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Ph.D. Thesis

QUALITY BY DESIGN DRIVEN DEVELOPMENT OF POLYMERIC AND LIPID-BASED NANOCARRIERS AS POTENTIAL SYSTEMS FOR ORAL DELIVERY OF GLP-1 ANALOGUES

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Szeged

2020

▪ PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS

I. **Ismail R**, Bocsik A, Katona G, Gróf I, Deli MA, Csóka I.
Encapsulation in polymeric nanoparticles enhances the enzymatic stability and the permeability of GLP1 analog- liraglutide across a culture model of intestinal permeability. *Pharmaceutics* 11,599,2019.
(IF: 4.773), Q1

II. **Ismail R**, Sovány T, Gácsi A, Ambrus R, Katona G, Imre N, Csóka I.
Synthesis and statistical optimization of poly (lactic-co-glycolic acid) nanoparticles encapsulating GLP-1 analog designed for oral delivery.
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III. Pallagi E, **Ismail R**, Paál T, Csóka I.
Initial Risk Assessment as part of the Quality by Design in peptide drug containing formulation development.
European Journal of Pharmaceutical Sciences 122,160–169,2018.
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IV. **Ismail R** and Csóka I. Novel strategies in the oral delivery of antidiabetic peptide drugs – Insulin, GLP1 and its analogs.
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V. **Ismail R**, Phan TNQ, Laffleur F, Csóka I, Schnürch AB. Hydrophobic ion pairing of a GLP-1 analogue for incorporating into lipid nanocarriers designed for oral delivery.
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▪ PUBLICATIONS NOT RELATED TO THE TOPIC OF THE THESIS

I. Akel H**, Ismail R**, Csóka I. Progress and perspectives of brain-targeting lipid-based nanosystems via the nasal route in Alzheimer's disease.
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Drug Discovery Today 25,158-194,2020.
(**IF 6.880), Q1**

- ## PRESENTATIONS RELATED TO THE TOPIC OF THE THESIS

Verbal presentation

I. **Ismail R** and Csóka I. Potential of polymeric and Lipid-based nanocarriers for oral GLP-1 analogue delivery. *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science*, Szeged, Hungary,2020.

II. **Ismail R** and Csóka I. Design of experiment DoE based methodology for designing polymeric NPs encapsulating Liraglutide for oral delivery. *12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs,* Szeged, Hungary,2018.

III. **Ismail R** and Csóka I. Quality by Design driven development of Liraglutide loaded nanocarrier system designed for oral delivery. *I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science*, Szeged, Hungary,2019

IV. **Ismail R** and Csóka I. QbD based strategy for the development of oral antidiabetic peptide containing nanocarrier system. *1st Young Technologists' Forum,* Budapest, Hungary,2018.

I. **Ismail R** and Csóka I. Up to date advances in nano-carrier systems for oral delivery of antidiabetic peptides. *13th International Conference and Exhibition on Nanomedicine and Pharmaceutical Nanotechnology,* Rome, Italy,2017.

Poster presentation

I. **Ismail R** and Csóka I. In vitro appraisal of polymeric nanoparticles designed for oral delivery of GLP-1 analog- liraglutide. *12th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology,* Vienna, Austria,2020.

II. Csóka I, Katona G, Dorina D, **Ismail R**, Manteghi R, Pallagi E. Colloidal systems as carriers for peptide drugs: possibilities and challenges. *EUFEPS*, Frankfurt, Germany,2019.

III. Csóka I, Katona G, **Ismail R**, Pallagi E. Expectations and limits of colloidal systems as carriers for peptide drugs. *3rd International Symposium on Scientific and Regulatory Advance in Biological and Non-Biological Complex Drugs: A to Z in Bioequivalence*, Budapest, Hungary,2018.

IV. **Ismail R** and Csóka I. Pharmaceutical applications of colloidal drug delivery systems: case studies for biological drugs. *11th Conference on Colloid Chemistry*, Eger, Hungary,2018.

V. **Ismail R** and Csóka I. Initial SWOT analysis of designing polymeric nanoparticles and lipid-based nanoparticles for oral GLP-1 analog delivery. *9th International Congress Nanotechnology in Biology and Medicine*, Graz, Austria,2018.

VI. **Ismail R** and Csóka I. Quality by design (QbD) oriented study for designing oral Liraglutide loaded polymeric nanoparticles. *11th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Granada, Spain,2018.

VII. **Ismail R** and Csóka I. Evaluation of techniques and possibilities for oral delivery of antidiabetic peptide drug -liraglutide- in a novel delivery system. *7th BBBB International Conference on Pharmaceutical Sciences*, Budapest, Hungary,2017.

CONTENT

ABBREVIATIONS

ADA	American Diabetes Association
ANOVA	Analysis of Variance
AUC	Area Under Curve
BA_R	Relative Bioavailability
BBD	Box-Behnken Design
cAMP	Cyclic Adenosine Monophosphate
CCD	Central Composite Design
CMAs	Critical Material Attributes
Cmax	Maximum Serum Concentration
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
DS	Design Space
DOC	Sodium Docusate
DoE	Design of Experiment
DPP-4	Dipeptidyl Peptidase-4
DPP-4i	Dipeptidyl Peptidase-4 Inhibitors
EE	Encapsulation Efficiency
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicine Agency
EASD	European Association for the Study of Diabetes
Exn	Exenatide
GI	Gastrointestinal
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLP-1	Glucagon-Like Peptide-1
GPCRs	G-Protein-Coupled Receptors
HLB	Hydrophilic-Lipophilic Balance
HPLC	High performance Liquid Chromatography
I.V.	Intravenous

ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
Lira	Liraglutide
log D $_{SEDDS/release\ medium}$	The distribution coefficient of the drug/ion pairs between the oily droplet phase of SEDDS and the release medium
MEM	Minimum Essential Medium Eagle
MS	Mean Square
NCs	Nanocarriers
NPs	Nanoparticles
O/W	Oil on Water
P.O.	Per Oral
PAA	Polyacrylic Acid
P_{app}	Apparent Permeability
PBD	Plackett-Burman Design
PCL	Poly E-Caprolactone
PDI	Polydispersity Index
PGA	Poly Glycolic Acid
PG	Propylene Glycol
Pgp	P-Glycoprotein
PLA	Poly (D, L -Lactic Acid)
PLGA	Poly (D, L -Lactic-Co-Glycolic Acid)
PVA	Poly (vinyl alcohol)
QbD	Quality by Design
QTPP	Quality Target Product Profile
RA	Risk Assessment
SC	Subcutaneous
SD	Standard deviation
SEDDS	Self-emulsifying Drug Delivery Systems
SGF	Simulated Gastric Fluid
SGFsp	Simulated Gastric Fluid without enzymes
SIF	Simulated Intestinal Fluid

SIFsp	Simulated Intestinal Fluid without enzymes
T2DM	Type 2 Diabetes Mellitus
TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
THA	Tetraheptylammonium Bromide
Tmax	Time to reach maximum serum concentration
TX-100	Triton X-100
USFDA	US Food and Drug Administration
W/O	Water to Oil ratio
W1/O/W2	Water in Oil in Water

1. INTRODUCTION AND AIMS

Type 2 diabetes mellitus (T2DM) is a progressive disorder characterized by the production of insufficient level of insulin due to β cell dysfunction and/or insulin resistance, accounting for roughly 90% of all diabetic cases (American Diabetes Association, 2015). With the expanding knowledge of the pathophysiology of T2DM, the incretin system has become a crucial target in the treatment of T2DM patients. There are two approved classes of incretin-based therapeutics: glucagon-like peptide-1 (GLP-1) analogues and dipeptidyl peptidase-4 inhibitors (DPP-4i).

Glucagon-like peptide-1 analogues liraglutide (Lira) and exenatide (Exn) are currently limited to subcutaneous injection (SC) in clinical protocols. Due to several drawbacks accompanied by this invasive route, the development of a patient-friendly delivery system should be aimed for (Araújo *et al.*, 2012). Herein, the oral route is likely the most desirable choice since it mimics the physiological GLP-1 secretion in addition to ensuring good patient adherence to the treatment (Lin *et al.*, 2016). Moreover, oral delivery appears to be feasible for such antidiabetic peptides due to the relatively larger safety window of GLP-1 analogues compared to insulin. Among the various strategies having been developed to conquer the barriers limiting oral peptide delivery, namely the harsh environment of the gastrointestinal (GI) tract and the absorption membrane barrier, the encapsulation of GLP-1 analogues into nanosystems seems to be very promising strategy (Ismail and Csóka, 2017).

Therefore, the aim of this work was to investigate the potential of polymeric and lipid-based nanocarriers (NCs) in overcoming the main challenges that block the oral delivery of Lira and Exn. Due to the complexity, biocompatibility and nanotoxicological concerns of nanopharmaceuticals, not to mention the risks the risks accompanied with peptide drugs formulation development, it was crucial to implement the Quality by Design (QbD) and risk assessment (RA) concepts aiming to classify the quality attributes and process parameters and ultimately develop a thorough understanding of the target product and process design (Pallagi et al., 2018). Following to setting up the Quality Target Product Profile (QTPP) and conducting the initial RA for designing oral GLP-1 analogues loaded nanocarriers (NCs), two different NCs were designed, optimized and assessed according to their critical quality attributes (CQAs). These developed NCs are (i) Lira loaded poly (d, l-lactic-co-glycolic acid) nanoparticles (PLGA NPs) and (ii) Exn loaded self-

emulsifying drug delivery system (SEDDS). Figure 1 presents the main experimental steps being followed in this work.

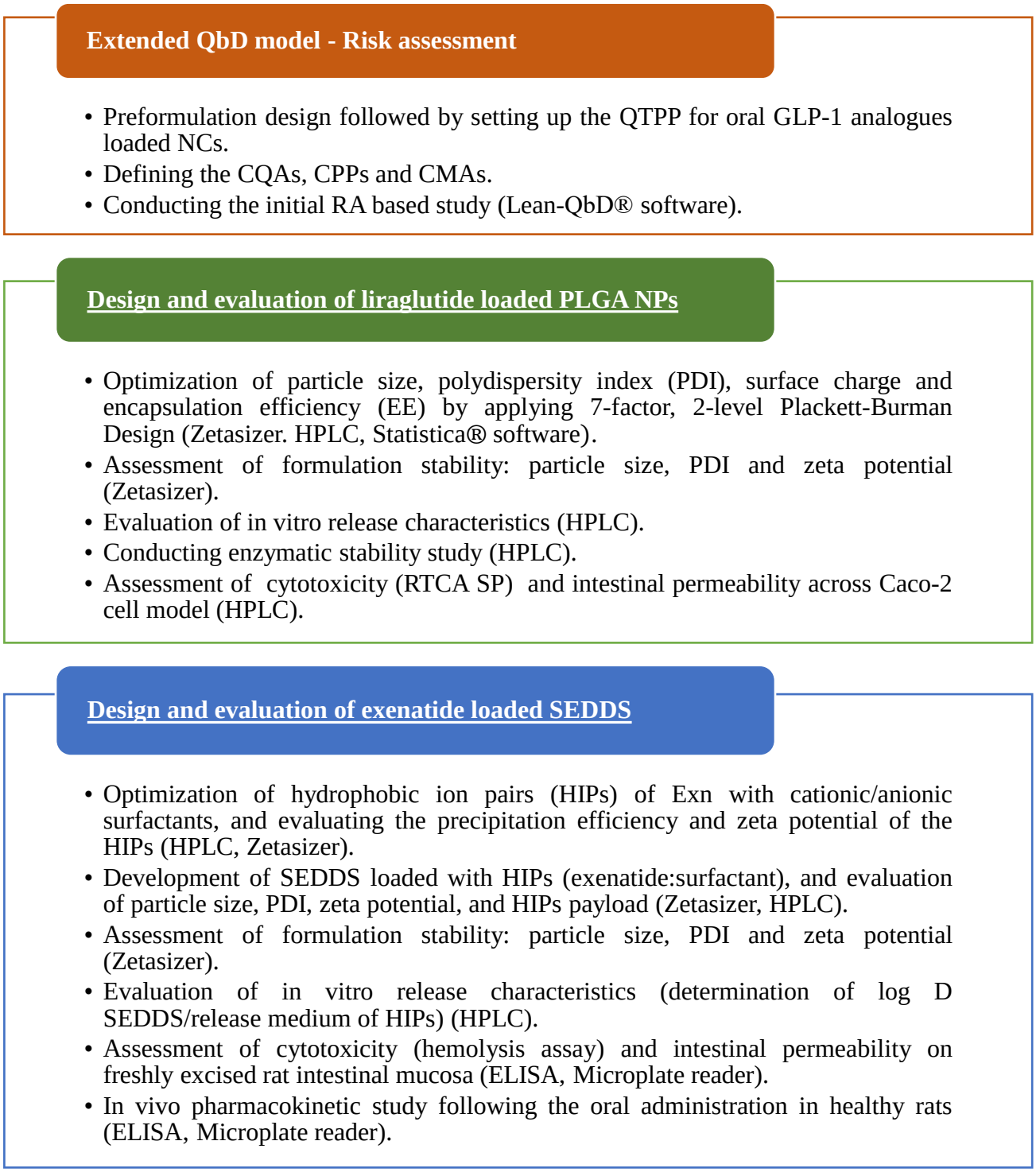


Figure 1. The main questions/tasks of this work to be answered.

2. THEORETICAL BACKGROUND

2.1.Incretin based therapies for T2DM

Incretin-based agents, including glucagon-like peptide-1 (GLP-1) analogues and dipeptidyl peptidase-4 inhibitors (DPP-4i), represent potential options for T2DM treatment (Łabuzek *et al*., 2013). These novel therapies have been recommended by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) as second line therapy for T2DM (Inzucchi *et al*.,2015).

The incretin effect refers to the amplification of insulin secretion that occurs when glucose is ingested orally as opposed to glucose given in any other way that bypasses the gut, under similar plasma glucose levels. In healthy individuals, the response to oral glucose is two to three-fold greater than the response to intravenous (i.v.) glucose (Hare *et al*., 2010). However, T2DM patients had shown to have a diminished or absent incretin effect, due to decreased secretion and insulinotropic action of incretin hormones namely; glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 (Nauck *et al*., 1986; Nauck, Baller and Meier, 2004). This suggests the significant contribution of these incretin hormones to glycemic control. Since the insulinotropic activity of GLP-1 is reported to be largely preserved in T2DM patients, this peptide has been considered as a potential therapeutic choice for T2DM. The hyperglycemia in patients with T2DM can readily be normalized by the i.v. administration of GLP-1, even at relatively low doses (Knop *et al*., 2007; Phillips and Prins, 2011). Unlike GLP-1, continuous i.v. infusion of GIP to T2DM patients has resulted in no significant glucose-lowering effect (Jones *et al*., 1989; Kazafeos, 2011).

2.1.1. Pharmacological effects of GLP-1

GLP-1 (7-36) (80% of circulating GLP-1) is a 30-amino acid polypeptide produced by post-translational processing of preproglucagon located on chromosome 17, and secreted from the intestinal L enteroendocrine cells of the ileum in response to food intake (Pacheco-López and Langhans, 2013). It binds to and activates the GLP-1 receptors belonging to class B family of G-protein-coupled receptors (GPCRs), and located in many tissues including the pancreas, stomach, duodenum, heart, muscles, liver in addition to several parts of the brain (Campos, Lee and Drucker, 1994; Bullock, Heller and Habener, 1996) (Figure 2). GLP-1 regulates the expression of β-cell genes as it stimulates pancreatic β-cell proliferation and differentiation, inhibits β-cell apoptosis and prevents β-cell glucolipotoxicity. Moreover, GLP-1 promotes insulin gene transcription,

blocks glucagon secretion from the pancreas, prevents acid secretion and gastric emptying and reduces food intake by inducing satiety, leading to body weight decrease (Tran *et al*., 2017).

Since its discovery in the early 1980s (Bell *et al*., 1983), GLP-1 has been regarded as possible potential key for T2DM treatment due to its unique antidiabetic functions. However, once in the circulation, GLP-1 undergoes a rapid N-terminal degradation by endopeptidases such as dipeptidyl peptidase-4 (DPP-4) enzyme which results in a short half-life of one to two minutes. The necessity of continuous i.v. infusion of native GLP-1 to normalize blood glucose levels in patients with T2DM is clearly not of any clinical potential for long term treatment (Nauck *et al*., 1993). To circumvent this issue, DPP-4i and GLP-1 analogues have been successfully developed to treat T2DM patients.

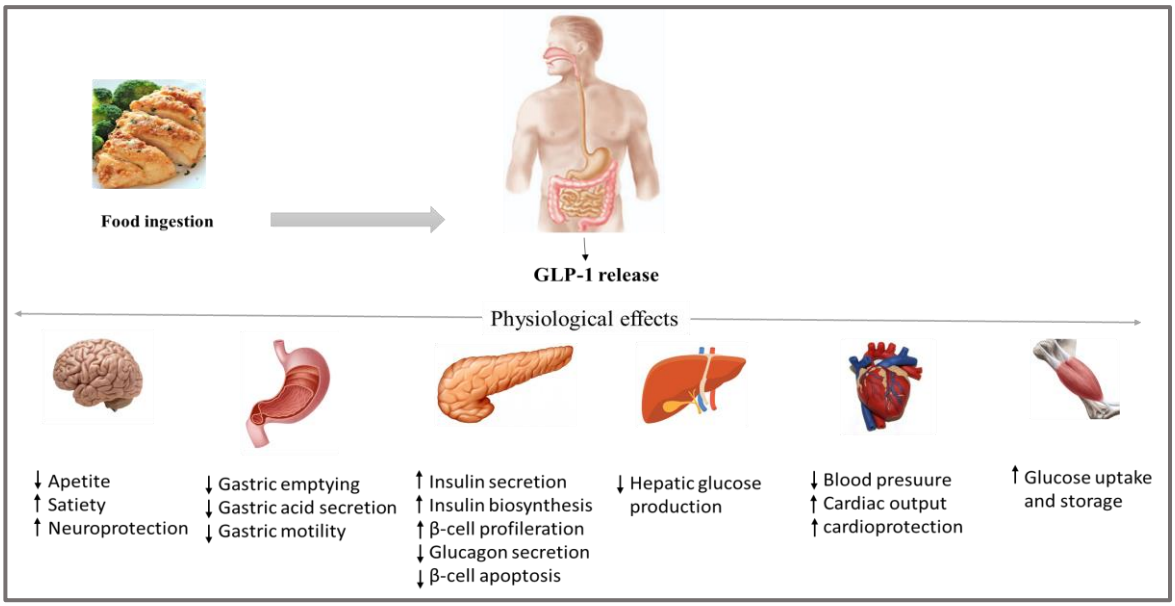


Figure 2. Schematic representation of GLP-1 effects in peripheral tissues.

2.1.2. GLP-1 analogues: Exenatide and Liraglutide

GLP-1 analogues have been recently acknowledged as a powerful treatment strategy for patients with T2DM. Exenatide (Exn), a 39-amino-acid peptide (molecular weight:4.186 KDa), is the synthetic form of exendin-4 which was isolated from the saliva of the lizard Heloderma suspectum in 1992 (Eng *et al.*, 1992). The substitution of Arginine at the 2_{nd} position in GLP-1 for Glycine in Exn (Figure 3) provides resistance against DPP-4 and thus a longer half-life of ~2-4 h (Shi *et al.*, 2018a). Exn is the first GLP-1 analogue to reach the market as Byetta® which

is administered twice daily at a dose of 5-10 µg through SC injection. Besides, Bydureon® is a sustained release Exn product prepared by microsphere-based technology where Exn is encapsulated in PLGA microspheres controlling the drug release over an extended period of time and has a 2-week half-life (Gupta, 2013).

Acylation is a process that was applied to prolong the half-life of GLP-1 through ensuring slow release from the injection site; promoting binding to human albumin, which can contribute to protecting it from metabolic degradation by DPP-4. Liraglutide (Lira) or NN2211 (MW: 3751.2 Da) is an is recombinant palmityl-acylated derivative of GLP-1 with two modifications (Figure 3): Arginine 34 for Lysine substitution, and a fatty acid side chain (16-carbon palmitate) has been attached to Lysine 26 via a glutamic acid linker (Araújo *et al.*, 2012). Lira retains the physiological activities of GLP-1 with a considerably longer half-life (approximately 13h) that supports once-daily dosing (Iepsen, Torekov and Holst, 2015). Lira once daily SC injection (Victoza®) was approved by both the European Medicine Agency (EMA) (2009) and the U.S. Food and Drug Administration (USFDA) (2010) for the treatment of T2DM. USFDA (2014) and EMA (2015) approved Lira under the name Saxenda® for the chronic weight management in patients with obesity or who are overweight with BMI $\geq$ 27 kg/m^2 and have a weight-related comorbid conditions (Mehta, Marso and Neeland, 2017). The dose of Lira SC injection is 3.0 mg once daily in case of weight management in contrast to the 1.2 or 1.8 mg once daily for T2DM management.

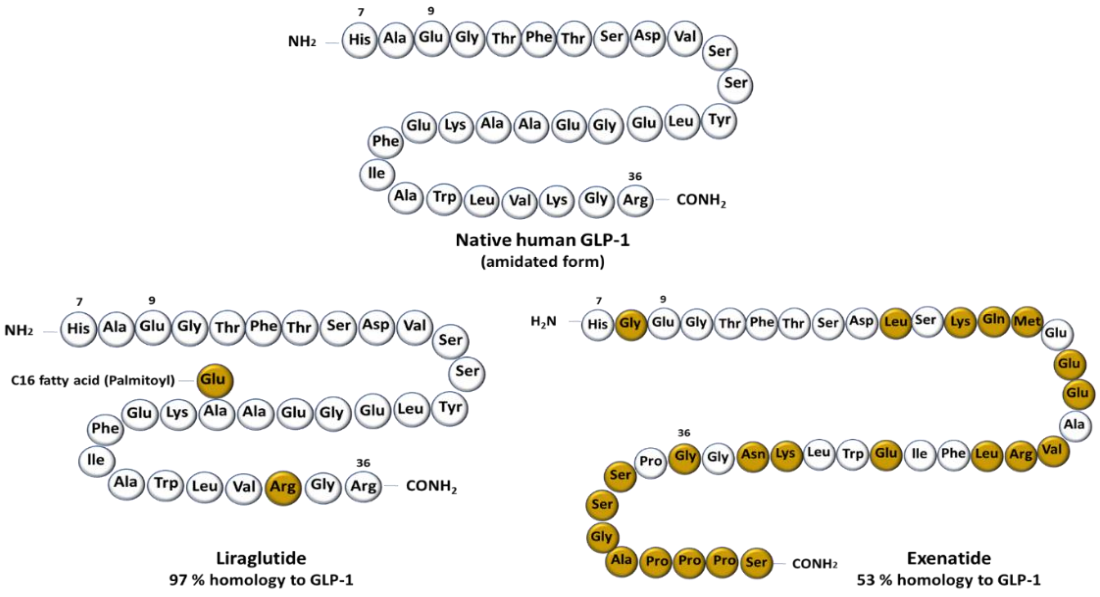


Figure 3. Amino acid structure of Exenatide and Liraglutide in comparision with native human GLP-1

2.2.Oral delivery of GLP-1 analogues

GLP-1 analogues Exn and Lira are currently administered through SC injection which is an invasive route that would adversely affect patients' adherence to the therapy (Lin *et al.*, 2016). Accordingly, a great effort should be devoted to developing a patient-friendly delivery system, and the oral route is considered the most widely accepted means of administration as it could prove safe and effective. Besides, the oral delivery stimulates the normal physiological pathway of the native GLP-1 peptide by targeting the liver, and subsequently minimizes potential side effects which would enhance patients' compliance to the treatment and thus increase the treatment efficacy (Shamekhi, Tamjid and Khajeh, 2018). However, oral delivery of peptide drugs constitutes a great challenge due to the harsh acidic environment in the stomach, the enzymatic barrier in the gastrointestinal (GI) tract, and sulfhydryl barrier that all lead to the low stability of such macromolecules. Additionally, the mucus barrier presented by the mucus gel layer covering the GI epithelium, and the absorption barrier presented by GI epithelial cells and tight junctions between adjacent epithelial cells contribute to the poor diffusion and penetration of the peptides across the intestinal epithelium into the blood circulation, resulting in low oral bioavailability (Leonaviciute and Bernkop-Schnürch, 2015).

A huge variety of strategies have been designed aiming to deliver these anti-diabetic peptides orally, from the structural modification of the peptide drug such as PEGylation, lipidization, inclusion of unnatural amino acids, palmitoylation and biotinylation to incorporation of enzyme inhibitors, absorption enhancers and cell-penetrating peptides (Ismail and Csóka, 2017; Kamei *et al.*, 2018), in addition to loading the peptide drugs into carrier systems. Among the several developed strategies, incorporation of GLP-1 analogues in nanocarrier systems could be the most promising choice attracting more and more academic and industrial research groups.

2.2.1. Nanocarriers for oral GLP-1 and its analogues delivery

The USFDA defines nanomaterials as "materials that have at least one dimension in the range of approximately 1 to 100 nm and exhibit dimension-dependent phenomena" (USFDA, 2014). This definition is even broader in the field of drug delivery, including particles up to 1000 nm in size (Jeevanandam *et al.*, 2018). The potential success of NCs as delivery systems for many small molecules has paved the way for the application of nanotechnology for peptides and proteins delivery (Navya *et al.*, 2019). Recently, several studies have been conducted using nanotechnology approaches to overcome the main biological barrier limiting GLP-1 administration, namely, its

short half-life (Araújo *et al.,* 2012; Pérez-Ortiz *et al.,* 2017). For efficient oral GLP-1 and its analogs delivery, a wide range of nanosystems have been explored. In addition to the protective effect towards the harsh environment in the GI tract, nanocarrier systems could control peptides release, facilitate their mucus permeation and enhance the cellular uptake, and accordingly, enhance the peptides oral bioavailability (Abeer *et al.,* 2019).

2.2.1.1. Polymeric nanoparticles

Utilizing polymers to form NPs provides simple elaboration and design by enabling modulation of physicochemical characteristics (particle size, surface properties, and hydrophobicity), drug release properties in addition to biological behavior (targeted drug delivery and improved cellular uptake) (Patel *et al.*, 2014). Polymeric NPs are stable in the GI environment and able to protect encapsulated therapeutics from the rapid pH variations and enzymatic degradation due to their capability of forming a steric hindrance for enzymes to attack to encapsulated peptide drugs (Suchaoin and Bernkop-Schnürch, 2017). They can also be excellent carrier systems to evade the efflux route limiting the intestinal transport since P-glycoprotein (Pgp) is incapable of recognizing NPs (Xu *et al.*, 2012). Providing protection towards degradation in the GI tract, enhancing cellular contact with the intestinal membrane, and promoting absorption in the small intestine, polymeric NPs are considered as promising carrier systems for oral peptide delivery.

Commonly used polymers include natural polysaccharides such as chitosan (CS), cellulose and alginate as well as synthetic biodegradable polymers, namely, poly ε-caprolactone (PCL), poly (d,l -lactic acid) (PLA), polyacrylic acid (PAA) and poly (d,l -lactic-co-glycolic acid) (PLGA) (Kamaly *et al.*, 2016). Since biodegradable polymers are degraded in vivo by either hydrolysis or other enzymatic mechanisms and producing nontoxic degradation products which are further eliminated by the normal metabolic pathways, these polymers are the preferred candidates for the design of polymeric nanosystems. Approved by USFDA for therapeutic use in human, PLGA (a co-polymer of PLA and polyglycolic acid (PGA)) is extensively utilized in nanodelivery for the entrapment of several therapeutics including biologics (Fredenberg *et al.*, 2011; Gutjahr *et al.*, 2016). It has been demonstrated that release kinetics of therapeutics from PLGA NPs are highly affected by molecular mass, the ratio of lactide to glycoside and drug loading. PLGA backbone contains labile bonds, ester linkage, being sensitive to hydrolysis (Mir, Ahmed and Rehman, 2017), and drug release from PLGA nanosystems can be mediated by different mechanisms: (i) desorption

of drug bound to the surface, (ii) surface erosion, (iii) polymer degradation at the surface or within the NPs matrix due to cleavage of ester bonds, (iv) drug diffusion through polymer matrix and/or (v) a combined erosion-diffusion process (Danhier *et al.*, 2012; Kamaly *et al.*, 2016).

Peptides encapsulation in polymeric NPs can be reached by several techniques such as nanoprecipitation, supercritical fluids, salting out, emulsification/solvent diffusion and emulsification/solvent evaporation in addition to ionic gelation and polymerization of monomers (Vandana and Sahoo, 2009; Nagavarma *et al.*, 2012; Gaudana *et al.*, 2013; Marin, Briceño and Caballero-George, 2013). Among these up-to-date applied techniques, the double emulsion W1/O/W2 solvent evaporation method is the most widely used one for loading peptides into PLGA NPs (Araújo *et al.*, 2016) (Figure 4).

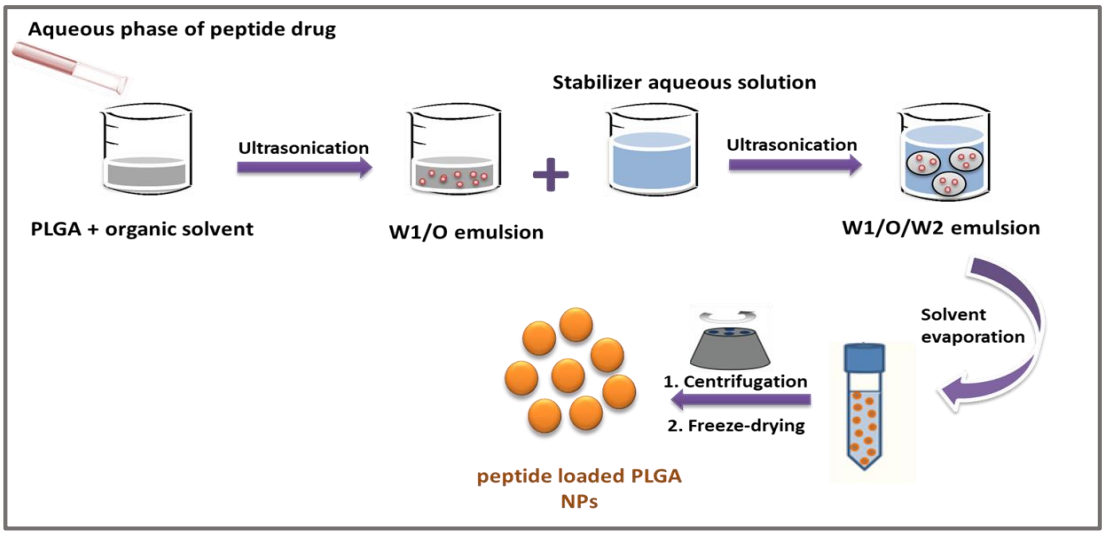


Figure 4. Schematic representation of preparation of PLGA nanoparticles (NPs) by water in oil in water (W1/O/W2) double emulsion solvent evaporation method.

Aiming to overcome the challenges limiting the oral GLP-1 delivery, different formulations of polymeric and lipid NPs were developed via modified double emulsion W1/O/W2 solvent evaporation technique, and results showed that CS-coated PLGA NPs was the system which could efficiently retain the GLP-1 from the harsh environment of the stomach simulated conditions and sustained the peptide release. However, this nanosystem was not as successful as CS-coated porous silicon NPs in enhancing the permeability across the intestinal Caco-2 and HT29-MTX cell monolayers (Araújo *et al.*, 2014). Another research group developed oral Exn -loaded CS NPs prepared using ionotropic gelation and modified with CSKSSDYQC peptide. This system was able

to significantly enhance the transport of Exn across a co-cultured Caco-2 and HT29 cell membrane, protect Exn from enzymatic degradation in the GI tract, and improve the relative bioavailability of the drug (versus SC) up in db/db mice up to 6.56% (Li *et al.*, 2015).

In a recent work, GLP-1 loaded PLGA-CS NPs were prepared by modified double emulsion W1/O/W2 solvent evaporation technique and conjugated to polyarginine (R9) using EDC/NHS coupling chemistry. This was followed by enteric encapsulation of the developed NPs within HPMC, and loading with the dipeptidyl peptidase-4 inhibitor (DPP-4i), using a double emulsion technique through a microfluidic flow-focusing glass device (Araujo *et al.*, 2016). Following the oral administration of the developed nanosystem in T2DM rat model, significantly higher plasmatic insulin levels and lower blood glucose levels with regard to the oral (GLP-1+DPP-4i) solution were obtained. Exn was recently encapsulated into Fc-modified polyethylene glycol (PEG)-PLGA NPs by double emulsion W1/O/W2 solvent evaporation method as well, and this nanosystem revealed improved Caco-2 cells uptake and could produce a sustained reduction in blood glucose levels following the oral delivery in db/db mice (Shi *et al.*, 2018b).

2.2.1.2. Self-emulsifying drug delivery systems

SEDDS are defined as isotropic thermodynamically stable mixtures of natural or synthetic oils, solid or liquid surfactants, solvents and co-solvents/surfactants which could emulsify spontaneously to produce oil-in-water (O/W) nanoemulsion of approximately 100 nm or less in size upon being introduced into aqueous phase such as GI fluids under gentle agitation (Khattab, Hassanin and Zaki, 2017; Mohd Izham *et al.*, 2019). This spontaneous formation of nanoemulsion in the GI tract presents the drug in a solubilized form inside small droplets of oil, all over its transit through the GI tract. SEDDS can offer the advantages of enhanced physical and/or chemical stability of the formulation, a high degree of patient compliance/tolerability due to the possibility of filling them into unit dosage forms such as soft/hard gelatin in addition to the ease of production and scale-up process (Date *et al.*, 2010).

SEDDS have emerged as a potential platform for the oral peptide delivery since they exhibited promising results for oral delivery of different therapeutic peptides (Ijaz *et al.*, 2016; Leonaviciute *et al.*, 2016; Zupančič *et al.*, 2017; Nardin and Köllner, 2018).). This can be due to their protective effect against peptidases and proteases that are too hydrophilic to enter the hydrophobic droplets of SEDDS (Pereira De Sousa and Bernkop-Schnürch, 2014). The mucus permeation enhancing properties of SEDDS should also be considered since they showed

promising results in overcoming the mucus gel barrier in a comparatively efficient manner facilitating the transport of incorporated peptide drugs to the underlying epithelium (Mahmood and Bernkop-Schnürch, 2018). The intestinal permeation enhancing effect of SEDDS for insulin has been proved and the results indicate that SEDDS can enable opening tight junctions of the Caco-2 monolayer. The relative oral bioavailability of insulin embedded in this formulation (versus SC) was also improved reaching up to 15% (Ma *et al.*, 2006).

Due to their hydrophilicity, hydrophilic macromolecules such as peptides and proteins cannot be directly loaded into SEDDS nanodroplet. Up to date, the hydrophobic ion-pairing (HIP) technique could be the most promising technique to increase the lipophilicity of hydrophilic peptide drugs upon co-precipitation of the peptide due to its full or partial association with an appropriate counterion in aqueous media (Chamieh *et al.*, 2019; Phan, Shahzadi and Bernkop-Schnürch, 2019).

Various anionic surfactants sodium glutamate, sodium dodecanoate, sodium deoxycholate and pamoic acid exhibiting mono- and di-carboxylic moieties were used for hydrophobic ion pairs (HIPs) formation with insulin, leuprolide and bovine serum albumin, and precipitated HIPs were further incorporated in SEDDS formulations. Findings showed that the stability of HIPs is an important controlling factor for improving their affinity towards SEDDS oily droplets and subsequently affecting the release behavior of these complexes upon dilution with intestinal fluids, which in turns affect their intestinal permeability (Nazir *et al.*, 2019). In another study, insulin was successfully into SEDDS formulation in the form of insulin:dimyristoyl phosphatidylglycerol HIPs, and the developed SEDDS could efficiently protect insulin from enzymatic degradation, prevent the burst effect and increase the mucus permeability (Karamanidou *et al.*, 2015).

2.3.Quality by Design (QbD) approach in pharmaceutical development

Due to the complexity of macromolecules containing nanosystems development, it is a priority to understand the potential risks to product quality, safety and efficacy associated with the nanosystem attributes (de Vlieger *et al.*, 2019). Pharmaceutical QbD is recommended by regulatory bodies to be applied as risk based approach which starts with predefined objectives and emphasizes product and process understanding and control based on sound science and quality risk management (USFDA, 2009). The elements of a pharmaceutical QbD approach are described in the relevant guidelines of the International Council of Harmonization (ICH), namely ICH Q8 (R2)

(Pharmaceutical Development), ICH Q9 (Quality Risk Management), and ICH Q10 (Pharmaceutical Quality System) (ICH, 2005, 2008, 2009). The ICH and USFDA strongly promote the implementation of QbD principles in drug product development since the quality must be built into the product (Mishra *et al.*, 2018). The flow chart in figure 5 illustrates the extended version of QbD model for R&D stage that was proposed by Csóka *et al*. (Csóka *et al*.,2018). The preformulation stage (labeled as zero phase) includes careful assessment of the available biopharmaceutical knowledge of the unmet therapeutic needs in addition to evaluating all the possibilities concerning the administration route–dosage form–drug substance triangle. This is followed by defining the QTPP that describes the targeted safety and efficacy aspects during product development. Identifying the characteristics that are critical to quality, CQAs forms the next step followed by the selection of CPPs and CMAs which are defined based on a thorough understanding of process design, previous practical investigations and evaluation of relevant literature. The Ishikawa diagram and Pareto charts can help in CQA, CMAs and CPP selection during the early development. The next step is to conduct RA to identify and rank the potentially high-risk attributes that warrant further optimization. Setting up the appropriate Design of Experiment (DoE) could enable the researcher to minimize the number of runs, rank the most influential parameters that could highly impact the quality of the product (Pallagi *et al.*, 2019) and select the optimum level of each parameter that assures the desired CQAs values, and ultimately generates the design space (DS) to comply with the predefined QTPP. One of the important questions when implementing DoE methodology is the selection of adequate experimental design that matches with the experimental objective. When estimating the main effects of a large number of factors are of interest to be investigated, screening designs such as 2- level Plackett-Burman design (PBD) is applied (Rahman *et al.*, 2010). The main advantage of applying such screening designs is the minimum number of observations needed to calculate the effect of several variables. If providing further information on direct and pairwise-interaction effects and curvilinear variable effects is desired, second-order designs: central composite designs (CCD) and Box-Behnken designs (BBD) are the most widely applied ones (Rakić *et al.*, 2014). The knowledge gained during previously designed development studies culminates in the establishment of a control strategy to measure the critical attributes and evaluate them according to the desired predefined value within the acceptance range, followed by evaluating the possibilities of continuous improvement.

