REVIEW

Chemically modified peptides and proteins - critical considerations for oral delivery

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ABSTRACT

Numerous approaches have been explored to date in the pursuit of delivering peptides or proteins via the oral route. One such example is chemical modification, whereby the native structure of a peptide or protein is tailored to provide a more efficient uptake across the epithelial barrier of the gastrointestinal tract via incorporation of a chemical motif or moiety. In this regard, a diverse array of concepts have been reported, ranging from the exploitation of endogenous transport mechanisms to incorporation of physicochemical modifications in the molecule, which promote more favorable interactions with the absorptive membrane at the cell surface. This review provides an overview of the modification technologies described in the literature and offers insights into some pragmatic considerations pertaining to their translation into clinically viable concepts.

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Introduction

An increasing number of peptides, proteins and their derivatives has been developed and approved for treatment of a variety of human diseases, while many others are still in different phases of development. So far, administration of the majority of these biological drugs is limited to invasive routes such as injections and infusions. The ability to administer at least some of these life-saving drugs by non-invasive routes, especially orally, would likely improve adherence to treatment regimens, limit hospital visits and thus reduce overall health care costs. Oral administration of peptides and proteins is, however, far from trivial as the gastrointestinal tract (GIT) is designed to prevent absorption of large molecules into the systemic circulation. Considerable efforts over the last several decades have been devoted to understanding the barriers preventing oral absorption as well as to developing strategies on how to overcome them.

In this review, we focus on oral absorption of peptides and proteins chemically modified to take advantage of the specific transport mechanisms evolved to ensure sufficient supply of nutrients, vitamins etc. for human metabolism. Firstly, the absorption routes that can be exploited are described and, secondly, the necessary technologies providing the peptides and

proteins of interest access to these specific absorption routes are discussed. Finally, an evaluation of the current status of the field from an industrial perspective is presented.

Exploiting absorption routes via chemical modifications

Passage across the epithelial barrier of the gastrointestinal tract (GIT) is governed by one or more pathways, namely, transcellular, paracellular, receptor mediated and carrier mediated transport.³⁸ While the inherent physicochemical and chemobiological properties of a peptide or a protein determines its propensity to be absorbed via specific routes, chemical modification of the native peptide or protein structure can afford the possibility to expressly target or exploit a particular route(s) of absorption and thereby promote improved permeation.

Cells of the epithelium lining the gastrointestinal tract are encapsulated by a phospholipid bilayer membrane. In addition, neighboring cells are connected at their apical surface via networks of tight junction proteins, which serve to restrict the absorption of substances residing in the luminal space through the paracellular route. In general, the vast majority of peptides exhibit a high degree of hydrophilicity, while also

being comparatively large in size. By virtue of these characteristics, absorption via the transcellular route is disfavoured, whereas their large size precludes significant passage via paracellular spaces. Thus, incorporation of specific motifs which can promote improved affinity for lipophilic biological membranes and /or modulate tight junction function provides a means to augment absorption via these routes, in addition to the more classical approaches of employing permeation enhancers and/or nanotechnologies.

Enterocytes also boast a multiplicity of native mechanisms which facilitate uptake of specific substrates. In the case of receptors, their substrate(s) are internalised in vesicles upon engagement for subsequent transportation into (and in certain cases, across) the cell. In the case of membrane-spanning carriers, they promote an energy-dependent shuttling of their cargo across biological barriers.

So-called receptor-mediated endo- and transcytosis refers to a collection of membrane deforming processes, which in some cases can yield successful trafficking of the substrate to the basolateral membrane surface. These events have been shown to be inducible in both enterocytes and M-cells of the GIT (Swaan, 1998). An array of ligands has been demonstrated to be transported via endocytotic/transcytotic mechanisms. Notable examples, which have been exploited for oral peptide and protein absorption, include the transferrin receptor (TfR), neonatal Fc receptors (FcRn) and the intrinsic factor receptor (IF).^{5,10,39} In such cases, the peptide or protein is conjugated to the relevant substrate (or derivative thereof), which serves to potentiate its trans-epithelial absorption. Despite being a promising approach, there remains many aspects which need to be more extensively addressed before their true potential can be evaluated.

