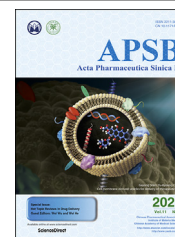




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REVIEW

Oral delivery of proteins and peptides: Challenges, status quo and future perspectives



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Abstract Proteins and peptides (PPs) have gradually become more attractive therapeutic molecules than small molecular drugs due to their high selectivity and efficacy, but fewer side effects. Owing to the poor stability and limited permeability through gastrointestinal (GI) tract and epithelia, the therapeutic PPs are usually administered by parenteral route. Given the big demand for oral administration in clinical use, a variety of researches focused on developing new technologies to overcome GI barriers of PPs, such as enteric coating, enzyme inhibitors, permeation enhancers, nanoparticles, as well as intestinal microdevices. Some new technologies have been developed under clinical trials and even on the market. This review summarizes the history, the physiological barriers and the overcoming approaches, current

Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BSA, bovine serum albumin; CAGR, compound annual growth; CaP, calcium phosphate; CD, Crohn's disease; COPD, chronic obstructive pulmonary disease; CPP, cell penetrating peptide; DCs, dendritic cells; DDVAP, desmopressin acetate; DTPA, diethylene triamine pentaacetic acid; EDTA, ethylene diamine tetraacetic acid; EPD, empirical phase diagrams; EPR, electron paramagnetic resonance; FA, folic acid; FcRn, Fc receptor; FDA, U.S. Food and Drug Administration; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; GIPET, gastrointestinal permeation enhancement technology; GLP-1, glucagon-like peptide 1; GRAS, generally recognized as safe; HBsAg, hepatitis B surface antigen; HPMCP, hydroxypropyl methylcellulose phthalate; IBD, inflammatory bowel disease; ILs, ionic liquids; LBNs, lipid-based nanoparticles; LMWP, low molecular weight protamine; MCT-1, monocarboxylate transporter 1; MSNs, mesoporous silica nanoparticles; NAC, *N*-acetyl-L-cysteine; NLCs, nanostructured lipid carriers; PAA, polyacrylic acid; PBPK, physiologically based pharmacokinetics; PCA, principal component analysis; PCL, polycaprolactone; PGA, poly- γ -glutamic acid; pHPMA, *N*-(2-hydroxypropyl)methacrylamide; *pI*, isoelectric point; PLA, poly(lactic acid); PLGA, poly(*l*-lactic-*co*-glycolic acid); PPs, proteins and peptides; PVA, poly vinyl alcohol; RGD, Arg-Gly-Asp; RTILs, room temperature ionic liquids; SAR, structure–activity relationship; sc, subcutaneous; sCT, salmon calcitonin; SDC, sodium deoxycholate; SGF, simulated gastric fluids; SGC, sodium glycocholate; STC, sodium taurocholate; SIF, simulated intestinal fluids; SLNs, solid lipid nanoparticles; SNAC, sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate; SNEDDS, self-nanoemulsifying drug delivery systems; TAT, *trans*-activating transcriptional peptide; Tf, transferrin; TfR, transferrin receptors; TMC, *N*-trimethyl chitosan; UC, ulcerative colitis; UEA1, ulex europaeus agglutinin 1; VB12, vitamin B12; WGA, wheat germ agglutinin.

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clinical and preclinical technologies, and future prospects of oral delivery of PPs.

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1. Introduction

With rapid advancement of biotechnology, more and more proteins and peptides (PPs) have been developed for treatment of various diseases¹. The PPs have become one of alternatives of small molecular drugs because they are highly selective and effective, but low toxicity², which stimulates interests of pharmaceutical industry. The statistical data of *Coherent Market Insights* revealed the global biologics market was approximately US \$ 255.19 billion in 2019 and was expected to be increasing over the forecast period (2019–2027) with a compound annual growth rate (CAGR) of 7.6%³. Similarly, biologics, including nucleotides and PPs, account for nearly 30% of all drugs approved by the U.S. Food and Drug Administration (FDA) between 2015 and 2018⁴. In addition, more than 90% of the recently approved biologics were monoclonal antibodies (mAbs) based drugs⁴.

PPs are constituted of lots of amino acids linked by peptide bonds. Generally, the short chains between two and fifty amino acids are defined as peptides. There are oligopeptides which have less than ten or fifteen amino acids, and polypeptides which have more than fifteen amino acids. It is known as a protein when the chains longer than fifty amino acids⁵. However, there is still controversy with respect to the use of proteins or peptides, for example, mature human insulin with 51 amino acid is confused to define as proteins or peptides⁶. Some references have also regarded the peptides as the smaller proteins with molecular mass less than 9000 Da^{7,8}. Therefore, PPs have large variations in molecular size and structure (Fig. 1). Besides, PPs have big differences in physicochemical characteristics with chemical drugs. Most of PPs are highly hydrophilic⁹, but some cyclic peptides exert hydrophobic properties, such as cyclosporine¹⁰. Owing to the ionization of amino and carboxyl groups, PPs have isoelectric point (pI) which leads to different charges under different pHs¹¹. The largest difference with chemical drugs is that the conformation is able to affect the pharmacological activity of PPs absolutely¹². Hence, unlike conventional small molecular drugs, it is impossible to develop clinical use of PPs without some sort of sophisticated pharmaceutical technology¹³.

Appropriate administration routes enable not only the therapeutic efficacy of drugs but also patient compliance. However, the administration route of PPs is usually parenteral injection due to their poor oral bioavailability¹⁴. The long-term continuous injection could pose a big challenge of medication adherence, including pain, aversion to injections, concerns about needle size and local irritation. Consequently, many scientific groups attempted to develop the alternative routes to deliver the PPs, such as oral, nasal, ophthalmic, buccal and transdermal administration^{4,15}, among which oral route is the most attractive alternative due to the higher safety and compliance^{16–18}. Furthermore, the oral route is able to mimic the physiological fate of the endogenous insulin which could achieve better glucose homeostasis than subcutaneous (sc) injection⁶. The oral route would enhance the health outcomes for the treatment of certain chronic conditions by improving the living conditions of patients. According to the

recent report from allied market research, the global market of oral PPs is expected to grow from US\$ 643 million in 2016 to 8.23 billion in 2028¹⁹. In addition, academic efforts are also focused on develop some novel technologies to improve the oral absorption of PPs^{20,21}. Since 1995, the number of publication about oral delivery of PPs was increasing exponentially (Fig. 2). However, the commercial products of oral proteins and peptides are very limited to some special peptides, such as Neoral[®] for cyclosporin A and Rybelsus[®] for semaglutide. The main obstacles to develop the oral delivery systems of PPs include the harsh environments of gastrointestinal (GI) tract, large molecular size, high hydrophilicity, and poor transmembrane permeability^{22–24}.

To be honest, there are still numerous excellent reviews about oral delivery of PPs, which however have different viewpoints. For instance, there are lots of reviews focused on oral delivery strategies of peptides^{7,17}, while more reviews focused on how nanoparticles improve the oral delivery of PPs^{25,26}. Some big reviews were written from the biologics which include a large amount of irrelative contents with PPs^{4,23}. This review aims to offer a comprehensive overview of the developing history of oral delivery of PPs, the major delivery challenges and the strategies of improving oral absorption, the current technologies in clinical and preclinical phases, as well as the future prospects.

2. The history

Although it is really tough to develop the oral delivery systems of PPs, various attempts have ever not ceased since the discovery of insulin (Fig. 3). Insulin was discovered by Dr. Frederick Banting and Dr. Charles Best in Canada in 1921 and developed by collaborators in the United States and Europe¹⁸. Only after one year, the first attempt of oral delivery of insulin was conducted in 1922²⁷, which opened prelude to develop oral formulations of PPs. Unfortunately, the results of the first attempt were negative, which makes the critical challenges of oral protein delivery become apparent. Therefore, it is necessary to employ novel delivery technologies to facilitate the oral absorption of PPs. The first paper about oral delivery of insulin was published in 1923²⁸, in which alcohol was used to improve the oral absorption of insulin. Edgar A. Ferguson firstly tried to mix anhydroformaldehyde-aniline with insulin as oral absorption enhancer and won the first patent of oral formulation of insulin in 1965²⁹. With the deep research of oral insulin, more and more scientists and companies kept eyes on other PPs drugs. In 1990, Sandimmune[®] approved by FDA was the first oral formulation of cyclosporin A which is a cyclic peptide with molecular weight of 1202 and also recognized as the first oral dosage form of peptides though it is usually sorted into poorly soluble drugs. After 5 years, the improved formulation of cyclosporin A, Neoral[®], was developed by Novartis and approved. Henceforth, self-nanoemulsifying drug delivery systems (SNEDDS) were regarded as an important strategy

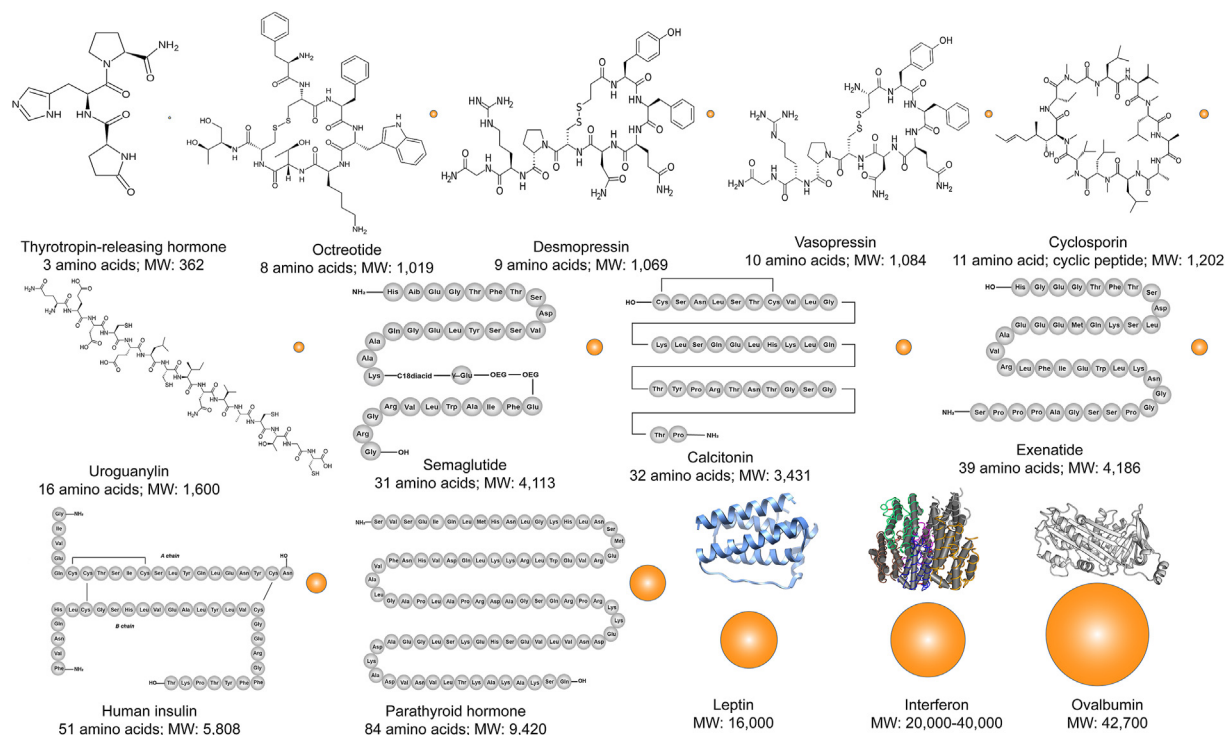


Figure 1 Schematic illustration of the molecular size and chemical structures of typical proteins and peptides (the conformational structures of leptin, interferon and ovalbumin are from Wikipedia). The spheres represent the relative size of PPs molecules.

for improving oral absorption of drug molecules²⁰. However, SNEDDS was not able to increase the oral bioavailability of hydrophilic PPs to large extent. Lots of companies have claimed to develop new delivery technologies to overcome the barriers of oral PPs. Nevertheless, some companies have vanished or been not interested in this field currently, such as AutoImmune, Biosante, Coremed, Coretechs, Eligen, Nobex and Protein Delivery. Five companies are always working on oral insulin for many years and have established some platforms, including Emisphere in USA, Biocon in India, Diabetology in UK, Diasome in USA and Oramed in Israel²⁷.

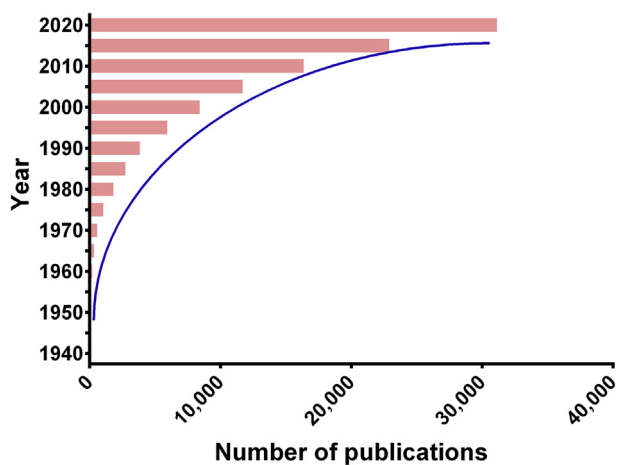


Figure 2 Chronological number of publications about oral delivery of PPs by searching Web of Science to Nov. 9th, 2020 using the phrases of “oral AND (absorption OR delivery) AND (protein* OR peptide*)”.

The first oral insulin formulation by Emisphere was allowed to conduct phase I clinical trials by FDA in 2001. In 2006, a 90-day double-blind phase II clinical study in India performed by Emisphere showed no significant differences from placebo. But the pace of development of oral insulin does not stop. In 2014, ORMD-0801 developed by Oramed was approved to perform phase III clinical trials by FDA³⁰. Emisphere developed an enhancer, SNAC (sodium *N*-[8-(2-hydroxybenzoyl) amino]caprylate)³¹, for improving oral delivery of insulin, which has been finally used to improve the oral absorption of semaglutide. In 2019, Rybelsus[®], an oral formulation of semaglutide developed by Novo Nordisk, was approved by FDA for treatment of type II diabetes. Subsequently, the sustained release capsule of octreotide (Mycapssa[®]) developed through transient permeability enhancer (TPE[®]) technology was also approved by FDA in 2020. These two successful oral products of peptides would bring about revolutionary changes in clinical administration of PPs and accelerate the development of oral delivery systems of PPs.

3. The barriers to the oral absorption of PPs

Though the oral delivery of PPs has attracted enormous interests of the pharmaceutical companies and the funding agencies, there are lots of factors impeding the development of oral PPs, such as instability in GI tract, poor permeability across intestinal epithelia and difficulty in development of formulation. The physiological barriers are the major obstacles to hinder the oral absorption of PPs due to the innate nature of GI tract which is not only the major position of food digestion and nutrient uptake but also is the first line defense against toxins and pathogens. Thus, it is necessary to

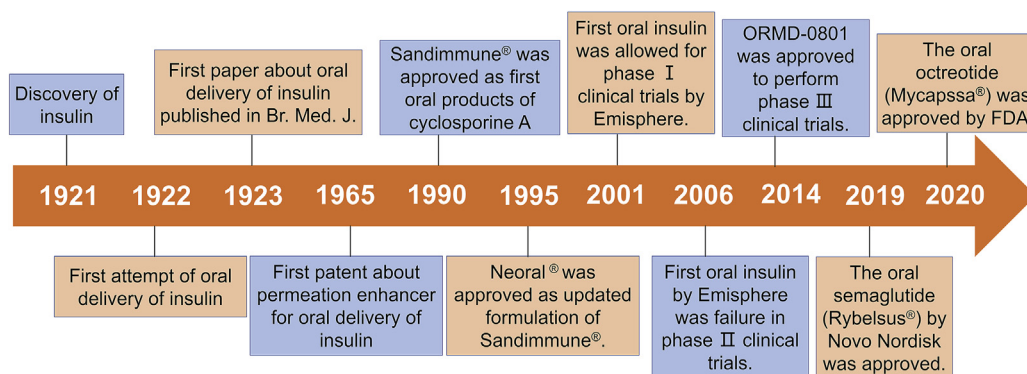


Figure 3 The historical major events in the development of oral delivery systems of PPs.

fully understand the physiological and formulation factors for overcoming barriers of oral delivery of PPs.

3.1. The physiological barriers

After oral administration, drugs suffer from gastric fluids firstly in stomach and then move into small intestine where most of drugs are absorbed. However, it is absolutely different of environments between stomach and intestines, including pH, enzymes, mucus and even epithelial permeability (Fig. 4), all of which influence the stability and absorption of PPs³².

