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Basics and recent advances in peptide and protein drug delivery

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Abstract

While the peptide and protein therapeutic market has developed significantly in the past decades, delivery has limited their use. Although oral delivery is preferred, most are currently delivered intravenously or subcutaneously due to degradation and limited absorption in the gastrointestinal tract. Therefore, absorption enhancers, enzyme inhibitors, carrier systems and stability enhancers are being studied to facilitate oral peptide delivery. Additionally, transdermal peptide delivery avoids the issues of the gastrointestinal tract, but also faces absorption limitations. Due to proteases, opsonization and agglutination, free peptides are not systemically stable without modifications. This review discusses oral and transdermal peptide drug delivery, focusing on barriers and solutions to absorption and stability issues. Methods to increase systemic stability and site-specific delivery are also discussed.

Peptides and proteins have great potential as therapeutics. Currently, the market for peptide and protein drugs is estimated to be greater than US$40 billion/year, or 10% of the pharmaceutical market [1]. This market is growing much faster than that of small molecules, and will make up an even larger proportion of the market in the future [1]. At present there are over 100 approved peptide-based therapeutics on the market, with the majority being smaller than 20 amino acids [1]. Compared with the typical small-molecule drugs that currently make up the majority of the pharmaceutical market, peptides and proteins can be highly selective as they have multiple points of contact with their target [1]. Increased selectivity may also result in decreased side effects and toxicity. Peptides can be designed to target a broad range of molecules, giving them almost limitless possibilities in fields such as oncology, immunology, infectious disease and endocrinology. For an overview of some popular therapeutic peptides/proteins, see **Table 1** [2–6,401–403].

These peptide and protein therapeutics have disadvantages as well, such as low bioavailability and metabolic liability. Oral bioavailability of peptides is limited by degradation in the gastrointestinal (GI) tract as well as their inability to cross the epithelial barrier. These therapeutics tend to have high MWs, low lipophilicity and charged functional groups that hamper their absorption [7]. These characteristics lead to the low bioavailability of most orally administered peptides (<2%) and short half-lives (<30 min) [8]. Intravenous (iv.) or subcutaneous (sc.) delivery of these therapeutics overcomes the issue of absorption, but other factors limit the bio-availability of peptide and protein therapeutics including: systemic proteases; rapid metabolism; **opsonization**; conformational changes; dissociation

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of subunit proteins; non-covalent complexation with blood products; and destruction of labile side-groups [1,9].

As oral delivery improves patient compliance, there is great interest in the development of systems that allow for the oral delivery of peptide and protein therapeutics [10]. This review will summarize the barriers to various noninvasive delivery methods with a focus on oral and transdermal delivery. Additionally, current methods to overcome these delivery barriers will be discussed. The final portion of this paper will cover schemes designed to overcome the problems of therapeutic targeting and systemic stability.

Oral delivery

Barriers to oral delivery

Oral delivery is the preferred route of drug administration, as the majority of patients see it as the most convenient way to take their drugs [11]. Drugs taken by the oral route have the highest level of patient compliance due to the ease and simplicity of taking medications [11,12]. Despite the large number of protein therapeutics being discovered each year, oral delivery continues to be a barrier. As a whole, protein and peptide drugs have low bioavailability when administered orally due to problematic barriers including gastrointestinal proteases, the epithelial barrier and efflux pumps. Common routes of administration for the systemic delivery of peptide and protein therapeutics are summarized in **Figure 1**. **Table 2** provides an overview of the delivery enhancers discussed in this paper with regards to where they act.

Proteins are degraded via enzymes and hydrolysis in the acidic environment in the stomach and in the GI tract by a number of proteases and peptidases [13–15]. The human degradome, a complete list of proteases in human cells, consists of at least 569 proteases [16]. There are five broad classes of proteases, including serine, cysteine, threonine, aspartic and metallo proteinases [17]. These proteases play roles in DNA replication, transcription, cell proliferation, fertility, stem cell mobilization, hemostasis, inflammation, senescence, apoptosis and many other vital cellular and regulatory processes [17]. Trypsin, carboxypeptidase and chymotrypsin are secreted from the pancreas into the small intestine, mostly in the duodenum, where they are present in gram quantities. These enzymes are responsible for 20% of the enzymatic degradation of ingested proteins and peptides [13,18,19]. The causes of the remaining 80% of enzymatic degradation are discussed below.

While peptide degradation is one obstacle to oral protein therapeutic delivery, the epithelial barrier of the small intestine poses an even greater challenge. This barrier consists of a single layer of columnar epithelial cells supported by lamina propria and muscularis mucosa [18]. Molecules can cross the epithelium by either transcellular or paracellular routes as depicted in **Figure 2**. Apical to the epithelial cell barrier is the mucosal layer, which contains glycocalyx, a layer of sulfated mucopolysaccharides [18], glyco-proteins, enzymes, electrolytes and water [18,20]. Additionally, most mucosal surfaces are coated by a hydrated gel consisting of mucins, which are high MW, heavily glycosylated proteins [21]. Bulk flow to the epithelial cells is limited, creating an unstirred layer near the epithelial surface [21]. This unstirred layer is protected from convective mixing forces, slowing the absorption of small molecules and ions. Once a molecule passes the mucosal layer, however, the unstirred layer may act as an absorption enhancer by allowing the particle more time exposed to the epithelial barrier [21].

The brush border membrane **(Figure 2)** is where the majority of peptide degradation occurs [18]. The brush border is the microvilli-covered surface of cells found in the small intestine, and the microvilli play a major role in nutrient digestion and absorption [8]. Tight junctions

(TJs) mediate the paracellular pathway of absorption in intact membranes **(Figure 2)**, and are the rate limiting step in transepithelial transport [21]. Adherin junctions, which are required for the assembly of TJs, are a multiprotein complex made of trans-membrane proteins, peripheral membrane proteins and regulatory molecules including kinases [21]. Adherin junctions work with desmosomes to provide the adhesive bonds that maintain cellular proximity and intercellular communication [21]. Both adherin junctions and TJs are supported by dense perijunctional rings of actin and myosin. The most important proteins to TJ assembly and maintenance are zonula occludens-1 and -2 along with transmembrane proteins in the claudin family [21,22].

A final barrier to protein drug absorption is efflux pumps, depicted in **Figure 2**. These are proteins belonging to the ATP-binding cassette superfamily that sit on the apical side of mature epithelial cells and mediate multidrug resistance in humans [23]. To date, 49 ATP-binding cassette proteins have been identified, many of which are overexpressed in multi drug resistance lines [24]. One specific example of an efflux pump is **P-glycoprotein** I (PGP-I; also known as MDR1) [25]. After peptides are absorbed in the GI, PGP-I can pump the drug or peptide back into the GI lumen [13]. It is known that linear lipophilic and cyclic peptides (including cyclosporine) are substrates of PGP-I [13,26].

Even after the drug is absorbed, first-pass metabolism can greatly reduce the fraction of drug that reaches systemic circulation. The first-pass effect, as it is known, is the phenomenon that accounts for the decreased fraction of drug systemically available compared with the fraction of drug that is absorbed. Once a drug is absorbed after oral administration it enters the hepatic portal system. It is then carried via the portal vein to the liver prior to reaching the rest of the body. The liver then metabolizes the drug, reducing the amount of the active, parent compound that enters systemic circulation [27]. Intramuscular (im.), iv., sc., sublingual, intrarectal, transdermal and pulmonary routes of administration avoid or minimize the first-pass effect [28].

While these barriers to absorption are large, much work has been done in order to overcome them. Methods to improve the bioavailability of protein therapeutics can be broadly classified into the following categories: structural modifications, enzyme inhibitors, absorption enhancers and carrier systems.

Strategies for oral delivery of peptides

Direct structural modification

One class of structural modifications under study is cyclization. The benefits of cyclization to oral peptide/protein therapy are evidenced by cyclosporine (CSA). CSA is a fungal-derived, non-ribosomal 11-amino acid peptide with a cyclic backbone and a single D-amino acid [1]. While most naturally occurring proteins and peptides are composed of L-amino acids, D-amino acids are found in some naturally occurring non-ribosomally synthesized peptides [29]. CSA is used most frequently as an immune system modulator for the prevention of solid organ rejection [30]. This cyclic peptide is resistant to proteolytic degradation and also has higher than expected absorption after oral administration [1]. The superior oral bioavailability is thought to be due to a number of properties including decreased flexibility and hydrogen bonding characteristics. The cyclic nature of CSA incorporates seven *N*-methyl groups that reduce the number of hydrogen bond donors, and the remaining four hydrogens bond intramolecularly. This reduction in intermolecular bonding reduces hydrophilicity. CSA has lipophilic side chain amino acids that further raise its lipophilicity and allows it to cross the gut wall [1]. Other peptides such as somatostatin and encephalin have demonstrated similar characteristics and improved oral absorption after cyclization [31,32]. Generically, cyclization is usually carried out between side chains or

ends of the peptide sequences through disulfide bonds, lanthionine, dicarba, hydrazine, or lactam bridges [1]. While cyclization is an option for some peptides, its widespread use is limited when larger peptides and proteins are needed for therapy.

PEGylation is a modification option for some peptides not amenable to cyclization. PEG is an amphipathic molecule that dissolves in organic solvents as well as in water [33]. Both PEG and its metabolites are nontoxic and US FDA approved [34,35]. PEG has been reported to be toxic at high parenteral doses, much higher than the amount of PEG a patient would be exposed to with current PEGylated therapies [36]. If PEG toxicity is seen it usually presents in the kidney, as unmodified PEG is mainly cleared through the kidneys. Interesting, even when pathological changes were seen, no functional deficits resulted [36]. Case studies exist that demonstrate high doses of PEG can induce acute tubular necrosis, and the use of PEG in colonoscopy bowel preparation is associated with an increased risk of acute renal failure in patients aged over 50 [37,38]. There is also evidence that repeat administration of PEGylated particles can lead to increased clearance rate, likely related to anti-PEG IgG and IgM antibodies [39,40]. The structure of the PEG molecules, properties of the molecule being PEGylated and method of PEGylation all play a role in determining immunogenicity [40].

