Intestinal permeation enhancers to improve oral bioavailability of macromolecules: reasons for low efficacy in humans

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Intestinal Permeation Enhancers to Improve Oral Bioavailability of Macromolecules: Reasons for Low Efficacy in Humans

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ABSTRACT

Introduction: Intestinal permeation enhancers (PEs) are substances that transiently alter the intestinal epithelial barrier to facilitate permeation of macromolecules with low oral bioavailability (BA). While a number of PEs have progressed to clinical testing in conventional formulations with macromolecules, there has been only low single digit increases in oral BA, irrespective of whether the drug met primary or secondary clinical endpoints.

Areas covered: This article considers the causes of sub-optimal BA of macromolecules from PE dosage forms and suggests approaches that may improve performance in humans.

Expert opinion: Permeation enhancement is most effective when the PE is co-localized with the macromolecule at the epithelial surface. Conditions in the GI tract impede optimal co-localisation. Novel delivery systems that limit dilution and spreading of the PE and macromolecule in the small intestine have attempted to replicate promising enhancement efficacy observed in static drug delivery models

Keywords: intestinal permeability, oral peptide delivery, intestinal permeation enhancers, intestinal epithelium, oral bioavailability

ARTICLE HIGHLIGHTS

- Intestinal PEs are currently the most common approach to enable oral delivery of macromolecules in clinical trials.
- PEs are most effective for delivery of small stable macromolecules that have a long plasma half-life.
- PEs are broadly categorized based on mode of action as either transcellular (acting via complexation on mild mucosal aberration) or paracellular (acting via direct interaction with the TJ or through endogenous intracellular signalling mechanisms), or a combination of both.
- Safety concerns related to modulation of intestinal barrier integrity have not yet emerged as a drawback for the narrow selection of PEs that have progressed to clinical trials.
- Conditions in the small intestine and standard dosage form designs limit the optimal co-localization of PE and macromolecule at the intestinal wall, and may be why there is persistence of low and variable BA in humans.
- A number of devices have been designed to promote optimal co-localization at the intestinal wall.

1. INTRODUCTION

Macromolecules including peptides and proteins are a structurally diverse drug category that are characterised by excellent safety, efficacy, and tolerability. The physiochemical properties that impart favourable therapeutic and pharmacokinetic (PK) properties following parenteral administration are, however, also associated with sub-optimal oral bioavailability (BA) [1, 2]. Macromolecules are typically formulated as injectable dosage forms, which is inconvenient for patients, while it adds costs for healthcare providers and pharmaceutical manufacturers. They may even be excluded from pharmaceutical R & D screening if they cannot be formulated as oral dosage forms [3]. There is therefore continued demand for practical solutions that enable oral delivery of macromolecules.

Macromolecules exhibit low oral BA due to pre-systemic degradation and low intestinal permeability. Efforts to address these hurdles are based on either drug modification or formulation optimisation. Several formulation and drug delivery approaches have attempted to address this problem, but with limited success to date. One approach is to co-formulate the macromolecule with excipients that prevent enzymatic degradation (e.g. acidifiers, peptidase inhibitors) and transiently alter the intestinal barrier to improve flux (permeation enhancers, PEs). PEs increase the fraction of drug extracted from the gut lumen (F_{abs}) to ultimately increase BA. In principle, simple ad-mixtures of macromolecules and PEs in a solid dosage form is less technically demanding relative to, for example entrapment in nanoparticles, where low payload loading, physical instability and batch-to-batch variability may prevent translation [4].

There are perceived safety concerns over the chronic use of PEs that compromise the integrity of the intestinal epithelium [5]. These safety concerns are magnified from a perusal of the vast range of PE types that alter barrier integrity, ranging from bacterial exotoxins to industry grade detergents, some of which will never be suitable for use in humans. A more immediate problem emerging from studies carried out over the last twenty years is the low and variable oral BA seen in clinical trials for orally-delivered macromolecules in

formulations containing PEs. These data relate in part to physiological hurdles and the difficulty in optimally co-presenting the macromolecule and PE at the epithelium in sufficient concentrations. Despite this, marketed oral formulations for four peptides have been approved for systemic delivery (cyclosporin, desmopressin, semaglutide, and octreotide), the latter two of which are PE-based. In many cases, there is no scope to change the macromolecule structure through medicinal chemistry, hence, optimisation is limited to formulation approaches. The discussion has matured from whether PE platforms can improve oral delivery to whether bespoke systems with or without PEs can improve the absorption of specific macromolecules in the dynamic GI conditions.

This review summarises the findings from selected pre-clinical studies and clinical trials where PEs were used to improve oral BA of macromolecules. We describe the physicochemical and pharmacological properties of the most promising macromolecule drug candidates and summarise the properties of leading PE categories. A brief discussion is provided on the physiological impediments to translation of oral macromolecule dosage forms along with strategies that are in development to improve the performance of PEs in the GI tract.

2. CANDIDATE MACROMOLECULES FOR ORAL DRUG DELIVERY

The term macromolecule encompasses a structurally diverse group of compounds including peptides, proteins, carbohydrate polymers, and large monoclonal antibodies. These substances range in molecular weight (MW) from 1 kDa for short cyclised peptides to over 150 kDa for antibodies. The majority of oral macromolecule delivery research has focussed on peptides ranging from 1 to 6 kDa (Table 1). There has been no concerted effort to facilitate systemic delivery of the larger biologics via the oral route because efforts to increase permeation of lower MW and cheaper macromolecules have had only modest success to date. Recently, Intract Pharma (London, UK) has developed excipients to stabilise antibodies in the colon (Soteria®) and a coating that triggers more reliable release in the colon (Phloral®).

These technologies facilitate regional delivery of antibodies to local GI targets, but not systemic delivery.

The most widely studied target for oral delivery is insulin (5.8 kDa, isoelectric point (pI): 5.5). Development of an oral formulation for insulin is often cited as the Holy Grail within the oral delivery field, but many consider it more as a model peptide and benchmark for oral delivery rather than a commercial target because of its low therapeutic index. A recent Phase II trial of a long acting insulin formulated in tablets with the PE sodium caprate (C10) [6] was accompanied by an editorial entitled "*oral insulin: time to rewrite text books*" [7], even though the oral BA was estimated at just 1-2%. The interest in oral insulin delivery has diminished by the development of oral formulations of glucagon-like peptide-1 (GLP-1) receptor agonists because they have a higher safety margin and may not require the same target level of oral BA as insulin.

The criteria for selecting a macromolecule that might be amenable to oral delivery (with or without PE) relates to physicochemical (LogP, MW, hydrogen bond donor (HBD)/hydrogen bond acceptor (HBA)), pharmacodynamic (PD) (e.g. potency), and PK properties (e.g. half-life ($t_{1/2}$)). Table 1 highlights the properties of peptides that influence choice of route of administration. A hydrophilic peptide that is potent, stable in GI secretions, of a relatively low MW, with a long $t_{1/2}$ may conceivably reach therapeutic levels without the need for a PE. For example, desmopressin relies on high potency, a degree of GI stability, and its relatively low MW to consistently achieve therapeutic levels, although its oral BA remains pitiful (0.16% [8]). The main difference between desmopressin and the recently-approved oral formulation of octreotide (Mycapssa®, Chiasma, Jerusalem, Israel) is a 50 to 200-fold decrease in comparable potency for the latter, necessitating the use of a delivery system. Cyclosporin is exceptional in that it exhibits oral bioavailability of ~27% and the fraction leaving the gut lumen (F_{abs}) is > 85% [9], i.e. its main problem is GI metabolism in epithelia, not permeability. Cyclosporin is a stable and lipophilic (Log $P_{oct/wat}$: 2.92 [10]) macrocycle peptide that undergoes passive transcellular permeation across the intestinal epithelium, offset by cytochrome P450-

3A4 metabolism and P-glycoprotein efflux. Its low aqueous solubility is addressed by lipid-based formulation (LBF) [11].

There are two core approaches to enable oral macromolecule administration, physical/chemical modification and / or use of a drug delivery approach. Both of these approaches can increase stability and intestinal permeability, while structural modification can also increase potency and extend t½. Structural modification and drug delivery technology can be combined to improve BA and achieve efficacy via the oral route. For oral delivery of semaglutide, Novo Nordisk combined chemical modification (to increase GI stability [6] or plasma $t_{1/2}$ [12]) and use of a PE (to increase solubility, stomach pH, and intestinal permeation, as well as to prevent the formation multimeric structures [12]). This led to approval of an oral tablet of semaglutide for Type II diabetes (T2D) treatment (Rybelsus®) [13]. A similar dual approach was also attempted with a long-acting insulin analogue [6], but the oral BA achieved was not commercially-viable even though it was equivalent or higher than that of oral semaglutide.

Chemical modification may increase stability in GI fluids and plasma, but it is unlikely to alter lipophilicity to the extent that the active undergoes sufficient passive intestinal permeation. This was the case for the alkylated and PEGylated insulin analogue IN-105, which ultimately failed to increase oral BA in Phase 3 trials [14]. A dramatic shift in lipophilicity can be achieved for ionisable macromolecules by creating insoluble salts using hydrophobic ion pairing (HIP) [15]. Here, ionisable groups are complexed with an amphiphilic counter ion (e.g. dodecyl sulphate, docusate) that favours association in aqueous fluid. This reduces aqueous solubility and improves partitioning in non-aqueous vehicles [16]. The rationale is that lipophilicity of hydrophilic macromolecules can be temporarily increased to levels observed with the non-ionisable lipophilic macromolecules, such as cyclosporin, which may lead to an increase in partitioning in self emulsified drug delivery systems (SEDDS) and a resulting increase in passive transcellular permeation.

There has been extensive evaluation of delivery approaches to protect macromolecules from pre-systemic degradation and to improve permeability. These include entrapment in particulates and lipoidal dispersions, use of devices, as well as incorporation of PEs and peptidase inhibitors in solid dosage forms. Several prototype macromolecules have been assessed in such systems, most notably insulin, salmon calcitonin (sCT), octreotide, leuprolide, parathyroid hormone (PTH), acyline, low molecular weight heparins (LMWHs) and GLP-1 receptor agonists. While there may appear to be a relatively narrow MW range among these candidates (apart from LMWHs), there are substantial differences in physicochemical and pharmacological properties. Insulin has a relatively high MW (5.8 kDa), relatively low potency, low permeability, and a short $t_{1/2}$, thus selecting it as a prototype puts a very high demand on the delivery system. Nonetheless, because of this high bar, a technology that can improve oral insulin BA could be effective with peptides with more favourable physicochemical properties and a wider therapeutic index.

3. PERMEATION ENHANCER CATEGORIES FOR ORAL MACROMOLECULE DELIVERY

A wide range of compounds have been shown to modulate intestinal barrier integrity [17]. These include microorganisms, toxins, industrial detergents, allergens, endogenous secretions (bile acids), synthetic TJ modulators, food additives and pharmaceutical excipients. Many of these substances have not progressed as candidate PEs for reasons of low efficacy, questionable safety, and lack of capacity to formulate in solid dosage forms. The lead PE candidates typically have a history of safe use in man as food additives, excipients, or presence in natural secretions. Pharmaceutical excipients that unintentionally increase permeation as a secondary action to their primary role in the formulation have been termed "*absorption modifying excipients* (AMEs [8])". Little is known about the direct effects of AMEs on oral bioavailability, and there are concerns that such substances may impact bioequivalence (reviewed in [18]). Excipients that are used in oral formulations such as wetting agents (sodium dodecyl sulphate), antioxidants (e.g. EDTA), and emulsifiers (e.g. macrogol glycerides) may be included in formulations as PEs to improve BA. PEs are broadly

categorised as to how they alter barrier integrity via paracellular or transcellular routes, and they can be further subdivided based on more detailed mode of action (Table 2).

3.1 Paracellular PEs

Paracellular PEs increase permeability through direct disruption of TJ proteins in adjacent epithelial cells or indirectly by targeting signalling processes involved in the opening and closing of TJs. Microbial toxins are among the most prominent TJ modulators in particular *zonula occludens* toxin (ZOT) [19], viral protein 8 (VP8) [20] and *Clostridium perfringens* enterotoxin (CPE) [21]. These toxins have helped elucidate the structure and function of TJs and have assisted in development of therapeutic strategies that promote TJ re-assembly in diseases characterised by increased GI epithelial permeability [22]. However, toxins in their native form cannot be used safely. Lower MW structural analogues can target motifs involved in TJ openings in the absence of virulence factors. Examples include the Carboxyl terminal of CPE (C-CPE [21]), PN159 [23], and AT1002 (Alba Therapeutics, Maryland, USA [24]), the latter being derived from ZOT. While these analogues have improved safety profiles, they may not be as potent in inducing permeability as native toxins or more established PEs [25] [26]. To our knowledge, only one such toxin-based system, the *Cholex* system of Applied Molecular Transport (San Francisco, CA, USA [27]) is currently in clinical trials for oral delivery of a macromolecule, IL-10, for potential treatment of ulcerative colitis. Its mechanism of action, however, is transcellular.

TJs have been further subdivided into bicellular (bTJ) and tricellular (tTJ), based on whether points of intercellular contact are between two or three epithelial cells, respectively [28] TJ modulators that specifically target proteins at tTJ, such as angulins (1 and 3) and tricellulin, represent a category that has been established recently [29]. The *Clostridium perfringens* iota toxin binds to angulin-1, and a shorted sequence corresponding to 421 to 664 termed angubindin-1 removed angulin-1 and tricellulin from tTJs. Angubindin-1 (50 µg/mL) increased permeation of fluorescein isothiocyanate- dextran of MW 4kDa (FD4), 10 kDa (FD10) and 40 kDa (FD40) across Caco-2 monolayers following chronic exposure [29]. There

was an increase in FD4 absorption between 30 to 120 min following rat intestinal instillations of high doses of angubindin-1. This PE is not selective for intestinal TJs, as it can increase flux of antisense oligonucleotides across endothelial TJs of the blood brain barrier [30], as is the case for some other TJ modulators [31].