3.1.1. pH gradient

The GI pH is absolutely different in each region of GI tract and influenced by various factors including presence of food, pathological conditions, even age and gender. Generally, in the healthy adult, the pH of gastric fluids is acidic (pH 1.5–3.5), and rises to around pH 5–6 in the duodenum due to neutralization of carbonate and bile juices, and then increases to pH 7–8 in the distal jejunum and ileum, while the colonic pH could be more than 8 or drop to pH 6 with high interindividual variability^{33,34}. The age growth is almost no effect on GI pH³⁵, which indicates the GI pH condition could maintain relative stable in whole life. However, The gastric pH is high shortly after birth, and rapidly reduces to pH 1 to 3³⁶. The intake of food would influence the GI pH transiently, such as elevation of gastric pH³⁷. In addition, the personal diet custom could be an important reason of individual variability in colonic pH. The GI pH could be changed by diseases significantly, such as inflammatory bowel disease (IBD) and GI cancers. The colonic pH in IBD patients differs from healthy adults and is characterized with big individual variability, but the general trend of pH was becoming more acidic. The average colonic pH was detected to be 5.3 in patients with Crohn's disease (CD)³⁸, while could even drop to 2–3 in patients under active ulcerative colitis (UC)³⁹. The GI local cancers could change the pH environment to a large extent. For example, the gastric pH was detected to be around 6–7 in 89 gastric cancer patients⁴⁰, which could be caused by reduction of secretion of gastric acid. The complicated pH environments in GI tract could lead to conformational alteration or enzymatic degradation of PPs, resulting in the loss of therapeutic efficacy. The extreme pHs could result in unfolding due to increase of electrostatic repulsions. Generally, proteins are stable in a narrow pH range which is not far away from their pI, such as pH 6.5–7.0 for recombinant factor VIII SQ (FVIII SQ)⁴¹. Thus, some proteins could be inactive in gastric fluids due to pH induced unfolding. What's more, the activity of enzymes is dependent on pH, for example, pepsin can exert the strongest ability of

degradation in pH 2–3, but be inactive completely in pH above 5³². Most PPs are degraded very fast in stomach of healthy adult⁴².

3.1.2. Enzymes

PPs are highly susceptible to various proteolytic enzymes including luminal enzymes from gastrointestinal and pancreatic secretions, bacterial enzymes in the colon and mucosal enzymes⁴³. They are primarily degraded by luminal enzymes (Table 1^{44,45}) before penetration across mucus. The entry of the protein could stimulate the gastric mucosa to secrete pepsins by the cells lining the stomach. Pepsin is able to degrade proteins into smaller fragments of peptides by hydrolyzing the peptide bonds⁴⁶. A great deal of proteolytic enzymes in the upper part of the small intestine are secreted by pancreas, such as trypsin, chymotrypsin, carboxypeptidase and elastase⁴⁷. Moreover, the remaining parts of the proteins are finally digested by various peptidases (e.g., aminopeptidase and dipeptidase) in brush border membrane into tripeptides, dipeptides and respective amino acids that are able to be absorbed into the blood capillaries from epithelium⁴⁸. However, most degradation data of PPs were obtained by *in vitro* simulated gastric or intestinal fluids with specific enzymes which are hard to be same activity as *in vivo* condition. For example, the pH 1.2 hydrochloride solution with 0.32% pepsin and the pH 6.8 phosphate buffer with 1% trypsin were often used to evaluate the stability of PPs *in vitro*⁴⁷. Most proteins can be degraded very fast in simulated gastric fluids (SGF), such as no insulin detected after 30 min incubation with SGF^{49,50}. Through comparison of human or pig GI fluids and simulated GI fluids, Wang et al.⁵¹ found there were good correlation between SGF and both human and pig gastric fluids for the stability of peptides, while the rate of peptide degradation in simulated intestinal fluids (SIF) was more rapid than that in human or pig intestinal fluids. What's more, there is a very interesting result that only 3 of 17 peptides left in human intestinal fluids after 30 min incubation. These 3 peptides are respectively cyclosporin (99%), desmopressin (25%) and octreotide (22%) which are all developed as oral therapeutic products named as Neoral®, Minrin® and Mycapssa®⁵¹. Therefore, it is the one of important prerequisites for successful development of oral PPs to protect the stability of PPs in GI tract.

3.1.3. Mucus

Mucus is a sticky and viscoelastic gel layer covering the entire GI tract. It is secreted by the goblet cells. Mucus can capture the foreign moieties and protect epithelia from the attack of exogenous pathogens⁵². The mucus in whole GI tract is composed of two layers including loosely and firmly adherent mucus layer from

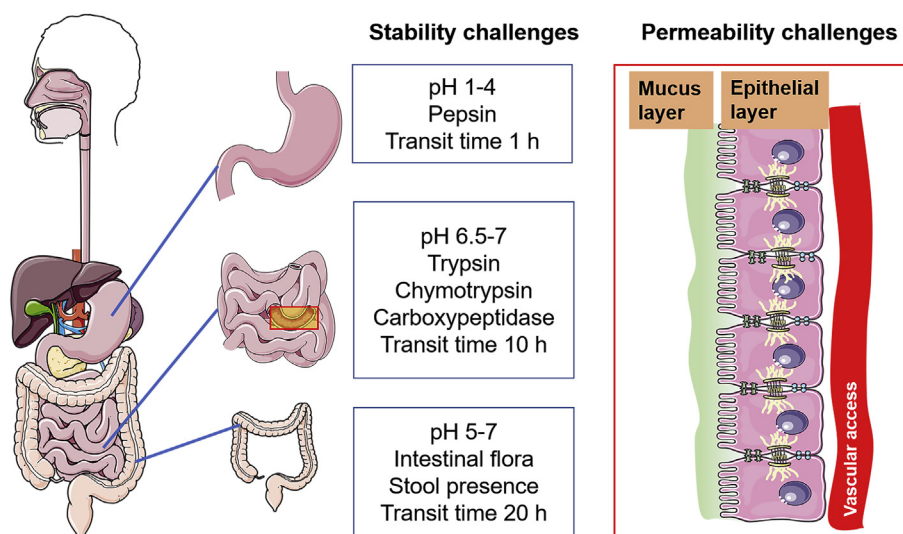


Figure 4 Schematic representation of physiological barriers in GI tract. PPs are prone to be degraded in harsh environments of gastrointestinal tract including pH gradient and various enzymes in lumen, which is stability challenge for oral delivery of PPs. In addition, most of PPs are hardly to transport across mucus layer and epithelial layer, which is permeability challenge.

lumen to epithelia (Fig. 5). The thickness of mucus layer varies significantly in different GI regions. Taking rat GI tract as an example, it ranges from 200 μm in upper part to 800 μm in lower part of GI tract⁵³. In humans, the thickest mucus layers are also located on the stomach (180 μm) and the colon (110–160 μm)⁵⁴. The components of mucus are very complex. The mucin glycoprotein is the major functional constituent and the other components include carbohydrates, proteins, lipids, salts, immunoglobulins, bacteria and cellular remnants⁵⁵. Mucins, including secreted and cell-bound types, are at least twenty subtypes encoded by the *MUC* genes. There are three secreted mucin types found in the GI tract, such as *MUC2*, *MUC5AC* and *MUC6*⁵⁶. The interactions between mucins make mucus gel layer viscoelastic, however, the viscoelasticity could be influenced by water, lipid or ion content^{57,58}. What's more, there is a pH gradient across whole mucus layer, especially gastric mucosa. The gastric mucus pH on the luminal surface is about 1–2 which is similar as gastric pH, but increases to neutral pH at the epithelial surface⁵⁹. This pH gradient could be main mechanism to protect gastric epithelial cell against digestion of pepsin.

The mucus exerts multiple barriers against the transport of drugs into the submucosal tissue. The high viscosity decreases the diffusivity of PPs through mucus, which directly affects the residence time of PPs in the small intestine²¹. In the intestine, the average mucus turnover time is around 50–270 min resulting in the removal of trapped particles in the mucus layer thereby, limiting the adhesion and holding time of the particles or PPs⁶⁰. The continuous secretion and replacement of mucus make it quite challenging for the PPs passing through the unstirred mucus layer by infiltration before reaching the surface of the epithelium. Greater interaction through electrostatic force may exist between mucin and drug particles which may be attributed to the fact that mucin is highly negatively charged due to the glycosylation of serine, and the presence of threonine and proline domain⁵⁴. Furthermore, mucins may function as a size-exclusion filter lowering the mobility of large compounds like proteins due to their brush-like scaffold structure^{61,62}. What's more, structural modification of proteins or entrapment of particles might occur

due to the fact that mucin fibers interact non-covalently with proteins or particles *via* van der Waals and electrostatic forces, hydrogen bonding, and hydrophobic interactions^{64–66}, thus hindering their absorption.

3.1.4. Epithelial barriers

The epithelial cells lying beneath the mucus also act as another predominant restrictions towards oral protein drug delivery. The intestinal epithelia include various types of cells with specific functions, such as enterocytes for absorption, goblet cells for secretion of mucus, paneth cells for secretion of enzymes and M cells for transporting foreign particles⁶³. The enterocytes are the major absorptive cells and also comprise around 90% of intestinal epithelium⁶⁴. A continuous monolayer is formed by these polarized epithelial cells, separating the intestinal lumen from the underlying lamina propria. The tight junctions (TJs), found between two neighboring epithelial cells, make the intestinal epithelium impermeable and a gatekeeper to macromolecules^{65,66}. TJs are elaborate networks formed by multiprotein junctional complexes, which is composed of peripheral membrane proteins like zonula occludens (ZO-1, ZO-2), transmembrane integral proteins like claudins, junctional adhesion molecules and regulatory proteins as well⁶⁷. Except for normal intestinal epithelium, there are some discontinuous follicle-associated epithelia (FAE) which is featured by few mucus, and the location of M cells, numerous intra-epithelial lymphocytes and macrophages⁶⁸. M cells are the most important epithelial cell types involved in the uptake and transport of a wide variety of particulates including intestinal antigens and large proteins, and thus recognized as immune cells of intestinal lumen⁶⁹.

The intestinal absorption of drugs is primarily dependent on transcellular pathway, while paracellular pathway is the main route of some small hydrophilic molecules²². According to Lipinski "Rule of 5"⁷⁰, PPs are predicted to be extremely low transcellular permeability because $\text{Log}P$ of PPs is likely to be below -1 that is far lower than 5, and PPs have a great number of hydrogen bond donors or acceptors, and molecular weight is far more than 500 Da. Thus, PPs are hard to be absorbed into portal vein by transcellular pathway. Moreover, the paracellular route

Table 1 Main digestive enzymes that degrade PPs along with their sites of action^{44,45}.

Secretion site	Enzyme	Specificity
Stomach	Pepsin	Asp, hydrophobic amino acids
Pancreas	Trypsin	Arg, Lys
	Chymotrypsin	Aliphatic amino acids (Phe, Tyr)
	Carboxypeptidase A	Aromatic amino acids in C-terminal (Tyr, Phe, Ile, Thr, Glu, His, Ala)
	Carboxypeptidase B	Arg, Lys in C-terminal
Small intestine	Elastase	Ala, Gly, Ser
	Aminopeptidase A	Asp, Glu in N-terminal
	Aminopeptidase N	Ala, Leu in N-terminal
	Aminopeptidase P	Pro in N-terminal
	Aminopeptidase W	Tyr, Tyr, Phe in N-terminal
	γ -Glutamyl transpeptidase	γ -Glutamic acid in N-terminal
	Dipeptidyl peptidase IV	Pro, Ala
	Peptidylpeptidase A	His–Leu
	Carboxypeptidase M	Lys, Arg in C-terminal
	Carboxypeptidase P	Pro, Gly, Ala in C-terminal
	γ -Glutamyl carboxypeptidase	γ -Glutamic acid
	Endopeptidase-24.11	Hydrophobic amino acids
	Endopeptidase-24.18	Aromatic amino acids
Enteropeptidase	(Asp) ₄ -Lys	

refers to the passage of drugs through water-filled pores of TJs, the pore sizes of which usually range between 3 and 10 Å⁷¹. The molecules larger than 500 Da are generally not recognized to be able to move through these small pores⁷². TJs can be regulated by some permeation enhancers, which makes pores larger⁷³. However, the width is still less than 20 nm even in fully opened state and the total surface of water filled pores only account for 0.01%–0.1% of entire intestinal epithelia⁷⁴. Therefore, the oral bioavailability of PPs is still extremely low even though the intestinal permeation enhancers have been added in formulation, such as transient permeability enhancer (TPE[®]) and SNAC^{18,75}. Compared with normal epithelia, lumen antigens, macromolecules and pathogenic particles are transported effectively and rapidly by M cells from the lumen to the underlying gut-associated lymphoid tissue (GALT) via pinocytosis and phagocytosis⁷⁶, which looks a favorable route for oral delivery of PPs. However, the numbers of M cell are very limited in human intestines, accounting for less than 1%⁷⁷. In addition, some endogenous PPs transported by M cells may stimulate the immune responses⁷⁸.

3.1.5. Inter-individual variability

The tremendous inter-individual variability is also a barrier to limit the development of oral PPs. Inter-individual variability in the anatomical and physiological properties of humans and animals is a common sense. For oral delivery, the inter-individual variability in the physiology of GI tract has significantly affected the bioavailability of oral PPs, such as the condition of mucus, the secretion of enzymes and gut motility⁷⁹. Especially in some disease states, the inter-individual variability is more evident. For example, gastric emptying and oesophageal motility have shown large variability in type 2 diabetic patients with different stages⁸⁰. The variability in gut motility could be particularly relevant to the difference in absorption rate of PPs, such as insulin for diabetes⁸⁰. Moreover, the pH and the expression of digestive enzymes in GI tract vary with individuals significantly, which leads to the inter-individual variability of degradation of PPs in GI tract. The relevant transporters potentially contribute to the extent and rate of transmucosal absorption of PPs, but the expression of transporters in the intestinal epithelia is dependent on individual genes⁸¹. In

addition, most of PPs are endogenous substances for regulating the physiological factors, but some physiological factors can also be influenced by other endogenous PPs. For example, glucose level can be regulated by insulin and glucagon simultaneously. The inter-individual variability of glucagon secretion also cause the differential hypoglycemic effect of oral insulin in different patients or animals⁸². Therefore, it is necessary to establish some models to evaluate the inter-individual variability of oral PPs for clinical development, such as physiologically based pharmacokinetics (PBPK) modelling⁸³.

3.2. Formulation factors

Except for physiological barriers, formulation is also a great challenge during the development of oral PPs commercial products. The chemical and physical stability of PPs are the most important considerations in formulation development, which aims to enable stability of PPs in manufacturing processes, transportation, storage and administration. There have been some excellent reviews to summarize the formulation factors influencing stability of PPs^{84–87}, especially for parenteral formulations. Unlike small molecular drugs, the major stability of PPs is generally referred to as their conformational integrity which is dominated by hydrophobic interaction, hydrogen bonding and electrostatic interaction^{88,89}. The formulation pH could change the protein's surface charge, density and distribution, which could cause the alteration of conformation of PPs⁹⁰. Meanwhile, the pH can also influence the colloidal stability of PPs and then changes the rate of protein aggregation and degradation^{91,92}. Ionic strength also affects the physical stability of PPs in solution as pH⁹³. For example, the increase of salts could improve the aggregation of proteins due to enhanced hydrophobic interactions⁹⁴. Hence, buffer solutions are usually employed to stabilize PPs in solution formulation. In addition, some excipients are very necessary to be added in formulation to improve solubility or suppress aggregation of proteins, such as arginine, histidine, glycine and so on⁹⁵. Arginine can reduce aggregation of proteins to stabilize the proteins' structure⁹⁶. The surfactant is also generally used to stabilize proteins through reducing molecular interactions in different

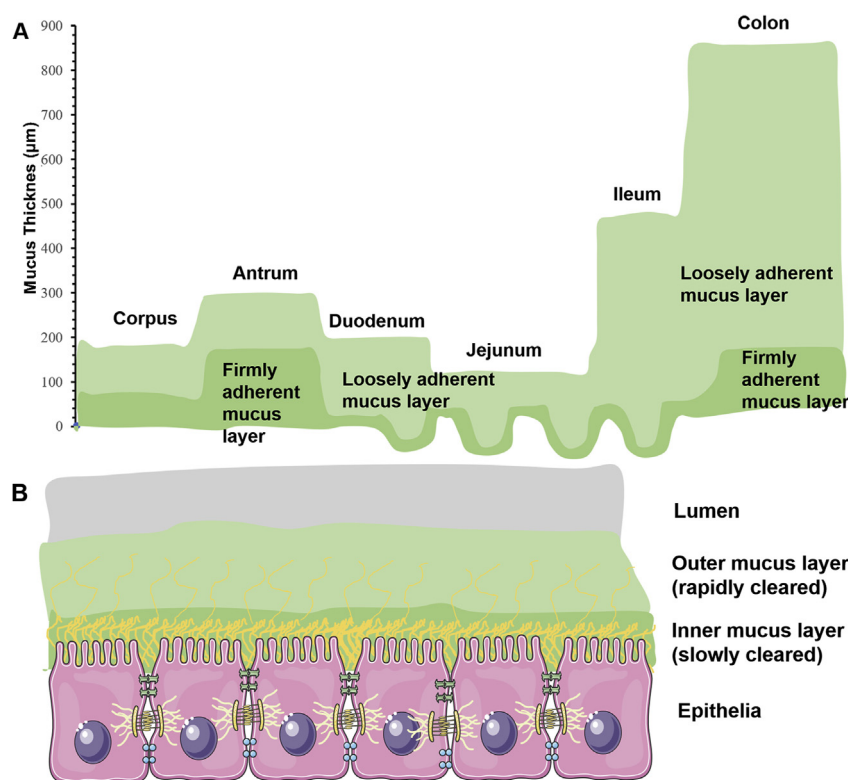


Figure 5 Schematic illustration of the mucus layers covering GI tract. Mucus layer is composed of two layers including outer mucus layer which is loosely adherent and inner mucus layer which is firmly adherent on epithelia. The mucus depth varies with GI sites (A). Outer mucus layer is cleared more rapidly than inner mucus layer (B). Reprinted with the permission from Ref. 54. Copyright © 2012 Elsevier.

interfaces, such as polysorbate⁹⁷. Chemical instability of PPs involves in product development, manufacturing and even post-administration. Oxidation is the most common factor inducing instability of PPs with residues of methionine, tryptophan, histidine, cysteine, phenylalanine or tyrosine⁹⁸, which could be hindered by anti-oxidants including methionine and ascorbic acid. However, enzymatic degradation is the biggest challenge in protecting PPs in GI environments after oral administration as described in Section 3.1.2. Most oral formulations are primarily to protect stability of PPs in GI tract against various digestive enzymes, such as enteric coating, encapsulation and enzymatic inhibitors, which will be described in detail in Section 4.1. In order to enhance epithelial permeability, some permeation enhancers are added in oral formulations, such as SNAC, bile salts and non-ionic surfactants (Section 4.3.2).