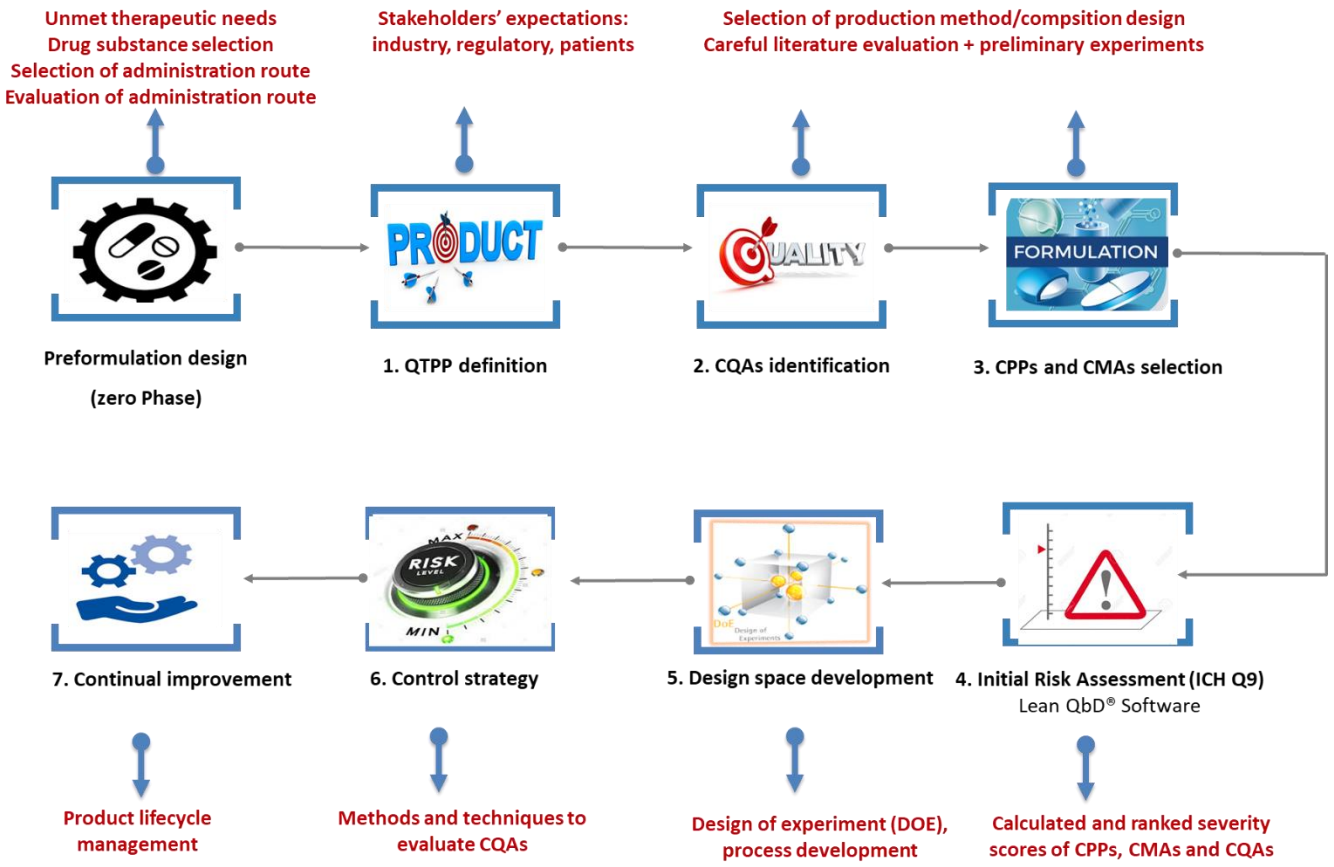


Figure 5. QbD flow chart for the research and development (R&D) stage.

3. MATERIALS

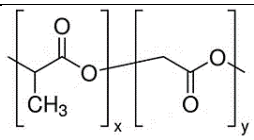
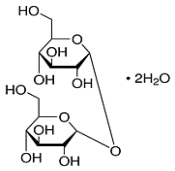
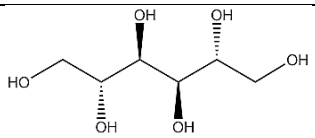
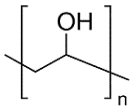
Liraglutide was purchased from Xi'an Health Biochem Technology Co., Ltd (Shaanxi, China). Exenatide was purchased from Chemos GmbH (Altdorf, Germany).

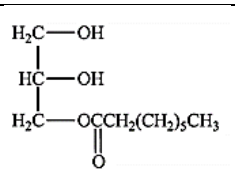
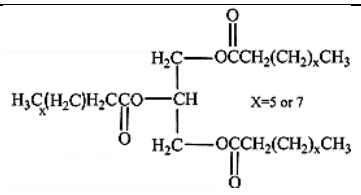
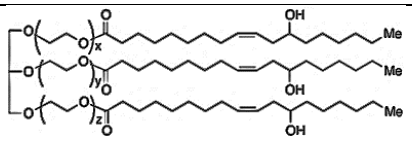
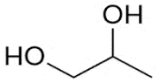
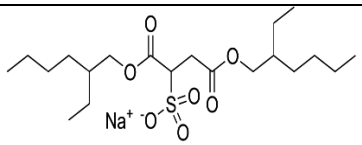
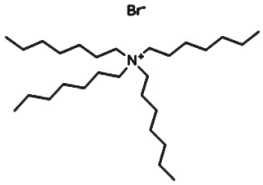
For PLGA NPs preparation, Poly (D,L-lactide-co-glycolide) (PLGA 50:50, Mw=30,000-60,000 Da), poly (vinyl alcohol) (MOWIOL 4-98®, MW~27000 Da) and D-(+)-Trehalose dihydrate (MW=378.33 g/mol) were purchased from Sigma-Aldrich (Munich, Germany). D-(-) -Mannitol was supplied from Molar Chemicals Ltd. (Budapest, Hungary). Sodium acetate anhydrous was supplied from Scharlau Chemie S.A. (Barcelona, Spain). Ethyl acetate used for dissolving PLGA was obtained from Reanal Labor (Budapest, Hungary). Pepsin from porcine gastric mucosa, powder (≥400 units/mg protein) and pancreatin from porcine pancreas (≥3×

USP specifications) were purchased from Sigma Aldrich (Budapest, Hungary). The Caco-2 intestinal epithelial cell line was purchased from ATCC (cat.no. HTB-37) at passage 60. Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) were used as cell culture supplements.

For SEDDS preparation, Capmul® MCM (mono/diglycerides of caprylic acid, HLB 5-6) and Captex® 355 (caprylic/capric triglycerides, HLB 0) were supplied from Abitec (Janesville, Wisconsin, USA). Kolliphor® RH 40 (polyoxyl hydrogenated 40 castor oil, HLB 14-16), sodium docusate (DOC) and tetraheptylammonium bromide (THA) were purchased from Sigma-Aldrich (Vienna, Austria). Minimum Essential Medium Eagle (MEM) and Dulbecco's phosphate-buffered saline were supplied from Biochrom GmbH (Berlin, Germany). Exendin-4 fluorescent ELISA kit was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Hanks' Balanced Salts (HBSS), propylene glycol and all other reagents and solvents were purchased from Sigma-Aldrich (Vienna, Austria). Table 1 shows the structures and applications of the excipients used in this work.

Table 1. Structures and applications of excipients applied for experimental work.

Excipient	Structure	Applications
PLGA		Biodegradable and biocompatible polymer used for PLGA NPs preparation
D- (+)-Trehalose dihydrate		Lyoprotectants used to stabilize PLGA NPs and protect them during freeze-drying and storage
D- (-)-Mannitol		
PVA		Surfactant used as stabilizer of PLGA NPs by minimizing surface tension of continuous aqueous phase

Capuml® MCM		Oils used for SEDDS preparation
Captex® 355		
Kolliphor® RH 40		Surfactant used for SEDDS formulation
PG		Co-solvent of hydrophobic ion pairs (HIPs) into SEDDS formulation
DOC		Anionic surfactant used to from hydrophobic ion pairs (HIPs) with exenatide
THA		Cationic surfactant used to from hydrophobic ion pairs (HIPs) with exenatide

4. METHODS

4.1. QbD based strategy for development of GLP-1 analogue loaded nanocarriers

At the preformulation design stage, all the possibilities concerning the administration route, dosage form and drug substance were carefully evaluated. This was followed by the identification of the QTPP for GLP-1 analogue loaded nanocarrier. Defining the CQAs, CMAs and CPPs was the next step, followed by performing the initial RA with Lean QbD® Software (QbD Works LLC. USA, CA, Fremont). At this stage, the interdependence rating was performed between CQAs and CMAs, CQAs and CPPs on a three-level scale and categorized as “high” (H), “medium,” (M) or “low” (L). The whole risk estimation also resulted in calculated and ranked severity scores of the CQAs that were presented in Pareto chart generated by the software.

4.2.Liraglutide loaded PLGA NPs

4.2.1. Preparation of liraglutide loaded PLGA NPs

The preparation of Lira loaded PLGA NPs was carried out by means of the double emulsion W1/O/W2-solvent evaporation method (Ismail *et al*., 2019b). PLGA was dissolved in ethyl acetate at room temperature to form the organic phase. The inner aqueous phase of Lira dissolved in 1% (m/v) sodium acetate aqueous solution was slowly added to the organic phase, and water in oil (W/O) primary emulsion was formed upon sonication at the power of 90 W for 30 s using a probe sonicator in ice bath. The obtained emulsion was re-emulsified with the external aqueous phase containing PVA as a stabilizer by sonication at the power of 90 W for 0.5-2 min using the probe sonicator in ice bath. The obtained W1/O/W2 double emulsion was subjected to magnetic stirring at room temperature over the night to allow the complete evaporation of ethyl acetate. The nanoparticles were then collected by centrifugation for 15 min at 16500 rpm, washed three times with water, resuspended in deionized water, and lyophilized at -40 °C at 0.01 mbar (Scanvac, CoolSafe 100-9 Pro freeze dryer). A 5-10% (m/v) of mannitol or trehalose was added as lyoprotectants. Samples were stored at -20 °C for further use.

4.2.2. Optimization of liraglutide loaded PLGA NPs: Plackett-Burman design (PBD)

PBD with a total of 8 runs involving 7 independent variables at two levels was carried out using STATISTICA 13® software (Table 2). Particle size (Y1), polydispersity index (PDI) (Y2), encapsulation efficiency (EE) (Y3) and zeta potential (Y4) were selected as dependent variables. The design was validated by 3 extra center checkpoint formulations and the bias (%) between predicted and observed values of response was calculated. The optimized formulation was prepared within the DS and compared with the predicted values of the responses.

Table 2. The input factor levels in 7-factor, 2-level, 8-run Plackett-Burman Design (PBD).

Run code	PLGA (mg)	Lira (mg)	2nd sonication time (min)	PVA (%)	Lyoprotectant type	Lyoprotectant (%)	W2/O ratio
PBD-F1	30	0.5	0.5	2	Trehalose	10	2
PBD-F2	60	0.5	0.5	0.5	Mannitol	10	5
PBD-F3	30	5	0.5	0.5	Trehalose	5	5
PBD-F4	60	5	0.5	2	Mannitol	5	2
PBD-F5	30	0.5	2	2	Mannitol	5	5
PBD-F6	60	0.5	2	0.5	Trehalose	5	2
PBD-F7	30	5	2	0.5	Mannitol	10	2
PBD-F8	60	5	2	2	Trehalose	10	5

4.2.3. Characterization of the developed liraglutide loaded PLGA NPs

4.2.3.1. Particle size, size distribution, surface charge and encapsulation efficiency measurements

The hydrodynamic diameter (Z-average), PDI and zeta potential of NPs reconstituted in demineralized water were measured in a folded capillary cell by using Malvern Nano ZS Zetasizer (Malvern Instruments Ltd. UK) equipped with He-Ne laser (633 nm). The EE of Lira loaded in PLGA NPs was determined directly using the centrifugation method (Ismail *et al.*, 2019b), and the supernatant was then collected and the amount of encapsulated Lira was measured using the HPLC method. Stability of the optimized Lira-PLGA NPs was also investigated by measuring the particle size, PDI and zeta potential after one week at room temperature. All the measurements were conducted in triplicate.

4.2.3.2. In vitro release study

The release behavior of Lira from PLGA NPs was evaluated in simulated gastric fluid without enzymes (SGFsp: 0.1N HCl at pH 1.2) over 2 hours followed by simulated intestinal fluid without enzymes (SIFsp: phosphate buffer saline at pH 6.8) over 4 hours. An aliquot of 500 µl was withdrawn from the release medium at predetermined time points and replenished with the same volume of the fresh preheated medium. Samples were centrifuged at 16,500 ×g and 4°C for 10 min, and the Lira concentration in the supernatant was determined by HPLC. The cumulative

percentage of Lira released was calculated and then plotted versus time. All experiments were conducted in triplicate.

4.2.3.3. Enzymatic degradation study

PLGA NPs containing 500 µg of Lira were resuspended in 2 ml of pepsin containing simulated gastric fluid SGF (3.2 g pepsin, 2 g of sodium chloride, 7 mL HCl, mixed and diluted with water to 1L, pH=1.2) or pancreatin containing simulated intestinal fluid SIF (10 g pancreatin, 6.8 g KH_2PO_4, mixed and adjusted with 0.2 N NaOH then diluted with water to 1L, pH=6.8) and incubated at 37°C under stirring of 100 rpm. Native Lira was used as control. Samples were withdrawn at specific time intervals over a 2-hour period, and an equal volume of ice-cold reagent was added: 0.1 M NaOH for SGF and 0.1 M HCl for SIF, to stop the enzymatic reaction. Samples were centrifuged at 16,500 ×g and 4°C for 10 min and the supernatant was analyzed by HPLC. All incubations were done in triplicates. Lira recovery in the withdrawn samples was calculated using the following equation:

Lira recovery (%) = (Remaining Lira amount/theoretical Lira amount) *100

4.2.3.4. Treatment of Caco-2 cells

The Caco-2 cells were grown, as previously reported (Bocsik *et al.*, 2019). The concentration of stock solutions for cell culture experiments was 1 mM for both the therapeutic peptide Lira and the PN159 peptide, which was used as a reference absorption enhancer. The final concentration of Lira encapsulated in the PLGA NPs was 100 µM and was diluted directly before using it. Lira was examined at 100 µM, while PN159 was examined at 3 µM concentration both for cell viability and permeability.

4.2.3.5. Cell viability measurement by impedance

Impedance was measured at 10 kHz by an RTCA SP instrument (RTCA-SP instrument, ACEA Biosciences, San Diego, CA, USA). Lira, Lira-loaded/free PLGA NPs, Lira and PN159 solution, and PN159 peptide were diluted in cell culture medium and the effects were followed for 24 h. Triton X-100 (TX-100) (1 mg/ml) was used as a reference positive control. The cell index was defined as Rn-Rb at each time point of measurement, where Rn is the cell–electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone.

4.2.3.6. Permeability study on the Caco-2 cell model

Transepithelial electrical resistance (TEER) monitoring was performed by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes. The final TEER was expressed relative to the surface area of the monolayers as $\Omega \times cm^2$ after the subtraction of TEER values of cell-free inserts. Caco-2 cells were seeded onto Transwell inserts (polycarbonate membrane, 3 μm pore size, 1.12 cm2 surface area) and cultured for three weeks. For transport experiments, the inserts were transferred to 12-well plates containing 1.5 mL Ringer-buffer in the acceptor (lower/basal) compartments. In the donor (upper/apical) compartments, the culture medium was replaced by 0.5 mL Ringer-buffer containing treatment solutions of Lira, Lira loaded in PLGA NPs, and Lira and PN159 solution at the concentration of 100 μM for Lira for 1 h. Treatment solutions from both compartments were collected and the Lira level was detected by HPLC.

The apparent permeability coefficients (P_{app}) and recovery (mass balance) were calculated as described previously (Hellinger *et al.*, 2012; Bocsik *et al.*, 2016).

4.2.3.7. Immunohistochemistry

Aiming to investigate the morphological changes in interepithelial junctions, immunostaining for the junctional proteins, zonula occludens protein-1 (ZO-1) and β-catenin, was carried out (Ismail *et al.*, 2019a). Cells were grown on glass coverslips (Menzel-Glaser, Braunschweig, Germany) at a density of 4 × 104 cells/coverslips for 4 days and treated with Lira (100 μM), Lira loaded in PLGA NPs, Lira and PN159 solution, and PN159 peptide (3 μM) solutions for 1 h. Hoechst dye 33342 was used to stain the cell nuclei. After mounting the samples (Fluoromount-G; Southern Biotech, Birmingham, AL, USA), the staining was visualized by a Visitron spinning disk confocal system (Visitron Systems GmbH, Puchheim, Germany).

4.2.3.8. Chromatographic equipment and conditions

Lira was analyzed by a reversed-phase HPLC (Shimadzu Corporation, NEXERA X2, Tokyo, Japan) method that was developed in our lab. A Kinetex® C18 column (5 μm, 150*4.6 mm, (Phenomenex, USA) was used as a stationary phase. The column temperature was set to 40 °C. The mobile phase consisted of 0.02 M aqueous KH2PO4 solution (pH = 7.0, eluent A) and acetonitrile (eluent B), and was pumped in a gradient mode from 80:20 (A:B, v/v) to 30:70 (A:B, v/v) in 12 min then set back to 80:20 (A:B, v/v) between 12.1-15 min at a flow rate of 1.5 ml/min over 15 min. Fifty microliters of sample volume were injected. The wavelength of UV detection

was 214 nm. Retention time of Lira was 8.65 min and the regression coefficient (R^2) of the calibration curve was 0.996 proving high linearity.

4.3.Exenatide loaded SEDDS

4.3.1. Optimization of hydrophobic ion-pairing of exenatide with THA and DOC

Exn acetate aqueous solution was prepared in a concentration of 2 mg/ml and the pH was adjusted with 0.1 M NaOH to 8.0. Increasing amounts of THA were dissolved in 1 ml of demineralized water: methanol and added slowly and dropwise to 1 ml of the Exn solution under light shaking. Exn to THA ratios of 1:1, 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12 were tested. Exn -DOC ion pairs were prepared as previously described (Menzel *et al.*, 2018), where the pH of 2 mg/ml Exn solution was adjusted to 3.0 with 2 M HCl followed by the addition of an equal volume of DOC aqueous solution slowly and dropwise to Exn solution under light shaking. Exn to DOC ratios of 1:1, 1:2, 1:4 and 1:6 were tested.

HIPs were collected by centrifugation for 5 min at 13400 rpm (MiniSpin®, Eppendorf Austria GmbH) and washing 3 times with water. The supernatant that contained the remaining amount of dissolved Exn was analyzed by HLPC to calculate the precipitation efficiency using the following equation:

$$\text{Precipitation efficiency (\%)} = \frac{\text{peptide concentration after HIP}}{\text{peptide concentration before HIP}} * 100$$

Zeta potential of HIPs with surfactant was determined with Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK). Precipitated HIPs were frozen, lyophilized at -30°C and 3-5 mTorr (Christ Gamma 1-16 LSC Freeze dryer) and stored at -20°C for further use.

4.3.2. Development of Exn-THA and Exn-DOC loaded SEDDS

HIPs with THA were prepared at a molar ratio of 1:8 (Exn: THA) at pH 8.0. HIPs with DOC were prepared at a molar ratio of 1:4 (Exn: DOC) at pH 4.0. SEDDS were formed by dissolving 2 mg of HIPs having been prepared as described above in 4 mg of propylene glycol (PG) by stirring for 2 min and homogenizing (1500 rpm) for 10 min at room temperature (Eppendorf ThermoMixer®, Hamburg, Germany). This was followed by addition of 41 mg of Capmul MCM (oil), 40 mg of Kolliphor RH (surfactant) and 15 mg of Captex 355 (oil) and homogenizing the mixture (1500 rpm) after the addition of each component.

4.3.2.1. Particle size, size distribution, surface charge and payload measurements

To determine the maximum payload of HIPs of Exn-surfactant in the formulation, the preconcentrate was analyzed by HPLC. Furthermore, the droplet size (Z-average), PDI and zeta potential of SEDDS having been dispersed in demineralized water (1:100) were measured via Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK). Stability of the SEDDS was also tested by measuring the droplet size, PDI and zeta potential after one week at room temperature. All the measurements were performed in triplicate.

4.3.2.2. Determination of log D $_{\text{SEDDS/release medium}}$ of Exn-THA and Exn-DOC

The release characteristics of Exn-THA and Exn-DOC from SEDDS were evaluated according to the distribution of the ion pairs between the oily droplet phase of SEDDS and the release medium (log $D_{SEDDS/RM}$) (Shahzadi *et al.*, 2018). Lyophilized Exn-THA (molar ratio 1:8) and Exn-DOC (molar ratio 1:4) were dispersed in SIF (50 mM phosphate buffer pH 6.8) and HBSS. After stirring for 3 hours at 1000 rpm, the suspension was centrifuged for 5 min at 13400 rpm. The supernatant was analyzed by HPLC to calculate the solubility of HIPs in each release medium (C_{RM}). The maximum payload of the complex represents the solubility of HIPs in SEDDS (C_{SEDDS}). Log $D_{SEDDS/RM}$ of HIPs was calculated according to the following equation:

$$\log \mathrm{D}\ SEDDS/RM = \log \frac{\mathrm{C\ RM}}{\mathrm{C}\ SEDDS}$$

4.3.2.3. In vitro hemolysis assay

The in vitro hemolysis assay on human erythrocytes can be used as a rapid and reliable method to evaluate cytocompatibility of drug delivery systems (Roointan *et al.*, 2018; Lam *et al.*, 2019). In-vitro hemolysis assay of Exn-THA SEDDS, Exn-DOC SEDDS and blank SEDDS was carried out according to a previously reported protocol (Lam *et al.*, 2019).

First, 50 µl of SEDDS was added to 1 ml of diluted blood to the final concentration of 0.1%, 0.25% and 0.5% (m/v). This was followed by an immediate shaking of the mixtures on thermomixer for 2 h at 300 rpm and 37 °C and additionally homogenized by inversion during the incubation. Then, the mixtures were centrifuged at 503*g for 5 min at 5° C. Tecan infinite M200 plate reader was used to read the absorbance of the supernatants at a wavelength of 420 nm. Triton-X-100 1% (m/v) in MEM and sterile Dulbecco's PBS pH 7.4 served as positive and negative control, respectively.

The percentage of hemolysis (H%) was calculated by the following equation

$$H\ (\%) = \frac{\text{Abs (test)} - \text{Abs (neg)}}{\text{Abs (pos)} - \text{Abs (neg)}}$$

where Abs_{test} is absorbance of the test sample, Abs_{neg} is absorbance of PBS (negative control) and Abs_{pos} is absorbance of TX-100 (positive control).

4.3.2.4. Ex-vivo permeability study

The ex-vivo permeability study was performed on fasted rats weighing 200-250 g. Rats were sacrificed and the freshly excised small intestine was preincubated in HBSS buffer for 30 min before being cut into strips of 2 cm mounted in Ussing-type chambers. The apical and basolateral sides were filled with 1 mL of HBSS pH 6.8 and incubated for 30 min in a water bath at 37 °C. Then, HBSS was replaced by fresh incubation medium at the apical side and by the following test samples: (1) 0.25% (m/v)of Exn-THA-SEDDS, (2) 0.6% (m/v) of Exn-DOC SEDDS and (3) 8 µg /ml of Exn solution in HBSS at the apical side.

Aliquots of 200 µl were withdrawn from the basolateral side at 1, 2, and 3 h and replaced with an equal volume of fresh HBSS preincubated at 37 °C. Exn concentration in the aliquots was quantified by fluorescent ELISA immunoassay (FEK-070-94; Phoenix Pharmaceuticals, Inc., USA) following the manufacturer's instructions. The absorbance was read at 450 nm using a microplate reader (Spark Multimode microplate reader, Tecan). Values are presented as means ± SD ($n = 4$).

4.3.2.5. In-vivo study

Male Sprague-Dawley rats with a mean bodyweight of 200-250 g were supplied by Janvier Labs (Saint Berthevin, France) and the in *vivo* study was approved by the Ethical Committee of Austria and performed according to the principles of Laboratory Animal Care. Rats were fasted 2 hours prior to drug administration and 6 hours thereafter but had free access to water during the entire experiment. Rats were randomly divided into four groups (n = 5). The first group was the positive control group where Exn solution was administered via SC injection at a dose of 50 µg/kg body weight. The second group was the negative control group where Exn solution was orally administered at a dose of 300 µg/kg body. The third and fourth group received 300 µg/kg body weight of Exn-THA and Exn-DOC SEDDS formulation via oral gavage, respectively. The formulations were diluted with water (1:2) before administration.

Blood samples (100 µl) were collected from the tail vein before drug administration and at predetermined time points after dosing. The collected blood samples were then centrifuged at 2000* g for 5 min at 4º C and the serum was separated and stored at -80 ºC for further analysis. Exn concentration was measured by fluorescent ELISA immunoassay (FEK-070-94; Phoenix Pharmaceuticals, INC., USA). The absorbance was read at 450 nm using a microplate reader (Spark Multimode microplate reader, Tecan). The relative bioavailability (BA_R) of Exn after oral administration was calculated using the following equation

$$BA_R\,(\%) = \frac{\text{AUC (oral)*Dose (SC)}}{\text{AUC (SC)*Dose (oral)}} * 100$$

4.3.2.6. Chromatographic equipment and conditions

Reversed-phase HPLC (Hitachi Chromaster HPLC-system equipped with 5430 photodiode array UV detector, Tokyo, Japan) method was developed in our lab to quantify exenatide. A Nucleosil® C18 100-5 column (5 µm, 250*4 mm, Phenomenex, USA) was used as a stationary phase. The mobile phase consisted of 0.1% TFA in water (eluent A) and 80% acetonitrile with 0.1% TFA (eluent B), and was pumped in a linear-gradient mode from 58:42 (A:B, v/v) to 24:76 (A:B, v/v) at a flow rate of 0.75 ml/min over 22 min. The column temperature was set to 40 °C. Twenty microliters of sample volume were injected. The wavelength of UV detection was 278 nm. The retention time of Exn was 16.5 min and the regression coefficient (R^2) of the calibration curve was 0.999 proving high linearity.

4.4.Statistical analysis

All data are presented as means ± SD. PB design of experiment was carried out using STATISTICA 13® software, and analysis of variance (ANOVA) was applied to determine the statistical significance of each model coefficient. The values of other tested responses were compared using ANOVA followed by the Dunnett test or the Bonferroni test. GraphPad Prism® 5.0 software (GraphPad Software Inc., San Diego, USA) was used. Changes were considered statistically significant at $p < 0.05$.

5. RESULTS and DISCUSSION

5.1.QbD based strategy for development of GLP-1 analogue loaded nanocarriers

The initial step of QbD-based study aiming to design oral GLP-1 analogue loaded nanocarriers was to set up the QTPP based on the relevant guideline (USFDA, 2009, 2017), and its extended version for early phase of R&D (Csóka et al.,2018) in addition to our initial experimental data (Figure 6). PLGA NPs and SEDDS were selected to be further investigated as potential carriers to deliver Lira and Exn orally.

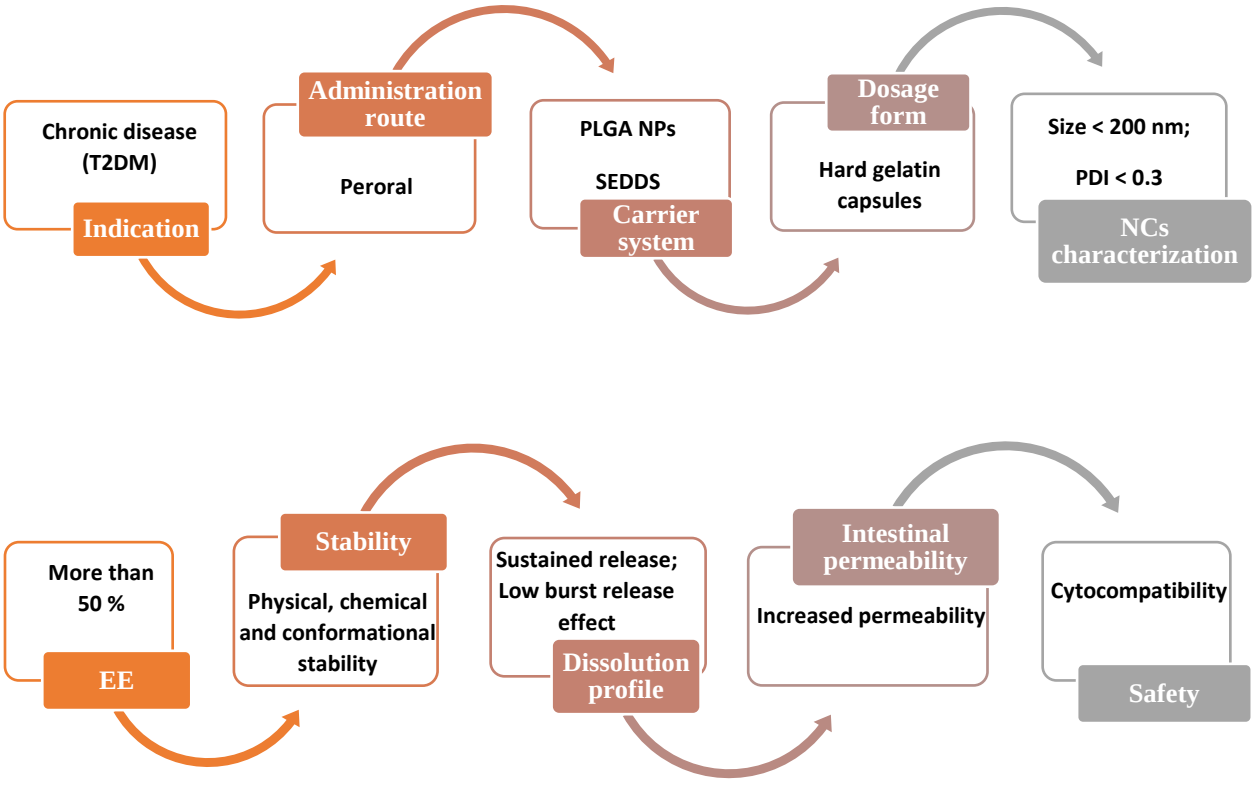


Figure 6. Defining the QTPP for GLP-1 analogue loaded nanocarriers (NCs).

Based on reviewing the most relevant production methods, Lira loaded PLGA NPs were selected to be prepared using double emulsion solvent evaporation method which is the most commonly used technique for the encapsulation of peptide drugs within PLGA NPs due to its simplicity and high encapsulation efficiency (Araújo *et al.*, 2016). For loading Exn into SEDDS formulation, the formation of HIPs (Exn with anionic or cationic counterion) was the selected

strategy to increase the lipophilicity of the peptide followed by incorporating the HIPs into SEDDS (Phan, Shahzadi and Bernkop-Schnürch, 2019).

The proposed CQAs, CMAs and CPPs regarding the preparation methods of PLGA NPs and SEDDS were also defined. Ishikawa fishbone diagram was established to configure the risk analysis process for defining the cause-effect relationship between the significant variables and the CQAs of the NCs (PLGA NPs, SEDDS) (Figure 7).

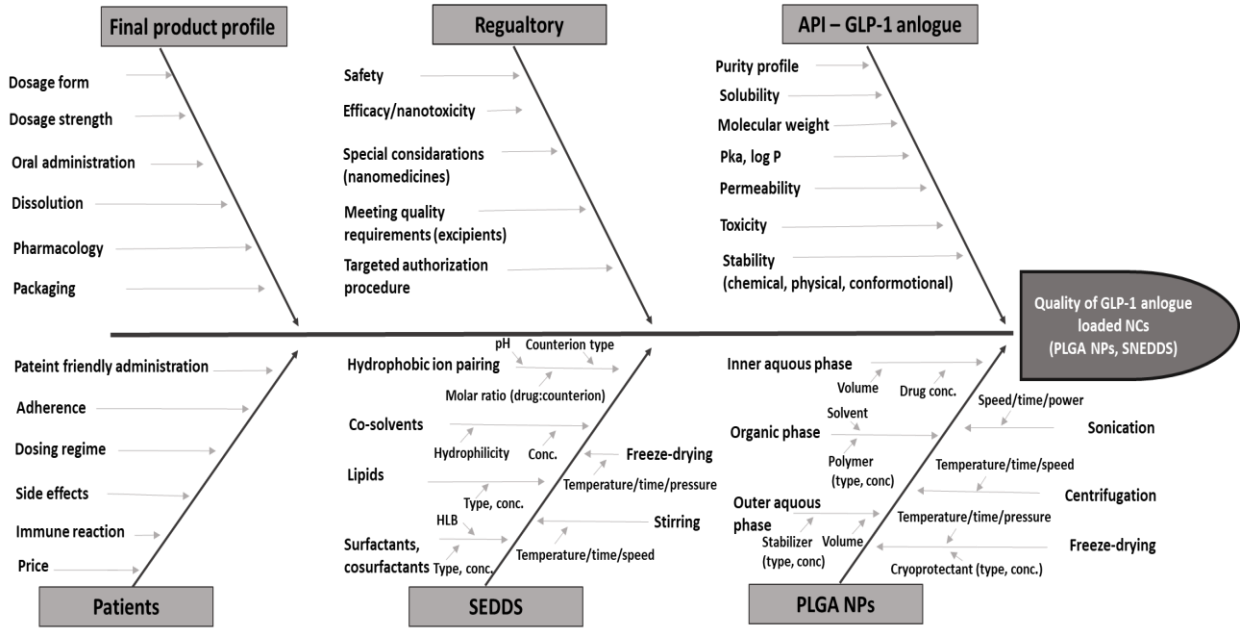


Figure 7. Ishikawa fishbone diagram for evaluating the risky factors related to the quality of GLP-1 analogue loaded into nanocarriers (NCs).

This was followed by the initial RA study achieved using Lean QbD® software. Figure 8 depicts the interdependence rating on the three-point scale between the selected CQAs and CMAs, and CQAs and CPPs, respectively. The calculated and ranked severity scores for the CQAs presented in a Pareto chart generated by the Lean QbD® Software are also shown in Figure 8E. Results of the RA study revealed that in the case of PLGA NPs, the most highly influential CPP is sonication time, and the most highly influential CMAs are polymer concentration, drug concentration, stabilizer concentration, cryoprotectant type, cryoprotectant concentration and external aqueous phase to organic phase ratio W2/O. Regarding SEDDS, the most highly influential CPP is the pH level during the HIPs formation, and the highly influential CMAs are

also related to HIPs formation (type of counterion, molar ratio of peptide to counterion) followed by the surfactant type and concentration.

A PLGA NPs development

Process	Double emulsion solvent evaporation							Freeze drying	
								Lyoprotectant	
CMAs / CQAs	Polymer type	Polymer conc.	Drug conc.	Stabilizer type	Stabilizer conc.	Organic solvent type	W2/O ratio	Type	Conc.
Particle size	H	H	H	H	H	L	H	H	M
PDI	M	M	L	M	L	M	L	M	M
Zeta potential	H	M	L	M	M	L	L	M	L
EE	H	H	H	H	H	M	H	H	H
Peptide stability	M	M	M	H	H	M	L	H	H
Release profile	M	M	M	M	M	L	H	M	M

B PLGA NPs development

Process	Double emulsion solvent evaporation					Freeze drying		
	Sonication		Centrifugation					
CPPS / CQAs	Time	Power	Time	Speed	Temp.	Time	Temp.	Pressure
Particle size	H	M	M	M	L	L	L	L
PDI	M	M	L	L	L	L	L	L
Zeta potential	H	L	L	L	L	L	L	L
EE	H	M	H	M	L	L	L	L
Peptide stability	H	M	M	M	M	H	H	H
Release profile	L	L	L	L	L	L	L	L

C SEDDS development

Process	Hydrophobic ion pairing			SNEDDS formulation					
CMAs / CQAs	Counter ion type	Counterion charge	Molar ratio (peptide:counterion)	Lipid type	Lipid conc.	Surfactant HLB	Surfactant conc.	Co-solvent type	Co-solvent conc.
Particle size	H	H	H	M	M	L	M	L	L
PDI	M	M	M	M	M	H	H	M	M
Zeta potential	H	H	H	L	L	M	M	L	L
HIPs payload	H	H	H	M	M	H	H	H	H
Peptide stability	H	H	H	M	M	H	H	M	M
Release profile	H	H	H	M	M	M	M	H	H

D SEDDS development

Process	Hydrophobic ion pairing						SNEDDS formulation		
	preparation	Centrifugation		Freeze-drying			Stirring		
CPPs / CQAs	pH level	Time	Speed	Time	Temp.	Pressure	Time	Speed	temp.
Particle size	L	M	M	L	L	L	M	M	L
PDI	M	L	L	L	L	L	M	M	L
Zeta potential	H	L	L	L	L	L	L	L	L
HIPs payload	H	M	M	L	L	L	L	L	L
Peptide stability	H	M	M	H	H	H	M	M	M
Relesae profile	M	L	L	L	L	L	L	L	L

E

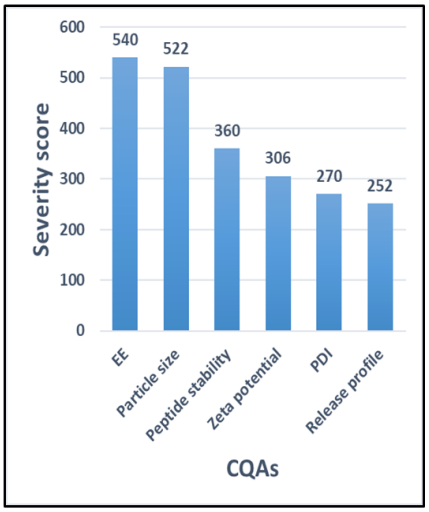


Figure 8. Risk assessment: Interdependence rating in case of PLGA NPs **(A)** between CMAs and CQAs, and **(B)** between CPPs and CQAs. Interdependence rating in case of SEDDS **(C)** between CMAs and CQAs, and **(D)** between CPPs and CQAs. **(E)** Pareto chart of estimated severity scores of the proposed CQAs.

5.2. Liraglutide loaded PLGA NPs

5.2.1. Optimization of liraglutide loaded PLGA NPs: Plackett-Burman design (PBD)

The high-risk formulation and process parameters resulted from RA study were further investigated regarding their effects on four responses namely: particle size (Y1), PDI (Y2), EE

(Y3) and zeta potential (Y4) by applying a seven-factor, two-level, eight-run PBD. The experimental data was validated by ANOVA for each factor. Depending on the selected parameters levels, the Z-average of the NPs ranged from 160.1±5.63 nm to 235.69±5.34 with an EE varying between 20.07±1.7% and 43.47±3.34%. Besides, NPs exhibited a practically monodisperse or narrow distribution as PDI ranged from 0.09±0.003 to 0.23±0.01 evidencing that the obtained NPs are homogeneous and stable with no aggregation. The zeta potential ranged from -31.15±1.23 mV to -23.83±0.95 mV and these expected negative values are attributed to the presence of carboxyl group end on PLGA (Table 3).

Table 3. Results of experimental responses in Plackett-Burman Design (PBD). Data are presented as mean ± SD (n=3).

Run code	Particle size (Z-Average) (nm)	PDI	EE%	Z-potential (mV)
PBD-F1	160.11±5.63	0.1±0.03	20.07±1.7	-30.57±1.79
PBD-F2	209.82±8.01	0.15±0.01	36.01±1.25	-24.97±1.64
PBD-F3	190.01±2.81	0.23±0.01	40.98±2.46	-27.35±0.67
PBD-F4	200.21±3.47	0.16±0.03	43.47±3.34	-31.15±1.23
PBD-F5	179.47±3.83	0.17±0.01	31.91±4.03	-23.83±0.95
PBD-F6	235.69±5.34	0.09±0.003	28.83±2.05	-29.24±0.41
PBD-F7	223.59±3.74	0.17±0.02	22.17±2.12	-30.42±0.39
PBD-F8	183.52±3.03	0.2±0.01	20.96±1.51	-26.95±0.148

The polynomial equations obtained for the fitted full model explaining the effect of formulation and process variables on the mean particle size, PDI, EE and zeta potential were as follows:

- Y1=197.8021+ 9.5079X1+1.5304X2+7.7638X3 - 16.9754X4 - 5.4712X5 - 3.5429X6 - 7.0791X7, with R^2 = 0.9745, adjusted R^2 = 0.9609, and Mean square (MS) = 22.3978
- Y2=0.1568-0.0061X1+ 0.0312X2+ 0.0005X3 - 0.0013X4 - 0.0032X5 - 0.0031X6 + 0.0291X7, with R^2 = 0.9235, adjusted R^2 = 0.8828, and Mean square (MS) = 0.0003
- Y3= 30.548 + 1.7683X1 + 1.3458X2 - 4.5825X3 - 1.4458X4 - 2.8408X5 - 5.7483X6 + 1.915X7, with R^2 = 0.9471, adjusted R^2 = 0.9189, and Mean square (MS) = 6.5021

- Y4= -28.0596 - 0.0154X1 - 0.9071X2 + 0.4496X3 - 0.0646X4 - 0.4679X5 - 0.1663X6 + 2.2846X7, with R^2 = 0.9001, adjusted R^2 = 0.8468, and Mean square (MS) = 1.1599

After establishing the polynomial equations describing the relationship between the CPPs, CMAs and the examined responses, particle size and EE were found to be significantly affected by almost all the tested CPPs and CMAs which is in accordance with the estimated severity scores of CQAs that was calculated previously in the initial RA step (Figure 8).

