Universidade de Lisboa

Faculdade de Farmácia

Development of lipid nanocapsules encapsulating exenatide for oral delivery in the treatment of type 2 diabetes mellitus

Ana Rita Barão Lemos

Mestrado Integrado em Ciências Farmacêuticas

2019

Universidade de Lisboa

Faculdade de Farmácia

Development of lipid nanocapsules encapsulating exenatide for oral delivery in the treatment of type 2 diabetes mellitus

Ana Rita Barão Lemos

Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

Orientadora: Doutora Ana Beloqui García, UCL

Co-orientadora: Doutora Helena Isabel Fialho Florindo Roque Ferreira, Professora Auxiliar, FFUL

2019

Université Catholique de Louvain

Louvain Drug Research Institute - Advanced Drug Delivery and Biomaterials

Development of lipid nanocapsules encapsulating exenatide for oral delivery in the treatment of type 2 diabetes mellitus

Ana Rita Barão Lemos

Master of Science (MSc) in Pharmaceutical Sciences

Supervisor: Doctor Ana Beloqui García, UCL

Co-supervisor: Doctor Helena Isabel Fialho Florindo Roque Ferreira, Assistant Professor, FFUL

2019

Resumo

Diabetes mellitus tipo 2 é o tipo mais comum de diabetes sendo considerado um dos principais problemas atuais de saúde mundial. Estima-se que em 2045, esta doença atingirá 629 milhões de pessoas, pelo que o desenvolvimento de terapias antidiabéticas se torna premente. O desenvolvimento de terapias antidiabéticas orais à base de péptidos, como os agonistas do receptor do péptido 1 semelhante ao glucagon, é essencial para evitar a administração de injeções diárias dolorosas, permitindo desta forma uma maior adesão à terapêutica por parte dos doentes. Atualmente, o maior desafio da indústria farmacêutica no que concerne à administração de fármacos prende-se com o desenvolvimento de formas farmacêuticas orais que permitam a absorção de péptidos terapêuticos até alcançar a circulação sistémica. As nanocápsulas lipídicas são sistemas promissores para a administração de agonistas do receptor do péptido 1 semelhante ao glucagon, o que pode permitir não só o aumento da absorção do péptido ao nível da circulação sistémica, mas também o aumento da secreção endógena do péptido 1 semelhante ao glucagon atuando como os ligandos nativos endógenos. Contudo, o desenvolvimento de nanocápsulas lipídicas que incorporem agonistas do receptor do péptido 1 semelhante ao glucagon constitui um desafio considerável, devido ao seu núcleo lipídico e à elevada temperatura utilizada durante a sua preparação, que teoricamente não é adequada à encapsulação de fármacos peptídicos hidrofílicos. O trabalho experimental conducente a esta tese centrou-se no desenvolvimento de nanocápsulas lipídicas que encapsulam micelas reversas que contêm exenatido (fármaco modelo) para a administração oral de agonistas do receptor do péptido 1 semelhante ao glucagon. Este nanosistema apresenta um efeito sinérgico de dupla ação entre o próprio efeito biológico (estimulação da libertação de péptido 1 semelhante ao glucagon) e o efeito da molécula bioativa encapsulada (exenatido), representando assim uma estratégia alternativa para o tratamento da diabetes mellitus tipo 2. Após a formulação das nanocápsulas lipídicas, foi inserido ácido propiónico na superfície das mesmas com o objetivo de aumentar o direcionamento para as células L enteroendócrinas de forma a melhorar a biodisponibilidade oral dos agonistas do receptor do péptido 1 semelhante ao glucagon. Começou-se por incorporar o exenatido em nanocápsulas lipídicas de diferentes tamanhos (30, 50, 100, 150 e 220 nm), utilizando um protocolo previamente estabelecido no nosso grupo de investigação. Os DSPE-PEG_{2k} e DSPE-PEG_{2k}-ácido propiónico foram posteriormente inseridos nas nanocápsulas lipídicas de 220 nm,

segundo o método desenvolvido no nosso grupo. Posteriormente, procedemos à caracterização das propriedades físico-químicas das nanocápsulas lipídicas, nomeadamente o diâmetro médio, índice de polidispersão, potencial zeta e eficiência de encapsulação do exenatido. A estabilidade *in vitro* das nanocápsulas lipídicas de 220 nm, que encapsulavam o exenatido com ou sem o ligando ácido propiónico, foi testada em diferentes fluídos gastrointestinais biomiméticos que simulavam as condições gástricas e intestinais nos estados pré e pós-prandial. Finalmente, avaliámos o perfil libertação do exenatido nestas nanocápsulas lipídicas de 220 nm nas condições anteriormente referidas. O diâmetro médio das nanocápsulas lipídicas variou entre os ~35 nm e os ~221 nm. O índice de polidispersão obtido foi reduzido (PdI < 0.15), atestando para a homogeneidade das nanocápsulas lipídicas no que diz respeito à distribuição dos seus diâmetros médios. No que concerne à estabilidade das nanocápsulas lipídicas de 220 nm, os resultados observados demonstraram que a sua estabilidade era mantida ao longo de 2 h em condições gástricas e ao fim de 6 h em condições intestinais, independentemente de ser no estado pré ou pós-prandial. Estes resultados apontam que as nanocápsulas lipídicas de 220 nm são potenciais nanosistemas gastrorressistentes para o tratamento da diabetes mellitus tipo 2. Quanto à avaliação do perfil de libertação do exenatido das nanocápsulas lipídicas de 220 nm em condições intestinais, foi observada uma libertação de cerca de 70% de exenatido ao fim de 6 h. No entanto, o perfil de libertação do exenatido das nanocápsulas lipídicas de 220 nm em condições gástricas apresentou valores indetetáveis. Esta diferença pode ter como causa a elevada afinidade do exenatido para os sais biliares presentes nos fluídos intestinais. Os sais biliares actuam como agente molhante que aumenta a dissolução e libertação do exenatido. Estes resultados indicam que estas nanocápsulas lipídicas de 220 nm representam uma estratégia alternativa às terapêuticas atuais, permitindo a redução de dose do exenatido ou de outros agonistas do receptor do péptido 1 semelhante ao glucagon, devido à sua dupla ação que permite a estimulação da secreção endógena do péptido 1 semelhante ao glucagon nativo, em sinergia com a função da molécula bioativa encapsulada nas nanocápsulas lipídicas. Por um lado, esta alternativa terapêutica apresenta vantagens ao nível do perfil de segurança (menor quantidade de fármaco que possa sofrer acumulação no organismo). Por outro lado, esta alternativa terapêutica permite a administração oral de agonistas do receptor do péptido 1 semelhante ao glucagon resultando numa maior adesão à terapêutica e, consequentemente, no aumento da eficácia terapêutica que se traduz na qualidade de vida melhorada dos doentes que sofrem diabetes mellitus tipo 2.

Palavras-Chave

Diabetes Mellitus Tipo 2, agonistas do receptor do péptido 1 semelhante ao glucagon, Nanocápsulas lipídicas, Exenatido

Abstract

Type 2 diabetes mellitus is the most common type of diabetes and it is considered a major global health care problem that, by 2045, is expected to reach 629 million people, leading to an increasing demand for anti-diabetes therapies. The development of oral peptide-based anti-diabetic treatments, such as Glucagon like peptides 1 receptor agonists, is crucial to avoid daily painful injections and achieve a better patient compliance. Currently, the biggest challenges towards the oral delivery of peptide therapeutics in the pharmaceutical industry are mostly related to the absorption of these peptide drugs into the systemic circulation. Lipid nanocapsules represent a promising oral delivery system for Glucagon like peptides 1 receptor agonists, which could not only increase the absorption of the peptide into systemic circulation, but also increase the endogenous Glucagon like peptides 1 secretion acting as the native endogenous ligands. However, the development of effective Glucagon like peptides 1 receptor agonists encapsulated lipid nanocapsules still faces big challenges due to their lipid core and the high temperature used during preparation, which in theory are not suitable to encapsulate hydrophilic peptide drugs. In this thesis, we developed lipid nanocapsules encapsulating exenatide (model drug)-loaded reverse micelles for oral delivery of Glucagon like peptides 1 receptor agonists. This nanosystem presents a dual-action effect synergizing its own biological effect (stimulation of Glucagon like peptides 1 release) and that of the encapsulated bioactive molecule (exenatide), thus representing an alternative strategy for the treatment of type 2 diabetes mellitus. We also grafted propionic acid on the surface of this nanocarrier aiming at increasing the targeting to enteroendocrine L cells to increase the oral bioavailability of Glucagon like peptides 1 receptor agonists. To be specific, first, exenatide-loaded reverse micelles lipid nanocapsules with different sizes, including 30, 50, 100, 150 and 220 nm, were prepared following a protocol already established in our research group. Secondly, DSPE-PEG_{2k} and DSPE-PEG_{2k}-propionic acid were post-inserted into 220 nm exenatide-loaded reverse micelles lipid nanocapsules following a methodology established in our group. We characterized the physicochemical properties of these lipid nanocapsules (mean average size, polydispersity index, zeta potential and encapsulation efficiency). The *in vitro* stability of PEGylated exenatide-loaded 220 nm reverse micelles lipid nanocapsules with or without ligand of propionic acid (EXE RM LNC-PEG and EXE RM LNC-PEG-Pro) were tested in different gastrointestinal

simulated fluids. Finally, the release profile of the exenatide from EXE RM LNC-PEG was also examined in gastric and intestinal media, respectively.