Direct PEGylation confers benefits in both protein absorption and systemic stability (described later in this paper). As an example, insulin PEGylated with a 750 Da version of PEG was formulated into a mucoadhesive tablet. After oral administration, insulin activity was demonstrated by the observed drop in blood glucose levels of approximately 50% 3 h after administration. Additionally, some activity of the orally administered insulin was seen up to 30 h after administration [41]. PEGylation of another peptide, salmon calcitonin (sCT), resulted in resistance to intestinal enzymes, a nearly sixfold increase in intestinal absorptionand slowed systemic clearance compared with the unmodified version of sCT [42].

Vitamin B12 has been used to increase the oral absorption of a number of therapeutic proteins including G-CSF, erythropoietin, insulin and lutenizing hormone releasing hormone [43]. By fusing therapeutic proteins to vitamin B12, it is possible to take advantage of the binding of vitamin B12 to IF, followed by the receptor-mediated absorption of the vitamin B12–IF conjugate. However, this system is limited by the quantity of B12 that can be absorbed, GI degradation, decreased activity of the protein therapeutic due to steric hindrances, and loss of IF affinity for conjugated vitamin B12 [43]. For more information on the use of B12 to improve the oral delivery of protein and peptides, please see the review by Petrus *et al.* [44].

Protein lipidization is another method that increases the bioavailability of orally administered proteins. Fatty acid conjugates of polypeptides demonstrate improved transport across biological membranes, higher stability and longer plasma half-lives [45,46]. sCT was lipidized using reversible aqueous lipidization and thus it is categorized as a prodrug in this case [46]. Compared with free sCT, the reversible aqueous lipidization sCT reported increased absorption and a 19-times higher AUC value [46]. Caprates, medium-chain fatty acids, promote paracellular diffusion of Class III (highly soluble, low permeability) molecules such as peptides [47]. In addition, triglycerides can be used to evade first-pass metabolism [47]. While irreversible methods of lipidization allow for increased membrane permeability, the activity of such modified proteins may be diminished due to steric issues with the fatty acid chain [47].

Recently, stapled peptides have garnered interest due to their enhanced biochemical properties in the context of drug delivery. More specifically, these are a-helical peptides that

contain a synthetic, hydrocarbon backbone linking various residues [48]. This backbone, known as the staple, locks the conformation of the peptide, increasing its helicity and stability in solution [49]. As an example, Walensky *et al*. have demonstrated the ability of a hydrocarbon-stapled BH3 helix to increase apoptosis *in vivo* [50]. The enhanced stability of these peptides, along with increased cellular penetration capabilities, makes these molecules ideal candidates for future study in peptide delivery.

A final method of peptide modification to increase oral bioavailability is the substitution of natural L-amino acids with D-amino acids. One study demonstrated that a variety of peptides cleaved by chymotrypsin, elastase, papain (a cysteine protease found in papaya), pepsin, trypsin, and carboxypeptidases are cleaved minimally or not at all by these enzymes when certain residues were replaced with D-amino acids [29,51]. Tugyi *et al*. investigated D-amino acid substitutions in MUC2, a mucin glycoprotein [52]. The authors noted that the substituted peptide demonstrated high resistance to proteolytic degradation *in vitro* in both human serum and lysosomal preparations. Their work illustrated that simultaneously modifying both N- and C-terminal regions with D-amino acids conferred the greatest stability increases [52].

The above mentioned direct modifications of peptides and proteins are key strategies that have been implemented to increase stability and oral bioavailability. Many other direct modifications have been carried out, including certain prodrug methods, an overview of which is given in **Table 3** [8,31,32,41,42,44,45,52–56].

Enzyme inhibitors

In addition to direct modifications, another method to increase oral peptide bioavailability is to coadminister with enzyme inhibitors. These enzyme inhibitors are usually more effective in the large intestine than the small intestine due to the large quantity and variety of proteases in the small intestine [57]. A leading enzyme inhibitor is soybean trypsin inhibitor, FT-448, a potent and specific inhibitor of chymotrypsin [57]. When coadministered with insulin to rats and dogs, levels of immunoreactive insulin rose proportionally to a decrease in blood glucose levels. Further, it is thought to play some role in increasing peptide absorption [57].

Aprotinin, originally branded as Trasylol™, and used to reduce bleeding during complex surgeries, is another enzyme inhibitor used [58]. When administered with insulin intraileally, blood glucose decreased by 30% over the next 3 h compared with administration of insulin alone [58]. Other enzyme inhibitors are summarized in **Table 4**. An alternative method to inhibit enzymes is to alter the pH at the site of action of the enzymes [59]. Most enzymes in the stomach, including pepsin, are only active at low pH (approximately 2) [60]. Therefore, if the pH in the stomach is increased, the enzymes are no longer able to degrade the peptides. Conversely, enzymes in the intestines often work at a higher pH; therefore, lowering the pH can decrease the activity of these enzymes [61,62]. These protease inhibitors do have shortcomings. First, they can disrupt the normal absorption of dietary peptides and may induce toxic shock after prolonged therapy [63,64]. It is believed this may cause the body to increase production of these proteases, which may lead to hypertrophy and hyperplasia of the pancreas [65]. The inhibitors themselves may also be toxic and damaging to the GI tract after prolonged administration [65]. Indeed, the majority of enzyme inhibitors are highly toxic. **Table 4** summarizes enzyme inhibitors with some promise of therapeutic translatability [57,58,65–70].

Absorption enhancers

The optimal absorption enhancer should be reversible, nontoxic at the effective concentration and provide a rapid permeation enhancing effect on the intestinal cell membrane. One such compound class of absorption enhancers is chitosans. Chitosans are nontoxic, biocompatible, FDA-approved polymer derivatives of chitin that enhance the absorption of hydrophilic macromolecule drugs [71]. In addition, due to their high MW, they are minimally absorbed from the gut, limiting the possibility of systemic side effects [72]. It is thought that varying degrees of deacetylation of chitin confer different amounts of absorption enhancement, with >80% deacetylation affording the greatest promoter effect in cell culture [73]. Chitosans have been used to enhance the absorption of molecules such as atenolol, insulin and 8-R-vasopressin [72]. Further, chitosans appear to be quite safe at their effective concentration [71,74]. Chitosans work by increasing paracellular permeability. By binding tightly to the epithelium via positive charges, chitosans cause redistribution of cytoskeletal F-actin and the zonula occludens 1 [75]. Chitosans are limited by their ability to diffuse across the mucous layer, as evidenced by their decreased activity on mucus-producing cells [76]. *In vivo* studies with chitosans demonstrated a threefold increase in octreotide absorption when the two were coadministered into the duodenum [72]. Another study with trimethyl chitosan chloride, a chitosan derivative, had many favorable characteristics. Trimethyl chitosan chloride was able to reversibly interact with TJs, leading to widening of the paracellular route, and at the same time did not damage cell membranes or alter the viability of intestinal epithelial cells. *In vivo* studies in rats demonstrated that it was able to increase the oral bioavailability of a peptide when the two were coadministered [71]. Overall, chitosans and their derivatives are a promising class of absorption enhancers.

Another class of absorption enhancers demonstrating potential includes the medium-chain fatty acids [77]. C8, C10 and C12 fatty acids (caprylate, caprate and laurate, respectively) can enhance paracellular permeability of hydrophilic compounds. First, caprate is thought to work by inducing dilation of TJs [78]. Interestingly, the lowest concentration that enhanced absorption was near the critical micelle concentration of each fatty acid [77]. The order of increased absorption *in vivo* is caprate>laurate>caprylate. Sodium caprate (C10) is the most studied of the medium-chain fatty acids. It is thought to increase absorption of hydrophobic molecules via the paracellular and transcellular route [79]. Unfortunately, a study reported that it can only significantly increase absorption for molecules up to 1200 g/mol, or 1.2 kDa (such as octreo-tide) [80]. At the effective dose of 13 mM, sodium caprate is nontoxic to epithelial cells [80].

Lectins are another type of absorption enhancer that have many of the characteristics of the ideal absorption enhancer. Lectins are proteins that specifically recognize and bind to sugar complexes attached to proteins and lipids [81]. Lectins are also naturally resistant to proteolytic breakdown, making inactivity before reaching their site of action unlikely [82]. They can be used to target luminal surfaces of the small intestine and trigger vesicular transport into or across epithelial cells [81]. Lectins are also mucoadhesive, which further leads to increased absorption [15].

Toxins can also be used for absorption enhancement, so long as they do not cause permanent cellular damage. Zonula occludens toxin (ZOT), is one such compound. ZOT, a 45 kDa toxin made by *Vibrio cholerae,* has been demonstrated to increase the permeability of small intestine mucosa by reversibly affecting the structure of TJs [83,84]. ZOT binds to ZOT receptors on the luminal surface of the intestine and causes cytoskeletal rearrangement related to changes in protein kinase C and binding to β-tubulin [84,85]. TJs can be perturbed enough to allow the transport of agents across the intestinal mucosa, although the increased bioavailability of insulin was only 20% [86]. In a study with Caco-2 cells, incubation with 4 μg/ml ZOT for 30 min increased the permeability to insulin by 6.3-fold [86]. Mediation of

TJs may not be the only method by which ZOT works; a study demonstrated that a fragment of ZOT was able to increase the bioavailability of hydrophobic drugs by interacting with PGP [87]. Additional work has been done to determine the smallest portion of ZOT that maintains activity [88].

Recently, coadministration of cell-penetrating peptides (CPPs; described later in more detail) with therapeutic peptides has been attempted in order to increase absorption of the therapeutic. In one study, insulin coadministered with CPPs consisting of six to ten repeats of argi-nine led to increased GI uptake of insulin [89]. Interestingly, the study investigated both D- and L-arginine-based CPPs, and the D-based CPPs allowed for greater increases in insulin absorption, assumed to be due resistance of D-amino acids to proteases [89]. It is important to note that the CPP was not fused to insulin; rather, they were co administered. A follow-up study demonstrated that electrostatic interactions between insulin and the CPP were responsible for the enhanced absorption of insulin [90]. Another study revealed that the CPP penetratin was best able to increase ileal insulin absorption [91]. Penetratin consists of basic amino acids (lys, arg) along with some hydrophobic regions. Use of CPPs as absorption enhancers represents a relatively new area of research that has the potential to add weapons to the absorptive enhancement arsenal.

