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Nanoparticles for oral delivery: Targeted nanoparticles with peptidic ligands for oral protein delivery

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Abstract

As the field of biotechnology has advanced, oral protein delivery has also made significant progress. Oral delivery is the most common method of drug administration with high levels of patient acceptance. Despite the preference of oral delivery, administration of therapeutic proteins has been extremely difficult. Increasing the bioavailability of oral protein drugs to the therapeutically acceptable level is still a challenging goal. Poor membrane permeability, high molecular weight, and enzymatic degradation of protein drugs have remained unsolved issues. Among diverse strategies, nanotechnology has provided a glimpse of hope in oral delivery of protein drugs. Nanoparticles have advantages, such as small size, high surface area, and modification using functional groups for high capacity or selectivity. Nanoparticles with peptidic ligands are especially worthy of notice because they can be used for specific targeting in the gastrointestinal (GI) tract. This article reviews the transport mechanism of the GI tract, barriers to protein absorption, current status and limitations of nanotechnology for oral protein delivery system.

Keywords

Oral protein delivery; Nanoparticles; Peptidic ligands; GI tract; Protein absorption

1. Introduction

Oral administration is most preferred because of the various advantages over other routes of drug delivery. The advantages include patient convenience and compliance, which increase the therapeutic efficacy of the drug. Oral formulations are also cheaper to produce because they do not need to be manufactured under sterile conditions [1]. Oral delivery of protein has become a pressing goal in recent years due to the increased availability of novel therapeutics through the advent of recombinant DNA technology. One of the holy grails of

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oral drug delivery is to deliver proteins, such as insulin, with the efficacy similar to the parenteral formulations [2].

The increasing importance of proteins can be attributed to three main developments. First, improved analytical methods have promoted discovery of numerous hormones and peptides that have found applications as biopharmaceuticals. Second, molecular biology and genetic engineering have enabled large-scale production of polypeptides previously available only in small quantities. Lastly, there is a better understanding of the role of regulatory proteins in the pathophysiology of human diseases [3, 4]. Consequently, pharmaceutical companies around the world have developed protein oral delivery technologies for producing therapeutically active ingredients in commercial scales, as listed in Table 1 [5]. Proteins have become the drugs of choice for treatment of numerous diseases as a result of their exquisite selectivity and their ability to provide effective and potent action [6]. Protein drug development, however, continues to be a formulation challenge to pharmaceutical scientists. Many protein drugs are currently used as parenteral formulations because of their poor oral bioavailability. This is due to several unfavorable physicochemical properties, such as large molecular size [7], susceptibility to enzymatic degradation, poor stability in the gastric low pH environment [8], poor penetration of the intestinal membrane, short plasma half-life, immunogenicity, and the tendency to undergo aggregation, adsorption, and denaturation [9, 10]. Enzymatic degradation and poor penetration of the intestinal membrane induce low oral bioavailability of biological molecules. The challenge here is to improve the oral bioavailability from less than 1% to at least 30–50% [11, 12]. These problems also remain unsolved. Unfavorable physicochemical properties of proteins present monumental challenges to pharmaceutical formulation scientists.

Designing and formulating a protein drug for delivery through the gastrointestinal (GI) tract requires innovative and practical strategies. Various strategies currently under investigation include chemical modification, formulation vehicles, protease inhibitors, absorption enhancers and muco-adhesive polymers. Among them, nanoparticles as a carrier or a device have become the focus of attention in this field recently. The nanoparticles possess certain advantages such as greater stability during storage, stability *in vivo* after administration and ease of scale-up without an aseptic process for oral administration [13]. The major goals in using nanoparticles as a drug delivery system are to control particle size, surface properties and release of active pharmaceutical ingredients for achieving the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Especially, nanoparticles with peptidic ligands as formulation hold out considerable promise for the future because all benefits collectively can make a significant synergistic effect. The following sections briefly review the transport mechanisms, barriers to absorption for oral protein delivery, targeted nanoparticles for protein oral delivery by using peptidic ligands.

2. Transport mechanisms in the GI tract

There are four distinct mechanisms for molecules to cross the cell membrane: via paracellular, transcellular, carrier-mediated, and receptor-mediated transport (Fig. 1). Absorption through each pathway is dependent on different physical characteristics, such as molecular weight, hydrophobicity, ionization constants, and pH stability of absorbing molecules as well as biological barriers that restrict protein absorption from the GI tract. Thus, an understanding of biomolecules and these distinct mechanisms are important in designing delivery systems for oral protein drugs.

2.1. Paracellular transport

Paracellular transport is the pathway of substances across an epithelium by passing through the intercellular spaces in between epithelial cells. Paracellular transport is passive and

results from diffusion. This transport is under the control of tight junctions. A tight junction constitutes the major rate limiting barrier towards the paracellular transport for permeation of ions and larger substances [14]. The dimension of the paracellular space is on the order of 10 Å. The average size of aqueous pores created by epithelial tight junctions is approximately 7–9 Å for the jejunum, 3–4 Å for the ileum, and 8–9 Å for the colon in the human intestine [15]. This data suggests that solutes with a molecular radius exceeding 15 Å (approximately 3.5 kDa) cannot be transported via this route [16]. Furthermore, tight junctions comprise only about 0.01% of the total absorption surface area of the intestine [17]. Consequently, one would conclude that protein delivery across mucosal epithelia using paracellular transport is severely restricted. However, paracellular transport varies enormously among epithelia in terms of electrical resistance and shows small differences in ionic selectivity. The paracellular transport complements the transcellular mechanism by defining the degree and selectivity of reverse leak for ions and solutes, making an important tissue-specific contribution to overall transport [18, 19]. The tight junction shares biophysical properties with conventional ion channels, including size and charge selectivity, dependency of permeability on the ion concentration, competition between permeant molecules, anomalous mole-fraction effects, and sensitivity to pH [20]. The paracellular pathway is not largely determined by the hydrogen bonding capacity and lipophilicity.

