Intestinal receptor targeting for peptide delivery: an expert's personal perspective on reasons for failure and new opportunities

The technology has been available more than 25 years that would enable the oral delivery of vaccines, proteins and peptides, thus avoiding the need for injection. To this day, injection is still the mode of delivery, yet not the main mode of choice. This review focuses on several of the potential modes for oral delivery of peptides, proteins and vaccines. Additionally, the review will provide the reader with an insight into the problems and potential solutions for several of these modes of oral delivery of peptides and proteins.

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Transport of peptides & proteins across the GI epithelial cell

There are many potential barriers for oral delivery of peptides and proteins, but perhaps the two greatest are the highly proteolytic environment encountered in the intestinal tract and the lack of uptake of peptides and proteins across the intestinal wall. The intestinal epithelial cell (the enterocyte) presents itself as a single cell barrier to the uptake of nutrients, minerals, vitamins, peptides and proteins from the intestine. Even the uptake of very small molecules such as water requires major intrinsic proteins that form pores in the cell membrane (aquaporins). These pores allow small hydrophilic molecules to pass through the hydrophobic medium, that is, the cell membrane [1–5]. Other molecules such as hexose are transported by the sodium–glucose transporter (SGLUT1), which allows uptake by co-transportation with sodium, and GLUT2 and GLUT5, which are involved in the absorption of fructose.

It was long thought that fatty acids did not require a transporter and that these molecules could 'phase partition' into the enterocyte membrane and, hence, gain access to the circulation. This is now known not to be the case and several fatty-acid transporters have been identified, one of which, FATP4, is expressed in high amounts on the apical membrane surface of enterocytes [6]. There are also sodium-dependent monocarboxylate transporters expressed in the small intestine [7], which are responsible for transport of molecules such as γ-hydroxybutyrate, L-lactate and pyruvate. Other smal-molecule transporters include amino acid transporters (LAT3, PROT, CSNU1, CSNU3, 4F2HC, CT1 and ASCI), peptide transporters (PEPT1 and HPT1) [8–11] and nucleoside transporters (CNT2 and SBC2). There are also organic cation transporters (SFXN1, OCT5 and OCTN2), organic anion transporters (NBC3, SDCT1, NADC1 and NBC1), bile acid transporter [11] and the fatty acid transporters FABP1 and FABP2 [12]. Additionally small atoms such as copper have their own transporter [13]. Essentially, there appears to be some sort of transporter for all small dietary molecules, but what about for larger molecules such as peptides and proteins?

Transport of peptides & proteins across the gut wall

Transport of large molecules, such as peptides and proteins, presents itself as a much bigger problem than transport of smaller nutrient molecules, such as amino acids, sugars and some small water-soluble vitamins. This problem is not characteristic of only the gut, but is also shared by all cells in the body, including the alveolar epithelium [14], vascular endothelium [15–18], renal proximal tubular endothelium [19–21] and blood–brain barrier [22–26]. The general phenomenon by which larger molecules cross these endothelia is that of receptor-mediated endocytosis, followed by transcytosis. In this process, a specific receptor, which is expressed on the luminal side of the intestinal epithelial cell (enterocyte), alveolar epithelium and vascular endothelium, binds to its target ligand, and once the ligand (in this case, the peptide or protein) is bound to this receptor, the ligand is internalized, transported across the epithelial cell and the ligand 'discharged' from the basal surface into the sub-endothelial

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Key Terms

Receptor-mediated transcytosis: Mechanism by which ligands, which are bound to surface receptors on one side of the cell are transported across the cell and are released on the other side of the cell.

Lactoferrin: Iron-binding glycoprotein found in milk and colostrum.

Transferrin: Glycoprotein that binds iron very tightly but reversibly.

Insulin receptor: Specific receptor that binds to insulin and which may thereby stimulate transport of the insulin across via receptor mediated transcytosis.

IgG-Fc receptor: Receptor expressed upon the surface of many cells, which specifically binds to the Fc fragment of the IgG class of antibodies.

interstitial space. From here the ligand can either pass into the draining venous or lymph systems.

Evidence of receptor mediated transcytosis in the gut

■ Receptor mediated transcytosis in the embryo

There is a considerable amount of evidence to show that early in embryonic development, the transport of vitamins and proteins into the developing embryo is dependent upon specific transporters. Thus, separate transporters have been identified that are required for the uptake of biotin [27–29], folate [30,31], riboflavin [32–36] and thiamine [35,37,38] in the developing embryo.

■ Receptor-mediated transcytosis in the neonate

During the growth of the fetus in the womb, one of the many changes that occurs is the development of **receptor-mediated transcytosis** (RMT) in the intestine, which matures near the time of birth [39]. Evidence suggests that the offspring of many suckling mammals obtain maternal serum proteins from maternal colostrum through the process of RMT in the neonatal intestine.

The major proteins identified in colostrum include immunoglobulins, particularly IgG, IgA and IgM; the cytokines IL1, IL2 and IL6; iron(Fe)-binding proteins, such as **lactoferrin** (Lf) and **transferrin** (Tf); the oligonucleotide-peptide transfer factors [40]; growth and maturation factors such as fibroblast growth factor, IGF-I, IGF-II, EPO and somatostatin [41,42]; and TGFα and β, insulin, PDGF and EGF [42,43]. It has been shown that there are receptors for many of these factors distributed along the intestinal epithelium and it has been postulated that these factors function as mediators of intestinal growth and development [42,44]. Receptors for these molecules appear to be fairly ubiquitous amongst mammals, and orally administered bovine colostrum has been shown to be effective in altering immune function in species as diverse as cattle, horses, pigs [45], sheep, cats, mice, rats, hamsters and ferrets. In humans, apically expressed receptors involved in uptake of EGF, insulin, IGF-I, hepatocyte growth factor, glucagon-like polypeptide receptor, and IgG Fc have all been identified [39,42]. Experiments in rats and mice have shown that many of the receptors present are active in RMT. Thus, suckling and weanling rats and mice have been found to transport corticosterone, prostaglandins, insulin, prolactin, EGF, IGF, TRH, TSH and somatostatin from the intestine into the circulation [39].

■ Receptor-mediated transcytosis in the adult

There are potentially three fates for molecules that bind to or are bound by the intestinal epithelial cells. First, the molecule can bind to the intestinal epithelium but is then not internalized (i.e., tomato lectin [46–49]). Second, the molecule can bind to the epithelial cell, be internalized by it, but not transported across the cell (i.e., cholera toxin). Third, the molecule can bind to the cell, be internalized by the cell, and then transported undegraded across the cell. Thus, even though the presence of an intestinal receptor for a particular protein can be detected, effective protein delivery using these molecules as carriers also requires that binding of the proteins to receptors involved in uptake is able to elicit receptor-mediated transcytosis. These transcytotic receptors are the major focus of this review.