Other classes of absorption enhancers have lost favor in recent years due to irreversible epithelial damage [92]. Surfactants such as sodium dodecyl sulfate were shown to cause increased permeability of the GI tract to hydrophilic compounds, but also cause altered cell morphology and cell membrane damage [93]. Sodium dodecyl sulfate shortened microvilli of cells and produced actin disbandment, structural separation of the TJs and damage to the apical cell membrane with even limited exposure [94]. Certain *in vivo* rat studies support the increase in absorption and revealed the damage caused to be reversible [95]. Bile salts such as sodium cholate and deoxycho-late were originally seen as safe and effective at increasing drug absorption; however, it is now understood that these particles are damaging after long-term use [96].

Carrier systems

Many drug carrier systems are currently being developed in an attempt to increase the oral bio-availability of peptide drugs. Some of these systems contain a combination of components listed above, while others have novel mechanisms.

The first group of carrier systems consists of hydrophilic mucoadhesive polymers (polyacrylates, cellulose, chitosan), which can be altered to suit the needs of the peptide/protein being delivered [97]. While chitosan has already been discussed under the absorption enhancers category, it has also been combined with EDTA in order to create a resin that binds bivalent cations [98]. It is thought that bivalent cations are essential for the activity of proteolytic enzymes; in fact, zinc proteases, carboxypeptidases and amino peptidases were strongly inhibited by this system, but serine proteases, trypsin, α-chymotrypsin and elastase were not inhibited [98].

Thiomers, thiolated polymers, have also been used as drug carrier systems. These mucoadhesive polymers display thiol-bearing side chains; disulfide bonds form between the polymer and cysteine-rich protein domains in the mucous glycoprotein layer. These polymers are available in both cationic and anionic varieties and can increase mucoadhesive properties of gels by up to 140-fold [99]. When adhered in the small intestine, mucoadhesion allows for a steeper concentration gradient across the epithelial barrier, which may lead to increased passive drug uptake and a prolonged therapeutic effect [99].

Next, polymer matrices can be used to protect proteins from proteolysis and antibody neutralization, resulting in increased protein activity *in vivo* [100]. It is very important that interaction between the protein and matrix be optimized; too little attraction and the protein will not be immobilized on the gel; too great an attraction will cause the protein to remain in the gel and thus not become systemically available. A sustained release system that protects the protein in the GI tract can be developed by tuning the cross-linkage and electrostatic interactions between matrix and protein [100].

Nanoemulsions are another carrier system for oral protein therapeutics. Nanoemulsions are defined as oil-in-water (o/w) or water-in-oil (w/o) emulsions with mean droplet diameters ranging from 50 to 1000 nm. The average droplet size is usually between 100 and 500 nm [101]. Generally, these emulsions are made from surfactants approved for human consumption and are generally recognized as safe. Nano emulsions have a much higher surface area and free energy than macroemulsions, thus making them an effective transport system. Further, nano emulsions do not cream, flocculate, coalesce or sediment. One such system in development is the 'self-nanoemulsifying drug-delivery system,' or SNEDDS. To test the concept, fluorescein isothiocyanate-labeled β-lactamase (BLM) was loaded into SNEDDS through solid dispersion. After an o/w emulsion was formed via addition of water, the nanoemulsion was able to increase transport of fluorescein isothiocyanate–bleomycin across MDCK monolayer of cells [102]. *In vivo* studies demonstrated a significant increase in SNEDDS–BLM absorption compared with free BLM [102].

Hydrogels are a network of cross-linked water-soluble polymer chains that are insoluble in water but have water as their dispersion medium. The porous nature of hydrogels can be finely tuned to allow for drug loading into the hydrogel. Further, pharmacokinetic properties for release of the loaded drug can be adjusted to the requirements of individual drugs [103]. Hydrogels can designed to deliver drugs to four sites after oral ingestion – mouth, stomach, small intestine or colon [104]. Newly developed homo- and copolymeric hydrogels are capable of protecting and delivering peptides and protein therapeutics [103]. For an overview on hydrogels, see Bindu Sri *et al*., and for more detail on the use of hydrogels for oral peptide drug delivery, the review by Peppas *et al*. is helpful [103,104].

While liposome systems have potential in oral drug delivery, there is a concern with stability of the vesicles under the physiologic conditions of the GI tract [105]. Adding to the problem, mucus may act as a barrier by blocking the diffusion of liposomes to the epithelial layer [106]. Despite this, orally administered liposomes have demonstrated some successes. Calcitonin was administered in a chitosan–aprotinin coated liposome and illustrated an increased pharmacological effect compared with free calcitonin [107]. Cyclosporine has also been delivered in liposomes; the egg lectin–cremophore–lactose liposome containing CSA had nine-times the bioavailability of free CSA and four-times that of the microemulsion on the market [108]. PEG coating, enteric encapsulation and the use of archaeosomes have been proposed to decrease degradation of the liposome in the GI tract [109].

Nanoparticles (NPs) are solid particles with sizes in the range of 10–1000 nm [110]. NPs allow for the encapsulation of proteins inside a polymeric matrix, thus protecting them against hydrolysis and enzymatic degradation [110]. These systems can be tuned in order to maximize encapsulation efficiency, bioavail-ability and retention time [111]. NPs, however, have a difficult time being absorbed from the GI tract; studies have demonstrated that cells lacking mucus (including M cells and Peyer's patches in general) are best at absorbing NPs [110]. Particles of 50 and 100 nm demonstrated the greatest absorption and detection in intestinal mucosa [112]. Furthermore, NPs smaller than 100 nm demonstrate a higher extent of uptake by absorptive enterocytes while those over 500 nm will rarely be taken up by absorptive enterocytes [110]. NPs are often made from poly(lactic acid), poly(lactic-co-

glyclic acid), chitosan, gelatin and poly-alkyl-acyanoacrylate, all of which are nontoxic, non-thrombogenic, non-immunogenic, non-inflammatory, stable in blood, biodegradable, avoid the reticuloendothelial system (RES), and are applicable to various biologics such as proteins, peptides and nucleotides [111]. While there are minimal scientific data on the toxicity of NPs, their size makes exposure during manufacturing almost guaranteed [113]. Impaired lung function and other respiratory symptoms have been seen in workers that were exposed to NPs [113]. iv. administration of NPs is followed by increased synthesis and release of cytokines. Furthermore, NPs passively target the liver through uptake by Kupfer cells, again followed by an inflammatory response [113]. Generally, the toxic effects of NPs are not fully understood, and care must be taken with the manufacturing and use of NPs as therapeutic agents. **Table 5** provides some details regarding the various polymers used to make NPs [111,114–121].

NPs can be targeted to certain sites based on particle size, surface charge, surface modification and hydrophobicity [111]. Surface charge is particularly important for cell internalization, as cationic surfaces increase the rate and extent of nanoparticle internalization [111]. Carboxylated polystyrene NPs demonstrate decreased affinity to intestinal epithelia and M cells compared with neutral and positively charged polystyrene NPs [122]. While hydrophobic polymer-based NPs are better absorbed than their hydrophilic counterparts [110], in order to avoid opsonization and the mononuclear phagocytic system, the use of hydrophilic-surfaced NPs is preferred over traditional hydrophobic-surfaced NPs [111,123]. Interestingly, negatively charged, hydro-philic NPs have increased bioadhesive properties and are uptaken by absorptive enterocytes and M cells [110,124].

Surface modifications such as PEG can create a steric barrier and reduce clearance by circulating macrophages in the liver as well as by the mononuclear phagocytic system [111]. PEG coating of NPs increases blood circulation half-life as well as reducing interactions between the NPs and digestive enzymes [125]. Lectins have been conjugated to NPs, which led to increased transport across intestinal mucosa, especially via M cells of Peyer's patches [110,111]. Finally, higher MW polymers will release the peptide slower than lower MW polymers [111].

Solid lipid NPs (SLN) are solid lipids that are stabilized with an emulsifying layer in an aqueous dispersion **(Figure 3)**. The colloidal size ranges between 50 and 1000 nm [105]. This system avoids the use of organic solvents and has the capacity to allow fast, effective, large-scale manufacturing of high-concentration suspensions. This system can be used to encapsulate peptides and proteins and thereby protect them against enzymatic degradation [126]. Another benefit of SLNs is that the drug can be incorporated into the matrix, onto the shell, or into the core of the particle [105]. A lectin-modified and insulin-coated SLN was able to deliver insulin to the system after administration to the small intestine [127]. SLNs have also been used for controlled release of sCT [128]. The systemic stability and GI absorption of SLNs and NPs as a whole make them promising protein carrier systems; research in this field continues to enhance the likelihood for oral delivery of systemically active peptides.

Many companies are attempting to develop carrier systems that will be able to deliver a wide variety of therapeutics with minimal modification [63]. Examples include Emisphere's Eligen™ system (NY, USA), which has the potential to deliver therapeutics from 0.5–150 kDa. The drug–carrier system known as SNAC (*n*-(8-[2-hydroxylbenzoyl]amino)caprylic acid) can be used to orally deliver active peptides into circulation [129,130]. The peptide/protein therapeutic is mixed with SNAC, which creates a noncovalently linked drug–carrier complex. The complex is highly lipophilic and is proposed to be able to directly cross the

epithelial membrane. After absorption, the complex dissociates by simple dilution, and the therapeutic is released, unchanged and in its active conformation [63,129]. This system has demonstrated promise in both human and animal models for the oral delivery of insulin, human growth hormone, and sCT [130].

A second such system is the gastro intestional mucoadhesive patch system (GI-MAPS), depicted in **Figure 4**. The GI-MAPS is composed of four layers contained in an enteric capsule, which when combined result in protection of the protein in the GI tract as well as increased absorption. The backing is made of ethyl cellulose, while the surface layer is made of an enteric, pH-sensitive polymer, in this case Eudragit® L100. The middle layer is a cellulose membrane that contains both the drug and absorption enhancers. The surface layer is attached to the middle layer via an adhesive layer made of Hiviswako 103 polymer [131].