2.2. Transcellular transport

Transcellular transport occurs through the intestinal epithelial cells by transcytosis, a particular process by which particles are taken up by cells. A typical example is the movement of glucose from the intestinal lumen to extracellular fluid by epithelial cells. This starts with an endocytic process that takes place at the cell apical membrane. Then, particles are transported through the cells and released at the basolateral pole [21]. The basolateral membrane is thinner and more permeable than the apical membrane because the protein-to-lipid ratio is very low in the basolateral membrane. Transport of particles by the transcellular transport depends on several factors: (i) various physicochemical properties of particles, such as size, lipophilicity, hydrogen bond potential, charge, surface hydrophobicity or the presence of a ligand at the particle surface; (ii) the physiology of the GI tract; and (iii) the animal model used to study the uptake [22, 23].

Enterocytes and M cells are the primary intestinal cells for transport. Enterocytes represent the majority of cells lining the gastrointestinal tract and M cells are mainly located within the epithelium of Peyer's patches and represent a very small proportion of the intestinal epithelium (5% of the human follicle-associated epithelium (FAE), i.e., about 1% of the total intestinal surface) [24]. M cells in the follicle-associated epithelium (FAE) of Peyer's patches are specialized for an antigen. M cells deliver proteins and peptides from the lumen to the underlying lymphoid tissues for the induction of immune responses. However, M cells are also exploited by a range of pathogens as a route for host invasion [25]. Furthermore, M cells represent a potential portal for oral delivery of proteins and peptides due to their high endocytosis ability. M cells possess a high transcytotic capacity and transport a wide variety of materials, including nanoparticles [26, 27]. M cells take up macromolecules, particles and microorganisms by adsorptive endocytosis via clathrin-coated pits and vesicles, fluid phase endocytosis and phagocytosis [28]. Although there has been some controversy in the literature on the extent of particle absorption, there is evidence that particle translocation can occur across enterocytes in the villipart of the intestine [29, 30]. However, the number of particles translocated through these routes is mostly very low because of the low endocytic activity of the enterocytes. It has been generally observed that the bulk of particle translocation mainly occurs in FAE [29, 31, 32]. As a result, many researchers have studied with great interest the Peyer's patches and M cells which have adapted to absorb a large range of materials. Nevertheless, this route is limited to the transport of relatively low-

molecular-weight lipophilic drugs. Furthermore, studies in humans have demonstrated that absorption by the transcellular route decreases significantly in the colon, whereas no such gradient exists for the paracellular route [33].

2.3. Carrier-mediated transport

Drugs are transferred across the cell membrane or entire cell and then released from the basal surface of the enterocyte into circulation [34]. The process is suitable and utilized by small hydrophilic molecules [35]. Active absorption requires energy-dependent uptake of specific molecules by carriers. The carriers recognize target molecules through membrane receptors and transport them across the membranes into the GI epithelium, even against the concentration gradient and in trace quantities. For example, small di/tripeptides (including β-lactam antibiotics and angiotensin-converting enzyme (ACE) inhibitors), monosaccharides, and amino acids are transported transcellularly by a carrier-mediated transportprocess [36]. Shah and Shen investigated the carrier-mediated transport of insulin across Caco-2 cell monolayers. They observed that transport of the conjugated insulin was mediated via the transferrin receptor and not through the insulin receptor. The authors found that insulin-transferrin (In-Tf) transport across the Caco-2 cell monolayers increased by 5- to 15-fold compared to free insulin [37].

2.4. Receptor-mediated transport

In receptor-mediated transport, protein drugs act either as a receptor specific ligand for surface-attached receptors or as a receptor for surface-attached ligands [38]. Receptor-mediated transport has also been exploited to increase the oral bioavailability of protein drugs by modification such as receptor specific ligands with peptide and protein drugs. This transportation entails cell invagination, which leads to formation of a vesicle. This transportation, in general, is known as endocytosis and comprises phagocytosis, pinocytosis, receptor-mediated endocytosis (clathrin-mediated), and potocytosis (nonclathrin-mediated) [39]. The first step in this process includes binding of the ligand to a specific cell-surface receptor, receptor clustering and internalization through coated vesicles into endosomal acidic compartments. The subsequent pathway is strongly dependent on the type of the receptor/ligand pair; the low endosomal pH may or may not trigger dissociation of the receptor and ligand, and sorting processes may lead to degradative lysosomal compartments. After protein drugs are transported to the GI tract, they take access to the systemic circulation via two separate and functionally distinct absorption pathways: portal blood and the intestinal lymphatics. The physicochemical and metabolic features of the protein drug and the characteristics of the formulation largely control the relative proportion of protein drug absorbed via these two pathways. Portal blood represents the major pathway for the majority of orally administered protein drugs. During this process, hydrophilic ligands are carried to the liver via the hepatic portal vein, and then by the hepatic artery gain access to the systemic circulation, for subsequent delivery to their sites of action. On the other hand, highly lipophilic ligands (log P >5) that cross the same epithelial barrier are transported to the intestinal lymphatics, which directly deliver them to the vena cava, thereby bypassing the hepatic first-pass metabolism [40].

3. Barriers to Protein Absorption

3.1. Gastrointestinal barriers

An understanding of the GI tract and of drug target sites offers an opportunity for targeted oral delivery of proteins [41]. The GI tract has various proteolytic enzymes such as trypsin, chymotrypsin, and elastase which are endopeptidases. Carboxypeptidase A and aminopeptidase are exopeptidases which are also involved as proteolytic enzymes [42]. Table 2 shows various proteases along with their sites of action [41, 43]. Endopeptidases

hydrolyze the bond internal to the terminal bonds of the peptide chain, while exopeptidases hydrolyze the bond linking the NH_2–terminal or the COOH-terminal amino acid to the peptide chain. Enzymatic degradation can occur at the lumen, brush border, the cytosol of the enterocytes, and even in the lysosomes and other cell organelles [44].

