International Journal of Peptide Research and Therapeutics (2024) 30:15
https://doi.org/10.1007/s10989-024-10592-z

Therapeutic Peptides and Proteins: Stabilization Challenges and Biomedical Applications by Means of Nanodelivery Systems

Elisa Berselli[1,2] · Carlotta Coccolini[1,2] · Giovanni Tosi[2] · Evren H. Gökçe[3] · M. Beatriz P. P. Oliveira[4] · Faezeh Fathi[4] · Karolline Krambeck[5] · Eliana B. Souto[1]

Accepted: 26 January 2024 / Published online: 28 February 2024

Abstract

The delivery of peptides and proteins usually faces formulation development challenges attributed to the difficulties encountered in their stabilization. Nanoparticles offer an alternative to improve the physicochemical stability of such biomacromolecules, while increasing their bioavailability by overcoming biological absorption barriers. With this review, we aim to discuss the stability problems of proteins and peptides that have driven the scientific community to find in nanotechnology a valid alternative for oral administration of biomolecules. In addition, we describe the most commonly used nanoparticles for this purpose (e.g., polymers such as polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, modified chitosan, and lipids such as oil-in-water nanoemulsions, self-emulsified drug delivery systems, solid lipid nanoparticles, nanostructured lipid carriers, liposomes, as well as hybrid systems like micelles), and we show some of the most important recent applications of these nanoparticles for the delivery of proteins and peptides, including for the treatment of diabetes, viruses (such as HIV), cancer, as well as in the development of vaccines.

Keywords Peptides · Proteins · Nanodelivery systems · Diabetes · Cancer · Vaccines

Introduction

Peptides and proteins are becoming increasingly important for the pharmaceutical market, especially because of their safety profile and low side effects in comparison to conventional drugs, which may encounter toxicological problems (Mahmood and Bernkop-Schnürch 2019). However, these macromolecules pose some stability challenges when it comes to formulation development. For instance, the gastrointestinal (GI) track, because of the stomach acidic pH and the presence of pepsin, constitutes an initial obstacle; then in the small and large intestine proteins and peptides have to face some degradation enzymes, such as peptidases and proteases (Smart et al. 2014). These biomolecules also have a short half-time, because of their rapid renal clearance from systemic circulation. For this reason, most of the drugs based on peptides and proteins available on the market are not for oral, but for injection, which is usually risky and painful (Abdelhamid et al. 2020; Haddadzadegan et al. 2022). There are a few proteins and peptides that can be orally administered (e.g., pancreatin, vancomycin, octreotide, desmopressin and linaclotide), yet still showing a low bioavailability (around 5% in conscious animals) (Smart et al. 2014). On the other hand, the oral delivery is preferred both from patients and pharmaceutical technologists. Therefore, technological designers have been motivated to develop new delivery systems that could overcome the biological barriers of biomolecules, and to find alternative

✉ Karolline Krambeck
karolline@ipg.pt

✉ Eliana B. Souto
ebsouto@ff.up.pt

1 Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

2 Department of Life Sciences, University of Modena and Reggio Emilia, Giuseppe Campi, 287 41125 Modena, Italy

3 Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, 35040 İzmir, Türkiye

4 REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira No. 280, 4050-313 Porto, Portugal

5 Health Sciences School, Guarda Polytechnic Institute, Rua da Cadeia, 6300-035 Guarda, Portugal

routes of administration to avoid the parental one, especially for chronic diseases situations (Durán-Lobato et al. 2021).

So far, the solution to these problems has been found in nanotechnology. In fact, many teams of research have devoted intense efforts to the study of nanoparticles for proteins and peptides delivery (Durán-Lobato et al. 2021; Haddadzadegan et al. 2022; Shahzadi et al. 2021; Zizzari et al. 2021). Nanoparticles (NPs) are solid particles with a size range between 1 and 100 nm, which, if dispersed in an aqueous phase, get a colloidal behaviour (Zizzari et al. 2021). NPs can be made both of biodegradable polymers (such as PLA, PLGA, polycaprolactone, modified chitosan) and lipids (such as oil-in-water nanoemulsions, self-emulsified drug delivery systems (SEDDS), solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), liposomes, micelles) (Blanco-Llamero et al. 2022; Haddadzadegan et al. 2022; Jain et al. 2013; Zielinska et al. 2020). An example of polymeric NPs used to improve the drug bioavailability through the intestinal route was patented by Gurny et al. (2007), who showed an increase of solubility of compounds in NP for oral administration. The authors used polymers such as Eudragit® L and S, and cellulose derivatives (hydroxypropyl methyl cellulose acetate succinate, hydroxypropyl methyl cellulose phthalate, cellulose acetate phthalate, and cellulose acetate trimellitate) in the development of pharmaceutical formulations for poorly water-soluble compounds (Gurny et al. 2007).

Lipid-based NP, on the other hand, are reported to be superior over polymeric ones for many reasons. First, most of the lipids and surfactants used for NP are listed in the pharmacopeia and are approved by the US Food and Drug Administration (FDA) for oral drug products (FDA 2022). Second, they are made of biodegradable and biocompatible materials, so that no toxicological risks are posed (Doktorovova et al. 2016; Niu et al. 2016). Third, and most important, they overcome the GI biological barrier that proteins and peptides have to face in the oral delivery, namely, the enzymatic degradation and the intestinal adsorption (Li et al. 2012). Moreover, lipids nanoparticles can overcome another important biological barrier for proteins and peptides: the intestinal epithelial barrier. This is possible because lipid NP can interact with the cells of the intestinal epithelium (Chu et al. 2023). This interaction can also be improved thanks to a combination with permeation enhancers, such as bile salts and fatty acids (Kommineni et al. 2023). Besides, lipids are known to be absorption enhancers which altogether will promote a synergistic effect towards improving oral bioavailability of drugs (Preeti et al. 2023).

As mentioned above, lipid-based nanocarriers have been used after the first cyclosporine formulation entered the market in 1980s. Since then, nanoemulsions, SLN, NLC and liposomes have been proposed for the oral administration of proteins and peptides (Shahzadi et al. 2021). Despite that, it has been shown that formulations with liquid lipids were not so functional because they have a difficult control of peptide/protein release (Bernkop-Schnürch and Jalil 2018). In contrast to liquid lipid formulation, there are solid lipid formulation such as SLN and NLC, which are obtained from lipids that melt above room and body temperatures (Doktorovova et al. 2014). This leads to a carrier with higher release control ability, thanks the lower drug diffusion in the solid lipid structure (Mehnert and Mäder 2012). To be more specific, SLN are composed of solid lipids only, whereas NLC are made of both solid and liquid lipids. The advantage of NLC over SLN is that, during their storage, NLC do not have drug expulsion problems caused by the crystallization and phase transition of the solid lipid. An example of successful loading was provided by Muntoni et al. (2021). The authors loaded glargine insulin with sodium dodecyl sulfate (SDS) and registered a decrease of blood glucose in induced diabetic rats after NLC oral administration (Muntoni et al. 2021). NLC and SLN however may also encounter some formulation challenges because their excipients and surfactants often degrade by gastrointestinal lipases. Also, the lipids may have problems of lipolysis and consequent degradation of peptides and proteins that are loaded (Olbrich and Müller 1999).

With this review, we aim to discuss the stability problems of proteins and peptides that have driven the scientific community to find in nanotechnology a valid alternative to improve the bioavailability of such biomolecules. In addition, we describe the most common delivery systems used, so far, for this purpose, and we show some of the most important recent discovery about proteins and peptides encapsulation into different types of nanoparticles.

Challenges Encountered in Peptides and Proteins Stability

The challenges, that can compromise proteins and peptides stability in vivo, depend on the administration route of the drug. For example, a relatively new route of administration is the nasal delivery. It has three mainly biological barriers that have to be faced: first, there is a low surface area available for the administration, therefore, only a limited volume of drug can be used; second, the high sensitivity of nasal mucosa to irritation; and third, the clearance mechanism of the mucociliary tissue (Durán-Lobato et al. 2021). Anyway, the most common, and troublesome, route of administration is the oral one. The oral delivery is full of biological barriers, due to the fact that, after the administration, the drug needs to cross the entire GI trait, which is full of obstacles.

Proteins and Peptides Stability Problems Through the Gastrointestinal (GI) Tract

As mentioned above, when orally administered, therapeutic proteins and peptides have to face numerous obstacles throughout the GI trait. To be more specific, they face a pH-dependent denaturation in the stomach, and an enzymatic degradation and weak uptake in the intestine (Hashim et al. 2023). Regarding the intestine in general, small and large, the most fearsome obstacle for peptides and proteins is to cross the intestinal epithelium, specifically the apical membrane of the intestinal epithelium. The problem is that peptides and proteins are generally too large to penetrate the cell membrane; the consequence is a low intestinal adsorption (Fan et al. 2018). In addition, between epithelial cells there are tight junctions, which are important seals that maintain epithelial tissue barriers and cell polarity. Tight junction obstacles the permeation of peptides and proteins through the intestinal epithelium (Lundquist and Artursson 2016).

Gastric Stability

The stomach is the first barrier that proteins and peptides have to face after the oral administration. The challenges encountered by the biological drugs in this environment are the acid pH caused by the gastric fluids, and the presence of pepsin, a type of proteolytic enzymes (Caffarel-Salvador et al. 2017). The acidic pH may lead to denaturation of proteins and peptides structure and consequent loss of function, because it alters the ionization of the amino acids in proteins and peptides. In this way, the biomolecules lose their secondary and tertiary structure, especially the biggest ones. Instead, pepsin causes a peptide structure degradation by breaking peptide bonds between hydrophobic amino acids (especially the aromatic ones) (Fig. 1). It can be deduced that proteins and peptides with a simpler structure and less hydrophobic amino acids are more stable in the stomach. Therefore, it is possible to relate the stability to the size of biomolecules: the smallest ones are more stable than the biggest one, because their structure is less influenced by the acid pH and pepsin (Smart et al. 2014). Peptides that have some hydrophobic amino acids in their structure, but a simple structure, such as vasoactive intestinal peptide, are degraded by pepsin, but are more stable under acidic pH (Cui et al. 2013).