When the capsule is swallowed, the enteric coating dissolves in the small intestine. Once this layer dissolves, the mucoadhesive layer of the patch is exposed. The patch therefore adsorbs to the mucus membrane of the small intestine, exposing the drug and absorption enhancer to the epithelial surface. When the patch attaches, it provides increased contact time, allowing more of the drug to be absorbed. In addition, a large concentration gradient is created across the epithelial cells, increasing the amount of drug absorbed [131]. While these are two examples of systems, **Table 6** has a more complete list of other similarly functional carrier systems.

The above has been a broad overview of the issues associated with oral administration of peptide and protein therapeutics. Many systems that increase stability and absorption of these therapeutics have been described. Until a more widely applicable system is developed, every protein therapeutic will require a unique system made of combinations of the above if the drug is to be orally bioavailable. While the oral route is a preferred method of administration, other routes, too, have their benefits. The next section will address the issues with transdermal peptide and protein delivery.

Transdermal delivery

Delivering peptides transdermally allows the avoidance of both GI degradation and hepatic first-pass metabolism of short half-life drugs, while still allowing administration via an easily accessible, non-invasive route. This not only diminishes the amount of potential drug–drug interactions with combined therapies, but can also lead to better patient compliance (compared with iv. injection) due to the ease of use, self-administration and less frequent dosing characterized by the prolonged, continuous and rate-controlled drug release unique to these systems [132–136].

First and foremost, the most important barrier for transdermal delivery is the skin itself [132]. Drugs that have been delivered transdermally for some time now, namely nicotine, estrogen and scopolamine, among others, are all small molecules and highly hydrophobic. Historically, it has been demonstrated that the skin tends to keep out drug molecules greater than 500 Da [137], especially those molecules of hydrophilic nature [138]. After all, the main biological function of the skin is to deny entry to foreign substances. Therefore, bypassing the skin to allow drug entry is a necessary step to successful transdermal delivery [139].

Anatomically, the skin is made up of three major layers. The outermost portion, and first line of defense to drug entry, is the stratum corneum [140]. This layer, mainly composed of dead cells (keratinocytes), is approximately 10–15 μm thick and surrounded by a lipid extra-cellular matrix. Below the stratum corneum lies the viable epidermis, which is approximately 50–100 μm thick. Taken together, these two layers are known as the full

epidermis. Below the full epidermis is the first sign of vasculature, present in a layer known simply as the dermis [136]. A fibrous layer, approximately 1–2 mm thick, the dermis comprises large capillary beds that are the site of drug entry into the circulation [141].

Due to these obstacles provided by the skin, successful transdermal delivery of large, potentially hydrophilic peptides, requires some type of physical and/or chemical enhancement. Conventional enhancements in transdermal delivery generally aim to bypass the main physical barrier, the stratum corneum [134,142–146]. Direct entry into the dermis, despite being the most direct way to get the drug into circulation, is often avoided as penetration of this layer would lead to patient bleeding and possible disruption of nerve endings [144].

Because the traditional transdermal patch is used solely to deliver small, hydrophobic drugs, and not peptides, it will not be discussed in this section. Instead, many of the currently 'in-development' transdermal enhancement methods will be briefly described, including microneedle technology, electroporation, iontophoresis, sonophoresis, thermal ablation and chemical enhancement.

Microneedle technology

Microneedle technology involves the use of small needles that create small pores in the skin, allowing drug passage across the outermost physical barrier [134]. Because one of the overall goals of transdermal delivery is to increase efficiency while still maintaining an easy, non-invasive technique, these microneedles are designed to breach only the stratum corneum [144]. By not reaching as far as the viable dermis, both the capillaries and nerve endings are avoided, leading to a painless feeling for the patient. These needles have been created using a number of materials, including silicon, various metals, or biodegradable materials such as polymers and sugars [141].

As described by Herwadker and Banga [133], multiple microneedle designs and drug introduction routes have been tested for efficient delivery. One such method involves a two-step approach, where the needles are used to puncture the skin to create pores, followed by topical administration of the drug. Another method includes coating the microneedles themselves with drugs, allowing the drug to then enter the body after the skin is treated with the needle. A third method includes encapsulating the drug in biodegradable microneedles, slowly releasing the drug as the needles degrade. Lastly, a final method includes creating hollow needles, through which drug can be infused following puncturing of the skin. Microneedles can be introduced via physical injection on the skin or in the form of a patch. One example utilizing this technology comes from Zosano Pharma (CA, USA), who have developed a patch containing drug-coated microneedles capable of delivering a variety of drugs including peptides and vaccines [141].

Thermal ablation

Like microneedle technology, thermal ablation aims to permeabilize only the stratum corneum, avoiding a breach of the deeper capillary and nerve-containing tissue layers [146]. However, instead of using needles to perforate the skin, this technique relies on short pulses of high heat (approximately 100°C) to create small, reversible channels in the micron size range [147]. Following the short bursts of heat, drug can be applied to the treated area for entry into the circulation. Multiple systems have been designed to successfully deliver drugs via thermal ablation, including PassPort® (Nitto Denko [Osaka, Japan]) and ViaDor® (Syneron Medical Ltd [Yokneam, Israel]). While these systems have demonstrated success with smaller drugs, delivery of peptides is still under study [141].

Electroporation

Electroporation utilizes very short pulses of high voltages (between 10 and 100 V) to perforate the skin. Similar to microneedles and iontophoresis (discussed later), application of electroporation breaches only the stratum corneum, characterizing it as another non-invasive method for drug introduction [148]. Instead of simply targeting the layer of dead cells, this method targets the surrounding lipid bilayers that are spread out throughout this layer. Application of an electric current disrupts the structure of these lipids, allowing molecules to penetrate the skin. In addition, delivery of drug can be increased using this method by increasing the voltage, number of pulses and duration of pulses to levels still viewed as safe for the patient [133]. Due to the high complexity of these systems, no peptides have been FDA approved for delivery by electroporation. However, multiple DNA-based vaccines are in clinical trials, which, if successful, could pave the way for peptide-based vaccines.

Sonophoresis

Sonophoresis, also referred to as cavitational ultrasound, relies on the application of sound waves to the skin to increase its permeability. Like electroporation, sonophoresis achieves this task by targeting the lipid bilayers embedded in the stratum corneum [133]. Sound waves, generally between 20–100 kHz, are believed to cause an increase in pore sizes on the skin (increased fluidity in these lipid bilayers), thus allowing drug penetration transcellularly through the stratum corneum [147]. Though nothing is currently FDA approved, delivery of insulin for Type I diabetes using the sonophoretic U-Strip system (Transdermal Specialties, Inc. [PA, USA]) is presently in clinical trials, parts of which are expected to be completed within a year [149].

Iontophoresis

Not all methods utilized for transdermal peptide delivery require physical disruption of the skin's outer barrier. Iontophoresis is one of those methods, which instead uses principles of both electrorepulsion (for charged peptides) and electroosmosis (for uncharged peptides) to act on the drug molecules themselves rather than the skin [133]. Generally speaking, iontophoresis utilizes a device placed on the skin capable of generating an electric current, similar to a battery. When delivering charged peptides (negatively charged peptides for instance), the battery builds up a strong negative charge at the anode, which would be placed on the same portion of the skin as the drug molecules. Utilizing charge–charge repulsion, this anode will drive the negatively charged peptide into the skin [132,150]. Using this method, the rate of drug release can be controlled as the release (entry into the body) is directly proportional to the current being administered on the skin [147]. Although peptides have yet to see FDA approval for delivery via iontophoresis, the system has been fine-tuned to deliver smaller molecules such as lidocaine (LidoSite®, Vyteris [NV, USA]). In addition, iontophoretic peptide delivery, including delivery of gonadotropin releasing hormone and insulin, has reached clinical trials on multiple occasions [141].

Biochemical enhancement

A final method involves the use of biochemical molecules to enhance permeation of peptide drugs across the skin. The ultimate goal in using biochemical enhancers is to increase the permeability of the skin, which provides a path for peptide drug delivery into the circulation [147], while remaining nontoxic, non-irritating and non-allergenic [140]. One such peptide used to enhance skin permeability is magainin, a 23-amino acid peptide known to form pores in bacterial cell membranes [151,152]. While previously demonstrated to increase the permeability of small molecules, its use for peptide delivery enhancement still requires optimization [153]. In addition, recent, work by Ruan *et al*. demonstrated the ability of a

small peptide known as TD1 to increase the transdermal penetration capability of hEGF when fused together [138,154]. This fusion system involving TD1 could have major implications in the near future for delivering hydrophilic peptides transdermally.

To summarize, all of the methods described above aim to make the drug delivery process as easy and as painless as possible. Painless, in these cases, requires avoiding a breach of the viable dermis layer of the skin, which includes vasculature and nerve endings. However, other barriers still exist to make this delivery process more efficient. Despite moderate success seen using the previously described physical and chemical enhancement methods, a recent study suggests that bypassing more than simply the stratum corneum is necessary for the most efficient transdermal delivery [136]. In addition, despite displaying low activity compared with other locations in the body, proteases do exist on the skin, adding another challenge to the transdermal delivery of peptides.

Other delivery routes

While this review has focused on delivery of peptides by oral and transdermal routes, delivery by other routes is also currently being researched. The next section of the review will give a brief overview and recommendations for readings on intranasal, buccal, pulmonary and rectal administration of peptide therapeutics. These routes of administration are illustrated in **Figure 1**.

The intranasal route for peptide drug delivery is an area that has already had some successes. For instance, desmopressin, calcitonin and the seasonal influenza vaccine are available via the intranasal route [155,156]. Advantages of the nasal route over injected medications include increased patient convenience and comfort, elimination of needle-stick related injuries and infections, and decreased syringe-related medical waste [156]. Disadvantages include nasal irritation, limitations on volume and milligram amount of drug that can be delivered nasally, the rapid renewal of nasal epithelium, acidic pH, endo- and exopeptidases, and large interpatient variability in absorption [156]. While the nasal route has traditionally thought to be an option only for small molecules, highly effective and non-irritating absorption enhancers have been developed [157]. For a more thorough review on intranasal peptide delivery, see Illum *et al.* [155].