The molecular mechanisms by which toxins alter permeability are often poorly defined. At the same time, elucidation of the structure and function of TJs, aided by empirical evaluations, has led to rational design of new chemical entities (NCE) that target molecular mechanisms. These TJ modulators have more specific modes of action, may be less sensitive to species -related toxicity and may have shorter development times relative to PEs with poorly understood mechanisms of action [4]. Examples include the PIP peptides (Permeable Inhibitor of myosin light chain (MLC) Phosphatase) [32]. These synthetic peptides target a physiological step involved in nutrient-induced TJ regulation via cytoskeletal contraction. They prevent dephosphorylation of the myosin light chain (MLC) via MLC phosphatase (MLCP) by mimicking the physiological regulation by MYPT1 or CPI-17. This action sustains phosphorylation induced by MLC kinase (MLCK), which was initially activated by elevation of intracellular Ca^{2+} via the Na+, Ca+ antiport in response to intracellular Na^{+} levels that become high during co-transport of Na^{+} with amino acids and glucose [33]. In addition to opening TJs, the phosphorylation of MLC increases expression of claudin 2, a TJ protein that has selectivity for cation uptake, thereby limiting permeation of anionic species, such as endotoxin. In Caco-2 monolayers, a decapeptide (PIP640) selectively increased paracellular permeation of diethylaminoethyl dextran relative compared to neutral dextran and carboxymethyl dextran [34]. There was also increased absorption of sCT compared to that of exenatide in rat jejunal instillations owing to their respective cationic and anionic charges at the pH of the small intestine. PIP-640 was moderately efficacious in improving the BA of insulin to 4% in rat intestinal instillations [32].

While the use of endogenous mechanisms to increase permeation may not be associated with cytotoxicity or inflammation, further studies assessing chronic repeat

exposure are required. The MLCP inhibitors highlight the potential for designing PEs based on physiological control of the TJ. Their development signals movement away from pharmacologically inactive excipients to molecules with drug-like mechanisms. Although several excipients have pharmacological actions, this is secondary to their intended purpose in the formulation. Examples in oral formulations include depression of the central nervous system (CNS) by co-solvents (e.g. ethanol), as well as inhibition of intestinal metabolic enzymes, efflux pumps, and transporters by surfactants e.g. (vitamin E TPGS [35], Kolliphor EL [36]). It may be argued that paracellular PEs that selectively target disruption of homophilic interactions between TJ proteins in adjacent epithelial cells (e.g. C-CPE, C1C2, Claudin-$1_{53\text{-}80}$ peptide) carry less development risk than progressing PEs that act on ubiquitously-expressed receptors or enzymes. This ultimately depends on whether the PE is itself absorbed. Whether a PE is a new chemical entity or excipient with established safety in humans, the risk of failure on the grounds of sub-optimal enhancement remains high. Given the overall poor translation record to date for PEs tested in clinical trials, an NCE TJ modulator must display improved efficacy relative to established PEs from the AME grouping to compensate for the potential safety risks and to justify investment.

The most clinically advanced paracellular PEs in development of oral formulation of macromolecules are chelators: EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid). Chelators increase paracellular permeation by sequestering extracellular Ca^{2+}, which is required by the Ca^{2+} dependent cell adhesion protein, E-cadherin. This leads to disruption of *adherens* junctions (AJ) [37]. As AJs are coupled to TJs via an interaction between ZO-1 from the TJ and afadin and α-catenin from the AJ [38], this accounts for an overall increase in paracellular permeability when AJs are disrupted. Initial studies showed that the protein kinase C (PKC) inhibitor, H7, attenuated the action of EDTA and this suggested that depletion of extracellular Ca^{2+} increases permeability via PKC [39]. EDTA is of particular interest because it is one of the constituents in the Protein Oral Delivery (POD™) technology developed by Oramed (Jerusalem, Israel) for oral delivery

of antidiabetic peptides (Table 3). Enteric-coated capsules delivering insulin in POD™ were administered three times daily to eight type 1 diabetics (T1D) [40]. There was a 17% mean plasma reduction in glucose over 24 h compared to measurements taken prior to treatment.

There are conflicting reports on the efficacy of EDTA as a PE. Apical addition of EDTA increased permeation of FD4 by 2-fold across Caco-2 monolayers, compared with a 10-fold increase when added basolaterally, and a 322-fold increase when added bilaterally [41]. These data suggest that Ca^{2+} at the AJ can be replenished from the serosal side, which might limit the overall potential of calcium chelation strategies administered via the intestinal lumen. EDTA may also be less effective when there is a high luminal concentration of competing divalent cations (Ca^{2+}, Mg^{2+}, Fe^{2+}). A potential drawback to the application of EDTA in dynamic conditions within the small intestine is the requirement of the PE to diffuse past the TJ to the AJ in order to alter permeability, which may result in a slower effect compared with PEs that initially act on the plasma membrane to open TJs (e.g. sodium caprate (C_{10})). Evidence in support of this was from a rat *in situ* intestinal instillation study where a faster maximal reduction (T_{min}) in blood glucose was detected with C_{10} and sodium glycocholate than EDTA at equivalent concentrations [42].

3.2 Transcellular PEs

A common approach to increasing permeation across the intestinal epithelium is using substances that alter barrier integrity via perturbation of the plasma membrane (Table 2, Table 3). Substances causing transcellular perturbation in pre-clinical models include surfactants (e.g. sodium dodecyl sulphate), co-solvents (e.g. ethanol), lipids (e.g. glycerol monocaprylate, capric acid, macrogol glycerides) and drugs (e.g. acetyl salicylate). Yet, when transcellular PEs are incorporated even at very high quantities into oral solid dosage forms, they have been poorly efficacious at increasing BA in clinical trials (Table 3).

3.2.1 Surfactants

The transcellular PE category is dominated by soluble surfactants that insert in the plasma membrane, altering packing density and fluidity at low concentrations and solubilising large membrane structures at higher concentrations [17]. Major categories include medium chain fatty acids, acylated amino acids, non-ionic surfactants bile salts, and acyl carnitines (Table 3). There is no correlation between surfactant structure and enhancement action, but there are associations. Soluble surfactants exist in either the free monomolecular form or as micelles. The free monomolecular form is responsible for detergent-like perturbation, whereas micelles are reservoirs for replenishment of free surfactant that in turn interacts with the plasma membrane. Micelles also solubilise membrane fragments that have been stripped from the plasma membrane. The critical micelle concentration (CMC) is a measure of the maximum free monomolecular form and defines the concentration above which micelles form. Hence, the most effective detergents have high CMCs owing to a high concentration of free surfactant that is available to engage the membrane. Surfactants with low CMCs exhibit low solubility in the free form, so there is less free surfactant available to insert into the membrane. However, it would be an over-simplification to conclude that all surfactants with low CMCs are weak detergents and that all surfactants with high CMCs are strong ones. This is because surfactants with high CMC values and high aqueous solubility also comprise hydrophobic moieties that can be less efficient at entering the plasma membrane. Therefore, consideration should also be given to hydrophilic-lipophilic balance (HLB) values. Optimal CMC and HLB combinations are can give rise to strong enhancement action. For many ionic and non-ionic surfactants, medium hydrophobic chain lengths (C_8-to-C_{12}) can provide a good balance between solubility in free form (high CMCs) and membrane penetration (low HLB), whereas short chain length (C_4-to-C_6) have high CMCs but inefficiently penetrate (high HLBs). Long chains (C_{14}-to-C_{18}) are more efficient at penetrating membranes (low HLBs) but have low solubility (lower CMCs).

Predictive patterns between HLB values and CMCs, with enhancement action are complicated when comparing surfactants with different hydrophilic head groups (e.g. carboxylates, sulphates, sulphonates, ethoxylates, and sugar esters). There can be different CMC/HLB combinations that achieve comparable enhancement. For example, medium chain ethoxylates can have very low CMCs (e.g. $C_{12}E_9$: 0.08 mM) and intermediate HLB values (HLB 13.6) compared to carboxylates with higher CMCs (e.g. C_{10}; CMC: 13 mM) and high HLB values (e.g. C_{10}, HLB: 19.4). However, both ethoxylates and carboxylates increase the P_{app} of [^{14}C]-mannitol, a paracellular small molecule flux marker, to the level of 1 x 10^{-5} cm/s across isolated rat colonic mucosae [43]. Despite variability, most studies report significant enhancement action for 10 carbon (C_{10}) and 12 carbon (SDS, sucrose laurate, $C_{12}E_9$) surfactant PEs compared to longer or shorter chain lengths, irrespective of the hydrophilic head group.

Surfactant PEs can be sub-categorised by chemical structure. Major categories include fatty acids, acyl carnitines, sulphates, sugar esters, ethoxylates, maltosides/glucosides and bile salts (reviewed in [17]). These have the capacity to perturb biological membranes. Some do so more efficiently than others and, while a lower quantity of a strong surfactant can elicit enhancement, epithelial barrier recovery may be slow and this raises safety concerns. The leading PEs in clinical trials are therefore mild perturbants used in high concentrations, which bring about only modest elevation of oral BA. For example, there was 550 mg of C_{10} in the oral basal insulin tablet and 300 mg of SNAC in the oral semaglutide tablet (Rybelsus®), both developed by Novo Nordisk [6].

The mechanism by which surfactants alter cell and tissue permeability has been assessed by immunocytochemistry, high content analysis (HCA), bioassays, western blotting and with pharmacological inhibitors. At low concentrations, a number of surfactants (e.g. C_{10}, lauroylcarnitine, SDS, lysophosphatidyl choline) increase intracellular mediators (Ca^{2+}, calmodulin, ATP [44] [45] [46]), alter expression and or localisation of specific TJ proteins, and upregulate receptor (PLC [44]) and enzyme activity (e.g. PKC, MLCK [47] [48]). Part of the

mechanism by which selected surfactants alter barrier integrity may involve activation of plasma membrane receptors and intracellular enzymes, modulation of intracellular mediators and/or the selective removal of TJ proteins from the plasma membrane. However, some studies make no reference to increased membrane perturbation at the high PE concentrations that are most likely responsible for enhancement [49] [50].

Soluble salts of medium chain fatty acids are the most widely studied surfactant PEs. These include sodium caprylate (C_8), C_{10}, and sodium laurate (C_{12}). Examples also extend to odd carbon chain lengths: (sodium nonanoate (C_9), sodium undecanoate (C_{11})), and unsaturated fatty acids (e.g. sodium undecylenate ($C_{11:1}$)) [43, 51]. The goal of investigating these variations is to identify PEs that may improve upon the efficacy of established even-chained medium chain fatty acids, but results to date are equivocal. The actions of C_{10} have been extensively reviewed [52, 53]. The 10-carbon chain length makes C_{10} less effective at inserting into plasma membranes than fatty acids of longer chain lengths. However, it has a much higher CMC than fatty acids with a longer chain length, which enables a higher free surfactant concentration in the gut lumen. C_{10} has been the subject of many mode-of-action studies, some of which downplay the contribution of membrane perturbation [49], whereas others show a correlation with flux increase [54]. This raises safety questions about using C_{10} and other perturbants as excipients in humans. Mild mucosal perturbation occurs when high concentrations of C_{10} (100 mM) are instilled into the intestinal lumen of rats [55]. Epithelial cells rapidly recover from the challenge of mild mucosal damage in rat instillations [56], and also following oral [57] and rectal [58] administration in humans. This is why clinical studies show that staggering the administration time of PE and permeability markers promotes less oral absorption in humans than when co-administered [57]. Rapid intestinal epithelial recovery is, however, not observed for all PEs (e.g. C-CPE [25]), and there may be greater safety concerns about these.

C_{10} was the main constituent of GIPET™ (GastroIntestinal Permeation Enhancement Technology™), a proprietary enteric-coated solid dose formulation that was developed by

Merrion Pharma (Dublin, Ireland). GIPET™ was licensed to Novo Nordisk who recently published results from a Phase 2 clinical trial involving oral delivery of their long acting insulin analogue, I338 (Table 3). The PD performance of this formulation was equivalent to subcutaneously-administered (SC) insulin, but the formulation was discontinued. Merrion evaluated GIPET™ with other macromolecules (e.g. acyline, desmopressin) and some poorly permeable small molecules (e.g. zoledronic acid, alendronate) [52, 53, 57, 59], yielding BA increases with very large coefficients of variation. Aside from GIPET™, C_{10} has also been tested by Ionsys (California, USA) for oral oligonucleotide delivery [60] and more recently by Biocon (Bangalore, India) for delivery of an oral insulin analogue (Tregopil™) [61] (Table 3).

C_8 is another medium chain fatty acid that is currently under clinical investigation for oral macromolecule delivery. This fatty acid has a higher CMC than longer chain carboxylates and is therefore more soluble, but it is at the threshold of chain length for membrane penetration and permeation enhancement. There is routinely ~ 500 mg of C_{10} in solid dosage forms in clinical trials so even more material may be needed if C_8 is used unless it is combined with other excipients. Chiasma developed an oily suspension for the oral administration of octreotide (Mycapssa®) to treat acromegaly. The Transient Permeation Enhancer (TPE™) technology reversibly increased permeability of macromolecules less than 10 kDa [62]. The reversibility seen for this delivery system is a minimum requirement for any PE used in humans. TPE™ is prepared by dispersing C_8, polyvinyl pyrrolidone (PV), and octreotide in water, followed by milling, lyophilisation, and suspension in a lipophilic vehicle containing polysorbate 80, glyceryl monocaprylate, and glyceryl tricaprylate [62]. The rationale of formulating an oily suspension has not been described, but given the difficulty in creating stable suspensions, there must be value beyond conventional solid dosage forms. In the first Phase 3 trial of Mycapssa™ performed in 150 acromegaly patients over 13 months, high doses of octreotide (20 to 80 mg) were required to achieve equivalent outcomes to the SC formulation (0.1 mg) (Table 3) [63]. Subsequently, additional trials led to approval by the FDA in 2020.

Some investigators include acylated amino acids on the list of surfactant PEs, including sodium salcaprozate (SNAC (reviewed in [17]) and (8-(N-2-hydroxy-5-chloro-benzoyl)-amino-caprylic acid) (5-CNAC). However, while SNAC has a reported CMC of 56 mM, this PE does not have the distinct hydrophilic and hydrophobic moieties typical of soluble surfactants. The presence of a carboxylate anion at one end of the molecule and a hydroxyl group in the more lipophilic moiety prevents surface action. Indeed, it is not clear if the structures formed at 56 mM SNAC are micelles or other colloidal structures. That SNAC does not undergo adsorption nor form micelles and is therefore not a classic surfactant has been acknowledged [12]. Although SNAC may induce some degree of transcellular perturbation in intestinal epithelia, it is among the least efficient perturbants that have been tested in pre-clinical models. There has long been controversy regarding the mode of action of this PE with initial studies arguing for an unusual non-covalent interaction with macromolecules leading to epithelial uptake of the complex [64, 65], while others argue that the evidence is more consistent with detergent-induced transcellular perturbation [66]. Novo Nordisk report that their biophysical investigations did not reveal evidence that SNAC formed non-covalent complexes with peptides [12].