Excipients can reduce the molecular interactions between PPs to avoid aggregations, but the interactions between PPs and excipients can also not be ignored. Understanding the protein–excipient interactions is indispensable to better design stable formulations of PPs. There is an excellent review to fully sum up the protein–excipient interactions in liquid formulations including mechanism and characterization⁹⁹. Owing to the complexity of PPs molecules, multiple interactions involved between PPs and excipients, such as electrostatic interactions, hydrogen bonding, preferential hydration and dispersive forces, which can be characterized by various technologies including Raman spectroscopy, circular dichroism, fluorescence, nuclear magnetic resonance, scanning probe microscopy and electron paramagnetic resonance (EPR) spectroscopy, and some advance numerical analysis methods including principal component analysis (PCA) and empirical phase diagrams (EPD). For example,

most of amino acids are able to stabilize proteins in liquid formulation through preferential hydration or direct binding¹⁰⁰, while sugars and carbohydrates can stabilize protein in solid state with the combining effect of specific interactions and formation of highly viscous glassy matrices¹⁰¹. In PPs formulations, some polymers and non-ionic surfactants are usually used to increase the stability. The non-ionic surfactants can compete the hydrophobic surface with protein molecules to avoid adsorbing-induced denaturation¹⁰². Some polymers are employed to encapsulate PPs for improving the stability or controlling the release. But the hydrophobic domain and charges of polymers can influence the stability of PPs, such as aggregation or adsorption^{103,104}.

4. Current strategies towards enhancement of the oral absorption of PPs

Despite multiple strategies to increase the oral absorption of PPs, the primary principles are based on three aspects including stabilization, mucus penetration or adhesion, and permeation enhancer (Fig. 6). These approaches are commonly integrated into one delivery system together.

4.1. Stabilization

Based on physiological and formulation factors, the stability of PPs after oral administration is primarily affected by pH and enzymes in GI tract. In addition, the structure of peptides influences their stability significantly. This section explores the stabilization strategies for oral PPs which have been widely used in formulation development.

4.1.1. pH modulation

The GI enzymes are the main sources to degrade oral PPs, but they need optimal pH to exert their effect. For example, pepsin can cleaves multiple proteins or peptides readily in the acidic environment, however, pepsin starts to lose their effect when the pH is over 3¹⁰⁵. Therefore, if we can modify the pH of microenvironment to 5, PPs can be protected against degradation in stomach. Nevertheless, enteric coating is generally used to overcome the degradation of PPs in stomach rather than pH modulation due to simpler formulation¹⁰⁶. Unfortunately, the proteolytic enzymes in the small intestine are also proficient at degradation of PPs. Similarly, these enzymes are also dependent on pH environment. Luminal proteases, such as trypsin and chymotrypsin, exhibit maximum activity at pH ≥ 6.5 ¹⁰⁷. Therefore, adjusting the pH of the intestinal contents has become an efficient approach to protect PPs in intestine. Some organic acids, such as citric acid, have been generally used as pH-lowering agents to inhibit the activity of intestinal enzymes¹⁰⁸. It has been proven that co-administration of citric acid and salmon calcitonin (sCT) is able to enhance the oral absorption of sCT in beagle dogs by reducing the activity of pancreatic serine protease trypsin¹⁰⁹. In addition, Tarsa Therapeutics (Philadelphia, USA) has successfully completed a phase III trial for oral delivery of sCT (ORACAL[®]) which comprises of an enteric coated capsule to bypass the stomach and citric acid to modulate the pH microenvironment in intestine¹¹⁰.

4.1.2. Enzymatic inhibitors

Except for pH modulation, the most important approach for inhibiting enzymes is using enzyme inhibitors. Enzyme inhibitors inactivate the target enzymes by binding to the specific site of the enzyme reversibly or irreversibly¹¹¹. There are multiple categories of enzyme inhibitors including non-amino acids, amino acids and modified amino acids, peptides and modified peptides. Many chemical molecules can inhibit the activity of enzymes, such as

cholic acids and their derivatives, diisopropyl fluorophosphates^{22,112}. However, these chemical molecules are rarely used due to their high toxicity. Besides, they could be absorbed faster than PPs itself due to low mass, leading to systemic side effects and loss of inhibition capacity. Amino acids and modified amino acids have the same problems as chemical inhibitors²². Hence, peptides and modified peptides derived enzymatic inhibitors have been extensively studied, such as aprotinin inhibiting trypsin and chymotrypsin and soybean trypsin inhibiting pancreatic endopeptidases. However, it is noteworthy that long duration administration of such enzymatic inhibitors could result in the deficiency of these enzymes in humans. The chicken and duck ovomucoids are recently developed and recognized as safer. They can efficiently inhibit the activity of α -chymotrypsin and trypsin and offer 100% protection for insulin¹¹³.

The enzymatic inhibitors have been extensively used in clinically developing products. For instance, soybean trypsin inhibitor and chelating agent which is a cofactor for many proteases have been used in ORM-0801 (developed by Oramed) as a formulation component for oral delivery of insulin¹¹⁴. The Chronotropic[™] platform technologies developed by Dexcel Pharma Technologies, Ltd. (Jerusalem, Israel) combine the protease inhibitor (camostat mesilate) and absorption enhancer (sodium glycocholate) to improve the oral bioavailability of insulin¹¹⁵. However, we have to consider their toxicities which are caused by high concentration and long duration.

4.1.3. Enteric coating and colon-specific delivery

The enteric coating can prevent the drug release in stomach but permit the drug release in the small intestine due to the dissolution of coating materials in higher pH¹¹⁶. Thus, the enteric coating is able to protect PPs against degradation by low pH and pepsin in stomach completely. Some pH-responsive polymers are usually used to coat the tablet, capsule or even micro-/nano-particles.

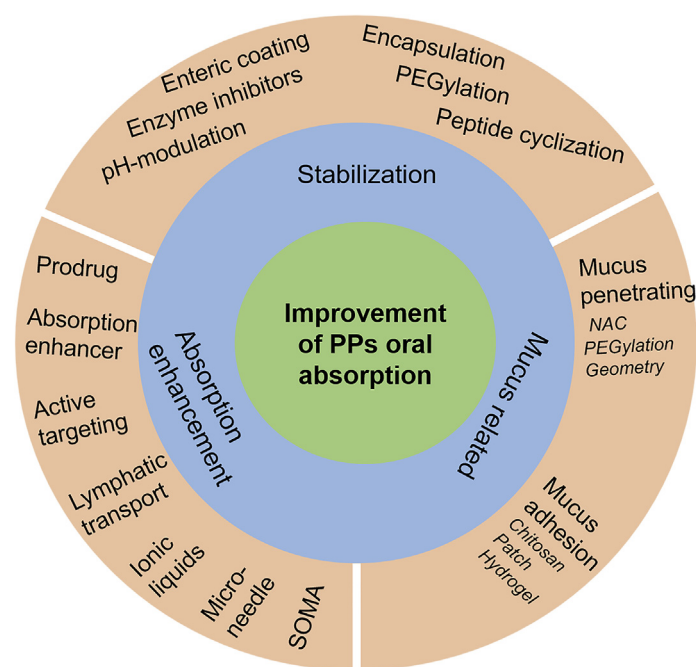


Figure 6 Flow chart of the general considerations in enhancement of oral bioavailability of PPs. There are various technologies based on three rationales including stabilization, absorption enhancement and mucus-related technologies.

Polyacrylic polymers have been widely used for enteric coating and shown to release insulin at different rates and different pH, such as Eudragit S100 or L100¹¹⁷. Most of oral PPs products have adopted enteric coating technology to bypass the stomach, such as enteric coating capsule for oral insulin by Oramed (ORMD 0801) and Diabetology (Capsulin™ OAD)¹¹⁸. However, the oral bioavailability of PPs can't be improved significantly if only enteric coating is used because there are still a great amount of digestive enzymes in intestine. Therefore, enteric coating is usually employed to improve the oral absorption of PPs by combination with protease inhibitors or permeation technologies¹¹⁹. Nanoparticles coated by enteric materials have shown significant enhanced effect for oral absorption of PPs. The relative bioavailability of insulin was found to be approximately 20% after oral administration of enteric coated capsules filled with chitosan/poly(γ -glutamic acid) nanoparticles¹²⁰. In addition, pH-responsive polymers can fabricate nanoparticles directly with other polymers which can enhance the permeability for oral delivery of PPs. Nanoparticles composed of hydroxypropyl methylcellulose phthalate (HPMCP) and chitosan increased the hypoglycemic effect of insulin by more than 9.8 and 2.8-fold as compared to oral insulin solution and chitosan nanoparticles without enteric materials¹²¹.

The colon acts as a more suitable absorption site for PPs compared to stomach and small intestine due to its decrease of enzyme activity and neutral pH value. Moreover, longer residence time and higher responsiveness to absorption enhancers make colon as ideal administration site of oral PPs¹²². There are a number of examples to develop colon targeted delivery systems for PPs, such as vasopressin, insulin, calcitonin, glucagon and so on¹²³. However, proper care must be taken to enable the release of the PPs at the target site. Among the various pH responsive polymers, Eudragit® enteric release polymers have been extensively exploited for oral delivery of PPs. It was reported that the oral bioavailability of insulin was enhanced by 1.73-fold *via* Eudragit S100®-coated chitosan nanoparticles loaded with insulin and *trans*-activating transcriptional peptide (TAT), compared to nanoparticles without enteric coating¹²⁴. Other carbohydrate polymers that are used to specifically deliver oral PPs to the colon include anionic carboxymethyl starch, cationic quaternary ammonium starch, gellan gum, retrograded starch and pectin etc^{125,126}. However, there are some challenges hindering the colon-specific delivery systems of PPs including lower surface area and tight junctions in colonic absorption site. What's more, alteration of enzymatic activity induced by some certain colonic diseases could affect drug release or stability¹²⁷.

4.1.4. Micro/nano-encapsulation

Therapeutic drugs or PPs can be protected from hydrolytic and enzymatic degradation in the harsh gastric milieu of the GI tract *via* encapsulation, by which a drug or protein of interest is engaged inside polymeric carriers, so as to improving their intestinal absorption¹²⁸. Particles can also enhance transport across epithelia except for protecting PPs against degradation²⁵. Therefore, the therapeutic PPs could be encapsulated in nanoparticles to improve the blood concentration after oral administration, while vaccines encapsulated in microparticles can be taken up by Peyer's patches for enhancing mucosal immunity¹²⁹. The particle transport is also affected by the stability in GI tract, surface properties, morphology and specific ligands^{130,131}. Both natural and synthetic materials can be used to encapsulate PPs, such as natural materials including chitosan, dextran, alginate, hyaluronic acid, and lipidic

materials, and synthetic polymers including poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA), polycaprolactone (PCL), and so on¹²⁸. The generally recognized as safe (GRAS) ingredients are highly recommended to prepare edible micro/nano-particles. So far, nanoparticles have been extensively developed for oral macromolecular drug delivery, such as polymeric nanoparticles, lipid nanoparticles, liposomes, nano-emulsions and inorganic nanoparticles, which were described in detail in Section 4.3.8.

4.1.5. PEGylation and peptide cyclization

The stability of PPs can be also improved by chemical approaches, such as PEGylation and peptide cyclization. PEGylation is generally used to reduce plasma clearance rate by increase the stability of PPs in the systemic circulation¹³². Several injectable PEGylated proteins have been launched to the market, such as growth hormone antagonist (Somavert®, Pfizer, USA), erythropoietin (Mircera®, Roche, Switzerland), and anti-TNF- α Fab (Cimzia®, UCB, Belgium)¹³³. Similarly, PEGylation can increase pH and thermal stability of PPs, and also resistance to intestinal proteolytic digestion¹³⁴. In addition, the branched chain PEGs demonstrate better than the linear PEGs¹³⁵. However, it is important to realize that PEGylation could lead to risk of different efficacy and side effect profiles with parent protein.

Cyclization makes peptide non-susceptible to enzymes by removing exposed N and C terminal from peptide molecules^{136,137}. It is inspired by many natural small cyclic proteins, such as cyclosporine and desmopressin¹³⁸. Desmopressin which is a cyclic analogue of vasopressin displays greater resistance to enzymatic degradation than vasopressin¹³⁹. Ring closure of a peptide can be attained by four different ways: head-to-tail (C-terminus to N-terminus), head-to-side chain, side chain-to-tail or side chain-to-side chain, depending on its functional groups¹⁴⁰. The typical example is Arg-Gly-Asp (RGD) which is highly susceptible to chemical degradation due to presence an aspartic acid residue in its structure, while the rigidity can prevent the Asp side chain carboxylic acid from positioning itself in the right position for attack on the peptide backbone after cyclization¹⁴¹. Furthermore, cyclization can also decrease the exposure of polar atoms to surroundings by folding peptides into bioactive conformations, leading to the increase of oral bioavailability¹⁴².

4.2. Mucus penetrating and mucoadhesive systems

As mentioned before, mucus lining along the intestinal membrane of the GIT serves major hurdle for protein absorption by presenting multiple barriers. However, mucus is a double-edge sword in design of drug delivery systems. There are two opposing approaches to improve the delivery efficiency, including mucus-penetrating and muco-adhesive systems. Mucus penetrating systems are able to pass through the unstirred layer rapidly to reach intestinal epithelium for absorption. In contrast, muco-adhesive systems can prolong drug residence time for absorption at the intestinal tract by avoiding mucociliary clearance. There have been lots of excellent reviews to clarify the mucus-penetrating and mucoadhesive systems^{143–145}.

4.2.1. Mucus penetrating systems

For mucus-penetrating systems, the mucolytics have been firstly used to disrupt mucus barrier. The mucolytics are generally used to remove abnormal mucus in pulmonary disease, such as chronic

obstructive pulmonary disease (COPD), while able to diminish the mucus barrier transiently for healthy mucosa¹⁴⁶. For example, *N*-acetyl-L-cysteine (NAC) is a commonly used mucolytic and can cause a 6-fold increase in the absorption of 3.2 μm polystyrene particles in Peyer's patches¹⁴⁷. Although mucolytics can facilitate the attachment of particles to intestinal absorptive cells by removing mucus covering surface of epithelium and further enhance the oral absorption, the depletion of mucus barrier could lead to the injury of intestinal epithelium due to direct contact with proteolytic enzymes and acid. Therefore, it is necessary to employ the particles with specific properties to penetrate through mucus for drug delivery.