The stomach produces gastric juice having hydrochloric acid (HCl), potassium chloride (KCl) and sodium chloride (NaCl). The acidic environment with a pH range of 1.5 to 3.5 induces proteolysis of proteins and peptides into constituent aminoacids, dipeptides, and tripeptides for absorption. Pepsin is the first in a series of enzymes that digest protein. In the stomach, protein chains bind in the deep active site groove of pepsin and are broken into smaller pieces. Pepsin acts within the stomach so its optimum pH is around 2, an acidic pH. When the enzyme passes into the duodenum it meets a higher pH and its enzyme activity ends. Rapid pH changes also affect the degradation of ingested proteins and peptides. The pH is increased from 2 to about 6 when proteins move from the stomach to the duodenum. This wide pH range covers the isoelectric points of many peptides and proteins to precipitate them. These precipitated proteins do not rapidly redissolve upon pH change [45–47].

The small intestine is generally the major place for absorption of food. But the enzymatic activity of proteases is also higher than in any other segment of the GI tract. The main parts of the small intestine are the duodenum, jejunum, and ileum. The brush border is the name for the microvilli-covered surface of the epithelial cells found in the small intestine. The brush border membrane contains sucrase and more than a dozen of peptidases. They together have a broad specificity and can degrade both proteins and peptides [42]. Brush border enzyme activity is generally greater in the duodenum and the jejunum than in the ileum. The duodenum has pancreatic proteases consisting of endopeptidases and exopeptidases. They can create severe conditions for ingested peptides and proteins. In the duodenum, pancreatic secretions increase the pH of the enteric juice for the action of other digestive enzymes, for example, trypsin. Apart from the areas these peptidases reach, there are areas in the jejunum and ileum where the aminopeptidases activity is about 20–30% of the aminopeptidases activity in other neighboring areas. Such areas are known as Peyer’s patches and are a potential targeting site for the delivery of proteins and peptides [36, 48].

3.2. Mucosal barrier

Mucus plays an important role in determining the absorption and bioavailability of orally administered drugs. The mucosal barrier consists of three protective components. These provide additional resistance for the mucosal surface of the stomach. The first is a compact epithelial cell lining which is bound by tight junctions that repel harsh fluids that may injure the mucosal lining. The second is a special mucus blanket. The mucus blanket is derived from mucus secreted by surface epithelial cells and mucosal neck cells. This insoluble mucus forms a protective gel-like coating over the entire surface of the gastric mucosa. The third consists of bicarbonate ions. The bicarbonate ions are secreted by the surface epithelial cells [49, 50]. Glycocalyx is one of the main mucosal barrier components against delivery proteins via the oral route. The glycocalyx, which is atop the epithelial cells, is a fuzzy and fibrous coat that is weakly acidic and consists of sulfated mucopolysaccharides. Goblet cells secrete mucus, which lines the top of the glycocalyx [51]. The mucus consists of mucin glycoproteins, enzymes, electrolytes and water [52]. The cohesive and adhesive nature of the mucus layer is due to the presence of mucin glycoprotein [53, 54]. These kinds of complex surfaces are protected by highly viscoelastic layers. In contrast to proteases, the mucin lining presents a physical barrier rather than a chemical one. It is reported that the mucin layer is thickest in the stomach and colon, whereas in the small intestine the thickness varies depending on the extent of digestive activity [55].

The mucus and glycocalyx layers are the first and foremost barriers to peptides and proteins, which must first diffuse through these layers to reach the cellular membrane. Due to the viscosity and the interactive nature of these layers, they offer a certain level of resistance to the protein drug diffusion. Electrostatic adhesive interactions with mucin fibers and particle aggregation affect the nanoparticle transport rate. Negatively charged carboxylate- and sulfate-modified particles showed a higher transport rate than near neutral or positively charged amine modified particles. The amine nanoparticle transport is severely limited, likely by particle aggregation and electrostatic adhesive interaction with mucin fibers [56]. The interaction of particle and mucus made by electrostatic/ionic interactions, van der Waals interactions, hydrophobic forces, and hydrogen bonding influence the nanoparticle retention at the mucosal surface [57]. After diffusing through the mucus and glycocalyx, the protein drug reaches the epithelial surface [58]. The protein drugs must adhere to the mucus and must cross the mucus layer. However, drugs delivered to mucosal surfaces are usually efficiently removed by mucus clearance mechanisms [59]. Mucus continuously traps and removes pathogens and foreign particles in order to protect the epithelial surface. For this reason, low tissue permeability is currently one of the biggest hurdles to orally administrated drugs. Nanoparticles as drug carriers are a good alternative to diffuse into the mucus layer and avoid elimination by mucilliary clearance. However, there is a size limit to cross the intestinal mucosal barrier because the mesh-pore spacing of the mucus is 50–1800 nm [60]. Many researchers have reported that the transport of nanoparticles at various mucosal sites is highly dependent on its size. The pore network accommodates the movement of a number of particles as long as hydrophobic and electrostatic mucoadhesive forces can be minimized. Many studies have shown that nanoparticles under 200 nm size effectively diffuse through the mucus [61]. Aoki et al. investigated the contribution of the mucus/glycocalyx layers in rat small intestine as a diffusional or enzymatic barrier to the absorption of insulin by *in vitro* studies [62]. Their studies also suggest the possibility of mucus/glycocalyx layers acting as an enzymatic but not a diffusional barrier, irrespective of the intestinal region. Morishita et al. reported using an in situ absorption study with different intestinal segment loops to increase the insulin absorption from the ileum, the distal part of the small intestine [63]. Lai et al. focused their research on mucoadhesive nanoparticles. Strong interactions with mucus could increase retention at the mucosal surface. These interactions are driven by hydrogen bonding, van der Waals interactions, polymer chain interpenetration, hydrophobic forces, and electrostatic/ionic interactions [64].

4. Strategies for oral protein delivery

4.1. Nanotechnology and protein delivery

Advances in biotechnology have resulted in discovery of a large number of therapeutic and antigenic proteins. Currently, more than 100 peptide and protein drug products are under clinical investigation and about 30 compounds have received FDA approval [65]. Each year new therapeutic proteins are introduced into the market. Many researchers have studied to find more suitable oral protein delivery systems. Important efforts have already been focused on the design of carriers for transport of proteins across mucosal and intestinal barriers. Nanotechnology has shown a potential for delivery of proteins [66–68]. Table 3 lists potential applications of nanotechnology for oral delivery and targeting of therapeutic and diagnostic agents [69].