Once the transported molecule crosses the intestinal epithelial cell, it may either enter the intestinal venous system directly, or it may enter the draining lacteal vessel. The route that the molecule follows will determine the speed at which the molecule appears in serum. Thus, direct transport into the blood leads to rapid appearance of the molecule in serum (i.e., glucose), whilst transport via the alternative, lymphatic route is much slower (i.e., vitamin B_{12} [VB_{12}] and Tf) and levels in serum may take several hours to peak.

Transport of macromolecules from the intestine into the circulation is further complicated by the type of receptor involved. In many cases, the receptor binds directly to the molecule to be transported, such as in the case of the **insulin receptor**, **IgG-FcRn**, Poly-Ig receptor and EGF receptor, whereas in other cases a separate, soluble protein is required to bind to the molecule to be transported, and this transport protein is then bound by a membrane receptor located on the intestinal epithelial cells. Such is the case for transport of VB_{12}, riboflavin and possibly biotin.

Conservative evolution

For many of the processes, an assumption could be made that the processes are evolutionarily conserved, and hence data from one species could be transferable to another species. It is important to note, however, that while the process is the same in different species, it may be quantitatively different between species. An

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example can be seen with VB_{12} uptake, where the process of uptake is the same in mice, rat, dogs, cats and man, but the levels of uptake are quantitatively different.

Oral immunogens

Early evidence that it may be possible to deliver peptides and proteins came from experiments on oral feeding of lectins [50–54,301]. These molecules bind to surface glycolipids and glycoproteins on the enterocyte and in some instances are internalized via receptor-mediated endocytosis, cross the enterocyte via RMT and can later elicit an immune response in serum [55–57,301]. It is this process, which has been postulated to be responsible for the allergic response to dietary allergens [58]. At the time when this process was initially described, it was not the accepted paradigm for oral vaccination, as it was generally thought that uptake of intact proteins in the gut occurred via nonspecific antigen-sampling activity of the intestinal M cells, which promoted uptake of these drug-laden particles from the intestine [59–61]. It was subsequently shown by Gabor, Russell-Jones and others [51,52–55,62–66] that effective oral immunization could be achieved using proteins that possessed lectin-like binding activity for the glycolipids and glycoproteins resident on the luminal membrane of the enterocyte [67,68]. The specificity of this phenomenon was demonstrated when the specific sugar molecule to which the lectins bound was co-fed with the lectin and a greatly reduced immune response was elicited to the lectin [52,55,67]. More recently, conjugates between lectins or lectin fragments have been used for oral vaccination [56,69–72]. Lectins have also been used to target orally administered liposomes [73–75] and nanoparticles [76–83]. Although these systems are highly effective in generating immune responses to the targeting agents, their continued use is potentially limited by the generation of an immune response to these highly immunogenic carriers and, as such, their utility for daily or weekly use as carriers for peptides and proteins is questionable [56]. For this reason, no further discussion of these systems will be included in this review.

Transferrin

Tf is one of the major proteins involved in uptake and transport of Fe from the intestine. The majority of Tf (~100 mg/day) is produced in the liver and secreted into bile, from which it subsequently travels into the small intestine. Once in the intestine, Tf binds to Fe^{3+} and the [$(Fe^{3+})_2$–Tf] complex is bound either by the Tf receptor on the surface of the duodenal enterocyte, or to cubulin [84], which has been shown to have receptor-binding activity for ($[Fe^{3+}]_2$–Tf) complex. The complex is then internalized and transported across the epithelial cell into the central lacteal lymph vessel and then on into the mesenteric lymph node eventually traveling via the thoracic duct and then into the circulation. The recycling of Tf from the liver, through bile, into the small intestine and then uptake across the intestine has lead many researchers to examine the potential to use Tf as an oral targeting agent for Tf conjugates and Tf fusion proteins, with initial estimates suggesting the potential delivery of more than 100 mg/day.

Widera and co-workers [85] found that disulfide-linked conjugates between GCSF and Tf were actively transported across rat alveolar epithelial cell monolayers [84,85]. Similarly, insulin–Tf conjugates were shown to be transported across Caco-2 monolayers [86,87]. Orally administered insulin–Tf conjugates have been found to have an anti-diabetic effect in diabetic rats [87–89].

Fusion proteins formed between GCSF and Tf were found to be active in inducing myelopoiesis following oral administration to rats [85,90,91]. Extensive work has been carried out by these researchers on fusion-linker technology [92–94]. Similarly, human growth hormone–Tf conjugates have been found to be active after oral administration to hypophysectomized rats [95].

Lactoferrin

One of the major protein species found in colostrum and milk is Lf, an immunomodulatory Fe-binding glycoprotein. Several studies have shown the presence of an intestinal receptor for Lf in both neonatal and adult animals, which is responsible for uptake of the molecule following oral administration. Thus, Takeuchi and co-workers demonstrated rapid uptake of bovine Lf from the duodenum of rats, with levels of 200 ng/100 gm body weight of rats seen within 60 min of administration [96,97]. Harada and co-workers have shown that the Lf contained in milk is transported into the circulation from the intestinal lumen and excreted into bile [98]. Absorption of Lf has been shown to occur in both neonatal [98,99] and weaned pigs [100,101], young cattle [102], adult mice [103–105] and humans [106–109]. Lf has been found at high concentrations in bovine colostrum (151 mg/l) [110] and a specific Lf receptor has been found in the

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intestines, including the duodenum, jejunum, ileum and colon in the adult cows [102]. Cox and others [111,112] have shown the Lf receptor on the brush border of the human GI tract.

Several studies have shown that Lf is able to resist degradation in the intestine and to be absorbed functionally intact from the intestine, following oral administration. Thus, oral administration of Lf to ovarectomized rats resulted in preserved bone mass and also improved bone microarchitecture [113]. Similar findings were observed following oral Lf administered to mice [104,114,115], rats [116] and oral Lf has been given for cancer treatment in rats [117,118] and in Phase I and II trials for cancer treatment in humans [108,109,119]. Increased resistance to degradation has been achieved by PEGylation of Lf, resulting in a tenfold increase in uptake [120,121] and following oral administration in enteric formulations [97,112].