The obtained LNC shown suitable colloidal stabilities. Moreover, 220 nm EXE RM LNC released an approximately 70% of the entrapped exenatide in intestinal conditions (FaSSIF) after 6 h of incubation. Therefore, these data indicate that the 220 nm EXE RM LNC constitute promising gastrorresistant nanocarriers, able to encapsulate and release exenatide, offering an alternative to the current available therapies for the treatment of T2DM, allowing for the oral delivery of these bioactive agents.

Keywords

Type 2 Diabetes Mellitus, Glucagon like peptides 1 receptor agonists, Lipid nanocapsules, Exenatide

Acknowledgements

This master thesis was achieved with perseverance, advices and guidance. For this reason, I want to thank all who worked with me during those three months in Brussels and the other six months in Lisbon.

Firstly, I would like to thank my Portuguese co-supervisor, Prof. Helena Florindo, and my Belgian coordinator, Prof. Véronique Préat, once they made it possible to proceed my work in the nanotechnology field at Louvain Drug Research Institute of UCL, as a continuation to the project I had already developed with Prof. Helena Florindo's team in the iMed.ULisboa, although in a different therapeutic area.

Secondly, I am grateful for my Belgian supervisor, Prof. Ana Beloqui García, for the opportunity to develop my master thesis' project in her research team.

Thirdly, I am thankful to Prof. Helena Florindo and Prof. Ana Beloqui García for both their guidance and support during the achievement of this thesis.

Finally, my gratefulness to my family and friends from Portugal and Belgium for always being on my side. A special thanks to my parents for encouraging me to follow my dreams.

Thank you!

Abbreviations

ACN: Acetonitrile

AE: Adverse Effects

API: Active Pharmaceutical Ingredient

cAMP: Cyclic Adenosine Monophosphate

DPP-IV: dipeptidyl peptidase IV

DSPE-PEG$_{2k}$: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]

DSPE-PEG$_{2k}$-Pro: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG-2000-propionic acid

Exenatide-LA: Long-Acting Exenatide

FA: formic acid

FaSSGF: Fasted State Simulated Gastric Fluid

FaSSIF: Fasted State Simulated Intestinal Fluid

Fc: Fragment crystallizable

FDA: Food and Drug Administration

FeSSIF: Fed State Simulated Intestinal Fluid

FG: Fasting Glucose

GI: Gastrointestinal

GLP-1: Glucagon-Like Peptide-1

GLP-2: Glucagon-Like Peptide-2

GLP-1 R: GPL-1 receptors

GLP-1 RA: Glucagon-like Peptide-1 Receptor Agonists

GPCR: G-protein-coupled receptors

GT: Glucose Tolerance

HbA_{1c}: Hemoglobin A1c

HPLC: High Performance Liquid Chromatography

IGT: Impaired Glucose Tolerance

LNC: Lipid Nanocapsules

O/W: Oil in Water

PdI: Polydispersity Index

PEG: Poly(ethylene glycol)

PIT: Phase-Inversion Temperature

PIZ: Phase-Inversion Zone

PYY: Peptide YY

RES: Reticuloendothelial System

SGLT2: Sodium-glucose Cotransporter-2

TFA: Trifluoroacetic Acid

T2DM: Type 2 Diabetes Mellitus

USD: United State Dollars

WHO: World Health Organization

W/O: Water in Oil

Z-Ave: Z-Average (size)

ZP: Zeta-potential (surface charge)

List of Contents

List of Figures

List of Tables

1. Introduction

1.1. Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM), also called non-insulin-dependent or adult diabetes, is the most common type of diabetes and it is considered a current major global health care problem.

T2DM is a complex endocrine metabolic disease, that typically appears as a result of excessive body weight, poor diet and physical inactivity (1,2). This disease is a consequence of both genetic and environmental factors that result in an abnormal response of the skeletal muscle, liver and adipose tissue to insulin and in the pancreatic ß-cells dysfunction (2,3). T2DM is marked by the dysregulation of the metabolism of carbohydrates, lipids and proteins, which leads to an insulin impaired secretion, insulin resistance or both (3). Insulin impaired secretion results from decreased beta cell mass and functional defects that leads to beta cell inability to provide enough insulin to fulfil the amount required due to insulin resistance (4). Insulin resistance is the acquired defect that results from the combination of environmental and genetic factors, and it does not necessarily develop to impaired glucose tolerance or diabetes because normal pancreatic beta cells can increase their secretion of insulin to make up for decreased physiological activity (4,5). Insulin resistance is related to tissue-specific inflammation induced by pro-inflammatory cytokines and oxidative stress mediators (6). Chronic exposure to inflammatory and oxidative substances contributes to the occlusion of insulin receptor in the pancreatic ß-cell islets (7). T2DM arises when the insulin secretion by pancreatic ß-cells is not suitable to overcome the insulin resistance in the tissues (6). Patients with insulin resistance display high levels of blood glucose (hyperglycaemia) that promotes oxidative stress, inflammatory pathways' activation and microvascular conditions, which may lead to macrovascular conditions, increasing T2DM's morbidity and mortality (2). Hyperglycaemia appears in the early stage of the disease and it is the crucial symptom that predisposes the development of T2DM, also known as prediabetes (3). Patients with prediabetes may have an increase in glycated hemoglobin (HbA1c) or an impairment in the fasting glucose (FG) or glucose tolerance (GT) levels (8).

The cardinal symptoms of T2DM are polyuria (frequent and abundant urination), polydipsia (excessive thirst), dry mouth, polyphagia (constant hunger), weight loss, tingling or numbness in hands and feet, frequent skin fungal infections, slow healing wounds, vision damages (blurred vision), fatigue and lack of energy (1). However, people

may live with T2DM for several years without being diagnosed because these symptoms can be mild or absent.

Diabetes major complications may arise with the disease progression and include heart attack, stroke, kidney failure, blindness and nerve damage, need of lower limb amputation, besides an increased risk of premature death (1).

T2DM has been associated with multiple risk factors including family history of diabetes, overweight, unhealthy diet, physical inactivity, increasing age, high blood pressure, ethnicity, impaired glucose tolerance (IGT), history of gestational diabetes, and poor nutrition during pregnancy (9).

1.1.1. Epidemiological context of Type 2 Diabetes Mellitus

The number of people living with diabetes has nearly quadrupled since 1980 to 422 million in 2014. The global prevalence of diabetes has almost doubled since 1980, increasing from 4.7% to 8.5% in the adult population, as a reflexion of an increased prevalence of obesity and overweight people, and a widespread lack of physical activity (1).

In 2016, diabetes was the seventh leading cause of death in the world and was the direct cause of an estimated 1.6 million deaths, according to the World Health Organization (WHO) (1).

In 2017, the International Diabetes Federation estimated that 425 million people between the ages of 20 and 79 had diabetes, and 90% of these are T2DM cases (9–11). However, there is still 1 in 2 people (212 million) with diabetes that were undiagnosed (9). That same year, diabetes caused 4 million deaths and there were spent 727 billion United State Dollars (USD) on diabetes treatment (9,10).

Currently, the proportion of people with T2DM is increasing in most countries. Therefore, as both diabetes diagnoses and life expectancy of people living with diabetes continue to increase, the global number of patients with this condition is expected to reach 629 million by 2045 (10), leading to an increasing demand for anti-diabetes therapies.

1.1.2. Current treatment strategies for Type 2 Diabetes Mellitus

T2DM treatment is focused on the achievement of optimal control of blood glucose levels (3). The core of T2DM management is the association of a healthy diet, an increased physical activity and the maintenance of a healthy body weight, with oral therapies, in combination, or not, with injectable therapy. However, T2DM has multiple

factors and its responsiveness to treatment highly varies accordingly to the complexity of the pathogenic mechanism (2).

