, Bouwstra JA, Junginger HE, Merkus FW, *et al.* Transport of peptide and protein drugs across biological membranes. Eur J Drug Metab Pharmacokinet. 1990;15: 83–93.
61. Yamahara H, Lee VHL. Drug metabolism in the oral cavity. Adv Drug Deliv Rev. 1993;12:25–39.
62. Kragelund C, Hansen C, Torpet LA, Nauntofte B, Brosen K, Pedersen AM, *et al.* Expression of two drug-metabolizing cytochrome P450-enzymes in human salivary glands. Oral Dis. 2008;14:533–40.
63. Xin Hua Z, Alain P, Wan L. Comparison of enzyme activities of tissues lining portals of absorption of drugs: species differences. Int J Pharm. 1991;70:271–83.
64. Aungst BJ, Rogers NJ. Site dependence of absorption-promoting actions of laureth-9, Na salicylate, Na_2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery. Pharm Res. 1988;5:305–8.
65. Walker GF, Langoth N, Bernkop-Schnurch A. Peptidase activity on the surface of the porcine buccal mucosa. Int J Pharm. 2002;233: 141–7.
66. Cui F, He C, He M, Tang C, Yin L, Qian F, *et al.* Preparation and evaluation of chitosan-ethylenediaminetetraacetic acid hydrogel films for the mucoadhesive transbuccal delivery of insulin. J Biomed Mat Res A. 2009;89:1063–71.
67. Xu H-B, Huang K-X, Zhu Y-S, Gao Q-H, Wu Q-Z, Tian W-Q, *et al.* Hypoglycaemic effect of a novel insulin buccal formulation on rabbits. Pharmacol Res. 2002;46:459–67.
68. Sahni J, Raj S, Ahmad FJ, Khar RK. Design and *in vitro* characterization of buccoadhesive drug delivery system of insulin. Indian J Pharm Sci. 2008;70:61–5.
69. Bird AP, Faltinek JR, Shojaei AH. Transbuccal peptide delivery: stability and *in vitro* permeation studies on endomorphin-1. J Control Release. 2001;73:31–6.
70. Rónai AZ, Timár J, Mako E, Erdóo F, Gyarmati Z, Tóth G, *et al.* Diprotin, an inhibitor of dipeptidyl aminopeptidase IV(EC 3.4.14.5) produces naloxone — reversible analgesia in rats. Life Sci. 1998;64:145–52.
71. Veuillez F, Deshusses J, Buri P. Synthesis and characterization of an acylated di-peptide (Myr-Trp-Leu) with modified transmucosal transport properties. Eur J Pharm Biopharm. 1999;48:21–6.
72. Yang C, Tirucherai GS, Mitra AK. Prodrug based optimal drug delivery via membrane transporter/receptor. Expert Opin Biol Ther. 2001;1:159–75.
73. Yamada K, Murakami M, Yamamoto A, Takada K, Muranishi S. Improvement of intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid. J Pharm Pharmacol. 1992;44:717–21.
74. Muranishi S, Yamamoto A. Modification of peptides for intestinal delivery. Top Pharm Sci. 1994; 373–3821994
75. Li C, Koch RL, Raul VA, Bhatt PP, Johnston TP. Absorption of thyrotropin-releasing hormone in rats using a mucoadhesive buccal patch. Drug Dev Ind Pharm. 1997;23:239–46.
76. Chinwala MG, Lin S. Application of hydrogel polymers for development of thyrotropin releasing hormone-loaded adhesive buccal patches. Pharm Dev Technol. 2010;15:311–27.
77. Bundgaard H, Møss J. Prodrugs of peptides. 6. Bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by the TRH-specific serum enzyme. Pharm Res. 1990;7:885–92.
78. Bundgaard H, Rasmussen GJ. Prodrugs of peptides. 9. Bioreversible N-α-hydroxyalkylation of the peptide bond to effect protection against carboxypeptidase or other proteolytic enzymes. Pharm Res. 1991;8:313–22.
79. Hassan N, Ahad A, Ali M, Ali J. Chemical permeation enhancers for transbuccal drug delivery. Expert Opin Drug Deliv. 2010;7:97–112.