Oral uptake via the insulin receptor

The ability of orally administered insulin to mimic the natural route of endogenous insulin secreted by the pancreas into the portal vein, and thence directly into the liver, has potential therapeutic advantages in diabetes therapy, particularly when compared with subcutaneously injected prandial insulin. Apart from this route being the more 'natural route', there are potential advantages of this route in controlling the weight gain and other undesirable sequelae that are seen with the 'non-natural' subcutaneous route administration of insulin [122–135]. Thus, orally administered insulin would potentially reach the liver in a similar fashion to naturally secreted insulin. In contrast, sub-cutaneously administered insulin is readily available to stimulate surrounding adipocytes, thereby stimulating differentiation and division of these cells with accompanying weight gain. In addition, subcutaneously administered insulin results in much higher circulatory levels of insulin than those seen following normal food ingestion [135]. This potential activity following oral delivery does, however, rely upon the premise that orally administered insulin will travel via the portal vein into the circulation and, hence, reach the liver first (first-pass metabolism), rather than enter the lacteal lymph vessel, where it will ultimately travel via the mesenteric lymph node and from there via the thoracic duct to the superior vena cava, thereby avoiding first-pass metabolism.

The insulin receptor has been found to be expressed on the luminal surface of both neonatal and adult small intestinea, and has a putative role in gut maturation in the neonate [136–145]. Furthermore, studies have shown active transcytosis of insulin though endothelial cells [146], which have been confirmed by microscopy and electron microscopy studies [147,148] suggesting the possibility of using this molecule either as its own delivery system or as an oral transporter.

There are several reports of abnormally high oral bioavailabilities of insulin (up to 22%) following incorporation within protective matrixes, such as nanoparticles and mucoadhesive polymers [59,149–166]. Similar results have been reported by Pan and others [68], who estimated an oral bioavailability of 14.9% for 250–400 nm chitosan NPs containing insulin. Chalasani and co-workers [167,168,302,303] reported an oral bioavailability of approximately 15% for insulin within dextran 'nanosponges'. The small size of these nanoparticle preparations means that the majority of the insulin entrapped within the structures is located on or near the surface of the nanoparticles. Such material has been found to be rapidly lost/displaced from the particles upon exposure to small intestinal juice (see section on VB_{12} nanoparticles, later in this article). This would then make the released insulin available for binding to the insulin receptor on the small-intestinal enterocyte, provided it was still in monomeric form, and had not been degraded by intestinal enzymes.

Other studies using solubilizing agents and 'permeation enhancers' have shown enhanced oral delivery of insulin, but not other peptides or proteins [169–172]. Similarly, Oramed Pharmaceuticals (Israel) claims to be developing an oral-insulin product, in which unmodified recombinant human insulin is mixed with 'adjuvants' that are purported to protect the insulin from enzymatic degradation in the GI tract and promote its absorption in the gut via some undisclosed mechanism [173–175]. A similar mode of protection is afforded in the formulations of Capsulin [176] or PEGylated insulin [177]; however, once again there has not been any proven mechanism for the mode of uptake of the orally administered insulin.

Various attempts to apply liposomes to the preparation of oral insulin have been reported [178–186]. The hypoglycaemic effect of liposomal insulin has been found to depend on the lipid composition, surface charge and physical state of phospholipid bilayer [178,181,183,186]. A marked decrease in blood-glucose concentration has been observed after intragastric

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administration of liposomes formed with high-melting dipalmitoyl phosphatidylcholine [184] or negatively charged phosphatidylinositol. Many of the liposomal formulations have questionable stability in the lipolytic milieu of the small intestine, and the observed reductions in serum glucose following oral administration could be explained by the release of entrapped insulin with subsequent binding to the insulin receptor. In this regard, Katayama and co-workers were able to increase the hypoglycemic effect of liposomally entrapped insulin through the co-entrapment of protease inhibitors, suggesting that normally the liposome-entrapped insulin is exposed to intestinal proteases, the action of which could be reduced by co-entrapped protease inhibitors [187].

Doyle and co-workers produced a VB_{12}–insulin conjugate that was found to lower blood glucose following oral administration of 100 nM (~600 µg insulin and 135.6 µg VB_{12}) dose to rats [188,189]. The uptake capacity for VB_{12} in rats is approximately 100 ng/dose, which is approximately 3 logs lower than the reported uptake and, therefore, VB_{12}-mediated uptake is not likely to be the uptake mechanism. The conjugate formed was via linkage to Lys-29 on the β-chain of insulin, which would have produced a monomeric molecule. Linkage to the Lys-29 inhibits formation of the insulin hexamer and the highly water soluble VB_{12} derivative would also increase the solubility of the conjugate, allowing binding to the IR (as found by Petrus *et al.* [189]. Similarly, linkage of PEG to the Lys-29 on insulin greatly increased the bioavailability of PEG-insulin (Nobex Coporation, USA) [190]. Thus, linkage of either VB_{12} or PEG to the Lys-29 residue of insulin would have a dual effect of increasing the resistance to intestinal enzymatic degradation as well as allowing for presentation of the insulin monomer to the insulin receptor in the intestine (see discussion below).

There are assorted data showing the potential for oral delivery of insulin via the neonatal insulin receptor. Insulin occurs in milk at approximately 5–50 ng/ml and in bovine colostrum (35 µg/l) [110]. The insulin receptor has been found to be present on the surface of the intestinal epithelial cells [191,192] and transcytosis of insulin though these cells has been described by King and Johnson [146] and Bendayan and co-workers [147,148], suggesting the possibility of using this molecule either as its own delivery system, or as an oral transporter.

Binding of insulin to its receptor requires the insulin to be present as a monomer. Unfortunately, the pH of the GI tract, particularly in the duodenum, is such that normal human insulin exists at best as a hexamer and at worst as an aggregate, thus preventing binding to the intestinal receptor. This potentially explains the high variability often seen in the experiments reported above, as the hexamer formation and insulin aggregation can be hard to control and is very dependent upon pH, which may be highly variable between animals and concentration, which may also be highly variable in the intestine of experimental animals.

Given the possibility that insulin is actually being taken up by its own receptor, it may just be a case of delivering 'protected' insulin in its monomer form to the receptor. In this regard, the majority of studies reported above did not optimize the formulation of insulin to ensure that it was presented in a monomeric form in the intestine and, furthermore, all studies used either standard human or bovine insulin, rather than an insulin preparation such as Lispro or Insulin Aspart, which would have presented as a monomer in the intestine. Proteolytic protection of insulin could be afforded either by chemical modification of the enzymatic sites for cleavage, which may explain the high levels of uptake seen by several researchers [177,187,189,190] or, alternatively, it could be physical protection, such as seen in liposomal preparations [178–185] or nanoparticulate structures [149–164]. These would not require additional targeting, but rather the monomeric form of insulin to be present for maximal receptor binding.