Small Intestine Stability

The intestine is, together with the stomach, an important barrier for proteins and peptides. In the small intestine, specifically, there are many different digestive enzymes that cause the degradation of different compounds (Durán-Lobato et al. 2021). In the small intestine, there are mainly two types of degradative enzymes, i.e., proteases and peptidases. They both break the peptide bond in proteins and peptides, leading to a structure degradation and loss of functionality. There is also another specific case for proteins and peptides that contain cysteine residues; these can be degraded by an exchange reaction (thiol-disulfide) between these cysteine residues and a reduced glutathione. Glutathione is an important compound for the organism, taking part in the antioxidant defence system. Glutathione can be oxidized or reduced in order to protect proteins and peptides from the free radicals. As in the stomach, also in the small intestine molecules with a simple structure and a small size show improved stability, because they have less enzyme cleavage sites. Similarly, proteins have a better stability than large peptides, attributed to the presence of a higher number of exposed peptides bonds in the latter, which can easily be substrate of the digestive enzymes. Proteins, due to their tertiary or quaternary structure, hide their peptide bonds from the enzymes. Anyway, after oral administration, proteins lose their structure and expose their peptide bonds, so a protective delivery system is necessary for both peptides and proteins (Smart et al. 2014).

Large Intestine Stability

In the large intestine, the main mechanism of proteins and peptides degradation is related to the presence of colonic microbes (Hua et al. 2015). Their activity is dual; on one side, they are responsible for the fermentation reaction and, on the other side, they secrete proteases that degrade biomolecules. Since fermentation is independent from the molecular size, in the large intestine there is no association between degradation and size (Smart et al. 2014). Compared with small intestine degradation, the large intestine

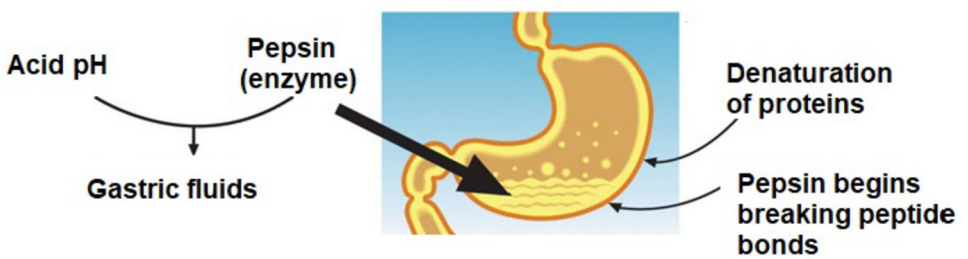

Fig. 1 Challenges in protein and peptide stability under gastric conditions. First denaturation of the macromolecule and then enzymatic digestion occurs

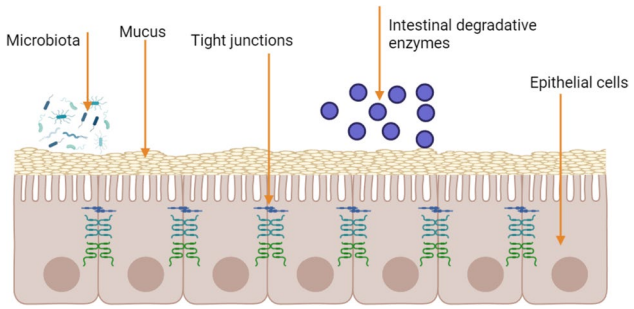

Fig. 2 Main barriers for peptides and proteins encountered in oral administration. Adapted from Lundquist and Artursson (2016)

degradation turned out to be lower, both in brushtail possums and rats (Tozaki et al. 1998; Wen et al. 2002).

Moreover, proteins and peptides also face a permeability problem caused by the mucus barrier in the intestine (Fig. 2). This is a protective barrier, which, via hydrophobic interaction, obstacles the intestinal penetration of many compounds, among which there are also some drugs that are immobilized in the mucus layer (Lundquist and Artursson 2016). To overcome this barrier, it is necessary to modify the delivery of drugs, for example using lipid or polymeric nanoparticles, which have a mucus-penetrating ability, especially if added with hydrophilic polymers, such as polyethylene glycol (PEG), that increase the mucus permeation (Zizzari et al. 2021). Insulin nanoparticles made of a core composed of insulin and a cell penetrating peptide (CPP), and covered with 2-hydroxypropylmetacrylamide copolymer (pHPMA), were compared with non-covered insulin (Shan et al. 2015). The covered one showed a better in vitro cellular internalization, 20-fold higher than the free insulin, due to the "mucus-inert" pHPMA and the increased absorption mediated by CPP. In addition, the authors evaluated the hypoglycaemic effect by in vivo oral administration of NPs on diabetic rats, and obtained a maximal blood glucose level reduction of 50%, in contrast with a non-reduction when free insulin was oral administered. A polymeric cover together with a cell penetrating peptide were shown to be able to avoid the intestinal mucus and epithelial barriers (Shan et al. 2015).

The effect of intestinal mucus layer on the absorption and bioavailability of orally administered drugs has been thoroughly studied by Haddadzadegan et al. (2023). The authors evaluated the effect of thiolated cyclodextrins in increasing the mucoadhesion through the intestinal route and thus their potential use to increase drug bioavailability. The study reported that protecting free sulfhydryl groups of the complexes, improved not only mucoadhesive strength but also their mucosal diffusion attributed to the reduced reactivity of thiol moieties (Haddadzadegan et al. 2023).

To improve the permeation, it is also possible to use specific surfactants, which works as permeation enhancers. Together with permeation enhancers and nanocarriers formulation, there is also another strategy that can be used to overcome the stability problems in the intestine: the cyclization of linear peptides (Zizzari et al. 2021). One example is desmopressin, which was obtained from cyclization of the peptide on a resin (Srivastava 2011).

Stabilization of Peptides and Proteins in Nanoparticles and Hydrophobic Ion Pairing

In the process of proteins and peptides loading into a nanocarrier, some technological problems may occur. In case of lipid nanoparticles, the main problem is related to the weak lipophilic character of the biological drug. In order to have a successful incorporation, the lipophilic character of proteins and peptides need to be enhanced. To do this there are some possibilities of chemical modification, namely, reversible aqueous lipidization, cyclization and bond formation between proteins or peptides and fatty acids (through an amide or ester bond). However, the best way to do that is the hydrophobic ion pairing (HIP) (Haddadzadegan et al. 2022). One of the reasons why this approach is better than the others, is the regulatory aspects: HIP does not modify any covalent bond of the starting molecules (Fig. 3), so the complexes obtained in this way do not need a full FDA approval, which is the opposite for the prodrugs approaches that require a full FDA authorisation (Pinkerton et al. 2013; Stella 2010).

HIP is, in general, a strategy used to incorporate charged hydrophilic molecules into hydrophobic complexes. In the specific and most common case of peptides and proteins loading into delivery systems, these small biological hydrophilic molecules interact with an oppositely charged counterion. The counterion is made of different domains, among

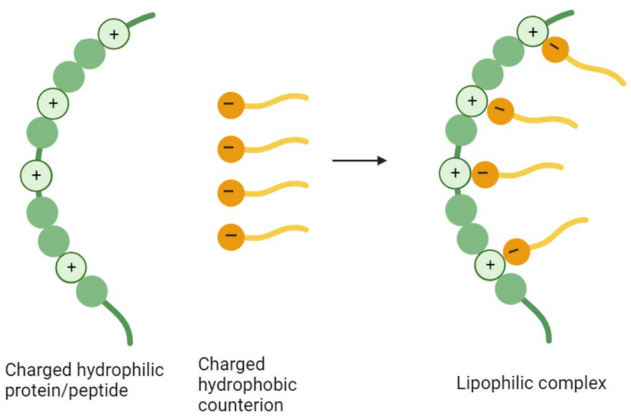

Fig. 3 Schematic representation of hydrophobic ion pairing mechanism

which at least one is hydrophobic (for example an alkyl tail or an aromatic ring). Since they are molecules with both an hydrophilic and a lipophilic domain, surfactants are usually employed as counterions, because they have this amphiphilic chemical structure described above (Ristroph and Prud'homme 2019). An example is provided by arginine derivates surfactants, namely Arg-diosgenyl ester (ADE) and Arg-cholesteryl ester (ACE), which resulted safe and efficient for HIP with insulin, compared to 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP), a non-degradable cationic surfactant used as a reference (Matteo Jörgensen et al. 2023). The complex formed in this way has a higher lipophilic character than the starting molecules, therefore, during the NP formulation, they can be dissolved into the lipophilic phase (Griesser et al. 2017). The lipophilic character is improved because of two reasons: firstly because the hydrophobic domain of the counterion covers the hydrophilic character of the starting molecules; secondly because the complex with the counterion disguises the original charge of the molecule, so it has a decrease in its aqueous solvent solubility (Ristroph and Prud'homme 2019). The ionic complexation between the original molecules and the counterions is possible because of the presence in proteins and peptides of anions on the C-terminal carbonic acid (as in glutamic acid and aspartic acid), and cations on the N-terminal primary amine (as in lysine and arginine). In order to have a successful ionic interaction, both the peptides/proteins and counterion have to be sufficiently charged. To do that, it is necessary to control the pH of the medium: it has to be at least two steps above or below the isoelectric point of the peptide or protein to have a noticeable, respectively, anionic or cation charge. Surfactants can either be charged depending on the pH, or they can be always ionized if they have a strong anionic or cationic permanent charge (for example, sulfates, sulfonates and quaternary ammonium). Anionic counterions commonly used for peptides and proteins are cholic acid, decanoic acid, oleic acid, pamoic acid. As cationic counterions arginine-based surfactants, chitosan, cetyltrimethylammonium bromide (CTAB), triethylamine (TEA) are often used (Ristroph and Prud'homme 2019).