A recent study in Caco-2 monolayers confirmed that both C_{10} and SNAC caused transcellular perturbation [67]. SNAC was originally from a library of Eligen™ carriers developed by Emisphere (New Jersey, USA) in the 1990s as oral delivery vehicles for macromolecules. It was eventually approved in 2012 under Medical Food regulations for the oral delivery of vitamin B_{12} (Eligen® B_{12}, Emisphere) [68]. Novo Nordisk subsequently assessed SNAC for an oral formulation of the long acting GLP-1 receptor agonist, semaglutide (Rybelsus®), where the daily non-enteric coated tablet containing 7 or 14 mg semaglutide and 300 mg SNAC was approved by the FDA in 2019 (Table 3) [4]. The efficacy of oral semaglutide was demonstrated in a group of clinical trials collectively termed PIONEER (Peptide InnOvatioN for Early DiabEtes tReatment) trials. These trials show a significant lowering of HbA1c and weight loss in T2D patients. The question of why to opt for SNAC over PEs that

might alter epithelial permeability at lower concentrations has not been discussed, but there is clearly more to PE selection than the capacity to modulate barrier integrity, and other factors include additional beneficial actions, less damage at the gastric mucosa, or better compatibility with other additives[68].

Aside from medium chain fatty acids and derivatives, there are three other noteworthy surfactant groups: acyl carnitines [69], bile salts [17], and non-ionic surfactants [4, 70]. The latter group comprises macrogol glycerides (e.g. Labrasol [71]), alkyl maltosides/glucosides [17], sucrose esters [72], and ethoxylates [17]. Chloride salts of lauroyl carnitine (LCC) and palmitoyl carnitine (PCC) are amphoteric surfactants capable of reacting as both an acid and a base. Acyl carnitines are constituents of iterations of Peptelligence™ (Enteris Biopharm, New Jersey, USA) [69]. For example, an unspecified acyl carnitine was present in a clinical evaluation of oral rhPTH(1-31)NH2 relative to injections of PTH(1-34)OH (Forsteo®, Eli Lilly, Indiana, USA) [73] [74]. More recently, Peptelligence™ has been investigated for oral delivery of Difelikefalin in a Phase II trial although it is not clear if acyl carnitines are present in the formulation (Korsuva™, Cara Therapeutics, Connecticut, USA). Behaviour of amphoteric surfactants is comparable to mild non-ionic surfactants if the former is presented as the non-ionised zwitterionic form at physiological pH. However, when acyl carnitines are formulated with citric acid to bring the luminal pH value to below the pKa of the strongest acid in the hydrophilic moiety of acylcarnitine (carboxylic acid: ~4.2), this surfactant may be cationic, which may cause more extensive mucosal perturbation. As was the case for C_{10}, there were *in vitro* studies with acyl carnitines in the 1990s that supported a paracellular mode of action [75], with more recent ones providing evidence for the primary action of transcellular perturbation [76, 77]. While acyl carnitines are not excipients, extensive safety studies were carried out on them by Merck (New Jersey, USA) in the 1990s.

Bile salts are a structurally distinct group of aromatic surfactants that differ from most linear aliphatic surfactants. Many improve macromolecule intestinal epithelial permeation (e.g. insulin, calcitonin, heparin), and as some of these emulsifiers are present in bile secretions at

concentrations of 20-30 mM in the fed state, there is less concern related to systemic toxicity. Bile salts are natural detergents that act via transcellular perturbation [78]. The local action on GI epithelia has been classified as having moderate efficacy with fast recovery [79]. Examples include sodium salts of taurodeoxycholate, taurocholate, cholate and deoxycholate. Bile salts are present in the POD™ technology of Oramed, although it is unclear if they have not been progressed in recent iterations. In another unrelated example, Sandoz (Basel, Switzerland) found hard gelatin capsules containing chenodeoxycholate (100 mg) and octreotide (4 mg) increased oral BA to 1.3% in humans [80]. Sodium taurocholate was also a PE in a prototype of Enteris's Peptelligence™ technology and in fact had a similar permeability enhancement effect on the oral BA of sCT as LCC [69].

Non-ionic surfactants are generally viewed as milder than ionisable ones and typically have lower CMCs, making them more efficient at micellar solubilisation. Although there is a lower free surfactant relative to ionisable surfactants, non-ionic surfactants may be more efficient at penetrating plasma membranes and can be potent PEs demonstrating efficacy on par with C_{10}. Examples include alkyl ethoxylates, macrogol glycerides, and sucrose/lactose esters. A recent article demonstrated significant intestinal absorption of insulin in rats in the presence of sucrose laurate [72]. This food additive and excipient is suitable for human consumption and is allowed at high daily ingestion limits.

Alkyl maltosides and glucosides are non-ionic surfactant that have also been assessed as vehicles for macromolecule delivery in pre-clinical studies [81]. Whether the hydrophilic group is ester- or ether- linked, disaccharide (sucrose, maltose) or monosaccharide (e.g. glucose), these surfactants do not differ much in terms of enhancement potency and efficacy. A common feature is low CMC values relative to ionisable surfactants, which may limit the quantity of free surfactant available for enhancement in the GI tract lumen. *In vitro* studies demonstrated that dodecyl maltoside (DDM) altered expression of TJ proteins [82], along with evidence of transcellular enhancement with mild mucosal perturbation [81]. Non-ionic surfactants are difficult to incorporate into oral solid dosage forms because they present as

liquids, semi solids, or waxy solids. They may however be included in liquid formulations for enhancement at other epithelia including nasal/ophthalmic delivery [83] or in non-aqueous vehicles [62]. The development of the oily suspension of octreotide, Mycapssa™, shows that manufacturers are not restricted to classic solid dose formulations for oral macromolecule delivery. At present, carbohydrate-based esters and glucosides have not progressed to clinical testing, although the alkyl maltosides form part of the Intravail™ delivery platform (Aegis Pharma, San Diego, CA, USA) that was recently used in an FDA-approved formulation for nasal delivery of sumatriptan (Tosymra™, Promius Pharma LLC, New Jersey, USA). Intravail™ has been assessed for oral peptide delivery in rodent studies [84].

Labrasol® (Gattefosse, Saint Priest, France) contains a mixture of medium chain macrogol-8 glycerides, glycerides and free PEG. The pre-concentrate spontaneously microemulsifies in water, and therefore the principle use for this vehicle has been to assist oral delivery of poorly soluble compounds. This versatile vehicle can be used directly as a solubilizer or in blends with other oils as an emulsifier. Labrasol® is an allowed excipient that has been shown to improve small intestinal BA of heparin in rodents [85] and, as it was recently approved for oral delivery of the BCS Class II drug, enzalutamide, it may be considered an AME. Additionally, in a recent comparison of several surfactants in isolated rat colonic mucosae, Labrasol® was the only PE where there was a high degree of separation between concentrations that caused enhancement and histological damage [43]. A recent study found that a number of constituents of Labrasol® contribute to enhancement, in particular the medium chain mono- and di- macrogol-8 glycerides [71]. There has been some debate whether it is the macrogol glycerides that elicit permeation enhancement or the medium chain fatty acids that are liberated during lipolysis in the GI tract, i.e. a prodrug concept. In theory, macrogol-8 glycerides are medium chain non-ionic surfactants that could be expected to behave like linear ethoxylates that exhibit strong enhancement (e.g. $C_{10}E_8$). Improvement of insulin BA following jejunal perfusion was similar for Labrasol® compared to when it was exposed to lipase inhibitors, suggesting preservation of enhancement action in the parent

molecules[71]. To our knowledge, Labrasol® has not yet progressed to clinical testing for oral peptide delivery, but there is renewed interest from Pharma [86].

3.2.2 Hydrophobic ion pairing

Investigators have sought to use non-aqueous vehicles to solubilise peptides that have been transiently hydrophobized via HIP [16]. Amphiphilic ion paring agents are sometimes referred to as a distinct sub-category of transcellular PEs, the distinction being that they do not act via perturbation [17]. Solubilisation of lipophilic salts in SEDDS offers the potential for both improved passive permeation of the lipophilic complex and transcellular perturbation from surfactants used to stabilise the SEDDS. The insoluble salts formed by HIP have improved partitioning in octanol and improved solubility in lipid-based formulations, although it remains to be seen if the complexes facilitate permeation across the intestinal epithelium. The main benefit of HIP may be to facilitate formulation and co-presentation of non-aqueous vehicles like Labrasol® with the macromolecule. The complexing agent sodium docusate has been assessed to improve permeation of desmopressin [87], leuprolide [87], exenatide [88] and insulin [87] in different LBFs. Oral administration of octreotide deoxycholate (equivalent to 50 mg of octreotide) to pigs in 10 mL of SEDDS (containing 60% Brij O10, 10% propylene glycol and octyldodecanol) followed by 10 mL apple juice had a bioavailability of 5.2%, nearly 18 fold higher than unformulated octreotide [89]. Here, enhancement may relate to protection from luminal peptidases in SEDDS, improved passive permeation and the capacity of the additives in the SEDDS (in particular polyoxyethylene-10 oleyl ether, Brij O10) to act as transcellular PEs.

3.2.3 Non-surfactant PEs that may act in part via the transcellular route

Not all transcellular PEs are surfactants. Such examples include cell penetrating peptides (CPPs), ionic liquids, and other solvents (see Table 2). Choline geranate (CAGE) is a liquid formed by mixing choline bicarbonate and geranic acid (1:2) which react to form the neutral choline geranate and free geranic acid. There is debate as to whether this is truly an ionic

liquid, i.e. a salt that forms a liquid below 100°C, or a eutectic mixture because of the presence of geranic acid and potential for free geranic acid to impede crystallization of the salt. Regardless of the classification, CAGE (80 mg) elevated relative oral BA of insulin (10 IU dose) to 45% from enteric-coated capsules following oral gavage to rats, a value that is much higher than those recorded for other PEs to date. Although there was no histological damage observed in rats, CAGE caused a partial reduction in Caco-2 cell viability and an increase in intracellular FITC-labelled insulin, suggesting some level of transcellular perturbation.

CPPs are widely studied for their capacity to efficiently assist cellular uptake of biologics. Research has mostly focused on their application as surface coatings for nanoparticles [90] or as conjugates [91]. There are a number of studies showing improved permeation of macromolecules with ad-mixtures of both natural (transportan, penetratin, HIV-TAT) and synthetic CPPs (polyarginines) [92]. The exact mode of action for CPPs is not clear: *in vitro* studies show they can improve permeation via endocytic pathways, membrane pore formation, physical complexation [93], and chemical conjugation [91]. The admixture of CPP with peptides is an approach that is unique to oral delivery. Proof-of-principle studies for CPPs have focused on oral delivery of insulin with polyarginine [94] and penetratin [95] in rats, but improved permeation of GLP-1 and larger proteins including gastrin has also been noted [96]. To date, CPPs have not been assessed clinically for oral macromolecule delivery as either ad-mixtures or components of nanoparticle formulations. More than 20 CPP conjugates of peptides have been tested clinically to improve cellular uptake following parenteral delivery, although most have been discontinued. The reasons cited for discontinuation include safety, low efficacy, and stability concerns for these peptides [97]. There are also stability concerns for application of CPPs in oral delivery. A recent attempt to improve enhancement involved loading CPPs in silica nanoparticles and then mixing with peptide-loaded nanoparticles electrostatically to create a nanoparticle dispersion [98]. This delivery system improved BA of recombinant human growth hormone. As CPPs are derived from microorganisms or are synthetic peptides with no prior history of use in humans, the lack of a defined mode of action

and failure to demonstrate improved efficacy relative to established PEs represent impediments to translation.

4. A PERSPECTIVE ON PE SAFETY

There are safety concerns relating to mechanisms of action and off-target pharmacological actions of PEs. These concerns are heightened for substances with known adverse actions in humans (e.g. microbial toxins) and for NCEs where there is a lack of human safety data. This was thought to be less of a concern for food additives, excipients, or substances with a history of safe use in humans, but a recent study found that chronic exposure to two common emulsifiers, polysorbate 80 and carboxymethyl cellulose caused mild inflammation in healthy mice as well as colitis in predisposed mice [99]. These inflammatory effects were associated with changes to the microbiome. The risks associated with unproven prospective excipients is an impediment to the development of new excipients, as there is no separate regulatory pathway for their approval. While all excipients have a purpose in a given formulation, only a small proportion exhibit pharmacological action. There are no excipients whose primary function involves modulation of biological processes, and thus the risk of off-target toxicity for PEs that pharmacologically modulate the intestinal barrier integrity presents an added development risk.

4.1 Concerns related to regional alteration in Intestinal barrier integrity

Aside from the risks associated with unidentified systemic actions, there are specific concerns for PEs relating to the regional alteration in intestinal barrier integrity. These concerns are greater for transcellular PEs that act initially via mild mucosal perturbation (SNAC, acyl carnitines, C_{10}), typically those with more established use in humans, rather than novel PEs that act via TJ modulation. It is noteworthy that regardless of whether the PE acts through transcellular perturbation or pharmacological opening of TJs, there is potential for common GI symptoms. Mild GI disturbance such as nausea and diarrhoea that are associated with several medications (e.g. metformin [100]) may relate to a physiological changes even in the absence

of intestinal lesions, so there are also similar concerns for paracellular PEs that do not cause mucosal perturbation.

Modulating intestinal permeability to improve BA of macromolecules has not revealed major adverse events in clinical trials to date and was not a major impediment to translation. Although most of the trials cited in Table 3 did not assess local mucosal perturbation, they suggest that the small improvements in permeability seen were attained without major adverse events in trial participants under controlled conditions. Safety data in clinical trials using PEs have mainly been limited to these mild established PE perturbants. The effect of mild perturbants on the intestinal wall still remains a cause for concern despite there being no immediately obvious manifestations in clinical trials. A study assessing several transcellular PEs in isolated rate colonic mucosae in Ussing chambers found an association between an increased P_{app} of [^{14}C]-mannitol and histological damage [43]. While these data cannot be extrapolated to the effects observed in humans, permeation enhancement tends to be associated with reversable perturbation. The question is what level of perturbation is within the routine range of damage and repair, and can thus be reversed by normal GI mechanisms? If delivery systems with new PEs designed to accentuate enhancement action are more effective than traditional ones, will there be a concurrent increase in mucosal perturbation to cause unacceptable GI side effects? A wide range of studies show that the gut has a remarkable capacity for regeneration [101], but repeated cycles of damage and repair may strain repair mechanisms [102].