There are several pulmonary delivery systems for insulin that have been developed including Exubera® (Pfizer), AERx®iDMS (Aradigm, USA), AIR (Alkermes, USA), Technosphere® (MannKind Corp, USA) and AERODOSE® (AeroGen Inc, USA) [173,193–198]. However, no mechanism has been proposed to explain uptake via this route. Receptor mediated uptake of insulin via an insulin receptor expressed on the alveolar epithelium is possibly the mechanism by which insulin gains access to the systemic circulation following pulmonary administration. A similar receptor-mediated uptake system for pulmonary Fc-fusion proteins has been demonstrated and is described below.

Oral uptake via the intestinal FcRn receptor

The commercial use of monoclonal antibodies (MAbs) or IgG Fc-fusion proteins is currently the fastest growing area of biophamaceuticals

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development. These are used in a range of treatment modalities with a wide range in specificities including MAbs against GMCSF, TNF, IL12, CDIIa, CD19, CD20, CD22, CD30, CD40, VEGF, ICAM, EGFR, HER-2 and many others. There is an equally impressive list of IgG-Fc fusion proteins that are being developed including Fc-EPO, TNF-receptor-Fc fusions, GLP-1-Fc and IFNβ-Fc fusions. Given the long list of MAbs and IgG-Fc fusion proteins currently in production or under development, it would be advantageous if a generic mode of oral uptake of these proteins could also be developed.

One potential strategy would be to utilize the IgG-FcRn receptor, which has been shown to be present on the intestinal epithelium, as a transporter. This receptor is able to bind IgG via the Fc portion of the molecule, in the acid milieu of the duodenal segment of the small intestine and transport the protein intact across the intestinal epithelium, into the circulation. Binding to the receptor is dependent upon an acidic pH and, thus, the pH difference between the apical (pH 5.5–6.5) and basolateral (pH 7.4) sides of the duodenal enterocytes facilitates the efficient unidirectional transport of IgG. Transport via this mechanism is well established for many colostrally fed neonates, and is particularly well-known in cattle [199], pigs [45,200,201], sheep [202] and rats [203–209].

The receptor appears to occur on the major mucosal sites and the FcRn appears to be relatively conserved across the species as it has been found in the lungs of humans and nonhuman primates [210–217] and in the lung of rats [218] and the human female genital tract [219].

There is considerable evidence for the presence of the human FcRn in the GI tract of humans, which has been reported by researchers such as Dickinson and others [204,216,217,220–226]. It is thought that the normal role for the IgG-FcRn is in the scavenging and processing of antigens. Its role is thus to 'bring/transport' IgG_1-antigen complexes from the intestine into the body for immunological processing [219,226].

Attempts to utilize the IgG-FcRn transport system for oral delivery of IgG and IgG-Fc fusion molecules in humans has been largely unsuccessful, possibly due to the high level of proteolysis in the small intestine, as well as several reports of the relative absence of the IgG-FcRn in the human intestine. In this regard, IgG_1 is particularly susceptible to pepsin-mediated cleavage and to trypsin cleavage at the hinge region between the Fab and Fc portions of the molecule. This results in the enzymatic cleavage of the IgG molecule into Fab and Fc fragments that can be readily detected in the intestinal lumen. In contrast, analysis of mucous isolated from the small intestine of rats and mice reveals high levels of intact IgG, which are present at levels much higher than IgA [55,64–66].

The alveolar cells of the lung have also been found to express the FcRn-transport pathways and several workers have attempted to overcome the barrier property of the intestine, as well as the enzymatic degradation of the IgG by administering IgG and IgG-Fc-fusion proteins via the pulmonary route [212], FSH-Fc fusions [215], EPO-Fc fusions [212–214]. There have, however, been various problems in pulmonary delivery [227], possibly due to the high concentrations of the IgG_1-Fc fusions. Thus pulmonary administration with Etanercept™ (Pfizer) resulted in an increased incidence of pulmonary granulomas [228] and fatal pulmonary Mycobacterium abscesses [229], and pulmonary Enbrel™ (Amgen) has been associated with increased interstitial lung disease [230].

Histological evidence of Fc-mediated uptake in adult mice has been obtained by our group. Thus, when fluorescently labeled IgG_1 monoclonal antibodies was administered into GI loops of anaesthetized, rapid binding of both Rhodamine-labeled IgG_1 and FITC-labeled IgG_1 was observed **(Figure 1).**

FcRn has recently been shown to also bind to albumen [215,231,232], which suggests the possibility of using albumen as a targeting agent for oral delivery. Intra-jejunal administration of rhodamine–BSA conjugates showed similar distribution to Rho-IgG in the adult mouse (GRJ and personal observations; **Figure 2**). This finding may explain some of the anomalous results found in studies on neonatal uptake, in which both albumen and IgG were found to be equally well transported from the gut of neonates [233].

Oral uptake via the VB_{12} intestinal uptake mechanism

VB_{12} (cobalamin) is the largest of the water soluble vitamins (1355.37 amu) and exists as two naturally occurring cofactors in the body, 5′-deoxy-adenosylcobalamin and methylcobalamin. Uptake of the dietary vitamin normally requires the release of the vitamin from food in the stomach, followed by binding of the vitamin to intrinisic factor (IF) in the stomach and proximal small intestine. The resultant IF–VB_{12} complex is then bound by an IF receptor [234],

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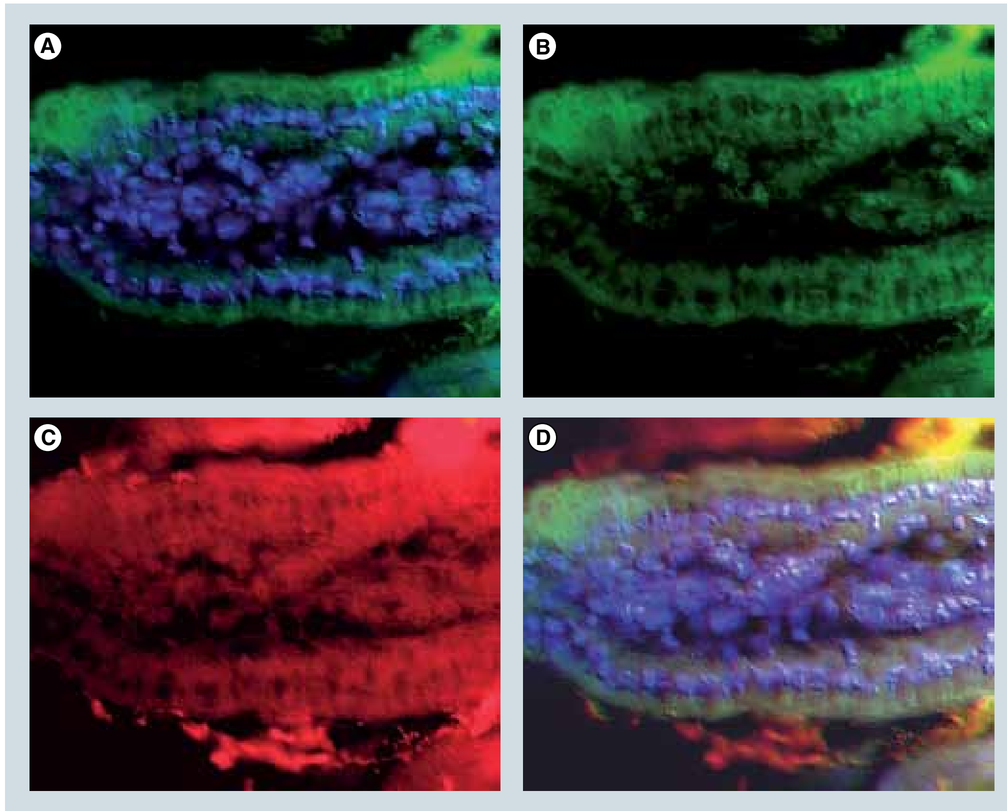