The US National Nanotechnology Initiative (NNI, http://www.nano.gov), launched in October 2000, provides a federal vision for nanotechnology-based investments through the coordination of 16 US departments and independent agencies. The potential research and development targets by 2015 for the NNI are shown in Table 4. They include no suffering and death from treated cancers, advanced materials and manufacturing, pharmaceutical synthesis and delivery, converging nanoscale technologies, and life-cycle biocompatible/

sustainable development. The targets are really ambitious and it may take much beyond 2015 to achieve them.

The major goals in designing nanoparticles as a delivery system are to control particle size, surface properties [70], and release kinetics of pharmacologically active ingredients in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen [71]. The advantages of using nanoparticles as a drug delivery system are listed in Table 5 [72]. The efficiency of drug delivery is directly related to particle size because particle size can enhance bioavailability and enable more precise targeting to the level of direct intracellular delivery [73]. Some investigators have observed that the number of nanoparticles which cross the intestinal epithelium is greater than the number of microspheres, and that not only the M cells but also the normal enterocytes are involved in the transport [74–76]. Nanoparticles allow penetration of cell membranes, binding, and encapsulation of the protein drugs inside a matrix and protect them against enzymatic and hydrolytic degradation [72]. There are many different techniques for making the nanoparticle with various biomaterials of polymers, lipids, and lectins. Emulsion polymerization, interfacial polymerization, emulsification evaporation, solvent displacement, salting out, emulsification diffusion, and desolvation are popular methods for making nanoparticles [77, 78]. The solvent displacement and salting out are more useful because they provide less stress to protein drugs. Chemical structures and surface characteristics have a significant influence on the physicochemical properties of nanoparticles and their behavior when they are exposed to physiological media [76].

4.2. Targeted nanoparticles with peptidic ligands

As stated earlier, there are a number of limitations to the oral delivery of proteins despite the progress of the knowledge in this field. The barriers to protein bioavailability after oral administration are intestinal membrane permeability, molecule size, intestinal and hepatic metabolism, and lastly solubility. Therefore, its administration has been restricted to the invasive route [79, 80]. The dosage form must initially stabilize the drug for oral delivery, making it easy to take orally [81]. It must then protect the drug from the extreme acidity and action of pepsin in the stomach. In the intestine, the drug must be protected from the many enzymes that are present in the intestinal lumen. In addition, the formulation must facilitate both aqueous solubility at neutral pH and lipid layer penetration for protein molecules to cross the intestinal membrane and then the basal membrane for entry into the blood stream. To ensure enteric protection and to improve bioavailability of proteins, diverse formulations have been developed, taking into account these restrictive parameters.

Modifying nanoparticles by coupling a targeting molecule at their surface could represent a more efficient way to enhance oral uptake of nanoparticles. Optimum contact between a carrier and a target biological surface is necessary to increase drug absorption. The nanoparticles should be able to make a strong interaction with the epithelial surface [82]. Extensive efforts have been devoted to achieving the so-called ‘active targeting’ of nanoparticles in order to deliver drugs to the right targets, based on molecular recognition processes such as ligand-receptor or antigen-antibody interactions. Targeting with small ligands appears more likely to succeed since they are easier to handle and manufacture. Furthermore, it could be advantageous when the active targeting ligands are used in combination with the long-circulating nanoparticles to maximize the likelihood of success in the active targeting of nanoparticles. In addition, ligands conjugated to the surface of engineered nanoparticles can influence the mode of cellular internalization. Ligands such as folic acid, albumin, and cholesterol have been shown to facilitate uptake through caveolin-mediated endocytosis, whereas ligands for glycol receptors promote clathrin-mediated endocytosis [83]. Ligands play important roles in dictating nanoparticle size, shape, and interparticle spacing, and also in determining the properties of the interface between the

ligands and the nanoparticle surface as well as the interface between the nanoparticle and its environment. Indeed, particles have been decorated by adsorption or covalent attachment of peptidic ligands interacting with surface receptors to target the epithelium, with an expectation that such interactions will lead to a greater uptake and delivery of nanoparticles [84–86]. Ligands specific for certain cell types can be incorporated into drug delivery platforms to localize delivery to specific cells and tissues, thereby reducing required dosage and minimizing side effects. Huang et al. demonstrated the effects of goblet cell-targeting nanoparticles on the oral absorption of insulin *in vitro*, *ex vivo* and *in vivo*, and identified the targeting mechanism as well as the influence of mucus. The nanoparticles were modified with a CSKSSDYQC (CSK) targeting peptide. The CSK peptide modified NPs facilitated the uptake in the villi. In transport studies across a Caco-2/HT29-MTX co-culture cell monolayer, the CSK peptide modification also showed enhanced transport ability, even if the targeting recognition was partially affected by mucus. CSK modified NPs produced a better effect with a 1.5 fold higher relative bioavailability compared to unmodified ones [86]. Angelo et al. have fused TNF with the ACDCRGDCFCG peptide, a ligand of α_V integrins by recombinant DNA technology. Subnanogram doses of this conjugate with melphalan were sufficient to induce antitumor effects in tumor-bearing mice, the ACGDRGDCFCG-mouse, TNF conjugate bound TNF receptors and trigger death signals. The Figure 2 shows that RGD-mTNF, although less active than NGR-mTNF on a molar basis, is capable of inducing antitumor effects in the pictogram range. This result indicates that a peptidic ligand improves antitumor activity of the drug while reducing side effects [87].

Table 6 shows various protein/peptidic ligands, functional activities, and characteristics. Figure 3 shows targeted nanoparticles with peptidic ligands.