Inspiring from viruses, scientists deduced some possible characteristics of mucus-penetrating particles, including small size, highly hydrophilic and net-neutral surfaces^{54,148}. A study has demonstrated that polymeric particle less than 230 nm could pass through mouse colorectal mucus rapidly¹⁴⁹, which is similar as the size of some viruses. In order to increase the hydrophilicity of particle surface, the particles are commonly modified by PEG to enhance mucus penetration¹⁵⁰. In addition to PEG, poly vinyl alcohol (PVA)¹⁵¹ and *N*-(2-hydroxypropyl)methacrylamide copolymer (pHPMA)¹⁵² can also engineer the mucus-inert particles to improve the oral absorption. The nanocomplex of insulin and cell penetrating peptide (CPP) demonstrated no evident hypoglycemic effect after oral administration to diabetic rats, while the blood glucose level can decline to around 50% by nanocomplex coated by pHPMA. Meanwhile, nanocomplex coated by pHPMA exhibited 20-fold higher transport than free insulin on mucus-secreting epithelium cells¹⁵². Recently, protein corona liposomes are also able to facilitate the penetration of mucus and transepithelial transport¹⁵³.

Another important factor influencing the mucus-penetrating is surface charge of nanoparticles. Both positive and negative charge are not good for mucus-penetrating, but nanoparticles with densely charges coated net-neutral surfaces which is like virus surface exhibited higher diffusion through mucus layer. A biomimetic virus-like or charge reversible nanoparticles are able to improve the oral insulin delivery by overcoming mucus barriers¹⁵⁴. In addition, particle geometry can affect the mucus-penetrating ability significantly by micromovement¹⁵⁵. It has been revealed that the nanorods diffused across mucus layer rapidly by rotation¹⁵⁶. Owing to the strong mucus-penetrating capacity, the rod shaped nanoparticles can penetrate into deep mucus and reside there to prolong the residence time in GI tract¹³⁰. What's more, SNEDDS produces droplets (≤ 50 nm) with hydrophilic surfaces and their shape deformability facilitates them suitable for diffusion through mucus¹⁵⁷. Better mucus diffusion was achieved by medium chain lipids (MC)-SNEDDS compared to lipids with short or long chains. For example, MC-SNEDDS produced 2-fold increase of oral bioavailability of enoxaparin¹⁵⁸.

4.2.2. Mucoadhesive systems

Mucoadhesion is a common phenomenon for particles, which was found by Florey in 1962 from India ink particles adhering intestinal mucus¹⁵⁹. Most of microparticles or nanoparticles exerted non-specific mucoadhesion with intestinal mucus. However, mucoadhesive polymers have to be used for improving residence time significantly. For example, mucoadhesive microspheres with a diameter of 680–850 μm fabricated by copolymers of fumaric acid and sebacic acid were able to significantly prolong retention time in the rat GI tract compared to that of non-adhesive polymers¹⁶⁰. The hydrophobicity, surface charge and chemistry

influence the mucoadhesive properties of polymers significantly. The hydrophobic particles were absorbed 100-fold more than particles composed of hydrophilic cellulose¹⁶¹, which indicated that hydrophobic interactions was also an important aspect for mucoadhesion. Due to the negative charge of mucus layer, the positively charged particles are strongly mucoadhesive. Chitosan, especially *N*-trimethyl chitosan (TMC), was commonly used to engineer or coat nanoparticles for improving the drug absorption through electrostatic mucoadhesive with mucins. TMC nanoparticles produced much higher antibody titers of IgG and secretory IgA after oral delivery of urease than the solution by increasing mucoadhesion and epithelial permeability¹⁶². Thiolation on the surface of polymer is a common strategy to increase the mucoadhesive ability owing to formation of disulfide between thiol group of polymer or cysteine-rich subdomains of mucus glycoproteins¹⁶³. The mucoadhesive properties of polymers could be enhanced by up to 100-fold after thiolation¹⁶⁴. TMC nanoparticles modified by cysteine increased insulin transport by 1.7–2.6-fold compared to TMC nanoparticles¹⁶⁵. Compared with the mucoadhesive polymers, the another category of molecules exert the bioadhesion on epithelial cells rather than mucus gel layer, such as lectins. They are able to specifically recognize receptor-like structures of the cell membrane and therefore bind directly to epithelium and hence called as the second generation of bioadhesives¹⁶⁶. The lectin modified nanoparticles are able to not only bind to intestinal epithelium for prolonging the residence time but also probably triggering the active transport by receptor mediated uptake. Many studies employed lectin modified nanoparticles to target M cell for enhancing transport of large molecules^{167,168}. The absorption enhancement of lectin was described in Section 4.3.3. in detail.

In addition to mucoadhesive micro-/nano-particles, intestinal patches have also been attempted to improve the oral delivery of PPs^{169,170}. Intestinal patches are like transdermal patches and millimeter sized patches composed of a pH sensitive layer, mucoadhesive drug reservoir layer and a backing layer¹⁷¹, which are suitable to deliver PPs orally because they can release PPs locally near the mucosa and protect it from proteolytic degradation^{172,173}. Insulin-loaded intestinal patches can significantly reduce the blood glucose level at the dose of 10 IU/kg after jejunal administration. The author attributed it that the intestinal micro-patches can be put into enteric capsules and strongly adhesive to the intestinal mucosa after entering small intestine (Fig. 7), which also facilitate oral absorption of insulin significantly¹⁷⁴. Similar as transdermal patches, intestinal patches can load various formulations to modify the drug loading, release or absorption, such as solid-in-oil formulation as a drug reservoir in intestinal patch for oral delivery of insulin¹⁷⁵.

Hydrogels have also been extensively explored for enhancing oral absorption of PPs¹⁷⁷. Hydrogels can enable PPs reside within specific gut regions for a prolonged residence time due to their mucoadhesive properties and resist enzymatic degradation simultaneously. Complexation hydrogels are the optimal choice for oral delivery of PPs due to their environment responsiveness. For example, complexation hydrogels composed of poly(methacrylic acid) grafted with poly(ethylene glycol) do not swell in acidic environment due to strong hydrogen bonds and hence prevent insulin release in stomach, while dissociate in small intestine, resulting in rapid swelling and release¹⁷⁸. The complexation hydrogels led to a drastic reduction of plasma calcium concentration by improving intestinal absorption of sCT. Moreover, the oral bioavailability of insulin-loaded complexation

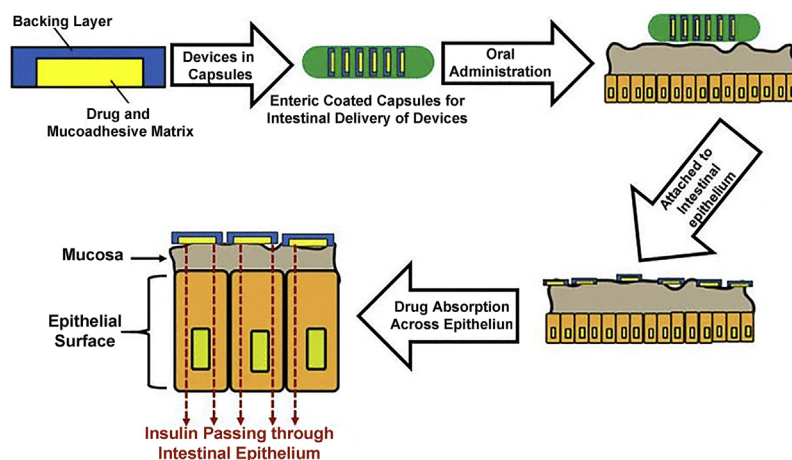


Figure 7 Schematic illustration of structure of intestinal patch and administration device, and *in vivo* mechanism of adhesion, drug release and absorption across intestinal epithelium. Reprinted with the permission from Ref. 176. Copyright © 2016 Springer.

hydrogels reached up to 7.2%¹⁷⁹. In addition, superporous hydrogels have also been used for enhancing intestinal absorption of PPs¹⁸⁰. They can swell to several hundred times within a few minutes and exhibit enhanced mucoadhesive force¹⁸¹. Oral administration of insulin-loaded superporous hydrogels leads to notable insulin absorption and hypoglycemic effect, which could attribute the prolonged residence time in high concentration of insulin within specific intestinal region and reversible opening of tight junctions¹⁸².

4.3. Absorption enhancement

In addition to instability in GI tract, another important factor limiting oral bioavailability of PPs is their extremely poor permeability across epithelial membrane due to large molecular weight and high hydrophilicity. It is indispensable to enhance the intestinal permeability of PPs by chemical or pharmaceutical approaches for development of oral products. So far, there are various strategies to enhance oral absorption of PPs, among which absorption enhancers could be the most commonly used in clinical or preclinical products.

4.3.1. Prodrugs

The prodrugs strategy is the most common approach to modulate physicochemical properties of drugs *via* chemical derivatization, such as improving stability, solubility or permeability. The prodrugs molecules are able to overcome barriers and then converted to be active form by *in vivo* degradation reactions¹¹. The bio-reversible cyclization of peptide backbone has been recognized as a promising prodrug methodology for PPs, which increases the intramolecular hydrogen bonding interactions, but decreases the intermolecular hydrogen bonding interactions with aqueous solvent¹⁸³. Borchardt et al.¹⁸⁴ used this method to develop the pheylpropionic acid based cyclic prodrugs of (Leu⁵)-enkephaline which have shown around 1680 folds higher permeability across Caco-2 cell monolayer than parent peptide. In the same study, coumarinic acid-based prodrugs of (Leu⁵)-enkephaline exhibited both high permeability and good stability. Lipidization is another promising approach to create prodrugs of PPs. Lipidization can increase hydrophobicity of peptides, leading to improved permeability. For example, two palmitoylated insulins lipidized by B1-monopalmitoyl and B29-dipalmitoyl showed higher lipophilicity

and greater stability, which leads to increased intestinal absorption¹⁸⁵. However, lipidization could reduce the biological activity of a peptide, which have been overcome by a reversible lipidization technique¹⁸⁶. This method can be carried out in an aqueous solution for conjugation of fatty acid and polypeptide, and can regenerate the original active polypeptides after oral absorption¹⁸⁷. The oral absorption of reversible lipidic prodrugs of salmon calcitonin was improved by at least 19 times compared to parent peptide¹⁸⁸. In addition, the prodrug design combining with lipid raft can generate site specific delivery by conjugation with targeting moiety. The combination of lipid and receptor targeting exhibited synergistic effect, leading to rapid transport through the cell membrane, which could be an alternative technology for enhancing absorption of hydrophilic biomacromolecules including PPs¹⁸⁹. However, the prodrug strategy is currently limited in modification of peptides. Proteins are hard to optimize their characteristics by chemical modification due to huge molecule, and conformational instability during chemical reaction.

4.3.2. Absorption enhancers

The largest obstruct for oral delivery of PPs is poor permeability across intestinal epithelium. The absorption enhancers are recognized to improve the intestinal permeability by altering the epithelial structure transiently, therefore extensively used in oral formulations of PPs. The possible mechanisms involved in current absorption enhancers are shown as Fig. 8. There are over 250 substances that have been used in preclinical studies as absorption enhancers for oral delivery of PPs according to an excellent review about intestinal permeation enhancers²¹, some of typical which have been listed in Table 2. Absorption enhancers have attracted more attention from pharmaceutical and biomaterial scientists since 1961 when a study found that sodium ethylene diamine tetraacetic acid (EDTA) was able to improve the oral absorption of heparin at dose of 50 mg/kg in dogs¹⁹⁰. Some semi-synthetic and synthetic substances have been developed as absorption enhancers including chelating agents, surfactants, polymers and bacterial toxins. These absorption enhancers can facilitate oral absorption of PPs either paracellularly *via* the opening of tight junctions or transcellularly through increasing membrane permeability, or a combination of both.

Chelating agents, like EDTA and citric acid, can generally enhance paracellular absorption by opening tight junctions which

is caused by reduction of intracellular calcium due to chelating properties¹⁰⁸. Diethylene triamine pentaacetic acid (DTPA), a novel chelator, has been approved to improve the oral insulin absorption with relative bioavailability of 20% by integrating into chitosan nanoparticles¹⁹¹.

Surfactants are main absorption enhancers in clinical studies, such as sodium caprylate/caprinate and their derivatives, and endogenous bile salts. Endogenous bile salts and their derivatives have been investigated to increase oral relative bioavailability of insulin by protecting stability of PPs and enhancing intestinal permeability^{47,192,193}. The advantages of endogenous bile salts and their derivatives include good biocompatibility and high drug loading for PPs when they are used in fabrication of liposomes¹⁹⁴. Sodium caprylate/caprinate and their derivatives are the most promising absorption enhancers and have been marketed for oral delivery of PPs, such as sodium caprylate in oral octreotide (Mycapssa[®], Chiasma Pharma, USA/Israel) and SNAC in oral semaglutide (Rybelsus[®], Novo Nordisk, Denmark). The SNAC was firstly approved using in Eligen[®] carrier for improving oral delivery of vitamin B₁₂ developed by Emisphere (USA). It is a derivative of sodium caprylate whose structure and mechanism of action are presented in Fig. 9. It was reported that SNAC were capable of enhancing permeation of heparin, sCT and insulin significantly^{195–197}. Most of studies regarded hydrophobic SNAC non-covalently associated with peptides improves their absorption across the intestinal epithelium. After transported, the peptide disassociated from the SNAC carrier and passed into the circulation freely¹⁹⁸. The success of Rybelsus[®] is related to its strong association with semaglutide¹⁹⁹. However, some studies also thought the SNAC enhanced transport of peptides through opening tight junctions because they caused significant decline of TEER and a 36-fold increase in mannitol permeability across Caco-2 monolayers²⁰⁰.

Chitosan and its derivatives are the most common polymers for enhancing oral delivery of PPs depending on the positive charge density and bioadhesive ability. They are generally fabricated as nano/micro particles to encapsulate PPs for improving oral absorption, which described in Section 4.4 in detail.

Some absorption enhancers emerging from toxins have gradually been used in improving oral delivery of PPs by altering paracellular or transcellular permeability²⁰². Due to safety consideration of native toxin, the common approach is that the short peptide sequence is developed by structure activity relationships (SAR) studies. For example, native ZoT (45 kDa) can enhance small intestine permeability *via* PKC-dependent cytoskeletal contraction which is exerted by its first six amino acids^{203,204}. Alba Therapeutics (USA) developed a short peptide sequence AT1002 (H-FCIGRL-OH) which can lead to 40-fold increment of lucifer yellow in Caco-2 monolayer²⁰⁵. The larazotide acetate, an 8-mer peptide that promotes tight junctions assembly, has been used in clinical development by Alba therapeutics (USA)²⁰⁶. In addition, some short peptides emerging from toxins could target tight junctions related proteins, such as claudins or occludins, to increase paracellular permeability^{207,208}. CPP are a sort of peptides derived from the transactivator of transcription (HIV-1 TAT) protein of the human immunodeficiency virus and can increase the membrane permeability of PPs²⁰⁹. The first CPP, Penetratin[®] (RQIKIWFQNRRMKWKK) consisting of 16 amino acids, was discovered in 1994²¹⁰. The therapeutic PPs are linked with the CPPs by chemical conjugation or complexed with the CPPs by non-covalent bonds²¹¹. The possible mechanism of CPPs on enhancing cellular uptake is that

they can increase paracellular and transcellular transport through endocytic pathway²¹². The low molecular weight protamine (LMWP) with a sequence of VSRRRRRRGRRRRRC is the one of CPPs, which can increase the intestinal cell membrane permeability and oral relative bioavailability of exenatide-Zn²⁺ by 29-fold²¹³. Both Penetratin[®] and its analog PenetraMax[®] exerted absorption enhanced ability for oral insulin in D-form by non-covalent approach; but there is no synergistic effect observed when using the combination of these two CPPs²¹⁴. Although CPPs have exerted excellent capacity in improving membrane permeability, they have not yet been validated in the clinic studies of oral delivery of peptides due to complex GI environment.

Absorption enhancers have been evidenced in improving oral absorption of PPs, even some enhancers have been used in marketed products, such as SNAC and EDTA. However, safety and regulation are the main concerns in the application of absorption enhancers in oral delivery. Toxicity has been considered as a potential drawback impeding the application of enhancers²¹. Fortunately, there have no significant adverse events reported for any absorption enhancers tested in clinical trials to date.

4.3.3. Active targeting

Increasing active transport has also become a promising approach to facilitate the oral absorption of PPs by targeting receptors, transporters and specialized cells in intestinal epithelia²¹⁵. The colloidal carriers decorated with a specific ligand (Table 3^{168,216–228}) have emerged as a promising technology to increase interaction with the epithelium by active targeting followed by higher transport.