Figure 1 Binding and uptake of an IgG monoclonal antibody to the endothelial cells of a mouse small intestine. Monoclonal antibodies were labeled with two different fluorescent molecules, rhodamine-isothiocyanate (Rho-IgG; red) and fluorescein isothiocyanate (FITC-IgG; green). The two preparations were mixed and administered to small intestinal loops prepared in anaesthetized mice. 60 min after instillation into the loops, the mice were euthanized and the loops removed for cryostate sectioning. Representative sections are presented. Significant binding and uptake was seen for **(A & B)** the FITC-IgG and **(C)** rho-IgG. **(D)** Co-incident staining (yellow) is indicative of specific binding of both molecules to the intestinal epithelial cells. Nuclei of cells are stained with bisbenzamide (blue) and are shown in A & D. Negative staining of nuclei can be seen in the upper right and lower left panels. No staining of the endothelial cells was observed in control loops into which untargeted rhodamine-labeled HPMA or FITC-HPMA were instilled (data not shown).

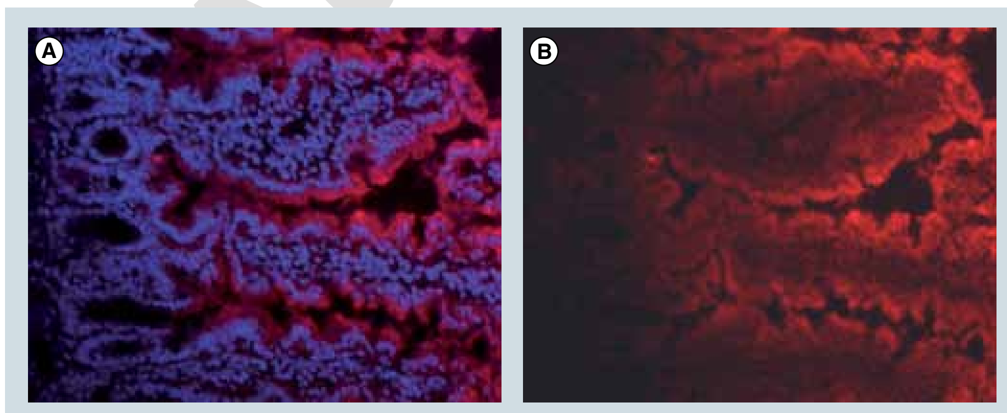

Figure 2 Binding of rhodamine-labeled bovine serum albumin to the endothelial cells of the small intestine of a mouse. Bovine serum albumin was rhodamine-labeled using rhodamine-isothiocyanate and administered to the small intestine of anaesthetized mice. Significant binding and uptake was seen for the rhodamine–bovine serum albumin. **(A)** Endothelial cell nuclei are stained with BisBenzamide (blue color). Methods are as described in **Figure 1**. **(B)** Binding of rhodamine–bovine serum albumin to the surface of the enterocytes is observed over the surface of the villous (red color).

cubulin, a 460 kD protein found on the intestinal epithelium and in the kidney [235–237]. The receptor is found located on the enterocytes of the duodenum, jejunum and ileum, with the largest concentration of receptors being found in the ileum. In humans and most other animals the number of receptors is very limited such that the amount of VB_{12} that can be taken up from the intestine is very low, approximately 1–2 µg/feed in humans, approximately 30 ng in the mouse and 100 ng in the rat (personal observations). Binding of the IF-VB_{12} complex to cubulin initiates endocytosis of the complex. The intrinsic factor is subsequently degraded within the cell and the VB_{12} then binds to another VB_{12} binding protein, transcobalamin II [238–241]. Eventually, the VB_{12}–TCII complex is secreted from the basal surface of the intestinal epithelial cell. From here the VB_{12}–TCII complex travels via the lacteal lymph vessel into the mesenteric lymph node and several hours after oral administration, VB_{12} can be measured in the circulation.

Experiments using VB_{12} as a transporter initially involved the direct conjugation of the VB_{12} to the peptide or protein. Thus, Russell-Jones and co-workers prepared conjugates to G-CSF [242,243,304,305], EPO [242–244,304,305], BSA [244,305], LHRH agonists (D-Lys6-LHRH) and antagonists (ANTIDE and derivatives) [245], calcitonin, GHRP-6 and vasopressin. The affinity of conjugates for IF was measured and many different spacers used in an attempt to optimize both IF affinity and biological activity. All conjugates bar the vasopressin conjugate were shown to be active following oral administration to mice, rats or pigs. VB_{12}–insulin conjugates have also been prepared and their utility discussed above [190].

Initially, conjugates were prepared by carbodiimide linkage of the e-VB_{12} carboxyl derivative to amino groups on the peptides or proteins. The e-VB_{12} carboxyl derivate was formed following acid hydrolysis of the VB_{12}, however the yield of this derivative was only around 5% of the starting material and, thus, deemed too expensive as a carrier. Later conjugates used VB_{12} derivatives prepared by activation of the 5′-hydroxyl of the ribose sugar of cyanocobalamin, resulting in more respectable yields (in excess of 80%) [246]. Despite the improvements in yield of VB_{12} derivatives, the resultant conjugates possessed several problems. First, the biological activity of the conjugated peptide or protein was generally slightly reduced or in the worst cases almost absent. Thus, a 25–50% reduction in biological activity was seen with EPO, G-CSF, IL-2 and D -lys$_6$-LHRH and growth hormone releasing peptide-6 (GHRP-1), with greater than 95% loss of activity with desmopressin. Second, the dose deliverable using VB_{12} as a targeting agent is very small (~1 nM per dose in humans) and, thus, the utility of the system is restricted to highly active compounds. Third, even if conjugates could be produced that had biological activities and which were suitably active for oral delivery via the VB_{12} transport system, the conjugates were still susceptable to proteolysis within the intestine.