After the administration, when the therapeutic peptides or proteins are released from the carrier, the lipophilic character gained with the HIP is lost: this is because, after the release from the NP, the original molecules and the counterions untie themselves and the original compounds are regenerated. The complex separation is due to the counterions competition with salts or a changing in the pH of the medium that cause the loss of the anionic or cationic charge. The re-complexation is impossible because both charged molecules are surrounded by the medium, which has a strong ionic character. At this point, the regenerated proteins or peptides are too hydrophilic to stick with the NP and will partition in the bulk; instead, the counterion can either stick to the NP or not, depending on its structure. This pH-dependent release can be useful for drug delivery and targeting to endosomes, tumors, or different traits of intestine (Ristroph and Prud'homme 2019).

Nanoparticles Used for Loading of Peptides and Proteins

The use of transport systems helps protect peptides and proteins from environmental conditions, reduces the immune response, and facilitates reaching the target site, thus they improve biocompatibility and efficacy. The most widely used transport systems to carry proteins and peptides are oil-in-water nanoemulsions, self-emulsifying drug delivery systems (SEDDS), solid lipid nanoparticles (SLN), nanostructure lipid carriers (NLC), liposomes and micelles, also due to the fact that they are administered orally, which clinically has the highest compliance (Griesser et al. 2017; Haddadzadegan et al. 2022).

With the formation of hydrophobic ion pairs (HIP), the hydrophilic character of the proteins and peptides to be loaded into the system drops dramatically, while their lipid properties increase, so it will be possible to load them into the lipid phase of transport systems (Griesser et al. 2017). Saleh et al. (2023) provided a recent example of peptide antibiotic (polymyxin B) polyphosphate nanoparticles (PMB-PP-NPs) formulated applying the ionic gelation technique: the PMB-PP-NPs showed protection against the enzymatic degradation of the peptide, resulting in an increased adsorption through the mucus gel layer due to the zwitterionic surface of the NPs, and an efficient release of the drug at the target epithelium thanks to an IAP-dependent cleavage of polyphosphate (Saleh et al. 2023). Encouraging results come also from a study, in which NPs are formulated based on polysaccharides: the study reported positive data about improved orally administered proteins/peptides bioavailability, improved proteins/peptides stability, and increased mucus barrier overcome and intestinal permeability (Yuan et al. 2023).

Self-Emulsifying Drug Delivery Systems

Self-emulsifying drug delivery systems (SEDDS) are systems that are easy to produce, the reason why they are preferred by the pharmaceutical industry (Kubackova et al. 2021). SEDDS are defined as mixtures of oils, surfactants, solvents and optionally co-solvents/surfactants that form nano-scaled oil-in-water (o/w) emulsions when they come into contact with an aqueous medium (Leonaviciute and Bernkop-Schnürch 2015). By combining HIP and SEDDS for oral administration of therapeutic peptides, some studies have shown an increase in bioavailability ranging from 5 to

25% (Friedl et al. 2021). Several studies have also shown that SEDDS can be an attractive way to deliver proteins and peptides since they protect the biomolecules from presystemic metabolism and facilitate permeation through the intestinal mucosa (Leonaviciute and Bernkop-Schnürch 2015). In fact, the use of these lipidic delivery systems, protects the proteins from attack by gastrointestinal peptidases, glutathione, and digestion proteins that are too hydrophilic to interact with the lipophilic surface: thus, peptides are protected from enzymatic degradation. In addition, to limit attack by lipases, excipients, that are poorly degraded by these enzymes, can be used. The nanocarriers that showed the best permeation properties through mucous membranes are those with size < 200 nm and muco-inert surface (PEG or zwitterionic surface). Moreover, once they reach the underlying membrane, nanocarriers are able to induce endocytosis, transcytosis or fuse with cell membranes and release their contents into the circulatory stream (Haddadzadegan et al. 2022). SEDDS have also been tested to find an answer to a new challenge, i.e., the delivery of vaccines by the oral route. In one of the studies conducted by Lupo et al. (2019), bovine serum albumin was considered as the model antigen to be loaded into the system, while *Salmonella minnesota* lipid A (MPLA) and squalene were used as adjuvants in the formulation, obtaining two different formulations SEDDS-BSA-MPLA and SEDDS-BSA-squalene. The formulation with MPLA elicited the best systemic and mucosal immune response (Lupo et al. 2019).

Lipid Nanoparticles

Solid lipid nanoparticles (SLN) are nanoparticles consisting of solid lipids, surfactants and cosurfactants in some cases, while nanostructured lipid nanoparticles (NLC) are nanoparticles consisting of both solid and liquid lipids (in the inner core). Protein-loaded SLN and NLC can be prepared through different techniques that are capable of avoiding modifying or damaging the peptide structures. The peptide content is complexed with HIP and dissolved in an appropriate solvent or fused with lipids and added during preparation. The techniques used can be high-pressure homogenization techniques, solvent emulsion-evaporation, solvent emulsion-diffusion, and microemulsion (Hu et al. 2004). The choice depends on the thermal stability of the proteins being carried and the desired final characteristics of the nanoparticles, which will be characterized based on size distribution, zeta potential, drug release, stability, ability to permeate through membranes, as well as efficacy (Almeida and Souto 2007; Üner 2006).

Since SLN and NLC are solid even after oral administration, they are more stable at the GI tract protecting the peptides from enzymatic attack (Almeida and Souto 2007; Muller et al. 2006). In addition, the solid nature of these carriers is useful in limiting the release of the contents into the GI fluids. However, the solid nature is a disadvantage once they reach the membrane since they cannot cross the epithelial barrier and they cannot reach the systemic circulation. Hu et al. (2016) showed that SLNs can strongly interact with intestinal epithelial cells. Furthermore, some studies have shown that the role of surfactants is central in ensuring resistance to lipid hydrolysis. Indeed, NLC ester-free and with PEG-ether on the surface do not undergo any hydrolysis, whereas those with PG-ester and PEG-ester do (Shahzadi et al. 2021).

Liposomes

Liposomes are spherical colloidal particles consisting of one (or more) phospholipid bilayers arranged to form a vesicle-like structure. They can be made up of natural or synthetic phospholipids, and due to both the hydrophobic (heads) and hydrophilic (tails) nature of phospholipids, liposomes are capable of encapsulating compounds that are hydrophilic in nature within the aqueous reservoir and lipophilic in nature within the bilayer (Pudlarz and Szemraj 2018; Suntres 2011; Teixeira et al. 2017). In fact, hydrophilic peptides will be carried within the aqueous core, while lipophilic HIP will be carried within the phospholipid bilayer; moreover, peptides or HIP can be linked to the nanoparticles' surface. It is referred to as colloidal solution because the liposomes approved in therapy are between 50 and 300 nm in size (Kraft et al. 2014).

To ensure protection of peptides from enzymatic degradation, it is essential to prevent lipolysis of the phospholipids that make up the wall. For this they can be replaced from tetraether lipids (TELs). To ensure efficacy by oral administration, it is necessary to increase stability in the GI tract, prevent protease action, and improve permeation through the mucosa. For this purpose, some studies described the formulation of surface-modified liposomes coated with mucoadhesive polymers e.g., chitosan (which increases stability and improves permeability) and also adding a protease inhibitor (aprotinin). In addition, surface PEGylation also increases the bioavailability of orally administered liposomes (Werle and Takeuchi 2009).

The permeation through biological membranes is increased due to the HIP formation. Authors therefore concluded that liposomes are only suitable for protecting peptides and proteins if they are coated with polymers such as chitosan or if they incorporate other agents such as bile salts (that increase its cellular uptake), so their protective effect is low. They are also produced through techniques that are more difficult to standardize than those of other lipid-based nanocarriers such as SEDDS (Song et al. 2005).

Micelles

Micelles are colloidal systems formed by self-assembly of amphiphilic molecules, constituting delivery systems for drugs and macromolecules that, in this way, are protected in their chemical and physical stability. Thus, micelles consist of a hydrophobic central zone and the hydrophilic surface portion: they are made of amphiphilic block polymers such as polyoxyethylene, polyethylene glycol, polyvinylpyrrolidone, and hydrophobic materials, such as polylactic acid, methyl methacrylate, polystyrene, polypropylene, etc. (Ommura et al. 2020).

By encapsulating amphiphilic peptides in micelles, aggregation can be avoided. Also, by associating PEG with membrane phospholipids, they can be sterically stabilized and form self-assembling Sterically Stabilized Micelles (SSM) (Cholkar et al. 2012). In addition, encapsulation of peptides in PEGylated micelles allows interaction between PEGs and the peptide that induces a conformational change in the peptide that assumes the more stable alpha-helical structure.

In addition, polymeric micelles ranging in size from 100 to 200 nm have also been tested for peptide delivery. For example, studies have shown that insulin-loaded polymeric micelles allowed effective uptake in liver cell lines and intestinal epithelium (Bahman et al. 2020); also other experiments conducted on rats, showed a reduction in blood glucose levels, after the administration of micelles containing insulin. As for the liposomes, however, micelles are effective in protecting peptides and proteins only when efficiently conjugated with auxiliary agents (Katsuda et al. 2010).