The buccal route, administration of drug through the mucosal membranes lining the cheeks, is another option for peptide delivery [158]. Drugs delivered by the buccal route are placed in the mouth between the gums and cheek [159]. Buccal delivery has many advantages including bypassing of the GI tract and possibly first-pass metabolism, ease of use, rapid onset, large contact surface area and is generally amenable to the delivery of hydrophilic macromolecules [159,160]. There are limitations to buccal delivery and patient adherence, such as irritation of the mucosa, low permeability to peptides and the bitter taste of many buccal drugs [159]. Absorption enhancers and bioadhesive polymers are being used to resolve these problems. Oxytocin, insulin, sCT and GLP-1 have all been successfully delivered via the buccal route [159,160]. For further reading on buccal peptide delivery, see Mujoriya *et al.* [160].

Rectal administration of drugs, while not patients' top choice, is sometimes necessary if other routes of administration (such as oral and iv.) are not possible. The rectum is composed of a one layer-thick epithelium complete with mucus and TJs [161]. While there are no villi, the surface area for drug absorption is approximately 200–400 cm^2 [161]. Rectal administration is useful due to the minimal amount of proteases and avoidance of the first-pass effect. However, the bioavailability of peptides is low without the use of absorption enhancers [161]. Both insulin and pentagastrin have been successfully delivered via the

rectal route. See Lakshmi *et al*. for a more in-depth discussion of rectal peptide delivery [161].

The pulmonary route can be utilized for the systemic delivery of peptide therapeutics. However, the anatomy of the lung creates many barriers to delivery including respiratory mucus, mucociliary clearance, alveolar epithelium with TJs, pulmonary enzymes, and macrophages that secrete peroxidases and proteases [162]. The alveolar epithelium and capillary endothelium have high permeability to many lipophilic substances, but passage of large hydrophilic molecules is limited [162]. Many absorption enhancers and enzyme inhibitors that have been used to increase peptide absorption have been demonstrated to be damaging to lung tissue [162]. Pulmonary delivery of insulin has been extensively studied and was FDA approved in 2006, but Pfizer (NY, USA) discontinued production in 2007 due to poor sales [162,163]. Calcitonin, human growth hormone, parathyroid hormone, and desmopressin have also been successfully delivered via inhalation [162,164]. A complete review of pulmonary peptide delivery can be found in the paper by Agu *et al.* [164].

Systemic peptide stability & site-specific delivery

Unfortunately, once the peptide has gained entrance to the systemic circulatory system, the task is only halfway complete. The protein must still reach its target site, and as many of the targets for protein drugs are intraceullular, this means transport through the circulation to the appropriate site, uptake by the appropriate cells and activity of the protein inside these cells. Therefore, the goals for the protein in the circulatory system include: avoidance of enzymatic degradation, opsonization and the RES, and non-selective accumulation of the protein, maintenance of protein solubility and activity, distribution to the site of action with targeting to certain cell types, cellular uptake, and release of the active protein. This portion of the paper will discuss many of the systems and methods mentioned earlier, but now focusing on issues within systemic circulation. Some of the systems discussed are not amenable to oral or transdermal delivery and would necessitate iv. delivery. Strategies discussed here include stability enhancers, drug carriers, endosomal escape and targeting moieties.

Systemic stability enhancement

Many of the stability enhancers discussed in the first portion of this review have a role in increasing the systemic stability of protein therapeutics as well. For example, fatty acid conjugation leads to extended plasma half-lives, site specific delivery and sustained release upon iv. administration [47]. As these drugs are lipophilic, they will likely be solubilized and stabilized by albumin and other serum lipoproteins [47]. Furthermore, these fatty acids can be removed from the protein via chemistry based on pH, reduction, peptidases, or esterases [47]. Non-reversible lipidization is also an option, and has been demonstrated to increase internalization and activity over non-lipidized counterparts [165].

PEGylation

PEGylation has also been used as a systemic stability enhancer. Direct PEGylation can aid in the stability of proteins for delivery, mainly leading to an increase in circulation time. PEG molecules are highly hydrated, and this increased size leads to decreased glomerular filtration [35]. Moreover, PEGylation of proteins is thought to reduce proteolysis and opsonization [166]. PEGylation also reduces uptake by the RES, decreases the formation of antibodies against the protein and decreases the apparent volume of distribution [34]. PEGylation, however, does have drawbacks. Due to the size of PEG, steric hindrance may decrease the activity of the protein. Also, increased protein aggregation after PEGylation has been noted [34]. Chronic iv. administration of PEG proteins has unintended consequences

such as vacuolation of the renal cortical tubular epithelium in laboratory animals. However, these side effects were noted only after exposure to toxic, supratherapeutic doses of PEG. Newer PEGylation methods such as living radical polymerization, free radical polymerization, atom transfer radical polymerization and reversible addition fragment transfer have allowed PEGylation with greater specificity and purity while making modification with PEG a simpler task [167].

Hyperglycosylation

Hyperglycosylation has many of the same benefits as PEGylation, namely increased half-life, improved solubility and reduced immunogenicity [34]. An additional benefit is that the oligosaccharides added via glycosylation are natural and biodegradable, thus skirting the possible problem of PEG accumulation with chronic administration. The increased stability of hyper-glycosylated peptides may be due to masking hydrophobic sites on the protein surface involved in non-covalent interactions that lead to aggregation, loss of activity and/or increased immunogenicity [168]. Hyperglycosylated therapeutic proteins may, however, see decreased activity due to steric hindrance [34].

Liposomes

Liposomes demonstrate great potential as a carrier system for systemically administered protein therapeutics. If constructed from biocompatible and biodegradable materials, liposomes cause very little to no antigenic, pyrogenic, allergic or toxic reactions [169]. Furthermore, liposomes can be nonimmunogenic and have already demonstrated delivery of a variety of active protein therapies to cells *in vivo* [9]. Liposomes have been used to cross the blood–brain barrier to deliver an active enzyme when injected in the tail vein of a rat [9,170]. While first generation liposomes are easily cleared from the bloodstream and accumulate in Kupfer cells of the liver and macrophages in the spleen, advances have begun to reduce these problems [169]. To start, PEG-grafted liposomes have increased circulation time, reduced aggregation and decreased capture by the RES. PEGylated liposomes, or Steath™ liposomes (Johnson & Johnson, NJ, USA), have been used to deliver the anthracycline chemotherapeutic doxorubicin and were able to deliver preferentially to the tumor site, likely via the enhanced permeability and retention (EPR) effect [171].

Fusogenic modifications to liposomes

Many modifications have been made to liposomes to increase intracellular delivery of proteins. When liposomes enter the cell they are contained in an endosome. Particles smaller than 300 nm usually do not enter cells through the endosomal pathway, but particles 500–700 nm are often taken up by endocytosis [172]. If the liposome or the contents of the liposome do not escape the endosome, the endosome will deliver its contents to the lysosome, where the therapeutic peptide will be digested. One method of facilitating endosomal escape is to include a pH-sensitive element into the liposome. The pH in the endosome is approximately 5, and many systems take advantage of this relatively low pH to allow liposomes to escape the endosome [172]. Methods for endosomal escape include pore formation in the endosomal membrane, the proton sponge effect, and fusion with the endosomal membrane [172].

Pore formation is based on a pore-forming or pore-enlarging molecule binding to the rim of a pore in the endosome. Once bound, the pore-forming agent reduces tension in the membrane, which then keeps the pore radius stable [172]. Therefore, these agents act to stabilize naturally forming pores rather than to form pores *de novo* [173]. Pore forming compounds include penton base, cholera toxin, melittin (the major ingredient in bee venom) and Shiga toxin [174–177]. The pH buffering effect, also known as the proton sponge effect, occurs when the low pH in the endosome leads to the protonation of molecules contained

inside the endosome. If the molecule has a high buffering capacity, protonation leads to an influx of H^+, Cl^- and H_2O, resulting in osmotic swelling and eventual endosomal rupture [172]. Examples of molecules causing the proton sponge effect include gp41 with polyethyleneimine, poly (L-histidine) and chloroquine [178–181]. Fusion within the endosome requires fusogenic peptides that undergo conformational changes with the lowered pH, allowing fusion with the lipid bilayer of the endosome [172]. For example, a decrease in pH converts hemagglutinin, a protein in the capsid of the influenza virus, from an anionic hydrophilic coil to a hydrophobic helical conformation, followed by fusion of the viral membrane to the endosomal membrane. Fusogenic peptides used in liposomes include the HA-2 subunit of hemagglutinin, influenza-derived diINF-7, the major envelope protein E of the West Nile Virus, glycoprotein H from herpes simplex virus, and KALA based on the HA-2 subunit of influenza hemagglutinin [172,174,182–185]. For a complete discussion of endosomal escape pathways, please see Varkouhi *et al.* [172].

One more fusogenic agent worth mentioning in detail is dioleyl phosphoethanolamine, or DOPE. This peptide exhibits a conical shape due to its small and minimally hydrated head group compared with its highly lipophilic tail [169]. It can be used as a stabilizer in cationic liposomal membranes, but its major activity concerns endosomal escape [169]. As the pH drops in the endosome containing a DOPE-liposome, it is hypothesized that DOPE displays an inverted hexagonal phase, which in turn destabilizes the endosomal membrane [186]. DOPE has been used to deliver Print3G, a hydrophilic 25-amino acid antagonist of an oncoprotein involved in breast cancer. Print3G was enveloped in a Stealth™ pH-sensitive liposome and was able to deliver the peptide to the cytoplasm of cancerous cells [169]. While PEGylation reduced the pH-dependent release, it did not hinder the cytoplasmic delivery of the liposomal cargo [187].