80. Smart JD. Buccal drug delivery. Expert Opin Drug Deliv. 2005;2: 507–17.
81. Bonferoni MC, Sandri G, Rossi S, Ferrari F, Caramella C. Chitosan and its salts for mucosal and transmucosal delivery. Expet Opin Drug Deliv. 2009;6:923–39.
82. Zhang J, Niu S, Ebert C, Stanley TH. An *in vivo* dog model for studying recovery kinetics of the buccal mucosa permeation barrier after exposure to permeation enhancers: apparent evidence of effective enhancement without tissue damage. Int J Pharm. 1994;101: 15–22.
83. Sohi H, Ahuja A, Ahmad FJ, Khar RK. Critical evaluation of permeation enhancers for oral mucosal drug delivery. Drug Dev Ind Pharm. 2010;36:254–82.
84. Aungst BJ. Absorption enhancers: applications and advances. AAPS J. 2012;14:10–8.
85. Hearnden V, Sankar V, Hull K, Juras DV, Greenberg M, Kerr AR, *et al.* New developments and opportunities in oral mucosal drug delivery for local and systemic disease. Adv Drug Deliv Rev. 2012;64:16–28.
86. Nicolazzo JA, Reed BL, Finnin BC. Assessment of the effects of sodium dodecyl sulfate on the buccal permeability of caffeine and estradiol. J Pharm Sci. 2004;93:431–40.
87. Gandhi R, Robinson J. Mechanisms of penetration enhancement for transbuccal delivery of salicylic acid. Int J Pharm. 1992;85:129–40.
88. Senel S, Hoogstraate AJ, Spies F, Verhoef JC, Bos-van Geest A, Junginger HE, *et al.* Enhancement of *in vitro* permeability of porcine buccal mucosa by bile salts: kinetic and histological studies. J Control Release. 1994;32:45–56.
89. Şenel S, Çapan Y, Sargon MF, İkinci G, Şolpan D, Güven O, *et al.* Enhancement of transbuccal permeation of morphine sulfate by sodium glycodeoxycholate *in vitro*. J Control Release. 1997;45: 153–62.
90. Xiang J, Fang X, Li X. Transbuccal delivery of 2′,3′-dideoxycytidine: *in vitro* permeation study and histological investigation. Int J Pharm. 2002;231:57–66.
91. Nicolazzo JA, Reed BL, Finnin BC. Enhancing the buccal mucosal uptake and retention of triamcinolone acetonide. J Control Release. 2005;105:240–8.
92. Hoogstraate AJ, Wertz PW, Squier CA, Bos-van Geest A, Abraham W, Garrison MD, *et al.* Effects of the penetration enhancer

glycodeoxycholate on the lipid integrity in porcine buccal epithelium *in vitro*. Eur J Pharm Sci. 1997;5:189–98.
93. Hoogstraate AJ, Senel S, Cullander C, Verhoef J, Junginger HE, Bodde HE. Effects of bile salts on transport rates and routes of FITC-labelled compounds across porcine buccal epithelium *in vitro*. J Control Release. 1996;40:211–21.
94. Starokadomskyy PL, Dubey IY. New absorption promoter for the buccal delivery: preparation and characterization of lysalbinic acid. Int J Pharm. 2006;308:149–54.
95. Kurosaki Y, Takatori T, Nishimura H, Nakayama T, Kimura T. Regional variation in oral mucosal drug absorption: permeability and degree of keratinization in hamster oral cavity. Pharm Res. 1991;8:1297–301.
96. Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. JNCI Monographs 2001;7–15.
97. Morishita M, Barichello JM, Takayama K, Chiba Y, Tokiwa S, Nagai T. Pluronic F-127 gels incorporating highly purified unsaturated fatty acids for buccal delivery of insulin. Int J Pharm. 2001;212:289–93.
98. Lee J, Kellaway IW. Combined effect of oleic acid and polyethylene glycol 200 on buccal permeation of [D-ala2, D-leu5]enkephalin from a cubic phase of glyceryl monooleate. Int J Pharm. 2000;204:137–44.