Biomedical Applications

Proteins and Peptides-Loaded Delivery Systems for Diabetes

Diabetes is a chronic condition classified into type 1 diabetes (autoimmune) and type 2 diabetes (insulin-resistant). In type 1 diabetes, patients lose the ability to secrete insulin and external administration is therefore necessary. Whereas, in type 2 diabetes, although insulin is produced, the receptors are insensitive. The early stages of type 2 diabetes are treated through behavioural therapy (exercise and diet) and through oral medications, such as metformin and alpha-glucosidase inhibitors (Souto et al. 2011). In the later stages, however, insulin injections are the only effective therapy (Wang et al. 2022). Diabetes is one of the 10 diseases that threaten world health the most, and is one of the fastest growing diseases in terms of patient numbers: 4.6 million people die each year from this disease (Bennett 2018).

Currently, the FDA has approved about 100 types of insulin-containing drugs. However, they are all intended to be administered by injection, which, in addition to having side effects such as erythema, abscesses, lipoatrophy or hypertrophy at the injection site and hypoglycemic reactions, is an invasive route of administration that has low patient compliance (Richardson and Kerr 2003).

In order to reduce the pain of injections and to promote greater therapeutic compliance, one of the aims of researchers in this regard has been to propose new routes of administration, such as sublingual, pulmonary, transocular and rectal (Fangueiro et al. 2013; Souto et al., 2019). However, the safest and most patient-accepted route is certainly the oral route, for which no hypoglycaemic or local reactions are observed (Wang et al. 2022).

However, the bioavailability of oral insulin is less than 2% due to many obstacles such as its hydrophilicity, poor stability, high molecular weight, and low tolerance to the action of proteases (Fig. 4). It also struggles to pass the membrane of the GI tract. For this reason, formulations with enzyme inhibitors, permeation enhancers and pH regulators were initially attempted, however, the safety and efficacy of these products was uncertain (Eldor et al. 2013). To date, the formulation of different delivery nanosystems has improved the oral bioavailability of insulin (Fangueiro et al. 2013).

Delivery nanosystems are produced through various techniques that lead to the formation of nanoparticles of different types, such as nanoliposomes, polymeric micelles, nanocapsules, nanospheres and microemulsions (Zhao et al. 2017). These delivery systems are able to improve drug bioavailability and half-life due to their characteristics such as small size, strong adhesion and easy crossing of mucous membranes and entry into cells, resistance to gastric juices and digestive enzymes.

A variety of pH responsive, biodegradable, biocompatible, and easy-to-synthesize materials are used for the formulation of nanocarriers. Both natural polymers (such as chitosan, sodium alginate, hyaluronic acid, and bile acids) and synthetics (such as PLA, PLGA, PCL) are used.

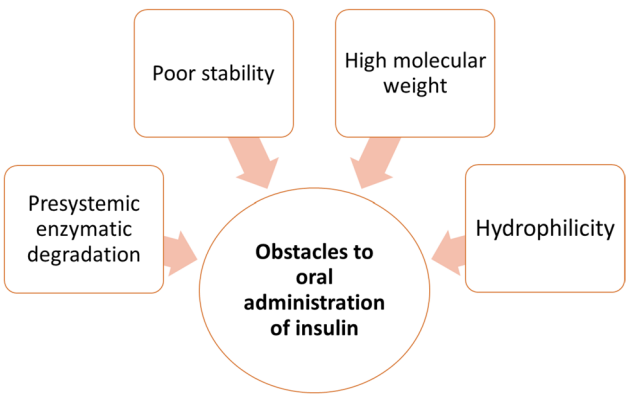

Fig. 4 The low bioavailability of oral insulin

Inorganic materials are also used mixed with organic materials (Ahmad et al. 2017; Kecman et al. 2020; Wang et al. 2022).

Various studies conducted in the laboratory confirm that nanosystems make numerous improvements in resistance to gastric juices, absorption through the intestinal mucosa, and drug bioavailability. However, many questions remain about the safety of the nanomaterials when administered in the clinic, the sub-optimal in vivo bioavailability and about safe and reproducible preparation processes.

Various nanocarriers have been produced to deliver insulin, such as liposomal nanoparticles, nanocapsules and nanospheres, nanogels and inorganic/organic nanohybrids.

Liposomes, as defined above, have advantages such as low toxicity, high biocompatibility, and reproducibility. They are also able to accumulate in some preferential organs such as liver, spleen and lungs, reducing toxicity in the heart and kidneys. Wang et al. (2022) (Wang et al. 2022) formulated cationic liposomes by thin film hydration technique and using egg yolk lecithin (EPC), cholesterol, and the cationic lipid DOTAP as carrier materials. In addition, bovine serum albumin has been inserted on the surface to make the charge of the system neutral and increase the hydrophilicity of the surface in order to facilitate the crossing of the epithelia. It has been shown that insulin in this formulation had a greater bioavailability than free insulin, and its ability to cross barriers increased by about 3 to 8 times. Other studies conducted on uncapped positive-charged liposomal nanoparticle (IPUL-CST) with a particle size of approximately 200 nm and then encapsulated with a chondroitin sulfate-taurocholic acid coupling (CST) recorded a 34% increase in protein bioavailability. In addition, the experiment is the first demonstration of an insulin delivery system from which insulin is released by direct activation by postprandial glucose increase in the intestine (Kim et al. 2018; Yu et al. 2019).

Increased membrane permeation has also been demonstrated in the case of insulin-loaded chitosan nanoparticles. These nanoparticles were made by ionic gelation method using tripolyphosphate sodium (TPP) or poly (acrylic acid) (PAA) (Sharma et al. 2015). In addition, these nanoparticles have made it possible to prolong the residence time of insulin in the intestine and to increase permeation via the paracellular pathway in the bloodstream. This is a great advantage since the biggest limitation of hydrophilic macromolecules, such as insulin, is their inability to cross the intestinal epithelium. This is possible due to the properties of chitosan, which is a cationic linear polysaccharide consisting of β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine (Sharma et al. 2015). Chitosan acts as a permeation enhancer, in fact it is able to adhere to the mucous membranes and transiently loosen the tight junctions between epithelial cells, allowing a significant increase in paracellular absorption. Chitosan regulates the expression of claudin-4 (transmembrane protein responsible for the integrity of tight junctions), in particular chitosan causes the redistribution of claudin-4 from the membrane to the cytoplasm where it is digested by lysosomes, thus weakening the junction. Cui et al. (2009) improved oral bioavailability by encapsulating insulin in the shell of pH sensitive carboxylated chitosan grafted poly (methyl methacrylated) nanoparticles. In this way a slow release at pH 2 of the stomach is obtained, while a faster release occurs at intestinal pH. Chitosan derivatives have also been introduced to improve water solubility, adhesion properties and permeability at neutral pH. However, it must be considered that chitosan shows a certain toxicity for the GI tract as it opens the tight junctions and allows the passage into circulation even of potentially harmful substances (Wang et al. 2022).

The oral insulin delivery was also proposed by the use of PEG-coated silica nanoparticles. Andreani et al. (2014) described a sol–gel technology for the production of silica nanoparticles followed by the coating with PEG. Everted rat intestine was used as the in vitro model to assess permeation profile. The authors concluded that the release of insulin is less prone to be influenced by the molecular weight of PEG, but rather to its affinity to silanol groups at silica surface. The released insulin kept its conformational changes under gastrointestinal simulated conditions.

Some researchers have also formulated micelles consisting of pH-sensitive cationic polymers. These core–shell structures self-assemble in aqueous medium and are able to prevent burst release of insulin at gastric pH (allowing a controlled release), but also promote adhesion and increase residence time in the gut (Wang et al. 2022). The formulated micelles have a hydrophobic core consisting of methyl methacrylate and methacrylic acid, while the hydrophilic and pH-sensitive surface consists of poly (2-aminoethyl methacrylate) (PAEMA). The PAEMA surface provides stability to the formulation by protecting the surface: amine residues fully protonate to the acidic pH of the stomach, conferring positive charge to the surface, which thus adheres better to mucous membranes, and improves bioavailability and membrane permeability by loosening tight junctions.

Other types of polymeric micelles have also been formulated by Han et al. (2020) who tried to mimic the surface characteristics of chlamydia virus to achieve its same ability to move in mucus. These micelles, consisting of an amphoteric betaine polymer conjugated to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), showed an increase in insulin bioavailability by proton-assisted amino acid transporter 1 (PAT1). In fact, in vitro experiments demonstrated a marked increase in the uptake of amphoteric cyclins in the Caco-2 cell line that over-expresses PAT1, while the entry of micelles into cells is inhibited by the receptor substrate. An increase in retention time in the intestine was also observed

for these micelles; animal studies showed a 42.6 percent increase of bioavailability. In general, polymeric monolayer micelles have smaller size and greater ability to permeate the cells of the intestinal epithelium than liposomes, although the drug loading capacity in micelles is lower. Micelles are of interest in controlling postprandial glucose levels because of their high release rate and responsive release capability (Wang et al. 2022).

One of the nanocarrier models for delivering insulin orally are also PLGA nanoparticles. PLGA is a synthetic biodegradable polymer approved by the FDA for drug delivery; studies have shown hydrophobic and hydrophilic interactions with insulin. The most encouraging results were obtained from the formulation of PLGA particles containing insulin and then embedded in a PVA hydrogels (Liu et al. 2007). Particles so formulated in the system showed a reduction in the rate of insulin release and in the total amount of drug released. Further modifying the surface of the nanoparticles with chitosan results in a better ability to interact with cell membranes and a consequent increase in endocytosis. This effect is due to the fact that chitosan as a polycationic polymer masks the negative surface of PLGA. PLGA nanoparticles and chitosan showed good bioadhesion and bioavailability. A complex of PLGA-insulin-sodium oleate (anionic surfactant that improves the liposolubility of the protein) prepared through emulsion solvent diffusion when administered in vivo to diabetic rats decreased glucose levels by 23.85% after 12 h and the effect was maintained for 24 h (Sun et al. 2010). These results are promising because they open the possibility to the treatment of diabetes with only one oral dose per day (Jain et al. 2012).