A peptide as a ligand has many advantages. Peptides can be synthesized by chemical methods on a large scale. These advantages and anticipated improvements in conjugation techniques can bring about a great improvement of their application in diagnosis and therapy. To screen peptide libraries produced by either chemical synthesis [109] or phage display [110–113] is a main method to select useful peptide ligands. The peptide library is widely applicable to both *in vitro* and *in vivo* studies. Moreover, the peptide library can be used to identify peptides even though receptors are unknown. Various peptide ligands have been found out for various types of receptors or cells, such as integrin receptors [114, 115], cardiomyocytes [116], thrombin receptors[117], tumor cells [116, 118–120], intestinal tissue, M cells, and pancreatic β cells [121]. *In vitro* and *in vivo* target specific ligand modified nanoparticle applications have been conducted by a large number of research groups to identify peptidic ligand administration routes including oral drug administration for targeting various organs and tissues. The sequence of peptidic ligands has been typically screened by analysis of comparative superiority in transcytosis efficacy across target cell layer *in vitro* and *in vivo* among a huge number of candidates [112]. Receptor-mediated endocytosis is a process that transports peptidic ligands into a target cell. The specificity results from a receptor-ligand interaction. The receptors on the plasma membrane of the target tissue specifically bind to peptidic lignads on the outside of the cell. The peptidic ligands and their receptors accumulate in coated pits and are internalized as receptor-ligand complexes. After internalized, endocytosed peptidic ligands are delivered into endosome. And then, endocytosed peptidic ligands are transported into lysosomes where hydrolytic digestion starts. The subsequent pathways are strongly dependent on the type of receptor/ligand pair [89]. Cho et al. reported to develop an efficient oral vaccine carrier which specifically targets the follicle-associated epithelium region of Peyer’s patch. M cell-homing peptide ligand was selected by the phase display technique. The CKSTHPLSC (CKS9) peptide sequence was immobilized chitosan nanoparticles (CKS9-CNs). The target specificity was evaluated by an *in vitro* transcytosis assay and *in vivo* assays. *In vivo*

localization of CKS9 was compared with CSK9 in rat small intestinal tissues. Their tissue specific localization was monitored under fluorescence-microscopy (Fig. 4). The CKS9-CNs were spread more effectively across the M cell model and accumulated more specifically into Peyer's patch regions in comparison with CNs [112].

Schneider et al. studied improving the transport of vaccine-loaded nanoparticles. The phage display screening was used to identify peptides targeting human M cells. Phage libraries have been used to select a peptide utilizing an *in vitro* model of the human follicle-associated epithelium (FAE) containing both Caco-2 and M cells. Clones were sequenced after five rounds of selection in the human FAE *in vitro* model. Three identical clones (CTGKSC, PAVLG and LRVG) appeared at high frequency after selection on both mono- and co-cultures (Table 7). The presence of LRVG and CTGKSC peptides modified nanoparticles significantly increased their transport across the cell layer by 8 and 4 times, respectively, when compared to non-modified nanoparticles. The transport of PAVLG-modified nanoparticles, on the other hand, was the same as that of non-modified nanoparticles. Two peptides could be used significantly to enhance the transport of vaccine-loaded nanoparticles across the intestinal mucosal barrier [113].

Different types of targeting molecules have been tested but the most studied has been the lectin family. Lectins are proteins that bind to highly-specific carbohydrate moieties of the glycocalyx of the intestinal enterocytes and the mucus layer [122]. They are involved in many cell recognition and adhesion processes. Their conjugation to polymeric nanoparticles significantly increases their transport across the intestinal mucosa by efficiently increasing interactions with the mucus [27, 123, 124] and/or the surface of the epithelial cells [125] and by promoting particle translocation [123, 126]. The association of lectins with nanoparticles can be achieved by adsorption or covalent coupling, with a definite preference for a covalent linkage, if conjugation does not affect lectin activity and specificity. As an alternative to injection, oral administration of lectin conjugated nanoparticles loaded with insulin, enhanced the intestinal absorption of insulin enough to drop the glucose level in blood [127]. Even if insulin is a hydrophilic peptide, it can be incorporated with high efficiency (about 98%) nanoparticles showing good physical stability and sustained drug release behavior [128]. YaShu et al. demonstrated that the highest amount of lectin conjugated nanoparticles was detected in the small intestine, suggesting an increase of intestinal bioadhesion and endocytosis. This result represented an increase of almost 1.4–3.1 fold across the intestine compared to <4.9% for the uptake of unconjugated nanoparticles.

Peptidic ligands like the well-known arginine-glycine-aspartic acid (RGD) and cell penetrating peptides have also been covalently linked on polymers before the formation of nanoparticles [129]. The RGD or CPP target β1 integrins localized at the apical pole of M cells [130]. Covalent binding on PEG chains favors RGD presentation and then targeting [131]. Ligands can also be non-covalently attached to PEG chains. The presence of RGD peptides on the surface has recently been shown to induce a 50-fold increase in transport across the human intestine epithelial cells compared to blank PS particles [132]. The RGD motif has previously been demonstrated to promote cell attachment to hydrophobic substrates [133, 134]. Recently, Gref et al. have grafted biotin molecules on PEG chains and exploited the strongest biological, non-covalent interactions. The non-covalent coupling method is an attractive method for modifying the surface of a nanoparticle such as a PCL-PEG-avidin-biotin ligand. A biotin–lectin ligand was incubated with nanoparticles in the presence of avidin. This process led to the formation of a nanoparticle–biotin–avidin/biotin–lectin complex. The main advantage of this technique lies in the variety of biotinylated ligands that could be grafted at the nanoparticle surface. Thus, the surface properties of the nanoparticles can be modified either by improving non-specific interactions with the cell apical surface or by grafting specific ligand targeting epithelial intestinal cells [102].