According to polynomial equations and Pareto charts (Figure 9), the most influential factors in terms of particle size were the PVA concentration, PLGA amount followed by sonication time and W2/O ratio at almost the same level of significance. Then the next important variables include lyoprotectant type and concentration. The tested levels of Lira amount were observed to have a non-statistically significant effect on the particle size. In the case of EE, the lyoprotectant %, 2_{nd} sonication time and lyoprotectant type were the most highly risky factors. This is followed by other factors which all showed a significant impact on the amount of Lira encapsulated in the PLGA NPs. However, results revealed that only Lira amount and W2/O ratio were critical in terms of PDI and zeta potential.

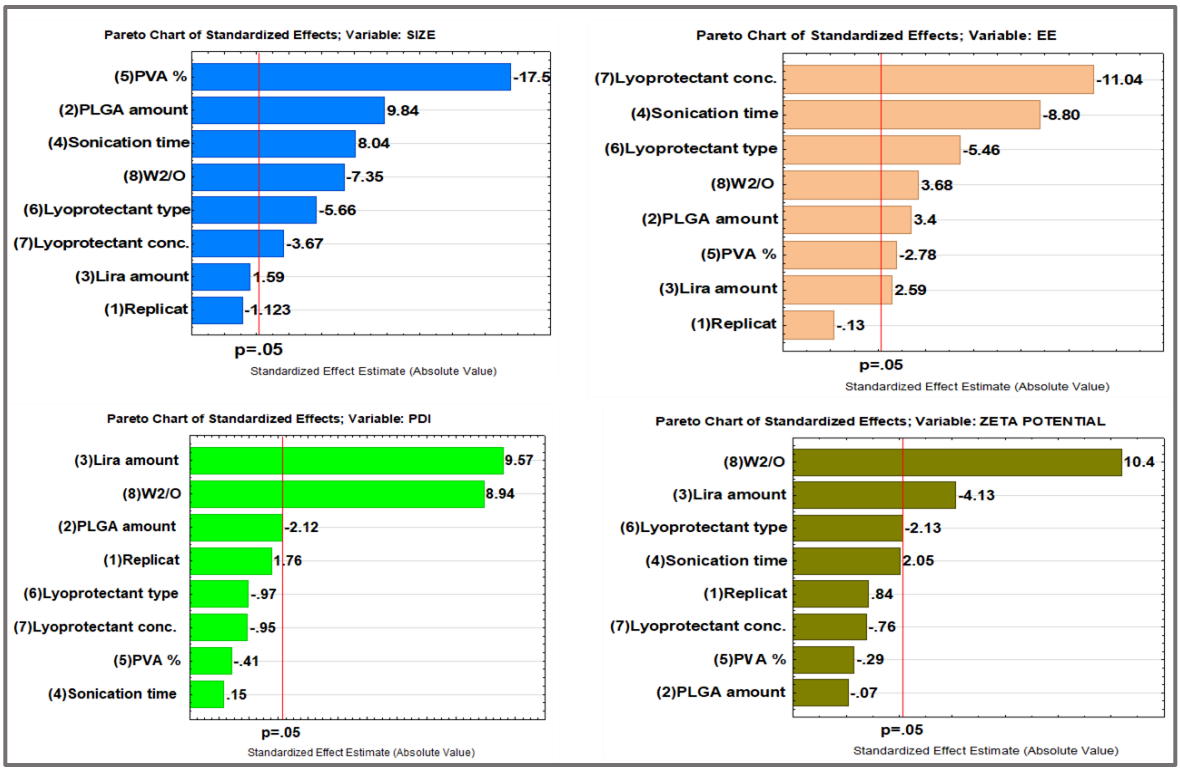


Figure 9. Pareto charts of the effects of the examined independent variables on particle size (Y1), PDI (Y2), EE (Y3), zeta potential (Y4) of Liraglutide loaded PLGA nanoparticles.

Aiming to validate the generated models and estimate the experimental error, three replications of center checkpoint formulations (F1, F2 and F3) were prepared and evaluated regarding the four responses. The minor differences between the predicted values and the average of experimental values confirm the validity of this design in providing a good prediction of the four tested responses (Table 4).

Table 4. The observed and the predicted values of the response values of the optimized Liraglutide loaded PLGA nanoparticles. Data are presented as mean ± SD (n=3).

Experimental response	Predicted value	Observed value (F1) (Bias%)	Observed value (F2) (Bias%)	Observed value (F3) (Bias%)
Particle size (nm)	197.8	195.8±2.5 (1.0)	202.72±5.8 (2.5)	196.41±4.06 (0.7)
EE (%)	30.5	29.46±1.83(3.3)	31.18±2.32 (2.3)	33.14±3.32 (8.5)
PDI	0.2	0.19±0.01 (5.5)	0.19±0.01(5.0)	0.18±0.003 (10.0)
Zeta potential (mV)	-28.1	-28.11±1.06 (0)	-28.84±2.51 (2.5)	-27.7±1.33 (1.4)

The DS was optimized targeting the following criteria: the particle size was minimized; EE was maximized while PDI and Zeta potential were excluded. Thanks to the knowledge obtained via the DS, the optimum levels of the formulation factors were determined: 60 mg of PLGA, 5 mg of Lira, 0.5 min 2_{nd} sonication time, 1.48% of PVA, 5% of mannitol and W2/O ratio of 5. As presented in Table 5, the observed values were comparable to the predicted ones, presenting another confirmation of the validity of the generated models and indicating that the optimized formulation is reliable. The optimized formula showed a sufficient stability after one week regarding particles size, PDI and zeta potential as shown in Table 6.

Table 5. The observed and the predicted values of the response values of the optimized Liraglutide loaded PLGA NPs. Data are presented as mean ± SD (n=3).

Experimental response	Predicted value	Observed value (Bias%)
Particle size (nm)	197.9	188.95±4.99 (4.5%)
EE%	48.31	51.81±2.39 (7.2%)
PDI	0.2	0.19±0.01 (7.8%)
Zeta potential (mV)	-26.5	-27.12±1.33 (2.2%)

Table 6. Particle size, polydispersity index (PDI), zeta potential of optimized Liraglutide loaded PLGA NPs in water after one week. Data are presented as mean ± SD (n=3).

Experimental response	Values
Particle size (nm)	193.17±6.51
PDI	0.21±0.03
Zeta potential (mV)	-27.83±1.49

5.2.2. Drug release study

The release behavior data presented in Figure 10A shows a biphasic release pattern starting by a moderate initial burst release during the first 2 hours in SGFsp, where 14.2±0.86% of Lira was released from the NPs. This was followed by a slow release profile until 6 hours in the SIFsp where only 18.5±2.39% of cumulative Lira release was reached.

At the burst release phase, PLGA NPs are exposed to the gastric simulated conditions and the surface of the NPs is hydrated. Then, non-capsulated Lira, or Lira existing close to the surface

having weak interactions with it is easily accessible by hydration and is released in the medium. At the second phase of slow Lira release, the degradation of the polymer matrix takes place, leading to diffusion of the encapsulated Lira. These findings prove that the PLGA nanosystem could hinder the release of encapsulated peptide in the simulated gastric medium, and later sustain the peptide release in the simulated intestinal medium.

5.2.3. Enzymatic degradation study

It is obvious that only 1.9±0.46% and 9.2±0.7% of the free Lira was recovered after 30 min incubation in SGF and SIF, respectively. Lira was completely degraded after incubation for 1 hour in SIF while only 5.7±0.53% was recovered after 2-hour incubation with SGF (Figure 10B). On the other hand, the encapsulation of Lira into PLGA NPs was able to successfully protect 71.2±1.49% and 87.6±1.3% of Lira from degradation in the SGF and SIF at the end of 2-hour incubation, respectively (Figure 10B). These results depict that PLGA NPs can provide a physical barrier between the encapsulated Lira and the harsh environment of GI tract thereby they could be promising for obtaining higher oral peptide bioavailability.

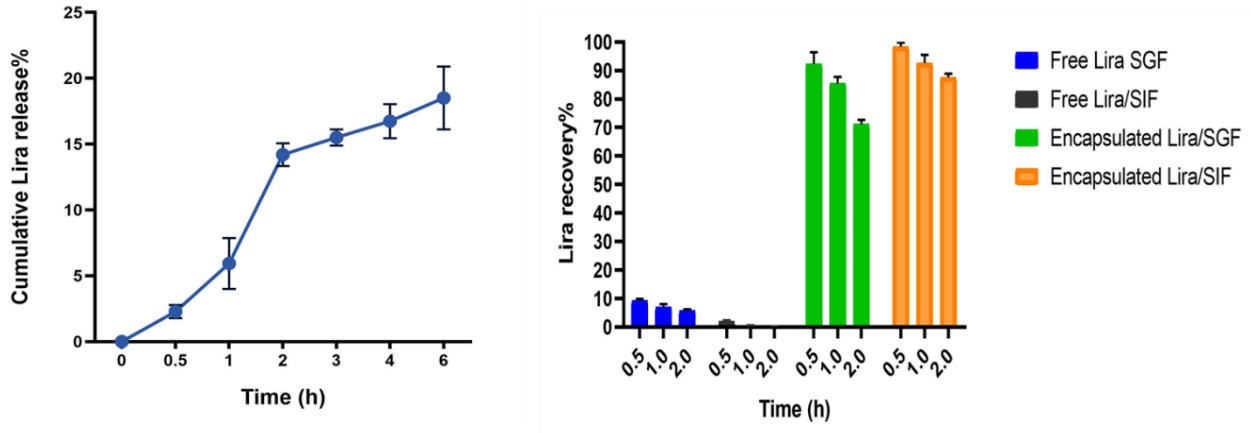


Figure 10. **(A)** Cumulative in vitro release profile of liraglutide (Lira) from PLGA NPs. **(B)** Enzymatic stability of Lira encapsulated in PLGA NPs in SGF and SIF media, with free Lira as a control. SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid.

5.2.4. Cell viability measurement by impedance

As illustrated in Figure 11, the impedance measurement did not exhibit any significant cell damage after treatments with Lira, Lira loaded in PLGA NPs, Lira with PN159 peptide, unloaded

PLGA NPs or PN159 peptide as reflected by the cell index values. These findings proved the biocompatibility of the PLGA NPs and showed that the composition of the nanosystem did not contribute to toxicity in Caco-2 cells. Figure 11A shows the kinetics of the cellular effects of treatment solutions, while the columns on Figure 11B show the effect of treatments at the 1-hour time point.

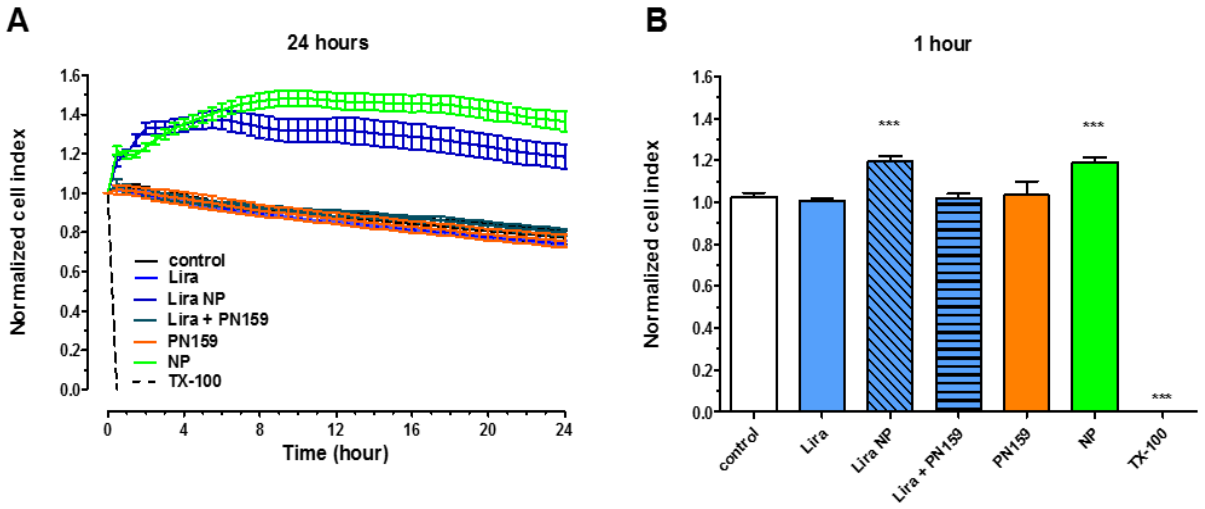


Figure 11. (A) Cell viability kinetics for 24 hours and **(B)** results of a 1-hour treatment of Caco-2 intestinal epithelial cells with liraglutide (Lira), Lira in PLGA NPs, Lira with PN159 peptide, blank PLGA NPs and PN159 peptide measured by impedance. Values are presented as means ± SD, n = 6–12. Statistical analysis: Analysis of Variance (ANOVA) followed by Dunnett's test. NPs, nanoparticles; TX-100, Triton X-100. ***p<0.001 compared to control.

5.2.5. Permeability study on the Caco-2 cell model

Free Lira solution at a donor concentration of 100 µM showed a good apparent permeability (P_{app}) of 16×10^{-6} cm/s through the Caco-2 cell model compared to what was reported for the native GLP-1 or Exn (Gupta, Doshi and Mitragotri, 2013) (Figure 12A), which could be due to the 16-carbon fatty acid chain that is attached to lysine at position 26 via a glutamic acid spacer (Knudsen and Lau, 2019). However, Lira encapsulated in PLGA NPs showed a 1.5-fold higher apparent permeability as compared to Lira alone (Figure 12A). Since PLGA NPs are more lipophilic compared to the free peptide drug, their transport across the lipid membrane of Caco-2 cells is better. Furthermore, these NPs showed a particle size of less than 200 nm which could

further enhance the cellular uptake compared to larger-sized (≥ 500 nm) particles (Lanza, Langer and Vacanti, 2013).

In the presence of PN159 peptide, our reference absorption enhancer, Lira permeability also increased (Figure 12A). This finding is consistent with previous results on the reversible tight junction opening and cell membrane permeability enhancing effects of the PN159 peptide through intestinal epithelial cells, which effectively improve the permeability of different drugs and hydrophilic marker molecules through the paracellular pathway (Cu,Kunyuan,Chen,iShu-Chih, Houston,Michael, Quay, 2006; Bocsik *et al.*, 2019). There was no statistical difference between Lira loaded PLGA NPs and Lira + PN159 groups in enhancing the permeability across Caco-2 cells. On the contrary of group containing PN159 peptide where the TEER values dropped after the 1-hour treatment (Figure 12A) (Cu,Kunyuan,Chen,iShu-Chih, Houston,Michael, Quay, 2006; Bocsik *et al.*, 2019), Lira alone or loaded in PLGA NPs did not change the ionic permeability (Figure 12B), suggesting no toxic effect on differentiated Caco-2 cells in agreement with the viability data (Figure 12) and no effect on the paracellular pathway.

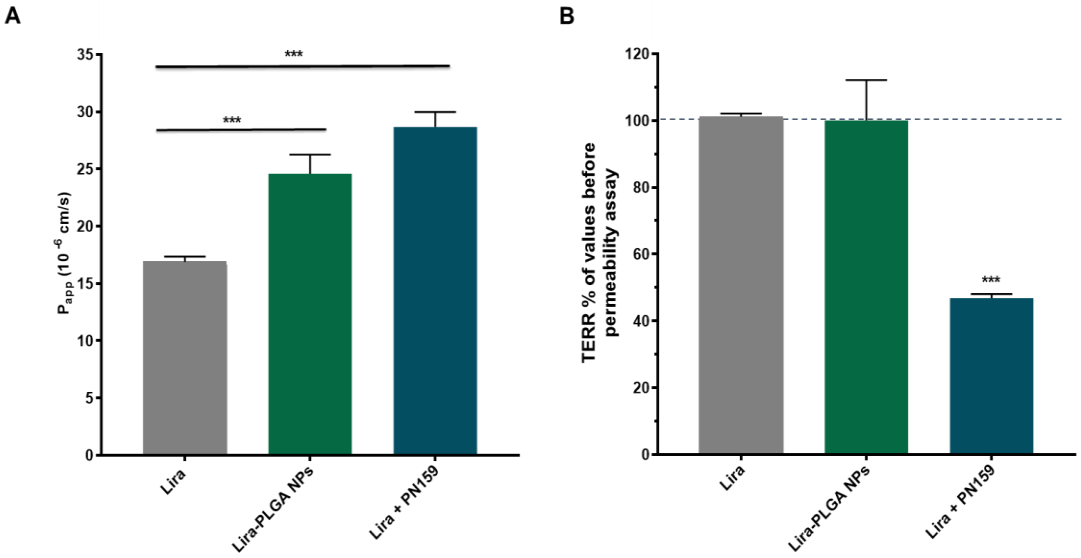


Figure 12. (A) Evaluation of permeability of liraglutide (Lira) (100 µM) across Caco-2 epithelial cell layers treated with different Lira formulations for 1 h. **(B)** Changes in transepithelial electrical resistance (TEER) values of Caco-2 cell layers after 1-hour treatment with different Lira formulations as compared to TEER values before treatment. Data are presented as means ± SD (n = 4). Statistical analysis: ANOVA followed by Bonferroni test, ***p<0.001 compared to Lira group.

There was a good recovery for Lira after the permeability experiments and no significant differences between the recovery values of the different investigated Lira groups were found (Table 7).

Table 7. Recovery (mass balance) calculation after liraglutide (Lira) permeability across Caco-2 cells. Data are presented as mean ± SD (n=4).

Group	Recovery (%)
Lira	80.9 ± 1.6
Lira-PLGA NPs	75.3 ± 2.3
Lira + PN159	81.3 ± 6.9

5.2.6. Immunohistochemistry

The cells were tightly apposed, and the junctional proteins were localized at the intercellular connections forming pericellular belts both in the control and the treated groups. No morphological changes of interepithelial junctions were observed with Lira-PLGA NPs treated group confirming the lack of a paracellular component in the transport mechanism. Only the PN159 peptide treated group demonstrated a visible change in the staining pattern of β-catenin adherens junctional protein which is also in accordance with the previous results confirming the tight junction opening effect of PN159 (Bocsik *et al.*, 2016) (Figure 13).

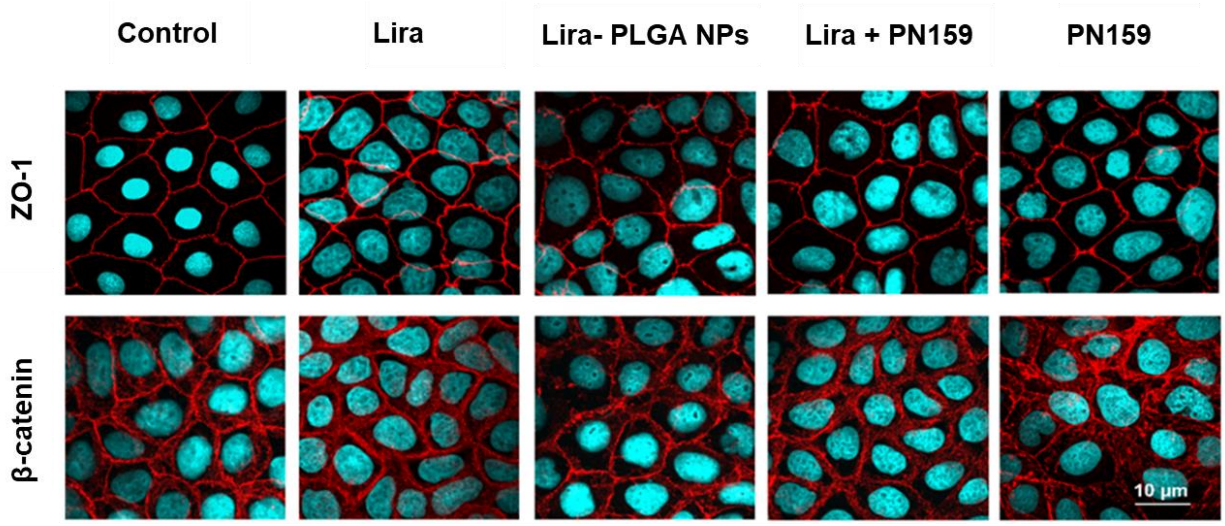


Figure 13. Effects of Lira, Lira-PLGA NPs, Lira and PN159 peptide, and PN159 peptide on the junctional morphology of Caco-2 epithelial cells. Immunostaining for zonula occludens-1 (ZO-1), and β-catenin junction proteins after a 1-hour treatment. Red color: immunostaining for junctional proteins. Blue color: staining of cell nuclei. Bar: 10 µm.

5.3.Exenatide loaded SEDDS

5.3.1. Optimization of hydrophobic ion-pairing (HIP) of exenatide with (THA)

The cationic surfactant THA and the anionic surfactant DOC were capable of forming HIPs with the anionic and cationic amino acids of Exn via non-covalent ionic interactions (Figure 14). The addition of THA or DOC to the Exn solution caused an immediate precipitation indicating the formation of HIPs.

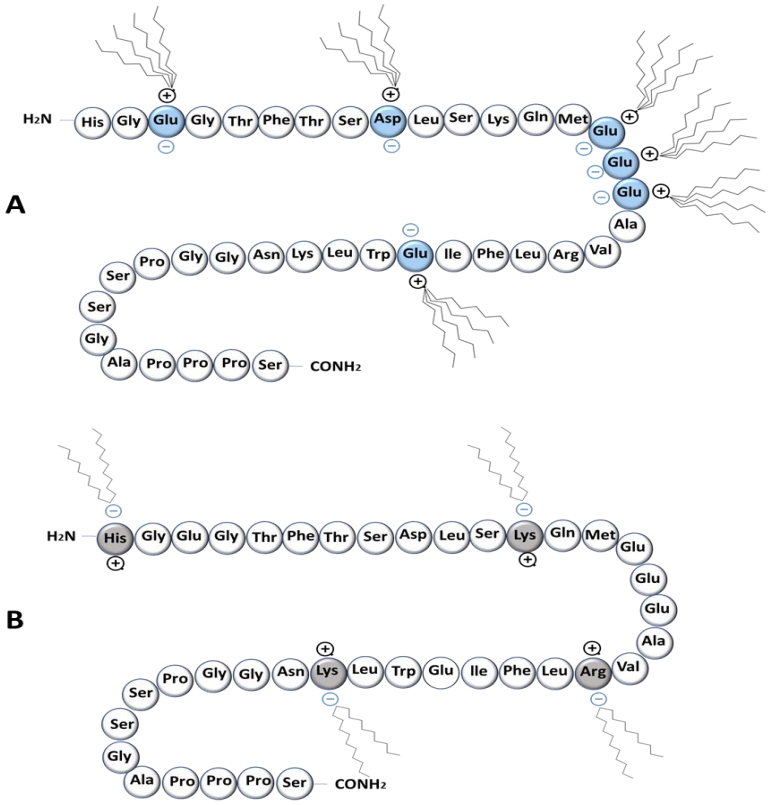


Figure 14. Graphical illustration of the chemical structure of **(A)** exenatide-tetraheptylammonium bromide (THA) ion pairs and **(B)** exenatide-docusate (DOC) ion pairs.

Different Exn to surfactant ratios were evaluated as illustrated in Figure 15. The more surfactant was added to Exn, the more HIPs were formed until the maximum precipitation efficiency of 95.3 ± 4.02% at a molar ratio of 1:8 (Exn: THA), whereas a maximum of 100% was reached in the case of DOC at a molar ratio of 1:4 (Exn: DOC). Higher concentrations of surfactant led to a lower amount of formed ion pairs. This observation was previously reported for other surfactants (Griesser *et al.*, 2017) and could be explained by the formation of micelles that re-dissolve the ion pair complex (Dai and Dong, 2007).

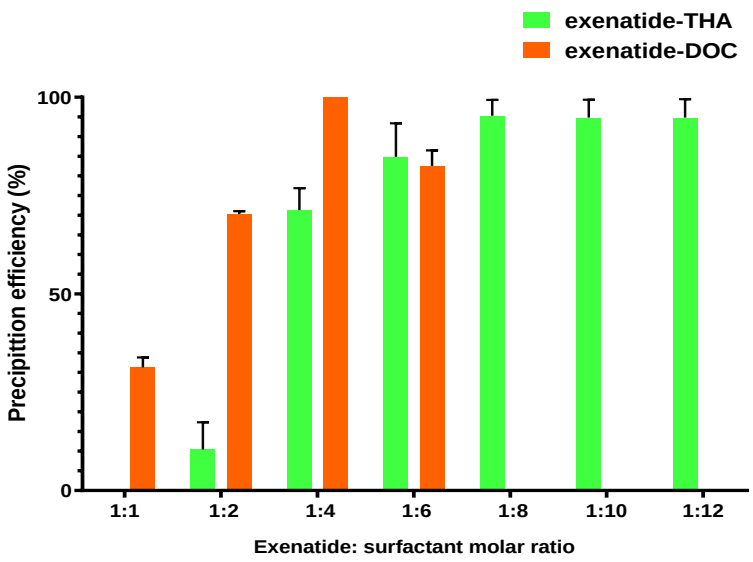


Figure 15. Precipitation efficiency of exenatide (2 mg/mL) with THA and DOC at different molar ratios. The precipitated exenatide-surfactant ion pairs were centrifuged and the remaining amount of exenatide in supernatant was quantified by HPLC. Data are presented as mean ± SD (n = 3).

The zeta potential of HIPs having been formed at different molar ratios of Exn to surfactant was determined in order to evaluate the surface charge of the complexes. Figure 16A shows that the negative charge of Exn at pH 8.0 decreased with the increase in THA having been bound to the peptide drug reaching the lowest negative zeta potential at a molar ratio of 1:10 (Exn: THA). At a higher molar ratio, the THA level could exceed the critical micellar concentration (CMC) and the resulting formation of micelles can explain the increase in negative zeta potential due to the solubilizing effect of the excess of THA. The formation of these micelles could cause reduction in the amount of THA being bound to the surface of exenatide causing a higher negative charge. In case of Exn -DOC, a shift in zeta potential toward negative values was observed where the positive charge of exenatide at pH 3.0 decreased with higher molar ratio (Exn:DOC) reaching almost 0 mV at a molar ratio of 1:4 (Figure 16B).

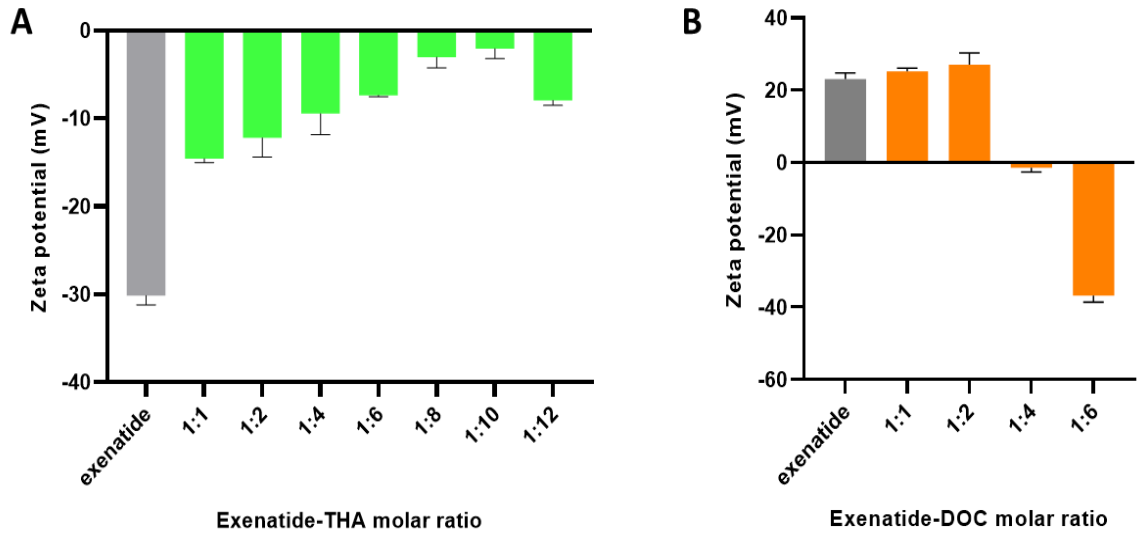


Figure 16. The shift in zeta potential values of **(A)** exenatide-THA HIPs formed at pH 8.0 in different molar ratios, and **(B)** exenatide-DOC HIPs formed at pH 3.0 in different molar ratios. Data are presented as mean ± SD (n = 3).

5.3.2. Characterization of exenatide-THA and exenatide-DOC loaded SEDDS

5.3.2.1. Particle size, size distribution, surface charge and payload measurements

Small droplet size (< 30 nm) with low PDI value was determined for loaded SEDDS (Table 8). The surface charge of Exn-THA loaded SEDDS was positive whereas negative values were measured in case of Exn-DOC loaded SEDDS. This observation can be explained by the cationic character of THA and the anionic character of DOC. Loaded SEDDS showed sufficient stability for one week regarding the droplet size, PDI and zeta potential as shown in Table 8. The maximum payload of Exn-THA and Exn-DOC that could be dissolved in the pre-concentrate was 0.54% and 0.17%, respectively. This payload was used to prepare SEDDS for further evaluation.

Table 8. Droplet size, polydispersity index (PDI) and zeta potential of reconstituted exenatide-THA SEDDS and exenatide-DOC SEDDS in water over one week. Data are presented as mean ± SD (n=3).

	Droplet Size (nm)	**PDI**	**Zeta potential (mV)**
Exenatide-THA NCs	25.71±2.48	0.24±0.48	10.11±1.07
Exenatide-DOC NCs	24.55±3.2	0.25±0.046	-20±2.2
		4 h	
	Droplet Size (nm)	**PDI**	**Zeta potential (mV)**
Exenatide-THA NCs	23.64±2.75	0.05±0.03	5.5±0.85
Exenatide-DOC NCs	25.17±5.22	0.2±0.07	-12.67±2.08
		1 week	
	Droplet Size (nm)	**PDI**	**Zeta potential (mV)**
Exenatide-THA NCs	22.49±4.55	0.081±0.11	9.24±3.42
Exenatide-DOC NCs	20.2±0.63	0.11±0.05	-9.37±0.15

5.3.2.2. Determination of log D $_{\text{SEDDS/release medium}}$ of exenatide-THA and exenatide-DOC

It was previously reported that the drug release from SEDDS formulation depends on a simple diffusion process from a lipophilic liquid phase into an aqueous liquid phase that occurs within a few seconds (Bernkop-Schnürch and Jalil, 2018). As the released HIPs are absorbed from the membrane, more HIPs will diffuse out of SEDDS until equilibrium is reached again. Since drug release is controlled by the partition coefficient of HIPs between the lipophilic phase (SEDDS) and the release medium (RM), log $D_{SEDDS/RM}$ was determined in two different media to assess affinity of Exn-THA and Exn-DOC ion pairs toward the oily droplets of SEDDS (Bernkop-Schnürch and Jalil, 2018; Shahzadi *et al.*, 2018). The log $D_{SEDDS/RM}$ of Exn-THA was 2.29 and 1.92, whereas the log $D_{SEDDS/RM}$ of Exn-DOC was 1.2 and -0.9 in simulated intestinal fluid and HBSS, respectively. These log D values proved that THA is capable of forming more

hydrophobic complexes than DOC. Accordingly, the amount of Exn immediately released from Exn-DOC SEDDS is likely higher than that from Exn-THA SEDDS.

5.3.2.3. Cytocompatibility

SEDDS loaded with different concentrations of either Exn-THA or Exn-DOC were tested regarding their hemolytic activity and compared to blank SEDDS. Figure 17 displays the hemolysis caused by increasing concentrations of blank and loaded SEDDS showing that this toxicity is concentration dependent. Both blank and loaded SEDDS displayed no significant hemolytic activity at concentrations of 0.1% (m/v) and 0.25% (m/v). Compared to SEDDS loaded with Exn-THA, SEDDS loaded with Exn-DOC showed higher toxicity as concluded from the hemolytic activity reaching more than 90% at a concentration of 0.5% (m/v) of this formulation.

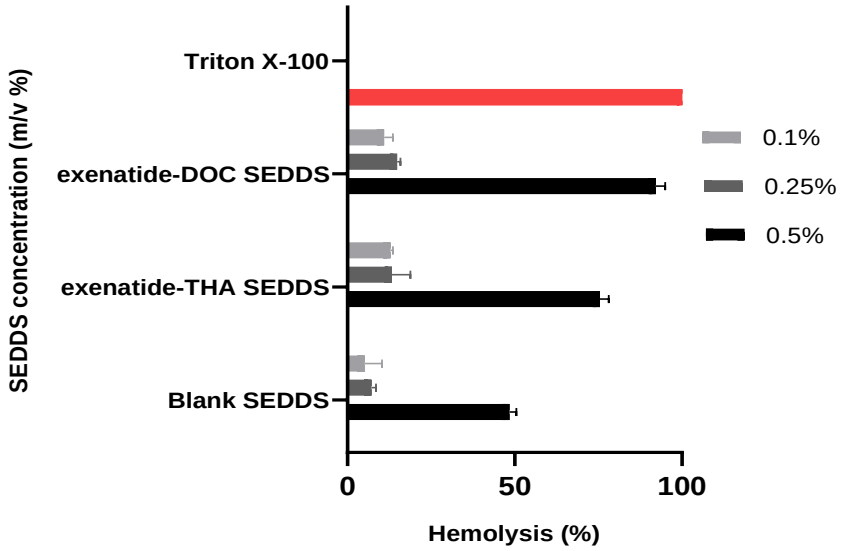


Figure 17. Cytotoxicity of different concentrations of blank SEDDS, exenatide-THA SEDDS, exenatide-DOC SEDDS. Hemolysis assay was conducted with human red blood cells (RBC). Data are presented as mean ± SD (n = 3).

5.3.2.4. Ex-vivo permeability study

Results presented in Figure 18 clearly demonstrate that Exn-DOC SEDDS and Exn-THA SEDDS yielded a 3-fold and a 10-fold enhancement of intestinal membrane permeability compared to free Exn. The lipophilic nature of SEDDS and their slippery surface in addition to a nanodroplet size of less than 30 nm are advantageous to facilitate their permeation through the intestinal mucus gel layer and likely also across the absorption membrane. The composition

of SEDDS could additionally contribute to an enhanced intestinal uptake of Exn. The main component of SEDDS having been tested in this study for instance is Capmul®MCM that was shown in previous studies to open tight junctions and to enhance permeation via the paracellular pathway (Holmes *et al.*, 2013; Keemink and Bergström, 2018).

Since Exn has six anionic substructures while only four cationic ones, the lipophilicity of HIPs with the cationic counter ion THA was assumed to be higher compared to those formed with the anionic surfactant DOC. This assumption could be confirmed by the higher solubility of Exn-THA than Exn-DOC complexes in the organic phase. Furthermore, the higher potential of Exn-THA SEDDS in their membrane permeation behaviour ($p < 0.05$) is in agreement with the higher lipophilic character of this complex. The positive zeta potential of Exn-THA SEDDS in comparison to the negatively charged Exn-DOC SEDDS might also contribute to their higher membrane permeating properties. This outcome provides evidence for the crucial role of the type of HIPs incorporated in SEDDS in enhancing permeability across the intestinal mucus gel layer and epithelial barrier strongly limiting oral peptide uptake.

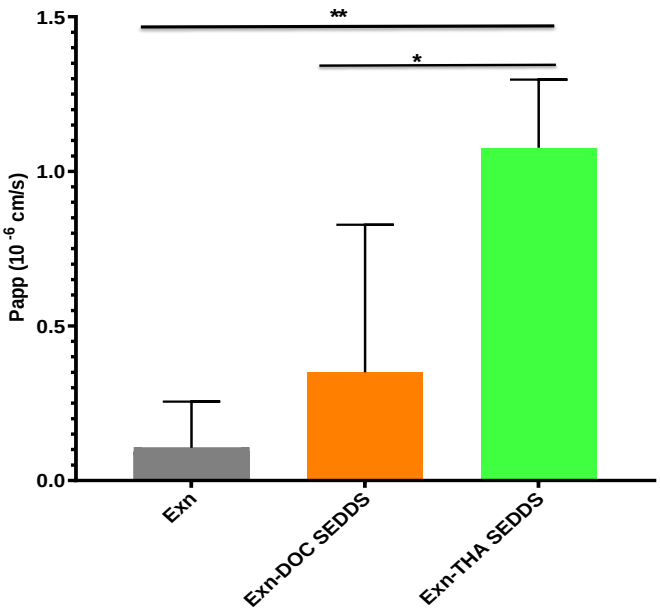


Figure 18. Evaluation of ex vivo permeability of exenatide (Exn) across rat intestinal mucosa following the 3-hour treatment with free Exn solution, Exn-THA SEDDS or Exn-DOC SEDDS. Data are presented as means ± SD (n = 3). Statistical analysis: Analysis of Variance (ANOVA) followed by Bonferroni test, * $p < 0.05$, ** $P < 0.01$.

5.3.2.5. In-vivo study

As illustrated in Figure 19, SC injection of free Exn solution elicited a maximum serum level at 1 hour after administration being in good agreement with previous reported studies (Nguyen *et al.*, 2011). The orally administered free Exn solution could not provide any systemic uptake of the drug. In contrast, high Exn serum levels were observed after oral administration of Exn-THA and Exn-DOC SEDDS reaching maximum after 3 hours.

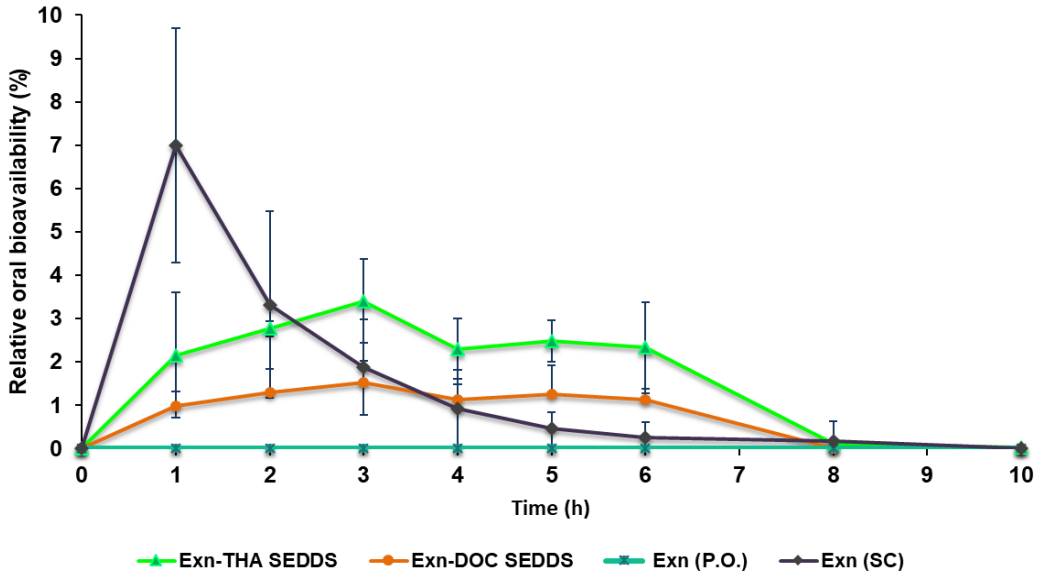


Figure 19. Exenatide (Exn) plasma concentration vs time profiles for SC free exenatide solution (50 µg/kg), oral exenatide solution (dose: 300 µg/kg), oral exenatide-THA SEDDS (dose: 300 µg/kg) and oral exenatide-DOC SEDDS (dose: 300 µg/kg). Data are presented as means ± SD (n = 5).