99. Tsutsumi K, Obata Y, Takayama K, Loftsson T, Nagai T. Effect of cod-liver oil extract on the buccal permeation of ergotamine tartrate. Drug Dev Ind Pharm. 1998;24:757–62.
100. Turunen TM, Urtti A, Paronen P, Audus KL, Rytting JH. Effect of some penetration enhancers on epithelial membrane lipid domains: evidence from fluorescence spectroscopy studies. Pharm Res. 1994;11:288–94.
101. Gershanik T, Benita S. Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs. Eur J Pharm Biopharm. 2000;50:179–88.
102. Coutelegros A, Maitani Y, Veillard M, Machida Y, Nagai T. Combined effects of pH, cosolvent and penetration enhancers on the invitro buccal absorption of propranolol through excised hamster-cheek pouch. Int J Pharm. 1992;84:117–28.
103. Jiang SJ, Zhou XJ. Examination of the mechanism of oleic acid-induced percutaneous penetration enhancement: an ultrastructural study. Biol Pharm Bull. 2003;26:66–8.
104. Riva R, Ragelle H, des Rieux A, Duhem N, Jerome C, Preat V. Chitosan and chitosan derivatives in drug delivery and tissue engineering. Adv Polym Sci. 2011;244:19–44.
105. Hu L, Sun Y, Wu Y. Advances in chitosan-based drug delivery vehicles. Nanoscale. 2013;5:3103–11.
106. Colonna C, Genta I, Perugini P, Pavanetto F, Modena T, Valli M, *et al.* 5-methyl-pyrrolidinone chitosan films as carriers for buccal administration of proteins. AAPS PharmSciTech. 2006;7:E107–13.
107. Portero A, Remunan-Lopez C, Nielsen HM. The potential of chitosan in enhancing peptide and protein absorption across the TR146 cell culture model-an *in vitro* model of the buccal epithelium. Pharm Res. 2002;19:169–74.
108. Sandri G, Rossi S, Bonferoni MC, Ferrari F, Zambito Y, Di Colo G, *et al.* Buccal penetration enhancement properties of N-trimethyl chitosan: influence of quaternization degree on absorption of a high molecular weight molecule. Int J Pharm. 2005;297:146–55.
109. Sandri G, Poggi P, Bonferoni MC, Rossi S, Ferrari F, Caramella C. Histological evaluation of buccal penetration enhancement properties of chitosan and trimethyl chitosan. J Pharm Pharmacol. 2006;58:1327–36.
110. Roldo M, Hornof M, Caliceti P, Bernkop-Schnurch A. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and *in vitro* evaluation. Eur J Pharm Biopharm. 2004;57:115–21.
111. Bernkop-Schnurch A, Guggi D, Pinter Y. Thiolated chitosans: development and *in vitro* evaluation of a mucoadhesive, permeation enhancing oral drug delivery system. J Control Release. 2004;94:177–86.
112. Roldo M, Hornof M, Caliceti P, Bernkop-Schnürch A. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and *in vitro* evaluation. Eur J Pharm Biopharm. 2004;57:115–21.
113. Bernkop-Schnurch A, Hornof M, Zoidl T. Thiolated polymers–thiomers: synthesis and *in vitro* evaluation of chitosan-2-iminothiolane conjugates. Int J Pharm. 2003;260:229–37.
114. Bernkop-Schnurch A, Thaler SC. Polycarbophil-cysteine conjugates as platforms for oral polypeptide delivery systems. J Pharm Sci. 2000;89:901–9.
115. Langoth N, Kahlbacher H, Schöffmann G, Schmerold I, Schuh M, Franz S, *et al.* Thiolated chitosans: design and *in vivo* evaluation of a mucoadhesive buccal peptide drug delivery system. Pharm Res. 2006;23:573–9.
116. Shinkar DM, Dhake AS, Setty CM. Drug delivery from the oral cavity: a focus on mucoadhesive buccal drug delivery systems. PDA J Pharm Sci. 2012;66:466–500.
117. Rai V, Tan HS, Michniak-Kohn B. Effect of surfactants and pH on naltrexone (NTX) permeation across buccal mucosa. Int J Pharm. 2011;411:92–7.