Cell function and viability testing in cell culture monolayers and isolated tissues provide a sensitive tool for the identification of potential toxicity. However, it can be difficult to model toxicity *in vitro* as lengthy exposure times do not represent dynamic conditions in the human small intestine. Studies comparing PEs to other dietary xenobiotics and endogenous secretions [103-105] in respect of potential for membrane perturbation emphasise that its occurrence is more widespread. Small intestinal epithelial cell turnover is high, and disruption to the capacity to of the intestinal epithelium to undergo repair, such as with the use of non-

steroidal anti-inflammatory drugs (NSAIDs) [106], can lead to serious GI side effects, thereby highlighting the central role of damage and repair cycles in the normal function of the GI tract.

4.2 Side effects of oral peptide dosage forms containing PEs in humans

Oral PE-based dosage forms have produced relatively mild and common GI side effects in both short and long duration clinical trials (Table 3). These include diarrhoea (e.g. I338 with C_{10} [6], sCT with 5-CNAC [107]), and constipation (e.g. sCT with 5-CNAC [107]). Other features are nausea (e.g. acyline with C_{10} [59] semaglutide with SNAC [108], sCT with citric acid [109] and octreotide with C_8 in an oily suspension [63]), as well as abdominal pain (e.g. sCT with 5-CNAC [107], PTH with lauroyl carnitine [74]) and vomiting (e.g. acyline with C_{10} [59], heparin with SNAC [110], sCT with 5-CNAC [107]). Some of these side effects may relate to specific drug classes rather the incorporated PE, as is the case with GLP-1 analogues [111]. These GI-related events can also be associated with underlying pathology or physiological/pharmacological actions caused by some medications [112]. For example, clinical manifestations of drug-induced gastritis include nausea and abdominal pain. Nausea is a non-specific symptom that may be the result of local gastric damage, gastroparesis, or chemical effects on the CNS. Drug-induced microscopic colitis is observed with selective serotonin reuptake inhibitors (SSRIs) and NSAIDs [113], which are also are associated with diarrhoea. Therefore, common GI disturbances observed with PEs in clinical testing warrant further evaluation particularly during long term use. Furthermore, mucosal injury can be asymptomatic for some substances that cause intestinal inflammation. The majority of inflammatory lesions resulting from diclofenac were asymptomatic as determined in a study of 40 healthy volunteers by capsule endoscopy [114].

The main pathologies of drug-induced GI disease are ulceration, stricture formation, inflammation and ischaemia. There has been no report of such pathologies induced by PEs in oral macromolecule dosage forms in clinical data presented to date (Table 3). Mucosal perturbation to at the intestinal wall may potentially cause microscopic colitis, but is less likely

to cause the extensive damage observed with NSAIDs. The capacity of NSAIDs to cause inflammation and ulceration in the stomach and small intestine is recognised, and there has also been studies associating NSAID use with inflammatory bowel disease (IBD) [115]. There is evidence that acidic drugs can perturb the gut wall. However, the ulcerogenic action of NSAIDs is also related to inhibition of cyclooxygenase [116], reduction in mucous and bicarbonate production, and decreased blood flow. Any suggestion that mucosal damage caused by PEs is acceptable based on precedence with approved products that cause GI disturbance could be a risky approach, so additional safety testing might be prudent. NSAIDs might not have a straightforward path to approval today.

Determining if PEs cause histological damage in the small intestine of humans is not practical. This is due to difficulty identifying the site of release and performing a biopsy at the enhancement site at the appropriate time. Capsule endoscopy was a useful tool to show gross tissue damage caused by NSAIDs, but it is unlikely that PEs cause macroscopic lesions. Toxicokinetics of PE dosage forms is more easily studied in the rectum, although differences in anatomy and physiology between the rectum and upper GI tract as well as major differences in formulation exposure time and dilution means it is not possible to extrapolate. Nonetheless, the first commercial application of intestinal PE was the use of C_{10} to assist rectal absorption of ampicillin. Doktacillin™ suppositories (250 mg ampicillin, 950 mg hard fat (Pharmasol B-105), 25 mg C_{10}) were initially developed by Astra Pharma (now AstraZeneca, Cambridge, UK) and are now part of the portfolio of Meda Pharma (Solna, Sweden), although the rectal formulation has since been discontinued. A clinical assessment of Doktacillin™ suppositories with or without C_{10} (25 mg) showed an increased in rectal BA of ampicillin from 13 to 23%, and there was an increase in the average histology score 25 minutes after administration from 0.62 to 1.94, which was partially ascribed to the hyperosmolarity of the formulation [58]. The rectal mucosa recovered to a score of 0.96 after 3 h, verifying fast recovery. Reversibility has also been shown further up the GI tract in rat intestinal instillations [56]. It would be interesting to see if there was progressively more damage and/or slower repair upon chronic repeat

exposure, as has been observed in reductionist models [102]. Doktacillin™ suppositories were intended for multiple daily administrations over a short period, but there may be more of an issue in chronic treatment of disease. Indeed, most clinical trials of oral macromolecules with PEs have been designed for once- or twice daily oral administration [13] [6] [117] [63]

4.3 Safety of modulating barrier integrity via the paracellular route

Modulation of TJs is a more physiological approach to altering GI permeability relative to the effects of transcellular perturbants. As TJ modulation is a natural response to nutrient absorption and fluid secretion, there seems to be less scope for TJ opening to permit microbial uptake or to result in inflammation over the length of the small intestine. The maximum pore diameter for TJ openings is below that of bacteria [118, 119]. A number of toxins alter TJ integrity (e.g. ZOT, CPE), but within the native structure there are moieties that may cause electrogenic chloride and fluid secretion (e.g. ZOT [19]). This is one of the reasons why analogues have been developed that omit segments of the protein responsible for adverse events. TJ modulators that target biological processes such as receptors, enzymes or messenger proteins present a greater potential risk beyond the mucosal surface in the systemic circulation. This risk can be sub-divided into unwanted permeability alteration at other TJs or off-target actions on receptors, enzymes, or messenger proteins that are ubiquitous in biological processes (e.g. PKC, PLC, calmodulin). In such instances, it may be difficult to distinguish side effects associated with the drug and excipients, a phenomenon that has been observed previously with novel excipients (e.g. Cremophor® EL [120]).

Not all investigators agree that improved permeability can be safely achieved by opening TJs, citing the role of barrier impairment in IBDs, bacterial infection and autoimmune disease [121]. There are concerns that modulating intestinal permeability may increase the risk of microbial infiltration of the sub-mucosa by opportunistic pathogens or increased permeation of microbial endotoxin. The clinical studies listed in Table 3 did not support the case that leading PE formulations permit entry of microorganisms into the systemic circulation. Additionally, there

is no convincing evidence from the study of intact intestinal tissue to support that bacteria or bacterial toxins are transported from the lumen via the paracellular route to the sub-mucosa [122]. Overall, there is likely to be greater potential for penetration of microbes and endotoxin into the sub-mucosa from transcellular perturbation relative to physiological modulation of TJs. In one study, co-incubation of octyl phenol ethoxylate (Triton® X-100) with *E. coli* increased bacterial translocation across Caco-2 monolayers, whereas C_{10} had negligible effect on microbial uptake [52]. The data shows the potential for microbes to move across compromised monolayers, but the same events may not occur *in vivo* due to a large reservoir of specialised resident macrophages in the sub-mucosa, as well as lines of defence against microbes and microbial products in the liver [123]. There are also additional luminal hurdles that are not found in Caco-2 monolayers, specifically the presence of a mucous gel layer that can restrict diffusion of microbes to the gut wall. As PEs cause only a modest uptake of co-localised peptides, there appears to be a low probability that bacteria or bacterial products that are spread diffusely would have the opportunity to translocate the gut wall at focal sites [123]. It is necessary to emphasise that the epithelial barrier is a major hurdle to translocation, which is perhaps why several pathogenic microorganisms produce enterotoxins capable of altering barrier integrity, and that virulence is often dependent on expression of these enterotoxins. Nevertheless, microorganisms that produce toxins that target TJs are more likely to cause fluid and electrolyte secretion rather than endotoxemia [122]. Although there appears to be a low risk of barrier alteration leading to microbial uptake, further studies are warranted. The interaction of microorganisms with the GI can result in asymptomatic infection, diarrhoea, gastroenteritis or systemic infections [124]. There is the view that many systemic infections start in the gut [125], although whether dietary substances cause such infections is less clear.

5. CURRENT REGULATORY STATUS OF PEs

Many excipients used in oral formulation have been shown to alter intestinal barrier integrity in pre-clinical drug delivery models including solvents (e.g. ethanol) and surfactants used as wetting agents (e.g. SDS) or emulsifiers (e.g. polysorbates, macrogol glycerides, sucrose

laurate). However, as these AMEs are included in low quantities and not intended to act as PEs, it is unclear if they reach a threshold concentration for barrier alteration or if they have any impact on oral bioavailability. Concerns have been raised that the presence of these excipients in innovator products may contribute to the BA values observed in clinical testing [18], and that their absence from generic products may lead to a reduction in BA. Any reduction in BA for generic products may go unnoticed if it is granted a BCS biowaiver. This would appear to be relatively low risk as there is currently no clinical data suggesting a generic product is less effective than an innovating due to the absence of an AME. PEs differ in that they are intentionally added to oral formulations to increase BA, and as the active will always be a BCS Class III drug (low permeability/high solubility), any generic product will not be suitable for a biowaiver. To date, there has been only three market authorisations for enteral products containing PEs; two oral products in the USA (SNAC and C_8) and one rectal suppository previously marketed in Sweden and Japan (C_{10}). Regulatory authorities in the USA, Japan and Europe review formulations on a case by case basis and do not provide general guidance relating to suitability of excipients. Nevertheless, as safety is considered a key aspect to any submission, there has been a cautious approach to PE selection, where the majority of PEs that progress have a history of use in humans as food additives or excipients. Examples include SNAC, C_8, bile salts, citric acid, C_{10}, and EDTA. selection of a PE from the FDA Inactive Ingredients List or substances with food additive or GRAS status does not provide an assurance on safety as the dose may be in excess of the recommended daily intake. Preclinical and clinical toxicology of the final dosage form consisting of the combined components can possibly address this, but separate PE toxicology studies may be required. There have been cases where prominent PEs have been omitted from the formulation that proceeds to phase III clinical trials (e.g. acyl carnitines in Peptelligence™), potentially due to safety concerns. As more safety data emerges from clinical trials, there may be greater confidence for inclusion of PEs that potentially offer greater efficacy especially as most products fail due to low and variable BA.

6. HURDLES TO TRANSLATION OF PEs IN ORAL DOSAGE FORMS FOR MACROMOLECULES

PE efficacy can be high in static drug delivery models such as Caco-2 monolayers, but becomes progressively less so in less reductionist models, to the point where there is sometimes negligible levels of BA in humans [126]. Use of Caco-2 monolayers to predict PE-assisted intestinal permeation of macromolecules is limited since they cannot accurately model the small intestinal response to PEs *in vivo*. While the concentration of the payload reduces as it moves along the lumen of the small intestine, permeation continues owing to the maintenance of a concentration gradient across the gut wall. The inclusion of a PE to assist movement of a macromolecule in static delivery models does not mirror the conditions within the GI tract because the PE does not act on the entire length of the intestine that the drug is exposed under *in vivo* conditions. Co-incubation of PE and active in static models shows what is possible if both substances are presented in the small intestine together for an extended duration in sufficient concentration. In the small intestine *in vivo*, there is a lag to onset of PE action that ranges from minutes (e.g. C_{10} [55]) to hours (e.g. peptides that directly target claudin proteins in TJs [127] [25]). Thus, continuous movement through the small intestine may reduce optimal PE concentrations, which is problematic for PEs that are slow to act. Transit of the macromolecule past the point where the PE achieves its highest concentration reduces exposure of the macromolecule at the altered epithelium. The concentration of PE is therefore reduced below a threshold for enhancement by dilution, spreading, and absorption (if it occurs). The simplest way to address these issues is to use a large excess of PE that quickly acts on the epithelium, so that there may be continuous exposure of the macromolecule to a compromised barrier, akin to a constant infusion. In this scenario, the PE concentration may remain above the required threshold concentration for an extended period. This has been the approach of a number of oral solid dosage forms, either inadvertently or by design.

The impact of fluid volume depends whether the dosage form is designed for release in the stomach or small intestine. The volume of liquid in each intestinal region depends on

the amount of water the formulation is administered and whether administered in the fasted or fed state. Variation in gastric and intestinal fluid volume impacts the luminal concentration of both PE and macromolecule. In the case of the macromolecule, dilution in a high fluid volume will reduce the concentration and ultimately decrease the concentration gradient, which slows diffusion across the gut wall. For the PE, dilution in a larger volume may result in reduced efficacy. For immediate release dosage forms intended to act in the stomach, variation in resting gastric fluid volume and the amount of water in which the formulation is taken in can lead to variations in fluid volume in the stomach. Small intestinal fluid is unevenly distributed in fluid-filled pockets rather than as a large reservoir of liquid [128]. Therefore, any prediction of luminal concentration based on the total fluid volume may underestimate the local PE concentration within a fluid pocket. A recent article reviews how dose volume, excipient dose level, volume of water chaser and gastric fluid volume influence luminal excipient concentrations for solubility and permeation enhancing excipients [129]. For PEs, the study summarises the disparity between efficacy in static in vitro models compared to in vivo, which was partly relating to fluid volume and the difficulty in maintaining a threshold concentration for enhancement. Additionally, the author notes a requirement for further studies collecting data on excipient concentrations in luminal fluids and determining how this effects drug absorption.