Various transport proteins, which are expressed along the GIT, may also be utilised for modulating the absorption of xenobiotics. Although nutrient transporters such as glucose transport (GLUT), peptide transporter (PEPT), apical sodium-dependent bile acid transporter (ASBT) etc., can potentially be utilised to facilitate the shuttling of drug molecules across the epithelial barrier of the GIT, the inherent large size of peptides and proteins likely precludes true exploitation of such mechanism(s) for the majority of biological drugs. Nevertheless, recent studies suggest that these transporters could potentially be exploited to function in a manner similar to that of membrane

receptors and thereby facilitate improved permeation of larger cargoes.³

Absorption enabling technologies

Cell penetrating peptide-conjugates (SPBY)

Cell penetrating peptides (CPPs) are peptide motifs of <30 amino acids, which can translocate biological membranes. Although relatively heterogenous in composition, they are typically classified into two groups, namely, cationic and amphiphilic peptides. Extensively characterized examples include HIV-TAT1 peptide, oligoarginine peptides and penetratin. A collective property of virtually all CPPs is their abundance of basic residues (e.g., Arg, Lys), which promotes interaction with negatively charged cell surface glycosaminoglycans (GAGs).⁴¹ This feature is believed to be central to their capacity to traverse membranes (together with their associated cargo(es)).⁴¹ Cumulative studies suggest that the uptake of CPPs is mediated via (receptor-independent) endocytotic processes and/or direct penetration,⁸ of which binding to the cell surface is a preceding step in the internalisation process. To achieve these effects, it has been proposed that the CPP should be chemically conjugated to its cargo.⁴⁶

The chemistry underpinning the conjugation process is governed by the cargo type, ease of synthesis and perceived impact on biological activity. Covalent conjugation may be achieved via chemical synthesis in the case of peptides, while larger proteins often necessitate recombinant techniques.⁴² In addition, a key feature, which must be incorporated into the conjugation strategy, is the necessity for the cargo to be efficiently cleaved from its corresponding CPP at the site of or upon absorption in order to ensure the desired therapeutic effect is achieved and a number of cleavage motifs have been identified.¹⁷

A number of studies have examined the performance of covalent conjugates of CPPs and their cargoes. Most notably, chemical modification of human insulin via conjugation to TAT peptide in position B29 (Lys) was shown to markedly improve its permeation across monolayers of Caco-2 cells, when compared with non-conjugated insulin.²⁸ However, some studies suggest that the conjugation process may in fact adversely impact the membrane penetrating ability of a CPP. For example, conjugation of parathyroid hormone (PTH) (1-34) with a panel of different CPPs (penetratin, Tat, VP22, R9) was shown to result in a reduction in permeation across Caco-2 monolayers

compared to native PTH (1-34).²⁶ This was also accompanied by a corresponding decrease in potency. Interestingly, conjugates employing the N-terminal were shown to perform better than those in which the conjugation was in the C-terminal. In contrast, co-administration of PTH (1-34) with the corresponding CPPs exhibited a trend for out-performing their conjugated counterparts. Thus, in cases where self-assembly of physical complexes can be successfully achieved, co-administration may be sufficient.²² Collectively, these findings highlight that the efficiency of such conjugates are likely heavily dependent on the specific properties of the individual CPP and cargo and their corresponding compatibility with each other. Moreover, it emphasizes the need to further expand our understanding of the precise fate of the CPP-cargo and the liberated cargo itself, so as to facilitate the identification and optimisation of existing conjugation approaches.

Ligand-modifications targeting transporters and receptors

The gastro-intestinal tract (GIT) is designed to digest nutrients into their building blocks and absorb these into the circulation. The building blocks are absorbed either by passive absorption or via multiple transporters expressed on the apical site of the GIT cells.¹⁵ As human metabolism is also dependent on a multitude of essential nutrients such as vitamin B12, biotin, folate, iron, etc., specific transporter systems ensuring absorption of sufficient amounts of these compounds have evolved. Furthermore, large amounts of bile acids are excreted into the GIT every day to aid the digestion process and the bile acids are subsequently re-absorbed and recycled; this represents another opportunity for oral delivery. Further inspiration from nature comes from bacteria or viruses, such as cholera or HIV that successfully penetrates the GIT epithelium and enters into the systemic circulation. Over the last decades, many studies have described attempts to hijack these systems for oral delivery of therapeutic peptides and proteins by conjugating the protein of interest to the natural ligands of these receptors in the hope of increasing oral absorption and this topic has been the subject of many focused reviews.^{2,27,37}