One of the objectives is also to obtain a release of insulin proportional to the amount of glucose in the blood, to have a more precise control and avoid hypoglycemic crises. To achieve this, studies were conducted on glucose-sensitive and glucose-responsive nanomaterials. An example was the formulation of a reverse microemulsion of dextran nanoparticles and Concanavalin A (Con A), that is a tetrafunctional glucose-binding protein. When in contact with free glucose, Con A releases polymer glucose and binds other free glucose, causing the system to disintegrate and insulin to be released (Sharma et al. 2015).

The oral administration of lectin-modified, insulin-loaded SLN was shown to reduce enzyme degradation and increase oral absorption (Zhang et al. 2006). Lectins are a class of proteins with adhesive properties for various carbohydrates and since lipids and proteins lining the intestinal epithelium are often glycosylated, lectins are responsible for increasing SLN adhesion and uptake. The hypoglycaemic effect of insulin-containing SLN synthesized by the solvent emulsification evaporation method was studied for 24 h and it was concluded that SLN increase oral insulin absorption (Sarmento et al. 2007). Other studies have shown that the uptake in the Caco-2 cell line of insulin-SLN modified with octaarginine is 18.44 times greater than with insulin solution. Other studies have formulated chitosan-coated SLN that have shown the ability not to be internalized in macrophages and escape phagocytosis. Lipid materials were also tested as constituents of SLN and the best in terms of hydrophobicity, drug availability was glyceryl palmitostearate. SLN have improved bioavailability, have high tolerability and low toxicity, are easy to produce on a large scale, are biodegradable. However, they have a low encapsulation capacity, low half-life and poor physical stability (Wang et al. 2022). An innovative technology has been tested by Chu et al. (2023), who formulated an in situ rearranged lipid nanoparticle encoding insulin for oral administration: insulin was solubilized in lipids via reverse micelles with the addition of functional components (chitosan, sodium deoxycholate and sulfobetaine). The lipid NP has been tested for insulin capacity, mucus and intestinal penetration, and in vivo absorption mechanism. The activity and structure of insulin don't change during the preparation process, so the protein is stable, the mucus penetration and the epithelial cell crossing are improved, leading to a pharmacological insulin bioavailability increased till 13.7% in diabetic rats.

These data are interesting starting points for oral insulin administration, but further studies will be needed to arrive at an effective, stable and safe formulation in vivo. Examples of nanocarriers for the loading of insulin and main outcomes are listed in Table 1.

To date, the research on oral insulin administration is running fast both with nanodelivery system and without. For example, a phase 3 clinical trial (NCT02954601) to assess the safety and efficacy of multiple doses of oral insulin (ORMD-0801) in subjects with type 2 diabetes is currently being evaluated. This phase 3 follows the positive results of the phase 2, in which a total of 373 subjects with glycated haemoglobin level > 7.5% were randomized to receive either placebo or ORMD-0801 at doses of 8 mg or 16 mg once or twice a day, or 32 mg once, twice or 3 times a day over 12-week period. Reduction in glycated haemoglobin levels, compared to placebo, were observed with 8 mg once and twice a day, and 32 mg once and twice a day, while no results were obtained with 16 mg once and twice a day. ORMD-0801 resulted safe and well tolerated, with no significant adverse events, in the same phase 2 trial (Eldor et al. 2023). Moreover, an oral treatment has been recently approved by FDA (NDA: 213,182) with the name of Rybelsus®: tablets composed of oral semaglutide co-formulate with the adsorption enhancer sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC). Semaglutide is a GLP-1 (glucagon-like petide-1) analog, so it promotes insulin secretion in a glucose concentration-depended manner, so it has a hypoglycemic effect. Rybelsus® is the first GLP-1 receptor agonist to be approved for oral administration, and it is

Table 1 Examples of studies describing the loading of insulin in nanocarriers

Type of nanocarrier	Surface functionalization	Results	References
Cationic liposomes	Bovine serum albumin	Increased epithelial permeability from 3 to 8	Wang et al. (2022)
Uncapped positive-charged liposomal NPs	Chondroitin-sulfate taurocholic acid	Increased bioavailability (34%)	Kim et al. (2018); Yu et al. (2019)
Poly (methyl methacrylate) NPs	pH sensitive carboxylate chitosan	Improved oral bioavailability	Cui et al. (2009)
Betaine polymeric micelles	DSPE	Improved insulin bioavailability (42.6%)	Han et al. (2020)
PLGA NPs	–	Decrease in glucose levels of 23.8% in 12 h	Sun et al. (2010)
In situ rearranged lipid NPs	Chitosan, sodium deoxycholate, sulfobetaine	Increased insulin bioavailability (13.7%) in diabetic rats	Chu et al. (2023)

efficient in patients with type 2 diabetes at various stages, either in monotherapy or in combination with other treatments (Miyasaka 2022).

Proteins and Peptides-Loaded Delivery Systems for Cancer

Despite the latest advancements towards the use of nanomedicines for the treatment of cancer (e.g., liposomal irinotecan, liposomal daunorubicin), some risk of toxicity and adverse effects still exists (van der Meel et al. 2019). That is why it is becoming necessary to develop new anti-cancer drugs with a higher efficacy/toxicity ratio. Among these new therapies, protein-based drugs are becoming increasingly popular, and, in particular, monoclonal antibodies (mAb) are emerging more than other proteins (Zielinska et al. 2023). The reason why they have been studied for so long is mainly because they can directly target cancer cells. Moreover, they can be receptors antagonist and inhibitors, and they can induce immune-related cell death in the cancerogenic tissue (Durán-Lobato et al. 2021). As mentioned above, proteins and peptides have many stability problems, when orally administered. That is why nanomedicine has emerged to face this problems, with significantly positive results for the anti-cancer therapy (Peer et al. 2007; van der Meel et al. 2019). In preclinical studies, in facts, nanomedicine has shown an increasing inhibition of tumoral growth compared with non-formulated drugs (van der Meel et al. 2019).

The formulation of nanocarriers is not always simple and may have some difficulty: for example, nanocarriers may go through an excessive alteration when get in contact with blood stream, and this could lead the loss of targeting ability and could induce an immune reaction against the biological drug inside the nanoparticle (Durán-Lobato et al. 2021). The solution to this problem may be the addition of PEG or other targeting ligands on the nanocarrier surface, so there are higher possibilities for the drug-loaded nanoparticles to reach the target (Cao et al. 2020).

So far, many clinical and preclinical studies have provided efficient results of nanomedicine for cancer therapy application, showing a tumor growth inhibition and raising the life time of patients (Iqbal et al. 2021). Some examples are listed in Table 2.

Fu et al. (2022) showed an efficient result of a nano-encapsuled protein for cancer therapy. They encapsulated a tetra-guanidinium (TG)-modified saporin, into tumor microenvironment (TME) pH-responsive polymeric NP. The formulation was administered and, after entering the systemic circulation, reached the tumor site. Here two different mechanism have been described: a pH of 6.5 provide the NP degradation and consequent protein release and penetration in the cancerogenic tissue with the aid of TG;

Table 2 Examples of studies describing the loading of proteins/peptides in nanocarriers against cancer

Protein/peptide	Delivery system	Type of tumor	Results	References
Tetra-guanidinium-modified saporin	pH-responsive polymeric NPs	Lung cancer	Decrease of lung cancer cell growth in vivo and in vitro	
Saporin	TME pH-responsive PEGylated polymer (PPMEMA)	Breast cancer	In vitro increase of cancer cells death, in vivo reduction of tumor size increasing	Shen et al. (2021)
Granzyme B, GALA	Platelet delivery platform	Post-surgical tumor recurrence	Prevention of tumor recurrence after tumor surgical resection	Fan et al. (2022)

some other proteins enter the cells by NPs endocytosis. The results showed an effective cytosolic delivery, and an excellent concentration-depending tumor inhibition at pH 6.5 (IC50 = 5.8 nM), so a decrease of lung cancer cells growth both in vitro and in vivo was efficiently performed. A similar TME pH-responsive polymeric NP loaded with saporin has shown an inhibition effect on breast cancer cells. A robust NP platform has been formulated with a TME pH-responsive PEGylated polymer (PPMEMA) and an amphiphilic cationic lipid-like compound (denoted as G0-C14), which form a complex with the saporin inside the NP. When the system reaches the TME, the pH induces the NP dissociation and exposure of protein/G0-C14 complex: in this way the saporin can induce cell apoptosis and the G0-C14 improves the endosomal escape of the internalized protein. The results showed a 60% of loaded saporin release from NP in 8 h at pH 6.8; an in vitro tumor cell death rate of ~ 50% (IC50 = 12.8 nM) after 24 h of NP treatment at saporin concentration of 10 nM on human breast cancer cells; and an in vivo reduction of tumor size increasing after 16 days of NP intravenous injection in tumor-bearing nude mice (Shen et al. 2021). Fan et al. (2022) formulated a platelet delivery platform to intracellular carry protein in post-surgical tumor recurrence. To be more specific, they first assembled a protein "backpack", which is a nanogel formed through the crosslinking of Granzyme B (the effective protease) and a pore-forming peptide (GALA) with a disulfide linker. The nanogels are conjugated on the surface of N_3-platelets with a benzoic-imine bond that is sensible to an acidic pH. When the system reaches the tumor acid environment, the benzoic-imine bond is cleaved and the protein backpack is released into the cells thanks to its nanoscale size. In the cells GALA and Granzyme B are decrosslinked, so the Granzyme B can induce cell apoptosis. The results showed that the platelets can quickly accumulate at the surgical tumor sites and release the backpack protein. Moreover, the Granzyme B demonstrated an efficient tumor recurrence preventions thanks to its cell apoptosis induction on mice models with incomplete metastatic melanoma resection (Fan et al. 2022).