The developed SEDDS incorporating Exn-DOC resulted in a greater BA_R (16.29±5.34%) when administered to rats (Table 9), compared to the previously reported formulation (Menzel *et al.*, 2018). This could be due to the difference in the above-mentioned SEDDS compositions that were able to efficiently protect Exn from enzymatic digestion. Besides, using a low concentration of PG could contribute to avoiding the trigger effect of such hydrophilic co-solvent in solubilizing HIPs and releasing them out of the oily droplet (Phan, Shahzadi and Bernkop-Schnürch, 2019). Exn-THA SEDDS, however, showed an even greater potential reaching a BA_R of 27.96 ± 5.24% (Table 9), which is in consistence with ex-*vivo* results

confirming the superiority of this system compared to Exn-DOC SEDDS. These results also demonstrate the great impact of the type of counter ions used for HIP formation on the oral bioavailability of the peptide drug of interest. They are in good agreement with previous studies showing that the oral bioavailability of octreotide strongly depends on the type of counter ion used for HIP formation. SEDDS containing octreotide-deoxycholate led to a 17.9-fold higher oral bioavailability than octreotide-docusate HIPs (Bonengel *et al.*, 2018).

Our study revealed the potential of cationic surfactants in increasing the lipophilicity of Exn before being embedded into SEDDS, which has a great impact on drug release characteristics and intestinal permeability. The developed Exn-THA SEDDS system holds high promise for oral delivery exceeding the results elicited from preceding reported studies (Jin *et al.*, Nguyen *et al.*, 2011; Zhang *et al.*, 2014; Li *et al.*, 2015; Menzel *et al.*, 2018; Zhang, Shi, Song, Duan, *et al.*, 2018; Zhang, Shi, Song, Sun, *et al.*, 2018; Song *et al.*, 2019).

Table 9. Pharmacokinetic parameters of exenatide in rats following subcutaneous (SC) injection of the free exenatide solution and per oral (P.O.) administration of exenatide solution, exenatide-THA SEDDS and exenatide-DOC SEDDS. C_{max}: maximum serum concentration; T_{max}: time at which C_{max} is reached; AUC 0-10: area under the serum concentration-time curve over 10 h, BA_R: relative bioavailability (n = 5).

Formulation	**Free exenatide solution**	**Exenatide-THA SEDDS**	**Exenatide-DOC SEDDS**
Route of administration	SC	P.O.	P.O.
Dose (µg/Kg)	50	300	300
Cmax (ng/ml)	7.0±2.70	3.4±0.97	1.53±0.50
Tmax (h)	1	3	3
AUC 0-10 (ng h/ml)	10.56±3.92	17.33±3.48	10.28±4.7
BA_R (%)	100	27.96±5.24	16.29±6.63

6. SUMMARY

Since the oral delivery of GLP-1 analogues Lira and Exn can improve the patient adherence to therapy, a smart carrier system that can tackle the challenges hindering the oral delivery of these peptides has been targeted. In accordance with our research goals, polymeric and lipid-based nanocarriers were designed for the oral delivery of Liraglutide and Exenatide. The extended QbD model-based development of GLP-1 analogues loaded nanocarriers was successfully implemented to identify and rank the potentially high-risk attributes.

I. Following the literature evaluation and preliminary experimental work, the QTPP encompassing the desired quality attributes in the GLP-1 analogues loaded nanocarriers for oral use was set up. To illustrate the identified CMAs and CPPs related to the selected production methods of PLGA NPs and SEDDS, Ishikawa diagram was constructed. According to initial RA study performed using Lean QbD® Software, the interdependence rating between CQAs and CMAs, CQAs and CPPs was conducted on a three-level scale. The calculated and ranked severity scores of CQAs were presented based on R&D QbD.

II. In the second part, PLGA NPs encapsulating Lira were prepared by double emulsion solvent evaporation method.

- A seven-factor, two-level, eight-run Plackett-Burman DoE was a validated statistical tool to be applied for the optimization of PLGA NPs containing Lira. The optimized formulation was prepared within the defined Design Space. Lira loaded PLGA NPs with a homogeneous distribution, particle size of 188.95 nm and encapsulation efficiency of 51.81% which were within the desired range. The physical stability of this formulation was also proved over one week.
- The PLGA nanosystem could hinder the release of loaded Lira in stomach since less than 15% of Lira was released from NPs in the SGFsp followed by a slow release in the SIFsp which is good for absorption. The ability of PLGA NPs to protect the encapsulated Lira from hostile environment of GI tract was further confirmed by enzymatic stability studies.

- The viability of Caco-2 intestinal epithelial cells was assessed by measuring cell impedance over 24 h. No decrease in cell impedance kinetics after treatment with Lira loaded/free PLGA NPs during the long-time treatment was observed. These findings proved the biocompatibility of PLGA NPs and showed that the composition of the nanosystem did not contribute to toxicity in Caco-2 cells.
- Compared to free Lira, Lira encapsulated in PLGA NPs showed a 1.5-fold enhancement in intestinal apparent permeability which was comparable to Lira + PN159 group.
- The immunostaining for the junctional proteins, ZO-1 and β-catenin was performed. On the contrary of PN159 containing group, no morphological changes of interepithelial junctions ZO-1 and β-catenin were observed with Lira loaded PLGA NPs treated group confirming the lack of a paracellular component in the transport mechanism.

III. In the third part, HIPs of Exn with cationic/anionic surfactant were formed before being incorporated into SEDDS.

- Aiming to sufficiently reduce hydrophilicity of the peptide, hydrophobic ion pairing of Exn with THA (cationic surfactant) and DOC (anionic surfactant) was applied. The maximum ion-pairing efficiency was reached after optimizing the molar ratio (Exn: surfactant) and pH levels. The formation of HIPs with THA and DOC was further verified by a shift in zeta potential values the more surfactant was bound to Exn.
- SEDDS formulations were developed with a droplet size of less than 30 nm with a homogenous distribution, and the physical stability of SEDDS formulations was proved over one week. The maximum payload of exenatide-THA and exenatide-DOC that could be dissolved in the lipophilic phase of the SEEDS was 0.54% and 0.17%, respectively.
- According to calculated log $D_{SEDDS/RM}$ in SIF and HBSS, the amount of Exn immediately released from Exn-DOC SEDDS is likely higher than that from Exn-THA SEDDS, showing the superiority of the cationic

surfactant in protecting the peptide from the GI environment by forming more hydrophobic complexes.

- In vitro toxicity studies with human red blood cells revealed that the toxicity is concentration dependent, and both blank and loaded SEDDS displayed no significant hemolytic activity at a concentration of 0.25% (m/v).
- Compared to free Exn solution, Exn-DOC SEDDS and Exn-THA SEDDS exhibited a 3-fold and 10-fold increment in intestinal membrane permeability.
- Orally administered Exn-THA and Exn-DOC SEDDS resulted in a relative bioavailability of 27.96 ± 5.24% and 16.29 ± 6.63%, respectively, confirming the comparatively higher potential of the cationic surfactant over the anionic surfactant.

New findings/Practical relevance of the work:

- This is the first practical evidence of the theoretical extended version of QbD model for R&D which was successfully applied to identify the critical material attributes and process parameters that highly affect the quality of the final target nanosystem designed for oral GLP-1 analogue delivery.
- The novel biocompatible PLGA nanoparticles could successfully protect the encapsulated Lira from the enzymatic degradation in simulated GI environment on top of significantly enhancing the permeability across the Caco-2 cell intestinal model.
- Hydrophobic ion pairing of Exn with a cationic surfactant is investigated for the first time, and results proved the superiority of cationic surfactant THA over the model anionic surfactant DOC regarding the tested quality attributes.
- The novel biocompatible Exn-THA SEDDS hold high potential for oral delivery exceeding the relative bioavailability results obtained from preceding studies.
- Polymeric and lipid based NCs aiming to deliver Lira and Exn orally were successfully designed with characteristics complying with the predefined quality target product profile. The developed NCs could offer novel possibilities for oral GLP-1 analogues delivery to ensure effective management of T2DM.

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ACKNOWLEDGEMENTS

Undertaking this PhD has been a truly life-changing experience for me, and it would not have been possible to do without the support and guidance that I received from many people.

Foremost, I would like to express my sincere gratitude to Assoc. Prof. **Ildikó Csóka** my supervisor, and the head of the Institute of Pharmaceutical Technology and Regulatory Affairs for the continuous support, motivation and valuable guidance throughout my Ph.D. study.

I am pleased to acknowledge to Prof. Dr. **Tamás Martinek** my co-supervisor, and the head of the Institute of Medical Chemistry for his great support.

I would like to extend my gratitude to Prof. Dr. **Piroska Szabó-Révész** and Dr. **Rita Ambrus** for being very kind and supportive from the beginning of my Ph.D. journey.

A special thanks to Prof. Dr. **Andreas Bernkop-Schnürch**, the head of the Faculty of pharmacy at Innsbruck University- who provided me an opportunity to join their research group as fellow and gave me the access to the laboratory and research facilities.

My deepest appreciation goes to **Erika Boda** and **Piroska Fekete** for their excellent technical assistance.

I am grateful to the **co-authors** for cooperation and their invaluable help that enabled this research to be possible.

Last but not the least, I would like to express my very profound gratitude to my beloved **parents**, my lovely **sisters** and my **brother in law** for supporting me spiritually to accomplish my personal goals. This accomplishment would not have been possible without their love, patience and encouragement!

FINANCIAL SUPPORT

This research was supported by Tempus Public Foundation- Stipendium Hungaricum Scholarship program 2016-2020.

ANNEX
RELATED PUBLICATIONS

PUBLICATION I.

pharmaceutics

MDPI

Article

Encapsulation in Polymeric Nanoparticles Enhances the Enzymatic Stability and the Permeability of the GLP-1 Analog, Liraglutide, Across a Culture Model of Intestinal Permeability

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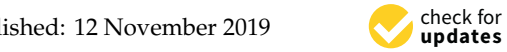
Received: 16 October 2019; Accepted: 11 November 2019; Published: 12 November 2019



Abstract: The potential of poly (lactic-*co*-glycolic acid) nanoparticles (PLGA NPs) to overcome the intestinal barrier that limits oral liraglutide delivery was evaluated. Liraglutide-loaded PLGA NPs were prepared by the double emulsion solvent evaporation method. In vitro release kinetics and enzymatic degradation studies were conducted, mimicking the gastrointestinal environment. The permeability of liraglutide solution, liraglutide-loaded PLGA NPs, and liraglutide in the presence of the absorption enhancer PN159 peptide was tested on the Caco-2 cell model. Liraglutide release from PLGA NPs showed a biphasic release pattern with a burst effect of less than 15%. The PLGA nanosystem protected the encapsulated liraglutide from the conditions simulating the gastric environment. The permeability of liraglutide encapsulated in PLGA NPs was 1.5-fold higher (24×10^{-6} cm/s) across Caco-2 cells as compared to liraglutide solution. PLGA NPs were as effective at elevating liraglutide penetration as the tight junction-opening PN159 peptide. No morphological changes were seen in the intercellular junctions of Caco-2 cells after treatment with liraglutide-PLGA NPs, confirming the lack of a paracellular component in the transport mechanism. PLGA NPs, by protecting liraglutide from enzyme degradation and enhancing its permeability across intestinal epithelium, hold great potential as carriers for oral GLP-1 analog delivery.

Keywords: liraglutide; GLP-1 analog; oral peptide delivery; enzymatic barrier; intestinal permeability; PLGA nanoparticles; Caco-2 cells

1. Introduction

The worldwide prevalence of type 2 diabetes mellitus (T2DM) has been increasing dramatically and has become a serious issue at an alarming rate. Because the incretin effect has been proven to be severely reduced or lost in relatively lean type 2 diabetic patients, incretin-based therapy, especially glucagon-like peptide 1 (GLP-1) receptor agonists, is now widely investigated for T2DM [1]. Long acting GLP-1 analogs have been developed to overcome the clinical limitations of the native GLP-1 due to its short circulating half-life [2,3].

Liraglutide (Lira; MW: 3751.2 Da) is an acylated derivative of GLP-1 that shares 97% homology to the native GLP-1, with two modifications: an Arg34Lys substitution, and a fatty acid side chain

(16-carbon palmitate) attached to Lys26 via a glutamic acid linker [4,5]. Lira retains the physiological activities of GLP-1 with a considerably longer half-life (approximately 13 h) that supports once-daily dosing. Subcutaneous Lira has been EMA and FDA approved for T2DM treatment, and soon after was approved for chronic weight management [6]. As Lira is limited to administration parenterally, the development of a patient-friendly delivery should be aimed for. Herein, oral administration is the most attractive choice as this route likely mimics physiological GLP-1 secretion in addition to ensuring good patient adherence to the treatment [7,8]. Moreover, oral delivery appears to be feasible for Lira due to the relatively large safety window of GLP1 analogs compared to insulin [9]. However, the oral delivery of Lira is still challenging due to low stability along the gastrointestinal (GI) tract and poor intestinal permeability that result in low oral bioavailability [10].

The encapsulation of peptides into nanocarrier systems, especially polymeric NPs, has arisen as a very promising alternative carrier system that has greater stability in biological fluids and during storage when compared to lipid-based nanosystems [11]. Polymeric NPs can not only protect the encapsulated peptide from the harsh environment in the GI tract but also control drug release and enhance its intracellular uptake [12,13]. Poly (lactic-*co*-glycolic acid) (PLGA) nanoparticles showed potential results as nanocarriers designed for the oral delivery of insulin and exenatide [14–17].

To enhance the delivery of protein or peptide molecules across biological barriers, several strategies can be used. One of them is the opening of intercellular tight junctions [18]. Another possibility is the use of cell penetrating peptides (CPPs). CPPs comprise a family of functional carrier peptides consisting of 5–30 amino acid residues that have been reported to have great potential in enhancing the peptide drugs permeability across the intestinal epithelium. Amphipathic CPPs, such as penetratin, are among the most widely promising ones. Many recent studies proved that the non-covalent intermolecular interaction between penetratin and insulin could be clinically promising as an absorption enhancer for successful oral insulin delivery [19–22]. Our group has recently demonstrated, that a permeability enhancing 18-mer amphiphilic peptide, PN159, also known as KLAL or MAP, has a dual action: by acting on claudin transmembrane tight junction proteins it opens the paracellular route in both epithelial and blood–brain barrier models [23] and at the same time it has cell permeabilizing and penetrating properties [24].

As realizing the dream of administering antidiabetic peptides such as liraglutide orally is still an elusive goal, we reported in our previous paper the potential of implementing the quality by design methodology from the early stage of product formulation, especially when dealing with complex nanosystems such as polymeric NPs designed for peptide delivery. We have successfully optimized the formulation of Lira encapsulated in hydrophobic PLGA NPs. For the present work, the purpose was to evaluate the effectiveness of our previously developed PLGA NPs in protecting the encapsulated peptide from the harsh environment in the GI tract. As there is no available literature regarding the intestinal permeability of Lira solution, we also evaluated the permeability of Lira through the Caco-2 cell model. Moreover, we investigated the potential of the optimized PLGA NPs in enhancing the permeability of encapsulated Lira through the Caco-2 cell model and compared it with the permeability enhancer PN159 peptide.

2. Materials and Methods

2.1. Materials

Liraglutide was purchased from Xi'an Health Biochem Technology Co., Ltd. (Shaanxi, China), Poly(lactide-*co*-glycolide) (PLGA 50:50, Mw = 30,000–60,000 Da), and PVA (MOWIOL 4-98, Mw ~ 27,000 Da) were purchased from Sigma Aldrich (Munich, Germany). D-mannitol was purchased from Molar Chemicals Ltd. (Budapest, Hungary). Sodium acetate anhydrous was purchased from Scharlau Chemie S.A. (Barcelona, Spain). Ethyl acetate was from REANAL Labor (Budapest, Hungary). Pepsin from porcine gastric mucosa, powder (≥400 units/mg protein) and pancreatin from porcine

pancreas (≥3× USP specifications) were purchased from Sigma Aldrich (Budapest, Hungary). All other chemicals in the study were of analytical reagent grade.

2.2. Human Caco-2 Intestinal Epithelial Cell Line

The Caco-2 intestinal epithelial cell line was purchased from ATCC (cat.no. HTB-37) at passage 60. The cells were grown, as previously reported [24], in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 50 μg/mL gentamycin in a humidified incubator with 5% CO_2 at 37 °C. All plastic surfaces were coated with 0.05% rat tail collagen in sterile distilled water before cell seeding in culture dishes and the medium was changed every 2 days.

2.3. Preparation of PLGA NPs

The Lira loaded PLGA NPs were prepared by the double emulsion solvent evaporation method and then lyophilized as described previously [25]. Following the initial risk assessment-based study [26], the Plackett–Burman screening design of the experiment was applied by our research group to optimize the lyophilized Lira loaded PLGA NP formulation regarding four critical quality attributes namely: particle size, polydispersity index, encapsulation efficiency and zeta potential [25]. The optimized formula is shown in Table 1.

Table 1. Optimized Lira loaded PLGA NPs; where PVA is the polyvinyl alcohol-stabilizer, and W2/O is the outer aqueous phase to organic phase ratio.

Formulation Parameters	
PLGA amount	60 mg
Lira amount	5 mg
2nd sonication time	0.5 min
PVA amount	1.48%
Lyoprotectant type	Mannitol
Lyoprotectant amount	5%
W2/O ratio	5

2.4. Preparation of Lira and PN159 Solutions

PN159 peptide (NH2-KLALKLALKALKAALKLA-amide) was previously synthesized and purified as reported by our research group [23,24]. To prepare Lira and PN159 solutions, liraglutide was dissolved in cell culture medium or Ringer-buffer (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, pH 7.4) in a plastic vial. PN159 was weighed in a plastic vial and dissolved in Ringer buffer as well. Equal volumes of Lira and PN159 solutions were gently mixed at room temperature. The final concentrations of liraglutide and PN159 were 100 μM and 3 μM, respectively, in all experiments.

2.5. In Vitro Drug Release Study

The in vitro release behavior of Lira from the prepared PLGA nanoparticles was assessed by dispersing Lira-loaded PLGA NPs (corresponding to 500 μg of Lira) in 10 mL of simulated gastric fluid without enzymes (SGFsp: 0.1 N HCl at pH 1.2). After 2 h, the NPs were centrifuged and transferred to simulated intestinal fluid without enzymes (SIFsp: phosphate buffer saline at pH 7.4).

The beaker was placed over a magnetic stirrer (100 rpm) and the temperature was kept at 37 ± 1 °C throughout the experiment. At specified time points (0, 0.5, 1, 2, 3, 4, 6 h), an aliquot of 500 μL was withdrawn from the release medium and replenished with the same volume of fresh preheated medium. Samples were centrifuged at 16,500× *g* and 4 °C for 10 min, and the Lira concentrations in the supernatant were determined by HPLC. The cumulative percentage of Lira released was calculated and then plotted versus time. All experiments were conducted in triplicate.

2.6. Release Kinetics Studies

To understand the in vitro release from PLGA NPs, the data were fitted into various kinetic models, which were the zero order (where the drug release rate is independent of its concentration), and first order model (where the drug release rate is concentration-dependent), and Higuchi (which describes the drug release from an insoluble matrix as a square root of the time-dependent process based on Fickian diffusion). The best-fit model was selected based on the coefficient of correlation R^2. Then the release mechanism was further confirmed by fitting the release data in the Korsmeyer–Peppas equation, where the exponent (η) value was used to describe the release mechanism of the drug through the PLGA matrix; where $0.45 \leq \eta$ corresponds to the Fickian diffusion mechanism, $0.45 < \eta < 0.89$ corresponds to anomalous (non-Fickian) transport, $\eta = 0.89$ corresponds to case II (relaxational) transport, and $\eta > 0.89$ corresponds to super case II transport.

2.7. Enzymatic Degradation Study

Stability analysis in the presence of pepsin and pancreatin was conducted and compared between native Lira and Lira loaded in PLGA NPs. Five hundred micrograms of native Lira, or the amount of NPs containing an equivalent amount of Lira, were added to 2 mL of pepsin-containing simulated gastric fluid SGF (3.2 g pepsin, 2 g of sodium chloride, 7 mL HCl, mixed and diluted with water to 1 L, pH = 1.2) or pancreatin-containing simulated intestinal fluid SIF (10 g pancreatin, 6.8 g KH_2PO_4, mixed and adjusted with 0.2 N NaOH then diluted with water to 1 L, pH = 6.8) and incubated at 37 °C under stirring at a speed of 100 rpm. The SGF and SIF were prepared as per USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). The same methodology was followed to assess the native Lira stability in SGFsp and SIFsp. The samples (750 µL) were withdrawn at specified time intervals for 2 h, and an equal volume of ice-cold reagent was added: 0.1 M NaOH for SGF and 0.1 M HCl for SIF, to stop the enzymatic reaction. The samples were centrifuged at 16,500× *g* and 4 °C for 10 min and the supernatant was analyzed by HPLC to calculate the residual Lira. All incubations were done in triplicates. Lira recovery in the withdrawn samples was calculated using the following equation:

$$\text{Lira recovery\%} = (\text{Remaining Lira amount/theoretical Lira amount}) \times 100$$

2.8. Treatment of Caco-2 Cells

The concentration of stock solutions for cell culture experiments were 1 mM for both the therapeutic peptide liraglutide and the PN159 peptide, which was used as a reference absorption enhancer [23,24]. The working solutions were diluted in cell culture medium or Ringer-buffer depending on the experiments. The final concentration of liraglutide encapsulated in the PLGA NPs was 100 µM and was diluted directly before using. Liraglutide was examined at 100 µM, while PN159 was examined at 3 µM concentration both for cell viability and permeability.

2.9. Cell Viability Measurement by Impedance

Impedance was measured at 10 kHz by an RTCA SP instrument (RTCA-SP instrument, ACEA Biosciences, San Diego, CA, USA). We have successfully tested the cellular effects of peptides and pharmaceutical excipients by impedance kinetics [23,27,28]. For background measurements, 50 µL cell culture medium was added to the wells. This was followed by seeding the cells at a density of 6×10^3 cells/well to 96-well plate with gold electrodes (E-plate 96, ACEA Biosciences) coated with collagen. Cells were cultured for 5 days in a CO_2 incubator at 37 °C and monitored every 10 min until the end of experiments. Cells were treated at the beginning of the plateau phase of growth. Lira, Lira-loaded PLGA NPs, blank PLGA NPs (without cargo), Lira and PN159 solution, and PN159 peptide were diluted in cell culture medium and the effects were followed for 24 h. Triton X-100 detergent (1 mg/mL) was used as a reference compound to obtain total cell toxicity. Cell index was

defined as R_n-R_b at each time point of measurement, where R_n is the cell–electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the medium alone.

2.10. Permeability Study on the Caco-2 Model

Transepithelial electrical resistance (TEER) reflects the overall tightness of cell layers of biological barriers. TEER monitoring was performed by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes. The final TEER was expressed relative to the surface area of the monolayers as $\Omega \times cm^2$ after subtraction of TEER values of cell free inserts.

Caco-2 cells were seeded onto Transwell inserts (polycarbonate membrane, 3 μm pore size, 1.12 cm^2 surface area; Corning Life Sciences, Tewksbury, MA, USA) and cultured for three weeks [29,30]. For transport experiments, the inserts were transferred to 12-well plates containing 1.5 mL Ringer-buffer in the acceptor (lower/basal) compartments. In the donor (upper/apical) compartments, the culture medium was replaced by 0.5 mL Ringer-buffer containing treatment solutions of Lira, Lira loaded in PLGA NPs, and Lira and PN159 solution at the concentration of 100 μM for liraglutide for 1 h. Treatment solutions from both compartments were collected and the Lira level was detected by HPLC.

The apparent permeability coefficients (P_{app}) were calculated as described previously [23]. Briefly, the cleared volume was calculated from the concentration difference of the tracer in the acceptor compartment ($\Delta[C]_A$) after 1 h and the donor compartments at 0 h ($[C]_D$), the volume of the acceptor compartment (V_A; 1.5 mL) and the surface area available for permeability (A; 1.1 cm^2) using this equation:

$$P_{app}(cm/s) = \frac{\Delta[C]_A \times V_A}{A \times [C]_D \times \Delta t}$$

Recovery (mass balance) was calculated according to the equation:

$$\%Recovery = \frac{C_f^D V^D + C_f^A V^A}{C_0^D V^D} \times 100\%$$

where C_0^D and C_f^D are the initial and final concentrations of the compound in the donor compartment, respectively; C_0^A is the final concentration in the acceptor compartment; and V^D and V^A are the volumes of the solutions in the donor and acceptor compartments [29].

2.11. Immunohistochemistry

Aiming to investigate the morphological changes in interepithelial junctions, immunostaining for the junctional proteins, zonula occludens protein-1 (ZO-1) and β-catenin, was carried out. Cells were grown on glass coverslips (Menzel-Glaser, Braunschweig, Germany) at a density of 4×10^4 cells/coverslips for 4 days and treated with Lira (100 μM), Lira loaded in PLGA NPs, Lira and PN159 solution, and PN159 peptide (3 μM) solutions for 1 h. After the treatment, the coverslips were washed with phosphate buffer (PBS) and the cells were fixed with 3% paraformaldehyde solution for 15 min at room temperature and incubated in 0.2% TX-100 solution for permeabilization. The cells were blocked with 3% bovine serum albumin in PBS and incubated with the rabbit primary antibodies, anti-ZO-1 and anti-β-catenin, overnight. Incubation with secondary Cy3-labeled anti-rabbit antibody lasted for 1 h. Hoechst dye 33342 was used to stain the cell nuclei. After mounting the samples (Fluoromount-G; Southern Biotech, Birmingham, AL, USA), the staining was visualized by a Visitron spinning disk confocal system (Visitron Systems GmbH, Puchheim, Germany).

2.12. Chromatographic Equipment and Conditions

Lira was analyzed by a reversed phase HPLC (Agilent 1200, San Diego, CA, USA) method that was previously developed and validated in our lab [25]. A Kinetex® C18 column with dimensions of (5 μm, 150 × 4.6 mm, (Phenomenex, Torrance, CA, USA) was used as the stationary phase. The mobile phase comprised 0.02 M aqueous KH_2PO_4 solution (pH = 7.0, solvent A) and acetonitrile (solvent B)

was pumped in a gradient mode from 80:20 (A:B, *v*/*v*) to 30:70 (A:B, *v*/*v*) in 12 min then back to 80:20 (*v*/*v*) between 12.1–15 min, at a flow rate of 1.5 mL/min. Fifty microliters of the sample was injected. The wavelength of UV detection was 214 nm. The retention time of Lira was 8.65 min.

The regression of the linearity (R^2) of the Lira calibration curve was 0.996.

2.13. Statistical Analysis

All data presented are means ± SD. The values were compared using the analysis of variance (ANOVA) followed by the Dunnett test or the Bonferroni test, using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Changes were considered statistically significant at $p < 0.05$.

3. Results

3.1. In Vitro Release of Lira from PLGA NPs

The release behavior data presented in (Figure 1) showed a biphasic release pattern starting by a moderate initial burst release during the first 2 h in SGFsp, where 14.2 ± 0.86% of Lira was released from the NPs. This was followed by a slow release profile until 6 h in the SIFsp, where only 18.5 ± 2.39% of cumulative Lira release was reached.

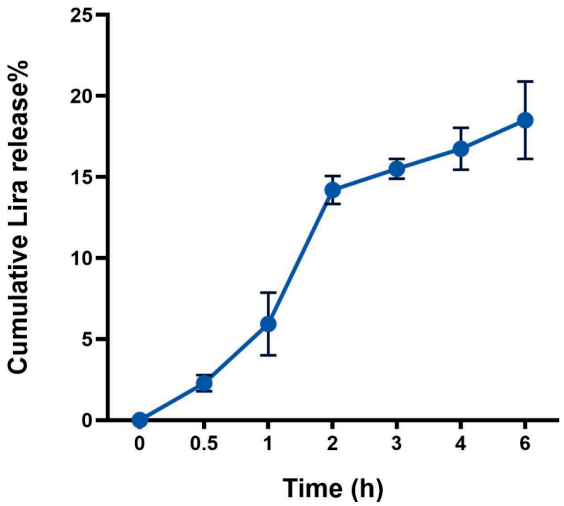


Figure 1. Cumulative in vitro release profile of liraglutide (Lira) from PLGA NPs.

3.2. Release Kinetics Studies

Based on the best fit with the highest correlation (R^2) value, it is concluded that Lira release from PLGA NPs follows the zero-order model (R^2 = 0.999) in SGFsp (pH = 1.2). When the release data is fitted into the Korsemeyer–Peppas equation (R^2 = 0.999), the exponent (n) value is 1.316, which is consistent with the zero-order release mechanism (Table 2).

Regarding the following 4 h in SIFsp, the release mechanism follows the Higuchi model (R^2 = 0.998), indicating diffusion-controlled release, and the exponent (n) value of the Korsmeyer–Peppas equation is 0.254, indicating that the release mechanism from PLGA NPs follows the Fickian diffusion mechanism (Table 2).

Table 2. Release kinetics data of Lira from PLGA NPs, in SGF and SIF (without enzymes).

Kinetic Model	SGF			SIF		
	N	*k*	R^2	*n*	*k*	R^2
Zero order	7.9857	1.84	0.9991	0.9836	12.658	0.9925
First order	0.038	2.01	0.998	0.0052	1.9418	0.9934
Higuchi	17.049	10.262	0.9854	4.1563	8.3559	0.9983
Korsmeyer-Peppas	1.3162	0.762	0.9993	0.2456	1.0696	0.9996

3.3. Enzymatic Degradation Study

It is obvious that only 1.9 ± 0.46% and 9.2 ± 0.7% of the free Lira was recovered after 30 min incubation in SGF and SIF, respectively. Lira was completely degraded after incubation for 1 h in SIF, while only 5.7 ± 0.53% Lira recovery occurred after 2 h incubation with SGF. On the other hand, the encapsulation of Lira into PLGA NPs was able to successfully protect 71.2 ± 1.49% and 87.6 ± 1.3% of Lira from degradation in the SGF and SIF at the end of the 2-h incubation, respectively (Figure 2). PLGA nanoparticles were claimed in previous research papers to be able to provide a protective and stable environment to encapsulate peptide drugs [11,31]. Encapsulation of GLP-1 into PLGA nanosystems could successfully shelter the peptide from the harsh environment of the simulated conditions of the stomach with sustained GLP-1 release [12].

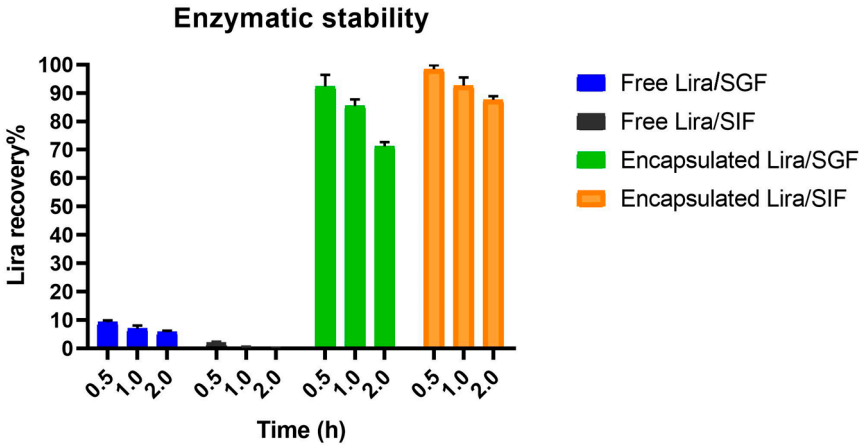


Figure 2. Enzymatic stability of liraglutide (Lira) encapsulated in PLGA NPs in both SGF and SIF mediums, with free Lira as a control.

3.4. Cell Viability Assay

Treatments with Lira, Lira loaded in PLGA NPs, Lira with PN159 peptide, unloaded PLGA NPs or PN159 peptide did not change the cell index values measured by impedance, a sensitive method to detect cellular effects, indicating good cell viability (Figure 3). Figure 3A shows the kinetics of the cellular effects of the treatment solutions, while the columns in Figure 3B show the effect of treatments at the 1-h time point. When cells were lysed with the detergent Triton X-100 the impedance dropped to zero (Figure 3B).

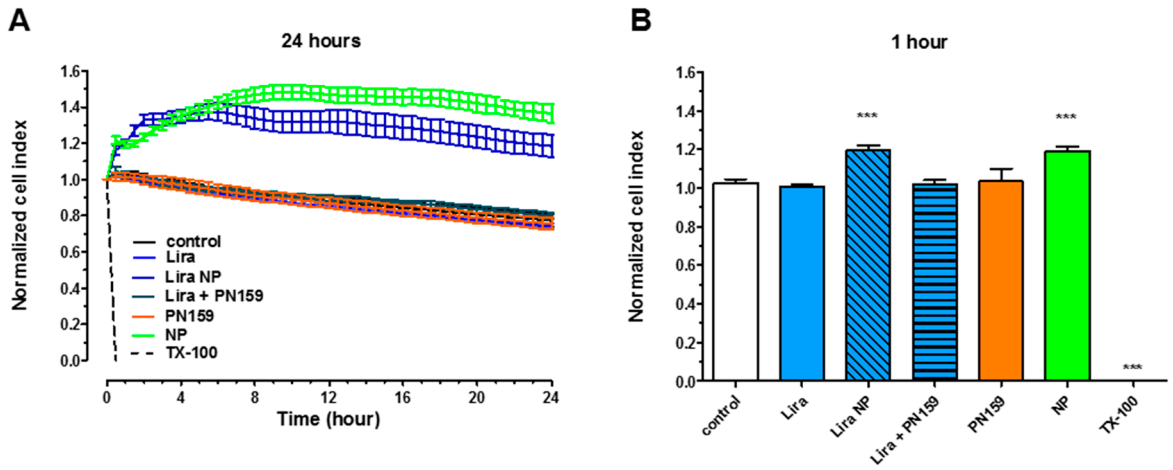


Figure 3. Cell viability kinetics for 24 h (**A**) and results of a 1-h treatment (**B**) of Caco-2 intestinal epithelial cells with liraglutide (Lira), NPs, liraglutide in NPs, liraglutide with PN159 peptide, and PN159 peptide measured by impedance. Values are presented as means ± SD, n = 6–12. Statistical analysis: Analysis of Variance (ANOVA) followed by Dunnett's test. NPs, nanoparticles; TX-100, Triton X-100. *** $p < 0.001$ compared to control.

3.5. Permeability Study on the Caco-2 Intestinal Barrier Model

Caco-2 monolayers showed high TEER values (893 ± 135 Ω × cm^2, n = 20) before permeability experiments indicating tight barrier properties. Because of the tight barrier the permeability was low for the marker molecule fluorescein (P_{app}: below 0.5×10^{-6} cm/s) as in our previous study [29]. The free Lira at a donor concentration of 100 µM showed good penetration as the P_{app} was 16×10^{-6} cm/s (Figure 4). The permeability of Lira encapsulated in NPs, 24×10^{-6} cm/s, was significantly higher than that for Lira solution. An increased Lira permeability (28×10^{-6} cm/s) was measured in the presence of PN159 peptide, our reference absorption enhancer (Figure 4A). There was no statistical difference between the liraglutide permeability values of the Lira-NP and Lira + PN159 groups. In contrast, the only group where the TEER values dropped after the 1-h treatment was the one containing PN159 peptide (Figure 4B) indicating opening of the paracellular pathway in agreement with observations from our previous studies [23,24]. Liraglutide alone or encapsulated in PLGA NPs did not change the ionic permeability (Figure 4B), suggesting no toxic effect on differentiated Caco-2 cells in concordance with the viability data (Figure 3) and no effect on the paracellular pathway.

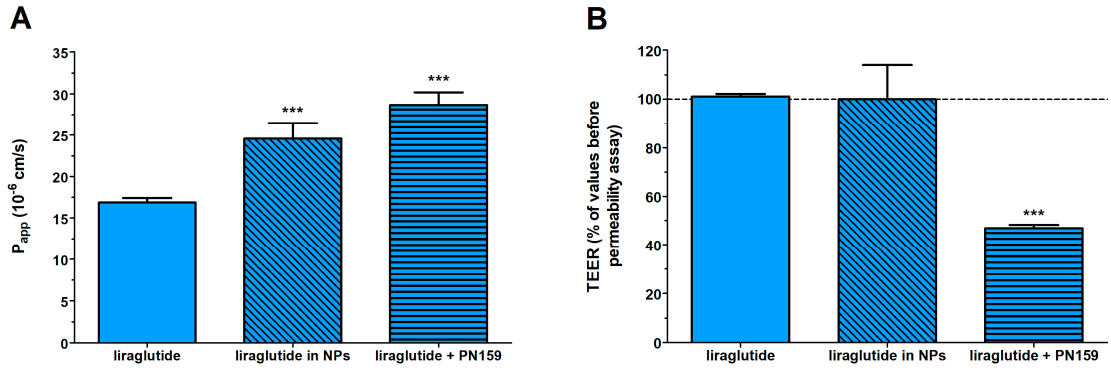


Figure 4. Evaluation of permeability of liraglutide (100 µM) across Caco-2 epithelial cell layers treated with different liraglutide formulations for 1 h (**A**). Changes in transepithelial electrical resistance (TEER) values of Caco-2 cell layers after 1-h treatment with different liraglutide formulations as compared to TEER values before treatment (**B**). Values are presented as means ± SD, n = 4. Statistical analysis: ANOVA followed by Bonferroni test, *** $p < 0.001$ compared to liraglutide group.

There was a good recovery for Lira after the permeability experiments and we found no significant differences between the recovery values of the different investigated Lira groups (Table 3).

Table 3. Recovery (mass balance) calculation after liraglutide permeability on Caco-2 cells.

Liraglutide	Recovery (%) Mean ± SD
Liraglutide	80.9 ± 1.6
Liraglutide in NPs	75.3 ± 2.3
Liraglutide + PN159	81.3 ± 6.9

3.6. Immunohistochemistry

The Caco-2 intestinal epithelial cells formed confluent layers visualized by the localization of the junctional proteins ZO-1 and β-catenin. The cells were attached to each other without gaps and had similar immunostaining patterns. An intact, belt-shaped continuous localization around the cell borders for the junctional proteins was observed both in the control and the treated groups. Only the PN159 peptide treated group showed a visible change in the staining pattern of β-catenin adherens junctional protein (Figure 5).

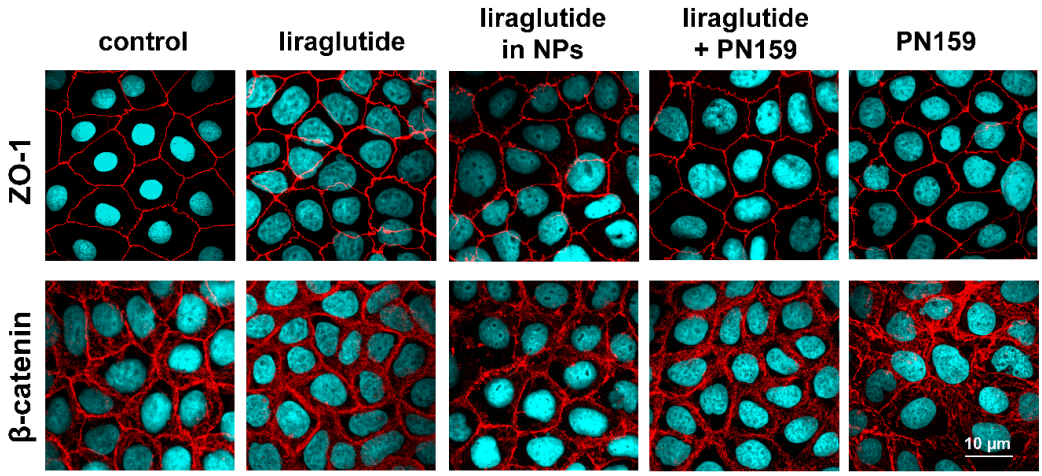


Figure 5. Effects of liraglutide, liraglutide in NPs, liraglutide and PN159 peptide together, and PN159 peptide on the junctional morphology of Caco-2 epithelial cells. Immunostaining for zonula occludens-1 (ZO-1), and β-catenin junction proteins after a 1-h treatment. Red color: immunostaining for junctional proteins. Blue color: staining of cell nuclei. Bar: 10 µm.