Receptor-mediated endocytosis can take up extracellular substances efficiently by internalization triggered by binding ligand molecules with receptors, which is a critical pathway to acquire sufficient essential nutrients for human body, such as vitamins, transferrin and hormones²²⁹. Therefore, some nutritional components including vitamins, saccharides and fatty acids have been extensively explored to decorate drug carriers for enhancing active transport. Some nutritional vitamins have to be transported by receptor mediated mechanisms from diet and other exogenous sources, such as vitamin B₁₂ (VB₁₂) and folate. VB₁₂ was used as a ligand to modify dextran-*g*-poly-ethyleneoxide cetyl ether micelles for oral delivery of cyclosporine A, which demonstrated increased permeability of cyclosporine A on Caco-2 monolayer. Moreover, VB₁₂ modified nanoparticles loading insulin produced 70%–75% blood glucose reductions²¹⁸. However, the limited absorption site of VB₁₂ in the distal ileum leads to slow uptake²³⁰, compromising its potential application. Folate and biotin are also the aqueous vitamin B family members and there are a large number of receptors in whole intestinal tract. A folic acid (FA)-pluronic 85-poly(lactide-*co*-glycolide) polymersome exhibited higher cellular uptake than unmodified polymersome and showed better enhanced absorption effect of insulin. The folate receptor mediated endocytosis pathway was also validated by cellular uptake mechanisms study²³¹. Insulin loaded liposomes modified by biotin showed significantly higher hypoglycemic effect with almost 2-fold relative bioavailability compared to the conventional liposomes²¹⁷. Like vitamins, there are a variety of saccharide receptors located on the intestinal epithelia for active transport, such as mannose, galactose and hyaluronic acid receptors²³². For example, the galactose-modified nanoparticles exhibited higher cellular uptake and *ex vivo* intestinal epithelial permeability compared with galactose free nanoparticles²³³. However, most saccharide receptors locate in M cells, by which oral delivery of vaccines can be enhanced²³⁴. Transferrin receptors (TfR)

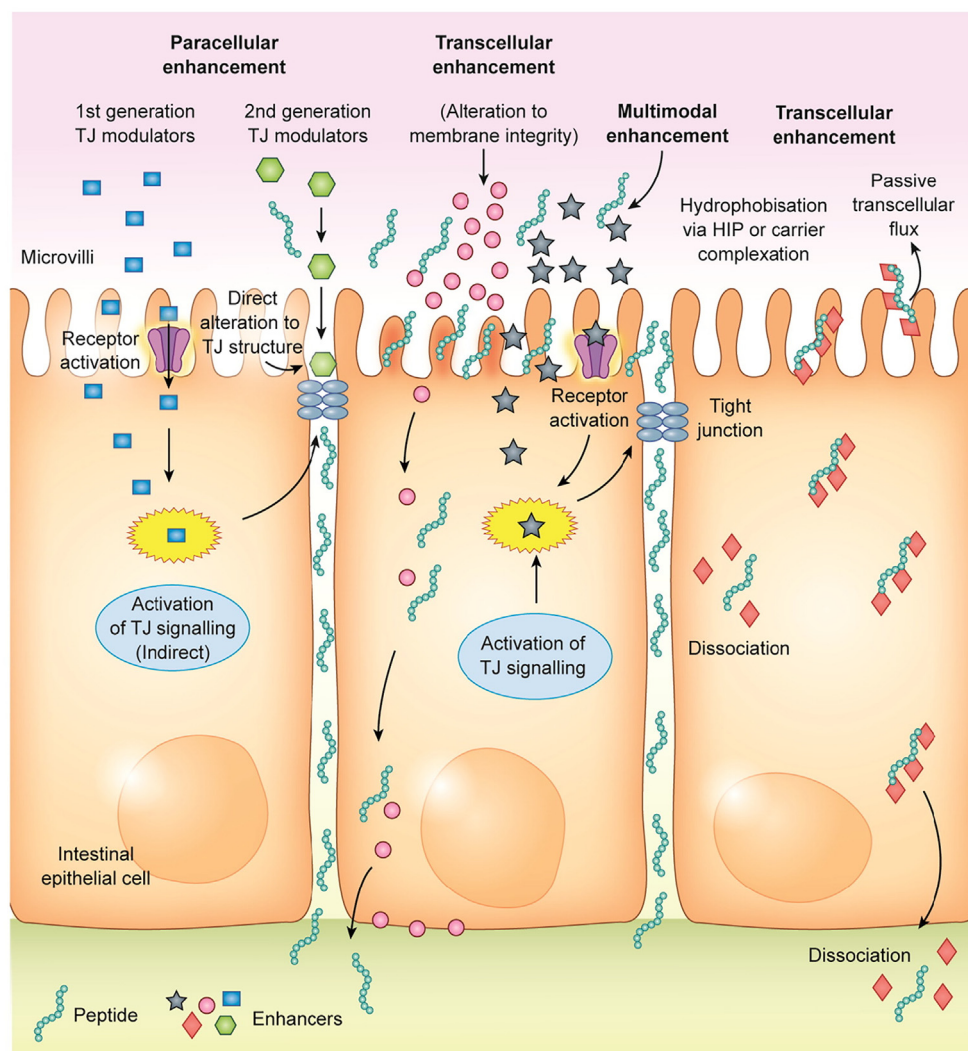


Figure 8 The schematic illustration of mechanisms of absorption enhancers including transcellular and paracellular pathways. Reprinted with the permission from Ref. 21. Copyright © 2016 Elsevier.

has recently become a promising target for oral delivery of PPs because they are distributed throughout the small intestinal epithelium²³⁵. In a recent study, Yong et al.²³⁶ found that nanoparticles modified by transferrin (Tf) were taken up by Caco-2 cells *via* TfR-mediated transcytosis more than the unmodified counterparts significantly. However, the increased number of endogenous Tf reduces the specificity of Tf-functionalized nanocarriers²³⁷. To

address this issue, Liu et al. developed a nanosystem modified by cycle peptide CRTIGPSVC (CRT) to target the Tf–TfR complex for circumventing the competitive inhibition, which significantly increased the Caco-2 cellular uptake *via* a non-canonical allosteric directed mechanism²³⁸. The Fc receptor (FcRn) is the most promising ligand candidate to actively transport biomacromolecules into circulation from small intestines due to its high efficiency in

Table 2 List of some typical permeation enhancers for oral absorption of PPs.

Enhancer	Mechanism	Application
EDTA	Chelating agents; paracellular	ORMD-0801; ORMD-0901 (Oramed Pharma, USA)
Citric acid	Chelating agents; paracellular	Peptelligence™ (Tarsa, USA)
Bile salts	Multimodal	IN-105 (Biocon, India)
Sodium caprate (C ₁₀)	Multimodal	GIPET® (Merrion Pharma, Ireland)
Sodium caprylate (C ₈)	Multimodal	TPE® (Chiasma, Israel)
SNAC/5-CNAC	Transcellular	Eligen® (Emisphere, USA)
Chitosan	Multimodal	Oral insulin (NanoMega, USA)
Penetratin/PenetraMax	Transcellular	Reported for various peptides

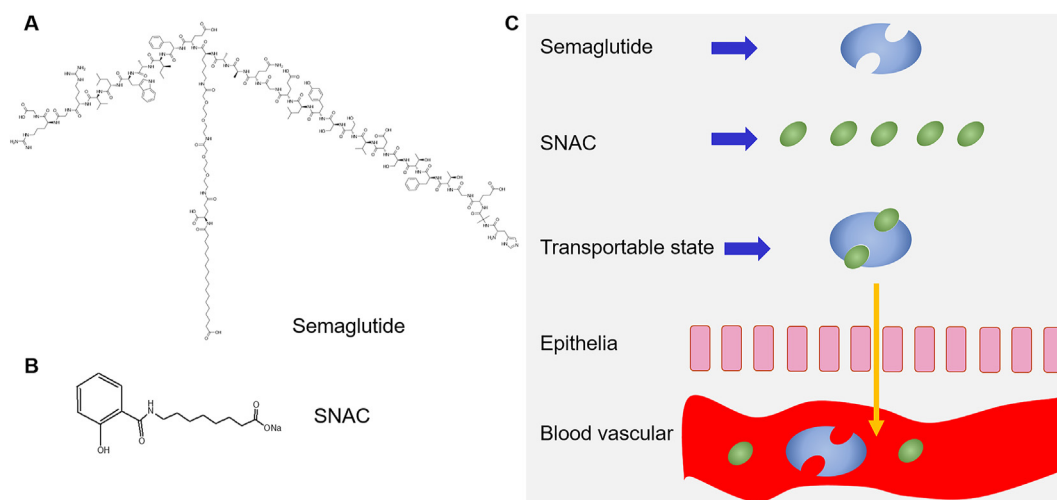


Figure 9 The chemical structure of semaglutide (A) and SNAC (B), and the rationale of permeation enhancing effect of SNAC on semaglutide (C). Reprinted with the permission from Ref. 201. Copyright © 2016 Springer.

Table 3 List of ligands for actively targeting the enteric epithelia.

Ligand	Target	Cell	Carrier	Biologic	Ref.
Folate	Folate receptor	Enterocyte	PLGA nanoparticles	Insulin	216
Biotin	Avidin	Enterocyte	Liposomes	Insulin	217
Vitamin B ₁₂	IF-CbI receptor*	Enterocyte	Dextran nanoparticles; calcium phosphate nanoparticles	Insulin	218,219
Galactose	Galactose receptor	Macrophage	PLGA nanoparticles	siRNA	220
Mannose	Mannose receptor (DEC-205)	Enterocyte	Proliposome	Glutathione	221,222
Hyaluronate	CD44 receptor	Enterocyte	CaCO ₃ nanoparticles	Insulin	223
Transferrin	Transferrin receptor	Enterocyte	Fuse protein	Human growth hormone	224
Fc fragments	Neonatal Fc receptor	Enterocyte	PLA-PEG nanoparticles	Insulin	225
lectin	Integrin receptor	M-cell	Liposome; solid lipid nanoparticles	Insulin	168,226
RGD	Integrin $\alpha v \beta 3$ receptor	Enterocyte; M-cell	PLGA-mPEG nanoparticles	Insulin	227
CSKSSDYQC (CSK)		Goblet cell	Chitosan nanoparticles	Insulin	228

IF-CbI: combination of gastric intrinsic factor (IF) and cobalamin (CbI, vitamin B12).

transporting immunoglobulin G antibodies across epithelial barriers²³⁹. FcRn targeted nanoparticles were able to increase a mean absorption efficiency up to approximately 13-fold and oral insulin-loaded FcRn targeted nanoparticles could lead to similar hypoglycemic effect as s.c. insulin at the same dose²²⁵. More recently, Martins et al.²⁴⁰ developed porous silica nanoparticles conjugated with Fc fragment of immunoglobulin G by microfluidics technology which exerted higher cytocompatibility and greater interaction with the intestinal cells, as well as ensued better absorption of glucagon-like peptide 1 (GLP-1).

Transporters located on the epithelia surface can selectively transport some specific molecules into the cytoplasm but rarely cause cell membrane active deformation to engulf the particles unlike receptor mediated endocytosis²⁴¹. Therefore, most transporters are used for improving oral bioavailability of small molecular drugs through development of prodrugs, but rarely for biomacromolecules. For example, the prodrug of zanamivir with amino acid groups increased intestinal jejunal permeation by transportation of amino acid transporter²⁴². However, some studies also demonstrated ligand modified nanoparticles were also transported by transporters. For example, the nanoparticles functionalized with deoxycholic acid are

able to overcome multiple obstacles and enhance oral absorption of insulin by targeting the apical sodium-dependent bile acid transporter (ASBT)²⁴³. Moreover, the insulin-loaded butyrate-PEG nanoparticles produced approximately 3.0-fold improvement of relative pharmacological bioavailability of oral insulin by targeting monocarboxylate transporter 1 (MCT-1) compared to the unmodified nanoparticles²⁴⁴.

Recently, targeting specialized cells in intestinal epithelia attracted lots of interests as an approach of improving oral delivery, such as M cells in peyer's patches, goblet cells and some immune cells. M cells are the most common target cell for oral drug delivery of antigens or proteins due to their special physiological functions²⁴⁵. Particles in intestinal gut can be transported by M cells very fast from apical side to basolateral side and then captured by immune cells in "dome trap" or further enter into lymphatic vessels⁶³. There are a variety of receptors expressed on the surface of M cells for targeting, such as intercellular adhesion molecule (ICAM)-1, L-fucose, $\beta 1$ integrin and glycoprotein 2 (GP2)²⁴⁶. Lectins are the most common used ligand binding reversibly to receptors of M cells, such as wheat germ agglutinin (WGA) and ulex europaeus agglutinin 1 (UEA1)^{226,247}. Lectin

conjugated microparticles and lipid nanoparticles loading insulin resulted in larger glucose level reduction and increment of residence time at intestinal membrane^{168,248}. The tripeptide RGD are extensively used for enhancing the transport of nanoparticles across M cells through targeting $\beta 1$ integrin²³⁴. Besides, some new ligands for targeting M cells were obtained by the phage display technique, such as CKS9²⁴⁹. Goblet cells, a mucus secretion cells, are rarely used to be as target sites for oral delivery. However, recent studies started to pay attention to drug delivery system based on targeting goblet cells. Nanoparticles modified with a peptide of CSKSSDYQC (CSK) which can target to goblet cells can facilitate the uptake in villi and higher internalization *via* calthrin and caveolae mediated endocytosis on HT29-MTX cells (goblet cell like model)²⁵⁰. Moreover, this nanoparticles loading insulin showed 1.5-fold improvement of relative bioavailability compared to the unmodified ones²²⁸. Besides, targeting dendritic cells (DCs) located on apical side of intestinal epithelia has been attempted to improve delivery of vaccines. Several DCs targeting peptides have been validated to increase oral delivery efficiency of antigen and enhance immunization, such as DC-pep that was screened out by phage display^{251,252}.

Although active targeting is able to increase the uptake in specific intestinal cell group, the insufficient absorption area limits the absorption extent of PPs, which is difficult to increase the oral bioavailability to a large extent. More targets which distribute more extensively in intestinal epithelia need to be explored for oral delivery.

4.3.4. Lymphatic transport

Lymphatic route is also an important way for oral absorption. There are various accesses to lymph depending on characteristic of drugs (Fig. 10). After transported across intestinal epithelia, small hydrophilic drug molecules or macromolecules that are smaller than 10 nm (or 16–20 kDa for proteins) are transported primarily into the blood capillaries^{253,254}. Nevertheless, the highly lipophilic drugs could be assembled into chylomicron with lipoproteins and subsequently transported into lymph. Particles or macromolecules (antigens or proteins) that are larger than 10 nm are hard to enter into blood capillaries due to small interstitial space of blood capillaries, but able to drain into lymph vessels. However, particles larger than 100 nm are poorly transported into lymph due to reduced diffusion and convection through the interstitium^{255,256}. Both lymphoid (Peyer's patches) and non-lymphoid tissue (villous) in intestinal lumen contribute to the lymphatic transport. Transferring into lymph vessels *via* non-lymphoid tissue depends upon the lipid pathway, vehicle effects, sieving mechanisms of the blood vessels and the application site. The proximal small intestine is the best lymphatic transport site, while the presence of lymphatic transport has also been proven in rectal administration. M cell in Peyer's patches can take up intestinal particles by phagocytosis and complete transcytosis very fast, which is the main route for highly potent compounds such as lymphokines and antigens. Some excellent reviews have showed various approaches for improving oral lymphatic delivery^{257,258}.

In order to increase absorption of lipid pathway, some lipid formulations were used to mimic the absorption process of dietary fats for improving oral lymphatic transport of PPs. For example, insulin-loaded solid lipid nanoparticles (SLNs) demonstrated significant drug accumulation within intestinal lymphatic system²⁶⁰. In addition, lipidization of peptides *via* chemical

modification with fatty acids is an important approach to increase the lymphatic transport by improving association with chylomicrons, which have been applied in oral delivery of several peptides, such as sCT, encephalin, tetragastrin and insulin²⁶¹. However, the flow rate of lymph through the intestinal lymphatic system is approximately 500-fold slower than that of blood through intestinal blood capillaries and portal vein, which leads to not sufficient quantity absorbed in systemic circulation for therapeutics²⁶². Therefore, it is very limited to elevate oral bioavailability of therapeutic PPs by targeting lymphatic systems. So far, there are no clinical and commercial products developed by lymphatic targeting technology. However, M cells route has been regarded as an effective pathway to oral deliver vaccine and protein therapeutics. The glucan microparticles incorporating with thermosensitive poloxamer 407 gel improved the oral absorption of insulin, hence producing mild reduction in blood glucose level for over 20 h in diabetic rats²⁶³. Meanwhile, the lymphatic transportation is highly correlated with pharmacological bioavailability, which indicates the lymphatic route plays critical role in oral absorption of insulin²⁶⁴. The M cell transport is likely related to the physicochemical characteristics of the particles, such as physical and chemical stability, size, surface charge, shape and elasticity. For example, the polystyrene particles with a range of 50 nm and 3 μ m have 6%–34% absorption ratios after oral administration²⁶⁵. But particles larger than 10 μ m are rarely able to be transported by M cell²⁶⁶. Meanwhile, the targeting ligands may influence the adhesive to M cell or uptake of M cell significantly, such as lectins and RGD peptides, which has been described in section 4.3.3 in detail. However, the GALT comprises less than 10% of whole intestinal epithelial surface, which limits the absorption extent of therapeutic PPs. In addition, the particles could be captured in dome trap after transcytosis by M cells to inhibit the entry of therapeutic PPs into systemic circulation *via* lymph vessels⁶³. Due to high potency of vaccines, M cell uptake of particulate oral vaccines demonstrated promising potential in clinical trials. The PLGA microspheres containing the *Escherichia coli* colonization factor antigen II as potential vaccine for enterotoxigenic *E. coli* can generate antibody responses in 5 out of 10 human subjects²⁶⁷. The PLGA microspheres encapsulating CS6 antigen also demonstrated effective vaccination in phase I clinical trials²⁶⁸. Nevertheless, these clinical trials have not been continued because of variability in immune response generation. Except for the immunology issues, the formulation design may be important hurdles for antigen-loaded particles, including stability of antigen, and release in intestinal lumen or Peyer's patches.