While a brief foray was made into amplifying the uptake capacity of the IF-mediated oral delivery of VB_{12} conjugates [306], the problem of proteolytic susceptibility of the conjugated peptide or protein still remained an issue. Additionally, conjugation in most instances leads to the generation of a new chemical entity, which was not considered desirable at that time. Furthermore, conjugation in most cases still led to loss of biological activity. For these reasons, experiments then concentrated on the development of a nanoparticle system for targeted oral delivery. Delivery via nanoparticles would enable the peptide or protein to be protected within a nanoparticle matrix and would mean that the peptide or protein would not need to be chemically modified and therefore would maintain its biological activity. In addition, the size of the nanoparticle would enable amplification of the dose deliverable via the VB_{12} uptake system. There were many problems associated with this line of research, as it was not known what size of nanoparticle would be optimal for delivery, how much targeting agent would be required, or how to entrap and release the pay-load from the nanoparticle. Furthermore, there was a great deal of scepticism that targeted nanoparticles would even enter or cross the intestinal epithelial cells.

■ Development of VB_{12}-targeted & drug-loadable nanoparticles

Early experiments on VB_{12}-targeting of nanoparticles was performed on fluorescently tagged latex nanoparticles of defined sizes (Fluoresbrite™ Polysciences). Using these particles it was quickly established in the Caco-2 cell model and OK cell models, that particles as large as 500 nm could be taken up into the cells via IF-mediated RMT [247]. Uptake was proportional to the amount of VB_{12} used to coat the nanoparticles and was increased in the presence of intrinsic factor. Similar IF-mediated uptake

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of polymeric micelles has also been observed by Francis and co-workers [248]. A series of experiments were then carried out on uptake in rats, dogs and pigs using intestinal loops surgically prepared in anaesthetized animals. Regardless of the species chosen good uptake of VB_{12}-coated Fluoresbrite yellow–green nanoparticles was observed from the intestinal loops. Uptake was found to peak at 200–400 nm size with particles larger than 400 nm being found entrapped within the mucous blanket and, thus, were not able to 'reach' the intestinal epithelium. Once the particles (50–400 nm) had been taken up from the intestinal they could be found accumalting in the central lacteal vessel of the villus (**Figure 3A**) and later could be seen in large numbers in the draining segment of the mesenteric lymph node (**Figure 3B**). Similar observations were found in repeat experiments using targeted Fluoresbrite yellow–orange and bright blue particles.

In further experiments in dogs and pigs (results not shown), uptake of nanoparticles from the intestinal loops was found to be rapid and VB_{12} was found to be much more effective as a targeting agent for nanoparticles than either LTB (*Escherichia coli* heat labile toxin, B subunit) or Con A (**Figure 4**) in these animals. Histological examination of the intestinal mucosa, following uptake experiments revealed that it was morphologically intact.

The experiments described above established that it was technically feasible for the **VB_{12} uptake system** to initiate the binding, uptake and transport of model latex nanoparticles to and across Caco-2 cells, OK cells and from the intestine of rats, mice, dogs and pigs. The problem with these experiments was that the latex nanoparticles were not suitable for loading with peptides or proteins. Attention switched to looking at uptake of nanoparticles that could be loaded with peptides and proteins. Initial work focused on the use of ^{125}I-insulin-loaded isobutylcyanoacrylate (IBCA) nanoparticles [155,156]. While it was relatively easy to load these particles, it was quickly found that the majority of the insulin was physically located on or near the surface of the nanoparticles and was rapidly degraded upon incubation with small-intestinal washout fluid. A surface cross-linking system utilizing esterase cleavable cross-linkers was devised and shown to protect the insulin from proteolysis (**Figure 5**) [307].

The IBCA nanoparticles were targeted with an octadecanoic-acid-5′O-VB_{12} surface coating and administered to mice. Nontargeted IBCA nanoparticles showed little uptake in the mice, and also showed little degradation within the intestine as evidenced by the very small quantity of ^{125}I detectable in the thyroid of the mice. In contrast, approximately 15% of the orally administered dose was recoverable in the tissues such as liver, spleen, lungs, skin and thyroid of the mice that had received the VB_{12}-targeted IBCA nanoparticles. This compared with only 2% of recovered counts in the control nanoparticles.

Despite the evidence of uptake of VB_{12}-coated IBCA nanoparticles, alternative nanoparticle systems were investigated as the isolation and washing steps required during the formation of the IBCA nanoparticles were not thought to be commercially viable. Additionally there was the potential that the cross-linking agent would also covalently link to the peptide or protein to be

Key Term

Vitamin B_{12} uptake system: Small molecule that has been shown to have massive potential for the oral delivery of peptides, proteins and drug-loaded nanoparticles.

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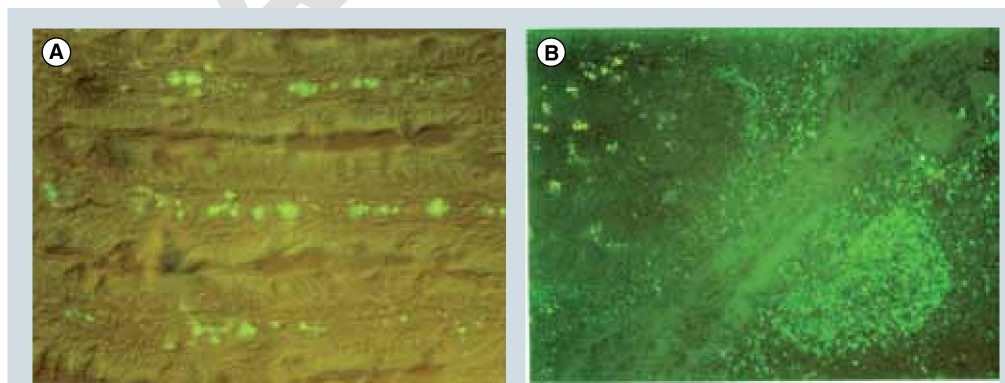

Figure 3. Uptake of 400 nm vitamin B_{12}-coated Fluoresbrite™ yellow–green nanoparticles from the intestinal loops installed in (A) rats and (B) pigs. Nanoparticles can be seen accumulating in the central lacteal vessel of the rat small intestine (T120 min), and in the mesenteric lymph node of pigs (T240 min). Little to no uptake of particles was observed using non-targeted particles.