4. Discussion

GLP-1 analogs represent a unique class of antidiabetic peptide drugs with potential clinical benefits over existing therapies for T2DM treatment [32]. Lira, a lipophilic long-acting GLP-1 analog, is still subcutaneously administered. Since the oral delivery of Lira can bypass the inconvenience zone of patients, a smart carrier system that can tackle the challenges hindering the oral peptide delivery has been aimed for. In a previous work, we formulated and statistically optimized the formulation and process parameters affecting the quality of Lira loaded PLGA NPs that are designed for oral delivery. Spherical shaped NPs with homogeneous distribution, 188.95 nm particle size and 51.81% encapsulation efficiency were obtained [25]. As a follow-up study, the aim of this work was to investigate the potential of the developed PLGA NPs in overcoming the main barriers limiting the oral peptide delivery, namely, the harsh environment through the GI tract and the absorption membrane barrier.

The behavior of drug release from polymeric NPs is a complex process attributed to diffusion followed by degradation and influenced by the drug physicochemical properties in addition to various formulation and process variables [33]. The Lira release from PLGA NPs showed a biphasic release

pattern, which was frequently reported for polymeric NPs by previous papers [12,34]. At the burst release phase (where less than 15% of Lira was released), PLGA NPs are exposed to the gastric media and the surface of the NPs is hydrated. Then, non-capsulated Lira, or Lira which exists close to the surface having weak interactions with it is easily accessible by hydration and is released in the media. Potentially, less than 15% of Lira was released from NPs in the gastric media following a zero-order model. At the second phase of slow Lira release, the degradation of the polymer matrix took place, leading to diffusion of the encapsulated Lira, and the release mechanism followed the Higuchi model, which further confirms the diffusion-controlled release. These results prove that the PLGA nanosystem is able to hinder the release of encapsulated peptides in gastric simulating conditions without enzymes (SGFsp), and later sustain the peptide release in intestinal simulating conditions without enzymes (SIFsp).

The enzymatic stability results were compared between the free Lira and Lira encapsulated in NPs. There was no Lira detected after the 2-h incubation of free Lira with SGF or SIF, which is due to the presence of amino acids, especially the aromatic ones, in the Lira structure, which makes it vulnerable to pepsin–pancreatin digestion [35]. The results revealed that PLGA NPs were effective in protecting 71% and 87% of Lira from pepsin and pancreatin digestion after a 2-h incubation with SGF and SIF, respectively. These findings confirm that PLGA NPs can provide a physical barrier between the encapsulated Lira and the harsh environment in the GI tract, and thereby are promising for obtaining higher oral peptide bioavailability [12,36].

There was no decrease in cell impedance kinetics regarding the five treatment groups; Lira, Lira loaded in PLGA NPs, Lira and PN159 peptide in solution, unloaded PLGA NPs or PN159 peptide, during the 24-h long treatment. These findings proved the biocompatibility of PLGA NPs and showed that the composition of the nanosystem did not contribute to toxicity in Caco-2 cells. This is in accordance with previous reports where PLGA nanosystems larger than 100 nm did not trigger any toxic effects at different concentrations [37–39]. Regarding PN159 peptide, the absence of cytotoxic effects at the concentration of 3 μM is also in accordance with our previous report [24,40].

To further evaluate the potential of the prepared nanosystem, permeability studies on the Caco-2 cell model were conducted. Lira showed a good apparent permeability through the cell model compared to what was reported for the native GLP-1 or exenatide, which could be due to the 16-carbon fatty acid chain that is attached to lysine at position 26 via a glutamic acid spacer [9]. This acylation leads to higher Lira hydrophobicity [41], which can enhance the intracellular permeability of this GLP-1 analog when compared to the native GLP-1 or exenatide [42]. The potential of the optimized polymeric NPs was also investigated to further enhance Lira permeability. Lira encapsulated in NPs showed 1.5-fold higher apparent permeability as compared to Lira alone. Because PLGA NPs are more lipophilic compared to the free peptide drug, their transport across the lipid membrane of Caco-2 cells is better. Furthermore, these optimized polymeric NPs showed a smaller size than 200 nm, and it was previously reported that NPs within the 100–200 nm size range showed the best properties for cellular uptake, while smaller-sized (50 nm) or larger-sized (≥500 nm) particles resulted in reduced uptake [43]. Moreover, the prepared NPs were spherical with a smooth surface, as previously confirmed by scanning electron microscopy [25], which is better for cellular uptake when compared to needle-shaped ones. The ability of PLGA NPs to enhance the in vitro permeability of the hydrophilic drug alendronate [44], salmon calcitonin [45], bovine serum albumin [46], and insulin [15,17] through the Caco-2 cell model has been reported in the literature. In the presence of PN159 peptide, our reference absorption enhancer, Lira permeability also increased. This finding is consistent with our results, and results from the literature, on the reversible tight junction opening by the PN159 peptide in biological barrier models, which effectively improves the permeability of different drugs and hydrophilic marker molecules through the paracellular pathway [23,24,40]. In addition, it was reported that this amphipathic CPP can strongly bind to and interact with biological membranes due to electrostatic and hydrophobic interactions [47,48], and we also confirmed the cell permeabilizing and penetration effects of PN159 on Caco-2 cells [23].

The immunostaining for the junctional proteins, ZO-1 and β-catenin, showed that Lira-PLGA NPs did not change the morphology of interepithelial junctions. The PN159 peptide treated group showed a change in the staining pattern of the β-catenin adherens junctional protein, which is also in accordance with our previous results [23,24]. We suppose that an increase in both the paracellular transport and the membrane permeability in Caco-2 cells by PN159 can contribute to the enhanced Lira permeability.

5. Conclusions

Being a relatively new GLP-1 analog, there are no reported studies for Caco-2 permeability of liraglutide alone or encapsulated into a carrier system. In this study we found that the developed PLGA nanosystem could efficiently protect the encapsulated liraglutide from the conditions simulating the harsh environment in the GI tract. This polymeric system seems to be promising as it can also enhance the permeability 1.5-fold compared to free liraglutide solution. These findings reveal that encapsulation in a polymeric nanosystem holds promise for oral GLP-1 analog delivery.

Author Contributions: Conceptualization, R.I., M.A.D. and I.C.; methodology, R.I, A.B, G.K. and M.A.D.; software, R.A., A.B. and G.K.; validation, R.I. and A.B.; formal analysis, R.I., A.B., G.K. and I.G.; investigation, R.I., A.B., G.K. and I.G.; resources, R.I. and A.B.; data curation, R.I., A.B., G.K. and I.G.; writing—original draft preparation, R.I. and A.B.; writing—review and editing, R.I., I.G., M.A.D and I.C.; visualization, R.I. and I.G.; supervision, M.A.D. and I.C.; project administration, I.Cs.; funding acquisition, I.C.

Funding: This work was supported by the Ministry of Human Capacities, Hungary (grant 20391-3/2018/FEKUSTRAT), the National Research, Development and Innovation Office, Hungary (GINOP-2.2.1-15-2016-00007) and the EU-funded Hungarian grant EFOP-3.6.1-16-2016-00008.

Conflicts of Interest: The authors declare no conflict of interest.

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PUBLICATION II.

Pharm Res (2019) 36:99
https://doi.org/10.1007/s11095-019-2620-9

RESEARCH PAPER

Synthesis and Statistical Optimization of Poly (Lactic-Co-Glycolic Acid) Nanoparticles Encapsulating GLP1 Analog Designed for Oral Delivery

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Received: 17 January 2019 / Accepted: 1 April 2019

ABSTRACT

Purpose To design and stabilize Liraglutide loaded poly (lactic-co-glycolic acid) nanoparticles (PLGA NPs) proper for oral administration.

Methods PLGA NPs were prepared by means of double emulsion solvent evaporation method and optimized by applying 7-factor 2-level Plackett-Burman screening design.

Results Spherical shaped NPs with homogeneous distribution, 188.95 nm particle size and 51.81% encapsulation efficiency were obtained. Liraglutide was successfully entrapped in the NPs while maintaining its native amorphous nature, and its structural integrity as well.

Conclusion Lira-PLGA NPs with the required Critical Quality Attributes (CQAs) were successfully designed by implementing a 7-factor 8-run Plackett Burman design into the extended Quality by Design (QbD) model, to elucidate the effect of formulation and process variables on the particle size, size-distribution, encapsulation efficiency and surface charge. As the developed nanoparticles maintained the native structure of the active pharmaceutical ingredient (API), they are promising compositions for the further development for the oral delivery of Lira.

KEY WORDS liraglutide · oral delivery · plackett Burman design · PLGA nanoparticles · quality by design

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11095-019-2620-9) contains supplementary material, which is available to authorized users.

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INTRODUCTION

A current scenario in pharmaceutical development is inclined towards employing rational Quality by Design (QbD) strategy (1,2) which has been adopted by the pharmaceutical industry to guarantee the quality of drug products (3). One of the key elements of QbD is to identify and thoroughly understand formulation and process variables and their effects on the critical quality attributes (CQAs), followed by the optimization of these variables by applying an appropriate statistical design of experiment (DOE) which enables the researcher to minimize the number of runs and helps in identifying the most influential parameters namely; critical process parameters (CPPs) and critical material attributes (CMAs) which may highly impact the quality of the product. In addition to that, DOE helps in identifying the optimum level of each factor that assures the desired CQAs values, to comply with the desired Quality Target Product Profile (QTPP) (4). One of the important questions when implementing DoE methodology is the selection of adequate experimental design.that matches the experimental objective. When estimating the main effects of large number of factors are of interest to be investigated, screening designs such as 2- level Plackett-Burman (PB) is applied. The main advantage of applying such screening designs is the minimum number of observations needed to calculate the effect of several variables. If providing further information on direct and pairwise-interaction effects and curvilinear variable effects is desired, second order designs: central composite designs (CCD) and Box Behncken designs (BBD) are the most widely applied ones (5,6). CCD provides better prediction capability than BBD, while the latter requires fewer runs in case of 3 or 4 variables and is applied when combined factor extremes should be avoided.

Liraglutide (Lira) or NN2211 is a recombinant palmitylacylated derivative of glucagon like peptide −1 (GLP1), which

Published online: 13 May 2019

was approved for the treatment of T2DM (6) in addition to chronic weight management (7). Lira is currently administered once daily through subcutaneous injection which is an invasive route known to be limited by insufficient patient adherence to the therapy in addition to the fact that this therapeutic route is not strictly mimicking the physiological secretion route of GLP1 (8). The oral route should be regarded as the most desirable choice for the delivery of Lira (9), and realizing the dream of administering a GLP1 analog such as Lira orally is still an elusive goal despite all advances in peptide delivery systems.

Based on our careful evaluation of literature regarding the emerging developments in oral delivery of antidiabetic peptides (10,11), we found out that PLGA nanoparticles showed promising results in improving the stability of peptides through the GIT in addition to other merits of nanocarrier systems, which can all lead to enhancing the oral bioavailability of these peptides (12). Among the applied techniques for preparing PLGA NPs, the double emulsion solvent evaporation method was found to be the most preferable one, which has been efficiently used for encapsulating peptides and proteins (13–15). Nevertheless, the physicochemical properties of nanoparticles may be affected by various formulation and process parameters, which influence the product quality.

To the best of our knowledge, there is no previously reported work considering the application of DOE as a part of the QbD strategy for the development of PLGA NPs encapsulating GLP-1 analog. Here we focus on the optimization of the size and EE of the NPs as a crucial demand along with the polydisperisity index (PDI) and surface charge. The design space (DS) was established to optimize the level of each of the examined factors, then surface morphology, compatibility studies as well as structural and conformational stability tests of Lira encapsulated in the optimized formula were conducted on the optimized formula.

MATERIALS AND METHODS

Materials

Liraglutide was purchased from Xi'an Health Biochem Technology Co., Ltd. (China), Poly(lactide-co-glycolide) (PLGA 50:50, Mw = 30,000–60,000 Da), PVA (MOWIOL 4–98, MW~27,000 Da) which is a soluble polymer, and D-(+)-Trehalose dihydrate (MW = 378.33 g/mol) were purchased from Sigma Aldrich (Germany). D-(−)-Mannitol was purchased from Molar Chemicals Ltd. (Hungary). Sodium acetate anhydrous was purchased from Scharlau Chemie S.A. (Spain). Ethyl acetate used for dissoloving PLGA was from REANAL Labor (Hungary). All other chemicals in the study were of analytical reagent grade.

Methods

Preparation of Liraglutide Loaded PLGA NPs Using Double Emulsion Solvent Evaporation Method

The preparation of Liraglutide loaded PLGA nanoparticles was carried out by means of the double emulsion W1/O/W2-solvent evaporation method, which is the most commonly used technique for the encapsulation of peptide drugs within PLGA NPs due to its simplicity and high encapsulation efficiency (15,16). The amount of PLGA (30 or 60 mg) was dissolved in ethyl acetate at room temperature to form the organic phase. Ethyl acetate was the organic solvent of choice here as it was reported to increase the rate of encapsulation of hydrophilic molecules (16). The inner aqueous phase of 0.5–5 mg liraglutide was dissolved in 0.5 ml of 1% sodium acetate aqueous solution, it was slowly added to the organic phase, then water/oil primary emulsion was formed upon sonication at the power of 90 W for 30 s using a probe sonicator in ice bath. The obtained emulsion was re-emulsified with external aqueous phase containing 0.5–2% PVA as stabilizer by sonication in ice bath at the power of 90 W for 0.5–2 min using the probe sonicator. The obtained water-in-oil-in-water (W1/O/W2) double emulsion was subjected to magnetic stirring at room temperature over the night to allow the complete evaporation of ethyl acetate. The nanoparticles were then collected by centrifugation for 15 min at 16500 rpm, washed three times with distilled water, and resuspended in deionized water.

For the lyophilization process, 1.5 ml of each nanoparticles suspension was poured into semi-stoppered glass vials with slotted rubber closures and freeze-dried at −40°C for 72 h. 5–10% mannitol or trehalose was added as lyoprotectants. The chamber pressure was maintained at 0.01 mbar, and the process was controlled by (Scanlaf CTS16a02) software.

Design of Experiment Study Using Plackett Burman Design

PB design is the most widely used method among the various screening designs used for the determination of the most influential factors affecting the pharmaceutical development as it has many advantages: it screens a large number of variables and identifies the highly influential ones with relatively few runs, while assuring a good degree of accuracy. PB design with a total of 8 runs involving 7 independent variables was carried out using STATISTICA 13 software, and analysis of variance (ANOVA) was applied to determine the statistical significance of each model coefficient, which was significant at 95% level ($P < 0.05$).

The linear equation of this model is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7$$

where Y is the response, b_0 is the constant and b_1, $b_2 \ldots b_7$ are the coefficient of factors X_1, $X_2 \ldots X_7$. It is known that a positive value of the regression coefficient is an indicator of a positive effect of the factor (X) on the response (Y), while a negative value refers to an inverse relation between the examined variable and the response (17).

Depending on our previous risk assessment-based investigation (18–20), the selected independent formulation and process variables were: PLGA amount (X1), liraglutide amount (X2), 2nd sonication time (X3), PVA concentration (X4), lyoprotectant type (X5), lyoprotectant concentration (X6) and external aqueous phase to organic phase w2/o ratio (X7). Particle size (Y1), PDI (Y2), EE (Y3) and zeta potential (Y4) were selected as dependent variables. The examined lower and upper levels of the independent factors X_1-X_7 (Table 1) were also determined depending on preliminary experiments and literature survey.. The design was validated by 3 extra center checkpoint formulations and the bias (%) between predicted and observed values of response was calculated. The optimized formulation was prepared within design space (DS) and compared with predicted results of the responses.

Table 1 Levels of the Selected Critical Factors

Critical factor		Low level	High level
PLGA amount (mg)	X1	30	60
Liraglutide amount (mg)	X2	0.5	5
2nd sonication time (min)	X3	0.5	2
PVA (%)	X4	0.5	2
Lyoprotectant type	X5	Mannitol	Trehalose
Lyoprotectant (%)	X6	5	10
W2/O ratio	X7	2	5

Characterization of the Prepared Liraglutide Loaded PLGA NPs

Particle Size, Size Distribution and Surface Charge Measurements. Approximately 5 mg of the prepared freezedried NPs was dispersed in 5 ml of double distilled water and sonicated to minimize the possible inter-particle interactions. The hydrodynamic diameter (Z-average), PDI and zeta potential of reconstituted NPs were measured in folded capillary cell by using Malvern Nano ZS Zetasizer (Malvern Instruments Ltd. UK) equipped with He-Ne laser(633 nm). The instrument allows the particle size measurement in the range of 0.3 nm-10.0 μm using patented *NIBS* (Non-Invasive Back Scatter) technology, with high accuracy of ± 2%. The samples were measured at 25°C, the refractive index was 1.445, and the number of scans was 17. All the measurements were conducted in triplicate and the average value of each was used.

Encapsulation Efficiency (EE). The encapsulation efficiency of liraglutide encapsulated in PLGA NPs was determined directly using the centrifugation method. In this method, 20 mg from each NPs formulation was dissolved in 2 ml of DCM, then liraglutide was extracted into 4 ml of PBS (pH = 8.1), soaked for 30 min and then centrifuged at 16500 rpm at 4°C for 15 min. The supernatant was then collected and the amount of encapsulated liraglutide in the supernatant was measured using the RP-HPLC method. Samples were run in triplicate.

The percentage of EE was calculated using the following equations:

$$EE\% = \text{encapsulated amount of liraglutide} / \text{total amount of liraglutide added}^{*} 100.$$

Chromatographic Equipment and Conditions. Reversed phase HPLC (Shimadzu Corporation, NEXERA X2, Tokyo, Japan) method was developed in our lab to analyze liraglutide. A Kinetex ® C18 column with dimensions of (5 μm, 150*4.6 mm, (Phenomenex, USA) was used as a stationary phase. The flow rate of 1.5 ml/min was set over 15 min with a mobile phase comprised of 0.02 M aqueous KH2PO4 solution (pH = 7.0, solvent A) and acetonitrile (solvent B). The mobile phase was pumped in a gradient mode as it was changed from 80:20 (A:B, *v*/v) to 30:70 (A:B, v/v) in 12 min then going back to 80:20 (v/v) between 12.1–15 min. The column temperature was set to 40°C, and the sample tray temperature was set to 15°C. Fifty microliters of sample volume was injected. The wavelength of UV detection was 214 nm.. The retention time of liraglutide is 8.65 min. In our HPLC method, some chromatographic parameters have been calculated. The limit of detection (LOD) value of the liraglutide is 0.175 ppm, the limit of quantification (LOQ) value is 0,530 ppm, respectively. Capacity factor (k') for liraglutide is 5.20, the asymmetry factor of the peak of liraglutide showed 1.40 value, respectively. The theoretical plate (N) value is 146,620, calculated by the Ph.Eur. guideline. The regression of the linearity (R^2) of the liraglutide calibration curve was 0.996, respectively.

Scanning Electron Microscopy Measurements (SEM). To investigate the surface morphology, sphericity, and discreteness of the freeze dried NPs containing lira, scanning electron

microscopy (SEM) (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan) at 10 kV was used. The samples were coated with gold-palladium (90 s) with a sputter coater (Bio-Rad SC 502, VG Microtech, Uckfield, UK) using an electric potential of 2.0 kV at 10 mA for 10 min. The air pressure was 1.3–13.0 mPa. 3 repetitions of the optimized formula were tested by SEM technique and images were captured from different surface regions of each sample and at two different magnifications (×15,000, ×45,000).

Compatibility Studies

To investigate the physicochemical compatibility between the drug and the polymer in the prepared PLGA NPs, FTIR, DSC and XRD analysis were conducted.

Fourier Transform Infrared Spectroscopy (FTIR). The FT-IR spectra of pure Lira, PLGA, Lira free/loaded PLGA NPs were recorded using FT-IR spectrometer (Thermo Nicolet AVATAR; LabX Midland, ON, Canada) in the range of 4000 and 400 cm^{-1} with an optical resolution of 4 cm^{-1}. The sample was mixed with 150 mg of dry KBr and compressed to prepare the pellet.

Differential Scanning Calorimetry (DSC). To define the physical state of the peptide drug in the nanoparticles and assess any possible intermolecular interaction between the drug and the polymer in the nanoparticles, DSC studies of pure Lira, PLGA, Lira free/loaded PLGA NPs were performed using (Mettler Toledo TG 821e DSC Mettler Inc., Schwerzenbach, Switzerland). Accurately weighed samples (3–5 mg) were sealed in an aluminum pan and an empty pan was used as a reference. The samples were analyzed at a scanning temperature from 25 to 300°C at a heating rate of 10°C/min under nitrogen purge. Data analysis was performed using the STAR^e software (Mettler Toledo Mettler Inc., Schwerzenbach, Switzerland).

X-Ray Diffraction Study (XRD). XRD is a useful technique applied herein to characterize the physical state of liraglutide entrapped in PLGA NPs and further to confirm the stability attributed to polymer-drug interaction. Powder X-ray diffraction (XRD) patterns of pure Lira, PLGA, Lira free/loaded PLGA NPs were obtained using an X-ray powder diffraction (XRPD) BRUKER D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany), supplied with a Cu K λ1 radiation source (λ = 1.54056 A°), with a voltage of 40 kV and a current of 40 mA, in flat plate θ/2θ geometry, over the 2θ ranges 3–40°, with a scan time of 0.1 s at step size of 0.007°. The sample was placed on a quartz holder and measured at ambient temperature and humidity.

Stability of Lira Encapsulated in PLGA NPs

Electrospray Ionization Mass Spectrometry. Electrospray Ionization Mass Spectrometry (ESI-MS) is a valuable tool to be used to provide information about the molecular weight of native Lira and compare it to Lira loaded in NPs. Lira was characterized by an Agilent 1100 LC-MSD trap mass spectrometer equipped with an electrospray ion source.

Circular Dichroism. CD was performed to evaluate the conformational stability of Lira loaded into the prepared polymeric NPs. CD spectra were obtained with a Jasco J-1100 spectropolarimeter (Tokyo, Japan). Aliquot of each of PBS (pH = 8.1), native Lira in PBS and Lira extracted from NPs in PBS was placed in a 10 mm pathway Far-UV quartz cuvette and the Far-UV CD spectra were collected by an PM-539 CD spectrometer. Spectra were collected at room temperature over the wavelength range of 260 nm to 195 nm with 0.2 nm interval. Ellipticity was recorded at scanning speed of 100 nm/min and 1.00 nm band with 5 accumulations. PBS solution subtraction, noise reduction and data analysis were performed using standard analysis and temperature/wavelength analysis programs (Jasco).

RESULTS AND DISCUSSION

Placket Burman Design: Risk Analysis

The QTPP that encompasses the desired CQAs was defined in our previous paper as following: stable, homogeneous and spherical shaped freeze dried NPs with particle size of 100-300 nm and maximum EE. Risk assessment was also conducted (using LEAN-QbD software) for ranking and prioritizing CMAs and CPPs likely to have an impact on the quality of Lira loaded PLGA NPs (20) and the highly influential parameters were prioritized and subjected to subsequent screening using a seven-factor, two-level, eight-run PB screening design (Table 2) in order to minimize their risk to a low level by controlling these variables in a specific accepted range.

Herein, the possible effects of these formulation and process parameters on four responses namely: mean particle size, PDI, EE and zeta potential were investigated by applying the PB screening design where the experimental data were validated by ANOVA for each factor. ANOVA parameters for predicting mean particle size (Y1), PDI (Y2), EE (Y3) and Zeta potential (Y4) are presented in Supplemental Table 1.Surface response plots are also useful diagrammatic representation of the values of the response, to project the significance of effects for each variable and can explain the relationship between tested independent factors and dependent responses. A color-scale object along with the surface plot serves as a legend, and the value of the response is dependent on the gradual

Table 2 The Input Factor Levels in 7 Factor, 2 Level, 8 Run PBD

Run code	PLGA (mg)	Lira (mg)	2nd sonication time (min)	PVA (%)	Lyoprotectant type	Lyoprotectant (%)	W_2/O ratio
PBD-F1	30	0.5	0.5	2	Trehalose	10	2
PBD-F2	60	0.5	0.5	0.5	Mannitol	10	5
PBD-F3	30	5	0.5	0.5	Trehalose	5	5
PBD-F4	60	5	0.5	2	Mannitol	5	2
PBD-F5	30	0.5	2	2	Mannitol	5	5
PBD-F6	60	0.5	2	0.5	Trehalose	5	2
PBD-F7	30	5	2	0.5	Mannitol	10	2
PBD-F8	60	5	2	2	Trehalose	10	5

color. Based on this color gradient, these plots can present the change in response value with different levels of the independent variable.

Influence of Investigated Parameters on the Z-Average Size, PDI, EE and Zeta Potential

Depending on the selected parameters levels, the Z-average ranged between 160.1 ± 5.6 nm and 235. ± 5.3 (Table 3). The corresponding coefficients are summarized in Supplemental Table 2, where the factors a *P* value<0.05 are regarded as highly significant, while the ones having nonsignificant response coefficients with a P value>0.05 are least contributing in the prediction of mean particle size. The polynomial equation obtained for the fitted full model explaining the effect of formulation and process variables on the mean particle size is:

$$Y_{1=}197.8021_{+}9.5079X_1 + 1.5304X_2 + 7.7638X_3 - 16.9754X_{4-}5.4712X_5 - 3.5429X_6 - 7.0791X_7$$

with $R^2 = 0.9745$, adjusted $R^2 = 0.9609$,

and Mean square (MS) = 22.3978

According to this polynominal equation and the Pareto chart (Fig. 1), the most influential factors in terms of particle size are the PVA concentration, PLGA amount followed by sonication time and W2/O ratio at almost the same level of significance. Then the next important variables include lyoprotectant type and concentration. The tested levels of Lira amount were observed to have a non-statistically significant effect on the mean particle size.

In all prepared formulations, NPs exhibited a practically monodisperse or narrow distribution (21) as PDI was ranged from 0.1 ± 0.003 in PBD-F6 to 0.22 ± 0.01 in PBD-F3 (Table 3) evidencing that the obtained NPs are homogeneous and stable with no aggregation. Figure S1 shows an example of PDI = 0.07 with Z-average = 157.1 nm obtained for PBD-F1. The polynomial equation obtained for the full model describing the effect of formulation and process variables on the PDI value is:

$$Y2 = 0.1568 - 0.0061X1 + 0.0312X2 + 0.0005X3 - 0.0013X4 - 0.0032X5 - 0.0031X6 + 0.0291X7$$

with $R^2 = 0.9235$, adjusted $R^2 = 0.8828$,

and Mean square (MS) = 0.0003

$$Y2 = 0.1568 - 0.0061X1 + 0.0312X2 - 0.0032X5 - 0.0031X6 + 0.0291X\,7 \ldots\ldots\ldots \text{Redcuced(model)}$$

with $R^2 = 0.92256$, adjusted $R^2 = 0.89522$, and

Mean square (MS) = 0.0002.

The statistical analysis (Table 3S) along with the Pareto chart (Fig. 3) revealed that only two of the examined CMAs namely Lira amount and W_2/O ratio were observed to have a significant effect on the PDI value.

Depending on the tested two levels of each factor, the results showed that EE varied between 20.1 ± 1.7 in formulation PBD-F1 and 43.5 ± 3.3in PBD-F4 (Table 3).The polynomial equation obtained for the fitted full model showing the impact of the seven examined variables on EE is:

Table 3 Experimental Responses Results in PBD

Run code	Z-AVE (nm)	PDI	EE%	Z-potential (mV)
PBD-F1	160.1 ± 5.6	0.10 ± 0.03	20.1 ± 1.7	−30.6 ± 1.8
PBD-F2	209.8 ± 8.01	0.15 ± 0.01	36 ± 1.25	−25 ± 1.6
PBD-F3	190.0 ± 2.8	0.23 ± 0.01	41 ± 2.46	−27.3 ± 0.7
PBD-F4	200.2 ± 3.5	0.16 ± 0.03	43.5 ± 3.34	−31.2 ± 1.2
PBD-F5	179.5 ± 3.8	0.17 ± 0.01	32 ± 4.03	−23.8 ± 1
PBD-F6	235.7 ± 5.3	0.10 ± 0.003	28.9 ± 2.05	−29.2 ± 0.4
PBD-F7	223.6 ± 3.7	0.17 ± 0.02	22.2 ± 2.12	−30.4 ± 0.
PBD-F8	183.5 ± 3.03	0.20 ± 0.01	21 ± 1.51	−26 ± 0.2

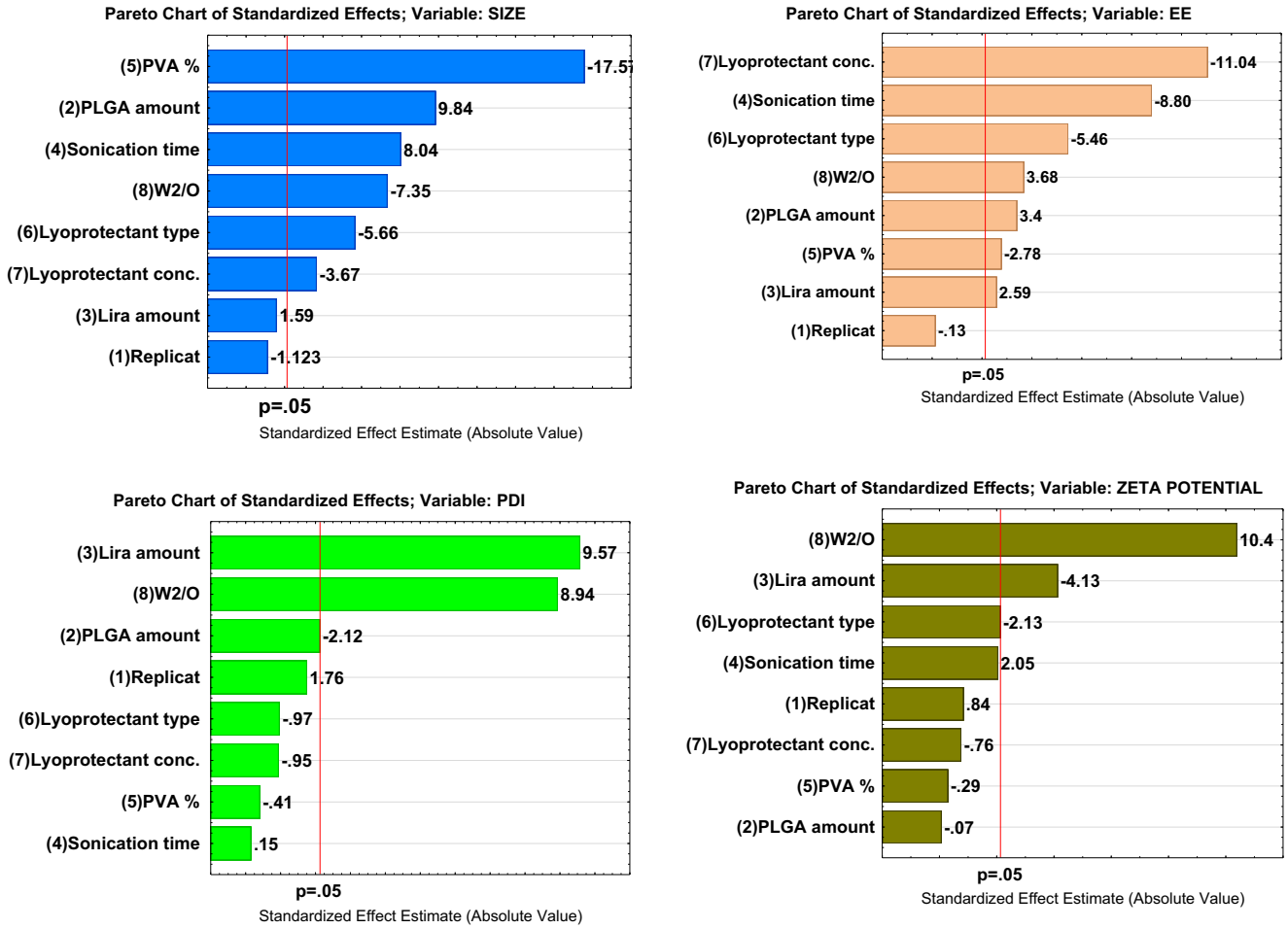

Fig. 1 Pareto charts of the effects of the examined independent variables on Z-average size (Y1), PDI (Y2), EE (Y3), zeta potential (Y4). Replicate refers to number of repetitions for each formula which was 3.

$$Y3 = 30.548 + 1.7683\,X_1 + 1.3458X_2 - 4.5825X_3 - 1.4458X_4 - 2.8408X_5 - 5.7483X_6 + 1.915X_7$$

with $R^{2=}0.9471$, adjusted $R^2 = 0.9189$,

and Mean square (MS) = 6.5021.

This equation along with the Pareto chart (Fig. 1) and statistical analysis (Table 4S) show that the lyoprotectant %, 2nd sonication time and lyoprotectant type are the most highly risky factors in terms of EE. This is followed by other factors which all show a significant impact on the amount of Lira encapsulated in the PLGA NPs.

The zeta potential was also monitored during the optimization steps and its values ranged from-31.2 ± 1.2 mV in PBD-F4 to −23.8 ± 0.95 mV in PBD-F5 (Table 3), and these expected negative values are attributed to the presence of carboxyl group end on PLGA. Figure S2 depicts the result of zeta potential obtained for formulation PBD-F1 as an example. The full model describing the effect of formulation and process variables on the zeta potential is:

$$Y_{4=} -28.0596 - 0.0154X_1 - 0.9071X_2 + 0.4496X_3 - 0.0646X_4 - 0.4679X_5 - 0.1663X_6 + 2.2846X_7$$

with R2 = 0.9001, adjusted R2 = 0.8468,

and Mean square (MS) = 1.1599

$$Y4 = -28.0596 - 0.9071X2 + 0.4496X3 - 0.4679X5 - 0.1663X6 + 2.2846X7 \ldots\ldots\ldots\ldots \text{Reduced (model)}$$

with R2 = 0.89946, adjusted R2 = 0.86397,

and Mean square (MS) = 1.0.297.

It is obvious from the statistical analysis (Table 5S) and the Pareto chart (Fig. 1) that only the W2/O ratio and the lira amount had a significant impact ($P < 0.05$) on the surface charge of PLGA NPs. Other examined variables were observed to have only a non-significant effect on surface charge.

The effect of the above-explained variables on Y1,Y2,Y3 and Y4 is discussed point by point in the following:

Effect of Polymer Amount

It is apparent from Fig. 2 that when the PLGA amount was increased, the Z-average increased correspondingly, as supported by many earlier published papers (22,23) That could be explained by increasing the viscosity of the organic phase which leads to a reduction in the net shear stress (24), in addition to a reduction in the evaporation rate; i.e. the dispersion rate of the organic phase toward the external aqueous phase will be slower, thus that incites the formation of larger particles (25,26). The formation of a more viscous organic phase was reported to push up the frequency of collisions between particles during the emulsification and droplet solidification step, which may lead to the aggregation of the semisolid particles (5). Regarding the PDI value, it was also observed that size distribution was slightly decreased by increasing the PLGA amount which means that a greater level of PLGA would promote the formation of much more homogeneous NPs.

The positive effect that the PLGA amount has on EE (Fig. 4) could be again due to the fact of increasing the viscosity of the organic phase with a higher amount of polymer, which can retard drug diffusion into the external aqueous phase and thus increase the amount of drug entrapped inside the NPs (27,28). It is also known that larger nanoparticles obtained with a higher PLGA level can provide sufficient surface for entrapping the peptide drug. Furthermore, higher PLGA levels give rise to more rapid polymer deposition as ethyl acetate is removed from the NPs, which is expected to hinder any undesirable Lira diffusion into the external phase (29).

Effect of Liraglutide Amount

The positive effect that Lira has on the 2nd emulsification and both final particle size (Fig. 2) and size distribution as a result (Fig. 3) would be explained by the influence of the drug on the droplet size of the inner aqueous phase within the organic phase in the first water-in-oil (w1/o) emulsion, which may modify its ability for dispersion in the outer aqueous phase. However, this effect was only limited and not significant in the case of particle size, while Lira theoretical loading amount was a significant influential parameter affecting the PDI and zeta potential values (Fig. 1, Fig. 3, Fig. 5). The Lira level was also shown to have a significant positive effect on EE; when loading higher amount of Lira, EE was even higher (Fig. 4). This positive trend was previously reported with other peptide drugs as insulin, and it is explained due to higher amount of peptide that is associated with the surface of nanoparticles and is electrooptically linked to a greater extent (30), thus resulting in a higher EE value. However, the studied levels of the Lira amount in our work were shown to be the least risky factor affecting EE as presented in the Pareto chart (Fig. 1).

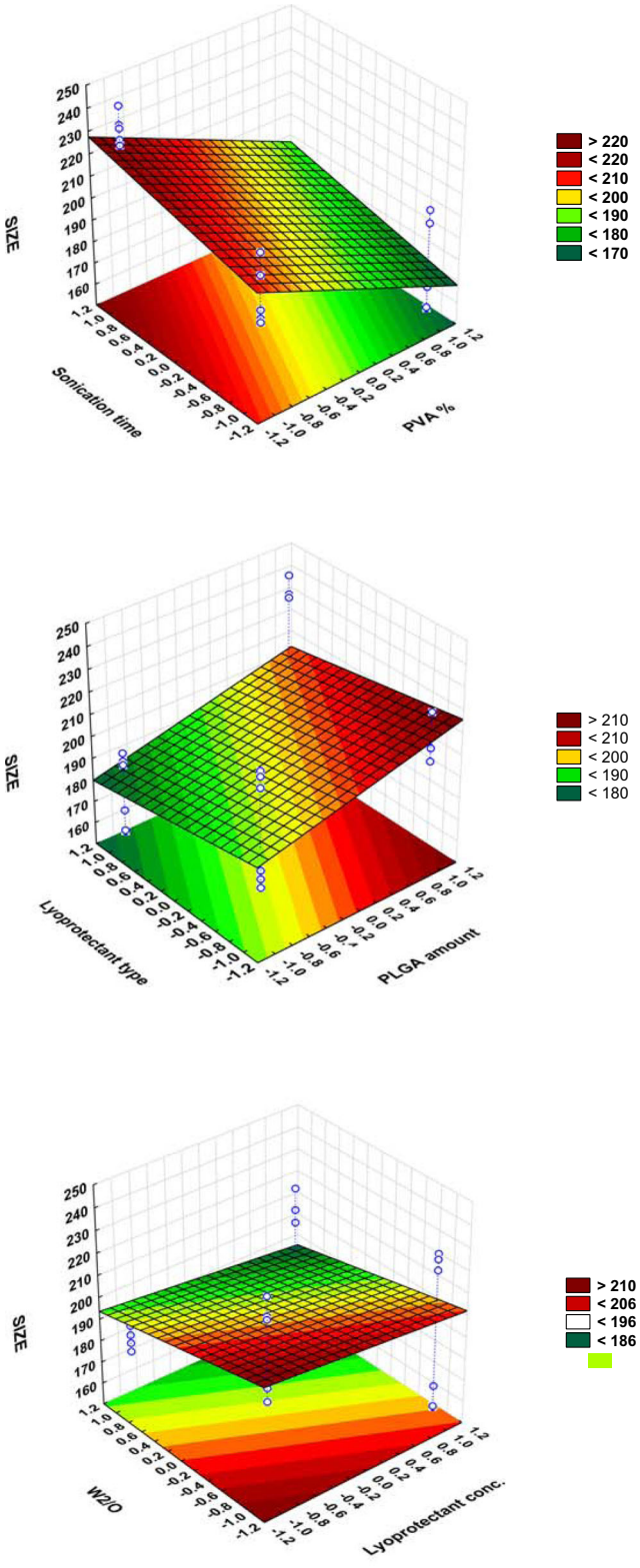

Fig. 2 Surface plots showing the effect of the significant examined variables on the Z-average size (Y1).