Micelles

Due to their large size, liposomes may have difficulty reaching the desired site of action, as the liposome may be larger than the vascular cutoff size in certain tumors [188]. If this is the case, micelles may be a better alternative. A study by Weissig *et al*. demonstrated this by comparing micelle and liposome protein delivery side-by-side in a Lewis lung carcinoma mouse model. The PEG–micelle delivered more of the therapeutic protein at the desired site than the long-circulating PEG–liposome [189]. Micelles, however, have inherent problems that may prevent them from being used in the delivery of therapeutic proteins, including low drug loading capacity, low stability in water (especially when diluted), short half-life in biological environments and possible *in vivo* toxicity [190].

Nanoparticles

NPs play a role in the protection and delivery of peptides in systemic circulation as well. One example, the carbon nanotube, is well-studied and has been used to deliver proteins [191]. A 2005 study by Wong and colleagues allowed for the pro-apoptotic protein cytochrome c to spontaneously adsorb onto carbon nanotubes. The nanotubes were then incubated with a variety of cell lines and were taken up via energy-dependent endocytosis. Once in the cell, cytochrome c was released from the nanotube and caused increased apoptosis over the empty control nanotube [191]. It has been consistently reported that ‘well processed, water-soluble nanotubes exhibit no apparent cytotoxicity to all living cell lines investigated thus far, at least in the timeframe of days’ [192]. In general, carbon nanotubes have a high propensity to cross cell membranes with the apparent mechanism being passive and endocytosis independent [193,194]. A proposed mechanism for cell entry is similar to that of nanoneedles, where the NPs perforate and diffuse through the lipid bilayer without causing damage or death to the cells [194]. However, nanoparticle targeting is not optimal,

and NPs often have poor tumor and tissue penetration. The EPR effect may also be overstated; thus passive targeting of NPs is not as good as once thought [195].

Functionalized NPs have been used to deliver antibodies, active proteins and epitope peptides to the immune system [194,196]. A recent study revealed new details on the mechanism of protein release from protein-loaded nanoparticle systems. The release of protein from an aliphatic polyester-based nanoparticle system was caused by bulk degradation of the nanoparticle. Moreover, intramolecular transesterification was followed by hydrolysis of the polymers, which caused the degradation [197]. Further, lyophilizing NPs led to a higher burst release of protein (40–50%) compared with nonlyophilized NPs (10–20%). The authors concluded therefore that freeze–drying forms pores in the NPs, thus facilitating burst release of encapsulated protein [197].

Many types of NPs other than carbon nano-tubes have been used in systemic drug delivery. A type of nanoparticle made of a PCL–PEO combination was able to demonstrate increased accumulation at the tumor site as well as reduced clearance by macrophages of the liver, thus increasing the possibility of the nanoparticle taking advantage of the EPR effect [198].

Cell penetrating peptides

While increased systemic circulation time and cargo stability are important factors, all of this is futile if the therapeutic is not internalized into the cells of interest. A promising and adaptable system for increased internalization is CPP. CPPs are short, water-soluble, poly basic peptides with a net positive charge at physiological pH [199]. CPPs are able to penetrate cell membranes at low micromolar concentrations without causing significant membrane damage [199]. The internalization method of these CPPs and their covalently attached cargo is still being debated; there is evidence of both energy-independent internalization and endocytosis as the mechanism of internalization. It is currently believed that endocytotic entry followed by endosomal escape is the most common entry pathway [200,201]. The endocytotic pathway is further broken down to include macro-pinocytosis and receptor-mediated endocytosis [200]. While receptor-mediated endocytosis relies on clathrin, caveolin, or both for internalization, macropinocytois may be internalized regardless of cell receptor status [200].

Targeting & membrane permeation

CPPs are a versatile tool that can be used for increased internalization of liposomes, NPs, or proteins themselves [202–204]. CPPs have been extensively researched as supplements to liposomes, and a few issues have been uncovered. First, CPPs such as TAT are susceptible to enzymatic cleavage by plasma enzymes when they are on the surface of liposomes [202]. Also, CPP-modified liposomes can cause severe toxicity and are rapidly cleared from the blood and accumulate in the kidney and liver; therefore, PEG modification is often necessary when using CPPs with liposomes [205–208]. Unfortunately, PEGylation of CPP-modified liposomes appears to decrease the effectiveness of the CPP [209]. TAT and arginine-rich CPPs have been used to target the kidney and spleen, respectively [210,211]. One study demonstrated that R8 (8 arginine repeat)-modified lipid NPs were able to efficiently deliver cargo (in this case siRNA) to the cytosol of cells in the liver [212].

CPPs have also been directly conjugated to proteins for delivery *in vivo* and *in vitro* [213–218]. One successful example of *in vivo* use delivered a single chain antibody Fv fragment to tumors, resulting in a decrease in tumor volume and neovascularization [216]. Targeted CPPs have also been discovered and designed. The specificity is often gained via activation of the CPP in the tumor environment or by conjugating a targeting moiety to the CPP [219,220,301]. Selectivity can also be obtained by having a homing motif in the CPP

sequence; Nishimura *et al*. have discovered a CPP screened by phage display that selectively transduces leukemia cells [221]. The CPP consists of a lymph-node homing motif (CAY) and the CPP motif (RLRR), with the full sequence being CAYHRLRRC [221]. This CPP is currently being utilized in our laboratory to deliver a protein therapeutic for chronic myeloid leukaemia therapy. CPPs are appealing, as they may be able to increase the delivery of protein therapeutics through the cell membrane, escape from endosomes and get into the cytoplasm of the desired cells. **Table 7** provides an representative example from a variety of CPP classes [217,219,221–223].

Antibodies are another modification strategy that has been implemented to increase the targeting ability of liposomes and NPs. One study by Kirpotin and colleagues demonstrated that monoclonal antibodies (MAb) directed against Her2/neu increased the cytoplasmic delivery of the liposomes contents [224]. Interestingly, in this study the MAb did not alter the biodistribution of the liposome, but rather increased MAb-mediated endocytosis, which increased drug delivery to the cytoplasm. Similar methods have been used with PLGA NPs [225]. Researchers were able to demonstrate *in vitro* selectivity and increased internalization of the MAb-adsorbed NPs [224]. As both liposomes and NPs can be loaded and/or coated with therapeutic peptides, targeting via antibodies can lead to increased peptide delivery to a specific site or cell type. The conjugation and adsorption of antibodies to liposomes and NPs is a promising field; further research will likely produce translatable results that will aid in the targeting of therapeutics.

Future perspective

While proteins and peptides have immense therapeutic potential, delivery and systemic stability currently limit their clinical use. The oral route is appealing, as it is a simple and often inexpensive route of delivery. Couple this with reduced consumption of the supplies needed for invasive delivery (iv., intramuscular, sc. and so on), and it is easy to see why patient compliance is highest for orally delivered drugs. Peptides and proteins are readily metabolized in the GI tract, and the hydrophilic nature of most natural peptides restricts movement across the epithelial barrier. Advances in the oral delivery of proteins and peptides have been made by the use of absorption enhancers, enzyme inhibitors and direct structural modification of the therapeutic. Muco-adhesive polymers, nanoemulsions and NPs have been utilized to increase the stability of peptides as well to increase their absorption. However, as of yet, no generalizable strategy for the delivery of peptide and protein therapeutics has been found; many of the strategies in this paper were customized to the peptide being delivered, as the complex nature and variety of peptides and proteins makes this difficult. Work on generalizable peptide delivery systems is ongoing; both the GI-MAPS and SNAC systems demonstrate encouraging data and appear to be well-suited to deliver a large range of peptides. With all of these systems, however, safety and efficacy questions loom large. Long-term safety has not been studied, and safety issues have arisen even with the short-term use of some of the compounds discussed in this review.

By delivering drugs transdermally, issues with GI stability can be avoided, but absorption still poses problems. Systems developed to overcome the barriers posed by the skin include micro-needle technology, thermal ablation, electro-poration, sonophoresis and iontophoresis. Each of these systems was designed with patient comfort and ease of use in mind. Despite some successes with transdermal delivery, most of the systems above have not been used to deliver therapeutic peptides or proteins to humans; further development and study in this area is necessary. While peptide delivery by other non-invasive routes (pulmonary, intranasal, buccal, rectal) is being studied, other reviews provide a more complete coverage of these topics.

After a peptide therapeutic enters the systemic circulation, it must remain active and reach the correct site/cell type in the body. Direct peptide modifications, liposomes, and NPs are used to increase stability, while the addition of antibodies and CPPs are used for targeting and to supplement cellular delivery. Endosomal escape, which threatens to inactivate the therapeutic peptide at the last stage, has also had some promising advances. It is important to note that some of the methods that increase systemic stability are not currently amenable to oral delivery (e.g., liposomes).

Peptide and protein therapeutics, with their high target specificity and broad applicability, have the potential to revolutionize medical therapy. Clearly, there are still challenges to overcome in each of the areas discussed. Optimally, in the future, there will be a system that can be used for the oral delivery and systemic stability of a variety of peptides and proteins. As delivery and systemic stability are two overarching issues with protein and peptide therapeutics, overcoming these would likely lead to even further development of peptide and protein therapeutics with great therapeutic potential.

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Key Term

Opsonization: The process by which an antibody recognizes and binds to foreign particles. After binding, the antibody–particle combination is phagocytosed or directly destroyed by via antibody–dependent cellular toxicity.

Key Terms

P-glycoprotein: 170 kDa transmembrane ATP-dependent drug efflux pump, expressed in various compartments throughout the body. Also known as MDR1, the main function of P-glycoprotein is the transport of xenobiotics across membranes, actively pushing them out of a cell.

Direct PEGylation: The covalent attachment of PEG to the protein or peptide therapeutic. This is accomplished by incubating functionalized PEG molecules with the protein. The linkage is often on the side groups of basic, acidic or polar amino acids.

Key Term

Hyperglycosylation: The addition of extra sugar molecules to the peptide to achieve many of the same benefits as PEGylation. This can be carried out by either specific chemical reactions *in situ* or by site-directed mutagenesis to create additional glycosylation sites into the sequence of the peptide.