Concerning the T2DM therapies available in the market, there are several alternatives including biguanides, first and second generations sulfonylureas, meglitinides, α -glucosidase inhibitors, thiazolidinediones, dipeptidyl peptidase IV (DPP-IV) inhibitors, glucagon-like peptide-1 receptor agonists (GLP-1 RA), insulin and sodium-glucose co-transporter 2 (SGLT2) inhibitors (2,3).

Metformin, one of the most used biguanides, has remained as the first-line therapy for T2DM in most guidelines. Metformin decreases insulin resistance and allows a more efficient insulin use (9).

Moreover, other therapeutic options used to help to manage glucose levels include DPP-IV inhibitors that are administered orally whereas the GLP-1 RA must be injected (12).

Furthermore, the treatment with insulin and sulfonylureas result in undesired effects like hypoglycaemia and weight gain (10).

Although there are several therapeutic alternatives, it remains challenging to some patients to maintain glucose homeostasis with the current available drugs. Therefore, developing more effective and safer therapies is essential to achieve optimal control of T2DM, allowing for a better management of the disease to the patients.

This thesis is focused on the GLP-1 RA class, more specifically on exenatide.

1.2. Glucagon like peptides 1 receptor agonists

The enteroendocrine L cells, present throughout the gastrointestinal (GI) tract, secrete essential peptides with physiological function, such as peptide YY (PYY) and glucagon like peptides 1 (GLP-1) and 2 (GLP-2). After the ingestion of glucose, other sugars, dietary fiber or fatty acids, these peptides are quickly released into the circulation (13).

L cells express many G-protein-coupled receptors (GPCR), that can be activated by nutrients, including lipids, found in the gut lumen (14,15). Therefore, any strategy that induce the endogenous peptide secretion constitutes a promising alternative for therapies of prevalent diseases such as T2DM and obesity (16–18). Thus, GLP-1 became a promising therapeutic class for the treatment of T2DM, particularly in the control of glucose and energy homeostasis, due to their ability to reduce food intake and to enhance

insulin secretion (incretin effect) (13). This incretin effect accounts for approximately 50% to 70% of the total insulin secreted after oral glucose administration (19).

GLP-1 was discovered in 1987 and consists in a 30 amino acid peptide that works as an incretin hormone. GLP-1 is released by intestinal L cells after food intake and plays an important role in glucose homeostasis (20,21). After GLP-1 release, it binds the GPL-1 receptors (GLP-1 R) present in pancreas, brain, heart, kidney and GI tract, activating them. Further, it promotes the increase of cyclic adenosine monophosphate (cAMP) and intracellular calcium levels leading to the glucose-dependent insulin exocytosis (20). GLP-1 promotes insulin secretion when the levels of glucose are high, but when the glucose levels are normal it almost exerts no effect, avoiding the risk of hypoglycaemia (10).

GLP-1 has potent antidiabetic effects for T2DM treatment such as: auto-limited insulinotropic effect, that decreases the risk of hypoglycaemia; regulation of postprandial glucose levels; stimulation of pancreatic ß-cells proliferation, neogenesis and inhibition of pancreatic ß-cells apoptosis, which can prevent or delay the ß-cells failure; prevention of gastric emptying, that leads to lower postprandial glucose levels; activation of GLP-1 Rs in the nervous system, that promotes satiety and inhibits energy intake and may help the loss of body weight; and decrease of glucagon secretion, leading to a reduction of glucose levels (20,22,23). However, native GLP-1 has a limited clinical application for T2DM once it has a short life-time of only 2 min due to the rapid degradation by DPP-IV (Figure 1) (20). Thus, peptides like incretins became a new drug category in clinical therapy for T2DM treatment, with special interest on GLP-1 R agonists (GLP-1 RA) that mimic the endogenous GLP-1 incretin action and, at the same time, present an extended half-life (24,25).

Exendin-4, a natural 39 amino acid peptide and hormone isolated from the saliva of a venomous lizard *Heloderma suspectum*, also known as Gila monster, was identified as a GLP-1 RA (10,20). Exedin-4 is only 53% homologous to human GLP-1, but comparatively to the native GLP-1, *in vitro* studies have shown that it is slightly more potent and more resistant to degradation by DPP-IV (10). Additional studies have demonstrated that exedin-4 is mostly metabolized by the kidneys and eliminated by glomerular filtration, whereas native GLP-1 is metabolized by kidneys, liver and peripheral tissues (10).

Although rapid degradation is still one of the greatest limits to the use of GLP-1 RA, several strategies have been successfully developed from exendin-4, including

sequential modification, fatty-acids linked to the peptide, plasma albumin binding to peptide, fragment crystallizable (Fc) region of a monoclonal antibody attachment to peptide, drug delivery systems, and PEGlylation (10). These strategies were employed to make GLP-1 RA resistant to DPP-IV degradation and to increase their metabolic stability, important aspects since their efficacy is dose dependent (10,20,21). The development of several half-life extension strategies leads to different pharmacokinetic profiles, efficacy, safety and use of these drugs, which increases the potential use of GLP-1 RA as a major therapeutic option for T2DM treatment (10).

On the other hand, GLP-1 RA present adverse effects (AE) that compromise the quality of the patient's life and, consequently, reduce the treatment adherence (10,24). The more common AE are nausea, vomiting, injection-site reactions and systemic allergic reactions (10,24). Additionally, they have shown carcinogenic potential and ability to enhance pancreatitis rate (10,24). The GI AE are not severe, transitory and dose dependent, limiting the administration of the maximum efficacious dosage, because the maximum tolerated doses are determined by the induction of nausea and vomiting (10,24,26). Moreover, GLP-1 RA induce antibodies' production, but they do not react with native GLP-1 and do not reduce the drug efficacy (10,24).

Accordingly to the strategy undertaken, it is possible to distinguish the GLP-1 RA in short-acting and long-acting ones (10,20). The major difference between the short-acting and the long-acting GLP-1 RA is that the plasma levels of the short-acting GLP-1 RA fluctuate, while this is not obtained for the long-acting ones, which can eventually result in the supra-activation of the GLP-1 R (10,27). This results in different action mechanisms, efficacy and tolerability (10,27). Therefore, the long-acting GLP-1 RA enable a better therapeutic adherence from patients, because they are more efficient in the same period, and also require lower injections frequency (10,27). Short-acting GLP-1 RA are administered once or twice daily, while long-acting GLP-1 RA are administered once weekly (10).

Exenatide was the first synthetic version of exendin-4 approved by the Food and Drug Administration (FDA) as a GLP-1 RA (10).

Currently, there are six GLP-1 RA (exenatide, liraglutide, lixisenatide, semaglutide, dulaglutide and albiglutide) approved by the FDA and others are under development.

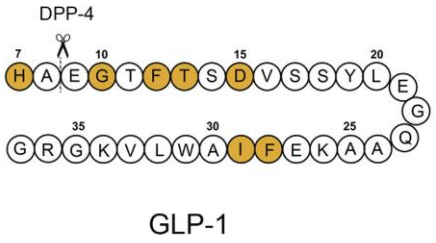

Figure 1 - GLP-1 degradation by DPP-4, adapted from (10)

1.2.1. Short-acting Glucagon like peptides 1 receptor agonists

Short-acting GLP-1 RA have shorter half-life, but they can prevent gastric emptying and lead to an identical glucagon secretion suppression as long-acting GLP-1 RA. On one hand, short-acting GLP-1 RA control more efficiently postprandial hyperglycaemia and increase or replace rapid-acting insulin during meals (10,28). On the other hand, they are more susceptible to cause AE, especially GI AE such as nausea (10,26). Nausea results from the high peak short-acting GLP-1 RA plasma concentrations (10).

Exenatide ($C_{184}H_{282}N_{50}O_{60}S$) (Figure 2) is natural resistant to DPP-IV proteolysis and presents a molecular weight of 4186.6 Daltons (10,29). As mentioned above, it was the first approved GLP-1 RA for human clinical use (21), as an additional treatment for patients who do not respond well to oral therapies and, therefore, do not reach an ideal glycaemic control (10,30). In spite of its natural resistance to the DPP-IV degradation, exenatide's half-life is only 2.4 hours, leading to a complex therapeutic scheme that requires two administrations per day, compromising the therapeutic adherence (10,31–33). Short-acting exenatide reduces HbA1c levels (mean 0.98%), fasting blood glucose (1.69 mmol/L), and body weight (1.5 kg) (10,17,34). Moreover, it has shown to be non-inferior to insulin glargine (long-acting insulin), in combination with metformin or sulfonylurea.