The dynamic conditions in the GI tract is another of the principle reasons why most PEs have only a modest effect on oral BA. There are segments of the GI tract where the PE and active may be co-localised for an extended period. They include the oral cavity (buccal, sublingual), rectum, and to a lesser extent the stomach and colon. Until recently, the stomach was not considered a target site for oral macromolecule delivery owing to potential for chemical/enzymatic degradation, a low surface area for absorption, and the difficulty in ensuring sufficient residence time. However, oral tablets of semaglutide containing SNAC that released in the stomach [12] challenged the consensus that enteric coating is a pre-requisite for oral peptide delivery. A slow release oral dosage form may contact the gastric mucosa for

an extended period, although any dissolved PE and active that enter bulk gastric fluid may still exit the stomach into the duodenum. There are additional hurdles to targeting the gastric mucosa. The patient must avoid food and drink for a time dictated by the release characteristics and enhancement kinetics, a minimum of 30 min. It is also not possible to predict consistent gastric emptying, but is less of a problem for GLP-1 receptor agonists that slow gastric emptying [130].

The small intestine has long been considered the preferred target site for delivery of peptides. While transit through this region is more predictable than the stomach and colon, the quick rate of movement under both fasted and fed states limits co-localisation of PE and active at any focal point. When both PE and macromolecule are confined to a segment of the small intestine via ligation there is a significant increase BA [131]. Confining the PE and active to a segment of the small intestine is difficult to achieve in humans. Initial efforts involved the use of mucoadhesive polymers [132] [133] [134], although they do not easily affix to mucous without force and, as there is a relatively high turnover of mucous, the formulation can detach. Fast GI transit of particulates at a flow rate of 2.5 to 3.5 cm/min (assuming a residence time of 3-4 [135] and length of 6.25 m [136]), highlights the challenge in localising a PE with a macromolecule.

Absorption may limit residence time of small molecule PE, although this is less of a problem for non-absorbable polymeric PEs, such as chitosan [137]. Recent clinical trials have highlighted that SNAC (T_{max}: ~30 min[12]) and C_{10} (T_{max}: 23 min [6]), are quickly absorbed from the stomach and small intestine, respectively. There have been efforts to control the release of PEs from oral dosage forms [60, 138], although in the absence of control over intestinal transit, this will not solve the problem of sub-optimal co-localisation. In immediate release systems, there is a requirement for synchronous release of PE and macromolecule. Efforts to release PE and active over an extended period may help to offset reduction in PE concentration due to its absorption, but such a release profile assumes that the PE reaches a threshold concentration for effective enhancement as soon as it is released, and that slow

release of the macromolecule does not slow diffusion across the intestinal wall as the concentration gradient falls. Yet, an oral formulation designed to exhibit a burst release of PE and macromolecule, followed by a supplemental release of PE, had only a modest effect on BA relative to the immediate release formulation [60]. There may be differences in optimal release kinetics from an immediate release tablet versus technologies that are designed to promote co-localisation, e.g. intestinal patches (Table 4). In the case of the patch, it may be appropriate to introduce a degree of asynchronous release, where there is initial release of PE followed by release of macromolecule and PE. This asynchronous approach may be effective because the patch is theoretically affixed to the gut wall, and hence the PE and macromolecule will still be presented at the same site.

The physicochemical properties of the PE and macromolecule must also be considered an area for optimisation, as are the properties of the formulation. Although the majority of macromolecules may exhibit properties similar to BCS Class III drugs, exhibiting high solubility and low permeability, an assumption that solubility and dissolution are not key factors in develop of oral formulations may not be accurate. Macromolecule drugs are often ionisable containing both acidic and basic side chains, and in some cases display amphipathic and zwitterionic behaviours, which has a significant impact on release properties in environments of high ionic strength. Zwitterionic peptides and proteins exhibit their lowest solubility at their pI. They are slow to dissolve at this pH and can precipitate if the bulk intestinal fluid has a high ionic strength or if there are divalent cations. Many peptides have good solubility in acidic conditions, but poor dissolution characteristics at the pH range in the small intestine (pH 6 to 7.5). Insulin is practically insoluble in water at its pI (5.4), but dissolves well at < pH 4 [139]. Insulin dissolves to a capacity of 5 mg/mL in water at pH 7, although solubility can decrease in the presence of organic species, electrolytes, and/or Zn^{2+} and Ca^{2+}.

Solubility must be considered relative to potency, as the higher the potency of the macromolecule the lower the impact of solubility at low doses. In recent clinical trials, the daily oral dose of long acting insulin (~16.4 to 98.2 mg [6]) was higher than semaglutide (7 – 14 mg

[4]), so dissolution considerations may be more of a consideration for insulin. Gradual dissolution may be problematic if there is fast dissolution of PE relative to the peptide, which in the context of intestinal flow may lead to a reduction in PE efficacy. There have been few studies assessing dissolution characteristics of PE-based dosage forms.

The physicochemical properties of the PE are equally important: solubility, compatibility, and stability are primary concerns. A soluble PE present in high concentration may compete with the macromolecule for water, thus slowing dissolution. Many PEs are weak acids, hence will exhibit better solubility above their pKa where they exist in the ionised conjugate base form. In this state, some PEs may be capable of forming ion pairs with weakly basic amino acid side chains, which may reduce aqueous solubility [140]. Stability maintenance of macromolecules in GI fluids is a widely cited concern, although it has been less considered for the PE.

Constituents of intestinal fluid are known to play a role in solubility and dissolution [141]. Solubility may change by several orders of magnitude at different pH values in the stomach and small intestine. Viscosity changes upon ingestion of food can slow dissolution [142], while ingested substances can interact with the macromolecule through chemical degradation and physical complexation/adsorption [143]. Detailed information about intestinal fluid composition has assisted in design of solubility enhancement strategies. The effect of luminal constituents on prediction of permeability or design of advanced drug delivery systems that improve permeation has not been studied to the same extent. There are two environments that must be considered, bulk intestinal fluid and the microenvironment at the epithelial surface. Bulk luminal fluid contains a mixture of endogenous secretions, sloughed cells and ingested substances, which creates a harsh environment for chemically and enzymatically labile macromolecule. There is the possibility that the free concentration of PE and/or active is reduced in both environments. Additionally, not all substances permeate mucous [144], and therefore will not have access to the microenvironment at the epithelial surface.

A variety of food interactions impact absorption, and there is potential for both soluble and insoluble substances to modulate enhancement. PEs are sensitive to the same chemical and enzymatic degradation as active macromolecules. Special consideration should be given to surfactants as the largest PE category. Transcellular perturbation closely mirrors detergency a process that is largely driven by the free monomeric surfactant, a factor that can change depending on the environment. For example, changes to ionic strength can reduce the CMC of ionic surfactants, thereby lowering the concentration of free surfactant available to interact with the membrane. Na^+ and K^+ counter-ions reduce repulsion between ionisable hydrophilic head groups, thereby creating more favourable conditions for micellization at lower concentrations [145]. Enhancement capacity of the anionic surfactant, SDS, was increased in hypotonic electrolyte solution in a rat single pass intestinal perfusion [146]. As surfactants preferentially adsorb at the interface between distinct phases such as solid-liquid, liquid-liquid (e.g. oil/water) or liquid air interfaces, free monomers may adsorb to undigested food particles, lipid globules, and colloidal structures in the GI lumen. Although the free surfactant can be replenished from micelles, the amount of free surfactant sequestration to other interfaces may play some part in reducing surfactant concentration at the epithelial surface. In the presence of divalent counterions, an insoluble complex may form between two surfactant monomers and the ion (e.g. calcium dicaprate) thereby preventing enhancement. Surfactants with low aqueous solubility are likely to have improved capacity to penetrate into plasma membranes, but their enhancement action is limited by low solubility. Any substance that increases surfactant solubility may potentially increase the amount of surfactant available to interact with the plasma membrane. There was an increase in enhancement action of methyl 10-hydroxydecanoate in the presence of PEG solvents [147]. The effects of luminal substances on surfactant action is not limited to ionisable formats. Constituents of simulated intestinal media reduced the effectiveness of maltopyranosides through the formulation of mixed micelles [148].

Recent investigations using rat single pass intestinal perfusions found that absorption was attenuated for PE-macromolecule combinations in fed state simulated intestinal fluid (FeSSIF). This was attributed to colloidal structures [149]. It is not possible to conclude that incorporation of a PE into a mixed micelle or other colloidal structure will attenuate enhancement. Surfactant blends containing PEG-8 glycerides (Labrasol®) with either C_{10} or $C_{11:1}$ had a greater effect on FD4 permeation across isolated rat colonic mucosae [150]. The CMC of Labrasol® was 0.01%v/v [151], thus it forms micelles at concentrations well below that of medium chain fatty acids. A reason why blends might be more effective is the combined detergent effects (from fatty acids) and efficient micellar solubilisation (from the PEG-8 glycerides) of membrane fragments. Fasted stated simulated intestinal fluid (FaSSIF) containing bile salts and phospholipids in buffered saline (pH 7.1) had no effect on efficacy of other PEs such as EDTA [152]. Studies evaluating the action of PEs in the stomach (e.g. SNAC) do not account for gastric pH, thus mode of action studies do not necessarily reflect the ionisation state of the PE.

It is difficult to predict the effect that luminal fluid composition might have on enhancement action due to variability in the composition of human GI fluid. Moreover, there may be subtle differences in the composition of bulk fluid and the microenvironment at the epithelial surface. While simulated intestinal fluids (SIFs) designed for dissolution testing focus on pH, ionic strength, and the presence of bile salts, lecithin and some lipids, other minor constituents can weaken predictions relating to permeation and/or the behaviour of PEs. Additionally, SIFs designed for dissolution testing contain species that are also capable of reducing barrier integrity including sodium taurocholate, lecithin, sodium oleate, and sodium monocaprylate [153]. They are therefore less suited for use in - bioassays used to identify PEs including Caco-2 monolayers, tissues mounted in Ussing chambers, and intestinal sacs. In a Caco-2 study, FaSSIF was considered a suitable vehicle for transport studies, whereas FeSSIF caused mucosal damage [154]. To date, efforts to develop biorelevant media for *in vitro* transport studies across monolayers or isolated tissue mucosae have involved reducing

the quantity of bile salts and lecithin in FaSSIF and FeSSIF that perturb the epithelium [155], while others have included a layer of biosimilar mucous [156] or have co-cultured Caco-2 cells with mucus- secreting HT29-MTX cells [157]. A major issue for implementation of SIF protocols with *in vitro* assays is data reliability. Perhaps assessing the impact of biorelevant media on PE action should begin in rat intestinal instillations or perfusions [149].

7. A PERSPECTIVE ON PE-BASED DOSAGE FORMS OF MACROMOLECULES

The majority of PEs that have progressed to clinical evaluation for oral macromolecule delivery are conventional ad-mixed powders that are formulated into immediate release or enteric-coated tablets/capsules. While the quantity of macromolecule required in oral formulations is much higher than in parenteral formulations, the quantity of the PE that is required in oral dosage forms is often 5 to 10 times higher than that of the macromolecule (Table 3). Perhaps the higher the quantity of PE that can be incorporated into a formulation, the more likely there is to be significant enhancement as the macromolecule moves through the small intestine [158]. The physical properties of the PE are therefore a key consideration in dosage form design. All poorly permeable macromolecules are solid substances, whereas the physical state taken by PEs can range from liquid (e.g. ethanol, CAGE, Labrasol®) to semi-solids and waxes (non-ionic surfactants), to solids. Liquid and semi solid PEs are more challenging to formulate with macromolecules.

The formulation of hydrophilic macromolecules in non-aqueous vehicles is challenging, so there must be a clear advantage for their use over PEs in solid formats. Some non-aqueous liquids like CAGE can be formulated in soft gelatin capsules [159], but stability concerns may arise when attempting to dissolve the macromolecule in the solvent where it could be sensitive to degradation by the solvent or from trace impurities from formulation additives. Insulin was stable in CAGE for 4 months at 4°C, although there was evidence of deterioration after six months [159]. Liquid PEs can be converted to solids using adsorbents (e.g. use of silica with Labrasol®), but the relatively large ratios of adsorbent required for liquid-to-solid conversion

reduces the overall quantity of PE that can be incorporated in the formulation [70]. It remains unclear if such solid variants retain permeation enhancement capacity.

If the macromolecule does not dissolve in the liquid PE, it may be possible to create a non-aqueous suspension in the vehicle, as was done by Chiasma with the oily suspension for Mycapssa®. It is unclear what advantage dissolution from an oily suspension has over dissolution from a solid dosage form. The macromolecule may be less prone to chemical instability in the solid state, but it can be difficult to achieve uniformity and suspended solids are sensitive to physical instability (e.g. coagulation, caking). Additionally, if the liquid is an oil (e.g. monoglycerides), this may result in a form of dissolution-controlled release, as is observed with granules coated with lipophilic coats or extended release oily suspensions. This could lead to more gradual dissolution and ultimately may give rise to asynchronous presentation of PE and macromolecule at the intestinal wall.

There has been effort to formulate peptides in coarse dispersions (including microparticles, water-in-oil emulsions (e.g. Macrulin™, Provalis, Flitshire, UK [160]) and multiple emulsions) and nanodispersions (e.g. microemulsions, reverse micelles). Some of these dispersions are difficult to formulate, and can suffer from physical instability in storage and when diluted in biological fluids (e.g. water-in-oil emulsions). These delivery systems may protect labile cargoes from enzymatic degradation and may offer potential for increased epithelial transport. However, to date there is not much evidence that current iterations enhance epithelial permeability beyond that of simple PE systems. Surfactants used to stabilise water-in-oil emulsions are typically insoluble surfactants that may act as PEs, but these lipophilic surfactants are inefficient detergents, thus the contribution to transcellular perturbation will be limited. Effort to formulate water-in-oil emulsions with soluble surfactant PEs is not feasible as these are more commonly used to formulate oil-in-water emulsions. As most water-soluble macromolecules preferentially partition in the aqueous external phase, there is little justification in formulating water-soluble peptides in oil-in-water systems despite greater efficacy from soluble surfactants used to stabilise oil droplets. Renewed interest in the

formation of lipophilic peptide salts via HIP has enabled greater loading in non-aqueous vehicles, offering the prospect of formulating peptides in conventional oil-in-water systems [161].