As the metabolic requirements for the different essential nutrients differ, so does the capacity of their transporter systems. For example, the capacity for iron uptake

via transferrin complex formation is high and it is estimated that up to 100 mg of transferrin gets secreted every day from the liver into the bile which is subsequently, upon binding of iron, reabsorbed into circulation via the lymph system.⁵¹ In contrast to the high capacity of the transferrin transporter, the capacity of the vitamin B12 absorption machinery is low (see section 4.1) and also vitamin B12 has been shown to travel to the circulation via the lymph.³⁷ The fate of the peptide or protein after oral absorption can differ significantly based upon which absorption pathway is being exploited. As mentioned above, the transferrin and vitamin B12 pathways lead to the lymphatic system before reaching the circulation, thus avoiding first pass metabolism in the liver. While circumventing first pass metabolism might be an advantage for many orally delivered peptides and proteins (e.g., PTH where the liver is a primary site of catabolism), it could also be disadvantageous; for example, in the case of oral delivery of insulin, where delivery approaches which permit direct delivery to the liver are preferred since they can more accurately mimic the physiological release of insulin from the pancreas directly to the vena porta. Thus, this should be carefully considered when pairing a peptide with a specific conjugate.

Another GIT absorption pathway utilizes the IgG-FcRn receptor. Although, this pathway is well functioning in neonates, attempts for its utilization for oral delivery of IgG-modified molecules in adults has been controversial so far.^{20,28,34} Albumin has been shown to bind to the IgG-FcRn receptor²⁵ and albumin-modified proteins, though having a significant molecular size, could also be potentially used to enhance oral absorption using this pathway. Recently, a truncated version of the Fc domain has been engineered that mimics full protein binding to the FcRn receptor. Its fusion with HIV-1 neutralizing antibody showed higher activity than fusion with the full length Fc domain.⁴³ This approach of reducing the size of the antibodies could potentially also hold promise for the oral delivery of peptides and proteins. The design of specific antibodies to any intestinal receptor attached to the protein of interest is an alternative way to hijack the intestinal receptors for oral delivery of proteins and peptides. Key to this approach is balancing the need for sufficient affinity of the antibody to the receptor to permit engagement and uptake, while at the same time ensuring that it is possible for its cargo to be released upon successful passage through the cellular barrier.⁵²

The attachment position of the protein of interest to the transport vehicle is also of crucial importance as both the activity of the protein of interest (i.e., binding to its physiologic receptor) as well as binding of the transport vehicle or ligand to its receptor need to be maintained. High resolution structural data exist for many peptides or proteins bound to their receptors, however, a further structural characterization of intestinal transporters bound to their natural substrates could improve the design of any conjugates designed for oral delivery. Furthermore, the stability of the ligand-modified peptide or protein in the GIT should be sufficient to allow for efficient oral delivery. Attachment of albumin/IgG/transferrin etc., to the peptide or protein of interest will very likely also change its circulation half-life, which again could be an advantage or a disadvantage depending on the therapeutic aim.

Apart from biological reasons, the mode of ligand attachment is also highly important from a production point of view. A separate production of the targeting vehicle (transferrin, IgG, vitamin B12, biotin, folate, etc.), the linker and the peptide or protein of interest followed by a chemical conjugation step is often used. The advantage of this sequential method is that optimal production of each component can be ensured, however, the chemical coupling step and the linker length and structure needs to be developed and optimized. The importance of the linker was described, for example, for a protamine-insulin conjugate, where the solubility of the resulting conjugate was highly dependent on the length of the linker.¹⁸ Production of a fusion construct comprising the protein targeting vehicle (transferrin, IgG, etc.), a linker and the peptide or protein of interest is perhaps the most optimal way if the right production host and process resulting in high expression yield can be identified. The order of the sequences in the fusion construct and the length and composition of the linker needs to be optimized as these will potentially affect both the biological properties (binding to the respective receptors, clearance, etc.) as well as the physicochemical properties (solubility, stability, etc.) of the resulting fusion protein.