(A) (B)

Figure 2 - (A) Peptide sequence and molecular structure of exenatide; (B) exenatide protein structure, adapted from (10) **and Drug Bank.**

Lixisenatide (Figure 3) is also a short-acting GLP-1 RA that differs from exendin-4 at C-terminal amino acids, and presents a half-life of 3-4 hours (10,27).

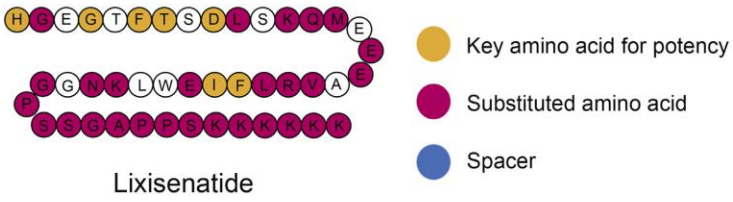

Figure 3 - Peptide sequence and molecular structure of lixisenatide, adapted from (10)

1.2.2. Long-acting Glucagon like peptides 1 receptor agonists

Long-acting GPL-1 RA result from the application of several strategies to improve the half-life of the drug. These GLP-1 RA mainly affect fasting plasma glucose levels, which may be the reason for a better efficacy in reducing HbA1c levels comparatively to the short-acting GLP-1 RA (10).

Liraglutide (Figure 4) displays a Lys instead of an Arg at 34 position and a linear fatty acid moiety bound to a glutamic acid, and presents an improved half-life of 12-14 hours and an affinity to serum albumin, enabling a daily administration (10,33).

Albiglutide (Figure 4) results from a combination between two copies of a modified human GLP-1 and recombinant albumin, enabling its reduced clearance, DPP-IV proteolysis resistance, and extended half-life of 6-8 days (10,35).

Dulaglutide (Figure 4) consists on two identical sulfidic chains (DPP-IV resistant) coupled to a G4 modified immunoglobulin (IgG4), providing a decreased clearance and the binding to the Fc receptors, and also to a reduced antibodies production (10,27).

The long-acting exenatide (exenatide-LA) consists on a biodegradable microsphere formulation, that offers the option for a once weekly subcutaneous injection, enabling the slow release of exenatide through diffusion (10,27). Exenatide-LA is the same molecular entity as exenatide twice daily, however, this formulation delays drug metabolism, which results in an extended half-life and a plasma concentration peak at 2 weeks after administration (10,36). Exenatide-LA reduces HbA1c (mean 1.4%), fasting blood glucose (mean - 1.94 mmol/L) and the body weight (mean 2.5 kg), with sustained effects for 5 years (10,34). Moreover, it has shown to be more efficacious than DPP-IV inhibitors, while no difference was obtained for its use and metformin administration; but, it has shown inferiority concerning pioglitazone (10,33). Comparing exenatide's twice daily and long-acting formulations, significant HbA1c reductions were achieved with

exenatide-LA, with lower incidence on GI AE, however with higher incidence on injection-site AE and antibody formation (10,17).

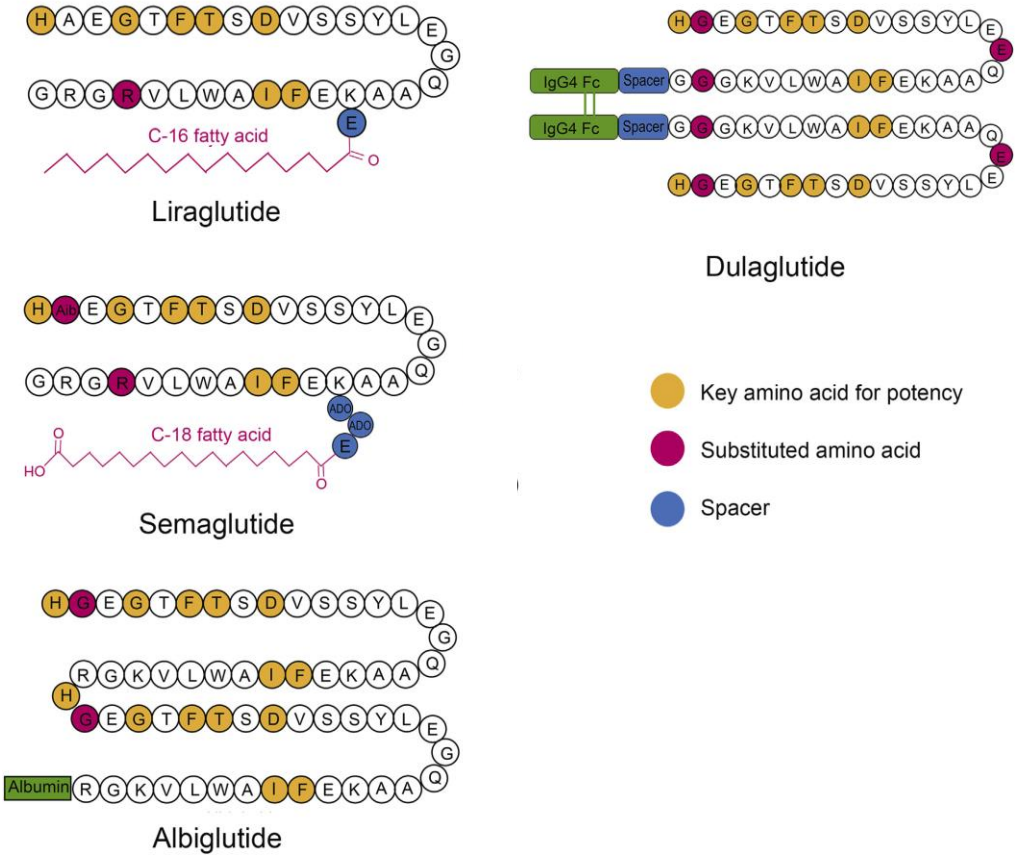

Figure 4 - Peptide sequences and molecular structures of FDA approved long-acting GLP-1 RA (10)

1.2.3. Glucagon like peptides 1 receptor agonists available on the market

Concerning GLP-1 RA class, there has been several strategies employed over the years resulting in many therapeutic options.

Based on a simple sequential modification, Byetta® (exenatide) and Adlyxin® (lixisenatide) emerged in the market as a twice daily and once daily treatments, respectively (10). Victoza® (liraglutide) and Ozempic® (semaglutide) resulted from the sequence modification associated with covalent fatty acid's link, and were approved as a once daily and once weekly drugs, respectively (10). More intricated molecular modifications, including the fusion of either recombinant human serum albumin or an antibody Fc moiety to a GLP-1 RA led to the development of once-weekly agents Tanzeum® (albiglutide) and Trulicity® (dulaglutide), respectively (10). Several GLP-1 modifications have also been developed, including the use of a recombinant peptide polymer XTEN® (VRS-859) or polyethylene glycol (PEG; LY2428757), which were

developed as a once-monthly treatment and a once-weekly treatment, respectively. However, none of these molecules proceeded beyond clinical trials (10).

In this market, it is also available controlled release GLP-RA agents as Bydureon® (exenatide), the once-weekly poly(lactide-co-glycolide) microspheres, and ITCA 650, an once-yearly titanium implant that has completed all Phase 3 and has been submitted to the FDA (10,37). Xultophy® (liraglutide and insulin degludec) and Soliqua® (lixisenatide and insulin glargine) were both approved as once daily treatments (10). All the above-mentioned drugs were developed to be administered by subcutaneous injections.

In September of the current year, FDA approved Rybelsus® (semaglutide), the first and only GLP-1 RA pill available in the market for the oral treatment of T2DM. It means that there is now a therapeutic option for adults with T2DM who cannot achieve a good management of their HbA1c levels with current antidiabetic therapeutic options. Rybelsus® is a once daily pill that will compete with the already marketed GLP-1 RA administered via injection. This therapy has proved to promote superior HbA1c reductions when compared with placebo, oral SGLT2 inhibitor empagliflozin and oral DPP-IV inhibitor sitagliptin (38).

1.3. Nanomedicine and development of orally delivered Glucagon like peptides 1 receptor agonists

One of the biggest challenges for the pharmaceutical industry is to develop oral dosage forms that enable therapeutic peptides' absorption to the systemic circulation.

Peptides are suitable and biocompatible therapies for several diseases since they present high efficiency, low toxicity and good tolerance. However, peptides are fragile in biological conditions, have large molecular weights and are hydrophilic, which results in low permeability and, consequently, poor bioavailability, reasons why most of them are administered by injection (39).