A nanodelivery system for cancer treatment is now in phase I clinical trial (NCT04751786): PRECIOUS-01 is an immunomodulating agent composed of the invariant natural killer T cells activator threitolceramide-6 and the New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1) cancer-testis antigen peptide, encapsulated in a PLGA nanoparticle. PRECIOUS-01 is being developed for the treatment of patients with NY-ESO-1-positive cancer. The NT-ESO-1 peptide already demonstrated to be safe and tolerated by patients with advanced cancer in previous clinical trials (Creemers et al. 2021).

Proteins and Peptides-Loaded Delivery Systems Against Viruses

In these last years, thanks to new research, the field of vaccines is being expanding. Traditional vaccines, made of live-attenuated or inactivated viruses, have been slowly replaced with vaccines containing proteins and peptides as main antigenic component. The advantages of this kind of antigens are in their safety profile and in the elimination of viral replication risk (Skwarczynski and Toth 2014). When protein or peptide-based vaccines are produced, it is, sometimes, necessary to add in the formulation appropriate adjuvant, because these biological antigens are less immunogenic. This means that, without adjuvant, they may not produce an enough strong immune response, so the vaccines do not give the desired results (Durán-Lobato et al. 2021). To delivery and target proteins and peptides antigens, nanotechnology has been often used, which has shown good results for some different types of vaccines. For example, Qi et al. (2018) have recently conducted a study with self-assembling protein nanoparticles made with ferritin and a conserved influenza matrix protein. The nasal administration of this nanoparticles provided promising results of protection against influenza. Moreover, other good results were obtained with the administration of nanofiber containing a peptide antigen against influenza virus: the nanofiber, in contrast with the non-coated peptide, showed an increased induction of the immune response (Si et al. 2018). Other types of nanocarriers for influenza vaccine have been produced. For example, a study reported the use of lipid nanoparticles, in particular liposomes made of lipid extracted from archaeal (archaeosomes), which have an adjuvant activity for the immune response. Into these liposomes were encapsuled an H1N1 influenza virus hemagglutinin protein, and the formulation was compared to a non-encapsuled one. The archaeosomes showed a strong immune response in intramuscular administered mice of different ages (Stark et al. 2019). In 2022, results of a in human dose-escalation open-label phase I clinical trial (NCT03814720) have been released. The study tested an HA stabilized stem ferritin nanoparticle vaccine (H1ssF) and evaluated the safety, tolerability, and antibody response of this vaccine. H1ssF demonstrated to be safe and tolerated, with only mild local and systemic reactogenicity. In addition, the results showed a durable antibody response: H1ssF stimulated the production of neutralizing antibodies against the conserved HA stem of group 1 influenza virus (Widge et al. 2023).

Nowadays, one of the most studied and challenging vaccine is the one against HIV. As for the influenza, also for this vaccine some researchers tried to use nanotechnology formulations. Dacoba et al. (2019) encapsulated an HIV peptide into a polysaccharide-based NP, specifically they used a peptide (PCS5) that is part of the 12 highly conserved

Table 3 Examples of studies describing the loading of proteins/peptides in nanocarriers against viroses

Protein/peptide	Delivery system	Disease	Results	References
Influenza virus matrix protein	Self-assembling protein NPs	Influenza	Protection against the disease	Qi et al. (2018)
Peptide antigen	Nanofiber	Influenza	Increased induction of immune response	Si et al. (2018)
H1N1 influenza virus hemagglutinin protein	Archaeosomes	Influenza	Strong immune response	Stark et al. (2019)
PCS5	Polymer/poly(I:C)-based NPs	HIV	Increased amount of anti-PCS5 antibodies	Dacoba et al. (2019)

protease cleavage sites (PCS1-12), which has recently demonstrated to be a strategical target to reduce the maturation and infectivity of HIV. To form the nanoparticles, PCS5 was first conjugated with chitosan and hyaluronic acid, and then with another oppositely charged polymer and poly(I:C). They intramuscularly injected this formulation to BALB/c mice. The results showed the ability of these NPs to elicit an IgG response that increases over the time, since it reaches its maximum values with an amount of anti-PCS5 antibodies 3 times higher than the levels detected in unvaccinated mice. Examples of works describing the loading of peptides and proteins into nanocarriers for the treatment of viroses are given in Table 3.

Conclusions

In recent years, the search for new strategies capable of overcoming physical and enzymatic barriers for the oral administration of proteins and peptides has been very promising, among them, nanoparticles have been proposed to improve the gastrointestinal stability of such macromolecules and thus their oral bioavailability. Besides, it has also been shown that combining different approaches, such as liposomes and hydrophobic ion pairing, as well as hybrid systems made of polymers and lipids, may lead to synergistic advantages in modifying the release profile and the uptake of peptides/proteins through the gut. The selection of the matrix composition of nanoparticles is key to improve the physicochemical stabilization and behaviour profile of loaded biomacromolecules intended for several biomedical applications. Several recent studies that evaluated the protein/peptide encapsulation into nanoparticles for oral administration, show positive and encouraging results, especially for the treatment of chronic diseases such as diabetes and cancer. Patients suffering from chronic diseases would certainly benefit from oral drug regimens as an approach to improve treatment compliance.

Acknowledgements EBS acknowledges FCT—Fundação para a Ciência e a Tecnologia, I.P., Lisbon, Portugal.

Author contributions EB, CC, KK and EBS contributed to the conceptualization, methodology, validation, formal analysis, investigation, and writing—original draft preparation. GT, EHG, FF, MBPPO, KK and EBS contributed to data validation, literature review, and software management. FF, KK and EBS contributed to the writing—review and editing, project administration, resources, supervision, and funding acquisition. All authors have made a substantial contribution to the work. All authors have read and agreed to the published version of the manuscript.

Funding Open access funding provided by FCT|FCCN (b-on).

Declarations

Competing interests The authors declare no competing interests.

Conflict of interests Authors report no conflict of interests with respect to this work.

References

Abdelhamid M, Jung CG, Zhou C, Abdullah M, Nakano M, Wakabayashi H, Abe F, Michikawa M (2020) Dietary lactoferrin supplementation prevents memory impairment and reduces amyloid-β generation in J20 mice. J Alzheimers Dis 74:245–259

Ahmad J, Singhal M, Amin S, Rizwanullah M, Akhter S, Amjad Kamal M, Haider N, Midoux P, Pichon C (2017) Bile salt stabilized vesicles (bilosomes): a novel nano-pharmaceutical design for oral delivery of proteins and peptides. Curr Pharm Des 23:1575–1588

Almeida AJ, Souto E (2007) Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Adv Drug Deliv Rev 59:478–490

Andreani T, de Souza AL, Kiill CP, Lorenzon EN, Fangueiro JF, Calpena AC, Chaud MV, Garcia ML, Gremiao MP, Silva AM, Souto EB (2014) Preparation and characterization of PEG-coated silica nanoparticles for oral insulin delivery. Int J Pharm 473:627–635

Bahman F, Taurin S, Altayeb D, Taha S, Bakhiet M, Greish K (2020) Oral insulin delivery using poly (styrene co-maleic acid) micelles in a diabetic mouse model. Pharmaceutics 12:1026