4.3.5. Ionic liquids

Ionic liquids (ILs) are a category of ionic compounds with melting point below 100 °C, while are called as room temperature ILs (RTILs) if the melting point declines to room temperature²⁶⁹. ILs have been extensively used in chemical engineering as solvents, catalysts, reagents and so on. ILs have good capacity for solubilizing poorly soluble drugs²⁷⁰ and strong permeation enhanced ability for biomacromolecules^{271,272}. However, most of ILs in chemistry are not biocompatible for drug delivery or other biological use²⁷³. Recently, a class of RTILs based on natural components were developed for improving drug delivery, such as choline and organic acids²⁷⁴. They demonstrated good biocompatibility and permeation enhanced capacity. They are firstly employed to improve transdermal delivery of PPs, such as insulin

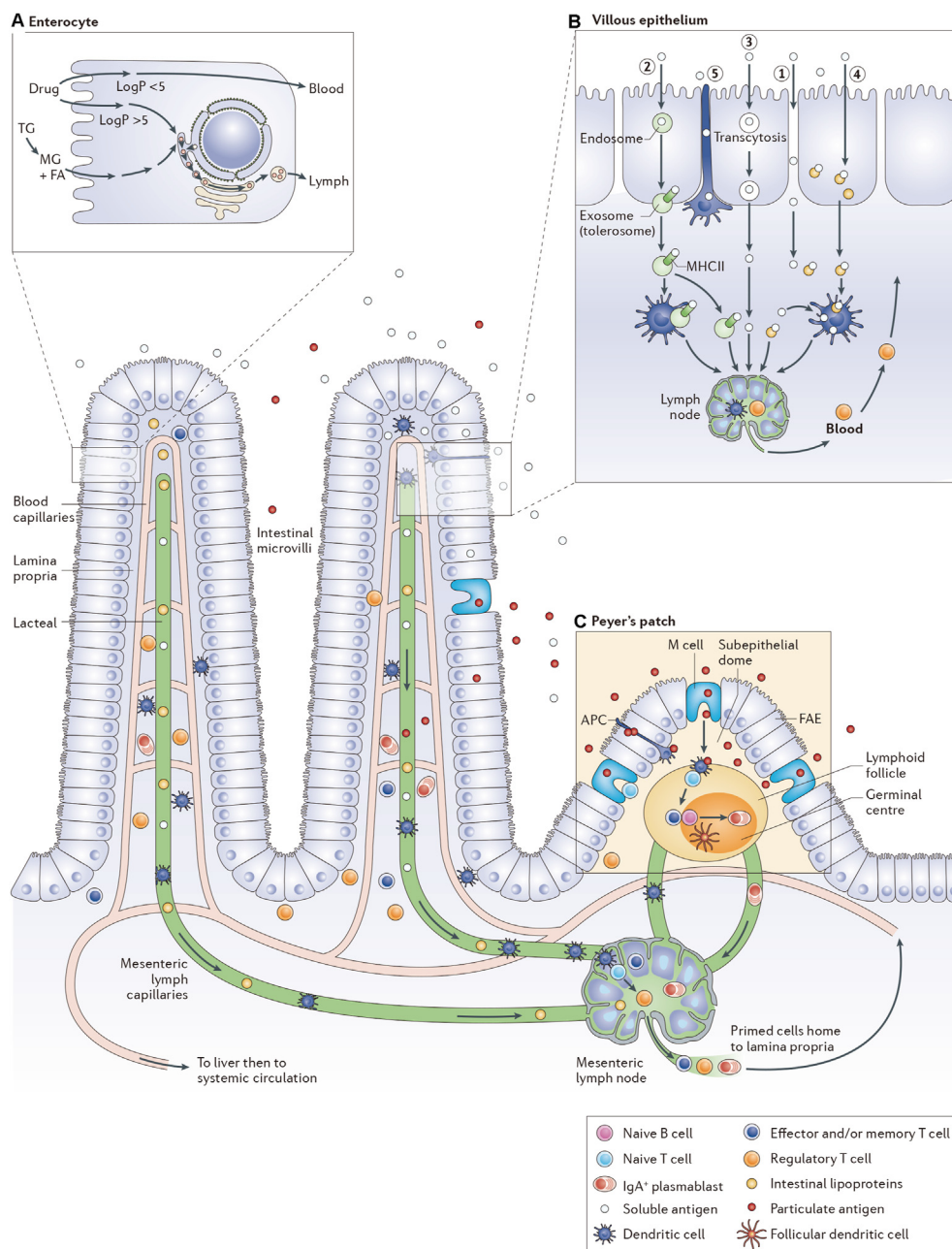


Figure 10 The schematic illustration of the intestinal lymphatic transport of chemical drugs or antigens (proteins) after oral administration. (A) Dietary lipids and some highly lipophilic drugs are taken up by enterocytes and then assembled as chylomicron with lipoproteins to be drained into mesenteric lymph. (B) Soluble antigens (proteins) access the mesenteric lymphatics directly or *via* phagocytosis by dendritic cells after transport by various routes including paracellular diffusion (①), uptake into endosome and then exocytosis by exosomes (②), transcytosis by enterocytes (③), transport by M cells (④) or dendritic cells (⑤). (C) Particulate antigens (proteins) are primarily transported by M cells and then processed by a large amount of immune cells under subepithelial dome. Reprinted with the permission from Ref. 259. Copyright © 2016 Nature.

and bovine serum albumin (BSA)²⁷⁵. Recent study indicated that choline and geranate (CAGE) ILs were able to enhance oral absorption of insulin and insulin-loaded CAGE ILs (3–10 IU/kg) produced a significant hypoglycemic effect after intrajejunal administration or oral intake of enteric capsules²⁷⁶. Meanwhile, insulin can maintain conformational and chemical stability in CAGE ILs. ILs could form a self-assembled nanostructure with gastrointestinal fluids spontaneously, which probably contribute to oral absorption or biodistribution *in vivo*²⁷⁷. Angsantikul et al.²⁷⁸ found that choline and glycolate ILs were also able to

reduce the viscosity of the intestinal mucus and enhance the paracellular transport. Therefore, they can effectively deliver TNF α antibodies into the intestinal mucosa as well as systemic circulation. ILs also act as permeation enhancer for biomacromolecules in other mucosal barrier, such as nasal delivery²⁷⁹. ILs can also combine with other formulations as a permeation enhancer. For example, Peng et al.²⁸⁰ fabricated a mucoadhesive ionic liquid gel patches that can improve the oral transport of insulin. However, the interaction between ILs and water has to be highly valued because water is the most common

substances in biological body. It is not clear whether water can attenuate the effect of ILs or not²⁸¹.

4.3.6. Intestinal microneedles

Microneedle-based technology has been broadly used in transdermal delivery in pharmaceutical and cosmetics products. Microneedles are able to overcome the main barriers hindering drug absorption, such as stratum corneum in transdermal delivery²⁸². In addition, microneedles can penetrate the physical barrier to improve drug penetration but bring about no damage to the tissue or nerves by tuning the needle length to appropriate size. Therefore, microneedle is a pain-free administration technology²⁸³. Recently, microneedles have gradually used in other mucosal delivery routes, such as ocular, oral and vaginal²⁸⁴.

Mucosal and epithelial barriers are the main factors influencing the oral absorption of PPs. Traverso et al.²⁸⁵ firstly demonstrated proof-of-concept experiments in swine that microneedles were capable to completely overcome the gastrointestinal mucosa and epithelia, and promote oral bioavailability of a biologically active molecule (Fig. 11A–C). This device was 2 cm in length and 1 cm in diameter, and the microneedles were made of metals. The drug can be loaded in hollow or solid microneedles for release (Fig. 11D). In spite of good safety and tolerability of this device indicating in experimental period, the biocompatibility is still a significant concern if it would be developed to be a clinical product. In order to avoid the toxicity caused by metals, biodegradable or dissolvable microneedles are fabricated for biomedical use. Abramson et al.²⁸⁶ employed polymers to fabricate a unfolding microneedle injector (LUMI) (Fig. 11E). This injector is composed of three flexible arms, each of which has a 0.5 cm² microneedle patch on the far end. The arms are initially bundled together and then the injector is filled in a capsule. The LUMI arms unfold outward after the injector is pushed out of capsule when the capsule reaches the intestine *via* intragastric administration. Then the arms press the microneedle patches against the intestinal wall to penetrate the epithelia barriers. A company (Rani Therapeutics, San Jose, CA, USA) is developing a related technology to deliver oral biologics, which has been studied in clinical trials²⁸⁷. However, future studies have to be determined whether the microneedle cause distension of small intestine. In addition, the small damage of intestine caused by microneedle could bring about large risk of systemic infection due to presence of a large amount of microorganisms in intestines.

4.3.7. Self-orienting millimeter-scale applicator (SOMA)

In order to efficiently deliver the biomacromolecules by oral routes, various administration devices were attempted. A self-orienting millimeter scale applicator (SOMA) was inspired by the leopard tortoise's ability to passively reorient (Fig. 12) and could deliver biologic drugs by penetrating gastric mucosa rather than intestinal gut²⁸⁸. The thickness of stomach's wall is approximately 4- to 6-mm which provides more space to design the length of needles for safety and efficacy. The hypoglycemic effect of insulin delivered by SOMA is similar as that of sc insulin. However, this oral device is intrinsically an injectable administration and the difference from the common injection is only injection site. Moreover, the injection in GI tract could cause larger risk than sc or intramuscular injection. Recently, a microjet vaccination system was developed to avoid the use of needles. It is a three-dimensional microelectromechanical systems-based drug delivery technology and can produce a high-pressure liquid jet of vaccine to penetrate the buccal mucosal layer²⁸⁹.

4.3.8. Nanocarrier-facilitated oral delivery of PPs

The largest barriers in oral delivery of PPs are mainly enzymatic degradation and poor permeability across intestinal epithelium. Almost most of strategies are based on overcoming these two barriers to improve oral bioavailability of PPs. Nanocarriers can entrap the active biomacromolecules into the matrix or the core to protect against enzyme degradation through GI lumen. In addition, nanocarriers can be taken up by epithelial cells or M cells in Peyer's patches. Nanocarriers are also able to entrap penetration enhancers together with PPs or be decorated by ligands to further enhance the oral absorption. Hence, various nanocarrier systems have been extensively investigated in oral delivery of biologics including PPs, vaccines and nucleic acids. According to materials of nanocarriers, there are primarily three categories of nanoparticles, such as polymeric, lipid-based and inorganic nanoparticles, some of which have demonstrated good delivery effect for PPs by oral routes (Table 4^{106,120,217,219,225,290–311}).

4.3.8.1. Polymeric nanoparticles. Polymeric nanoparticles have been widely explored to circumvent multiple barriers hindering oral delivery of PPs. Except for protecting PPs from degradation in harsh GI environments, polymeric nanoparticles can increase the epithelial transport by improving cellular uptake and inhibiting efflux of P-gp³¹². In addition, some polymers can reversibly open tight junctions, allowing transport of PPs through paracellular pathway, such as chitosan and its derivatives³¹³. The active transport through enterocytes, goblet and M cells can be enhanced by decorating ligands on the surface of nanoparticles²¹⁵. Polymeric nanoparticles are generally composed of synthetic, semi-synthetic or natural polymers with diameter ranging from 10 to 1000 nm. Natural polymers are abundantly present in nature and have good compatibility, hence, have gained extensive interests in oral delivery of PPs, such as chitosan, gelatin, alginate and hyaluronic acid¹. Chitosan is the most common materials as nanoparticulate delivery system for PPs due to its mucoadhesive and permeation enhancement characteristics. In addition, chitosan is also easily modified to achieve various aims, such as pH-responsive release, increasing hydrophilicity and positive charges or improving mucoadhesive ability^{314–316}. A pH-responsive nanoparticles developed by conjugating chitosan and poly- γ -glutamic acid (PGA) can enhance the paracellular transport of insulin by opening the tight junctions between adjacent cells³¹⁷. Chitosan/alginate nanoparticles can increase pharmacological availability of insulin to 6.8% and 3.4% for the 50 and 100 IU/kg dose respectively³¹⁸. The *N*-trimethyl chitosan (TMC) can significantly enhance the permeability of peptides or proteins in comparison of normal chitosan³¹⁹, hence, has been used as permeation enhancer to coat other nanocarriers³²⁰ or engineer nanocarriers together with other materials³²¹. Recently, chitosan oligomers were found to reduce the coulombic repulsion between anionic calcitonin and negatively charged intestinal epithelial cells, which facilitates the oral absorption of calcitonin³²². In terms of synthetic polymers, the PLGA, PCL, PLA and polyacrylic acid (PAA) are frequently studied for PPs delivery due to good biocompatibility. These polymeric nanoparticles exhibited better capacity to protect against GI digestion, but it is necessary to combine with other permeation enhancers for improving intestinal epithelial permeability of PPs³²³. However, lack of self-regulating release of insulin is still a big issue for oral delivery. Recently, Paul et al.^{324,325} developed a biomimetic imprinted nanoparticles to increase self-regulating adhesion and release of insulin by molecular imprinting technique. The imprinted

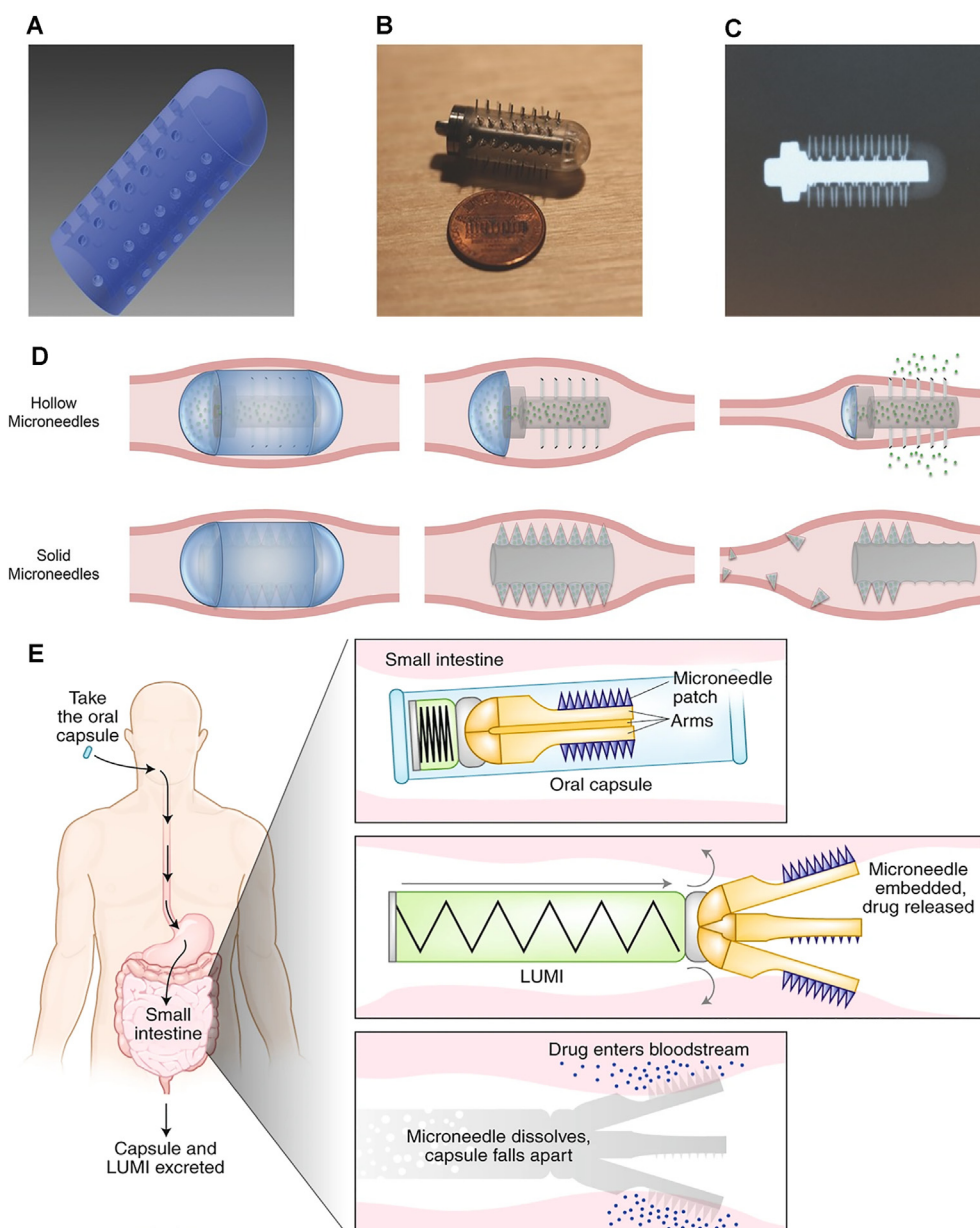


Figure 11 Schematic illustration of the rationale of orally administered microneedles. (A) Computer-aided design of the radial prototype; (B) The produced microdevice with metal endcap and pin; (C) Radiography of the microneedle; (D) Therapeutic use concept of both hollow and solid microneedles; (E) The concept of oral delivery of PPs *via* microneedle patch. Reprinted with the permission from Refs. 286 and 288. Copyright © 2015 Elsevier and 2019 Nature.

nanoparticles significantly prolonged hypoglycemic effect for up to 24 h and increased insulin transport *via* transcellular pathway. Yet, the oral bioavailability of PPs delivered by polymeric nanoparticles is still very limited and not sufficient for clinical therapy. Meanwhile, long-term exposure of these nanoparticles in GI tract is still a concern.