Executive summary

Oral delivery of peptide therapeutics

- Barriers to oral delivery:
- Gastrointestinal proteases, tight junctions, efflux pumps and the intestinal epithelial layer limit the oral delivery of peptides.
- Methods to overcome the barriers:
- A variety of absorption enhancers, direct peptide modifications, enzyme inhibitors and carrier molecules have been used to increase the bioavailability of orally delivered peptides;
- Current modifications must be customized to the peptide being delivered, but versatile carrier systems are being developed.

Transdermal delivery

- Barriers to transdermal delivery:
- The skin itself physically acts as the major barrier to transdermal delivery, generally prohibiting passive entry of hydrophilic molecules and hydrophobic molecules greater than 500 D.
- Methods to overcome the barriers:
- Microneedle technology, thermal ablation, electroporation, sonophoresis, iontophoresis and biochemical enhancers are techniques and methods that are currently under study as a means to overcome transdermal peptide delivery barriers;
- Systemic stability.

Systemic inactivation of peptide & protein drugs

- After peptides enter systemic circulation they are susceptible to enzymatic degradation, opsonization, agglutination and poor solubility, all of which can lead to decreased protein activity.
- Methods to increase systemic stability and site-specific targeting:
- Combinations of PEGylation, hyperglycosylation, liposomes and nanoparticles can be used to increase peptide stability;
- Cell-penetrating peptides and antibodies can be used for targeting and increased uptake of the therapeutic at the site of action.

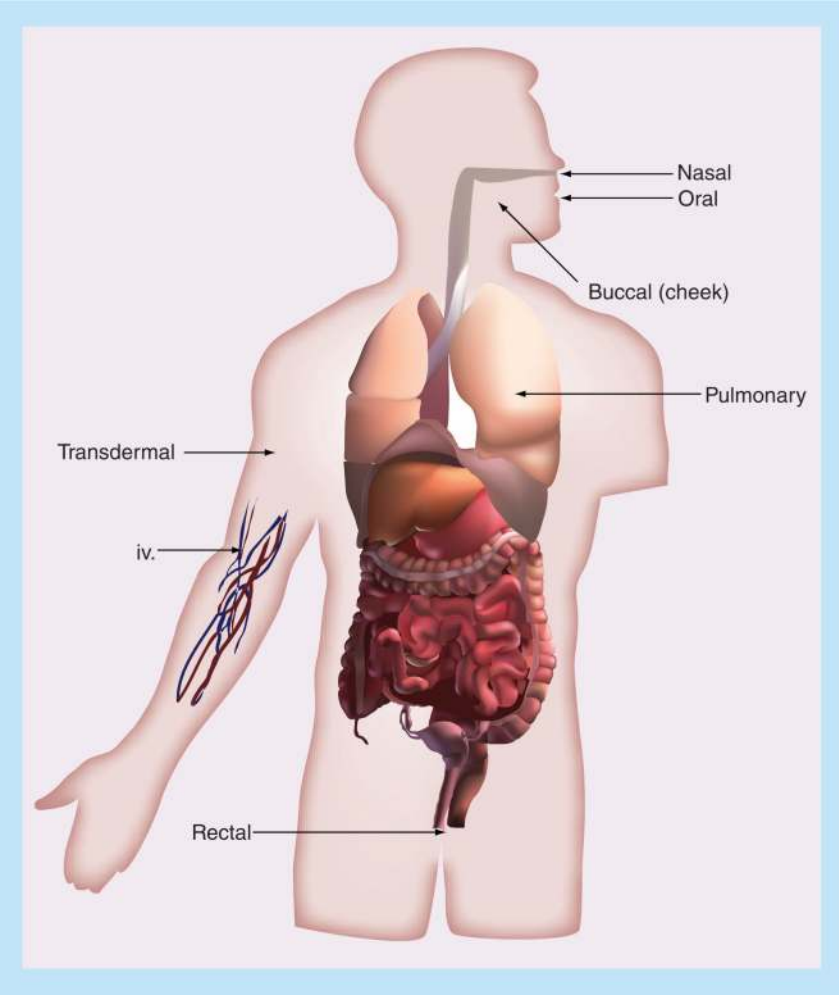

Figure 1.
Common routes of administration for systemic delivery of peptides and proteins.

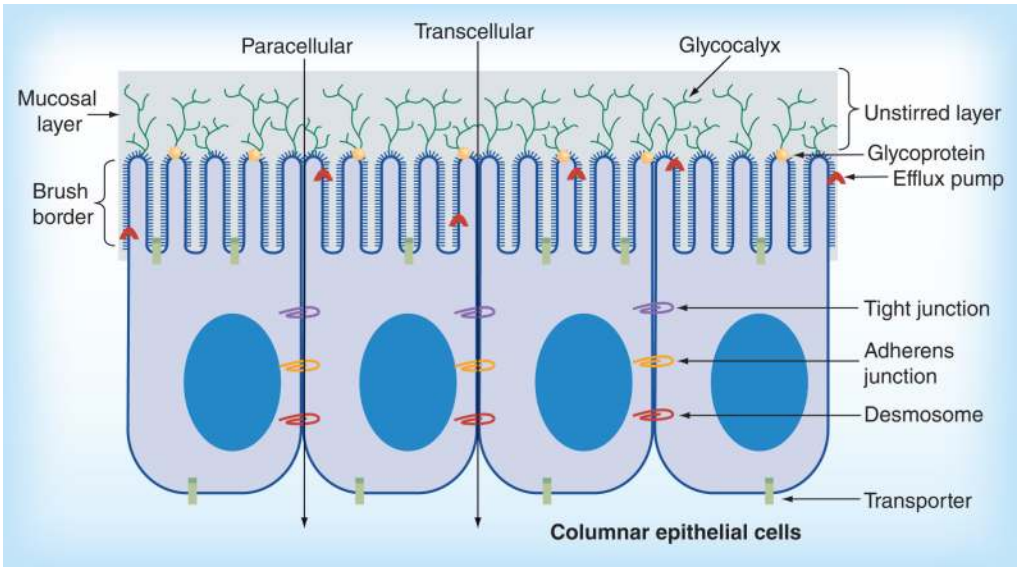

Figure 2. Intestinal barriers to peptide delivery

The epithelial intestinal barrier is made up of a single layer of columnar epithelial cells. The apical side of the barrier contains the mucosal layer. Drugs may penetrate the epithelial barrier either through the transcellular route (across the cell) or the paracellular route (between tight junctions). See text for other term explanations.

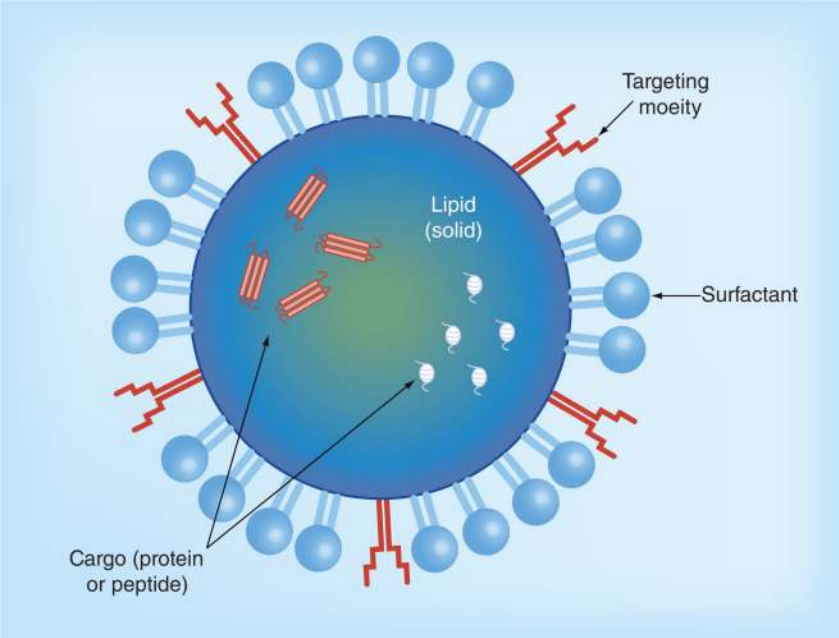

Figure 3. Solid lipid nanoparticle

Solid lipid nanoparticles have a solid lipid core and is coated with surfactant. Targeting moieties may be added to decorate the surface of the solid lipid nanoparticle. Cargo for solid lipid nanoparticle are illustrated as peptides or proteins, but may also include siRNA or small-molecule drugs.

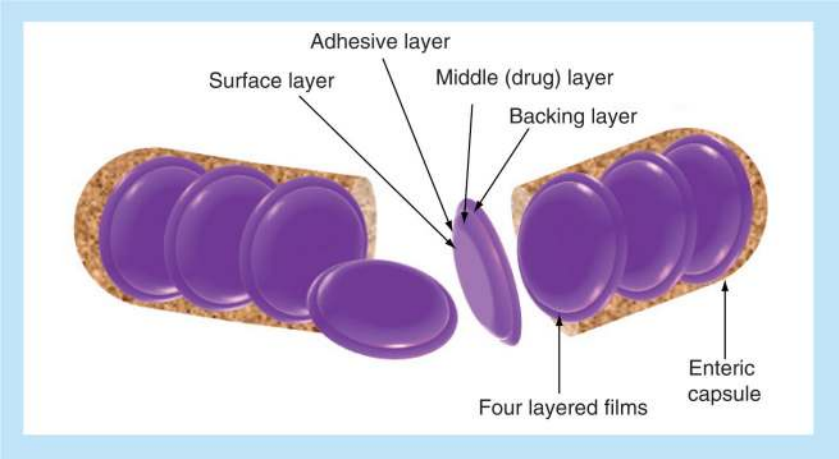

Figure 4. Gastrointestinal mucoadhesive patch system

The system contains four layered films in an enteric capsule. Layer one is the backing layer, layer two is the middle or drug layer, layer three is the adhesive layer, and layer four is the surface layer.

Table 1

Overview of a selection of currently available peptide/protein therapeutics.