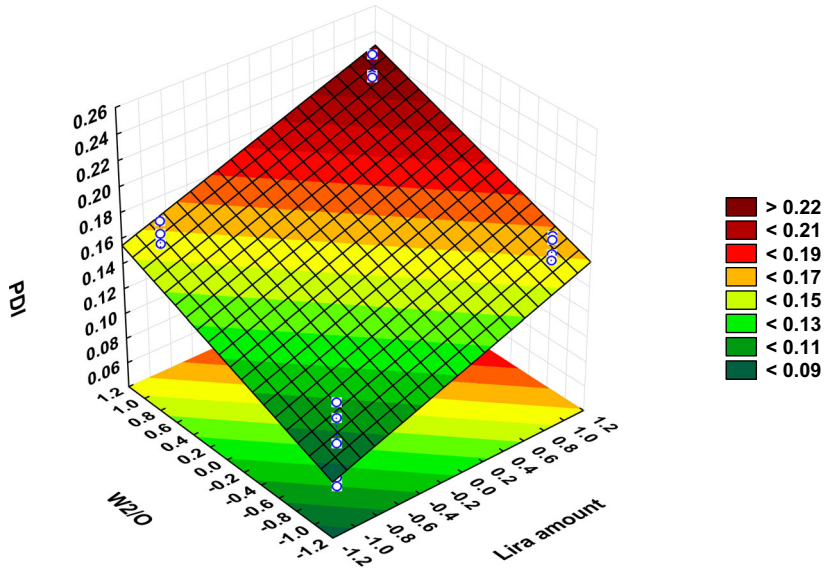

Fig. 3 Surface plot showing the effect of lira amount and W_2/O ratio on PDI (Y2).

Effect of PVA Concentration

PVA has previously proved to be a good choice as a surfactant used to prepare stable PLGA NPs with a small size and a narrow PDI due to its ability of minimizing the surface tension of the continuous phase which is an aqueous phase in our work. The statistical analysis of the results showed that increasing PVA level played a crucial role in decreasing particle size

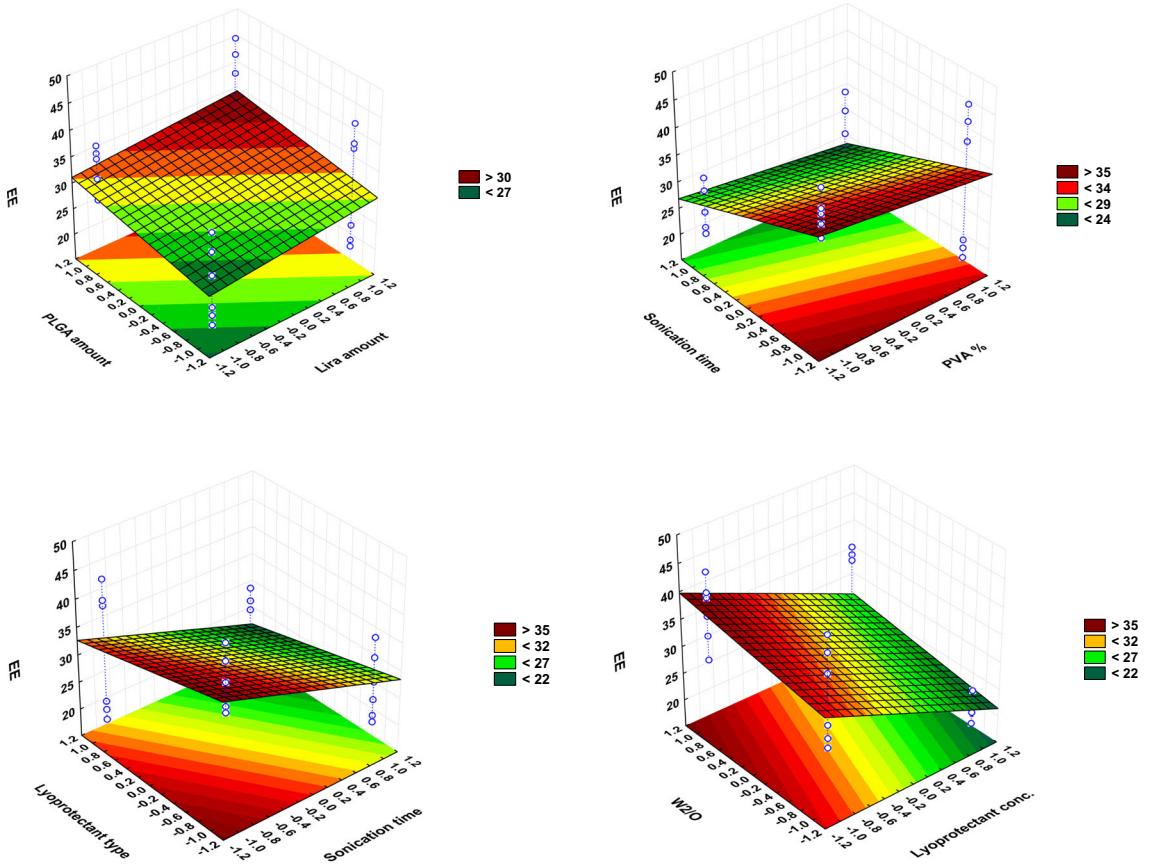

Fig. 4 Surface plot showing the effect of examined variables on EE (Y3).

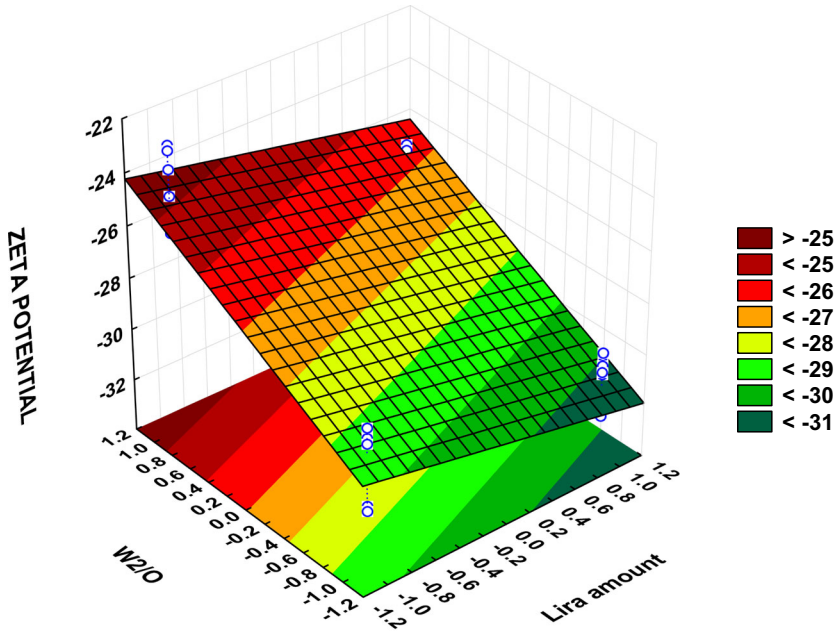

Fig. 5 Surface plot showing the effect of lira amount and W_2/O ratio on zeta potential (Y3).

(Fig. 2), and it was the leading factor impacting the size of NPs as can be seen in the Pareto chart (Fig. 1) which is in agreement with previous reports (31,32) .This result can be expected from the stabilizing function of PVA molecules that tend to align themselves at the droplet surface lowering the free energy at the interface between two phases and avoiding coalescence between nanodroplets, thus stabilizing the smaller droplets and preventing coalescence into a larger one (33,34). Hence, at a low PVA concentration, a larger particle size was obtained due to insufficient reduction in interfacial tension. It

Table 4 The Observed and the Predicted Values of the Response Values of the Center Checkpoints

Experimental response	Predicted value	Observed value (F1) / Bias%	Observed value (F2) / Bias%	Observed value (F3) / Bias%	Intermediate precision (%)
Z-average (nm)	197.8	195.8±2.5 / 1.0	202.7±5.8 / 2.5	196.4±4.1 / 0.7	3.8
EE%	30.5	29.5±1.8 / 3.3	31.2±2.2 / 2.3	33.1±3.3 / 8.5	1.8
PDI	0.20	0.19±0.01 / 5.0	0.19±0.01 / 5.0	0.18±0.003 / 10.0	0.01
Zeta potential (mV)	-28.1	-28.1±1.1 / 0	-28.8±1 / 2.5	-27.7±1.3 / 1.4	0.6

was also reported that a fraction of PVA remains associated with the surface of nanoparticles even after the washing of nanoparticles (33). Thus, the presence of the PVA layer on the surface of nanoparticles may also improve their stability during the freeze-drying process.

It is also clear that as PVA level increased from 0.5% to 2%, EE decreased (Fig. 4). A possible explanation of this negative impact was discussed in a preceding published work, as it was proved that the breakdown of the inner aqueous droplets containing Lira took place along with the fragmentation of the organic phase because of the cavitation occurs in the complex system of three phases, the higher level of PVA in the external aqueous phase is attributed to enhancing the breakdown of inner aqueous droplets and a higher amount of Lira can escape to the external phase as a result (34). This is supported by a previous paper in which increasing the emulsifier concentration had led to lower entrapment of the protein drug in PLGA NPs, which was explained as a result of increasing the partitioning of the drug from the inner to the outer phase (35).

Effect of 2nd Sonication Time

The results revealed that larger and less homogeneous particles were yielded when increasing 2nd sonication time from 30 s to 2 min (Fig. 1, Fig. 2). This could be explained as follows: at the beginning, increasing the sonication time led to the formation of smaller droplets due to the production of higher energy and higher shearing rates, which are more efficient in breaking large droplets into smaller ones (36). However, a further elevation in this sonication period resulted in the re-aggregation of these particles. This trend is in accordance with results obtained by others where they observed the formation of larger droplets as an outcome of longer sonication or homogenization time (37). Besides, the longer the 2nd sonication time, the higher the shear energy input, thus the higher the leached amount of peptide from W1/O to the external aqueous phase i.e. the lower the EE. The Pareto chart (Fig. 1) demonstrates that the prolonged 2nd sonication time was the 2nd highest risky factor regarding the influence on EE.

Effect of Lyoprotectant Type and Concentration

Lyoprotectant are commonly used to stabilize the particles and protect them from degradation during freeze-drying and storage (38). Regarding the type of lyoprotectant used in this study, these significant changes in particle size may be related with the behavior of each lyoprotectant during freeze-drying, and the adsorption of lyoprotectant on the surface of nanoparticles. It is clear that trehalose is more effective in obtaining smaller nanoparticles (Fig. 1, Fig. 2).This is in accordance with previous papers that confirmed that trehalose which is a non-reducing sugar could be the most preferable lyoprotectant of choice because of its merits over the other sugars; including a very low chemical reactivity, a higher glass transition temperature Tg, less hygroscopicity, in addition to the absence of internal hydrogen bounds, allowing a more flexible formation of hydrogen bonds with nanoparticles during the freeze-drying process (39). However, mannitol was proved to be more effective in obtaining a higher EE value according to our experimental work. Trehalose was investigated before regarding its effect on the secondary structure of insulin, and the results showed that it highly affected the conformational stability of the peptide; so it might not be the best choice to encapsulate peptide drugs (40). In addition to that; mannitol is able to form crystal morphology (which is confirmed later in this paper by DSC and XRD) and this might be attributed to the stability of peptide.

When it comes to the lyoprotectant level, results revealed that increasing this level up to 10% significantly reduced the Z-average and slightly minimized the PDI of the obtained NPs (Fig. 1,Fig. 2), which means that at this level the used lyoprotectants are more efficient in preventing the aggregation and stabilizing the PLGA NPs the use of an excess amount of lyoprotectant might eventually make it reach the limit of its stabilization ability and thus the agglomeration of NPs is likely to increase (41). It is also apparent from the statistical analysis presented in the Pareto chart and surface plot (Fig. 1, Fig. 4) that the lyoprotectant level was the most influential formulation variable impacting EE. When the level of lyoprotectant continued increasing, the amount of entrapped Lira significantly decreased, which could be the result of smaller NPs obtained with a higher lyoprotectant level, and thus less sufficient surface area for entrapping the drug.

Effect of W_2/O Ratio

As the volume ratio of external aqueous phase to organic phase W2/O went on increasing, which was achieved by increasing the volume of the external aqueous phase, the average particle size was significantly decreased as can be seen from the Pareto chart and surface plot (Fig. 1, Fig. 2). This formation of smaller droplets may be due to the higher amount of stabilizer present as compared to the non-sufficient amount of stabilizer when using a lower amount of this phase. It was also recently reported that increasing the continuous phase volume:organic phase ratio had led to particle size reduction (27).

Regarding PDI values, a significant increase in size distribution was observed when the volume of the external aqueous phase was higher, as shown by the Pareto chart and surface plot (Fig. 1, Fig. 3). This observation might be attributed to a reduction of shear stress during the homogenization process

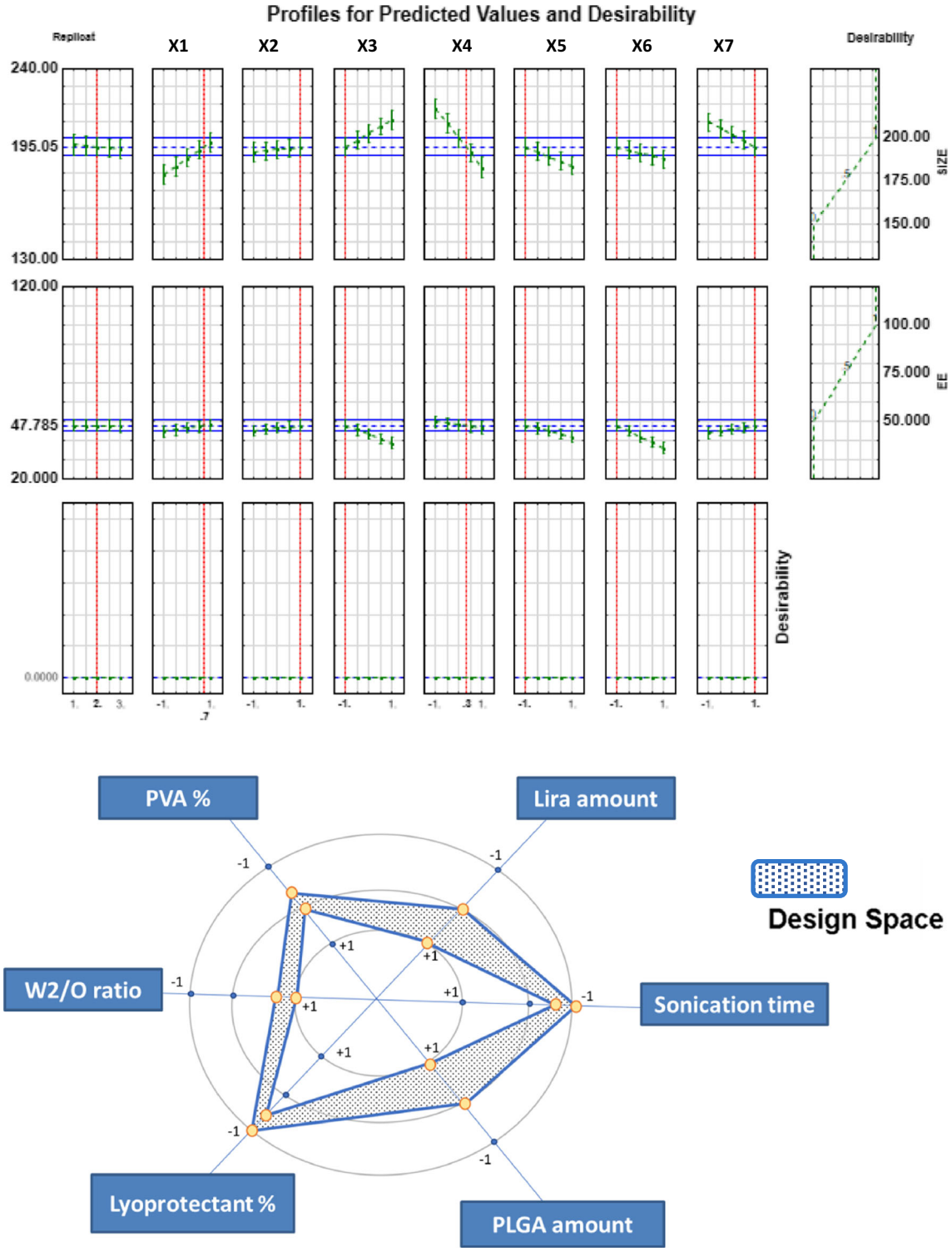

Fig. 6 The desirability plots and graphical design space representing the optimum levels of factors required to prepare the optimized formula.

(42). Besides, the phase ratio was the highly influential factor affecting the surface charge as increasing the external aqueous phase volume led to a significant increase in the zeta potential value (Fig. 5).

The EE exhibited a significant upward trend when increasing the volume of the external aqueous phase, as presented in the Pareto chart and surface plot (Fig. 1,Fig. 4). The impact of the external aqueous phase/organic phase is controversial, as

Table 5 The Observed and the Predicted Values of the response values of the Optimum Lira Nanoparticle

Experimental response	Predicted value	Observed value	Residual	Bias (%)
Z-average (nm)	197.9	189 ± 4.99	8.95	4.5
EE%	48.3	51.8 ± 2.39	3.5	7.2
PDI	0.21	0.19 ± 0.012	0.034	7.8
Zeta potential (mV)	−26.5	−27.1 ± 1.33	0.58	2.2

many papers reported that increasing the W2/O ratio can lead to minimizing the amount of the encapsulated drug (31,42). However, other published papers assumed that a relatively higher volume ratio of the external aqueous phase was beneficial for maximizing the drug encapsulated in the NPs as a higher outer aqueous phase volume can speed up the solidification time (evaporation of ethyl acetate and formation of NPs), while the smaller the volume of this outer aqueous phase, the longer the time required for solidification, thus over this time Lira may leak to the outer phase due to its hydrophilicity (43).

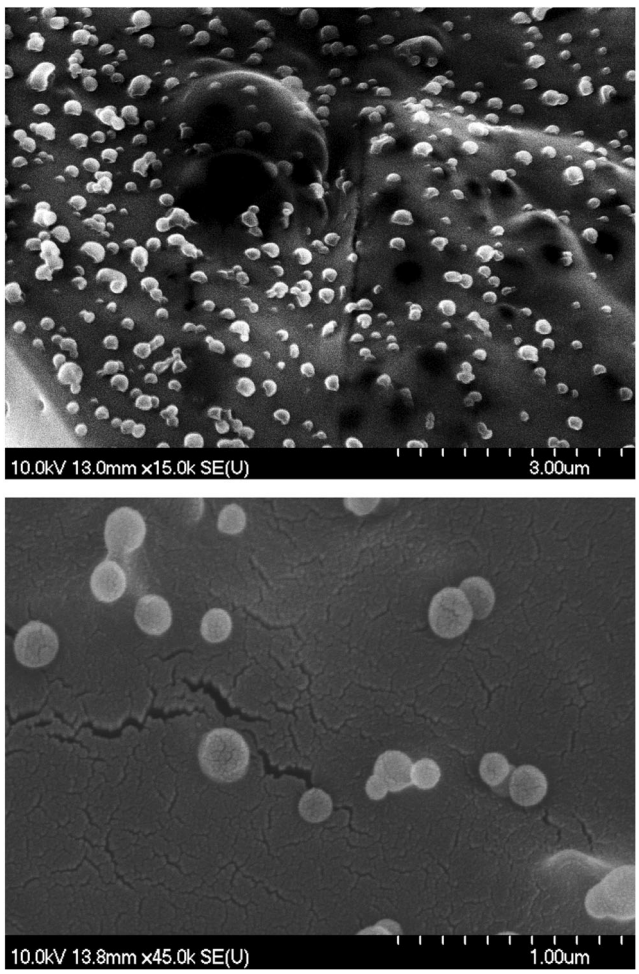

Fig. 7 SEM images of liraglutide loaded PLGA NPs.

Placket Burman Design: Model Validation

The three replications of center checkpoint formulations were prepared and evaluated for the particle size, EE, PDI and zeta potential to evaluate the reproducibility of the generated models and estimate the experimental error. Table 4 presents the percentage of bias between predicted and observed values for each response was calculated by means of the following equation .

$$\text{Bias (\%)} = (\text{Predicted value} - \text{observed value})/\text{Predicted value}^{*}100$$

The minor differences between the predicted values and the average of experimental values confirm the validity of this design in providing a good prediction of the four tested responses.

In addition to that, the calculated relative standard deviation RSD% values that are presented in Table 4 prove the repeatability and intermediate precision regarding the 4 responses that further confirms high analytical process variability.

Placket Burman Design: Design Space and Optimization

After establishing the polynomial equations describing the relationship between the CPPs, CMAs and the examined responses namely; particle size, EE, PDI and zeta potential, the optimization process was conducted. Among the four responses, size and EE were the highly critical quality attributes of nanoparticles being significantly affected by almost all the tested variables which is in accordance with the estimated severity scores of CQAs that was calculated previously at the initial risk assessment process. Therefore, the deign space (DS) was optimized (Fig. 6) targeting the following criteria: the particle size was minimized, encapsulation efficiency was maximized while PDI and Zeta potential were excluded. Thanks to the knowledge obtained via the DS, the optimum levels of the formulation factors were determined: 60 mg of PLGA, 5 mg of Lira, 0.5 min 2nd sonication time, 1.48% of PVA, 5% of mannitol and W2/O ratio of 5.As shown in Table 5, the observed values were comparable to the predicted ones, presenting another confirmation of the validity of the generated

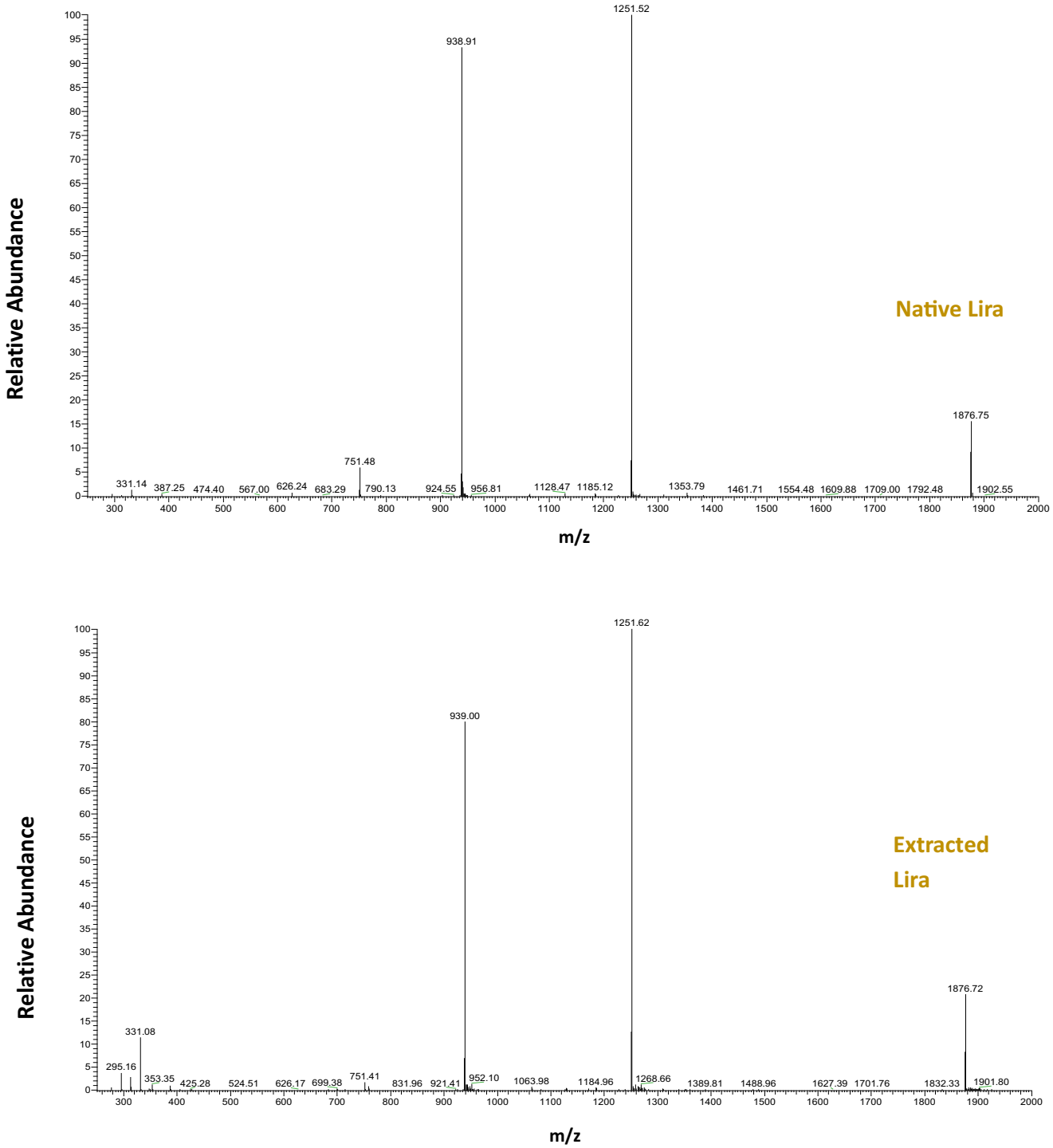

Fig. 8 Mass spectra of lira extracted from PLGA NPs compared to native lira.

models and indicating that the optimized formulation is reliable.

Scanning Electron Microscopy (SEM)

Figure 7 depicts the shape and surface morphology of the optimized Lira loaded PLGA NPs visualized by SEM. Since the optimized formula was homogeneous (in accordance with low PDI) we selected two images as representative for the sample. The results revealed that Lira loaded PLGA NPs were spherical with quite a smooth surface and they had homogeneous distribution which is in agreement with the above-mentioned results that demonstrated low PDI values for all formulations.

Compatibility Studies

Fourier Transform Infrared Spectroscopy (FTIR)

Figure S3. represents the FTIR spectra of pure Lira, pure PLGA and Lira free/loaded PLGA NPs.

The amide I region (1710–1590 cm^{-1}) is the most representative region of the spectra to assess peptide or protein based drug secondary structure (44). In the FTIR of pure Lira, the amide I band was located at 1655 cm^{-1} and was assigned to C=O stretching, while the amide II band was observed at 1541 cm^{-1} (in-plane N–H bending component and C–N stretching bands of the amide bond). Besides, the typical peak at 2928 cm^{-1} was ascribed to C-H stretching of CH3, and the peak at 1396 cm^{-1} is attributed to amide III. When analyzing the FTIR spectra of Lira loaded PLGA NPs, it was found that the major peaks of pure Lira assigned to amide I and II and III were masked by the PLGA bands, and this seems to be a logical result since the amount of PLGA was much higher than the Lira amount in these NPs. There were no clear differences between the spectra of the blank NPs and Lira loaded NPs which is also expected as the drug loading is very small when compared to the polymer amount. These observations suggest that Lira was successfully loaded into the PLGA NPs.

Differential Scanning Calorimetry (DSC)

Figure S4 represents the DSC thermograms for pure Lira, PLGA, liraglutide free/loaded PLGA NPs. For the temperature range examined, the PLGA thermogram exhibited a glass transition point at 49.22°C and no melting endothermic peak was observed, as PLGA appears amorphous in nature. The DSC thermogram of pure Lira revealed a peak at 275.46°C, which is attributed to the thermal degradation of this peptide drug, and no endothermic peak of melting was shown, which proved the amorphous nature of the drug. Since the thermogram of Lira loaded PLGA NPs did not display any extra endo/exothermic peaks compared to the blank NPs, this is an indicator of the presence of Lira in the amorphous phase and this drug is successfully encapsulated into the PLGA matrix.

X-Ray Diffraction Study (XRD)

XRD studies further verified the amorphous nature of both PLGA and pure Lira as they showed no characteristic peaks in their diffractograms which is in accordance with the results of DSC thermograms (Fig. S4). As depicted in Fig. S5, mannitol remained in crystalline state after freeze drying which is due to the property of mannitol to recrystallize at low cooling rates rather than rapid cooling. The crystallization of this lyoprotectant could have a negative effect on the stability of NPs as it is able to limit the formation of these hydrogen bonds (30), and this can explain why trehalose was more efficient than mannitol at preventing the aggregation of NPs and thus minimizing the Z-average. There was no difference between the diffractograms of the loaded and blank PLGA NPs which is explained in literature as a result of the successful encapsulation of the peptide drug inside the polymeric nanoparticles without change in its physical state (45), and this is in accordance with the DSC results.

Structural Stability

ESI-MS

MS was used to compare the molecular weight MW of lira standard (native) to lira extracted from PLGA NPs. As the spectra in Fig. 8 depicts, the measured MW of native Lira and Lira loaded in NPs are almost equal, and the spectra confirmed the presence of Lira with a molar mass of 3751 Da which is an evidence of the integrity of Lira loaded in PLGA NPs prepared using the optimized formulation and process parameters.

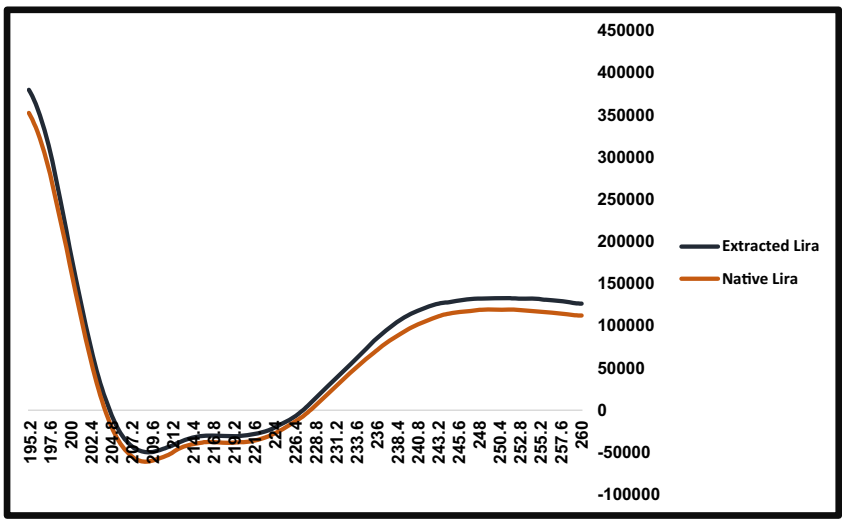

Fig. 9 CD spectra of lira extracted from PLGA NPs compared to native lira.

CD

Since the preservation of the secondary structural integrity of a peptide drug in the nanocarrier is critical for its biological efficacy, the secondary structure of Lira extracted from NPs was compared to that of native Lira. The CD spectra of native Lira (Fig. 9) showed two minima at 208.8 nm and 218.4 nm indicating the presence of alpha helix elements in the structure, which is in consistent with previous studies on the typical structure of the glucagon-like peptide-1 family. No significant conformational change was recorded for Lira extracted from PLGA NPs (in PBS, pH = 8.1) as the far UV CD spectra for it showed two minima at 209.4 nm and 219.2 nm, and almost entirely overlapped with the CD spectrum for the standard.

CONCLUSION AND FUTURE PERSPECTIVES

The present study is the first published work that substantiated the application of rational QbD-based methodology for the optimization of a GLP-1 analog loaded nanocarrier system. This work demonstrated the importance of implementing DOE within QbD philosophy in the early stage of Liraglutide containing NPs development due to the complexity of this system.

After establishing the design space, with the minimum particle size and maximum EE, the optimized formula was successfully prepared meeting the targeted CQAs. This optimized Lira loaded PLGA NPs formula was also successful in maintaining the native structure of Lira and could be promising for the oral delivery. Thus, *in vitro* release kinetics, cytotoxicity, intestinal permeability and *in vivo* studies will be further conducted on this formula.

ACKNOWLEDGEMENTS

This research was supported by the EU-funded Hungarian grant EFOP-3.6.1-16-2016-00008.

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PUBLICATION III.

European Journal of Pharmaceutical Sciences 122 (2018) 160–169

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Initial Risk Assessment as part of the Quality by Design in peptide drug containing formulation development

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ARTICLE INFO

Keywords:
Quality by Design
Pharmaceutical Risk Assessment
Peptide formulation
Oral peptide delivery
GLP 1 analog
Liraglutide
Polymeric nanoparticles

ABSTRACT

Risk Assessment (RA) is the key element of the Quality by Design (QbD) approach recommended by the pharmaceutical regulatory bodies. This research paper aimed to implement the regulatory requirements, the QbD thinking and the RA from the first steps of the oral peptide formulation development. The authors intended to give a general recommendation about the application possibilities of this methodology, to demonstrate the risk factors and the required decision points. Later, this paper presents a concrete development in practice. This case study shows the QbD and RA based early phase development of the GLP 1 analog, Liraglutide, an antidiabetic peptide drug mainly used in the treatment of type 2 Diabetes Mellitus. The objective here was to design Liraglutide encapsulated polymeric nanoparticles for oral delivery and the progress of their RA based development is presented. In this case, the particle size, the encapsulation efficiency, and the drug loading were found as the most critical quality attributes, the polymer concentration, the drug concentration, the w2/o ratio, the stabilizer concentration and polymer type were identified by the criticality rating as having the greatest impact on the product quality among the critical material attributes, finally the sonication time and sonication power were selected as the most critical elements of the production process. The results showed the importance of the risk factor-focused development in the oral peptide pharmaceutical formulations, and underlined the importance of the profound planning phase even in such cases. The formulation of an oral peptide delivery system is associated with several risks, but their priority ranking helps to focus on the resources (human, financial, time) related to the final product quality aimed at.

1. Background

1.1. Risk Assessment and the Quality by Design

The QbD approach is a holistic, systematic, knowledge and risk based methodology of pharmaceutical development approach, which is proactive and focuses on profound preliminary design (Yu et al., 2014; Yu, 2008). It represents the main stream of the pharmaceutical technological formulation development nowadays (Pallagi et al., 2015; Bhise et al., 2017; Pallagi et al., 2016; Lee et al., 2017; Kovács et al., 2017). QbD has several steps, which are described in the relevant guidelines of the International Council of Harmonisation (ICH), namely in the ICH Q8R2, Q9, Q10 papers (Pharmaceuticals International Conference on Harmonisation of Technical Requirements for Registration for Human Use, 2009; T. I. C. on Harmonisation, 2008; ICH Expert Working Group, 2005). The implementation of QbD in the manufacturing of medicinal products is often called the “GMP of the 21st century”; regulatory authorities strongly force the pharmaceutical industry to apply this strategic planning approach (Lee et al., 2017; Kovács et al., 2017). The adaptation and application of this methodology into the early research phases within the R&D activities holds many advantages, as mentioned in our previous papers. QbD based early development brings scientific results closer to the practical requirements and has a facilitating effect on industrial scale up and product transfer to the market. The main elements of QbD are: (1) the definition of the Quality Target Product Profile (QTPP), (2) the identification of the quality attributes and the selection of the Critical Quality Attributes (CQAs) related to the target product, (3) the prior selection of the production method and the identification of the Critical Process Parameters (CPPs), (4) performing of the initial Risk Assessment (RA). The results of RA will be the ordered CQAs and CPPs according to their calculated risk severity. The next step is the (5) setting up of the Design of Experiments (DoE) based on the highly risk related factors calculated previously in the RA step. This DoE is generally a factorial design and the aim is to determine the process and product Design Space (DS) (6), which means a multidimensional space and is

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https://doi.org/10.1016/j.ejps.2018.07.003
Received 19 February 2018; Received in revised form 2 July 2018; Accepted 2 July 2018
Available online 03 July 2018
0928-0987/

determined by ranges of process elements and material attributes. Then it is needed to determine the Control Strategy (7), followed by the evaluation of the possibilities of Continuous Improvement (8). Risk Assessment is a key element in general, and it is especially advantageous in the case of complex drugs (e.g. peptides) and/or carrier systems of special risk (e.g. nano-delivery systems).

1.2. Peptide drug delivery and new formulation possibilities

The interest in the pharmaceutical technology utilizing therapeutic peptides in the treatment of a variety of diseases has dramatically increased over the past few years. However, these peptides suffer from several drawbacks, including metabolic liability, poor permeability across biological barriers, and fast hepatic clearance and subsequent inherent short half-lives, which all lead to the low bioavailability of such drugs. Hence, peptide administration is usually limited to parenteral routes such as subcutaneous, intravenous, and intramuscular administration, which are regarded as invasive routes associated with many downsides leading to insufficient patient adherence. Thus, a patient friendly non-invasive route of administration such as the oral route is desired in order to overcome the various drawbacks associated with the invasive delivery route. However, an orally administered peptide drug encounters numerous formidable obstacles, including chemical and enzymatic instability in addition to the limited ability to traverse biological barriers (Ahn et al., 2013). Besides these barriers, the stability of the peptide drug during formulation and storage is a crucial aspect to be investigated when developing a peptide delivery system as peptides are sensitive drugs that can be damaged or become inactivate almost in every step of the production method (Patel et al., 2011).

The selected drug for this case study is Liraglutide, a fatty acid modified glucagon like peptide-1 (GLP-1), which shares 97% amino acid sequence identity with human GLP-1, and is produced by recombinant technologies using yeast. This peptide drug was approved for the treatment of type 2 diabetes mellitus (Bode, 2012), and Novo Nordisk has recently begun marketing it for the chronic weight management for obese or overweight adults who have associated comorbidities, such as hypertension, diabetes and dyslipidemia (Mehta et al., 2017). Since Liraglutide is still delivered parenterally, the oral route should be aimed at, providing the patient friendly administration in addition to mimicking the physiological route of GLP-1 from intestine to circulation. Still, enhancing the oral bioavailability of Liraglutide presents an interesting challenge and the development of a novel oral Liraglutide delivery system is regarded as a high risk and high reward process.

Tremendous efforts have been devoted over the past decades to the oral administration of antidiabetic peptides. Based on our previous review and the evaluation of recently published papers in the field of antidiabetic peptide oral delivery (Ismail and Csóka, 2017; Ismail and Csoka, 2017a), it can be concluded that among several techniques to improve antidiabetic peptide oral bioavailability, much of the success was recorded when using polymeric nanoparticles (NPs). And among the numerous polymers used to obtain polymeric NPs, poly lactic-*co*-glycolic acid (PLGA) is the most widely used one, as it is a biodegradable and biocompatible synthetic polymer approved by the Food and Drug Administration (FDA, USA) (Malathi et al., 2015). PLGA NPs were found to be successful in the protection of peptides from harsh environment in the gastrointestinal tract, thus enhancing stability, in addition to other merits of nanocarrier systems, which can all lead to improving the oral bioavailability of these drugs as in insulin, GLP-1 or its analogs (Ismail and Csoka, 2017a; Sharma et al., 2015; Araujo et al., 2016; Ismail and Csoka, 2017b).

The aim of this study was to evaluate the role of RA in the case of the development of a Liraglutide drug delivery system for oral administration. Based on a careful collection, selection and evaluation of the relevant literature together with the previous developments of the research group in this field (Kovács et al., 2017; Karimi et al., 2016; Kovács et al., 2016), we aimed to set up a strategic flow chart with proposed RA function and decision steps. The selected pilot study focused on novel Liraglutide loaded PLGA NPs prepared by means of the double emulsion solvent evaporation method and the application of the QbD concept in order to optimize the formulation by evaluating the effect of different formulation and process parameters on the quality of the aimed product.