Bennett PH (2018) Diabetes mortality in the USA: winning the battle but not the war? Lancet 391:2392–2393
Bernkop-Schnürch A, Jalil A (2018) Do drug release studies from SEDDS make any sense? J Control Release 271:55–59
Blanco-Llamero C, Fonseca J, Durazzo A, Lucarini M, Santini A, Senorans FJ, Souto EB (2022) Nutraceuticals and food-grade lipid nanoparticles: from natural sources to a circular bioeconomy approach. Foods 11:2318
Caffarel-Salvador E, Abramson A, Langer R, Traverso G (2017) Oral delivery of biologics using drug-device combinations. Curr Opin Pharmacol 36:8–13
Cao J, Huang D, Peppas NA (2020) Advanced engineered nanoparticulate platforms to address key biological barriers for delivering chemotherapeutic agents to target sites. Adv Drug Deliv Rev 167:170–188
Cholkar K, Patel A, Dutt Vadlapudi A, Mitra K (2012) Novel nanomicellar formulation approaches for anterior and posterior segment ocular drug delivery. Recent Patents Nanomed 2:82–95
Chu C, Deng Y, Liu H, Wei M, Xu X, Gou J, He H, Yin T, Zhang Y, Tang X (2023) In situ rearranged multifunctional lipid nanoparticles via synergistic potentiation for oral insulin delivery. Int J Pharm 636:122811
Creemers JHA, Pawlitzky I, Grosios K, Gileadi U, Middleton MR, Gerritsen WR, Mehra N, Rivoltini L, Walters I, Figdor CG, Ottevanger PB, de Vries IJM (2021) Assessing the safety, tolerability and efficacy of PLGA-based immunomodulatory nanoparticles in patients with advanced NY-ESO-1-positive cancers: a first-in-human phase I open-label dose-escalation study protocol. BMJ Open 11:e050725
Cui F, Qian F, Zhao Z, Yin L, Tang C, Yin C (2009) Preparation, characterization, and oral delivery of insulin loaded carboxylated chitosan grafted poly(methyl methacrylate) nanoparticles. Biomacromolecules 10:1253–1258
Cui X, Cao D, Qu C, Zhang X, Zheng A (2013) A study of the chemical and biological stability of vasoactive intestinal peptide. Drug Dev Ind Pharm 39:1907–1910
Dacoba TG, Omange RW, Li H, Crecente-Campo J, Luo M, Alonso MJ (2019) Polysaccharide nanoparticles can efficiently modulate the immune response against an HIV peptide antigen. ACS Nano 13:4947–4959
Doktorovova S, Souto EB, Silva AM (2014) Nanotoxicology applied to solid lipid nanoparticles and nanostructured lipid carriers—a systematic review of in vitro data. Eur J Pharm Biopharm 87:1–18
Doktorovova S, Kovacevic AB, Garcia ML, Souto EB (2016) Preclinical safety of solid lipid nanoparticles and nanostructured lipid carriers: current evidence from in vitro and in vivo evaluation. Eur J Pharm Biopharm 108:235–252
Durán-Lobato M, López-Estévez AM, Cordeiro AS, Dacoba TG, Crecente-Campo J, Torres D, Alonso MJ (2021) Nanotechnologies for the delivery of biologicals: historical perspective and current landscape. Adv Drug Deliv Rev 176:113899
Eldor R, Arbit E, Corcos A, Kidron M (2013) Glucose-reducing effect of the ORMD-0801 oral insulin preparation in patients with uncontrolled type 1 diabetes: a pilot study. PLoS ONE 8:e59524
Eldor R, Francis BH, Fleming A, Neutel J, Homer K, Kidron M, Rosenstock J (2023) Oral insulin (ORMD-0801) in type 2 diabetes mellitus: a dose-finding 12-week randomized placebo-controlled study. Diabetes Obes Metab 25:943–952
Fan W, Xia D, Zhu Q, Li X, He S, Zhu C, Guo S, Hovgaard L, Yang M, Gan Y (2018) Functional nanoparticles exploit the bile acid pathway to overcome multiple barriers of the intestinal epithelium for oral insulin delivery. Biomaterials 151:13–23
Fan X, Wang K, Lu Q, Lu Y, Liu F, Li L, Li S, Ye H, Zhao J, Cao L, Zhang H, He Z, Sun J (2022) Surface-anchored tumor microenvironment-responsive protein nanogel-platelet system for cytosolic delivery of therapeutic protein in the post-surgical cancer treatment. Acta Biomater 154:412–423
Fangueiro JF, Gonzalez-Mira E, Martins-Lopes P, Egea MA, Garcia ML, Souto SB, Souto EB (2013) A novel lipid nanocarrier for insulin delivery: production, characterization and toxicity testing. Pharm Dev Technol 18:545–549
FDA (2022) Drug approvals and database, https://www.fda.gov/drugs/development-approval-process-drugs/drug-approvals-and-databases (Accessed 30 Oct 2023)
Friedl JD, Jörgensen AM, Le-Vinh B, Braun DE, Tribus M, Bernkop-Schnürch A (2021) Solidification of self-emulsifying drug delivery systems (SEDDS): impact on storage stability of a therapeutic protein. J Colloid Interface Sci 584:684–697
Fu L, Hua X, Jiang X, Shi J (2022) Multistage systemic and cytosolic protein delivery for effective cancer treatment. Nano Lett 22:111–118
Griesser J, Hetényi G, Moser M, Demarne F, Jannin V, Bernkop-Schnürch A (2017) Hydrophobic ion pairing: key to highly payloaded self-emulsifying peptide drug delivery systems. Int J Pharm 520:267–274
Gurny R, Allemann E, Leroux J-C (2007) Nanoparticles for oral administration of pharmaceutical agents of low solubility. US20040018236A1
Haddadzadegan S, Dorkoosh F, Bernkop-Schnürch A (2022) Oral delivery of therapeutic peptides and proteins: technology landscape of lipid-based nanocarriers. Adv Drug Deliv Rev 182:114097
Haddadzadegan S, Knoll P, Wibel R, Kali G, Bernkop-Schnürch A (2023) Three generations of thiolated cyclodextrins: a direct comparison of their mucus permeating and mucoadhesive properties. Acta Biomater 167:309–320
Han X, Lu Y, Xie J, Zhang E, Zhu H, Du H, Wang K, Song B, Yang C, Shi Y (2020) Zwitterionic micelles efficiently deliver oral insulin without opening tight junctions. Nat Nanotechnol 15:605–614
Hashim LE, Sabri AH, Athirah Mohamad M, Kurnia Anjani Q, Mustaffa MF, Abdul Hamid K (2023) Circumventing the gastrointestinal barrier for oral delivery of therapeutic proteins and peptides (PPTs): current trends and future trajectories. Curr Drug Deliv 21:211
Hu F, Hong Y, Yuan H (2004) Preparation and characterization of solid lipid nanoparticles containing peptide. Int J Pharm 273:29–35
Hu X, Fan W, Yu Z, Lu Y, Qi J, Zhang J, Dong X, Zhao W, Wu W (2016) Evidence does not support absorption of intact solid lipid nanoparticles via oral delivery. Nanoscale 8:7024–7035
Hua S, Marks E, Schneider JJ, Keely S (2015) Advances in oral nanodelivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. Nanomed: Nanotechnol Biol Med 11:1117–1132
Iqbal H, Yang T, Li T, Zhang M, Ke H, Ding D, Deng Y, Chen H (2021) Serum protein-based nanoparticles for cancer diagnosis and treatment. J Control Release 329:997–1022
Jain S, Rathi VV, Jain AK, Das M, Godugu C (2012) Folate-decorated PLGA nanoparticles as a rationally designed vehicle for the oral delivery of insulin. Nanomedicine 7:1311–1337
Jain A, Jain A, Gulbake A, Shilpi S, Hurkat P, Jain SK (2013) Peptide and protein delivery using new drug delivery systems. Crit Rev Ther Drug Carrier Syst 30:293–329
Katsuda C, Niiyama K, Obana E, Yamamoto T, Katou Y, Kataoka M, Ohkura K, Shinohara Y (2010) Specific formation of trypsin-resistant micelles on a hydrophobic peptide observed with Triton X-100 but not with octylglucoside. Biochim et Biophys Acta Biomembr 1798:2090–2093
Kecman S, Škrbić R, Badnjevic Cengic A, Mooranian A, Al-Salami H, Mikov M, Golocorbin-Kon S (2020) Potentials of human bile acids and their salts in pharmaceutical nano delivery and formulations adjuvants. Technol Health Care 28:325–335

Kim KS, Kwag DS, Hwang HS, Lee ES, Bae YH (2018) Immense insulin intestinal uptake and lymphatic transport using bile acid conjugated partially uncapped liposome. Mol Pharm 15:4756–4763

Kommineni N, Sainaga Jyothi VGS, Butreddy A, Raju S, Shapira T, Khan W, Angsantikul P, Domb AJ (2023) SNAC for enhanced oral bioavailability: an updated review. Pharm Res 40:633–650

Kraft JC, Freeling JP, Wang Z, Ho RJ (2014) Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. J Pharm Sci 103:29–52

Kubackova J, Holas O, Zbytovska J, Vranikova B, Zeng G, Pavek P, Mullertz A (2021) Oligonucleotide delivery across the Caco-2 monolayer: the design and evaluation of self-emulsifying drug delivery systems (SEDDS). Pharmaceutics 13:459

Leonaviciute G, Bernkop-Schnürch A (2015) Self-emulsifying drug delivery systems in oral (poly) peptide drug delivery. Expert Opin Drug Deliv 12:1703–1716

Li P, Nielsen HM, Müllertz A (2012) Oral delivery of peptides and proteins using lipid-based drug delivery systems. Expert Opin Drug Deliv 9:1289–1304

Liu J, Zhang S, Chen P, Cheng L, Zhou W, Tang W, Chen Z, Ke C (2007) Controlled release of insulin from PLGA nanoparticles embedded within PVA hydrogels. J Mater Sci Mater Med 18:2205–2210

Lundquist P, Artursson P (2016) Oral absorption of peptides and nanoparticles across the human intestine: opportunities, limitations and studies in human tissues. Adv Drug Deliv Rev 106:256–276

Lupo N, Tkadlečková VN, Jelkmann M, Laffleur F, Hetényi G, Kubová K, Bernkop-Schnürch A (2019) Self-emulsifying drug delivery systems: in vivo evaluation of their potential for oral vaccination. Acta Biomater 94:425–434

Mahmood A, Bernkop-Schnürch A (2019) SEDDS: a game changing approach for the oral administration of hydrophilic macromolecular drugs. Adv Drug Deliv Rev 142:91–101

Matteo Jörgensen A, Knoll P, Haddadzadegan S, Fabian H, Hupfauf A, Gust R, Georg Jörgensen R, Bernkop-Schnürch A (2023) Biodegradable arginine based steroid-surfactants: cationic green agents for hydrophobic ion-pairing. Int J Pharm 630:122438

Mehnert W, Mäder K (2012) Solid lipid nanoparticles: production, characterization and applications. Adv Drug Deliv Rev 64:83–101

Miyasaka K (2022) New drug for type 2 diabetes: introduction of oral Semaglutide (Rybelsus (®) tablets), an oral GLP-1 receptor agonist. Nihon Yakurigaku Zasshi 157:146–154

Muller RH, Runge S, Ravelli V, Mehnert W, Thunemann AF, Souto EB (2006) Oral bioavailability of cyclosporine: solid lipid nanoparticles (SLN) versus drug nanocrystals. Int J Pharm 317:82–89

Muntoni E, Anfossi L, Milla P, Marini E, Ferraris C, Capucchio MT, Colombino E, Segale L, Porta M, Battaglia L (2021) Glargine insulin loaded lipid nanoparticles: Oral delivery of liquid and solid oral dosage forms. Nutr Metab Cardiovasc Dis 31:691–698