Use of semi-solids such as Tween 80 and $C_{12}E_{9,}$ as well as soft solids such as sucrose esters also present challenges for oral formulation. It is difficult to create a uniform suspension of macromolecules in a semi-solid PE. The semi-solid can be melted to facilitate better dispersion of the macromolecule (either as a solution or suspension) provided it is stable at the melting point of the semi-solid (e.g. 44°C for Gelucire^{TM} 44/14). An alternative is to freeze-dry an aqueous dispersion of PE and macromolecule. PEs that are waxy soft solids may be admixed with macromolecule, but heat and moisture increases cohesion, which reduces flowability and may impeding optimal mixing. It may be feasible to incorporate these materials in to capsules, but waxy malleable solids are difficult to incorporate into tablets.

PEs that have progressed to clinical testing or evaluation in large animals are often salts of weak acids (e.g. C_{10}, C_8, SNAC, sodium cholate), weak bases (chitosan hydrochloride), strong bases (trimethylated chitosan chloride), or amphoteric compounds (e.g. acyl carnitine chlorides). These substances are more readily incorporated into solid dosage forms. Soluble salts typically exhibit good dissolution in both acidic and basic environments, because the adjustment of the pH around the tablet favours ionisation of weakly acidic or basic functional groups. Exceptions are the salts of ionisable polymers like chitosan, which can undergo gelling in the environment around the tablet resulting in slow dissolution [162]. Depending on the pKa, the PE may lose its charge in bulk gastric (for weak acids) or intestinal fluid (for weak bases), which could lead to precipitation if it has low intrinsic solubility. It could also increase the likelihood of PE absorption or alter the predicted enhancement efficacy, as the ionised form is often responsible for alteration to barrier integrity. As the majority of PE dosage forms are enteric-coated formulations, release of PE will occur at pH values greater than 6, thus a high proportion of weakly-acidic PEs will be ionised in the small intestine, and

enhancement will mirror at least the pH conditions observed in Caco-2, Ussing chambers and intestinal instillations.

There are few studies detailing the excipients used in the formulation of PE dosage forms or the conditions for optimal release. Patent embodiments show the presence of excipients common to other solid dosage forms (e.g. binders, disintegrants), as well as others that are unique to oral dosage forms for macromolecules (e.g. peptidase inhibitors). There is limited information on the justification for selecting formulations with specific release characteristics, although there has been some rationale presented for targeting stomach, jejunum or ileum for specific PE-macromolecule formulations. The rationale for quick release from enteric formulations can be to target receptors in the upper GI tract or where there is a requirement for timely drug onset (e.g. prandial insulin). The oral semaglutide formulation (Rybelsus®, Novo Nordisk) is an immediate release dosage form that specifically targets the gastric mucosa. A unique presentation of semaglutide with SNAC and formulation additives at the gastric mucosa over 1 h is considered essential for delivery [12].

As SNAC is incorporated as the soluble salt, sodium salcaprozate, there will be an increase in pH in the interfacial region around the tablet, which affords some protection from pepsin in proximity to the tablet. Slow erosion of the tablet in close proximity to the gastric mucosa may provide a static location, conditions that may help SNAC sustain a concentration gradient that promotes uptake of semaglutide. Therefore, the kinetics of gradual release is considered an important property of the formulation [12] and patent embodiments suggest that increasing the proportion of microcrystalline cellulose and povidone increased the disintegration time of semaglutide tablets [163]. Scintigraphy studies in humans and dogs suggest the semaglutide tablets erode over 60 min, although there has been no published dissolution testing in simulated gastric fluid. Since SNAC is rapidly absorbed(T_{max}: 30 min [12]) in humans, this suggests that the erosion time of the semaglutide tablet indicated by scintigraphy does not correlate with the release and absorption kinetics for SNAC. It is unclear to what extent SNAC and semaglutide diffuse into bulk fluid and pass into the small intestine,

and whether there is any absorption of the latter from the small intestine in humans. While the tablet is taken on an empty stomach, there may be rapid emptying into the small intestine, the consequences of which are unclear. Overall, the semaglutide formulation is a relatively simple design, which relies heavily on the favourable physicochemical properties of this potent peptide, which impart its long t½.

It is not clear whether it is the protonated or deprotonated form of SNAC that reaches the gastric epithelial surface. For C_{10}, pH-dependent protonation at stomach acid pH results in precipitation as capric acid, an insoluble surfactant (HLB 4.8) that does not form micelles and exhibits little or no detergent characteristics. Unlike C_{10}, SNAC is not a conventional amphiphile, hence it is difficult predict how its ionisation state will impact efficacy. SNAC is an acidic PE and so the inclusion of the basic sodium salt form (sodium salcaprozate) in oral semaglutide suggests that the PE may exist in the ionised state in the interfacial layer around that tablet. If the tablet rests against the gastric mucosa prior to dissolution, SNAC may be presented to the epithelium in the ionised form because the pH in the vicinity of the tablet is relatively high, whereas if the tablet initially dissolves in bulk gastric fluid, there will be pH-dependent conversion to the less soluble more permeable acidic form, which may interact with the gastric mucosa differently from the ionised form. There is greater potential for passive transport of the non-ionised form of weak acids into gastric cells, but once inside gastric cells may donate a proton to acidify the intracellular environment and cause ion trapping, a potential contributing factor to damage caused by acetylsalicylic acid [164]. The possibility of intracellular acidification by the acidic form of SNAC has not been assessed in pre-clinical delivery models as the PE is normally tested at pH values close to neutral, where it predominantly exists in the conjugate base form. Further mechanistic studies are warranted at pH values that represent conditions in the stomach.

The inclusion of excipients that prevent both enzymatic degradation and facilitate permeation in an oral dosage form is a rational approach, but the capacity of PEs to improve permeation further would benefit from delivery modalities that address the aforementioned

hurdles. Devices (e.g. microcontainer, intestinal patch, Fig. 1) that improve efficacy of an excipient is a relatively novel concept, although there are cases where one excipient improves the function of another excipient. For example, a drug may be solubilised in an oil, but in the absence of other excipients (emulsifiers, co-surfactants and/or co-solvents), there may be poor oral absorption from the vehicle. A delivery system or simple excipient approach that promotes efficient co-localisation may help to improve translation of oral peptide dosage forms.

8. STRATEGIES TO IMPROVE PERFORMANCE OF PEs IN DYNAMIC INTESTINAL CONDITIONS

Despite several hundred studies identifying novel PEs, along with their safety and mode of action, there are relatively few studies assessing effects of PEs formulated in immediate release or enteric dosage forms. There is a paucity of data assessing the impact of co-presentation and release kinetics on permeation enhancement, and consequently, delivery technologies that attempt to improve uptake are mostly based on trial-and-error under the premise that localising the macromolecule and PE for an extended period will improve permeation. Initial attempts to manipulate intestinal conditions to extend the duration of the absorption window (e.g. mucoadhesives) did not factor in PEs and the same is true of first iterations of intestinal patches and microcontainers. Absorption of molecules that are slowly or incompletely absorbed in the upper GI tract may also be increased by motility inhibitors, although whether the change alters the clinical performance depends on the properties of the drug [165]. For some drugs, like metformin, slowing or increasing gastric and small intestinal residence time has no effect on PK metrics [166]. For most macromolecules, where absorption is negligible *per se*, extending residence time alone is expected to have little impact on BA.

Due to limitations in the use of mucoadhesive polymers for promoting oral peptide delivery, bioadhesives including lectins can be used that target the epithelial surface rather than mucus. There is the added hurdle of ensuring penetration through mucous. If efficient adhesion to the mucosal surface could be achieved, bioadhesives may promote co-

localisation of PE and macromolecule, but only if there is gradual release from the tablet. An adherent tablet may localise at the gut wall and release constituents to the epithelial surface and also into bulk intestinal fluids. If there is fast disintegration and rapid dissolution of the formulation into bulk intestinal fluid, mucoadhesion may have only a negligible effect.

Nanoparticles may also promote co-localisation of PE and macromolecule at the intestinal epithelium (Fig. 1). There has been extensive research on the application of nanoparticles to protect macromolecules from pre-systemic degradation and shuttle entrapped cargoes across the epithelial surface. Although, many studies report improved oral BA in rodent models, there have been few clinical trials [4]. The underpinning rationale that nanoparticles will shuttle entrapped cargoes across the gut wall in a substantial and quantifiable manner has not yet been verified *in vivo*, although research targeting uptake pathways with ligands on the nanoparticle surface has shown promise [167] as have recent studies showing transcellular uptake of insulin via zwitterionic polymer micelles [121]. Mucopermeant nanoparticles may protect labile macromolecules from degradation and help to co-present PE and payload at the epithelial surface [168, 169]. Release from nanoparticles is however, multidirectional, i.e. PE and macromolecule will be released towards to epithelial surface and bulk lumen, which may cause dilution and ultimately sub-optimal enhancement. Additionally, it can be difficult to avoid premature release from nanoparticles in small intestinal luminal fluid.

A device that affixes to the intestinal mucosa and release constituents in the direction of the epithelial surface may offer an environment that co-localises the macromolecule and PE at the gut wall (Fig. 1). These technologies have similarities to transdermal patches including unidirectional release, unique geometry, and in some cases adhesive properties. Devices in the category include intestinal patches, micropatches, microcontainers, and microdevices (Table 4). Adhesion of a patch to a mucous coated epithelial layer in a fluid-filled environment that is under powerful shear forces is more difficult than affixing a patch to dry skin, especially as only a fraction of the pressure is available to affix the device to the surface.

Additionally, there are currently only a narrow range of experimental mucoadhesives (e.g. chitosan, thiomers) and bioadhesives (e.g. tomato lectin) that can be used promote mucoadhesion. A number of adhesives that strongly adhere to wet surfaces are also in development [170, 171]. Devices with more obscure geometries, such as rectangular cuboid, may be more resistant to movement compared to capsules and tablets resulting in less demand on the adhesive, but there still must be efficient adhesion to the epithelial surface in order to ensure optimal co-presentation.

The presence of PEs in more recent iterations of GI devices emphasises that low permeation cannot be efficiently addressed through presentation of macromolecule at the gut wall in the absence of PE. The dimensions of an intestinal device and loaded dose of PE/active are constrained by size of the dosage form that can be ingested, although there may be a reduction in the quantity of PE and macromolecule required if efficient co-presentation is achieved. Confining a PE and macromolecule to only a small fraction of the total surface area for absorption may result in a more gradual increase in plasma concentration, reducing the C_{max}, and increasing T_{max}, which may impact therapeutic efficacy if the macromolecule fails to reach a therapeutic threshold in plasma or if time to onset is too slow. There may be an increase in surface area coverage if a greater number of small patches (mm to micron) are administered instead of a small quantity of large patches (cm) [172]. However, miniaturising patches may lead to some of the difficult issues arising regarding development of micro- and nanoparticles, such as physical instability, and sub optimal loading efficiency/release kinetics.

As the PE and macromolecule are released from the patch into the mucous gel layer or binding directly to the epithelial surface, there will be a low volume of fluid for dissolution compared to bulk intestinal fluids. Therefore, dissolution of PE and macromolecule can be slow in low fluid volume environments, which could lead to sub-optimal co-presentation. One approach used to address dissolution in low fluid environments is to incorporate the payload in a lipophilic or hydrophilic semi-solid base that melts at body temperature, as is the case with rectal suppositories. In this case, dissolution of drug is dependent on the vehicle. Another

important factor in patch design is premature release of PE or macromolecule prior to attachment to the epithelial surface. This is more of a problem if there is fast dissolution of PE and macromolecule. Premature release in the stomach can be avoided using an enteric outer layer or insertion into an enteric capsule. Release can be slowed from solid dosage forms using controlled release technology. However, the success of any controlled release approach to the epithelial surface will depend on whether the PE reaches a threshold for enhancement before it itself is absorbed.

There are devices that cause physical aberration to the epithelial surface in a similar way to how microneedles bypass the stratum corneum. These are distinct from intestinal patches and microcontainers, as they improve permeation through physical disruption. Disruptive devices aim to bypass the epithelial surface, thus there is no requirement for a PE. However, some fabricated devices may provide a reservoir for unidirectional co-presentation of PE and active at the gut wall rather than physical penetration. One such example is the self-orientating millimetre scale applicator (SOMA), a device that contains a spring loaded millipost that is actuated by fluid ingress [173]. Actuation of the device causes insulin loaded milliposts to be accelerate through the epithelial layer, although such penetration was not consistently achieved for each device. Another spring-actuated device is the luminal unfolding microneedle injector (LUMI), an array of 32 drug entrapped microneedles that are released at the pH in the small intestine. Overall, any device that is adapted to promote co-presentation must be practical, scalable, and cost effective, and hence must exhibit a higher BA than conventional PE-based dosage forms.

The choice of PE included in a patch or microcontainer device is not as straightforward as the selection for a conventional dosage form where historical use is usually based on established use in humans. The quantity of macromolecule and PE that can be administered via an intestinal device is less than what can be administered in a conventional solid dosage form as the constituents of the device will take up a major fraction of the dosage form. As the PE may be more effective under static conditions created by the device, a lower quantity of

PE may exhibit greater efficacy than in conventional oral dosage forms. Hence, there may be justification for inclusion of a lower dose of a stronger PE, such as SDS [174]. In the case of SDS, use of a lower quantity in a device may lie within its acceptable use concentration as a surfactant in oral formulations as listed on the US FDA Inactive Ingredients List for Approved Drug Products [175].

9. EXPERT OPINION

Oral macromolecule delivery has been a prominent area of drug delivery over the last 30 years. Addressing oral delivery of insulin and low MW heparin was a priority for many years, but more recently, focus has switched to GLP-1 receptor agonists, somatostatin analogues and analgesic peptides (see Table 1). Novel technologies may assist in reformulation of some marketed injectable peptides for oral administration and could help to promote broader screening of peptides in Discovery, where they can be either excluded from testing or be discontinued due to sub-optimal physicochemical properties. There have been only four approvals in oral dosage forms to date with desmopressin, cyclosporin, semaglutide and octreotide. Oral formulations of cyclosporin and semaglutide rely on a combination of physicochemical/pharmacological properties and additives to ensure adequate oral BA. A similar trend was observed in a recent clinical trial with a modified oral insulin delivered in a formulation containing a PE [6]. There is extensive research on how peptide structure impacts bioavailability. This branch of medicinal chemistry was recently reviewed in tandem with delivery approaches [4], an article that highlighted the importance of both areas to successful development of oral peptide formulations.