2. Materials and methods

2.1. Materials

Liraglutide was purchased from Xi'an Health Biochem Technology Co., Ltd. (China), Poly(lactide-*co*-glycolide) (PLGA 50:50, Mw = 30,000–60,000 Da), PVA (MOWIOL 4–88, MW = 27,000 Da), and D-(+)-Trehalose dihydrate (MW = 378.33 g/mol) were purchased from Sigma Aldrich (Germany). D-(-)-Mannit was purchased from Molar chemical KFT (Hungary). Sodium acetate anhydrous was purchased from Scharlam Chemie S.A. (Spain). Ethyl acetate which used for dissolving PLGA was from REANAL Labor (Hungary).

2.2. Methods

2.2.1. Knowledge space development

According to the QbD nomenclature, the collection and systematization of the relevant scientific literature and experience from the previous research is called “knowledge space development”. The methodology was the analysis of the relevant scientific literature, structural development of data-collection. Modern quality management tools were used for visualization, such as preparation of Ishikawa diagrams (Ishikawa, 1976) and building up of flow charts for process description and systemic evaluation (Iso Iec, 1985).

2.2.2. Definition of QTPP

The QTPP forms the basis of product development design. It should include patient-relevant product performance and characteristics related to the aimed therapeutic or clinical use. Considerations for QTPP selection are described in the ICH Q8R2 guideline, e.g. the route of administration, dosage form, delivery system, pharmacokinetic and other product quality criteria (e.g. sterility, stability and drug release), etc. The QTPP is always unique depending on the target.

2.2.3. Determination of the CQAs

Those factors which have critical influence on the QTPP linked with safety, quality or efficacy are CQAs. CQAs are generally associated with the drug substance, excipients, intermediates (in-process materials) and drug product. A CQA is a physical, chemical, biological, or microbiological property or characteristic of the output material (product) that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are connected strictly to the product. The selection of CQAs needs carful design and a holistic view of the formulation development and is based on previous knowledge and experience.

2.2.4. Determination of the CMAs and CPPs

CMAs are critical material attributes, physical, chemical, biological, or microbiological properties or characteristics of an input material (Zhang and Mao, 2017). CPPs are process parameters whose variability has a critical effect on the aimed product performance. CPPs and CMAs

are linked to the selected production/formulation process. The selection of the CMA and CPP is based on prior knowledge, previous practical investigations and data from the relevant literature.

2.2.5. Initial Risk Assessment

Initial Risk Assessment aids in identifying which material attributes and process parameters potentially have an effect on product CQAs (ICH Q8R2). On the basis of prior knowledge and initial experimental data, RA tools can be used to identify and rank parameters (e.g. process, equipment and input materials) with the potential of impacting on final product quality. The initial RA was performed with Lean QbD Software (Lean QbD® Software, QbD Works LLC. USA, CA, Fremont) to evaluate the risks and get the Risk Priority Number (RPN) data. First, the interdependence rating was performed between CQAs and CMAs, CQAs and CPPs on a three-level scale and categorized as “high” (H), “medium,” (M) or “low” (L). After the interdependence rating, an occurrence rating related to the selected CQAs, CMAs and CPPs was also made. The whole risk estimation resulted in calculated and ranked severity scores of CQAs, CMAs and CPPs, presented in Pareto charts generated by the software. The software also generates the relative occurrence-relative severity chart, depicting the critical factors in four different groups according to their estimated occurrence and severity (or the degree of their impact if they occur) estimated as the result of the RA. This affords a different presentation mode of the RA results. In this manner the upper right corner of the generated figure needs the highest attention as it represents those critical factors which have the highest risk of occurrence and have great impact on quality.

Generally, RA results show the factors with the highest impact on product quality, which are usually the key elements of the systemic designed experiments in practice.

2.2.6. Preparation of nanoparticles by double emulsion solvent evaporation technique

The process of the Liraglutide encapsulation by double emulsion solvent evaporation technique includes the following steps: first, formation of a primary w1/o emulsion, where the aqueous solution of the peptide drug is added to the polymeric organic solution upon sonication in ice bath. This was followed by the formation of a double emulsion (w1/o/w2) by dispersing the primary emulsion in an external aqueous phase containing poly vinyl alcohol PVA as a stabilizer, with the use of sonication in ice bath. Finally, organic solvent evaporation resulted in the formation of Liraglutide loaded nanoparticles.

3. Results

3.1. Theoretical evaluation of the Risk Assessment based oral peptide drug formulation development - General considerations

Oral peptide drug delivery has several challenges and many risks because peptide drugs are more complex and heterogeneous in nature than chemical drugs (Fosgerau and Hoffmann, 2015; Muheem et al., 2016; Lennernäs et al., 2014). Every product is unique with several risks related (Bak et al., 2015). Also, the European Medicine Agency (EMA) says that: “Biological products possess such a large number of quality attributes that it might not be possible to fully evaluate the impact on safety and efficacy of each one. Risk assessments can be performed to rank or prioritize quality attributes”. So RA is a key element in the formulation of biological and even in the oral peptide delivery preparations. Fig. 1 presents the collected potential risk factors which should be taken into consideration when developing a new oral peptide dosage form (Fig. 1A).

It is very important to note that besides the guidelines which describe the classical QbD (ICH Q82R, Q9 and Q10), an extension has to be made with the ICH Q11 guideline (European Medicines Agency, 2016) about the development and manufacturing of drug substances as shown in Fig. 1, part B., since special attention needs to be paid when the active pharmaceutical ingredient is biological, like peptide.

In the next step of the knowledge space development phase of this study, the following Ishikawa diagram was set up as a result (Fig. 2.). The Ishikawa diagram, as a quality management tool, can help to explore the cause and effect relationships. The diagram marks all the influencing factors related to peptide formulation for oral delivery in a detailed manner. These influencing factors can be the potential risks, namely CQAs, or CPPs depending on the aimed product. On the other hand, in the case of biologicals, the effect of the CQAs and CPPs on the QTPP are more difficult to understand, and the interactions are more complex with other risk aspects related to the safety, efficacy, immunogenicity, pharmacokinetics, bioactivity, etc. of the final drug product (Rathore, 2009). Rathore A. gives a thorough description of the relationships between the quality criteria of the biotechnology product and their safety and efficacy (Rathore and Winkle, 2009). On the other hand, the monoclonal antibody formulation study by Awotwe-Otoo et al. draws attention to the special elements of a biologic QTPP, such as elements regarding the reconstitution of previously lyophilized formula (e.g. reconstitution time, isotonicity, aggregation, etc.), which underlines the need for complex and careful thinking in this field (Awotwe-Otoo et al., 2012).

Besides the several different CQAs of a peptide formula, the formulation process development could have many additional problems, i.e. the technology transfer may be difficult due to the sensitive nature of the peptide drug as small changes in either the formulation composition or in the manufacturing steps can have great effect on the final quality (Winkle and Nasr, 2011; Jain, 2014).

The flow chart, prepared by the authors illustrates the suggested decisions and their order when formulating a new dosage form containing a peptide drug substance (Fig. 3).

The therapeutic target area (“unmet clinical need”) should be defined first, which gives the main framework for the drug substance (active pharmaceutical ingredient, API) selection. The peptide type drug has to be characterized next, namely the physio-chemical properties, therapeutic activity profile, physical, chemical or structural stability, impurities, and the fact whether a surface modification is needed or not in order to fit the purpose. In connection with API profile building, it is required to define the desired therapeutic targeted place, either local or systemic. If the aimed effect is local, the drug size is not critical, immunogenicity is reduced, and there is no need for using permeability enhancers. If the new peptide formula is designed to have a systemic effect, the size is critical because of absorption, and the risk of immunogenicity is also increased. Immunogenicity is one of the most critical elements, so each aspect that has an influence or a potential influence on it needs special attention. Excipients could also be risk factors, as it is necessary to use permeability enhancers for better absorption, and enzyme inhibitors in order to protect the peptide from inactivation. This is also the point where the administration route of the planned new dosage form has to be decided. If the oral route is targeted, the following characteristics could be critical, for example: size, charge or electrostatic interactions, surface polarity, bioadhesive properties, lipophilicity, PEGylation, surface ligands. The dosage form selection is also part of the development design, and will be part of the QTPPs at the end as well. Depending on the targeted dosage form (e.g. conventional capsule, tablet like solid forms or emulsion, suspension, liquid formulas etc.), special considerations have to be made regarding excipients (permeation enhancers, protease inhibitors, enteric coating materials, artificial proteins, protective antibodies, etc.) and the proper production process has to be selected. Related to that phase of the design, the preliminary definition of other requirements is essential. These

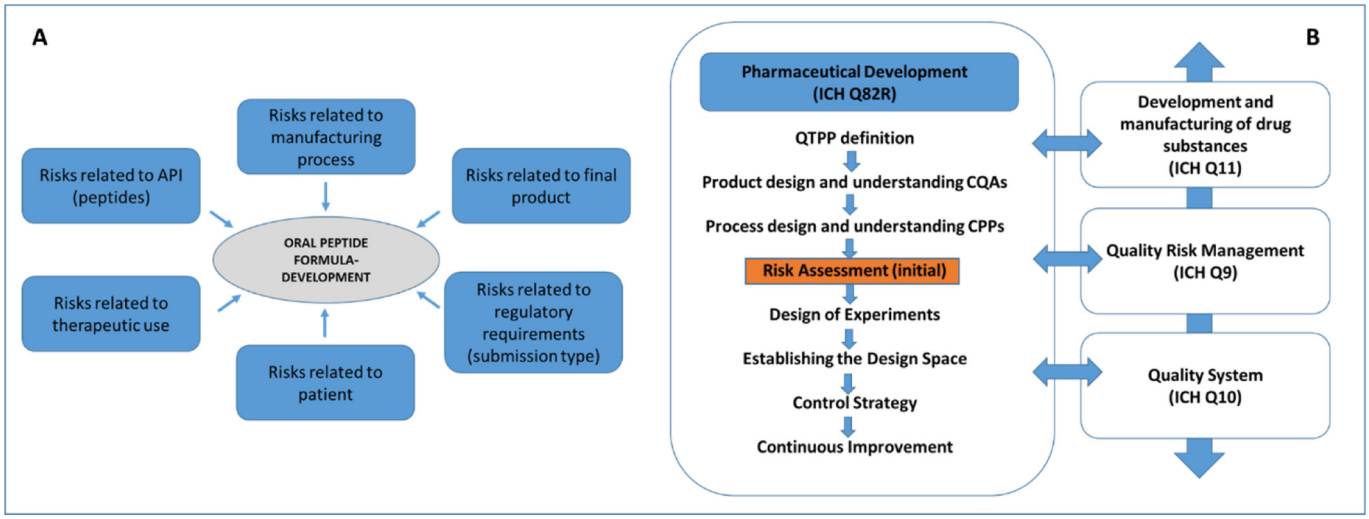

Fig. 1. Potential risks to be considered in the development of a new oral peptide containing drug (A) and steps of the extended QbD method (B).

requirements include the dissolution profile, stability, impurities, permeability related characteristics, etc. The production process is also highly critical in the case of peptide formulation, due to their sensitivity. In this consideration, the selected manufacturing process has to be evaluated carefully from the risky aspects and the CPPs have to be selected. Other decisions should be made concerning regulatory and industrial expectations. From these aspects, costs planning can be a critical point of decision as there could be great differences in the marketing authorization costs between an originator and a biosimilar medicinal product.

The left part of Fig. 3 will generally give the basis of the QTPP definition, and the other sections of the chart could give the basis of the CQA and CPP selection for the different targeted peptide formulations. After the selection step the RA can be performed.

3.2. *Practical implementation: Results of the Risk Assessment of a Liraglutide containing drug formulation development for oral delivery - Practical findings*

The study presented below is intended to show how the previously introduced risk based theoretical model can be applied in the early phase of peptide drug containing formulation development. Based on reviewing the most relevant strategies (Ismail and Csóka, 2017), Liraglutide loaded PLGA NPs are to be prepared by using the double

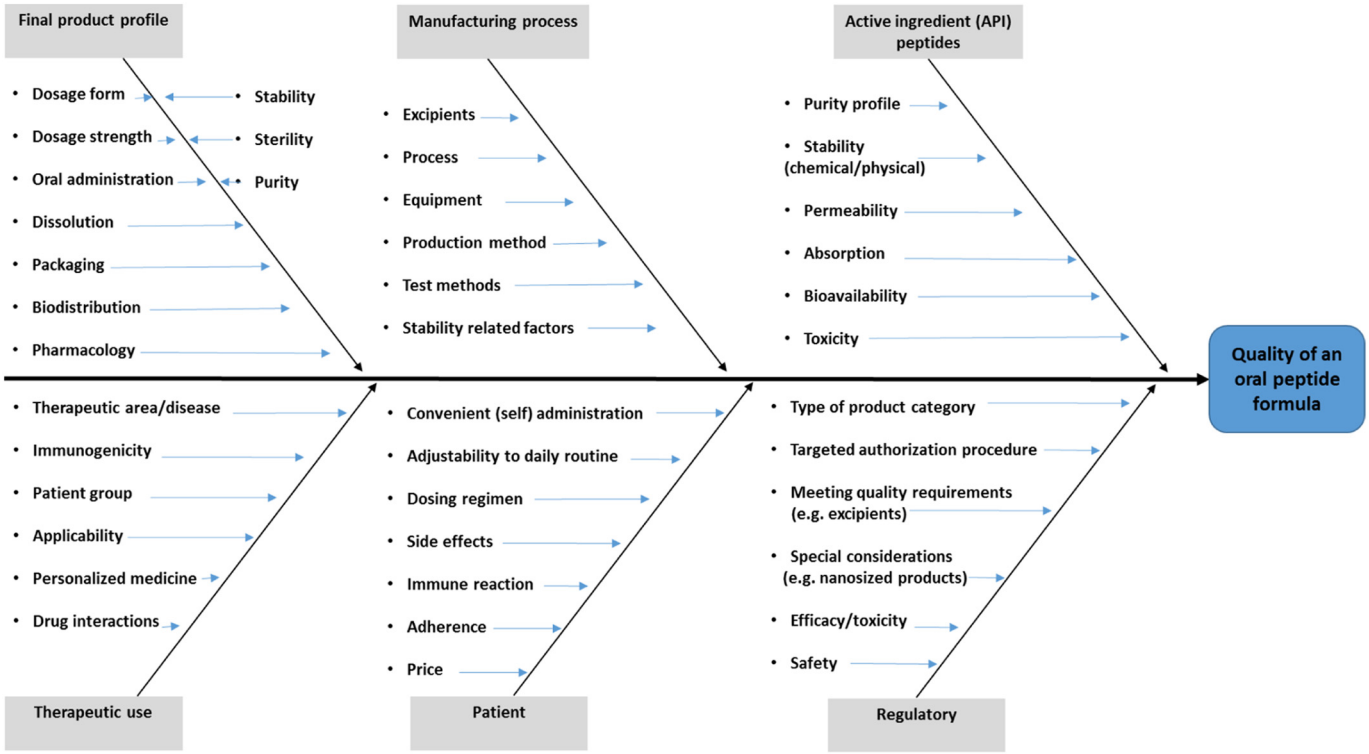

Fig. 2. Ishikawa diagram for evaluating risks in general, related to the quality of an oral peptide drug development.

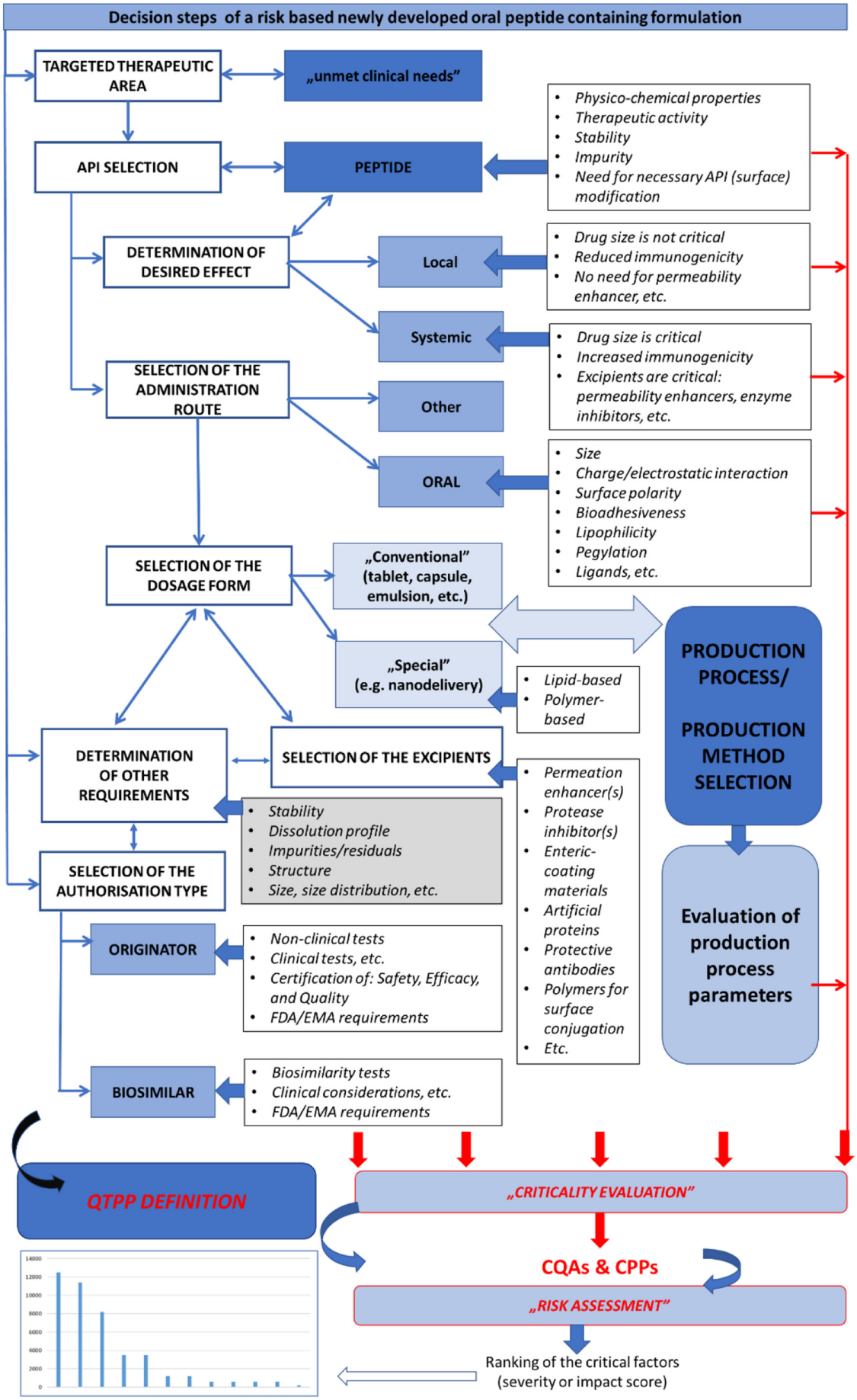

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Fig. 3. Flow chart of decision steps involving Risk Assessment for the development of a peptide formulation.

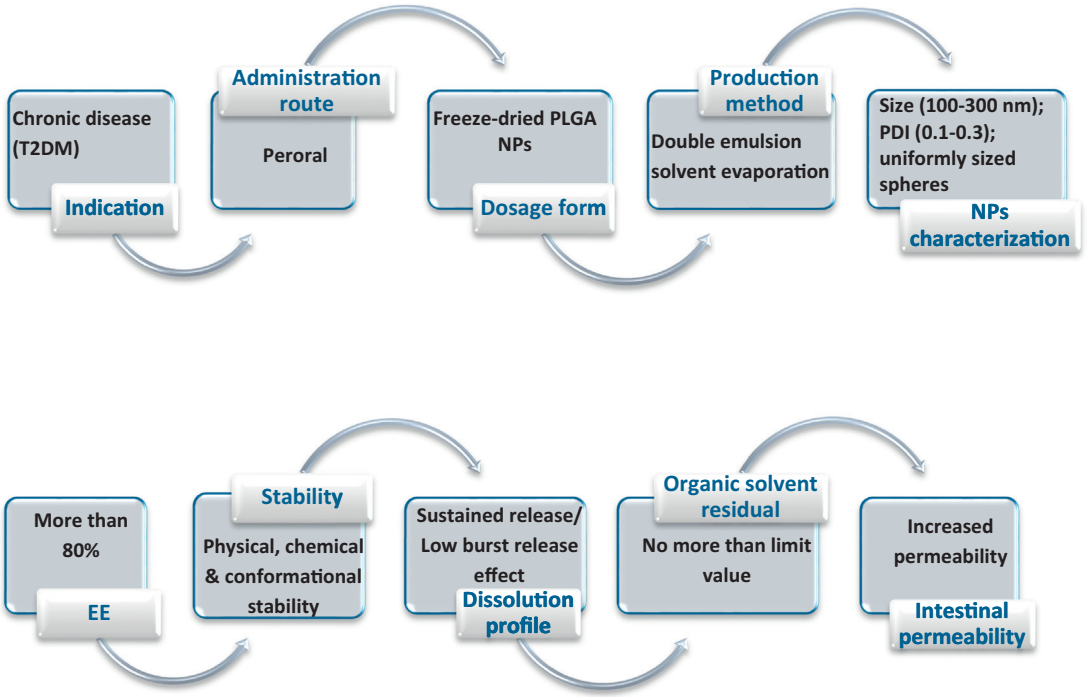

Fig. 4. Defining the QTPP for Liraglutide loaded PLGA NPs.

emulsion solvent evaporation method, well-known (Sipos et al., 2005) as a suitable choice for encapsulating hydrophilic drugs, particularly protein and peptide drugs (Ramalho and Pereira, 2016; Iqbal et al., 2015).

According to the guideline, the initial step of this QbD based study aiming to design oral Liraglutide loaded PLGA NPs was to set up the QTPP as shown in Fig. 4.

Then, the proposed CQAs that could critically affect the desired QTPP were identified. This was followed by selecting CMAs and CPPs that may have a significant effect on the CQAs of the lira-PLGA NPs, and the Ishikawa fish bone diagram (Wang et al., 2014) was constructed to illustrate these potential formulation and process variables likely to impact the quality of the lira-PLGA NPs (Fig. 5).

The initial RA study was achieved by means of the "Lean QbD" software. Fig. 6 depicts the interdependence rating on the three-point scale between the selected CQAs and CMAs on the one hand, and CQAs and CPPs on the other hand.

The calculated and ranked severity scores for the CQAs, CMAs and CPPs are presented in Pareto charts generated by the Lean QbD Software as shown in Fig. 7.

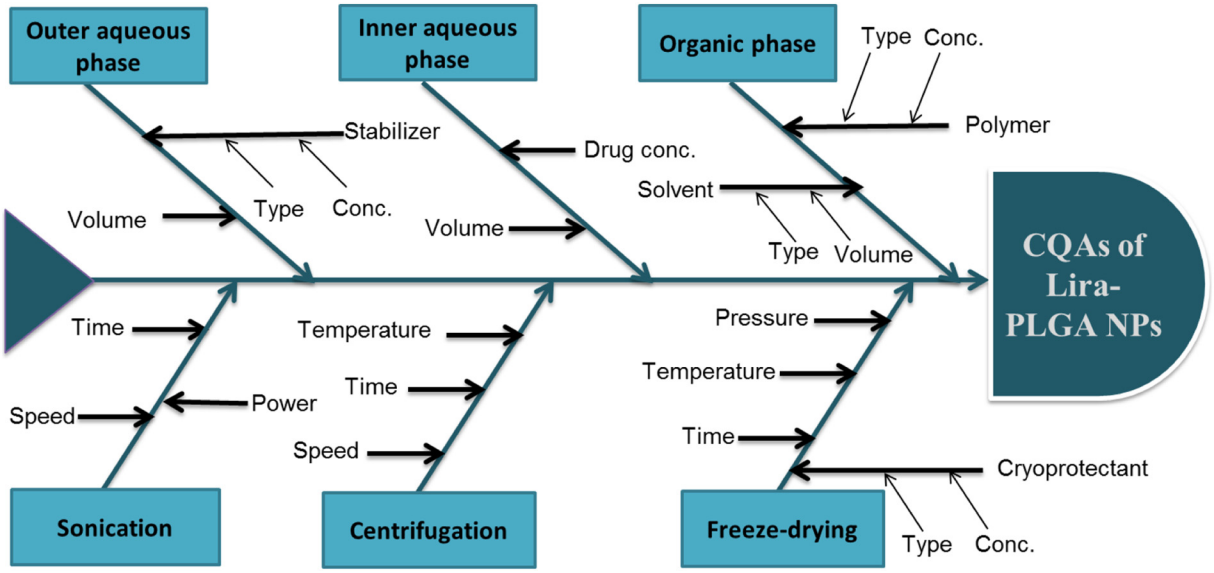

Fig. 5. Formulation and process parameters affecting the CQAs of lira-PLGA NPs.

A

Process	Double emulsion solvent evaporation							Freeze drying	
CMAs / CQAs	Polymer type	Polymer conc.	Drug conc.	Stabilizer type	Stabilizer conc.	Organic solvent type	W2/O ratio	Cryoprotectant type	Cryoprotectant conc.
Particle size	M	H	H	L	H	L	H	H	H
PDI	M	L	L	M	L	M	L	L	L
Zeta potential	H	L	L	M	L	M	L	L	L
EE	H	H	H	H	H	H	H	M	M
DL	H	H	H	H	H	H	H	M	M

B

Process	Double emulsion solvent evaporation					Freeze drying	
CPPs / CQAs	Sonication time	Sonication power	Centrifugation time	Centrifugation speed	Centrifugation Temp.	Freeze-drying time	Freeze-drying temp.
Particle size	H	M	L	L	L	L	L
PDI	M	H	L	L	L	L	L
Zeta potential	H	L	L	L	L	L	L
EE	H	M	M	M	L	L	L
DL	H	M	M	M	L	L	L

Fig. 6. RA interdependence ratings: (A) the interdependence rating matrix between the selected CQAs and CMAs, and (B) between the CQAs and CPPs.

Besides, Fig. 8 presents the occurrence rating of the CMAs and CPPs, and here we selected the most potential factors with the highest occurrence and severity rate to be subjected to further investigation, applying a suitable design of experiment that enables the statistical optimization of the Liraglutide containing nanoparticles by defining the optimal value of each examined variable.

4. Discussion

The main steps and elements of oral peptide formulation development were evaluated as peptide type drug substance containing formulations can be handled as high risk dosage forms due to their complexity in both composition and preparation design. Initial Risk Assessment should be considered as a crucial part of the development process.

Classic drug development works with small, chemically manufactured active substance molecules. Biologic drugs, such as peptides, are biologically produced large molecules. Compared with conventional small drug formulation, peptide containing dosage form development has many challenges. For example, oral delivery systems for peptides need special considerations as the active agent has to be protected against digestion in the stomach and intestines, whereupon they become ineffective. The right technological formulation technique in addition to the proper selection of the excipients (permeation enhancers, protease inhibitors if needed, coating materials, etc.) could be the solutions to peptide stability and protection. The sensitivity of the biologicals should be the most focused area during their formulation development. In this regard, the main findings are the following: the prior ranking of the CQAs and CPPs which are mainly related to stability in the peptide formulation helps in more effective experimental design and the prioritization of the limited development sources. The identification of the relative risk levels at the beginning of product development has great advantages in such complex formulation.

The highly focused RA fields in peptide drug formulation are the following: (1) The drug substance, especially characteristics related to its stability and quality. (2) The manufacturing process, also related to the drug substance and product stability. Small changes in the production process could have a great effect on quality thus influencing the safety, efficacy and side effects related to the therapeutic application. These are strongly connected to the immunogenicity aspects (3).

Regarding the RA based study of Liraglutide encapsulated in PLGA NPs system prepared by double emulsion solvent evaporation technique for enhancing the oral bioavailability of this peptide drug, the QTPP was set up as presented in Fig. 6 based on prior knowledge regarding the peptide delivery, PLGA NPs formulation and methods of preparation, in addition to our initial experimental data. This was followed by the identification of CQAs, namely: particle size, zeta potential, polydispersity index (PDI), encapsulation efficiency (EE) and drug loading (DL). Then, initial RA was performed by identifying the CMAs and CPPs (regarding the double emulsion evaporation method) that may have a high risk of impacting the CQAs of the Liraglutide loaded PLGA nanoparticles (Yerlikaya et al., 2013). Here, the Ishikawa fish bone diagram was constructed to configure the risk analysis process for defining the cause and effect relationship between the significant variables and the CQAs of the desired lira-PLGA NPs (Dhat et al., 2017), and it can be seen from Fig. 7 that six main formulation (outer aqueous phase, inner aqueous phase, organic phase) and process (homogenization, centrifugation, freeze-drying) causes were identified. Further RA using

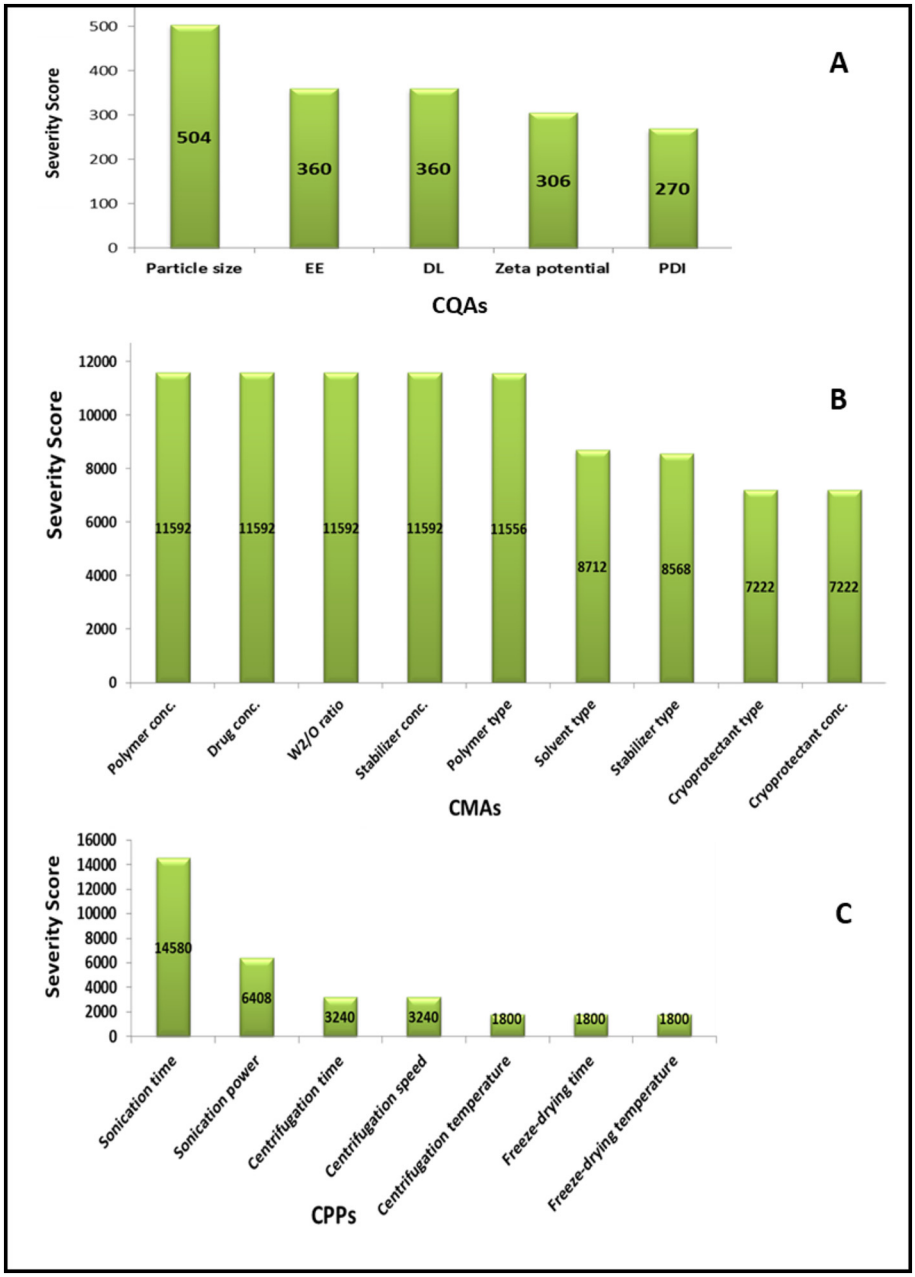

Fig. 7. Pareto charts A- Estimated severity scores of the proposed CQAs, B- Estimated severity rating of the proposed CMAs, C- Estimated severity rating of the proposed CPPs (PDI: polydispersity index, EE: encapsulation efficiency, DL: drug loading).

Lean QbD software showed that the most highly influential CPP (Figs. 6B, 7C, 8B) is sonication time, while the most highly influential CMAs (Figs. 6A, 7B, 8A) are polymer concentration, drug concentration, stabilizer concentration, cryoprotectant type, cryoprotectant concentration and external aqueous phase to organic phase ratio w2/o.

To optimize process and formulation parameters, all these potential parameters will be subjected to further investigation with the use of a screening statistical design of experiment (Narayanan et al., 2014) in order to minimize their risk to a low level by controlling theses variables in a specific accepted range in order to obtain design space, thus assuring the desired CQAs values, to comply with the QTPP.

5. Conclusion

Several challenges and many risks are entailed in formulating dosage forms containing peptide drugs, which are more complex and heterogeneous in nature than chemical drugs. Thus, it is advisable to apply QbD based formulation development in order to save time and effort by directing the effort toward building the quality in each step of peptide delivery system development, which includes RA as an initial part of this QbD based process. This RA focused approach of the peptide pharmaceutical formulation development is essential as it results in ranked and prioritized risk factors, thereby leading to an effective formulation development. This study can help researchers to implement RA focused thinking and the QbD approach in their peptide formulation development if they follow the general steps of decisions presented

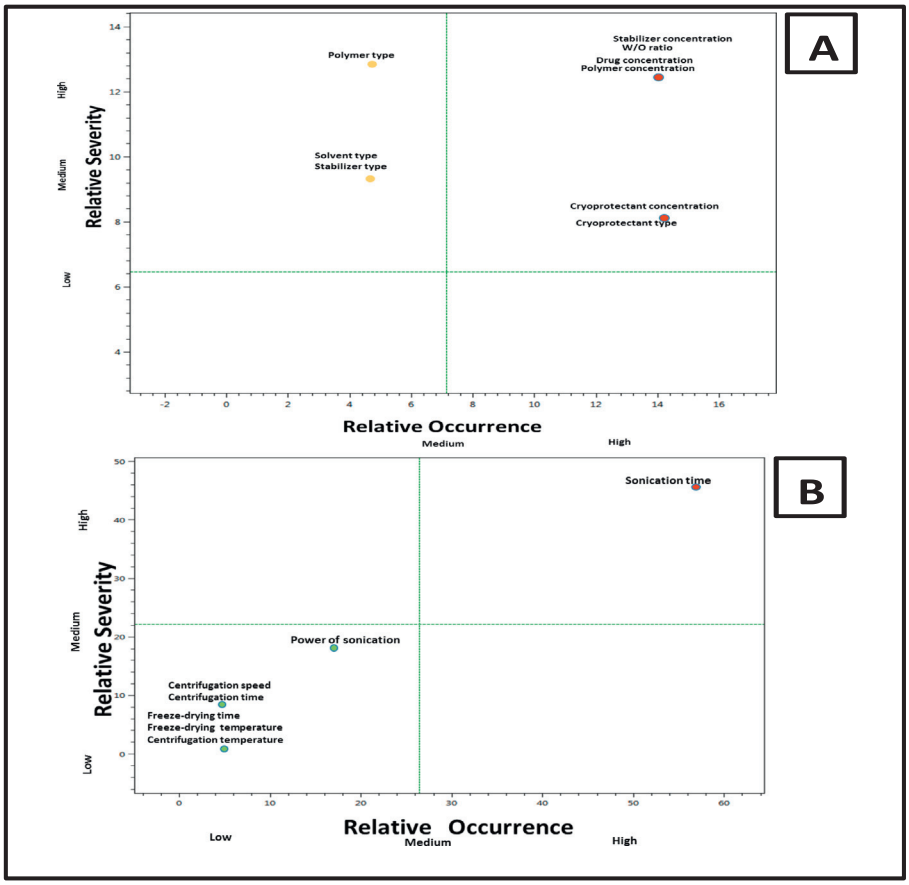

Fig. 8. Relative Occurrence/Relative Severity of A- proposed CMAs, B- proposed CPPs.

previously. In our pilot study, QbD oriented development was successfully implemented to gain understanding of critical parameters influencing the quality of the Liraglutide loaded PLGA NPs. Furthermore, the next step will be the application of a reliable and robust statistical DOE to investigate the effect of the most critical factors on CQAs with minimum number of runs, then determining the best optimum level of each variable which should be used to prepare an optimized formulation that assures the required CQAs.

Abbreviations

API	Active Pharmaceutical Ingredient
CMAs	Critical Material Attributes
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
DL	Drug loading
DoE	Design of Experiments
DS	Design Space
EE	Encapsulation Efficiency
EMA	European Medicine Agency
FDA	Food and Drug Administration
GLP-1	glucagon like peptide −1
ICH	International Council of Harmonisation
Lira	Liraglutide
NPs	Nanoparticles
PDI	Polydispersity Index
PLGA NPs	Poly lactic-*co*-glycolic acid -nanoparticles
PLGA	Poly lactic-*co*-glycolic acid
QbD	Quality by Design
QTPP	Quality Target Product Profile
RA	Risk Assessment

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PUBLICATION IV.

European Journal of Pharmaceutics and Biopharmaceutics 115 (2017) 257–267

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review article

Novel strategies in the oral delivery of antidiabetic peptide drugs – Insulin, GLP 1 and its analogs

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ARTICLE INFO

Article history:
Received 8 December 2016
Revised 30 January 2017
Accepted in revised form 19 March 2017
Available online 21 March 2017

Keywords:
Diabetes
Antidiabetic peptides
Insulin
GLP-1
Nano-carriers
Polymeric nanoparticles
Solid lipid nanoparticles
Liposomes
Micelles
Quality by design

ABSTRACT

As diabetes is a complex disorder being a major cause of mortality and morbidity in epidemic rates, continuous research has been done on new drug types and administration routes. Up to now, a large number of therapeutic peptides have been produced to treat diabetes including insulin, glucagon-like peptide-1 (GLP-1) and its analogs. The most common route of administration of these antidiabetic peptides is parenteral. Due to several drawbacks associated with this invasive route, delivery of these antidiabetic peptides by the oral route has been a goal of pharmaceutical technology for many decades. Dosage form development should focus on overcoming the limitations facing oral peptides delivery as degradation by proteolytic enzymes and poor absorption in the gastrointestinal tract (GIT). This review focuses on currently developed strategies to improve oral bioavailability of these peptide based drugs; evaluating their advantages and limitations in addition to discussing future perspectives on oral peptides delivery. Depending on the previous reports and papers, the area of nanocarriers systems including polymeric nanoparticles, solid lipid nanoparticles, liposomes and micelles seem to be the most promising strategy that could be applied for successful oral peptides delivery; but still further potential attempts are required to be able to achieve the FDA approved oral antidiabetic peptide delivery system.

Contents

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http://dx.doi.org/10.1016/j.ejpb.2017.03.015
0939-6411/

1. Introduction

Proteins and peptides are the building blocks of life and are now evolving as a very promising brand of therapeutic drugs [1]. They have gained much interest because they can be used for the treatment of several diseases due to their ability to provide effective and potent action [2–4]. More than that, peptides and proteins are highly selective [5] that can lead to a significant decline in toxicity. "Diseases that might be treated with this type of therapeutics include auto immune diseases, cancer, mental disorder, hypertension, and certain cardiovascular and metabolic diseases" [6]. A wide variety of peptide and protein drugs is now developed as a result of advances in the biotechnology field [7].