Conclusions

Poor intestinal absorption of the protein drugs is due to their unfavorable physicochemical properties, such as high molecular weight and susceptibility to enzymatic hydrolysis. In addition, there are several biological barriers to intestinal absorption of protein drugs in the GI tract. The low bioavailability of protein drugs remain to be an important issue requiring active research. The nanoparticles with peptidic ligands have many advantages to solve the limitations mentioned above. They can be used to target the epithelium, with an expectation that such interactions will lead to a greater uptake and delivery of the drug. Much research, however, is yet to be done to determine the exact mechanism of the nanoparticulate uptake and subsequent clearance, associated potential for *in vivo* nano-toxicology, tissue specific targeting, and modulation of GI transit. To translate the potential into real products, practical formulations need to be developed. The nanoparticles with peptidic ligands would be a promising candidate for oral protein delivery. The potential of nanoparticles in drug delivery has remained. Challenges to developing protein formulations for oral delivery are still significant, and the quest to overcome the problem is ongoing.

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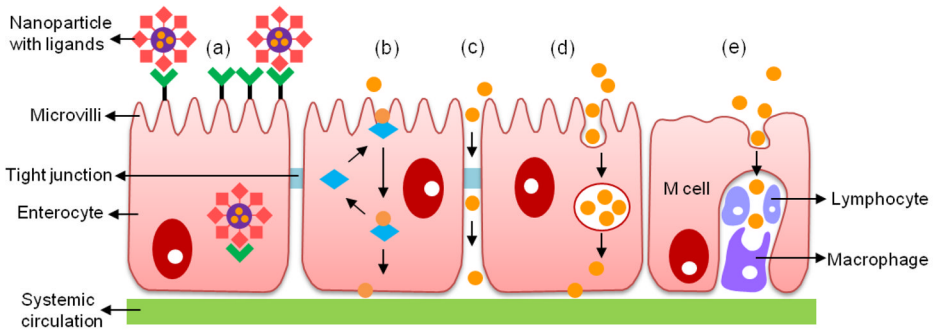

Figure 1.
Schematic representation of the transport mechanisms: (a) receptor-mediated transport; (b) carrier-mediated transport; (c) paracellular transport; (d) transcellular transport; and (e) M cell mediated transport (i.e., phagocytosis by M cells).

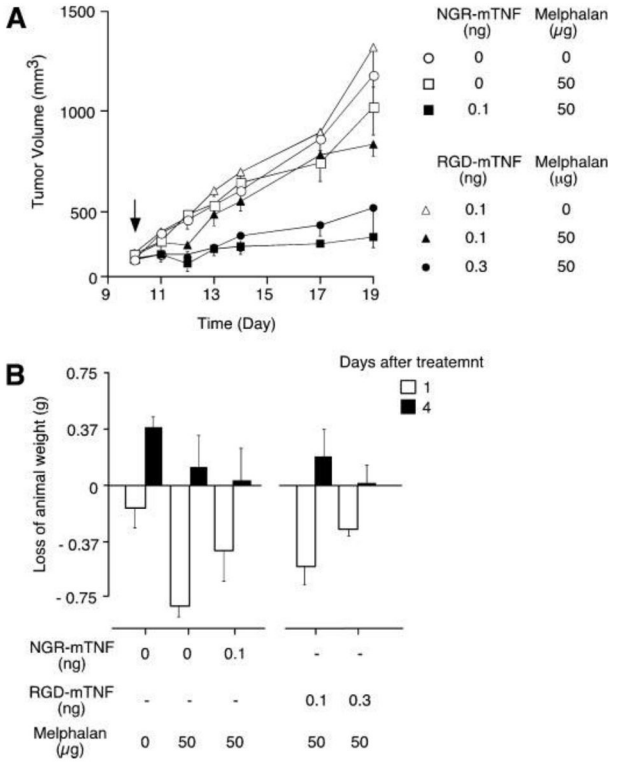

Figure 2.

The effect of ACGDRGDCFCG-murine tumor necrosis factor-conjugate (RGD-mTNF) on tumor growth and body weight of animal-bearing RMA tumors. 5 mice/group were treated intraperitoneal at day 10 with melphalan alone or in combination with RGD-mTNF or CNGRCG-mTNF conjugate (NGR-mTNF) at the indicated doses (A). Loss of animal weight at day 1 and day 4 after treatment (B) [87].

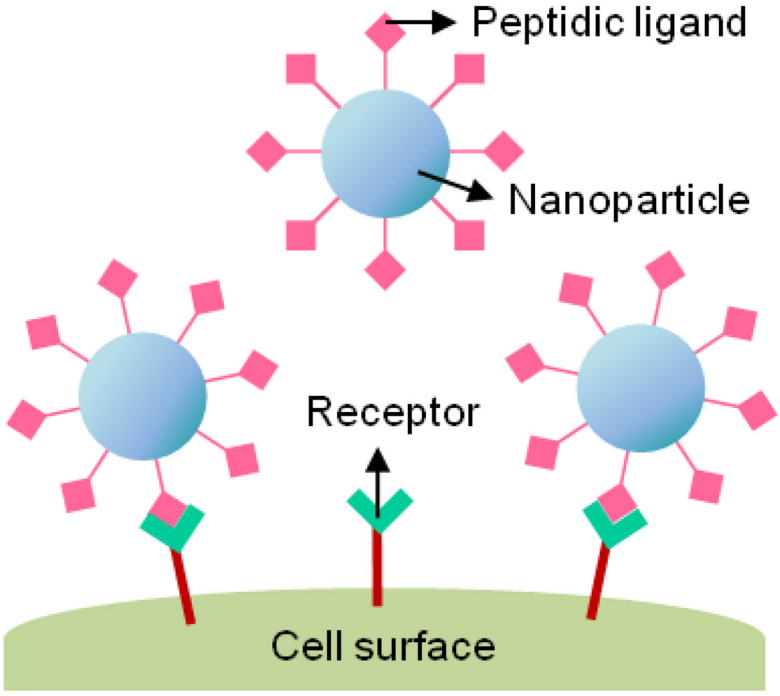

Figure 3.
Schematic illustration of targeted nanoparticles with peptidic ligands.