Niu Z, Conejos-Sánchez I, Griffin BT, O'Driscoll CM, Alonso MJ (2016) Lipid-based nanocarriers for oral peptide delivery. Adv Drug Deliv Rev 106:337–354

Olbrich C, Müller R (1999) Enzymatic degradation of SLN—effect of surfactant and surfactant mixtures. Int J Pharm 180:31–39

Ommura Y, Imai S, Takenaka M, Ouchi M, Terashima T (2020) Selective coupling and polymerization of folded polymer micelles to nanodomain self-assemblies. ACS Macro Lett 9:426–430

Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2:751–760

Pinkerton NM, Grandeury A, Fisch A, Brozio J, Riebesehl BU, Prud'homme RK (2013) Formation of stable nanocarriers by in situ ion pairing during block-copolymer-directed rapid precipitation. Mol Pharm 10:319–328

Preeti SS, Saharan R, Narwal S, Malik R, Gahlot V, Khalid A, Najmi A, Zoghebi K, Halawi MA, Albratty M, Mohan S (2023) Exploring LIPIDs for their potential to improves bioavailability of lipophilic drugs candidates: a review. Saudi Pharm J 31:101870

Pudlarz A, Szemraj J (2018) Nanoparticles as carriers of proteins, peptides and other therapeutic molecules. Open Life Sci 13:285–298

Qi M, Zhang XE, Sun X, Zhang X, Yao Y, Liu S, Chen Z, Li W, Zhang Z, Chen J (2018) Intranasal nanovaccine confers homo-and hetero-subtypic influenza protection. Small 14:1703207

Richardson T, Kerr D (2003) Skin-related complications of insulin therapy. Am J Clin Dermatol 4:661–667

Ristroph KD, Prud'homme RK (2019) Hydrophobic ion pairing: encapsulating small molecules, peptides, and proteins into nanocarriers. Nanoscale Adv 1:4207–4237

Saleh A, Akkuş-Dağdeviren ZB, Haddadzadegan S, Wibel R, Bernkop-Schnürch A (2023) Peptide antibiotic-polyphosphate nanoparticles: a promising strategy to overcome the enzymatic and mucus barrier of the intestine. Biomacromolecules 24:2587–2595

Sarmento B, Martins S, Ferreira D, Souto EB (2007) Oral insulin delivery by means of solid lipid nanoparticles. Int J Nanomed 2:743–749

Shahzadi I, Fürst A, Knoll P, Bernkop-Schnürch A (2021) Nanostructured lipid carriers (NLCs) for oral peptide drug delivery: about the impact of surface decoration. Pharmaceutics 13:1312

Shan W, Zhu X, Liu M, Li L, Zhong J, Sun W, Zhang Z, Huang Y (2015) Overcoming the diffusion barrier of mucus and absorption barrier of epithelium by self-assembled nanoparticles for oral delivery of insulin. ACS Nano 9:2345–2356

Sharma G, Sharma AR, Nam JS, Doss GP, Lee SS, Chakraborty C (2015) Nanoparticle based insulin delivery system: the next generation efficient therapy for Type 1 diabetes. J Nanobiotechnology 13:74

Shen Q, Xu L, Li R, Wu G, Li S, Saw PE, Zhou Y, Xu X (2021) A tumor microenvironment (TME)-responsive nanoplatform for systemic saporin delivery and effective breast cancer therapy. Chem Commun (camb) 57:2563–2566

Si Y, Wen Y, Kelly SH, Chong AS, Collier JH (2018) Intranasal delivery of adjuvant-free peptide nanofibers elicits resident CD8+ T cell responses. J Control Release 282:120–130

Skwarczynski M, Toth I (2014) Recent advances in peptide-based subunit nanovaccines. Nanomedicine 9:2657–2669

Smart AL, Gaisford S, Basit AW (2014) Oral peptide and protein delivery: intestinal obstacles and commercial prospects. Expert Opin Drug Deliv 11:1323–1335

Song K-H, Chung S-J, Shim C-K (2005) Enhanced intestinal absorption of salmon calcitonin (sCT) from proliposomes containing bile salts. J Control Release 106:298–308

Souto SB, Souto EB, Braga DC, Medina JL (2011) Prevention and current onset delay approaches of type 2 diabetes mellitus (T2DM). Eur J Clin Pharmacol 67:653–661

Souto EB, Souto SB, Campos JR, Severino P, Pashirova TN, Zakharova LY, Silva AM, Durazzo A, Lucarini M, Izzo AA, Santini A (2019) Nanoparticle delivery systems in the treatment of diabetes complications. Molecules 24:4209

Srivastava KS (2011) Synthesis of desmopressin. WO2011011342A9.

Stark FC, Akache B, Ponce A, Dudani R, Deschatelets L, Jia Y, Sauvageau J, Williams D, Jamshidi MP, Agbayani G, Wachholz K, Harrison BA, Li X, Krishnan L, Chen W, McCluskie MJ (2019) Archaeal glycolipid adjuvanted vaccines induce strong influenza-specific immune responses through direct immunization in young and aged mice or through passive maternal immunization. Vaccine 37:7108–7116

Stella VJ (2010) Prodrugs: some thoughts and current issues. J Pharm Sci 99:4755–4765

Sun S, Liang N, Piao H, Yamamoto H, Kawashima Y, Cui F (2010) Insulin-SO (sodium oleate) complex-loaded PLGA nanoparticles: formulation, characterization and in vivo evaluation. J Microencapsul 27:471–478

Suntres ZE (2011) Liposomal antioxidants for protection against oxidant-induced damage. J Toxicol 2011:152474

Teixeira MC, Carbone C, Souto EB (2017) Beyond liposomes: recent advances on lipid based nanostructures for poorly soluble/poorly permeable drug delivery. Prog Lipid Res 68:1–11

Tozaki H, Odoriba T, Iseki T, Taniguchi T, Fujita T, Murakami M, Muranishi S, Yamamoto A (1998) Use of protease inhibitors to improve calcitonin absorption from the small and large intestine in rats. J Pharm Pharmacol 50:913–920

Üner M (2006) Preparation, characterization and physico-chemical properties of solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC): their benefits as colloidal drug carrier systems. Die Pharmazie-an Int J Pharm Sci 61:375–386

van der Meel R, Sulheim E, Shi Y, Kiessling F, Mulder WJM, Lammers T (2019) Smart cancer nanomedicine. Nat Nanotechnol 14:1007–1017

Wang M, Wang C, Ren S, Pan J, Wang Y, Shen Y, Zeng Z, Cui H, Zhao X (2022) Versatile oral insulin delivery nanosystems: from materials to nanostructures. Int J Mol Sci 23:3362

Wen J-Y, Ledger R, McLeod B, Davies N, Butt A, Tucker I (2002) Protein and peptide degradation in the intestine of the common brushtail possum (*Trichosurus vulpecula*). J Comp Physiol B 172:553–559

Werle M, Takeuchi H (2009) Chitosan–aprotinin coated liposomes for oral peptide delivery: development, characterisation and in vivo evaluation. Int J Pharm 370:26–32

Widge AT, Hofstetter AR, Houser KV, Awan SF, Chen GL, Burgos Florez MC, Berkowitz NM, Mendoza F, Hendel CS, Holman LA, Gordon IJ, Apte P, Liang CJ, Gaudinski MR, Coates EE, Strom L, Wycuff D, Vazquez S, Stein JA, Gall JG, Adams WC, Carlton K, Gillespie RA, Creanga A, Crank MC, Andrews SF, Castro M, Serebryannyy LA, Narpala SR, Hatcher C, Lin BC, O'Connell S, Freyn AW, Rosado VC, Nachbagauer R, Palese P, Kanekiyo M, McDermott AB, Koup RA, Dropulic LK, Graham BS, Mascola JR, Ledgerwood JE (2023) An influenza hemagglutinin stem nanoparticle vaccine induces cross-group 1 neutralizing antibodies in healthy adults. Sci Transl Med 15:4790

Yu J, Zhang Y, Wang J, Wen D, Kahkoska AR, Buse JB, Gu Z (2019) Glucose-responsive oral insulin delivery for postprandial glycemic regulation. Nano Res 12:1539–1545

Yuan H, Guo C, Liu L, Zhao L, Zhang Y, Yin T, He H, Gou J, Pan B, Tang X (2023) Progress and prospects of polysaccharide-based nanocarriers for oral delivery of proteins/peptides. Carbohyd Polym 312:120838

Zhang N, Ping Q, Huang G, Xu W, Cheng Y, Han X (2006) Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin. Int J Pharm 327:153–159

Zhao X, Meng Z, Wang Y, Chen W, Sun C, Cui B, Cui J, Yu M, Zeng Z, Guo S (2017) Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers. Nat Plants 3:956–964

Zielinska A, Carreiro F, Oliveira AM, Neves A, Pires B, Venkatesh DN, Durazzo A, Lucarini M, Eder P, Silva AM, Santini A, Souto EB (2020) Polymeric nanoparticles: production, characterization, toxicology and ecotoxicology. Molecules 25:3731

Zielinska A, Eder P, Karczewski J, Szalata M, Hryhorowicz S, Wielgus K, Szalata M, Dobrowolska A, Atanasov AG, Slomski R, Souto EB (2023) Tocilizumab-coated solid lipid nanoparticles loaded with cannabidiol as a novel drug delivery strategy for treating COVID-19: a review. Front Immunol 14:1147991

Zizzari AT, Pliatsika D, Gall FM, Fischer T, Riedl R (2021) New perspectives in oral peptide delivery. Drug Discovery Today 26:1097–1105