118. Shore PA, Brodie BB, Hogben CA. The gastric secretion of drugs: a pH partition hypothesis. J Pharmacol Exp Ther. 1957;119:361–9.
119. Leskovac V. The pH dependence of enzyme catalysis. In Leskovac V, editor. Comprehensive enzyme kinetics. Springer: US; 2004. p. 283–315.
120. Nakane S, Kakumoto M, Yukimatsu K, Chien YW. Oramucosal delivery of LHRH: pharmacokinetic studies of controlled and enhanced transmucosal permeation. Pharm Dev Technol. 1996;1:251–9.
121. Park H, Robinson JR. Physico-chemical properties of water insoluble polymers important to mucin/epithelial adhesion. J Control Release. 1985;2:47–57.
122. Schoellhammer CM, Blankschtein D, Langer R. Skin permeabilization for transdermal drug delivery: recent advances and future prospects. Expert Opin Drug Deliv. 2014;11:393–407.
123. Gratieri T, Kalaria D, Kalia YN. Non-invasive iontophoretic delivery of peptides and proteins across the skin. Expert Opin Drug Deliv. 2011;8:645–63.
124. Singh P, Maibach HI. Iontophoresis in drug delivery: basic principles and applications. Crit Rev Ther Drug Carrier Syst. 1994;11:161–213.
125. Shidhaye SS, Saindane NS, Sutar S, Kadam V. Mucoadhesive bilayered patches for administration of sumatriptan succinate. AAPS PharmSciTech. 2008;9:909–16.
126. Dhote V, Bhatnagar P, Mishra PK, Mahajan SC, Mishra DK. Iontophoresis: a potential emergence of a transdermal drug delivery system. Sci Pharm. 2012;80:1–28.
127. Rojanasakul Y, Wang LY, Bhat M, Glover DD, Malanga CJ, Ma JK. The transport barrier of epithelia: a comparative study on membrane permeability and charge selectivity in the rabbit. Pharm Res. 1992;9:1029–34.
128. Guy RH. Current status and future prospects of transdermal drug delivery. Pharm Res. 1996;13:1765–9.
129. Huang Y-Y, Wu S-M. Stability of peptides during iontophoretic transdermal delivery. Int J Pharm. 1996;131:19–23.
130. Chou W-L, Cheng C-H, Yen S-C, Jiang T-S. The enhanced iontophoretic transport of TRH and its impedance study. Drug Dev Ind Pharm. 1996;22:943–50.
131. Riviere JE, Heit MC. Electrically-assisted transdermal drug delivery. Pharm Res. 1997;14:687–97.
132. Kalia YN, Naik A, Garrison J, Guy RH. Iontophoretic drug delivery. Adv Drug Deliv Rev. 2004;56:619–58.
133. Patel MP, Churchman ST, Cruchley AT, Braden M, Williams DM. Electrically induced transport of macromolecules through oral buccal mucosa. Dent Mater. 2013;29:674–81.

134. Lee V, Lee VHL, Hashida M, Mizushima Y. Trends and future perspectives in peptide and protein drug delivery. Taylor & Francis; 1995.
135. Sage BH BC, Denuzzio JD, Hocke RA. Technological and developmental issues of iontophoretic transport of peptide and protein drugs. In: Lee VHL HM, Mizushima Y, editors. Trends and future perspectives in peptide and protein drug delivery. Switzerland: Harwood Academic; 1995. p. 111–34.
136. Patel MP, Churchman ST, Cruchley AT, Braden M, Williams DM. Delivery of macromolecules across oral mucosa from polymeric hydrogels is enhanced by electrophoresis (iontophoresis). Dent Mater. 2013;29:e299–307.
137. Choi EH, Lee SH, Ahn SK, Hwang SM. The pretreatment effect of chemical skin penetration enhancers in transdermal drug delivery using iontophoresis. Skin Pharmacol Appl Skin Physiol. 1999;12: 326–35.
138. Dixit N, Bali V, Baboota S, Ahuja A, Ali J. Iontophoresis - an approach for controlled drug delivery: a review. Curr Drug Deliv. 2007;4:1–10.