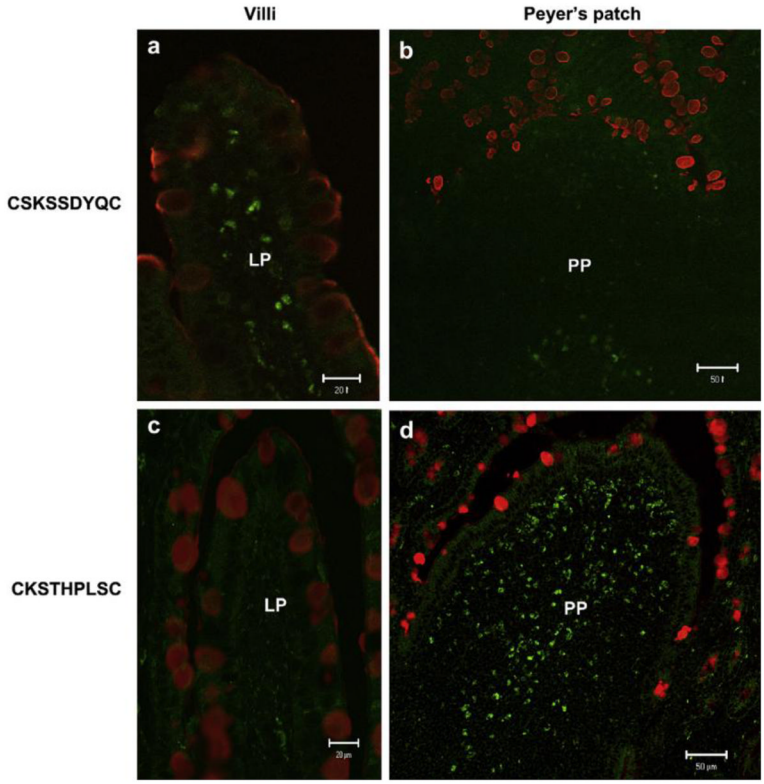

Figure 4.

In vivo localization of CKS9 compared with CSK9 in rat small intestinal tissues. Chemically synthesized CKS9 or CSK9 was injected into closed ileal loops and their tissue specific localization was monitored under fluorescence-microscopy; (a and b) *in vivo* localization of CSK9 peptides in Peyer's patches and Non- Peyer's patches. (c and d) *in vivo* localization of CKS9 peptides in Peyer's patches and Non- Peyer's patches. Green and red fluorescent signals in each panel indicate the location of the peptides and mucus layer in rat small intestinal tissues (closed ileal loops), respectively. Scale bars indicate 50 mm in Peyer's patches and 20 mm in Non- Peyer's patches, respectively [112].

Table 1

Protein oral delivery technologies under development by companies [5].

Company	Product	Systems	Characteristics and advantages	Products currently available or under development
Emisphere	Eligen®	Carrier molecules	Facilitates the absorption of small molecules without altering chemical form, biological integrity or pharmacological properties Passive transcellular transport enables drug molecules of all sizes to cross the cell membrane	Calcitonin, GPL-1, PYY, insulin, growth hormone, parathyroid hormone, heparin
Altus	CLEC®	Protein crystallization	Catalysts containing the enzyme alcohol dehydrogenase (ADH) Protein stabilization against proteolysis and self-digestion	Calcitonin, other polypeptides, lipases, esterases, and proteases
Generex	Oral-Lyn™	Spray device and aerosol particles	Penetrate the buccal epithelium Treatment of Type 1 & 2 diabetes	Insulin, Macrotonin
NOBEX/Biocon	HIM2	Amphiphilic oligomers	Resist enzyme digestion and increase membrane permeation	Insulin, enkephalin, calcitonin, parathyroid hormone
Apollo Life Sciences	Oradel™	Nanoparticles	Protection of the drug payload from digestive enzymes and transport of protein-based drugs and antibodies across the intestinal wall transporting both small and large molecules (up to 150 kDa in size) for protein-based drugs and antibodies	Insulin and oral delivery of anti-inflammatory proteins(TNF blocker)
Autoimmune Incorporated/Eli-Lilly	AI-401	Oral formulation	Protect proteins from enzyme digestion. Oral tolerance therapy Treatment of Type 1 diabetes but also for prevention of progression	Insulin
Provalis PLC	Macrulin™	Lipid-based water-in-oil microemulsion	Protect proteins from proteolysis or acidic degradation, and enhance the protein absorption in GIT treatment of Type 2 diabetes	Insulin, salmon calcitonin
Endorex	Orasome™	Polymerized liposomes	Protect proteins from the stomach and upper GIT	Insulin and growth hormone, vaccines

Table 2

A list of various proteases along with their sites of action [41].

Types	Enzymes	Major Site of Action
Gastric proteases	Pepsins (aspartic proteases)	Broad activity, hydrolyzes many peptide bond peptides
Brush border proteases	Aminopeptidase A	Aminopeptidases are N-terminopeptidases, degrading mostly 3–10 amino acid residue-dipeptides and amino acids
	Aminopeptidase N	
	Aminooligopeptidase	
	Dipeptidylaminopeptidase IV	
	Carboxypeptidase	
Cystosolic proteases	Di- and tripeptidase	2–3 aminopeptide amino acids
Intestinal pancreatic proteases	Trypsin (endopeptidase)	Peptide bonds of basic amino acids/peptides
	α-chymotrypsin (endopeptidase)	Peptide bonds of hydrophobic amino acids/peptides
	Elastase (endopeptidase)	Peptide bonds of smaller and nonaromatic amino acids/peptides
	Carboxypeptidases (exopeptidase)	A: C-terminal amino acid
		B: C-terminal basic amino acid
Brush border proteases	Aminopeptidase A	Aminopeptidases are N-terminopeptidases, degrading mostly 3–10 amino acid residue-dipeptides and amino acids
	Aminopeptidase N	
	Aminooligopeptidase	
	Dipeptidylaminopeptidase IV	
	Carboxypeptidase	

Table 3

Potential applications for nanotechnologies in drug delivery [69].