The relevance of oral peptides formulations development is to meet the need of a viable alternative to patients who show a poor compliance due to a chronic parenteral administration, resulting in a lower treatment efficacy. Actually, some studies have estimated that more than 5% of the population suffers needle phobia (40).

Oral administration is a non-invasive and painless solution to improve the use of therapeutic peptides and to promote a better patient compliance. Moreover, the administration of bioactive agents by the oral route for the treatment of T2DM better mimics the physiological condition than their injection, as the delivery by the portal vein is similar to the pancreatic release (41). Furthermore, from a commercial perspective, the

oral dosage forms may allow the extension of patents with the development of new products for expiring parenteral administered peptides (41).

Currently, they are several strategies under development to achieve this goal, including protease inhibitors, penetration enhancers and nanotechnology-based drugs (41).

Nanotechnology consists on the use of materials that can result in improved physical, chemical or biological outcomes due to the sizes within the nanoscale (42). Nanomedicine is the application of nanotechnology to medicine and is a promising and emerging field for the development of effective targeted treatments (39), such as orally delivered T2DM therapies. Nanomedicine comprises the use of nanocarriers as nano-sized systems that may entrap, load, conjugate or deliver one or several active pharmaceutical ingredients (API) in order to fulfil their delivery more efficiently (42). The nanocarrier should be biodegradable, biocompatible, non-toxic and with a suitable size that can be up to 500 nm in diameter, accordingly to pharmaceutical literature since there is no consensus among the different regulatory agencies (39).

In the development of orally delivered peptides, nanomedicine has been considered a potential delivery tool. Nanocarriers can protect the drugs entrapped from the biological conditions along the GI tract and have the ability to cross the epithelia improving their uptake (39). Moreover, nanocarriers present useful advantages comparatively to conventional treatments, including: entrapment of one or several API; improvement of API solubility and pharmacokinetic profile; API protection from chemical and enzymatic degradation; higher cellular and tissue penetration; specific targeting resulting in reduced side effects and improved tolerability; controlled release; better API biodistribution and increased local concentration in target tissues; and evasion of API resistance mechanisms (42). All these properties offer a possibility of therapies with an improved bioavailability and greater treatment efficacy.

However, it remains difficult to produce nanocarriers encapsulating oral peptides at an industrial scale, while achieving a suitable peptide loading and maintaining their intestinal stability (41)

Nanocarriers' world comprise liposomes, polymeric nanocapsules and nanospheres, micelles, lipid solid nanoparticles, lipid nanostructured carriers, nanotubes, dendrimers, cyclodextrin-based nanoparticles and viral nanoparticles, among others.

In this thesis, we want to focus on a class of nanocarriers that has been considered as a promising delivery strategy for T2DM treatment, the lipid nanocapsules (LNC) (43).

1.4. Lipid nanocapsules

Lipid nanocapsules (LNC) have been developed and patented by Prof. Benoit and his research group from University of Angers in 2000. They are hybrid nanocarriers that mimic lipoproteins and display a structure with properties between polymer nanocapsules and liposomes (43). However, when compared to liposomes, LNC present greater physical stability (up to 18 months at 4ºC) and are prepared by a solvent-free and soft-energy process (43).

LNC are spherical and colloidal monodispersed systems with sizes that vary within a range of 20 to 100 nm (43). They usually have high rates of encapsulation efficiencies (43). Besides, LNC are able to encapsulate hydrophilic and lipophilic drugs, nucleic acids within lipoplexes or radiopharmaceuticals, while enabling a sustainable release of these drugs (44).

1.4.1. Preparation of Lipid nanocapsules

LNC formulation requires at least three components: an oily liquid phase, an aqueous phase and a non-ionic surfactant. The oily core is made of medium-chain triglycerides of capric and caprylic acids, commercially known as Labrafac® WL 1349 (43). The surrounding rigid membrane is made of a lipophilic surfactant (Lipoid® S100) linked to the oily phase and a hydrophilic non-ionic surfactant with the chain of polyethylene glycol (PEG) water phase oriented (Solutol® HS15) (43). Lipoid® S100 consists of 94% of phosphatidylcholine soybean lecithin and is used in small proportions to increase the LNC stability, which is essential for LNC formulations between 50-100 nm (45). Solutol® HS15 consists in a mixture of free PEG 660 and PEG 660 hydroxystearate. The aqueous phase is composed by MilliQ® water and sodium chloride salt (NaCl) (43). All the previous components are FDA-approved for parenteral, topical and oral administration (43).

The formulation and the LNC stability are influenced by several elements, such as: the amount of non-ionic surfactant (Solutol® HS15), which plays a major influence on LNC formation and stability (46,47); the temperature cycles that promote LNC formation and improve the dispersion of LNC (48,49); the oil proportions (Labrafac® WL 1349) responsible for increasing LNC size (50); the NaCl that decreases phase-inversion temperature (PIT) (51,52); and the lipophilic surfactant (Lipoid® S100) able to stabilize the LNC rigid shell and promote the process of freeze-drying (53,54).

1.4.2. Phase-inversion temperature method

The LNC preparation is a two-step process based on the phase-inversion temperature (PIT) method of an emulsion resulting in monodispersed LNC formation, as described in the patent No. WO02688000 (55). This method consists on variation of nonionic surfactant solubility with temperature. At temperatures above the phase inversion zone (PIZ), the surfactant becomes less hydrophilic resulting in the formation of water in oil (W/O) emulsion (51). At the PIT, there is a balance between both hydrophilic and lipophilic surfactant behaviour (43).

Firstly, all components are mixed under magnetic stirring and heat from room temperature up to pre-determined temperature (T2, ~15ºC above the PIT), in order to form a W/O emulsion (43). The amounts of the components vary for each study. It follows a cooling process to the T1 temperature (~15ºC below the PIT), to obtain an O/W emulsion (43). Afterwards, multiple temperature cycles crossing the PIZ are performed, between T2 and T1 (43). The range of temperatures should be chosen accordingly to the medium salinity and the encapsulated API thermostability, to avoid its degradation during the process.

The second step consists in adding cold water to the mixture during the last cooling process, at a temperature ~1-3ºC from the beginning of the O/W emulsion, followed by 5 minutes under low magnetic stirring (50). This last step breaks irreversibly the microemulsion obtained in the PIZ and enables the formation of stable nanocapsules (43).

1.4.3. Physical characteristics

LNC size and surface characteristics can be adjusted to increase their plasma half-life for passive targeting, to recognize specific receptors for active targeting or to be used in oral and local delivery of drugs (43).

LNC particle size and dispersity are highly influenced by the constituent proportions, so it was established a ternary diagram to allow the optimization of these proportions (50,51). When the NaCl and lipophilic surfactant (Lipoid®) amounts are fixed at 1.75% and at 1.5% respectively, the feasibility region for the LNC formation consists on 10-40% of hydrophilic surfactant (Solutol®), 35-80% of water and 10-25% of oil (50). Nanocapsules average size ranges between 20-100 nm and its polidispersity index (PdI) has a narrow variation (PdI < 0.3).

In this feasibility zone, the amount of Solutol® plays an important role on the average diameter of LNC, as higher percentages, decreases the LNC average diameter. This is caused by its characteristics at the triglyceride/water interface (56). On the other hand, an increased oil proportion results in an increased particle size, whereas water has no consequence on LNC diameter (Table 1).

Moreover, the temperature cycles have a major effect on LNC formulation. LNC formation and quality of LNC size and dispersion are improved by an increased number of cycles. The number of temperature cycles required to LNC stabilization are as higher as less the amount of surfactant added. When used greater amounts of surfactant, it seems that there is not a need to apply several temperature cycles (49). When working within the values of the feasibility region, it appears that an increase on the number of temperature cycles to more than 3 is not useful to adjust LNC size and decrease of PdI.

Concerning the zeta potential, it represents the electrical potential at the shear surface of LNC, being relevant to predict and control LNC stability (57). It is measured by a laser Doppler method. Commonly, LNC present a negative surface charge as a result of the presence of phospholipids and PEG dipoles in their surface (58,59).