Any covalent modification of peptides or proteins (fusion proteins, conjugates, etc.) presents a potential risk of increased immunogenicity. Assessment of the immunogenicity of such a new peptide or protein is highly complicated; although considerable improvements in immunogenicity prediction algorithms have recently been achieved^{30,53} and some cellular based

assay are available to test immunogenicity,⁵⁰ the ultimate answer will come from the human clinical trials and clinical practice.

Modification of physicochemical properties

The solubility, hydrophilicity and hydrophobicity physicochemical properties of peptides or proteins are often not optimal for oral absorption. Covalent modifications with polyethylene glycol (PEG) or low molecular weight chitosan are often used to circumvent low solubility. The same modifications also often improve enzymatic stability in the GIT as the hydrophilic PEG/chitosan shields the surface of the molecule from enzymatic degradation.⁴⁵ These improvements in stability and solubility are, however, often accompanied by a loss of potency, especially for smaller peptides and proteins.⁶ Recently, conjugation of polycarboxybetain to chymotrypsin improved both stability and bioactivity significantly in comparison to conjugation with PEG of corresponding size.²³ A novel single short-chain PEG-modified insulin analog (IN-105) is currently tested in clinical trials.²⁴ This modification is claimed to improve stability toward enzymatic degradation, solubility and absorption.

A diametrically opposite approach has been adopted by others to modify proteins with lipids to afford more hydrophobic surfaces and thereby induce more favorable interactions with the intestinal cell membranes. Calcitonin,⁴⁸ desmopressin⁴⁷ and Bowman-Birk inhibitor¹³ were modified by reversible lipidisation.¹⁴ Modification using this method generally led to increased stability, longer plasma half-life and improved epithelial absorption. Furthermore a hexyl modified insulin had been developed, tested in clinical trials¹² and later abandoned.

Industry perspective

Absorption capacity

At a first glance, few approaches appear as straightforward as targeting the natural endocytotic/transcytotic uptake mechanisms of the gastrointestinal tract and piggy-back on these to increase absorption and uptake. Academic groups as well as biotech companies have explored this strategy by synthesising tailor-made peptide or protein therapeutics conjugated to ligands which target receptors and transporters expressed along the luminal side of the intestine for

endocytic uptake. As stated above, a key requirement for this strategy to be successful is to maintain the affinity for the transporter or receptor of the attached ligand while retaining therapeutic potency of the peptide or protein cargo.³⁵ Even if cellular uptake activity of the bioconjugate can be preserved, gaining entry into the epithelial cell via endocytosis is often only the first step of many for a successful oral absorption process as the bioconjugate needs to escape the endosome prior to endosomal recycling or lysosomal degradation as well as an exit route from the intestinal epithelial cells for systemic exposure. An example highlighting this is the use of glycosceramides as molecular carriers for mucosal delivery of peptides as reported by the group of Wayne Lencer.^{11,49} Linking glucagon-like-peptide1 (GLP-1) to the naturally occurring ceramide domain of the glycosphingolipid GM1, the receptor for cholera toxin that mediates cell entry, a marked dependence on ceramide lipid chain length and saturation on intracellular trafficking, localization and, ultimately, fate of the bioconjugate was elegantly demonstrated thus highlighting that cell entry via endocytosis is not on its own a guarantee of subsequent transcytosis.

The highly effective and endogenous vitamin B12 uptake pathway has been the target of a wide variety of bioconjugate cargoes and therapeutic peptides or proteins from luteinizing hormone releasing hormone (LHRH) to erythropoietin (EPO) and insulin have been sought delivered.^{4,10,36} Despite significant efforts over the years, none of the vitamin B12 peptide bioconjugates have so far made it into clinical trials, despite successful demonstration of both increased transport through the vitamin B12 receptor uptake pathway and subsequent biological activity on the target receptors. One of the underlying reasons for failure to reach therapeutic utility could lie with the limited absorption capacity of the vitamin B12 receptor system that has a modest daily capacity in humans of only 1-2 μg .¹⁶ To put this capacity into perspective in the case of the proposed vitamin B12 bioconjugated insulin therapy, many diabetes patients have a daily dose requirement of approximately 0.5 insulin units per kg of body weight, resulting in the daily need for 1-2 mg of insulin, a factor 1000 higher than what the vitamin B12 system can accommodate for. An additional limitation is the daily intake of B12 from food which might further limit the absorption of any therapeutic bioconjugates through binding site competition