139. Giovino C, Ayensu I, Tetteh J, Boateng JS. Development and characterisation of chitosan films impregnated with insulin loaded PEG-b-PLA nanoparticles (NPs): a potential approach for buccal delivery of macromolecules. Int J Pharm. 2012;428:143–51.
140. Cui F, He C, He M, Tang C, Yin L, Qian F, *et al.* Preparation and evaluation of chitosan-ethylenediaminetetraacetic acid hydrogel films for the mucoadhesive transbuccal delivery of insulin. J Biomed Mater Res A. 2009;89:1063–71.
141. Moebus K, Siepmann J, Bodmeier R. Alginate-poloxamer microparticles for controlled drug delivery to mucosal tissue. Eur J Pharm Biopharm. 2009;72:42–53.
142. Gombotz WR, Wee S. Protein release from alginate matrices. Adv Drug Deliv Rev. 1998;31:267–85.
143. Peppas NA, Bures P, Leobandung W, Ichikawa H. Hydrogels in pharmaceutical formulations. Eur J Pharm Biopharm. 2000;50:27–46.
144. Perumal VA, Govender T, Lutchman D, Mackraj I. Investigating a new approach to film casting for enhanced drug content uniformity in polymeric films. Drug Dev Ind Pharm. 2008;34:1036–47.
145. Luo Y, Xu H, Huang K, Gao Z, Peng H, Sheng X. Study on a nanoparticle system for buccal delivery of insulin. Conf Proc IEEE Eng Med Biol Soc. 2005;5:4842–5.
146. Serra L, Doménech J, Peppas NA. Design of poly(ethylene glycol)-tethered copolymers as novel mucoadhesive drug delivery systems. Eur J Pharm Biopharm. 2006;63:11–8.
147. Zheng C, Zhang XG, Sun L, Zhang ZP, Li CX. Biodegradable and redox-responsive chitosan/poly(L-aspartic acid) submicron capsules for transmucosal delivery of proteins and peptides. J Mater Sci Mater Med. 2013;24:931–9.
148. Yang TZ, Wang XT, Yan XY, Zhang Q. Phospholipid deformable vesicles for buccal delivery of insulin. Chem Pharm Bull. 2002;50: 749–53.
149. Duchêne D, Touchard F, Peppas NA. Pharmaceutical and medical aspects of bioadhesive systems for drug administration. Drug Dev Ind Pharm. 1988;14:283–318.
150. Smart JD. The role of water movement and polymer hydration in mucoadhesion. Bioadhesive drug delivery systems. CRC Press; 1999, p. 11–23.
151. Smart JD. The basics and underlying mechanisms of mucoadhesion. Adv Drug Deliv Rev. 2005;57:1556–68.
152. Lee JW, Park JH, Robinson JR. Bioadhesive-based dosage forms: the next generation. J Pharm Sci. 2000;89:850–66.
153. Silberberg-Bouhnik M, Ramon O, Ladyzhinski I, Mizrahi S, Cohen Y. Osmotic deswelling of weakly charged poly(acrylic acid) solutions and gels. J Polym Sci Pol Phys. 1995;33:2269–79.
154. Smart JD. Drug delivery using buccal-adhesive systems. Adv Drug Deliver Rev. 1993;11:253–70.
155. Andrews GP, Laverty TP, Jones DS. Mucoadhesive polymeric platforms for controlled drug delivery. Eur J Pharm Biopharm. 2009;71:505–18.
156. Morales JO, Ross AC, McConville JT. Protein-coated nanoparticles embedded in films as delivery platforms. J Pharm Pharmacol. 2013;65:827–38.
157. Portero A, Teijeiro-Osorio D, Alonso MJ, Remunan-Lopez C. Development of chitosan sponges for buccal administration of insulin. Carbohyd Polym. 2007;68:617–25.
158. Ayensu I, Mitchell JC, Boateng JS. Development and physicomechanical characterisation of lyophilised chitosan wafers as potential protein drug delivery systems via the buccal mucosa. Colloids Surf B: Biointerfaces. 2012;91:258–65.
159. Matthews KH, Stevens HN, Auffret AD, Humphrey MJ, Eccleston GM. Lyophilised wafers as a drug delivery system for wound healing containing methylcellulose as a viscosity modifier. Int J Pharm. 2005;289:51–62.