Material/technique	Characteristics	Medical applications
Ligands attached to nanoparticles	Surface modification with functional groups High degree of engineering precision Control the size of the nanoparticles	Labeling, tracing and imaging Sensing and detection Recognition and attachment to damaged or diseased tissue followed by release of therapeutic compound
Quantum dots	Emit different wavelengths over abroad range of the light spectrum from visible to infrared, depending on their size and chemical composition Influence the fluorescence properties of the particles	Fluorescent probes Detection and targeting
Nanocapsules	Consists of a shell and a space Can be made in specific sizes, shapes, and in reasonable quantities Control the release of substances or protect them from the environment Higher safety and efficacy Evasion of the host immune system and delivery of therapeutic agent to target sites	Slowly release loading drugs Lipid nanocapsules as nanocarriers e.g. Buckyball-based treatment for AIDS
Nanoporous materials	Ability of nanopores of certain sizes to let some substances pass and others not, or to force molecules	Nanoporous membranes for molecules like DNA and RNA Can be coupled to sensors or used for drug-delivering implants
Polymers	Allow for judicious selection for targeting and delivery Can be used to improve the function of the nanoparticle High degree of engineering precision	Drug carrying devices or implants Combining multi-modal therapy and imaging
Sorting biomolecules and precise sorting	Nanopores capable of rapid and precise sorting	Gene analysis and sequencing

Table 4

Targets for the US National Nanotechnology Initiative.

Research and development targets related to drug delivery/diagnosis
Advanced materials and manufacturing: one-half from molecular level
Converging technologies from nanoscale
Life-cycle biocompatible/sustainable development
No suffering and death from cancer when treated
Pharmaceuticals synthesis and delivery: one-half on nanoscale level
Research and development targets not directly related drug delivery/diagnosis
Control of nanoparticles in air, soils, and waters
Education: nanoscale instead of microscale based
Nanoscale visualization and simulation of three-dimensional domains
New catalysts for chemical manufacturing
Transistor beyond/integrated CMOS < 10 nm

Table 5

Advantages of using nanoparticles as a drug delivery system [72].

1	Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after various routes of administration.
2	Nanoparticles control and sustain the release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve an increase in drug therapeutic efficacy and a reduction in side effects.
3	Controlled release and particle degradation characteristics can be readily modulated by the choice of matrix constituents. Drug loading is relatively high and drugs can be incorporated into the systems without any chemical reaction; this is an important factor for preserving the drug activity.
4	Site-specific targeting can be achieved by attaching targeting ligands to the surfaces of particles or by using magnetic guidance.
5	The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular, etc.

Table 6

Proteins/targeting ligands and functional activity [88].

Protein/ligand	Functional activity and characteristics	References
Transferrin	Iron uptake occurs via the internalization of iron-loaded transferring mediated by the interaction with the Transferrin receptor Widely applied as a targeting ligand in the active targeting of anticancer agents, proteins and genes to primary proliferating cells via transferrin receptors	[89–93]
Insulin	A hormone that regulates blood glucose levels, A small protein	[94]
Elastin	A cross-linked protein in the extracellular matrix that provides elasticity for many tissues	[95]
Albumin	The major serum protein, binds a wide variety of lipophilic compounds including steroids	[96]
RGD peptide	Increases cell spreading, differentiation, and enhances DNA synthesis The RGD sequence can bind to multiple integrin species and also minimizes the risk of immune reactivity or pathogen transfer, particularly when xenograftor cadaveric protein sources are utilized	[97–101]
Lectin (WGA)	Binds to the Caco-2 cell surface and human enterocytes	[84, 102–108]

Table 7

Phage selection. Clones were sequenced after five rounds of selection in the human FAE *in vitro* model. T5C were clones selected for their ability to induce phage transcytosis on co-cultures and T5M on monocultures. Numbers 1 and 2 represent both sequencing in which 12 and 50 clones were analyzed respectively. Bold type represents identical clones recovered at high frequency after selection on mono- and co-cultures.

Clone name	Sequence/peptide	Frequency
T5C1-2/3/5/11-T5C2-1/4	**L-R-V-G-stop**	**6**
T5C1-1 - T5C2-3/5/8/10/12/14/17/19/22/24/26/28/38/40/42/45/47	C-**T-G-K-S**-c-stop	**25**
T5C1-4	C-stop	1
T5C1-6	C-E-G-P-L-K-P-stop	1
T5C1-7	C-G-G-X-D-N-S-C	
T5C1-8 - T5C2-2/13/20/30/41	S-stop	6
T5C1-9	C-A-P-I-L-F-P-R-C	1
T5C1-10 - T5C2-7/29/34/36/37	P-A-stop	6
T5C1-12 - T5C2-9	C-L-E-S-K-K-K-T-C	2
T5C2-21/27	**P-A-V-L-G**	**2**
T5C2-6	C-R-M-K	1
T5C2-18	C-E-K-R	1
T5C2-25	C-I-G-K-R-D-A-K-H	1
T5C2-32	C-R-R-stop	1
T5C2-33	C-K-**S-G-G-T-S**-A-C	1
T5C2-35	C-R-**S-G-T-S**-R-S-C	1
T5C2-46	C-R-D-stop	1
T5M1-2/4/8 - T5M2-9/11/18/23	**P-A-V-L-G**	**7**
T5M1-1 - T5M2-1/3/7/20/24/26/28/30/35/38/45/48/49	C-**T-G-K-S**-C	**25**
T5M1-3 - T5M2-4	S-A-stop	2
T5M1-5 - T5M2-16/21	P-A-stop	2
T5M1-6	C-I-E-V-P-C	1
T5M1-7	C-G-E-K-K-M-R-C	1
T5M1-9	C-**G-K-S**-T-K-N-W	1
T5M1-10 - T5M2-5/8/10/17/22	S-stop	6
T5M1-11	P-A-R-L-A-R-L	1
T5M2-2/13/14/19/25	**L-R-V-G**	**5**
T5M2-36/46/47	C-P-F-D-S-stop	3
T5M2-6/12	C-K-stop	2
T5M2-29	L-V-G-G-H-C-G-E-C	1
T5M2-15	C-Q-E-A-T-N-R-K-C	1
T5M2-37	C-**T-G-K**-R	1