with the endogenous substrate. This discrepancy between therapeutic need and intrinsic transport capacity highlights the need for careful consideration of the transporter or receptor system which should be aligned with both the therapeutic requirement as well as the physiological consequences of targeting the system. A commonly used strategy to mitigate the limited capacity of the GIT uptake pathways has been to use formulation approaches to increase the uptake of therapeutic agent per receptor. In case of the vitamin B12 uptake pathway, vitamin B12 ligands were used to decorate the exterior of dextran nanoparticles loaded with several insulin molecules. Nanoparticle binding and transport via a single VitB12 receptor increased the amount of absorbed insulin and this strategy resulted in a reduction in blood glucose when the nanoparticle system was administered orally to diabetic rats.¹⁰ The nanoparticle system has not entered into clinical trials, however, this suggests that the intrinsic endocytotic/transcytotic uptake mechanisms under the right circumstances can be utilised to ensure physiological relevant oral absorption of therapeutic peptides. For more on the use of targeted nanoparticles the reader is referred to a recent review elsewhere.³³

Given that many chemically modified peptides seek to exploit endogenous transport mechanisms (i.e., receptors, transporters etc.) to achieve improved absorption, it is also relevant to consider their efficacy, performance and safety under various physiological and diseased states as the expression levels of intestinal transporters are not necessarily constant and can be either upregulated or down-regulated upon starvation or under certain pathological conditions.²⁹ As examples, chronic inflammation has been reported to result in up-regulation of PEPT1 expression in colon,²¹ whereas lower expression levels of PEPT1 were found in type 2 diabetes and obesity animal models.¹⁹ Furthermore, uptake of even small amounts of folic acid by its receptor can lead to its down-regulation and thereby cause a slowing down of the internalisation/externalisation rates of the receptor, and consequently reducing any subsequent uptake via this route. Clearly, in such instances where changes in expression levels are manifested, a marked alteration in the behavior of the modified peptides or proteins exploiting these absorption routes can be expected.

Finally, consideration of the regional expression profile of the target receptor or transporter in the GIT

is also highly important. For instance, as the apical sodium-dependent bile acid transporter (ASBT) is primarily expressed in the terminal ileum,⁷ bile acid bioconjugates targeting this transporter should ideally be administered into the distal part of the small intestine for optimal co-localization of ligand and transport system. Furthermore, the selection of animal model also needs to be considered carefully as differences in both physiology and expression patterns exist. As an example, Sprague-Dawley rats have very short ileal segments compared to other species which could prevent direct translation of results obtained in rats using ASBT ligand bioconjugates.

Toxicity/disease states

On account of the chemical modifications applied to any given native peptide, such drug molecules will be considered by regulatory authorities as a novel pharmaceutical ingredients. Thus, extensive evaluation of safety and toxicity parameters is necessary. In particular, the potential for eliciting unwanted immune response(s) should be carefully addressed. For instance, failure to sufficiently remove lipopolysaccharides during purification processes following employment of bacterial expression systems for the production of covalently conjugated CPP-drug entities, has been reported to give rise to unwanted immune responses.²⁶ Strategies to improve the efficiency and performance of chemically modified peptides can also be potentially confounded by increased risk for possible undesirable side effects. Although improving the metabolic stability and thereby the half-life of modified peptides may conceivably be attractive from a pharmacological perspective, such approaches are also associated with a corresponding reduced clearance rate and thus prolonged circulation of the novel active pharmaceutical ingredient (API). On account of this, in such cases, a more extensive safety assessment is typically necessary. This is further underscored by the fact that many of these peptides or proteins are indicated for chronic administration and thus long-term safety considerations are especially pertinent. Interestingly, there are reports which indicate that covalent conjugation of a cargo (peptide) to a delivery vector may be associated with an increased risk of cellular toxicity. For example, a CPP-modified version of PTH (1-34) was shown to exhibit toxic effects in Caco-2 cells, while a corresponding physical mixture of the peptide and CPP was not associated with any adverse effects.²⁶ However, the precise reasons underlying these

observations remain to be clarified, and it is unclear if this is also the case for other covalently modified peptides. Thus, one should be cautious in over-interpreting such findings at this point in time.