Table 1. Influence of the components proportion in the PIZ and size of LNC

Components	Amount	PIZ (ºC)	Size (nm)
Labrafac® WL 1349	↑	-	↑
Peceol®	↑	↓	↓
Solutol® HS15	↑	↑	↓
Lipoid® S100	↑	↓	↓
NaCl	↑	↓	-

1.5. PEGylation as a strategy to enhance lipid nanocapsules diffusion in mucus

LNC surface is the first layer in contact with the biological environment, thus, it is clear that a suitable LNC surface design can improve its stability *in vivo* (39). Several LNC surface modifications can be performed, namely by coating components such as hydrophilic polymers (e.g. PEG) or surfactants after formulation, or by performing an LNC structure or affinity modification during its formulation. Surfactants or hydrophilic polymer coatings (e.g. PEG) can be grafted on the LNC surface to avoid its opsonization by plasma membranes, prevent clearance by the reticuloendothelial system (RES) and to

increase its plasma half-life. These strategies will influence LNC surface properties like zeta potential, hydrophilicity, and therefore its stability in GI fluids, resistance to digestion, mucus interaction and bioavailability, determining the LNC path in biological conditions (39). However, it is crucial to control physicochemical characteristics, so it does not cause immunological responses.

Mucus consists in a viscoelastic and sticky gel layer that coats exposed mucosal membranes, like lung airways, GI tract, female reproductive tract, nose and eyes (60). The viscoelastic mucus secretions can be responsible for the fast elimination of drug delivery systems, therefore jeopardizing the sustained and targeted drug delivery at mucosal surfaces (61). To increase particle distribution across the mucosa and improve delivery to the underlying epithelial cells, there is a need to develop nanocarriers capable of crossing mucus. Therefore, these drug delivery systems should be capable of: (a) penetrate mucus at faster rates than mucus turnover (62); (b) be internalized into cells or be retained at the mucosa (63); (c) promote sustained and/or controlled drug release (64); and (d) protect the drug content from early degradation (65). Besides that, once mucus presents steric and adhesive barrier properties, mucus-penetrating nanocarriers must be: (a) small enough to escape steric obstruction by the dense mesh of mucin fibers and (b) muco-inert enough to avoid mucins association (66).

In the last decade, it has been shown that PEGylation strategies based on covalently grafting PEG to nanocarriers surface, are successful in the development of mucus-penetrating drug carriers for targeted drug delivery, achieving mucus diffusion rates approaching their rates in water (67).

PEG is a hydrophilic and non-ionic polymer, capable of reducing particle adhesion to the mucin fibers of mucus, thus providing a rapid diffusion of the nanocarriers through the interstitial fluids between these fibers (68). The PEG ability to increase mucus diffusion results in an enhanced distribution and retention of nanocarriers at mucosal surfaces, promoting the achievement of sustained and targeted mucosal drug delivery systems. Moreover, PEG also plays an important role in pharmaceutical formulations, due to its ability to enhance colloidal stability, limit LNC recognition, decrease macrophage uptake and complement activation, and reduce elimination via liver which results in an increased blood circulation time (69,70).

The design of these PEG coatings depends on the exact physicochemical properties and PEG conformation on the nanocarriers surface required to escape the association with mucins and to promote fast nanocarrier diffusion through mucus. The

length of PEG must be adapted to its application, due to its tendency to present a globular conformation. Therefore, PEG length decreases nanocarrier accessibility to ions. Hence, there has been an increased interest among the scientific community on these alternatives and it appears that a PEG with a length of 2000 or 3000 g mol^{-1} is suitable when the shell accessibility is essential. However, if the nanocarriers must be protected from the external environment, longer PEG lengths are needed.

Recently, several studies (71–73) have shown that PEGylated LNC are able to provide a suitable stability in physiological environment and an improved intracellular delivery.

Propionate or propionic acid is a naturally occurring short-chain fatty acid (SCFA), produced by fermentation of polysaccharides, oligosaccharides, long-chain fatty acids, protein, peptides and glycoprotein precursors by the microbiota of colon (74,75). Propionic acid is present in the milk and other dairy products, due to bacterial fermentation, and it is also used as a food preservative once it is a potent mold inhibitor (74,75).

On one hand, some studies have related several beneficial metabolic effects of dietary fibers, like the increase of postprandial satiety and reduce of body weight and fat mass, to the formation of SCFAs from fermentation (76). Particularly, propionic acid has been considered to present inhibitory effects on the metabolism of free fatty acids and inflammation. Therefore, propionic acid was potentially able to enhance insulin sensitivity once high free fatty acid levels cause inflammation and these two factors results in insulin resistance (75). Moreover, there was evidence that propionic acid resulted in satiety and reduced food intake in ruminants (75).

On the other hand, a recent study conducted in mice and humans reported that the exposure of mice to propionic acid caused the prompt activation of sympathetic nervous system and a rise of glucagon and FABP4 (fasting insulin counter-regulatory hormones), in postprandial conditions (74). These higher levels of hormones led to an increase of endogenous glucose production, which may be the result of a primary hepatic glycogenolysis, causing hyperglycaemia and, consequently, hyperinsulinemia (74). Additionally, this study has shown that chronic exposure of mice to a similar daily propionate amount caused increased plasma concentrations of glucagon and FABP4 and, consequently, insulin resistance, hyperinsulinemia and gradual weight gain (74).

The metabolic disadvantages reported using lower concentrations of propionic acid contrast with the beneficial metabolic effects demonstrated in previous studies, using higher concentrations of propionic acid than the ones used as a food preservative.
However, the direct metabolic and physiological effects of propionic acid are not well established and the causal connection between oral propionic acid consumption and human obesity and metabolic abnormalities is still under study.

For all the reasons discussed above, we have chosen to formulate 220 nm EXE RM LNC-DSPE-PEG_{2k} and EXE RM LNC DSPE-PEG_{2k}-Pro to evaluate these two types of nanoparticles as carriers for the oral delivery of peptides.

1.6. Simulated gastrointestinal fluids

Orally administered drugs are the most common pharmaceutical forms; however, the prediction of oral drug absorption remains complex, due to the different biopharmaceutical characteristics of drug formulations, and to the intricacy of GI physiology (77). Drug absorption in the GI tract is determined by the rate of drug dissolution, which is influenced by drug physicochemical characteristics, such as pKa, solubility, crystalline energy and surface area, and by the GI tract biological conditions (78). These GI conditions namely pH, surface tension, solubilization, buffer capacity, and volume of luminal content, vary after food ingestion and change along the GI tract (78). For example, after food ingestion there are higher concentrations of amphiphilic bile components such as bile salts and lecithin leading to an increased dissolution rate for several poorly soluble drugs (78). This may happen due to an increased solubility by micellar solubilization, at higher concentrations than critical micelle concentration, and/or to a raised drug wettability (78).

As known, gastric and intestinal enzymes can quickly digest lipids, that are later absorbed within micelles by enterocytes. Therefore, the GI tract can be considered as a barrier that may reduce the absorption of LNC due to disruption. Thus, it is essential that orally delivered drugs cross the first barrier of the GI tract, in order to further cross the intestinal epithelium.

It has been demonstrated that the choice of suitable simulated GI fluids, containing enzymes that mimic the human body physiological conditions, have an important application to forecast the *in vivo* performance of formulations and the food effects, concerning oral dosage forms. Moreover, these dissolution media help to correlate the *in vitro* and the *in vivo* dissolution during the pharmacokinetic studies. Therefore,

biorelevant dissolution testing leads to an optimization of dosing conditions and drug formulation (77).

The most relevant goals of a biorelevant *in vitro* dissolution testing are the comparison of formulations and the forecasting of the *in vivo* behaviour of drug formulations before and after food ingestion. The forecast of intralumenal performance in the proximal gut requires a correct simulation of the conditions in the stomach and the proximal part of the small intestine (79). Therefore, in order to design a biorelevant dissolution test there was the need to develop a global fasted state and a global fed state medium that enable the correlations between *in vitro* and *in vivo* drug dissolution.

Dr. Jennifer Dressman's research team of the J. W. Goethe University (Germany), has developed biorelevant GI media that simulate the fasted and the fed states to simulate the important physiological conditions in the GI tract (78,80–84). Fasted State Simulated Gastric Fluid (FaSSGF) was developed as the medium that mimics the fasted stomach, which contains pepsin and low quantities of bile salt and lecithin (85). At fasted state, there are two essential physiological factors that influence drug dissolution and absorption, consisting in: GI tract hydrodynamics; and GI fluids components. The first factor depends on GI motility, such as the gastric emptying. Concerning the key GI fluid components, these are pH, bile salts and buffer species, volume, enzymes, osmolarity and calcium amounts (77). FeSSGF represents the postprandial stomach (79). About twenty years ago, there were introduced Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF) (83).