4.3.8.2. Lipid-based nanocarriers. Lipid-based nanocarriers (LBNs) are composed of natural lipids or phospholipids to form emulsion, solid particles or vesicles³²⁶. Due to high biocompatibility, LBNs have received much attention as oral delivery systems. However, LBNs are difficult to entrap hydrophilic macromolecules with high efficiency, which is a primary hurdle to be developed for oral delivery of PPs³²⁷. In addition, LBNs can be

degraded by lipolysis in GI tract, leading to poor protecting ability for entrapped PPs³²⁸. Therefore, SLNs appear to be better than emulsions or liposomes. Nevertheless, nanoemulsions and liposomes have also exerted certain superiority for oral delivery of some PPs by regulating formulation.

The nanoemulsions are generally used to incorporate lipophilic drugs into the oil droplets for improving oral absorption. Although the incorporation of hydrophilic PPs in the internal phase of o/w nanoemulsions is difficult to achieve, some poorly water soluble peptides have been successfully developed to be oral products by SNEDDS, such as Sandimmun Neoral[®] for cyclosporine A³²⁹. In order to effectively entrap hydrophilic PPs in nanoemulsions, the w/o nanoemulsion was employed for oral delivery of PPs. The PPs can be completely encapsulated in the core of w/o nanoemulsions

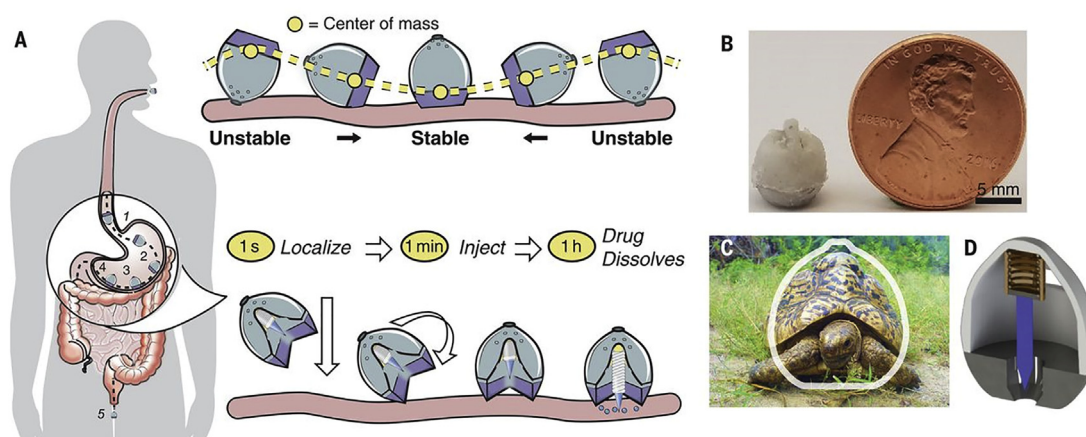


Figure 12 The schematic diagram of SOMA. (A) The mechanism of localization, orientation and injection of SOMA in stomach; (B) The fabricated product of SOMA; (C) The shape comparison between SOMA and the leopard tortoise (*S. pardalis*); (D) The injection device in SOMA. Reprinted with the permission from Ref. 289. Copyright © 2019 Science.

to protect against degradation in the harsh GI environments³³⁰. A insulin-loaded w/o nanoemulsions with the diameter of 161.7 ± 24.7 nm enhanced oral bioavailability of insulin by 10-fold compared with plain insulin solution³³¹. Moreover, the w/o microemulsion exhibited more significant effect in oral delivery of other proteins, such as earthworm fibrinolytic enzyme which are increased to 208-fold higher bioavailability than that of control solution by intraduodenal administration³³². However, the w/o nanoemulsions definitely occur phase conversion due to a large amount of water in GI tract, which could cause the leakage of encapsulated PPs. Therefore, Li et al.³⁰⁴ employed chitosan and alginate to coat the nanoemulsion to avoid fast leakage of insulin from the core of nanoemulsion.

Liposomes are simulated natural cell membrane structure and have been successfully used in delivery of anticancer drugs to decrease the toxicity, such as Doxil[®] for doxorubicin. Likewise, multiple remarkable advantages of liposomes received much interests in oral drug delivery, such as good biocompatibility, flexible encapsulation and tunable characteristics³³³. Liposomes have been attempted to be investigated in oral drug delivery of insulin as early as the late 1970s^{334,335}. Unfortunately, the results are always indistinct. For example, a study indicated that oral insulin-loaded liposomes were able to exhibit hypoglycemic effect in only 54% of the normal rats and 67% of the diabetic rabbits³³⁶, which could be ascribed to poor stability of conventional liposomes in GI tract^{112,337}. Recent modification technologies facilitate the development of liposomes in oral delivery by addition of polymer coating and modulating liposomal compositions. Liposomes containing bile salts have revealed better stability in GI tract than conventional liposomes^{192,193} by avoiding the destructive effect of physiological bile salts. In addition, bile salts, such as sodium glycocholate (SGC), sodium taurocholate (STC) and sodium deoxycholate (SDC), are capable of inhibiting the activity of GI enzymes including pepsin, trypsin and α -chymotrypsin¹¹². Moreover, liposomes containing bile salts can improve the *trans*-enterocytic internalization compared with conventional liposomes, which could be an important mechanism for enhanced oral absorption of PPs¹⁹². Besides, cholesterol in conventional liposomes replaced by other sterols

(*i.e.*, ergosterol) is also a promising strategy for improving stability and transmembrane ability of liposomes in GI tract⁴⁷. Liposomes containing bile salts, also named as bilosomes, have been widely used in the fields of vaccine delivery³³⁸. The general sense of bilosomes refers to bilayer vesicles fabricated by surfactants with the incorporation of bile salts which is to stabilize the vesicles in GI tract by preventing membrane destabilization. For example, bilosomes constructed from monopalmitoylglycerol, cholesterol, dicetyl phosphate and SDC showed promoted effect in reduction of viral cell load in an influenza challenge study by increasing uptake within the Peyer's patches³³⁹.

Lipid nanoparticles are composed of solid lipids or mixtures of solid and liquid lipids with a diameter size smaller than 1000 nm, including SLNs and nanostructured lipid carriers (NLCs)³⁴⁰. Due to highly hydrophilic nature of PPs, they are poorly encapsulated in the matrix of SLNs and could distributed in the water phase or interface between oil and water owing to the presence of surfactants³⁴¹. In addition, the drug expulsion usually occurs in SLNs after polymorphic transition during storage. NLCs can improve the loading capacity and avoid drug expulsion due to the involvement of liquid lipids compared with SLNs³⁴². Since the mid 1990's, many groups have explored the encapsulation of various PPs in lipid nanoparticles through optimization of formulation and preparation, such as BSA, insulin, sCT, LHRH and protein antigens³⁴¹. Although entrapment efficiency can be improved significantly, the loading capacity for most of PPs is still not more than 10%. Sarmiento et al.³⁴³ engineered an insulin-loaded SLNs with entrapment efficiency of over 43% demonstrated a considerable hypoglycemic effect during 24 h after oral administration to diabetic rats. Various surface modification technologies were employed to further increase the delivery efficiency of SLNs, such as chitosan coating³⁴⁴, lectin¹⁶⁸ and octaarginine modification³⁴⁵. In addition, surface charge of lipid nanoparticles is a crucial factor determining the *in vivo* behaviors after oral administration. Anionic charges can slow down the lipolysis of SLNs, while cationic charge can accelerate this process. The absorption of intact SLNs into blood circulation with the fastest and largest absorption was observed for net neutral

Table 4 Representative examples of nanotechnology-based strategies used for enhancing oral absorption of PPs.

Nanotechnology	Material	Modification	Size (nm)	PPs	Absorption	Ref.	
Polymeric nanoparticles	PLGA	TMC	247.6	Insulin	2-Fold higher relative bioavailability for insulin	290	
		WGA	231.9	Thymopentin	Enhancing the interaction with M cells by 1.8–4.2-fold and increasing the values of CD4 ⁺ /CD8 ⁺ ratios	291	
	PLA-PEG	RGD-PEG	200	Ovalbumin	Concentrating in M cells particularly	292	
		No	302–328	β -LGDP	Increasing mucosal immunity and milk allergy prevention	293	
		Fc fragments	55	Insulin	Producing a prolonged hypoglycemic effect in wild-type mice at a clinically relevant insulin dose of 1.1 U/kg	225	
		Chitosan	PGA	232.9	Insulin	Approximately 20% of relative oral bioavailability	120
	Lipid-based nanoparticles	Dendrimer	PEG	200–250	PTH1-34	Improving oral bioavailability of PTH remarkably	294
			Dextran/TMC	200–300	Lipoprotein	Increasing the humoral immunity and the level of antibody	295
		Liposomes	Eudragit L100/mannosylated	558.2	BSA	Eliciting strong systemic IgG antibody and mucosal IgA responses	106
			PEG	1100	Insulin	Controlling the blood glucose levels	296
SLN		WGA-carbopol	100	sCT	Enhancing oral absorption of sCT by more than 20-fold	297	
		Biotinylated	150	Insulin	The relative oral bioavailability achieved to 12.09%	217	
		Sodium caprate	200–250	hGH	Leading to a relative bioavailability of hGH of 3.4%	298	
		PLFE	500	OVA	Improving the immune response of OVA	299	
		MPC	300	BSA	Higher levels of IgG in the sera and IgA in the mucosa	300	
		UEA-1	400–500	BSM	Higher sIgA level and cytokines level	301	
	Cationic lipids	300	Insulin	Protecting insulin from the enzymatic degradation	302		
	CSK/IRQ	244/410	sCT	Leading to the absolute bioavailability of 12.41% and 10.05%, respectively	303		
Nanoemulsions	Alginate/chitosan	500	Insulin	The oral relative bioavailability was 8.42%	304		
	Oil-structured nanoemulsion	250.8	Exenatide	Improving the bioavailability of exenatide <i>via</i> intestinal lymphatic transport	305		
Inorganic nanoparticles	Silica	Chitosan	226	Exenatide	Enhancing transport across Caco-2 monolayer by 1.7-fold	306	
		CPP-PEG	173.2	hGH	Leading to 4.91-fold increase in pharmacodynamics	307	
		No	200	Insulin	Showing sustaining hypoglycemic effect.	308	
		No	175–321	OVA	Enhancing the phagocytosis by dendritic cells and the production of specific antibodies	309	
	Calcium phosphate	VB ₁₂	<250	Insulin	Enhancing oral bioavailability by 4-fold and showing sustained hypoglycemic effects up to 12 h	219	
		Polysaccharide	14–35	OVA	Enhancing the mucosal IgA and serum IgG responses	310	
Gold	Chondroitin sulfate	123	Insulin	The oral absorption was enhanced with 6.61-fold	311		

Abbreviations: PLGA, poly(D,L-lactide-co-glycolide); TMC, N-trimethyl chitosan chloride; WGA, wheat germ agglutinin; RGD, arginylglycylaspartic acid; PLA-PEG, poly(lactic acid)-poly(ethylene oxide); PGA, poly(γ -glutamic acid); PTH1–34, parathyroid hormone 1–34; β -LGDP, beta-lactoglobulin-derived peptides; BSA, bovine serum albumin; sCT, salmon calcitonin; hGH, human growth hormone; PLFE, polar lipid fraction E; UEA-1, ulex europaeus agglutinin 1; BSM, bovine submaxillary mucin; MPC, mannose-PEG-cholesterol conjugate; CPP, cell-penetrating peptide; OVA, ovalbumin; VB₁₂, vitamin B₁₂.

SLNs³⁴⁶. The absorption amount of intact SLNs is highly significant for therapeutic PPs.

4.3.8.3. Inorganic nanoparticles. Inorganic nanoparticles have attracted increasing attention in drug delivery systems and also been used in improving oral delivery of PPs, such as silica nanoparticles²⁶, gold nanoparticles³¹¹, calcium carbonate or phosphate nanoparticles³⁴⁷.

Silica nanoparticles are the most commonly used drug carrier in inorganic nanoparticles due to good biocompatibility and tunability in size or morphology. Mesoporous silica nanoparticles (MSNs) have been widely used in improving oral bioavailability of poorly water soluble drugs³⁴⁸. PPs can be loaded in MSNs by physical absorption and covalent conjugation. Physical absorption is difficult to achieve higher loading capacity which is generally lower than 15%. Whereas, covalent conjugation can bring about environmental sensitive release for PPs²⁶. MSNs can protect PPs against degradation by harsh GI environments. In addition, the surface of MSNs can also be coated or modified for improving stability or intestinal transport. PEGylated MSNs are able to protect the conformation of insulin under simulated gastric and intestinal condition³⁴⁹ and polymethacrylates coating enables pH responsive release of insulin from MSNs³⁵⁰. The SBA-15, a kind of MSNs, were used to deliver hepatitis B orally, indicating that they were able to protect and release the hepatitis B surface antigen (HBsAg) and offer better antibody response than intravenous delivery³⁵¹. Calcium based nanoparticles have attracted more interests in nanocarrier for PPs, such as calcium phosphate (CaP) and calcium carbonate (CaCO₃)³⁵². Similarly as MSNs, calcium based nanoparticles were generally coated using some polymers to improve the oral absorption and biocompatibility, for instance, hyaluronic acid coated CaCO₃ nanoparticles exerted satisfied hypoglycemic effect for oral insulin delivery²²³ and PEGylated CaP nanoparticles were found to be able to protect the insulin and sustain the release at the physiological pH³⁵³. Further, vitamin B₁₂ was grafted to chitosan as a ligand to coat CaP nanoparticles for improving oral insulin absorption by 4.3-fold compared with naked nanoparticles²¹⁹. Gold nanoparticles capped with chondroitin sulfate or apple polysaccharide are able to increase plasma insulin concentration and anti-diabetic capability^{311,354}. The composites of insulin/zirconium phosphate (ZrP) coated with titanium dioxide (TiO₂) were found to be non-toxic to biological environment and used for oral insulin delivery, which indicates promising *in vitro* drug release for a long time³⁵⁵.

However, biosafety is a big concern for inorganic nanoparticles in drug delivery since they are non-degradable in biological environments³⁵⁶. Most of PPs are needed to be administered for a long time due to short half-life, which could lead to accumulation of inorganic nanoparticles in human body³⁵⁷. Thus, inorganic nanoparticles could be not ideal carriers for oral delivery of PPs.

5. Oral delivery systems of PPs commercially available and under clinical and preclinical studies

5.1. Current oral PPs on the market

Since the approval of first recombinant insulin in 1981, a great number of PPs have been developed in the recent years as new therapeutics. However, few PPs are administered *via* oral route

due to great challenges in oral delivery³⁵⁸. Moreover, current oral PPs on the market include both systemic delivery and local retention in the GI tract. For instance, cyclosporine A is one of the marketed oral peptides and formulated as SNEDDS³⁵⁹. While, numerous oral enzyme products are delivered to local GI tract to treat metabolic disorders. Some oral PPs commercially available are listed in Table 5.