Diabetes mellitus is an endocrinological and/or metabolic disorder [8], and the worldwide prevalence and incidence of it has continued to increase dramatically. Currently, more than 250 million people in the world have diabetes and it is predicted that this number will double over 20 years [9]. The disease results in hyperglycemia which may cause multi-organ damage. The debilitating effects of diabetes mellitus include various organ failures, progressive metabolic complications such as retinopathy, nephropathy, and/or neuropathy [9–11]. The main aim for the treatment of type I and type II diabetes is to cure the symptoms related to hyperglycemia [12].

Over the last years, the interest of the pharmaceutical technology utilizing therapeutic peptides in diabetes treatment has been increased. However, these peptide drugs have several drawbacks, including low bioavailability, metabolic liability and short half-lives [13–15]. Hence, these drugs are usually administered by parenteral route [16–18]. However, since injections are associated with pain and low patient compliance [19], researchers in the pharmaceutical technology field believe that alternative routes of non-invasively delivery ways such the oral, pulmonary, nasal, transdermal and buccal routes are highly desirable for peptides and proteins delivery [20–22]. Among these non-invasive routes, the oral route is often the most preferred route.

2. Delivery of the most common antidiabetic peptides

2.1. Insulin

Insulin is a life-saving drug for diabetes as it has the ability to control the blood glucose level by facilitating the uptake of glucose [23]. Until now, insulin is the first line treatment of type 1 diabetes. It is also used to treat type 2 diabetic patients (especially in late-stage disease) [24]. Human insulin is consisted of two amino acid chains: A chain (21 amino acid residues) and B chain (30 amino acid residues) linked by disulfide bonds. Insulin can be isolated from human, porcine, bovine or sheep sources [25]. Currently; insulin is still administered by subcutaneous injection because of its instability in the gastrointestinal route, due to stomach acid and proteolytic enzymes present in the intestine, and low permeability through the intestinal mucosa [26] in addition to the hepatic first pass effect [27].

However, multiple daily insulin injections can lead to infection at the injection site in addition to psychological stress leading to poor patient compliance [26,28], and as a result non-effective treatment.

All of these drawbacks have motivated researchers to develop a safe and effective noninvasive route for insulin delivery. Among different noninvasive routes, oral insulin delivery not only improves the quality of life of diabetes patients who routinely receive insulin by the subcutaneous route, but also offers many advantages: rapid hepatic insulinisation in addition to avoidance of peripheral hyperinsulinemia and other adverse effects such as possible hypoglycemia and weight gain [29,30].

2.2. Glucagon-like peptide-1 and its analogs

The incretin hormone glucagon-like peptide-1(GLP-1) is a 30 amino-acid peptide derived from a proglucagon gene that is secreted by neuroendocrine L cells of the ilium and colon [31]. Food intake is the primary physiological stimulus of GLP-1 release from enter-endocrine cells [32]. The main effect of incretins is the reduction of blood glucose, mediated by the regulation of hormonal pancreatic secretions, inhibiting gastric emptying and reducing appetite and food intake [33]. The half-life of bioactive GLP-1 in the circulation is less than 2 min due to rapid inactivation by the ubiquitously expressed dipeptidyl peptidase-4 (DPP-4) which is mainly located on the luminal surface of the endothelial cells [31]. To overcome premature GLP-1 metabolism, long-acting GLP-1 analogs have been developed to resist DPP-4 degradation. Currently, six glucagon-like peptide-1 receptor agonists (GLP-1RAs) are approved for treating type 2 diabetes. These fall into two classes based on their receptor activation: short-acting exenatide twice daily and lixisenatide once daily, and longer-acting liraglutide once daily, exenatide once weekly, albiglutide once weekly and dulaglutide once weekly [34].

Both exenatide and liraglutide are delivered parenterally and have been proven to improve glycemic control. Therefore an oral route is preferable as it could prove safe and effective, it would mimic physiological route of GLP-1 from intestine to circulation to avoid potential side effects, and provide more convenience, ease of administration, and comfort, which would increase patients' compliance to the treatment and thus increase the treatment efficacy [35,37].

3. Major obstacles associated with oral delivery of therapeutic peptides

Peptides and proteins could be transported across the GIT epithelium via either transcellular or paracellular pathway. However, these molecules are hydrophilic with large size. So, they would not be expected to follow the transcellular route of absorption by means of passive diffusion. Alternatively, the paracellular route is an aqueous extracellular route that may be interesting for peptides and proteins delivery due to possible deficiency in proteolytic enzymes. It was demonstrated that the paracellular route is not an option for absorption of proteins and peptides because they cannot fit in these spaces [38–40]. Another major obstacle that contributes to the extremely low bioavailability of proteins and peptides is the presystemic enzymatic degradation in the harsh environment of the GI tract as well as presystemic elimination in the liver [38–42]. Besides, efflux transporters such as P-glycoprotein (P-gp), a 170-kDa protein, might contribute significantly to the poor bioavailability of peptides and proteins [43,44] as this protein acts in reverse to transcellular drug absorption [45,46].

Thus, the barriers that limit oral peptide delivery can be summarized as follow:

- Low pH environment of the gastric media.
- Enzymatic barrier.
- Viscous mucous layer.
- The intestinal epithelium cells.

As the possibilities in their oral delivery and the main challenges in their development, current and future prospects, with focus on technology trends in the market has been summarized

recently in general [1]; therefore now we focus on antidiabetic peptides and their challenges.

4. Most recent attempts for oral antidiabetic peptide delivery

Tremendous efforts have been devoted over past decades for oral administration of antidiabetic peptides. Up to date, several strategies - from very simple to more complex ones - have been developed to improve the oral bioavailability of those peptides. These include incorporating absorption enhancers, enzyme inhibitors into the formulations, development of mucoadhesive polymeric and particulate delivery systems; modification of the structure of macromolecules in addition to site specific delivery to colon and recently by mean of cell penetrating peptides.

As the oral route of administration is closer to the natural physiological route of insulin, therefore the most recent developments in this domain are detailed and evaluated in the following section.

These developments should solve at least one from the two main challenges: (1) to protect the protein/peptide from enzymatic degradation and (2) to enhance absorption without altering biological activity.

This review goes from the simplest to the most complex solutions available in the literature, where examples contained antidiabetic peptide examples for possible oral administration.

4.1. Modifying the structure of peptides

Chemical modification offers great potential for enabling the oral delivery of a macromolecule drug [47]. Many strategies of chemical modification were developed such as cyclization, lipidation, inclusion of unnatural amino acids, PEGylation and biotinylation.

4.1.1. Pegylation

Even cyclization can stabilize oligopeptides and improve their lipophilicity by reducing their charges and hydrogen bonding potential, its widespread use is limited when larger peptides and proteins are needed for therapy. Thus, PEGylation is one possible modification strategy for peptides that could not be cyclized [48,49]. This technology was first reported in the 1970s, and it is achieved by attaching polyethylene glycol (PEG) covalently to a protein or peptide drug [50,51]. There are several reasons for PEGylation of peptides and proteins such as preventing recognition and degradation by proteolytic enzymes, enhancing the stability and potency of proteins and peptides [52–54]. PEGylation of compounds have also proved to reduce immune responses [53] which usually minimize half-life of drugs in the body. Researchers concluded that the PEGylation of insulin was able to remove the resultant conjugate's immunogenicity, allergenicity, and antigenicity. Moreover, the (monomethoxypoly (ethylene glycol) mPEG attachment was observed to remarkably improve insulin's resistance to aggregation [55].

The effect of PEG on biotinylated GLP-1 (DBP-GLP-1) was shown to be better in enhancing absorption and enzyme resistance than just biotinylation (its plasma concentration increased quickly 30 min after oral administration), with as good results as native GLP-1 in peptide bioactivity and decreased glucose concentration [56]. Other study proved that loading of PEGylated insulin (in which a 5000 Da PEG chain conjugated to the PheB1 site of insulin) into a pH responsive poly(methacrylic acid-g-ethylene glycol) (P (MAA-g-EG)) hydrogel maintained the bioactivity of the insulin and significantly sustained the duration of the hypoglycemic effect [57]. A recent study showed that insulin loaded deoxycholic acid conjugated PEGylated polyhydroxybutyrate co-polymeric nanoparticles was able to sustain the release of insulin and showed enhanced its oral bioavailability [58].

However; even PEGylation is a crucial strategy for chemical modifications of peptides, safety and efficacy of newly developed PEGylated peptides should be well investigated. On one side, PEG polymers are mixture of polymers of different molecular mass, and on the other side, peptide drugs contain several accessible sites for PEGylation which allow product heterogeneity that could lead to changing of the efficacy of the drug.

4.1.2. Lipidization

By increasing the lipid character of the hydrophilic peptide drug, both the membrane penetrative and stability properties may be enhanced, and as a result the oral bioavailability will increase. Peptide lipidization has been applied as a strategy to enhance the oral bioavailability of peptide based drugs [59,60]. For example, long fatty acid chains have been commonly used to improve peptides uptake by increasing the lipophilicity of these drugs [59], protecting an unstable peptide from enzymatic degradation [61], and demonstrating higher stability. In addition to that being lipophilic, these peptides will most likely be solubilized by albumin or serum lipoproteins, both of which can contribute to stabilization and increased circulatory half-life [62]. The lipid is attached via either a stable linkage, or a labile one, creating a pro-drug [63,64]. An example of hydrophobization to increase lipophilicity of insulin is palmitoylation. Results of one study of demonstrated that the attachment of 1,3-dipalmitoylglycerol to insulin by an ester bond could lead to remarkable improvement in both intestinal penetration and stability against enzymatic degradation [65].

All of the studies have shown that, compared to unmodified peptide, the lipidized peptidyl drugs proved enhancement in the pharmacological activity in addition to an increased mucosal permeability, plasma concentration, and area under the curve (AUC) [66]. But one of the major obstacles of using lipidized peptides for oral delivery is the lack of a systematic study of the mechanism of enhanced oral absorption. In addition, there are many factors, such as the linker used in lipid-conjugation, the formulations for oral administration, and the presence of other excipients that can influence the oral bioavailability of a lipidized peptide.

4.1.3. Unnatural amino acids

The physio-chemical properties of peptides can be improved by the substitution of natural amino acids with unnatural ones [66,67]: D-conformation, N-methylation, tetra-substitution, -amino acids, and side chain methylation(s). Such modifications creates a peptide sequence which is more resistant to enzymatic degradation as naturally occurring proteases are designed to catalyze reactions involving natural peptides. Studies showed that Glucagon-like peptide-1 is enzymatically cleaved at ala2. So, replacement of ala2 with D-ala2 was proved to improve drug stability, prolong half-life and increase activity [68]. One difficulty in this approach is the activity of the drug must be retained when altering of the amino acids sequence.

4.1.4. Biotinylation

Another reported technique for successful oral GLP-1 delivery was by peptide surface modification using biotin (vitamin H), which proved to actively traverses the intestine membrane via sodium-dependent multivitamin transport. Based on a published study, Lys34- and Lys26,34–biotin–GLP-1 derivatives were prepared and evaluated to investigate their protective effect against enzymatic degradation in addition to oral absorption enhancing ability. Results depicted that Lys26,34–biotin–GLP-1 proved oral hypoglycemic efficacy approximately 9 folds compared to the native GLP-1 [69]. One year later, another research was published

on designing different biotinylated exendin-4 analogs as oral exenatide carrier systems. Based on in vitro and in vivo studies, Lys12, 27-Biotin-Exendin-4 showed the best stability efficacy against intestinal enzymes and was remarkably effective in increasing the intestinal absorption of GLP-1 and its hypoglycemic effect as a result [70].

4.2. Absorption enhancers

The use of permeation enhancers that modify the properties of the intestinal epithelium by either paracellular or transcellular permeation is one possible method to promote permeation of peptides and proteins across the epithelial membrane [69]. These absorption enhancers may break up the structural integrity of the intestinal barrier, open the tight junctions, decrease the mucus viscosity or increase the membrane fluidity [70–72]. Ideally, the absorption enhancer should be reversible, safe and it should be able to enhance the permeation immediately and coincidentally with the presence of the drug at the absorption site [71]. And it is suggested that the most common drawback of absorption enhancers in the case of long-term usage is that they may damage the bio-membrane and lead to local inflammation [15,73,74], in addition to the lack of specificity, i.e., they facilitate the penetration of all contents of the intestinal tracts including toxins and pathogens so they easily access to the systemic bloodstream [74–76].

That is why many studies aimed to modulate tight-junction permeability in order to increase paracellular transport [77–79]. Many available absorption enhancers can cause opening of tight junctions in order to allow hydrophilic peptides and proteins to pass; including: for example, chitosan has shown good permeation enhancing capacity via the paracellular pathway [80,81] through opening the inter-epithelial tight junction and thus facilitates systemic peptides and proteins transport. Chitosan and its derivatives have been used, as we mentioned before, as penetration enhancers for oral insulin delivery, and have resulted in an effective reduction of glucose levels in the body [82–90]. Moreover, the permeation of peptides through the GI mucosa can be improved by the use of thiolated polymers or so-called thiomers. Thiomers have been studied as penetration enhancers for oral insulin delivery, and have resulted in effective reduction of glucose levels in the body [76,91].

Besides, fatty acids and bile salts, surfactants, tri-sodium citrates, glycerides and chelating agents, Labrasol have also shown the capacity to enhance the permeability across the mucosal walls [92,93]. Surfactants can improve the transcellular transport of macromolecules by breaking up the lipid bilayer and making the cell membrane more permeable [77,92]. For example, both distearyl-dimethyl-ammonium bromide (DSAB) or soybean phosphatidylcholine (SPC) were proved to be able to improve insulin loading into different lipid-based drug delivery systems, facilitate the oral insulin absorption and hence increase its oral bioavailability when complexes between insulin and one of these surfactants were prepared by freeze-drying [94].

Chelating agents like EDTA form complexes with calcium ions and break tight junctions to enhance paracellular transport of proteins and peptides [95]. Besides; diethylene triamine pentaacetic acid (DTPA) which belongs to the polyamino carboxylic acid family of complexing agents, was proved to possess a protease inhibitory activity, due to the withdrawal of essential metal ions out of the enzyme structure, and a tight junction opening capacity, as DPTA can chelate metal cations through its amines and carboxylates [96]. Incorporation of DTPA in functional NPs which were prepared by mixing cationic chitosan (CS) with anionic γPGA-DTPA conjugate was able to enhance the absorption of insulin throughout the entire small intestine in addition to providing a prolonged reduction in blood glucose levels [97]. Salts of Medium chain fatty acids including caproate, caprylates, caprates and laureates, have also proven capacity to improve absorption via transient opening of tight junction [98–100].

4.3. Enzyme inhibitors

One of the well-known key challenge in oral peptide and protein delivery is to ensure their protection against the degradation by numerous types of endopeptidases (such as pepsin, trypsin, chymotrypsin, elastase) and exopeptidases (such as carboxypeptidases A and B) along the GIT [101,102]. Hence each and every peptide bond in a molecule had to be protected from these proteolytic enzymes. Thus, one strategy to improve the oral bioavailability of peptides and is to use enzyme inhibitors [78].

Several enzyme inhibitors were used to improve oral insulin stability against enzymatic degradation including trypsin or a-chymotrypsin inhibitors, such as soybean trypsin inhibitor [103], FK-448 [104], camostat mesylate [104], and aprotinin [103–105].

However, based upon previous scientific papers, using enzyme inhibitors in long-term therapy is still controversial due to the probability of absorption of undesired proteins and peptides, disturbance of nutritive proteins digestion in addition to enhancing protease release [106].

4.4. Cell penetrating peptides

Cell penetrating peptides (CPP) have obtained increased importance due its ability to improve the delivery of proteins and peptides over the plasma membrane and thus CPP demonstrate promise for therapeutic purposes [107–109]. Many recent studies have demonstrated that the combination of peptides with CPPs is a potential approach for oral delivery of these macromolecular drugs [110]. CPPs can be grouped according to their physicochemical properties, such as being cationic or amphipathic, or according to their origin, being naturally derived, synthetic or chimeras. acids), To generalize, the CPPs are Short peptides sequences rich of basic residues [i.e. arginine (Arg) and lysine (Lys)], which enables electrostatic interactions with negatively charged cell surface molecules [16]. In addition to the cationic Arg and Lys residues, the presence of hydrophobic amino acid residues, especially tryptophan (Trp), positively influences membrane translocation of the CPP through interaction with the lipid bilayer [17]. Trp residues may furthermore improve the interaction with cell surface GAGs, thereby facilitating cellular CPP uptake by endocytosis [18]. Not only the presence, but importantly also the specific positioning of Trp residues in a CPP sequence influences the cell-penetrating propensity of the CPP [17].

One recent research aimed to evaluate the effectiveness of one amphipathic CPP (penetratin) (RQIKIWFQNRRMKWKK) in enhancing the epithelial permeability of insulin. It was proved that physically blending penetratin with insulin could be successful way for oral delivery of insulin under harsh intestinal environment, and the electrostatic and hydrophobic interactions between CPP and the drug is related to the enhancing effect of the CPP on the intestinal absorption of therapeutic peptides and proteins [111].

Recently, GLP-1 loaded PLGA nanoparticles were prepared through the modified solvent emulsification–evaporation method, and these NPs were added into chitosan (CS) solution. These prepared NPs were conjugated to CPP which was R9 using EDC/NHS coupling chemistry in order to conjugate the free amine groups in the CS structure with the carboxylic group of CPP. After that; the GLP-1 loaded NPs were encapsulated within the HPMC-as pH-sensitive polymer, loaded with the dipeptidyl peptidase-4 inhibitor (iDPP4), using a double emulsion technique through a microfluidic flow focusing glass device [112]. It was reported that Glp-1/iDPP4 delivery multifunctional composite system was useful in providing enhanced, controlled and prolonged hypoglycemic

effects in diabetic rats. Thus researchers suggested that this novel system has a potential in development of oral protein/peptide delivery systems for Type 2 diabetes mellitus therapy. Another research showed that conjugation of insulin with CPP that is low molecular weight protamine (VSRRRRRRGGRRRR) followed by encapsulation in mucoadhesive (N-trimethyl chitosan chloride-coated PLGA) NPs was able to protect insulin and enhance its permeability as well. Thus, this developed system provided high oral bioavailability of insulin in diabetic rats compared with that for insulin loaded MNPs [113].

However, using CPPs has a limitation due to their inherent chemical instability as they could degrade by extracellular and intracellular enzymes [109]. Several strategies have been employed for improving the CPP stability by alteration of the amino acid stereochemistry which may negatively affect the resulting cell-penetrating propensity of the CPP and/or its ability to act as a delivery vector. For example, a previous study reported the ability of L- and D-penetratin (RQIKIWFQNRRMKWKK) to mediate delivery of exendin-4, GLP-1 across nasal and intestinal epithelia. It was observed that L-penetratin increased the plasma concentration of exendin-4 and GLP-1 to a greater extent than D-penetratin, as Cmax for orally administered exendin-4 and GLP-1 (with L-penetratin) was 57 and 5 times higher than the Cmax values of these two peptides, respectively, when using D-penetratin. Thus, changing CPP amino acid stereochemistry from L to D may improve the stability and membrane permeation of the CPP, but does not necessarily increase delivery of all cargo drugs [114].

4.5. Mucoadhesive polymeric systems

Mucoadhesive polymeric systems are the most promising approach among other developed ones [115]. These systems demonstrate noticeable alteration in mucoadhesion properties with regards to changes in environmental factors, including pH, temperature, enzymes, light, electric field or ionic strength. In addition to that, Mucoadhesion may be affected by hydrophilicity, molecular weight, cross-linking and swelling. The lower the cross-link density, the higher the flexibility and hydration rate, the higher the degree of swelling, the larger the surface area of the polymer, and thus, the better the mucoadhesion [116].

Mucoadhesive polymeric system can be also used to reach site-specific drug delivery, as mucoadhesive properties can maintain a close contact with the mucosa at the site of drug uptake hindering a presystemic metabolism and increasing the oral bioavailability of peptides and proteins [117,118]. Besides, these systems are able to decrease the drug clearance rate from the absorption site, and as a result extend the time available for absorption [119].

One of the most widely used polymers is chitosan which is a mucoadhesive polymer that has shown good permeation enhancing capacity via the paracellular pathway [119,120] through opening the inter-epithelial tight junction and thus facilitates systemic peptides and proteins transport. Chitosan is nontoxic, biocompatible and biodegradable FDA-approved polymer and it is able to improve the absorption of hydrophilic macromolecule drugs [84,85,119]. Chitosan and its derivatives have all been studied as penetration enhancers for oral insulin delivery, and have resulted in effective reduction of glucose levels in the body [82,87,88]. Besides, oral exenatide-loaded nanoparticles were prepared using e ionotropic gelation method with modified chitosan which was conjugated with CSKSSDYQC (CSK) peptide. Results of this research depicted that the transport of exenatide across a co-cultured Caco-2 and HT29 cell membrane was significantly enhanced and the relative bioavailability of CSK-chitosan was also improved when compared to non-modified chitosan. That could be due to the ability of chitosan to adhere well to the mucus coat of epithelial tissues in addition to the affinity of CSK for the globet cells, an important component of intestinal epithelial cells [121]. In another recent research, the oral negatively charged insulin carboxymethyl chitosan/chitosan nanogels prepared using the simple ionic gelation method proved to have excellent mucoadhesion on the jejunum and ileum which were important intestinal segments for nutrient absorption and thus were effective in controlling the blood glucose levels in diabetic rats [122].

Also, researchers reported a self-assembled trimethyl chitosan (TMC) nanoparticles with a dissociable "mucus-inert" hydrophilic coating of N-(2-hydroxypropyl) methacrylamide copolymer (pHPMA) derivative as a promising oral delivery of insulin by facilitating the permeation through mucus layer and epithelium via opening tight junction [89]. Based on a new published paper, a multistage dual-drug delivery nanosystem was developed to deliver GLP-1 and DPP4 inhibitor simultaneously. This developed system composed of chitosan-modified porous silicon (CSUn) nanoparticles, which were further coated using hydroxyl-propyl-methylcellulose acetate succinate MF. The orally delivered NPs in diabetic rats exhibited hypoglycaemic activity without initial increase in the blood glucose levels as observed for controls [123].

Furthermore, the potential of mucoadhesive devices prepared with a blend of Carbopol 934/pectin/sodium carboxy-methyl-cellulose in enhancing intestinal absorption of both exenatide and insulin was investigated. Results demonstrated that the novel devices not only increased systemic oral peptide drug absorption, but also extended glycemic control by controlled insulin/exenatide release [124].

Thiolated polymers (thiomers) have been considered as a hopeful alternative for non-invasive peptides and proteins delivery [125,126]. It was shown that the additional covalent bonds between thiol groups of thiomers and cysteine-rich subdomains of mucus glycoproteins are responsible for strong mucoadhesive properties of thiolated polymers [126,127]. Thiolated insulin delivery system was developed using Chitosan–6 mercaptonicotinic acid (chitosan–6-MNA) which is a strong mucoadhesive polymer with pH-independent reactivity. This developed system resulted in a significant serum insulin concentration and a blood glucose reduction after oral administration as the areas under the concentration–time curves (AUC) of human insulin in plasma was improved up to 21-fold compared with unmodified chitosan and up to 6.8-fold compared with aliphatic thiolated chitosan [90]. Researchers suggested that chitosan 6-MNA could be a useful tool for the oral delivery of other peptide drugs as well.

In addition to that, alginate (Alg) is one of the most common water-soluble anionic biopolymers, which is a biocompatible, mucoadhesive, biodegradable, pH sensitive polysaccharide that was used as oral carrier for peptides and proteins [84]. However, there is a problem of drug by leaching through the pores in the microbeads during the preparation of calcium alginate microbeads [128]. Thus; many researchers tried to overcome this obstacle by coating the alginate microbeads with chitosan layers in order to be able to control both of diffusion and release rates of the entrapped drugs/proteins. It was shown that the coated alginate microbeads containing higher concentrations of aminated chitosan were more stable at the higher pH 7.4 when compared to alginate microbeads, and this new pH sensitive system could be successfully administered orally for site-specific release of insulin and other protein drugs in the intestinal and colon tracts [129].

4.6. Oral microparticles delivery systems

Microparticles can be classified as particles between 1 and 1000 μm. It is known that microencapsulation of therapeutic peptides have proved to be able to deliver various peptide and drugs efficiently by protecting them from harsh gastric milieu of the gastrointestinal tract and enhancing their intestinal absorption

[1,130]. β-cyclodextrin insulin microparticles that were prepared by one-step spray-drying technique and stabilized using enteric retardant polymers (ethylcellulose and EC and hydroxypropyl methylcellulose acetate succinate HPMCAS), were effective in lowering the blood glucose in a diabetic rat model and had almost comparable hypoglycemic activity when compared to insulin administered by SC injection [131]. In another study, PLGA microparticles containing insulin Poly (ε-caprolactone) PCL nanoparticles (so-called composite microparticles) were prepared by the W/O/W solvent extraction method. The results confirmed that the prepared composite microparticles have succeed in obtaining high insulin encapsulation efficiency, limited burst effect in addition to controlled insulin release which was extended up to 24 h [132].

Moreover, exenatide was encapsulated in microparticles prepared by chemical cross-linking of alginate and hyaluronate. After the oral administration, the prepared system could keep intact in stomach and then release loaded exenatide in small intestine for absorption, depending on pH responsive mechanism. Besides, this system showed a blood glucose lowering activity that mimic the effects of SC injection of exenatide. However, the authors suggested further systemic studies to evaluate the safety of the prepared microspheres for human [133].

4.7. Oral nanoparticle delivery systems

According to the definition from NNI (National Nanotechnology Initiative), nanoparticles are structures of sizes that range from 1 to 100 nm in at least one dimension [134]. Nano-carriers composed of biocompatible, stable and nontoxic polymers [134,135] with optimized physicochemical and biological properties can facilitate transport across intestinal epithelial cells [134–136], so they can be successfully used as a promising method for oral delivery of macromolecules [137,138]. Additionally, an optimized nanoparticle carrier offers the prospect of protecting the peptide or protein from hydrolysis and enzymatic degradation in the GIT [139–142], and it was demonstrated that polymeric nanoparticles (NPs) can be an excellent drug delivery systems to evade the efflux route as P-glycoprotein (Pgp) is incapable of recognizing NPs [143,144]. Actually, many factors can affect the absorption of proteins or peptides incorporated in nanoparticles such as particle size, surface charge, ligands in addition to the dynamic nature of particle interactions in the gut [142].

Different nanoparticles were developed to formulate stable and efficient peptides delivery system. Indeed, the potential of polymeric nanoparticles, solid lipid nanoparticles, liposomes and micelles for oral peptide and protein administration has been recently reported in many research papers [28,145].

Many attempts to develop oral polymeric nanoparticles for exenatide were reported, for example, the oral bioavailability of exenatide which was embedded in NPs composed of chitosan and poly (γ-glutamic acid) was found to be 14% when compared to SC injection, and the prepared nanoparticles were able to provide a prolonged glucose-lowering effect [146].

Moreover, polyethylene imine-based nanoparticles applying three-layer release technology platform which consists of a flexible film composed of neutral polymethacrylate Eudragit® NE and superdisintegrant sodium starch glycolate Explotab®, added as a pore former, applied to a hydroxyl-propyl methylcellulose (Methocel® E50, HPMC) coating [147]. In the three-layer system, the hydrophilic layer based on Methocel® E50 was demonstrated to delay the drug release by a swelling/erosion mechanism, while the Eudragit® NE/Explotab® CLV film was aimed at prolonging the duration of the lag phase as imparted by the underlying HPMC coat. Results showed that this system was successful in oral colonic insulin delivery in diabetic rats.

In order to enhance the oral delivery efficiency of insulin, researchers in another study prepared insulin-loaded glucose responsive nanocarriers that were further encapsulated into a three-dimensional (3D) hyaluronic acid (HA) hydrogel environment for multi-protection of insulin through the GI tract. The released insulin from prepared systems showed an effective hypoglycemic effect for longer time when compared with insulin-loaded nanocarriers [148]. Another L-valine modified chitosan-based multifunctional nanoparticles exhibited a promising oral insulin delivery system as the prepared nanocarriers are glucose-sensitive and capable of preventing the rapid release of insulin from nanocarriers in the stomach, and increase the oral insulin bioavailability by enhancing the transportation and absorption in the intestinal cells [82]. Other efforts were reported to increase the permeability of insulin across the intestinal wall as applying double coating with chitosan (CS) and albumin (ALB) to the surface of insulin-loaded alginate/dextran sulfate (ADS)-nanoparticles, and this approach approved enhancing of the NPs' electrostatic interactions with the intestinal cells [83].

Solid lipid nanoparticles (SLN) are submicron colloidal carriers (50–1000 nm) that contain solid lipids and distributed either in water or in an aqueous surfactant solution [149]. Many examples of these lipids include highly purified tri-acyl-glycerols, waxes or complexes of acyl-glycerol mixtures [150]. Frankly, SLN depict more merits as drug delivery system when compared with other nanosystems, including a good tolerability, biodegradation and the possibility of production on large scale [151,152]. This system can act as an effective tool for protecting peptides and proteins against enzymatic degradation [153]. However, their hydrophobic nature generally accounts for low peptide entrapment efficiency (EE%). So there has been much efforts to solve the problem of poor peptide entrapment and stability. Researchers prepared a novel oral insulin delivery system that aimed to improve the peptide EE% by incorporating a hydrophilic viscosity-enhancing agent: propylene glycol (PG), polyethylene glycol (PEG) 400 and PEG 600, within SLN cores to increase the viscosity of the aqueous internal phase of the w/o/w double emulsion system, and thus develop viscosity enhanced nanocarriers (VEN). These agents. and the highest EE% was achieved by using 70% w/w of PG and the prepared systems were able to reach a good hypoglycemic effect in fasted rats with an oral bioavailability of about 5% and a lower cellular toxicity compared to conventional SLN [154]. In another study, incorporating of Methocel in insulin SLN has proved to enhance the peptide entrapment and provide the advantages of SLN system as well. Researcher suggested that this mentioned hybrid system could be effective for oral delivery of other peptide drugs [155].

Three different nanosystems loaded with GLP-1 and composed of (poly(lactide-co-glycolide) polymer (PLGA), Witepsol E85 lipid (solid lipid nanoparticles SLN) and porous silicon (PSi) were prepared in order to deliver GLP-1 orally [156]. Those systems were compared according to their ability to sustain the release of GLP-1 through the simulated GI tract conditions, and the interactions between these nanosystems and the intestinal cell lines were studied. Results showed that chitosan-coated nanosystems were better than uncoated nanoparticles as they were more able to sustain GLP-1 release and improve the interaction with the intestinal cells. Chitosan CS was reported in previous reports to have good permeation enhancing capacity via the paracellular pathway through opening the inter-epithelial tight junction and thus facilitates systemic peptides and proteins transport, in addition to improving the positive surface charge that facilitate the interaction with the negative charge moieties of the cell membrane and lead to internalization [119,157]. In addition to that, PLGA CS and PSi CS were the best nanocarriers as novel oral GLP-1 delivery systems and the highest GLP-1 permeation across the intestinal in vitro models

Table 1
Advantages and Limitation of several techniques utilized for oral antidiabetic peptides delivery.

Technique	Advantages	Limitations
Chitosan/PLGA/PCL based nanoparticles	– Biodegradable polymers that enhance the stability – Control and target the drug release – Control the pharmacokinetic parameters – Reduced toxicity in the peripheral healthy tissues [171,172]	– Chitosan: instability in acidic environments of GI tract [173,19] – PLGA/PCL: poor drug loading, higher cost of production, protein or peptide drugs instability problems like denaturation or aggregation [174–176]
Solid Lipid Nanoparticles	– Protection of peptides from degradation, controlling drug release – Biocompatibility – Ease of large scale production [151,177,178]	– Low peptide entrapment efficiency (EE%) [154] – Lack of an in-depth understanding interaction with biological barriers [179]
Liposomes	– Improve stability against oxidation and deamination – Provide protection against enzymes degradation [1,171] – Safety and low toxicity – Flexibility, biocompatibility – Entirely biodegradability and non-immunogenicity [171,179]	– Low stability against acidic pH of stomach, bile salts and pancreatic lipase [42] – Low hydrophobic drug loading – Leakage of entrapped drug and high production cost [180]
Polymeric Micelles	Increase the intestinal absorption of the peptide [74,181]	Nucleic acids are more stable incorporated into polymeric micelles than peptides and proteins [182]
Microspheres	Controlled and Targeted peptide delivery, protection of encapsulated peptides against degradation [183,184]	Limit precise control and possible burst release [1,185]
Microemulsions	– Increase protection against luminal enzymatic degradation – Enhance induced structural and fluidity changes in the mucosal membrane [186] – No intestinal tissue damage or irritation is detected [187]	– Stability is influenced by environmental parameters such as temperature and pH [40,188,189] – Suffer from limitations of phase separation [189,190]
Mucoadhesive polymers	– Site–specific delivery – Improve membrane permeation [191]	Mucus turnover in absorption sites (intestine) [71,192]
Cell penetrating peptide	Enhance intracellular permeation of peptides [193]	– High cost – Toxic side effects and immunological response – Requirement of knowledge of conjunction chemistry in addition to indiscriminate transduction of CPPs [194,195]
Absorption enhancer	Enhance oral bioavailability by increasing membrane permeation [196]	– Cause altered cell morphology and cell membrane damage – Lack of specificity [197,198]
Enzyme inhibitors	Retard the rate of peptide degradation by enzymes [199]	– Severe side effects in the long term therapy, may affect the normal digestion of nutritive proteins [42,198,199]
Modifying the structure of peptide	Increase peptides oral bioavailability by reducing the enzymatic degradation of peptides/improving the permeability across membrane [168,200,201]	– PEG its self has some major safety risks: PEG is both immunogenic and antigenic [201,202] – Conjunction method could be complex [200]

was noticed with PSi CS nanoparticles. In another study, Exendin-4-loaded SLNs using precirol, a glycerol palmitostearate, were prepared and results showed low particle size, high encapsulation and loading efficiency, and long-term physical stability with similar effects on insulin secretion compared to those of native Ex-4. Thus, it was suggested that the prepared SLNs can be applied for successful oral Exendin-4 delivery [36].

Recently, polymeric micelles have received growing scientific attention. Micelles are formed by amphiphilic copolymers which self-assembled to nano-sized aggregates above the critical micellar concentration. The hydrophobic moiety forms the core of micelles whereas the hydrophilic moiety forms the corona in the shell of micelles [158]. It was concluded that using N-octyl-N-Arginine chitosan (OACS) micelles for the enhancement of oral insulin delivery is a promising approach for the oral insulin delivery [159].

Moreover, liposomes – closed bilayer vesicles made up of lipids containing an aqueous core – are considered to be the best class of organic nanoparticles for the treatment of many diseases [160]. Liposomes have been widely prepared as oral insulin delivery systems due to its ease of preparation, versatility of its composition, in addition to its biocompatibility [161]. However, the conventional liposomes suffer from instability in the gastrointestinal tract and poor permeability across the epithelial membrane. Thus, modification of this system was developed to overcome these challenges [162]. Many studies aimed to investigate the potential and effectiveness of liposomes containing bile salts as oral peptide delivery systems. One examples of these bile salts is sodium glycocholate that is reported to have good permeation-enhancing properties, with relatively low toxicity, in addition to protease-inhibiting effect. Insulin loaded sodium glycocholate liposomes were prepared by a reversed-phase evaporation method followed by homogenization. Results showed that the both conformation, and bioactivity of insulin was preserved under preparative stress, and the prepared liposomes were able to protect the drug against enzymatic degradation by pepsin, trypsin, and α-chymotrypsin depending on in vitro studies [163]. Another published paper showed that when comparing insulin loaded liposomes containing different types of bile salts [sodium glycocholate, sodium taurocholate, or sodium de-oxy-cholate] or cholesterol, the highest oral bioavailability can be achieved by SGC-liposomes as they were the most effective in protection against enzymatic degradation [164]. In addition to incorporating bile salts in liposomes structure, researchers examined the effectiveness of biotinylated liposomes as oral insulin delivery systems. Results of this study proved a significant hypoglycemic effect and enhanced absorption after treating diabetic rats with the BLPs, and the oral bioavailability was double compared to that of conventional liposomes [165].

4.8. Other particulate delivery systems (emulsions, microemulsions)

Microemulsions are defined as "isotropic, thermodynamically stable transparent systems composing of oil, water, surfactant and sometimes, co- surfactant forming particles with droplet size of <200 nm" [166]. These systems are typically grouped into three classes: oil-in-water (o/w), water-in-oil (w/o) and bicontinuous [167]. Microemulsions encapsulating insulin was prepared using a reverse micellar approach under low shear process conditions that are able to maintain the secondary structure of insulin in the short term. In this study. Di-doceyl-dimethyl-ammonium bromide (DMAB) was the surfactant, propylene glycol (PG) was the co-

surfactant, triacetin (TA) was the oil phase and insulin solution was the aqueous phase. This developed device was able to improve the oral insulin bioavailability and displayed a potential hyperglycemia controlling activity in streptozotocin induced diabetic rats [168]. In addition to that, researchers invented a novel stable water-in-oil microemulsion formulation for oral delivery of many therapeutic peptides including insulin and exenatide that was able to enhance their absorption via mucous membrane [169]. Generally, the oil phase (20–40% v/v) was composed of a medium-chain triglyceride with a low HLB surfactant (pH between 3 and 8) and an aqueous phase consisting of the drug dissolved in protein stabilizing buffer. Another novel patent demonstrated that preparing oil-in-water microemulsions could be 'an interesting approach for improving both the bioavailability and permeability of insulin and GLP-1 analogs [170].

5. General opinion and comments

Based on previous published papers, each of the aforementioned technologies, which were developed to provide successful oral antidiabetic peptides delivery, has its own merits in enhancing the oral bioavailability of peptide drugs either by protecting the peptides from enzymatic degradation within the GIT or by enhancing their transport across intestinal cell barriers.

However, each strategy also has disadvantages that may limit its efficacy in oral antidiabetic peptide delivery as shown in Table 1, which summarizes the advantages and limitations of the most recent applied techniques.

A proper combination of these techniques seems to be the solution for this complex problem, e.g. carefully selecting the excipients or additives for absorption enhancement, parallel with incorporating into a carrier system with long term stability and patient acceptance for chronic therapy.

There is an urgent need to ensure and prove that the benefits of these developed oral carrier systems overweight their risks, further in vivo and human studies must be conducted to know more about the effect of these carriers on the pharmacokinetics and efficacy of antidiabetic drugs, and their possible interactions with biological barriers as well.

The complexity in these formulations is the consequence partly of the nature of the drug (peptide) and also the challenge in incorporating the modifiers and/or formulating these carrier systems.

Based on the recent research experiences of our research group [203–208], a model is proposed after reviewing the relevant articles in the field. This model is briefly summarized as the graphical abstract of this work. Quality by Design approach - implemented by the International Conference on Harmonization (ICH) Q8 (R2), Q9, and Q10 guidelines [208–212] – is advised to use, when evaluating and selecting among the possibilities and prior risk assessment as well, before product development.

6. Conclusion and future perspectives

Current progresses in pharmaceutical biotechnology have led to the discovery of numerous antidiabetic peptides. However, these drugs like insulin, GLP-1 and its analogs are still delivered parenterally due to numerous potential physiochemical and enzymatic barriers that limit their absorption in the GI tract.

Because of its several advantageous properties, many attempts have been made to achieve oral delivery of these aforementioned peptides using various strategies. There has been a remarkable advance in this direction as seen based on the articles collected in this review.

It can be concluded, that much of the success was recorded using nanoparticles as carrier systems for these peptides, each of these developments is still associated with further challenges and have limitations.

Despite these significant research efforts in this field, there is still no FDA approved oral insulin or Glp-1 analog on the market. Thus research in this area should be continued at even a higher pace to investigate novel methods to enhance the stability and safety of these developed delivery systems in addition to overcoming other possible limitation accompanied, and researchers must avoid repeating others' findings because we undoubtedly need novelty in oral antidiabetic peptides delivery in order to access a safe and effective delivery system that could be completely accepted for chronic treatment of diabetes; the epidemic of the 21st century.

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