Bile salts are able to rush the adsorption of lipase on particle surfaces and consequently, the complex lipase/co-lipase can degrade the particles (86). Sodium taurocholate represents the bile salts, once it has a low pKa value and due to its good solubility at all pH values considered. Media that only comprise sodium taurocholate as the bile salt have been successful in the solubility prediction of poorly soluble drugs (79). The surfactants allow the evaluation of strength and integrity of extended release formulations' coating (77).

1.7. Previous data from our research group studies and the aim of this thesis

Recently, it has been described that LNC are able to trigger GLP-1 secretion by L cells (87). In this project, I continued to develop the idea of our research group that LNC

with appropriate size would be able to deliver peptides such as GLP-1 RA into blood circulation and, at the same time, mimic endogenous ligands that stimulate the secretion of GLP-1. A previous study of our research team (88) demonstrated that the LNC have the potential to work as a dual-action oral delivery system, since they were capable of targeting and stimulating the enteroendocrine L cells to secrete the endogenous GLP-1, while being also able to act as an oral carrier for GLP-1 RA, exenatide and liraglutide. However, in this study they also concluded that these LNC needed to be further optimized to surpass the biological barrier of the mucus layer, without compromising the targeting effect on the enteroendocrine L cells. Increasing the mucous diffusion could potentially provide increased GLP-1 levels, it was not known if the obtained levels would be therapeutically relevant. They found that there was no significant pharmacological blood glucose lowering effect observed for neither exenatide-LNC nor liraglutide-LNC in normal mice. It was further observed in an *ex vivo* study that this occurred as LNC were mainly confined to the mucus layer covering the intestinal cells.

Another study from our research group (87) tested five different sizes (25, 50, 100, 150, and 200 nm) of LNC obtained by several proportions of the different components. Small nanocapsules appeared to be more toxic than large ones, probably because smaller particle sizes required a higher amount of surfactant (Solutol® HS15) to stabilize their large surface area. The amounts of Solutol® HS15 decrease with increase particle sizes. It has been reported that the toxic effect of the surfactants mainly depends on the interactions with both the polar head groups and the lipophilic tails of the cellular lipid bilayer, resulting in the disruption of the plasmatic membrane (89,90). In addition, their results showed that the GLP-1 secretion was induced only by the 200 nm size LNC, emphasizing how important the LNC particle size is on the secretion of GLP-1 by L cells. Therefore, LNC with suitable size would be able to deliver drugs into blood circulation and mimic endogenous ligands thus inducing a combined incretin effect essential for T2DM treatment. The different formulations had no effect on proglucagon mRNA expression, suggesting that there was not an increased in the GLP-1 synthesis. They further showed that 200 nm LNC administration in normoglycemic mice increased the GLP-1 levels by 4- and 3-fold compared to untreated control mice 60 and 180 min after the administration, respectively. Finally, this study suggested that 200 nm LNC may be a potential gastro-resistant nanocarrier to encapsulate drugs and a promising ligand to induce a stimulation of GLP-1 secretion for T2DM treatment.

In this thesis we wanted to further explore the 220 nm LNC encapsulating exenatide as a potential dual-action nanocarrier of GLP-1 RA.

2. Materials and Methods

2.1. Materials

Solutions were prepared from analytical grade reagents using Millipore Milli-Q® ultrapure water.

Solutol® HS15 (mixture of free PEG 660 and PEG 660 12-hydroxystearate, M_w 870 Da), Span® 80 (sorbitan oleate), lecithin, sodium taurocholate, sodium hydroxide (NaOH), sodium monobasic phosphate ($NaH_2PO_4.H_2O$), acetic acid and pepsin were purchased from Sigma-Aldrich (St. Louis, USA).

Labrafac® WL1349 (caprylic/capric acid triglycerides) and Peceol® (oleic acid mono-, di- and triglycerides) were kindly provided by Gattefossé (Saint-Priest, France).

Lipoid® S100 (soybean lecithin at 94 % of phosphatidylcholines) was bought from Lipoid GmbH (Ludwigshafen, Germany).

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG_{2k}) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG-carboxylic acid (DSPE-PEG_{2k}-Pro) (customer service) were purchased from NANOsoft polymer.

Sodium chloride (NaCl) and trifluoroacetic acid (TFA) were bought from Carl Roth GmbH & Co. Kg (Karlsruhe, Germany).

Acetonitrile and methanol were purchased from VWR Chemicals (Pennsylvania, USA).

Dichloromethane was bought from Fisher Scientific UK (Loughborough, UK).

All chemical regents used in this study were of analytical grade.

2.2. Preparation of reverse micelles-loaded lipid nanocapsules differing in particle size

Reverse micelles-loaded lipid nanocapsules (RM LNC) were prepared in two steps. The encapsulation of exenatide within reverse micelles, and further encapsulation of exenatide-loaded reverse micelles (EXE RM) within LNC. Initially, the EXE RM were prepared with a mixture of a surfactant (Span® 80) and an oil (Labrafac® WL1349) (1:5 weight ratio) under high speed stirring. Afterwards, 50 µL of exenatide (30 mg/mL in MilliQ water) were added dropwise in the mixture and maintained under stirring. Exenatide-loaded reverse micelles lipid nanocapsules (EXE RM LNC) were prepared following a phase inversion process previously described by Heurtault *et al* (51).

In summary, all components, such as lipophilic Labrafac® WL1349, Lipoid® S100, Solutol® HS15, sodium chloride (NaCl) and Milli-Q® water, were mixed together at 40 °C at 200 rpm for 5 minutes. Three temperature cycles of progressive heating/cooling were conducted from 45 °C to 60 °C (30, 50, 100 and 150 nm LNC) and from 50 °C to 67 °C (220 nm LNC). Within the last cycle, pre-heated 500 µL of exenatide-loaded RM were added to the mixture at approximately 3 °C above the phase inversion (PIZ; 50 to 52 °C for 30, 50, 100, 150 nm LNC, and 59 to 61.5 °C for 220 nm LNC). After cooling the solution to reach the phase inversion zone (PIZ) temperature, 2.5 mL of cold water (0 °C) were added at 51.5 °C (30, 50, 100 and 150 nm LNC) and at 60.5 °C (220 nm LNC) under high speed stirring for two minutes. The EXE RM LNC were filtered using a 0.45 µm filter and stored at 4 °C until use.

The final composition for the different sizes formulations is summarized in Table 2.

Table 2. Composition of reverse micelles LNC

LNC size (nm)	**Reverse micelles (RM)**	**30**	**50**	**100**	**150**	**220**
Drug solution (µL)	50	/	/	/	/	/
Span® 80 (mg)	100	/	/	/	/	/
Labrafac® WL 1349 (mg)	500	144	219.8	633.6	761.5	769.5
Solutol® HS15 (mg)	/	700	400	300	220	120
Lipoid® S100 (mg)	/	13.4	13.4	13.4	13.4	13.4
Peceol® (mg)	/	176	146.6	158.4	150.5	85.5
Sodium chloride (mg)	/	50	50	50	50	50
MilliQ® Water (µL)	/	980	1234	908	868	1025
MilliQ® Water at 0 ºC (µL)	/	2500	2500	2500	2500	2500

Temperature / Cycle (º C)		45-60	45-60	45-60	45-60	50-67
PIZ (º C)		50-53	50-52	50-52	50-52	59-61

2.3. Post-insertion of DSPE-PEG$_{2k}$ and DSPE-PEG$_{2k}$-propionic acid into 220 nm EXE RM LNC

The 220 nm EXE RM LNC-DSPE-PEG$_{2k}$ and EXE RM LNC-DSPE-PEG$_{2k}$-Pro were prepared following a post-insertion method modified by our research group (91). Firstly, 5 mg of DSPE-PEG$_{2k}$ and DSPE-PEG$_{2k}$-Pro were weighted into two different tubes. Then, 1 mL of EXE RM LNC was added to each tube (5 mg/mL), followed by vortexing for 5 minutes. The formulations were incubated at 38-39 °C at 150 rpm, for 15-20 minutes. Each 15-20 minutes, the formulations were vortexed, and then quenched in an ice bath for 1 minute and centrifuged, followed by another incubation for 15-20 minutes. The total incubation time was around 4 h. The 220 nm EXE RM LNC-DSPE-PEG$_{2k}$ and 220 nm EXE RM LNC-DSPE-PEG$_{2k}$-Pro were stored at 4 °C until use.