Cyclosporine A is the one of the most successful oral peptide products in market. The combination of the cyclic lipophilic undecapeptide with SNEDDS technologies enables the oral bioavailability achieve 19%–40%³⁶⁰. Meanwhile, SNEDDS formulation (Neoral[®]) overcome the high intra- and inter-patients pharmacokinetics variability in a high percentage of patients by better control of droplet size, increasing intestinal permeability and inhibiting P-glycoprotein efflux and P450 metabolism³⁶⁰. Desmopressin acetate (DDVAP) is also a cyclic peptide and an analog of arginine vasopressin. Its stability is improved by chemical modifications including deamination of the first amino acid and substitution of the eighth amino acid L-arginine by D-arginine³⁶¹. It was developed as an oral tablet by Ferring Pharmaceuticals (Denmark) as early as 1995 and subsequently a variety of generic products were also approved by FDA. However, the oral bioavailability of DDVAP is only around 0.1% because there is no any permeation enhanced technology used in these oral tablet³⁶². Octreotide is another cyclic peptide which has been commercially available oral products on the market. It is a synthetic analog of the endogenous hormone somatostatin and exerts higher stability than somatostatin in SGF with pepsin due to cyclic structure^{49,363}. The oral enteric capsule of octreotide developed by an oily suspension containing the permeation enhancer sodium caprylate was approved in June 2020 by FDA. However, the phase I and II clinical pharmacokinetics showed the oral bioavailability was only 0.5%³⁶⁴. As a peptide drug treating diabetes, semaglutide was successfully developed as oral formulation by Novo Nordisk. Semaglutide is a GLP-1 analog consisting of 31 amino acid residues and far larger than DDVAP and octreotide. The oral semaglutide (Rybelsus[®]) was formulated as a tablet combining with permeation enhancer SNAC developed by Emisphere technologies⁷⁵. The clinical trials demonstrated that 40 mg oral dose was comparable with the 1 mg sc dose³⁶⁵. In addition, taltirelin and reduced L-glutathione can also be administered orally to exert therapeutic efficacy.

Some proteins are not needed to be delivered systemically, but needed to retain in GI tract to treat some local diseases, such as linaclotide for irritable bowel syndrome and oral enzyme products for GI metabolic disorders. Linaclotide acts as a guanylyl cyclase C agonist locally in the small intestine and was developed as an oral hard capsules to treat chronic idiopathic constipation and irritable bowel syndrome with constipation³⁶⁶. The oral linaclotide is almost not absorbed into systemic circulation. Vancomycin is a glycosylated tricyclic heptapeptide antibiotics and poorly absorbed due to high hydrophilicity and large cyclic structure³⁶⁷. Thus, oral vancomycin capsule was approved for treatment of pseudomembranous colitis by FDA. Some enzymes related with GI function prefer to be administered by oral route. There are a variety of oral enzyme products on the market for GI metabolic disorders such as Creon[®], Lacteeze[®], Sucraid[®] and DAOSin[®]³⁶⁸. However, most of them are approved as dietary supplements and only pancreatin was approved as drug by FDA.

5.2. Overview of the current clinical studies

In the past decades, the non-invasive routes have attracted more attention for delivery of PPs. A number of oral PPs developed by various new technologies are being clinically evaluated (Table 6) for both systemic and local delivery. The majority of the technologies for systemic delivery of PPs under clinical trials are for oral insulin¹³³.

There are two typical oral insulin products under phase I clinical trials which are developed by Novo Nordisk (Denmark) and NOD Pharmaceuticals Inc. (China). Novo Nordisk employed gastrointestinal permeation enhancement technology (GIPET) to formulate an oral insulin tablet with Merriam Pharmaceuticals (Ireland)³⁶⁹. The formulation is comprised of micelles with absorption enhancers (sodium caprate). Moreover, NOD encapsulated insulin into enteric coated bioadhesive calcium phosphate nanoparticles which were filled into a capsule (Nodlin™). Nodlin™ has completed phase I clinical trials (ChiCTR-TRC-12001872) on healthy volunteers, which exhibited similar hypoglycemic effect compared with sc insulin³⁷⁰.

An oral insulin enteric capsule containing protease inhibitors and permeation enhancers developed by Oramed Pharmaceuticals Inc. (Israel) has completed phase II clinical evaluation for the treatment of T1DM and T2DM. In phase II trial (NCT00867594), the ORME-0801 capsule is able to reduce blood glucose level on eight T1DM patients significantly³⁷¹. The capsules showed good tolerance and no hypoglycemic phenomenon on all patients even though a high dose of insulin was administered. Inorganic silica nanoparticles were firstly used for oral insulin delivery by the Oshadi Drug Administration Ltd. (Israel), which is under phase II clinical trial. The formulation is developed by inert silica nanoparticles (1–100 nm) loading oily suspended insulin together with the branched polysaccharides³⁷². Biocon limited (India) developed an oral insulin named Tregopil based on chemical modification. Tregopil employed chemical modification to conjugate the β 29-Lys-amino group of human insulin with a single methoxy triethylene glycol propionyl unit through amide linkage³⁷³. Recently, Tregopil has completed phase II clinical trials for two doses (45 mg and 30 mg). Tregopil demonstrated well toleration with the patients, and rapid and prolonged hypoglycemic effect at different dosing interval. Besides, the composition of the observed meal has no influences on pharmacodynamic effect of the insulin³⁷⁴. In addition, there are some other oral peptides under phase II clinical trials, such as PTH for osteoporosis and leuprolide for endometriosis.

It is very interesting that a hepatocyte targeting liposomes of oral insulin developed by Diasome Pharmaceuticals (USA) has completed phase II clinical trials and is preparing for phase III clinical trials. This product is formulated by nanoparticle technology and comprised of liposomes smaller than 150 nm containing insulin conjugated with hepatocyte-targeting moieties (biotin-phosphatidyl-ethanolamine)³⁷⁵. These liposomes are transported by intestinal epithelia into portal vein and then captured by hepatocytes to mimic the physiological insulin delivery. Although recent results of clinical trials have not yet been disclosed, the preliminary results revealed that it showed well toleration with the patients and better control of blood glucose level even after oral administration of a low dose of insulin (5 IU)³⁷⁶. sCT is also one of the most developed peptides for oral delivery except for insulin. Emisphere technologies Inc (NJ, USA)

employed their Eligen® technology to develop oral sCT (SMC021) which is under phase III clinical trials. This formulation used permeation enhancer 8-(*N*-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid (5-CNAC) to improve the oral absorption of sCT, which is similar rationale with oral semaglutide³⁷⁷.

For local delivery, most of products primarily employed new technologies to make them resistant against enzymatic degradation in GI tract. For instance, the Vectrix™ platform developed by Protagonist Therapeutics Inc. designed highly constrained and stable peptides by molecular design tools and libraries of scaffolds. Currently, there have been two products in clinical trials based on Vectrix™ platform, including PN-10-943 for UC and PTG-200 for the treatment of CD³⁷⁸. Following a similar rationale, Avaxia Biologics, Inc developed a milk-derived antibody (AVX-470) to treat UC, which is now in phase I³⁷⁹. In addition, biomimetic drug delivery systems are broadly used in local delivery of PPs. The cellulose wall of plant cell is able to protect the recombinant proteins from degradation along the GI tract. The company Protalix Biotherapeutics have developed several candidates in clinical trials based on the ProCellEx delivery platform, such as a recombinant human tumor necrosis factor receptor II fused to an IgG1 Fc domain (TNFRII-Fc) for UC in phase II and the glycosylated glucocerebrosidase enzyme (prGCD) for the treatment of Gaucher disease also in phase II³⁸⁰. Furthermore, more and more new technologies are emerging for oral delivery of PPs to treat local diseases in GI tract, such as Vorabodies™ or ActoBio Therapeutics™²³.

5.3. Recent status of preclinical strategies

As mentioned above, there have been a variety of strategies to improve oral delivery of PPs, some of which have been widely used in marketed products and clinical trials, such as enteric coating, enzyme inhibitors, permeation enhancers, nanotechnology, colonic targeting, as well as chemical modification. There are hundreds of papers published about new oral delivery systems for PPs every year. Table 7 lists partial projects of oral delivery systems for PPs in preclinical phase.

Currently, nanotechnology have become as the main research hotspot to improve the oral bioavailability of PPs. The nanoparticle system of NanoMega (CA, USA) is capable of decreasing the blood glucose over 8 h in diabetic rats. What's more, an oral suspension of nanoparticles demonstrates a relative bioavailability of 15%, while an enteric capsule containing freeze-dried nanoparticles shows a relative bioavailability of 20%. This nanoparticle is composed of chitosan and PGA¹²⁰. Chitosan could act as a permeation enhancer by transient opening of tight junctions. Meanwhile, this technology has also been used for oral delivery of exendin-4³⁸¹. Nanoparticles are further conjugated with ligands on surface to facilitate intestinal absorption by targeting intestinal receptors or transporters. Transgene Biotek Ltd. (India) conjugated VB₁₂ or Tf on SLNs to establish a TrabiOral™ platform for oral delivery of PPs. A oral insulin project (TBL-1002OI) led by Transgene has revealed prolonged hypoglycemia in rats for 10 h after oral administration³⁸².

Additionally, the intestinal microdevices attract more and more attention in oral delivery of PPs, such as microneedles. A robotic pill was developed by Rani Therapeutics (USA) for oral protein delivery. It is actually a balloon-like structure of sugar microneedles encapsulated by PLGA which is a degradable polymer.

Table 5 Oral products of PPs on markets.

PPs	Trade name	Technology	Indication	Company
Cyclosporin A	Neoral [®] /Sandimmune [®]	SNEDDS	Immunosuppression; systemic delivery	Novartis AG (Switzerland)
Desmopressin acetate	DDAVP [®]	Chemical modification	Central diabetes insipidus; systemic delivery	Ferring Pharmaceuticals (Switzerland)
Octreotide	Mycapssa [®]	Enteric coating; permeation enhancer	Long-term maintenance treatment in acromegaly patients; systemic delivery	Chiasma (USA)
Semaglutide	Rybelsus [®]	Permeation enhancer	Type 2 diabetes mellitus; systemic delivery	Novo Nordisk (Denmark)
Taltirelin hydrate	Ceredist [®] /Ceredist OD [®]	Chemical modification	Spinocerebellar degeneration; systemic delivery	Mitsubishi Tanabe Pharma Co. (Japan)
Linacotide	Linzess [®]	Acts locally	Irritable bowel syndrome, chronic idiopathic constipation; local delivery	Actavis, Inc. (USA)
Vancomycin	Vancocin [®]	Acts locally	Infection	ANI Pharmaceuticals, Inc (USA)
Colistin sulfate	Koolistin [®]	Acts locally	Infection	Biocon Ltd. (India)
Tyrothricin	Lozenges [®]	Acts locally on the throat	Pharyngitis	The Boots Company PLC (UK)
Pancrelipase	Creon [®]	Delayed release; acts locally	Exocrine pancreatic insufficiency	AbbVie Inc. (USA)
Tilactase	Lacteeze [®]	Chewable tablets; acts locally	Lactose intolerance	Lacteeze (USA)
Sacrosidase	Sucreid [®]	Oral solutions; acts locally	Congenital sucrase-isomaltase deficiency	QOL Medical, LLC (USA)
Diamine oxidase	DAOSiN [®]	Acts locally	Histamine intolerance	SCiOTEC (Austria)

Table 6 Oral PP products under clinical trials (clinical [trials.gov](https://clinicaltrials.gov)).

PPs	Phase	Technology	Company
Insulin	I	Enteric coating; bioadhesive calcium phosphate nanoparticles (Nodlin TM)	NOD Pharmaceuticals, Inc. (China)
Insulin	I	Gastrointestinal permeation enhancement technology (GIPET TM)	Merrion Pharmaceuticals Ltd. (Ireland) with Novo Nordisk (Denmark)
Insulin	I	Permeation enhancer (Eligen [®])	Emisphere (USA) with Novo Nordisk (Denmark)
GLP-1 analog	I	Permeation enhancer (sodium caparate)	Merrion Pharmaceuticals Ltd. (Ireland) with Novo Nordisk (Denmark)
Insulin	II	Silica-based nanoparticles	Oshadi (Israel)
Insulin	II	Enteric coating; enzyme inhibitor; permeation enhancer	Oramed (Israel)
Insulin	II	Permeation enhancers (Axess TM , Capsulin TM)	Proxima Concepts Ltd/Diabetology (UK)
PTH	II	Permeation enhancers (Axess TM , CaPTHymone TM)	Proxima Concepts Ltd./Diabetology (UK)
rhPTH(1–31)	II	Permeation enhancers; enzyme inhibitor (Peptelligence TM)	Enteris Biopharma, Inc. (USA)
CsA	II	Oil in water emulsion	Sigmoid Pharma (Ireland)
Dolcanatide	II	Chemical modification	Synergy Pharmaceuticals Inc. (USA)
Acyline	II	Gastrointestinal permeation enhancement technology (GIPET TM)	Merrion Pharmaceuticals Ltd. (Ireland)
Leuprolide	II	Permeation enhancer; pH modulator; enzyme inhibitor	Enteris Biopharm (USA)
Insulin	III	Liver-targeted liposomes	Diasome Pharma (USA)
Insulin	III	Chemical modification (PEGylated insulin)	Biocon (India)
sCT	III	Permeation enhancers (Axess TM , Capsitonin TM)	Proxima Concepts Ltd./Diabetology (UK)
sCT	III	pH modulator (Peptelligence TM)	Tarsa therapeutics, Inc. (USA)
sCT	III	Permeation enhancer (5-CNC)	Emisphere (USA)
Plenacatide	III	Chemical modification; local delivery	Chiasma (Israel)

Table 7 Examples of preclinical projects of oral delivery systems for PPs.

Company	PPs	Technology
Emisphere (USA)	hGH PTH(1–34) PYY	Permeation enhancer (Eligen®)
NanoMega Medical Corp. (USA)	Insulin Exendin-4	Chitosan nanoparticles
Transgene Biotek Ltd. (India)	Insulin	Solid lipid nanoparticles
Aegis Therapeutics, LLC. (USA)	Octreotide D-Leu-OB-3 (leptin) PTH GLP-1 analogs	Permeation enhancer (Intravail®)
Rani Therapeutics, LLC (USA)	Insulin	Microneedles; pH modulator; Robotic pill
Oramed	Exenatide (ORMD-0901)	Enteric coating; permeation enhancer; enzyme inhibitor
I2O therapeutics (USA)	Insulin	Ionic liquids

Insulin loaded the microneedles exhibited encouraging hypoglycemic effect with oral bioavailability of 50% in preclinical trials³⁸³. Likewise, intestinal patches have also been attempted to orally delivery sCT, exenatide, insulin and interferon- α . The patches can be prepared as the size of millimeters or micrometers coated with pH-responsive polymer and contain drug reservoir. This device could also integrate absorption enhancers or enzyme inhibitors to further improve oral absorption of PPs¹⁷⁰.

Permeation enhancers are the main components in products of oral PPs in market or clinical trials. Until now, a number of companies are still developing various permeation enhancers. For instance, Emisphere developed a series of derivatives of caprylic acid as permeation enhancers, such as SNAC and 5-CNAC³⁸⁴. Aegis Therapeutics (CA, USA) developed a category of permeation enhanced excipients named Intravail® which are a group of alkylsaccharides composed of disaccharides and alkyl chain substituents with lengths between 10 and 16 carbons. They have been used in oral delivery of various peptides including octreotide and D-Leu-OB3. The results showed high systemic bioavailability after combination of peptides and Intravail® in rodents compared to sc administration³⁸⁵.

6. Conclusions and future perspectives

Oral delivery of PPs has become more attractive in drug research and development since the increasing market share in the last decade. Therefore, various new technologies are emerging for improving oral bioavailability of PPs by overcoming obstacles of PPs in terms of stability and permeability. Some technologies have been successfully used in oral marketed products of PPs, such as enteric coating, enzyme inhibitors, permeation enhancers, as well as chemical modification. Currently, nanotechnology-based approaches have shown potential for developing oral PPs formulation. Moreover, intestinal microdevices were also developed for delivering PPs by oral route, such as intestinal microneedles. However, the safety, efficacy and reproducibility in preparation technique of these new technologies are needed to be improved and evaluated thoroughly. Furthermore, the oral bioavailability of PPs for systemic delivery is still very low, even lower than 1% for some products despite using new technology to improve the stability and permeability.

Nanoparticles are able to facilitate the intestinal transport of PPs, which has been reported by numerous papers. However, it is more

important to shed light on well-understanding the detailed mechanism of interaction between these nanocarriers and PPs or the intestinal milieu. Moreover, elucidation of PPs transport in the intestinal epithelium and the influence of physiological factors on absorption of PPs are also indispensable. Therefore, elucidating the *in vivo* fate of nanoparticles and PPs after oral administration is the necessary prerequisite to development of highly efficient oral systems of PPs.

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Author contributions

Quangang Zhu, Zhongjian Chen and Pijush Kumar Paul collected references and drafted the manuscript. Yi Lu collected references. Jianping Qi and Wei Wu proposed the concept and revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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