Molecule-specific requirements/limitations

As described above many parameters need to be considered before the optimal peptide or protein conjugate is obtained. It appears that optimization of these parameters is highly specific for any given protein and the fact that a particular strategy works for one peptide or protein does not guarantee it will work for another. Arguably, modifications of peptides or smaller proteins are more critical as these are more likely to affect the biological properties as opposed to larger proteins that have more surface space to accommodate these changes. Nonetheless, in either case, the modification sites need to be tested and optimized through a series of structural, stability and binding experiments. An increasing amount of this screening could be done *in silico* using constantly improving algorithms and thereby reduce the actual price of development programmes.

As previously mentioned, protein modifications to improve oral delivery will almost certainly affect PK parameters such as half-life, distribution and clearance of the delivered peptide or protein in the body and the resulting change in PK parameters could be either positive or negative and must be known and considered before proceeding with the development program. The changes in physicochemical, immunologic and PK properties due to chemical conjugation of therapeutic proteins is a definite disadvantage when compared to approaches using native peptides or proteins combined with a platform consisting of physical mixtures of native proteins, permeation enhancers and other excipients. Unlike in the physical mixture approach, where a separation of the excipients from the “naked” peptide or protein will likely occur during the passage through the GIT, conjugated proteins carry their own “carrier vehicles,” hopefully improving their bioavailability. Ultimately, this improvement of bioavailability afforded by the conjugation of native peptide or proteins to carrier vehicles must outweigh the uncertainty related to introduction of a new API with potentially modified activity, PK and immunologic parameters.

Full manufacturing cost considerations

Any potential pharmaceutical peptide or protein must be safe, stable and its production costs must be in-line with the market opportunity. The production costs need to take into account the very low bioavailability observed for oral delivery of peptides and proteins in humans, which in the case of calcitonin is estimated to be around 0.8%.⁹ The physical mixture approach will typically consist of relatively inexpensive ingredients and the native protein with established production methods. In contrast, the production of the conjugated protein needs to be established by either producing the native protein and the carrier vehicle separately followed by a chemical conjugation step or by producing a fusion protein containing all of the active parts in a single construct. The expected production scale is also important to consider to ensure a sufficient supply of the necessary raw materials, e.g., vitamin B12. The long-term stability and sufficient purity of such conjugates is also a critical factor.

To provide some perspective, given the world-wide epidemic of type 2 diabetes and the relatively high amount of insulin needed by many type 2 diabetes patients each day, insulin is produced annually in the multi-ton scale. The typical daily requirement of insulin for a type 2 diabetes patient is ~30 insulin units (~1.1 mg), and assuming a 1% oral bioavailability, each tablet should correspond to ~110 mg of insulin or

reduced potency due to such modification. Should an insulin-transferrin conjugate be preferred, the amount of this conjugate in each dose would be 360 mg (assuming 5% bioavailability, 30 insulin units/dose, unchanged potency and 95.8 kDa molecular weight of the conjugate; Table 1). This insulin-transferrin conjugate including any excipients, etc. should cost <\$20 in order to be competitive with the approximate current daily cost of insulin therapy. Table 1 provides an interesting perspective with regards to the combination of the size of the conjugate, required daily dose of the active pharmaceutical ingredient and bioavailability given that a typical size of a tablet including the API and all excipients is smaller than 1g. As seen in Table 1, the average daily dose of the API is very important in selecting the delivery vehicle, for example the amount of transferrin conjugate necessary to deliver high daily dose drugs such as insulin or Victoza® far exceeds 100 mg/dose unless the bioavailability of the resulting conjugate approaches 100%. The situation is more favorable for low daily dose APIs such as PTH or calcitonin in which case bioavailability of 5% requires “only” ~10 mg of API per dose. Similarly, lower amounts are required for conjugates with smaller size delivery vehicles such as penetratin or vitamin B12.

Critical perspectives on data and models

While significant inroads have been made with regards to designing and developing chemically-

Table 1. Estimated amount of API in dosage form for selected conjugates.