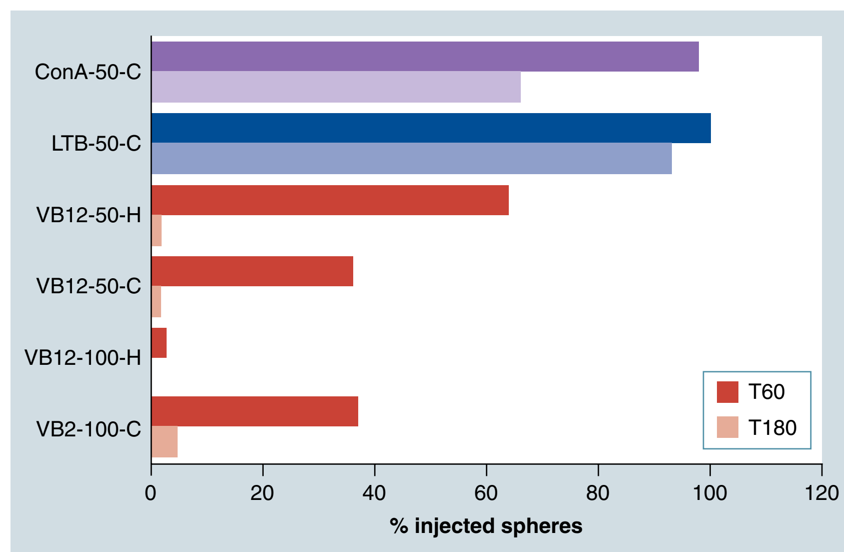

Figure 4. Recovery of 50 and 100 nm fluorescent Polysciences latex nanoparticles from intestinal loops instilled in anaesthetized dogs. Particles were injected into separate loops then at 60 and 180 min the dogs were euthanized and the contents of the loops recovered following saline washout. Particles were either coated with Conconavlin A (Con A), *Escherichia coli* heat-labile toxin B subunit (LTB) or adipylhydrazidyl-e-VB12 (VB12). Particles were surface modified following activation with EDAC/NHS and were subsequently blocked with glycine (C) or ethanolamine (H). Due to the cost, animal ethics requirements and technical feasibility of these experiments, they were performed as single-dog experiments, which were repeated 3-times. Data is presented for one representative experiment.

delivered. In an attempt to overcome these problems, Chalasani and co-workers [167,168,302,303] developed a drug-loadable dextran 'nanosponge'. These porous nanosponges were formed by epichlorhydrin cross-linking of emulsified dextran. The resultant nanosponges are dried and then surface modified with succinic anhydride. Finally, aminohexyl-VB_{12} was linked to the surface using a suitable carbodiimide. The resultant VB_{12}-targeted nanosponge can be 'loaded' with peptides or proteins by swelling dried particles in the drug to be delivered. When insulin loaded VB_{12}-targeted nanosponges were fed to diabetic rats, a significant reduction in serum glucose levels was observed [168]. The reduction in serum glucose persisted for several hours before returning to pre-treated levels approximately 12–16 h later. Repeated daily feeding for 5 days resulted in similar levels of blood glucose reduction. Interestingly, repeated feeding of the particles also resulted in a reduction in food intake, as well as a reduction in intake of water. One major problem with the commercial development of this system is that considerable optimization is required to make the method scalable and, furthermore, the ester-linked succinate group was found to hydrolyze upon storage.

VB_{12} has also been used to coat 200 nm Gantrez nanoparticles [249], which were loaded with ovalbumin. Oral feeding of the nanoparticles to mice resulted in a higher anti-OVA mucosal response than either sub-cutaneously injected material or plain OVA-loaded Gantrez particles.

Initial problems of developing a scaleable, targetable nanoparticle system, which enables good entrapment of both small peptides and large proteins were solved by the development of a carboxymethyl-dextran (CM-D) nanolattice system [308]. In this system CM-D is dissolved in water and dispersed in an oil/surfactant/cosurfactant mix to form a stable water-in-oil microemulsion (W/OME). The water soluble peptides or proteins are added to the W/O ME, stirred gently and nanolattices formed by cross-linked using a divalent cation such as Ca^{++}, Mg^{++} or Zn^{++}. The extent of cross-linking is controlled by the amount of divalent cation added, which in turn controls the rate of release of the entrapped material from the nanolattice matrix. These nanolattices can be surface-modified with VB_{12} as a targeting agent using N-e-Lysyl-5′-O-VB_{12} or other suitable chelating targeting agent. Formation of the nanolattices within the microemulsion leads to the production of small (~200 nm diameter) nanolattices, which are readily isolated from the ME by ethanol precipitation and washing. Particles can be isolated following resuspension in distilled water and freeze-drying. The resultant powder is stable at room temperature and stability

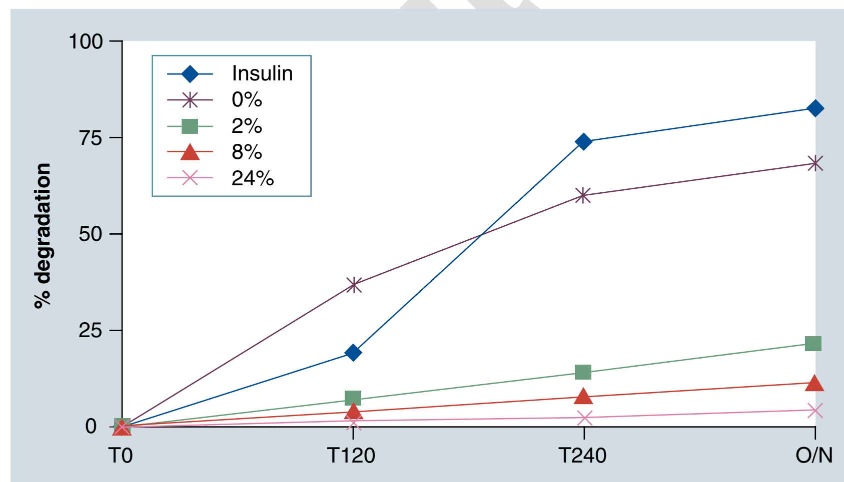

Figure 5. Resistance of 125-labeled insulin to enzymatic degradation following incorporation into isobutylcyanoacrylate nanoparticles and surface cross-linking with esterase-cleavable cross-linkers. 125-labeled insulin containing isobutylcyanoacrylate nanoparticles were surface cross-linked with an esterase-cleavable dipeptide and were incubated with small intestinal washout at 37°C. At various times samples were removed and the percentage of released insulin determined.

testing has shown little change in particle structure following 9 months storage of both insulin and IgG within the nanolattices.

Nanolattices containing human insulin formulated as described above were administered to diabetic rats, and serum glucose monitored over 8 h **(Figure 6)**. A mean drop in serum glucose levels of 40% of the original values was obtained, which persisted for more than 8 h. A slight variation in blood glucose lowering activity of the nanolattices was observed depending upon the pH of preparation of the insulin added to the nanolattices.