160. Boateng JS, Auffret AD, Matthews KH, Humphrey MJ, Stevens HN, Eccleston GM. Characterisation of freeze-dried wafers and solvent evaporated films as potential drug delivery systems to mucosal surfaces. Int J Pharm. 2010;389:24–31.
161. Morra G, Meli M, Colombo G. Molecular dynamics simulations of proteins and peptides: from folding to drug design. Curr Protein Pept Sc. 2008;9:181–96.
162. Vendruscolo M, Dobson CM. Structural biology: protein self-assembly intermediates. Nat Chem Biol. 2013;9:216–7.
163. Gorbenko G, Trusova V. Protein aggregation in a membrane environment. Adv Protein Chem Struct Biol. 2011;84:113–42.
164. Nault L, Vendrely C, Brechet Y, Bruckert F, Weidenhaupt M. Peptides that form beta-sheets on hydrophobic surfaces accelerate surface-induced insulin amyloidal aggregation. FEBS Lett. 2013;587:1281–6.
165. Bauer HH, Aebi U, Haner M, Hermann R, Muller M, Merkle HP. Architecture and polymorphism of fibrillar supramolecular assemblies produced by *in vitro* aggregation of human calcitonin. J Struct Biol. 1995;115:1–15.
166. Katakam M, Banga AK. Aggregation of insulin and its prevention by carbohydrate excipients. PDA J Pharm Sci Technol. 1995;49: 160–5.
167. Gibson TJ, Murphy RM. Inhibition of insulin fibrillogenesis with targeted peptides. Protein Sci. 2006;15:1133–41.
168. Arora A, Ha C, Park CB. Inhibition of insulin amyloid formation by small stress molecules. FEBS Lett. 2004;564:121–5.
169. Brange J, Andersen L, Laursen ED, Meyn G, Rasmussen E. Toward understanding insulin fibrillation. J Pharm Sci. 1997;86: 517–25.
170. Grau U, Saudek CD. Stable insulin preparation for implanted insulin pumps - laboratory and animal trials. Diabetes. 1987;36: 1453–9.
171. Hoogstraate AJ, Coos Verhoef J, Pijpers A, van Leengoed LA, Verheijden JH, Junginger HE, *et al. In vivo* buccal delivery of the peptide drug buserelin with glycodeoxycholate as an absorption enhancer in pigs. Pharm Res. 1996;13:1233–7.
172. Hoogstraate AJ, Verhoef JC, Tuk B, Pijpers A, van Leengoed LA, Verheijden JH, *et al.* In-vivo buccal delivery of fluorescein isothiocyanate-dextran 4400 with glycodeoxycholate as an absorption enhancer in pigs. J Pharm Sci. 1996;85:457–60.
173. Aungst BJ, Rogers NJ. Comparison of the effects of various transmucosal absorption promoters on buccal insulin delivery. Int J Pharm. 1989;53:227–35.
174. Oh CK, Ritschel WA. Biopharmaceutic aspects of buccal absorption of insulin. Methods Find Exp Clin Pharmacol. 1990;12:205–12.
175. Aungst BJ. Site-dependence and structure-effect relationships for alkylglycosides as transmucosal absorption promoters for insulin. Int J Pharm. 1994;105:219–25.

176. Steward A, Bayley DL, Howes C. The effect of enhancers on the buccal absorption of hybrid (BDBB) alpha-interferon. Int J Pharm. 1994;104:145–9.
177. Langoth N, Bernkop-Schnürch A, Kurka P. *In vitro* evaluation of various buccal permeation enhancing systems for PACAP (pituitary adenylate cyclase-activating polypeptide). Pharm Res. 2005;22: 2045–50.
178. Johnston TP, Rahman A, Alur H, Shah D, Mitra AK. Permeation of unfolded basic fibroblast growth factor (bFGF) across rabbit buccal mucosa–does unfolding of bFGF enhance transport? Pharm Res. 1998;15:246–53.
179. Jasti BR, Zhou S, Mehta RC, Li X. Permeability of antisense oligonucleotide through porcine buccal mucosa. Int J Pharm. 2000;208:35–9.