API	daily s.c. dose in mg ^a	API per dose (mg) ^b				Assumed bioavailability(%)
		Unmodified API	Transferrin:API 1:1 construct	Penetratin:API 1:1 construct	B12:API1:1 construct	
Insulin MW = 5800	1.1	110	1816.9	155.5	135.7	1
		22.0	363.4	31.1	27.1	5
		11.0	181.7	15.6	13.6	10
		1.1	18.2	1.6	1.4	100
PTH(1-34) MW = 3700	0.02	2	50.6	3.3	2.7	1
		0.40	10.1	0.7	0.6	5
		0.20	5.1	0.3	0.3	10
		0.02	0.5	0.03	0.03	100
Calcitonin MW = 3400	0.016	1.6	44	2.7	2.2	1
		0.32	8.8	0.6	0.5	5
		0.16	4.4	0.3	0.0	10
		0.02	0.4	0.03	0.02	100
Liraglutide MW = 3700	1.2	120	3030.9	197.8	163.9	1
		24.0	607.8	39.6	32.8	5
		12.0	303.9	19.8	16.4	10
		1.2	30.4	2	1.6	100

Notes. ^aDaily doses were calculated based on the following: insulin; average dose of 30 U for type 2 diabetes patient (<http://www.drugs.com/dosage/insulin-regu-lar.html>); PTH average doses of 20 µg (<http://www.uptodate.com/contents/parathyroid-hormone-therapy-for-osteoporosis>); calcitonin, average dose of 100 U (<http://www.drugs.com/dosage/calcitonin.html>); Victoza®, average dose of 1.2 mg.³²

^bCalculations are based assuming no change in potency and no linker connecting the API with the respective conjugates. Molecular weights used in calculations are 90,000 Da (transferrin), 2,400 Da (penetratin) and 1,355 Da (vitamin B12).

modified peptides with traits that confer an enhanced capacity for improved absorption via the oral route, there remain a number of key aspects which must be further addressed in order to drive these concepts toward true realization. Thus far, the vast majority of studies report efficacy in either in vitro experimental setups or murine models. While such studies represent an initial proof-of-concept, it is crucial that promising concepts are further explored in large animal models, which more closely resemble human physiology. Specifically, the issue of scaling across species must be appropriately addressed. This includes validating the capacity and efficiency of any specific transporter/receptor being exploited together with ensuring sufficient homology across the species in which the peptide is to be tested. For example, there are reported discrepancies in the homology of e.g., TfR, when comparing murine species and human,⁴⁴ while it is known that the affinity of ligands for their receptor can vary widely across species.¹ Moreover, although administration of liquid-based formulations via oral gavage or direct instillation into the intestinal tract is an efficient approach for confirming the efficacy of a novel chemically-modified peptide; subsequently, it is also necessary to carefully address (pre-)formulation considerations as they relate to development of a solid dosage form (i.e., tablet, capsule). This should include appropriate stability studies, together with development of prototype formulations using appropriate and compatible excipients. Subsequently, characterization of lead formulations as it relates to their disintegration and dissolution is necessary, followed by comprehensive evaluation of the final formulation in vivo.

Conclusion

Remarkable progress has been achieved in recent times with respect to the suite of approaches and technologies available for the purpose of delivery of peptides or proteins via the oral route. As highlighted in this review, achieving efficient oral delivery of a peptide or protein is not a trivial task. While employment of chemical conjugation and modification to exploit the endogenous transport mechanisms of the GIT would appear to be a seemingly attractive and logical approach, it is nevertheless associated with a myriad of challenges, which are diverse in both their nature and level of complexity. Fundamentally, it is essential that a sound biological rationale exists for exploiting

the chosen transport route/mechanism(s) and that it offers desirable benefits and a notable edge over comparable existing technologies. In addition, the translational merit of any given concept must be assiduously evaluated. Many of the delivery principles described here offer significant promise in terms of their capacity to provide efficient delivery of orally administered peptides or proteins. In this regard, it is hoped that a judicious evaluation of the key aspects discussed here, can permit a pragmatic framework for ensuring advancement of those conjugation/modification principles, which offer greatest promise.

Disclosure of potential conflicts of interest

The authors declare the following competing financial interest (s): S.T.B., F.H. and U.L.R. are employees and shareholders of Novo Nordisk A/S. The views and opinions expressed are those of the authors and do not necessarily reflect that of Novo Nordisk A/S.

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