Concluding remarks on VB_{12}-mediated delivery of peptides & proteins

Despite over 27 years of research into the use of VB_{12} uptake system as a transport system for peptides and proteins (reviewed by Clardy *et al.* [250]), there is, as yet, no commercially available product utilizing this system for oral delivery. There are two main reasons for this. The first is theoretical and the second is technical. From a theoretical point of view, the lack of a similar transport mechanism that had been characterized and used for transport of molecules has meant that many researchers have had trouble in believing in the theoretical possibility that the VB_{12} transport system could be used for uptake and transport of peptides, proteins and nanoparticles.

Technically, many issues have had to be resolved, including:

- Development of conjugation chemistry – initial conjugation was via acid hydrolysis of VB_{12} to generate a free carboxyl group for chemistry. Following extensive optimization the yield of the required 'e' VB_{12}-carboxylate was only 5% [243,245,251,252]. The development of the 5′O-VB_{12} conjugation greatly increased the yield to over 80%, thus making conjugation economically feasible [242,243,256,253,306,309,310]. In this regard the structural basis of binding of VB_{12} to the two transporters, intrinsic factor and transcoblamin II was not determined for many years after the initiation of the conjugation chemistry [253,254].
- Development of uptake models – initially uptake models were poor, however the development of the Caco-2 cell culture, and OK cell lines, plus gastro-intestinal loop models in rats, mice, dogs and pigs, combined with digital fluorescence microsopy greatly aided in the demonstration of VB_{12}-mediated transport of peptides, proteins and nanoparticles [242,247,255,256].
- Development of nanoparticle technology – early experiments examining the ability of VB_{12} to act as an oral transporter of nanoparticles utilized commercial nanoparticle preparations that were unsuitable for drug loading, particularly with peptides or proteins. Thus, whilst it was demonstrated that it was technically feasible to use VB_{12} as an oral targeting agent, no-one could make nanoparticles, or even small microparticles, at commercial scale with any reproducibility. Even then there was no technology to measure the size and so on of the particle, apart from electron microscopy. The development of the ζ-sizer greatly over-came these problems, however, it was not until the development of the dextran nanosponges of Chalasani and coworkers [168,303], and more recently the dextran nanolattices, that a commercially, scaleable, VB12-targetable, peptide and protein loadable system for oral delivery has become a reality [250,256].

Future perspective

The various carrier systems described in the current review for oral delivery of peptides and proteins are by no means all of the potential carrier systems that are available to the pharmaceutical scientist. They do, however, share one thing in

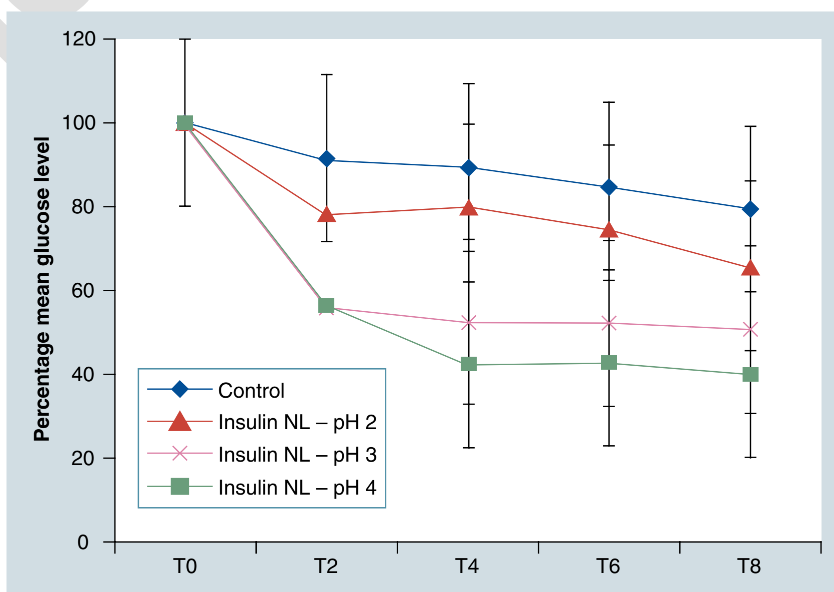

Figure 6. Reduction in serum glucose levels following oral administration of insulin-loaded, vitamin B_{12}-targeted dextran nanolattices to diabetic rats. The data is represented as the mean glucose level (n = 4), expressed as a percentage, in comparison to T0 over the period of 2, 4, 6 and 8 h. Nanolattices were formed from insulin solutions, which were prepared at pH 2.0, 3.0 or 4.0, using methods described in Russell-Jones and Luke [257]. Statistically significant differences are found for NL-pH3 and NL-pH 4 T2, T4, T6 and T8.

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common in that they all use natural transport systems present within the body. Technological advances, which have occurred in the past two decades should, in the future, enable an off-the shelf 'cassette-type' approach to the oral delivery of peptides and proteins. Thus, molecules that can survive proteolysis within the milieu of the small intestine can be genetically linked to carriers such as Tf, Lf, lactalbumin, albumin or the IgG_1–Fc fragment, and can be produced in large quantities at relatively low cost and administered orally to patients. Peptides and proteins that are highly protease susceptible can be readily entrapped within nanolattices to which are linked small targeting molecules such as VB_{12} and others currently being developed. These targeted nanolattice structures can be engineered to include delayed and prolonged release mechanisms to enable subtle control of the release of the entrapped material. Oral delivery of peptides and proteins is, thus, a reality and delivery via this route, plus the recent advances in transdermal delivery [257–259,311] may spell the end of subcutaneous injection for all but those medications requiring immediate release profiles via direct, intravenous injection.

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Executive summary

- In order for effective oral delivery of peptides and proteins across the GI tract wall, a specific transporter molecule is required, which must bind to a specific receptor on the gut epithelium.
- Five such transport systems are described: an insulin receptor and transporter, the IgG-Fc transporter, transferrin and the transferrin receptor, lactoferrin and the lactoferrin receptor, and the vitamin B_{12} transport protein, intrinsic factor and the intrinsic factor receptor.
- Use of any of the transporters requires that the molecule to be transported and the transporter must be resistant to proteolysis in the small intestine.
- The technology is currently available to entrap any peptide or protein within a protective nanolattice system, to coat it with a transporter and to achieve effective delivery to the circulation following oral feeding.
- A scaleable nanolattice system using vitamin B_{12} as the delivery agent has already been produced and shown to be stable upon storage for over 6 months.
- Future work will concentrate on the identification of yet more transporters and upon bringing these systems to commercial reality.

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