Inclusion of a PE in an oral macromolecule formulation is a simple and attractive approach to address low GI permeability, however, there have been only low single digit levels of BA observed in publications of clinical trials, which is part of the reason why peptides that are now being engineered to exhibit improved potency, stability, plasma t½, and permeability. Ongoing research continues to identify novel PEs with data for ionic liquids (e.g. CAGE [159]), non-ionic surfactants (e.g. sucrose laurate [72]), amino acids (e.g. tryptophan [176]), peptide

constructs (e.g. PP1 [177]), PCB-DSPE (polycarboxybetaine (5kDa) conjugated to distearoylglycero-30phosphoethanolamine) [121], analogues of toxins [178], and PEs from natural sources [179]. These PEs may have improved efficacy relative to established PEs like C_{10}, SNAC, EDTA and bile salts. However, there is potentially more to be gained by engineering around the physiological hurdles that impede optimal co-localisation of existing PEs. This research falls under two categories i) assessing the behaviour of PEs in dynamic environments and with simulated intestinal fluids and ii) evaluating the performance of PEs in systems that promote unidirectional release resulting in co-presentation of PE and macromolecule.

The limitations of cell, tissue and animal models in predicting bioavailability and other PK metrics has been highlighted. Differences in fluid volume, transit times, luminal diameter composition of intestinal fluid, diet, anatomy have been implicated as sources of variability of these models to predict BA in humans from PE-based dosage forms. However, few studies in animals assess the performance of PEs in complete oral dosage forms, thus the physiological and formulation challenges that are impeding optimal performance have not been fully assessed. A faster small intestinal transit time in dogs can reduce the efficacy of PEs in standard formulations, whereas a longer gastric residence time may accentuate it in the stomach. The potential impact of species differences on enhancement was summarised in a recent review [70]. Few would disagree that *the best model for humans is humans* [180], although such studies are time consuming and costly to perform.

There are several possible reasons why PE-based oral dosage forms for macromolecules have largely failed to translate to humans, with just a few exceptions. Most studies disseminated in patents, where PE formulations are administered orally to animals, predicted low and variable absorption. There is high permeability observed in cell monolayers, isolated tissues, and intestinal instillations/perfusions, but these models offer close-to-optimal presentation formats. As efforts begin to identify better PEs and to develop formulation strategies that recreate optimal conditions, these models will still continue to have value.

Kinetics of permeation enhancement and novel devices can be assessed in cell culture monolayers, isolated tissues, and in rat intestinal instillations/perfusions, prior to testing complete dosage forms in large animals. Tablets and capsules of comparable dimensions to those used in humans can be tested in dogs and pigs, although the dose/kg should be adjusted so that PD metrics are not exaggerated, and because the level of enhancement may be dose-independent. Attempts to address specific physiological hurdles that are limiting effectiveness in humans, should be assessed in animals that most closely represent humans. Pigs more closely align to humans in terms of diet, GI residence, fluid volume and gastric pH than dogs or rodents [70].

The shift towards development of devices to improve performance of PEs in oral dosage forms creates a complexity issue that will only be acceptable if there is an appreciable increase in BA over conventional admixed formulations without causing intestinal blockage and epithelial perforation. Some of the fabrication techniques used for devices may be suitable for large scale manufacturing, which may be an advantage over nanotechnologies [4]. How will constituents of luminal fluid, in particular protein or surfactant adsorption, impact adhesion of intestinal devices to the gut wall, and can premature burst release be avoided? There may need to be a trade-off between optimal device size/shape, given the constraints presented by administration via the oral route.

As focus shifts to understanding how to improve performance of PEs in dynamic environments, there is continued demand for studies that elucidate the mode of action of PEs. An understanding of the mechanism by which a PE alters epithelial barrier integrity may help to predict undesirable off target toxicity, especially for PEs that do not have a history of safety use in humans. Mode of action studies may therefore help to predict toxicological outcomes and potentially limit the scope of safety studies required during product development. There have been many studies assessing the mode of action of leading PEs, although the mechanism by which these PEs alter barrier integrity often remains unclear, but are now being resolved with advanced tools (e.g. HCA). There is additional ambiguity for some of the first-

generation PEs as to whether they act via paracellular or transcellular mechanisms. For many surfactants there are concentration-dependent effects which contributes to the difficulty in determining how they alter barrier integrity. It is difficult to state with certainty whether low or high concentrations prevail at the gut wall in humans, and therefore not possible to conclude that a PE acts via paracellular TJ openings (generally observed in cell culture monolayers) or transcellular perturbation (usually observed in tissues). Surfactants that alter barrier integrity are more likely to act via transcellular perturbation, whereas EDTA, C-CPE and PIPs are PEs that act via the paracellular route.

Cytotoxicity assays may assist in distinguishing between a molecular mechanism leading to TJ openings and transcellular perturbation. More recently, HCA techniques have been used to simultaneously assess several cytotoxic metrics of medium chain fatty acids in live cells [54]. That study highlighted that C_{10} increases permeability when there is an alteration to membrane integrity in Caco-2 cells. It places more context around the initial work showing that C_{10} also acts through activation of PLC, increasing intracellular Ca^{2+} and subsequent activation of MLCK, which in turn phosphorylates MLC leading to contraction of the peri-junctional actinomyosin ring and disbandment of TJs [17]. The HCA study showed evidence for an increase in intracellular Ca^{2+} in Caco-2 cells, but this was only observed at concentrations below the threshold for permeation enhancement. The latest molecular approaches to epithelial TJ opening have targeted the physiological mechanisms by which TJ are modulated, such as when glucose transporters are saturated [33]. The rational design of TJ modulators over screening families of candidate PEs aligns excipient development closer to how drug candidates are selected. Although targeting specific molecular pathways may reduce off-target toxicity, there may still be off target toxicity at junctions beyond the target cell population, a factor that may contribute safety and tolerability. These molecular candidates offer the potential to limit adverse effects at the gut wall. There have been no inflammatory or cytotoxic actions observed for the rationally-designed PIP peptides, which mimic the endogenous opening of TJs in response to saturation of Na+ glucose cotransporters [177].

Elucidation of the structure and function of the TJ has been assisted in party by studies assessing the effects of microbial toxins and metabolites that alter barrier integrity. Substances including ZOT, cytochalasin D, and CPE were identified through cell and tissue screening, and although they have evolved to target specific pathways, these mechanisms are often not well defined. Toxins are a rich source of potential target pathways that if fully elucidated may lead to development of novel PEs that target endogenous pathways. To date, the majority of research effort on these substances has focused more on structural variants that preserve enhancement action and eliminate off target deleterious actions. Toxins like CPE directly target homophilic interactions between TJ proteins in adjacent epithelial cells, rather than physiological signalling mechanism. A PE that directly targets the interaction between intestinal TJ proteins rather than an upstream, ubiquitous cell signalling molecule may be a more selective approach.

Performance of PEs in animal models is commonly assessed via a combination of PK and PD metrics. BA is a commonly cited metric that when taken in isolation does not provide an indication of whether an oral formulation will be effective in humans. For example, an oral insulin formulation may yield a BA of between 1 to 10% in clinical trials, and fail to meet clinical outcomes and economic viability, whereas an oral dosage form of desmopressin with a BA of ~0.2% is acceptable and satisfies clinical assessments. One of the key questions not assessed in clinical trials is the overall efficacy of associated PEs in the dosage form. PK data from clinical trials of oral octreotide or semaglutide formulations show BA of approximately 1 %, although the extent by which the PE is improving BA is unclear in the absence of testing an unassisted formulation. The dose of macromolecule may be several orders of magnitude higher than in equivalent injectable forms highlighting only a modest benefit from PE formulations. BA of macromolecules administered orally in such dosage forms relies on high doses of the macromolecule, which has become more commercially- feasible today, at least for some peptide candidates.

10. CONCLUSIONS

Development of oral delivery systems that address low intestinal permeation for macromolecules has lagged behind solubility enhancement strategies for small molecules. Approaches to improve permeation of macromolecules have become progressively more complex, from inclusion of PEs to encapsulation in nanoparticles and devices. The majority of delivery systems that have progressed to clinical testing include PEs, although success has been limited to small, potent, stable peptides. Approaches that combine optimisation of the structure of the macromolecule tailored to a delivery system will lead to greater oral absorption. While pre-systemic degradation and poor permeation are key impediments to oral absorption, other physiological variables such as GI transit, fluid volume, and composition of GI fluid prevent optimal performance of current PEs. Few studies have assessed the effect of PEs in complete dosage forms, which means that some of the hurdles to translation have not been widely studied. Delivery systems that promote co-localisation of macromolecule and PE at the intestinal wall could help to replicate the delivery seen in static delivery models. The safety data amassed for leading PEs in clinical trials or approved products has so far not led to cause for concern, although chronic studies are warranted to determine if transcellular perturbants cause histological damage at the site of enhancement in real time as well as the long-term consequences. Although several hundred molecules alter epithelial permeability, relatively few are suitable PEs. There should be continued efforts to identify potent PEs that cause rapid reversible alteration to barrier integrity. This may be facilitated by novel robotic screening tools using isolated porcine jejunal tissue [181].

Declaration and competing interests

DB acts as consultants to Pharma researching oral peptides. Past collaborative research in DB's lab has been funded by Sanofi and Novo-Nordisk.

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Table 1: Properties of selected macromolecules that have a MW of less than 10 kDa with potential for oral delivery. Dose ranges for insulin aspart and cyclosporin were estimated for a 70 kg adult. * estimated LogP values (XlogP AA). Information sourced from Drugbank, ChEMBL-EBI, Pubchem and/or from the Summary of Product Characteristics (SmPC) or Package Inserts downloaded from the Health Products Regulatory Authority (HPRA) database (Ireland), European Medicines Agency and/or the US Food and Drug Administration (FDA). Excludes peptides that are intended for regional delivery in the GI tract (e.g. linaclotide).

Macromolecule	**MW**	**$T_{1/2}$**	**LogP**	**Route**	**BCS**	**BA**	**Dose**	**Frequency**
LMWHs Tinzaparin Dalteparin Enoxaparin Nandroparin Clivarin Ardeparin	 5866 Da 5819 Da 4371 Da 4855 Da 4653 Da 6000 Da	5 h	-13.2*	sc	-	-	20 mg	Daily
Insulin aspart	5832 Da	80 min	-14.3*	sc	-	-	> 1 mg	Prandial
Insulin degludec	6108 Da	25 h	-4.9*	sc	-	-	0.35 mg	Daily
Exenatide	4186 Da	2 h	-21*	sc	-	-	5-10 µg	Twice daily
Liraglutide	3751 Da	13 h	-3.4*	sc	-	-	0.6-1.8 mg	Daily
Teriparatide	4118 Da	1 h	-18.7*	sc	-	-	20 µg	Daily
Semaglutide	4114 Da	168 h	-5.8*	sc Oral	- III	- 0.4-1.0 % (oral)	0.25-1 mg 7-14 mg	Weekly Daily
Salmon calcitonin	3432 Da	1 h	-16.6*	sc Nasal	- -	- -	15 µg 30 µg	Daily Daily
Vancomycin	1449 Da	7 h	-3.1	Infusion Oral	- III	- -	0.5-1 g 125-500 mg	Every 12 h Every 6 h
Nafarelin	1322 Da	4 h	0.8*	Nasal	-	2.8%	200 µg	Twice daily
Buserelin	1239 Da	1 h	-0.1	sc Nasal	- -	- TBC	500 µg 300 µg	Daily Thrice daily
Leuprolide	1209 Da	3 h	-1.4	Depot	-	-	3.75 mg	monthly
Cyclosporin	1203 Da	6 h	1.4	Infusion Oral	- IV	- 27%	50 – 350 mg 70-525 mg	Daily Twice daily
Octreotide	1019 Da	2 h	-1.4	sc Oral	-	- 0.25 %	0.1 mg 40-80 mg	Thrice daily Daily
Desmopressin	1069 Da	3 h	- 4*	sc Nasal Sub-lingual Oral	- - - III	- 3-5% 0.25% 0.16%	1-4 µg 10-20 µg 60-240 µg 100-200 µg	Daily Twice daily Thrice daily Thrice daily
Difelikefalin	680 Da	2 h	- 0.6	IV oral	-	-	0.25-1 mg	TBC

Table 2: Categorisation of PEs based on mode of action

Category	Sub category	Description	Examples
Paracellular	Direct TJ alteration	▪ PE category that alters permeability by directly disrupting homophilic interactions at cell adhesion recognition (CAR) sequences between TJ or AJ proteins in adjacent epithelial cells. ▪ One of the first and most effective PEs in this category was CPE. A truncated analogue, C-CPE, has been categorised as a claudin modulator. ▪ Several synthetic peptides have been developed that target claudin [21] and occludin [182] at the TJ and E-cadherin [183] at the AJ. EDTA may act in part through complexation of Ca^{2+} required to maintain junctional integrity.	EDTA [37] C-CPE [21] Claudin-1_{53-80} peptide [127] C1C2 [184] Occludin peptides (OPs) [182]
	Endogenous signalling processes	▪ PEs that alter TJ integrity through intracellular signalling pathways. These can be subdivided into PEs that have defined or poorly defined modes of action. ▪ A number of microbial toxins and metabolites have been shown to modulate endogenous signalling pathways that result in TJ opening (e.g. ZOT). The mechanism by which these substances modulate permeability is poorly understood, and they often exhibit off target deleterious actions making them unsuitable PEs in the native form. ▪ Investigators have attempted to modulate paracellular permeability by specifically targeting enzymes involved in physiological modulation of barrier integrity. PIP peptides directly target phosphatase enzymes involved in regulation of carbohydrate absorption.	**Poorly defined MoA** ZOT [185] Cytochalasin [186] VP8 [20] Tryptophan [176] PN159 [23] **Defined MoA** PIP peptides [177]
Transcellular	Complexation	▪ The formation of a physical complex between a PE and macromolecule through either electrostatic bonding or dipole-dipole interaction may transiently increase lipophilicity, which may improve passive permeation. ▪ An insoluble salt formed between an ionisable macromolecule and amphiphilic counterion increase lipophilicity through charge neutralisation and introduction of a lipophilic moiety. ▪ Hydrophobic ion pairing may facilitate transcellular permeation of some species, but it does not solve all impediments to passive permeation.	**HIP** Sodium docusate [88] Sodium dodecyl sulphate [16] Sodium deoxycholate [89] Linoleic acid [89] Sodium oleate [16] **Dipole-dipole interactions** SNAC [64] 5-CNAC [187]
	Mild mucosal Aberration (and multimodal effects)	▪ A large proportion of PEs tested are surfactants, including bile salts, fatty acids, and non-ionic surfactants. These surfactants have a range of actions that include alteration to the fluidity of the plasma membrane, selective removal of proteins from lipid rafts, increased exposure of ligands to receptors and at higher concentrations mucosal perturbation [17]. ▪ When tested at low concentrations, some studies have shown that these PEs may act in part through a paracellular mechanism. CPPs have demonstrated the ability to alter TEER [188] and increase transcellular permeation of insulin [189], although the exact mechanism by which they alter permeation needs requires further studies.	C_8, C_{10}, $C_{11:1}$ [51, 52, 190] Lauryl carnitine [191] Labrasol® [71] Sodium taurocholate [192] $C_{12}E_9$ [43] Tetradecylmaltoside [193] Sucrose laurate [72] Caproyl 90 [194] PentraMax [189] Penetratin [188]