Generic drug name	Size	indications†	Recent sales numbers	Route delivered†	Ref.
Etanercept	150 kDa	RA, psoriatic arthritis, plaque psoriasis, ankylosing spondylitis	US$7.9 billion (2012)	sc.	[403]
Insulin glargine	53 AA, 6.1 kDa	Type 1 and 2 DM	$6.6 billion (2012)	sc.	[403]
Pegfilgrastim	39 kDa	Neutropenia	$4.1 billion (2012)	sc.	[403]
Salmon calcitonin	32 AA	Osteoporosis	1,700,000 Units (2011)	im., sc., intranasal	[2]
Cyclosporine	Cyclic, 11 AA	Prophylaxis, solid organ rejection	$579 million (2012)	Oral, iv.	[3]
Octreotide	8 AA, somatostatin analog	Acromegaly, gigantism, symptomatic relief of carcinoid syndrome	Estimated $1.5 billion (2011)	iv., sc., im. (depot)	[4]
Liraglutide	31 AA, 3.8 kDa	Type 2 DM (GLP-1 agonist)	Estimated $843 million (Q3 2012–Q2 2013)	sc.	[401]
Bivalirudin	2.2 kDa	Anticoagulant	$481 million (2011)	iv.	[5]
Desmopressin	9 AA (8 D-AA)	Nocturnal enuresis	610,000 Rx US (2009)	iv., im., sc., intranasal	[6]

AA: Amino acid; DM: Diabetes mellitus; GLP: Glucagon-like peptide; im.: Intramuscular; iv.: Intravenous; RA: Rheumatoid arthritis; sc.: Subcutaneous.

† *Data taken from* [402].

Table 2

Overview of peptide modifications and delivery systems associated with their site of action.

Goal of modification	Peptide
Stomach	
Increased Stability	PEG, D-amino acids, nanoparticles, SLN
Small intestine	
Increased Stability	Cyclization, PEG, lipidization, D-amino acids, polymer matrices, nanoparticles, esterification, *N*-acetylation
Enzyme Inhibitors	Soybean trypsin inhibitor, aprotinin, puromycin, bacitracin
Absorption Enhancers	Chitosans, fatty acids, lectins, ZOT, CPP, liposomes, nanoemulsions, mucoadhesive polymers, nanoparticles, SLN
Circulation	
Increased Stability	PEG, hyperglycosylation, liposomes, nanoparticles
Targeting	Antibody CPP

CPP: Cell-penetrating peptide; SLN: Solid lipid nanoparticle; ZOT: Zonula occludens toxin.

Table 3

Direct modifications of peptides and proteins. Overview of direct modifications made to peptides and the resulting change in bioavailability.

Method of modification	Subtype of modification	Successfully modified compound(s)	Outcome	Ref.
Cyclization		CSA, somatostatin, encephalin	Reduces hydrophilicity, decreases conformational flexibility, enhances membrane permeability and increases stability to proteolysis	[8,31,32]
PEG	Direct, permanent modification	Insulin, salmon calcitonin	Resistance to intestinal enzymes, slowed systemic clearance, increased intestinal absorption, decreased glucose levels (insulin)	[41,42]
	Prodrug	IFN-B-1b	Decreased aggregation, maintained activity, dimished IgG response, 100-fold increase in AUC, improved protection from enzymatic degradation	[41,53]
B12 conjugation	ε position on the corrin ring	Albumin, G-CSF, EPO, LHRH and analogues, DP3, dextran nanoparticles	Increased oral absorption via vitamin B12-intrinsic factor binding	[44]
	5′-hydroxy on ribose unit of the α tail	IFN, insulin	Efficacy is hindered by naturally low capacity for B12 absorption	[44]
	Phosphate unit of the α tail	Albumin, γG-globulin	Steric hindrance limits absorption, activity of the therapeutic	[44]
Lipidization	Reversible aqueous lipidization with n-palmitoyl cysteinyl 2-pyridyl disulfide	Salmon calcitonin	AUC 19-times higher than unmodified, marker for bone resorption reduced	[45]
N-acetylation		Salmon calcitonin	Improved oral bioavailability, marginally improved resistance to trypsin and leucine aminopeptidase, enhanced membrane permeability	[54]
D-AA	6 out of 11 L-AA substituted for D-AA	MUC2	Resistance to proteolytic cleavage by chymotrypsin, elastase, papain, pepsin, trypsin and carboxypeptidase	[52]
Prodrug	Esterification	Desmopressin	Increased permeation of Caco-2 cell layer, active in plasma after cleavage	[55]
	Perbuytrylation	Glycovir	Increased bioavailability	[56]

AA: Amino acid; AUC: Area under the curve; CSA: Cyclosporine; DP3: Octapeptide (Glu-Ala-Ser-Ala-Ser-Tyr-Ser-Ala); EPO: Erythropoietin; LHRH:Lutenizing hormone releasing hormone.

Table 4

Enzyme inhibitors, their targets and effects on peptide delivery. An overview of potentially clinically relevant enzyme inhibitors.

Enzyme inhibitor	Molecules inhibited	Effect on peptide drugs	Ref.
Soybean trypsin inhibitor, FK-448	Chymotrypsin	Enhanced intestinal absorption of insulin in rats and dogs. Suppressed digestion of insulin by pancreatic enzymes	[57]
Aprotinin	Serine proteases, specifically trypsin, chymotrypsin, and plasmin	Intraileally administered insulin with aprotinin led to decrease in blood glucose of 30% compared with controls	[58]
Puromycin	Serine and metallopeptidases	Improved stability of leucine encephalin, and stability and permeability of D-Ala2, D-Leu5 enkephalin (DADLE)	[66–68]
N-acetylcysteine	Inhibits aminopeptidase N and has mucolytic properties		[65,69]
Bacitracin	Trypsin and pepsin, aminopeptidase N	Used to increase delivery of insulin, met-kephamid and buserelin	[65,69,70]

Table 5

Polymer carrier systems. Commonly used polymers for construction of nanoparticles, their biocompatibility and use in peptide delivery.

Polymer	Biocompatibility†	Example of use	Ref.
PLA	Biocompatible and biodegradable	BSA loaded with 71 % efficiency, BSA was stable after release	[114]
PCL	Degraded by hydrolysis	Preparation of long-term implantable device Insulin loaded with 96% efficiency, improved response to OGTT Has mucoadhesive properties AmB loaded PCL nanoparticles two- to three-times more effective than free AmB	[115,116]
Chitosan	Nontoxic, biocompatible	Insulin-loaded chitosan nanoparticles enhanced intestinal absorption of insulin through a combination of insulin internalization in enterocytes and insulin-loaded particle uptake by Peyer's patches	[117]
Gelatin	Nontoxic, biodegradable	Encapsulated paclitaxel, oligonucleotides, chloroquine	[118–120]
Poly(alkyl-cyano-acrylates)	Biodegradable and biocompatible, degraded by esterases. Produce some toxic metabolites, not suitable for human use	Encapsulated doxorubicin, ampicillin, indomethacin	[111]
PLGA	Biodegradable, excellent toxicological profile	PLGA nanoparticles with influenza HA incorporated throughout the matrix, increased uptake via M cells	[121]

AmB: Amphotericin B; BSA: Bovine serum albumin; HA: Hemagglutinin; OGTT: Oral glucose tolerance test; PCL: Poly(ε-caprolactone) PLA: Poly(lactic acid); PLGA: Poly(lactic-co-glyclic acid).

†*Data taken from* [111].

Table 6

Multi-component carrier systems. These systems are composed of multiple parts and chemicals. They are designed to allow for the delivery of a variety of peptides and proteins.

System	Product name	Effect on absorption	Therapeutics tested with the system
GI-MAPS	Eudragit® L100, Eudragit S100, HP-55	Protection of peptide, increased exposure to GI tract	Granulocyte colony-stimulating factor
SNAC carrier molecule	Emisphere™	Increased membrane permeability	Insulin, calcitonin, growth hormone, GLP-1
Amphiphilic oligomers	HIM2	Resists GI enzyme degradation and increases membrane permeability	Insulin, calcitonin, enkephalin, parathyroid hormone
Lipid-based microemulsion	Macrulin™	Protects peptide against acidic and proteolytic degradation; increased GI absorption	Insulin, sCT
Protein crystallization	CLEC™	Increased stability against proteolysis	Calcitonin, lipases, polypeptides

GI: Gastrointestinal; GI-MAPS: Gastrointestinal mucoadhesive patch system; GLP: Glucagon like peptide; sCT: Salmon calcitonin; SNAC: n-(8-[2-hydroxylbenzoyl]amino)caprylic acid.

Adapted from [63].

Table 7

Sample cell-penetrating peptides and their applications in targeted and untargeted peptide delivery.

Name/Description	Sequence	Category	Outcomes of interest	Ref.
TAT	CGRKKRRQRRRPPQC	Protein derived CPP from HIV-1	Able to deliver peptides, proteins, nanoparticles and oligonucleotides intracellularly	[222]
Penetratin	RQIKIWFQNRRMKWKK	Protein derived CPP from *Drosophila* antennapedia domain	Able to deliver a variety of cargoes to many cell types	[223]
LS-CPP	CAYHRLRRC	Dual-motif CPP	Specific delivery to leukemia cells	[221]
Tumor prodrug CPP	EEEEEDDDDK/ARRRRRRRRR	Prodrug with tumor environment activation	Proteases in the tumor environment cleave the sequence, removing the acidic (negative) amino acid residues	[219]
Antibody–CPP conjugate	Penetratin+MAb CC49	MAb targeted CPP	Increased tumor: normal tissue delivery ratio	[217]

This table contains representative CPPs that represent the major classes of targeted and untargeted CPPs. TAT and Penetratin are generally untargeted, although their biodistribution may cause site-selective accumulation. LS-CPP is a leukemia-specific CPP. The tumor prodrug CPP is cleaved at the '/' when in the tumor environment, thus separating the negatively charged amino acids from the positively charged CPP-cargo conjugate. Antibody-targeted CPPs can be used to selectively deliver a CPP–cargo conjugate to desired cell types.

CPP: Cell-penetrating peptide; LS-CPP: Leukemia-specific cell-penetrating peptide; MAb: Monoclonal antibody; TAT: Trans-activating transcriptional activator.