Table 3: Summary of PEs in macromolecule oral dosage forms in clinical trials

PE	Active(s)	Proprietary name(s)	Progress summary
SNAC	Heparin Insulin PYY GLP-1 Cobalamin Semaglutide PTH	Eligen™	▪ A member of Emisphere's Eligen™ family, SNAC was initially progressed to clinical trials for oral delivery of heparin (PROTECT). In those trials, high doses of heparin (90000 IU) and heparin (2.25g) meant that the dose was delivered as a liquid (15 mL) every 8 hours [110]. There was low compliance due to the bitterness of SNAC in solution and the formulation failed to meet its primary endpoint. Formulation in soft gelatin capsules was also unsuccessful [195]. ▪ Much lower quantities of SNAC were tested with more potent macromolecules [196]. SNAC was eventually developed as a constituent of a cobalamin food supplement (Eligen™ B_{12}, Emisphere, USA). ▪ The recent inclusion of SNAC in semaglutide tablets has renewed interest in SNAC for oral macromolecule delivery. Oral semaglutide is formulated as an immediate release solid dosage form containing 300 mg of SNAC and 7-14 mg of semaglutide. The formulation was designed to slowly erode over 30-60 minutes in the stomach [12]. There is evidence from dog studies suggesting enhancement occurs at the gastric mucosa. Oral semaglutide consistently lowered plasma HBA1c in the PIONEER Phase III trials (Reviewed in [4]). The product was licensed in the USA in 2019 (Rybelsus®). ▪ EnteraBio (Jerusalem, Israel) has also listed SNAC as a constituent in formulations designed for oral delivery of PTH [197].
C_{10}	Ampicillin Acyline Desmopressin Alendronate Nucleic acids Insulin tregopil Insulin I338	GIPET™	▪ C_{10} was one of the first PEs used in a marketed product to improve permeation within the GI tract, albeit as a component of a rectal ampicillin suppository [58]. There was a 10% increase in BA from 10 to 20% when C_{10} (25 mg) was included in the triglyceride base. ▪ Although C_{10} has been extensively evaluated in a broad range of pre-clinical delivery models, most of the clinical trials performed are based on the proprietary enteric coated solid dosage form, GIPET™, by Merrion Pharma (Ireland). This technology was licensed by Novo Nordisk for oral delivery of antidiabetic peptides. This culminated in an 8-week phase II trial of a long acting insulin analogue (I338) formulated in a derivative of GIPET™ containing 550 mg of C_{10} [6]. There was a comparable drop in fasting plasma glucose for I338 (-2.4 mm/L) compared to a lower dose of insulin glargine (-2.6 mm/L). The decrease in HBA1c was -0.75% for I338 and -1.05% for insulin glargine. The estimated oral BA of I338 is 2% relative to sc-administered insulin glargine. ▪ C_{10} has also been tested to improve oral delivery of a PEGylated alkylated insulin termed tregopil™ (Biocon, Bangalore, India). The quantity of C_{10} in the formulation has not been specified, but C_{10} and insulin tregopil have no effect on metformin absorption, thereby reducing the potential for a drug interaction [61].
C_8	Octreotide	TPE™	▪ C_8 is a slightly shorter medium chain fatty acid compared to C_{10}, meaning it has a lower CMC, and less favourable interaction with biological membranes than longer chain fatty acids. ▪ C_8 is a constituent in Chiasma's oral octreotide formulation (Mycapssa™). The formulation consists of a suspension of octreotide and C_8 dispersed in an oily vehicle [62]. In a phase III trial, patients receiving Mycapssa™ (20-80 mg) had improved acromegaly related symptoms and 65% of patients achieved a primary outcome of plasma IGF-1 of less than 2.5 ng/mL after 7 months, rising to 85% after 13 months [63]. ▪ Mycapssa™ was approved by the in 2020 [198].
5-CNAC	sCT	Eligen™	▪ 5-CNAC was another Eligen® carrier that was extensively assessed for oral delivery of sCT by Nordic Biosciences and Novartis. Daily administration of an immediate release tablet containing sCT (0.8 mg) and 5-CNAC (200 mg) was assessed over 3 years in osteoporosis patients [199]. The primary endpoint of preventing new fractures was not reached, although there was an increase in spinal bone mass density. ▪ The formulation was also assessed for treatment of osteoarthritis [117], where there was no significant effect on joint space narrowing or WOMAC (despite a 4% reduction).
Acyl carnitines Citric acid	sCT	Peptelligence™	▪ Enteris Biopharm (USA) developed Peptelligence™ technology to improve oral peptide delivery, in particular sCT. ▪ Peptelligence™ is an enteric coated tablet containing citric acid and, in early iterations acyl carnitine and taurodeoxycholate. In order to facilitate optimal dissolution of Eudragit® coatings in the small intestine, granules containing CA and peptide are sub-coated to slow dissolution and ultimately limit interference is dissolution of enteric polymers. Citric acid lowers the pH optimum for peptidases and if high concentrations can be achieved at the gut wall, may alter barrier integrity (reviewed in [17]), although has no effect on intestinal permeation in dilute solutions [200]. ▪ The iteration of Peptelligence™ tested in the Phase 3 ORACAL (oral calcitonin in post-menopausal women) trial contained only CA [109]. In this clinical trial, TBRIA™ contained 0.2 mg sCT and an undisclosed quantity of CA (presumed to be up to 500 mg [69]). There was a significant reduction in lumbar spine bone mineral density in patients receiving TBRIA™ (1.5%) versus placebo (0.5%), although the formulation did not receive market authorisation.
EDTA	Insulin	POD™	▪ Oramed has performed a number of clinical trials for oral delivery of antidiabetic peptides using the POD™ platform. While the exact composition of POD has not been disclosed, patents, conference presentations and allude to additives such as EDTA, soy bean trypsin inhibitor, ovomucoid, soya bean trypsin inhibitor, aprotinin, omega 3 fatty acids, and taurocholate [201]. ▪ In an open label assessment of 8 healthy volunteers, 5 iterations of POD™ containing 8 mg insulin and varying proportions of formulation additives were administered after an overnight fast. There were reductions in blood C-peptide (27-90%) and glucose (11-35%) in all volunteers, and no significant difference in the performance between formulations [202]. ▪ Another iteration of this formulation (ORMD-0801) was tested in uncontrolled T1D patients (HbA1c: 9.4%) [40]. In addition to their existing insulin regimen, patients received oral insulin (8 mg) three times daily to 8 patients, 45 minutes before meals. There was a 24% decrease in the frequency of glucose readings above 200 mg/dL compared to a pre-treatment period and a 17% decrease in glucose AUC in 6 of 8 patients.

Table 4: Examples of devices that may be used to promote optimal luminal presentation of PE and macromolecule. PE external to the patch*

Device Name	Design features	Actives	PEs	Example of enhancement action	References
Patch	Enteric polymer: - Mucoadhesive: Carbopol 934 Core: Drug loaded BSA microspheres, active Backing layer: Ethyl cellulose	Rhodamine Phenol red FD70	-		[203]
GI-MAPS™	Enteric polymer: L100 Mucoadhesive: Carbomer Core: PE blend, PE, active Backing layer: ethyl cellulose	Fluorescein Caffeine FD4 G-CSF	CA HCO-60 Salicylic acid	Pharmacological availability of 23% following oral administration to dogs	[204] [205] [206]
Patch	Enteric polymer: - Mucoadhesive: Carbopol 934 Core: Pectin, CMC, active Backing layer: Ethyl cellulose	Insulin sCT Exenatide	Sodium glycocholate* PPS*	Sustained reduction in plasma Glc over 5 h following direct insertion in to the small intestine of pigs	[207] [208] [209]
Patch	Enteric polymer: - (Coated capsules, Eudragit L100) Mucoadhesive: Eudragit E PO Core: pectin, CMC, PE, active, PE Backing layer: Ethyl cellulose	BSA Lysosome Insulin	PPS	Sustained reduction in plasma Glc over 8 h following oral administration of enteric capsules to non-diabetic rats	[210]
GI MAPS™	Enteric polymer: Eudragit L 100 Mucoadhesive: - Core: gelatin, Pharmasol, PE blend, active Backing layer: Cellulose acetate	EPO IFN	Gelucire™ 44/14 Labrasol HCO-60 SDS CA	Improved jejunal BA of EPO to 12% following insertion in to rat jejunum	[211] [212]
Patch	Enteric polymer: Eudragit S100 Mucoadhesive: Carbopol 974P Core: Sylysia 550, PE, active Backing layer: Cellulose acetate	EPO	Labrasol®	Oral BA of EPO was 2% following oral administration in dogs	[213]
Patch	Enteric polymer: Eudragit L100 Mucoadhesive: Polycarbophil cysteine Core: Polycarbophil, glutathione, mannitol, active Backing layer: Ethyl cellulose	FD4 Insulin	-	Oral BA of insulin was 2.2% in rats	[214] [215]
Micropatch	Enteric polymer: - Adhesive: Lectin Core: active Base: Silicone dioxide wafer	BSA	-	81% adhesion of lectin modified micropatches to Caco-2 monolayers	[216] [217]
Micropatch	Enteric polymer: - Adhesive: Lectin Core: PEGDMA hydrogel, active Base: SU-8	Camptothecin Fluorescein BSA	-	There was a 10-fold increase in fluorescein permeation through a diffusion flow cell from micropatches compared to a free solution form.	[218] [219]
Microdevice	Enteric polymer: - Adhesive: Lectin Core: hydrogel, active Base: PMMA	Acyclovir BSA	-	A 4.6-fold increase in BA of acyclovir in mice compared to an oral solution.	[220] [221]
Micropatch	Enteric polymer: Eudragit L100 Mucoadhesive: Core: pectin, CMC, Eudragit E PO, active Backing layer: Ethyl cellulose	Insulin Coumarin	PPS CA*	Patches did not reduce blood glucose level following oral administration to rats	[172]
Microcontainers	Enteric polymer: Adhesive: chitosan Core: active, PE, PLGA (lid), PEG (lid) Base: SU-8, PCL	Insulin Paracetamol Lysozyme Ovalbumin	SDS, C_{10}	Microcontainers reduced TEER in Caco-2 monolayers to a greater extent than a solution containing an equivalent amount of PE and macromolecule. *In vivo* studies in rats failed to deliver insulin due to mucus interference [174]	[222] [223] [174] [224] [225]

CA, citric acid; CMC, carboxymethyl cellulose; EPO, erythropoietin; IFN, interferon; PCL, poly(caprolactone); PEG, poly(ethylene) glycol; PEGDMA, polyethylene glycol dimetacrylate; PLGA, poly(lactic-co-glycolic acid); PMMA, poly(methyl)methacrylate; PPS, amidosulfobetain-16; SU-8, epoxy-based photoresist. SDS, sodium dodecyl sulphate.

FIGURES

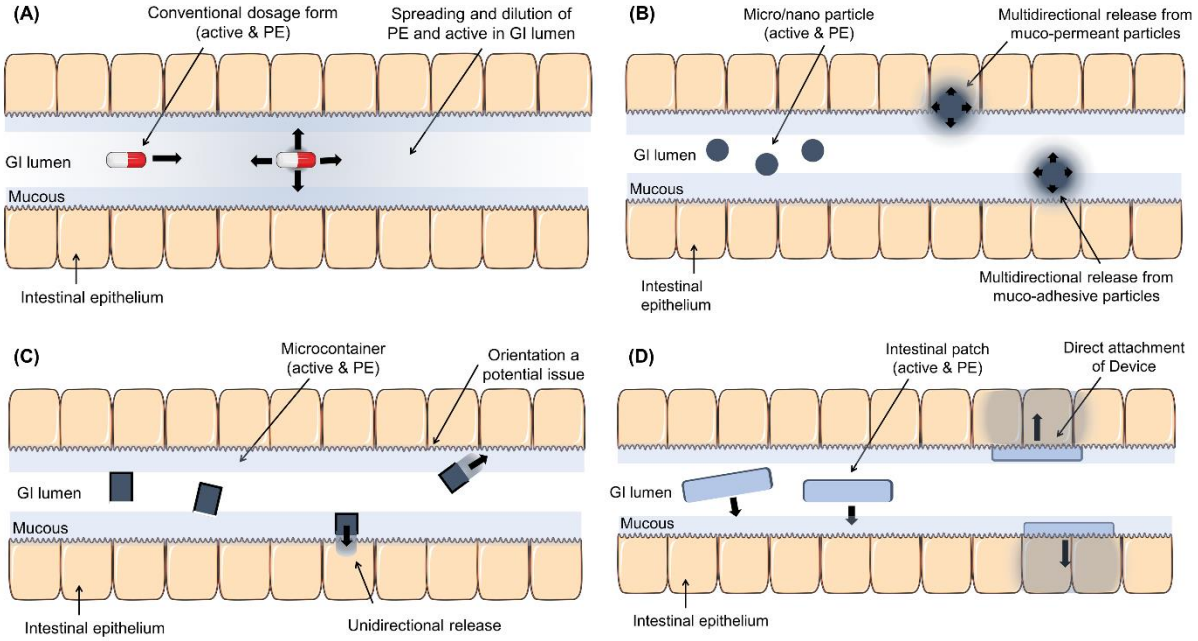

Fig. 1: Delivery systems that may promote optimal co-localisation of macromolecule and PE at the intestinal wall. Release from (A) conventional capsule, (B) muco-permeant or mucoadhesive micro/nano particulate, (C) microcontainer, (D) intestinal patch/device.

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