2.4. Quantification of exenatide

The exenatide encapsulated within RM LNC was quantified by high performance liquid chromatography (HPLC, Shimadzu, Japan) using a gradient method as previously described by Shrestha *et al.* (88). Briefly, a Kinetex® EVO C18 column (100Å, 2.6 μm, 150 x 4.6 mm) (Phenomenex, USA), with a security guard column (Phenomenex, USA) was used at room temperature. The aqueous mobile phase comprised of 0.05% (v/v) trifluoroacetic acid (TFA) in water and the organic mobile phase consisted of 0.05% (v/v) TFA in acetonitrile. A gradient system was developed with an initial ratio of 10:90 (v/v, aqueous:organic phase) at flow rate of 1 mL/minute, which was linearly changed to 90:10 (v/v) over 10 minutes, and kept constant for the next minute. Then the ratio was linearly changed to initial composition in the next 1.5 minutes and was stabilized for the last minute. The injection volume used was 20 μL and the detection wavelength used was 220 nm. The retention time was 5.9 min and the limit of detection and limit of quantification was 1.1 ± 0.4 μg/mL and 3.3 ± 1.1 μg/mL, respectively.

2.5. Characterization of the EXE RM LNC

The EXE RM LNC particle size and polydispersity index (PdI) were characterized by dynamic light scattering (DLS) using a Zetasiser Nano ZS (Malvern Instruments Ltd.,

Worcestershire, U.K.) (0.5% LNC dispersed in 10 nM of NaCl). The zeta potential was measured by laser Doppler velocimetry (LDV) also using a Zetasiser Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.) (0.5% LNC dispersed in 10 nM of NaCl). All measurements were performed in triplicate.

The EXE RM LNC drug encapsulation efficiency (EE, %) was also characterized. To calculate the total drug content, 50 µL of EXE RM LNC were dissolved in 950 µL of methanol followed by strong vortexing. Free and encapsulated exenatide were separated by ultrafiltration using Amicon® centrifuge filters (MWCO 30 kDa, 4000g, 4°C, 20 minutes) (Millipore). Filtrates were further diluted using a 1:2 dilution factor. The exenatide in the filtrate and dissolved in methanol was quantified using the above-described HPLC method. The EE was calculated using the following equation:

$$EE\ (\%) = \frac{Total\ exenatide\ amount\ (\mu g) - Unencapsulated\ exenatide\ amount\ (\mu g)}{Total\ exenatide\ amount\ (\mu g)} x\ 100$$

2.6. *In vitro* stability study of 220 nm EXE RM LNC in simulated gastrointestinal fluids

2.6.1. Preparation of simulated gastrointestinal fluids

The preparation of the simulated GI fluids used in the *in vitro* dissolution testing was based on the instructions for the formulation and preparation for the biorelevant media developed by Dressman *et al.* (78), with some adjustments that were already optimized by Beloqui's research group (80–84).

FaSSGF pH 1.6 was prepared by dissolving 1 g of NaCl in 500 mL of MilliQ® water (A). To adjust the pH value, it was used HCl or NaOH. Then, 0.02 g of sodium taurocholate were dissolved in 3 mL of A (B). The next step was to dissolve 0.01 g of lecithin in 200 µL of dichloromethane (C). Then, C was added into B and maintained under magnetic stirring at 60 °C, which resulted in a clear micellar solution, having no perceptible odour of methylene chloride (D). After cooling to room temperature, D was added into the rest of A (E). To prepare FaSSGF with pepsin, all the previous steps were performed, followed by taking out the appropriate volume of E and add pepsin, in order to the final concentration be 0.1 mg/mL. For example, 100 mL of E plus 10 mg of pepsin.

FaSSIF pH 6.5 was prepared by dissolving 2 g of NaH2PO4.H2O, 0.17 g of NaOH and 3 g of NaCl in 500 mL of MilliQ® water (A). To adjust the pH value, it was used HCl or NaOH. Then, 0.8 g of sodium taurocholate were dissolved in 100 mL of A (B). The next step was to dissolve 0.3 g of lecithin in 1.5 mL of dichloromethane (C). Then, C was

added into B and maintained under magnetic stirring at 60 °C, which resulted in a clear micellar solution, having no perceptible odour of dichloromethane (D). After cooling to room temperature, D was added into the rest of 400 mL of A (E).

FeSSIF pH 5.0 was prepared in the same way as FaSSIF. However, in the first step, instead of adding NaH2PO4.H2O it was added acetic acid. The other difference is that it results in a slightly hazy, micellar solution, having no perceptible odour of dichloromethane.

The detailed composition of FaSSGF, FaSSIF, and FeSSIF is presented in Table 3. All the fluids were stored at 4 °C in the fridge until use.

Table 3. Composition of FaSSGF with and without pepsin, FaSSIF and FeSSIF

Composition	**FaSSGF without pepsin**	**FaSSGF with pepsin**	**FaSSIF**	**FeSSIF**
Sodium taurocholate (g)	0.02	0.02	0.8	4.035
Lecithin (g)	0.01	0.01	0.29	1.445
Sodium chloride (g)	1	1	3.093	5.935
Pepsin (mg)	-	50 (0.1 mg/mL)	-	-
Sodium hydroxide (g)	-	-	0.174	2.02
Acetic acid (g)	-	-	-	4.325
Sodium monobasic phosphate (g)	-	-	1.977	-
pH	1.6	1.6	6.5	5.0

2.6.2. *In vitro* stability of 220 nm EXE RM LNC in simulated gastrointestinal fluids

The stability of 220 nm EXE RM LNC was performed *in vitro* using various biomimetic GI fluids with enzymes to predict the *in vivo* LNC integrity under different stress conditions.

LNC stability was evaluated in four different biomimetic media: Fasted State Simulated Gastric Fluid (FaSSGF) with and without pepsin, Fasted State Simulated Intestinal Fluid (FaSSIF), and a Fed State Simulated Intestinal Fluid (FeSSIF) (Table 3).

The 220 nm EXE RM LNC were incubated in the four media referred above, at 37 °C (80 µL of LNC in 8 mL of FaSSIF and FeSSIF, and 60 µL of LNC in 6 mL of FaSSGF). At predetermined time points, 0, 0.5, 1 and 2 h for FaSSGF and 0, 0.5, 1, 3, and 6 h for FaSSIF and FeSSIF, samples were withdrawn and then analysed by DLS. At each time point, the particle size and the PdI were measured to evaluate the influence of gastric and intestinal conditions on LNC stability. The stability studies were performed in triplicate.

2.7. *In vitro* drug release studies of 220 nm EXE RM LNC

The drug release from 220 nm EXE RM LNC was evaluated in FaSSGF in the absence of pepsin and in FaSSIF media, for 2 h and 6 h, respectively. The studies were performed using the dialysis method. Briefly, 1 mL of EXE RM LNC was placed in disposable dialysis membranes (MWCO 100 kDa) (Float-A-Lyzer G2, Microfloat, Spectrum labs, USA) and introduced into 50 mL falcon tubes containing 25 mL of medium at 37 °C under magnetic stirring. At predetermined times (0, 0.5, 1, 1.5 and 2 h for FaSSGF, and 0, 0.5, 1, 1.5, 2, 3, 4, and 6 h for FaSSIF), 50 µL of sample were withdrawn and dissolved in 950 µL of methanol. The concentration of exenatide was determined by the HPLC method above described.

2.8. Statistical analysis

The GraphPad Prism program (version 8) (San Diego, CA, USA) was used for statistical analysis. The results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by Student's *t* test or Mann-Whitney test. Differences were considered statistically significant at $*p < 0.05$.

3. Results and Discussion

Therapeutic levels of exenatide enables pancreatic ß-cells apoptosis and delays the gastric emptying, thus, providing satiety and body weight loss. However, exenatide suffers enzymatic digestion and has low intestinal permeability, resulting in poor bioavailability (92). Therefore, the main goal of this project is to investigate oral delivery systems that can overcome exenatide's poor oral bioavailability and weak therapeutic adherence to the current exenatide subcutaneous treatments. The best oral dosage forms should present a highly potency and a wide safety margin, so it is crucial to evaluate all the properties of the oral nanocarriers to fulfil these goals.

3.1. Preparation and characterization of the different EXE RM LNC formulations

LNC of different sizes were prepared by a phase inversion process, being the size highly influenced by the proportions of the different excipients (93).The increase of the oily phase (Labrafac® WL 1349) results in the increase of LNC size, while higher surfactant (Solutol® HS15) concentrations leads to LNC of smaller diameters (50,56). The temperature cycles crossing the phase inversion region are also crucial for the development of LNC (49). The number of temperature cycles needs to be increased if the amount of surfactant is reduced, in order to stabilize the nanoparticle dispersion (93,94). However, the water amount seems to have no significant influence on particle size (69,93).

The preparation of the LNC with different sizes (Figure 5), ranging from 30 to 220 nm, followed a protocol already established in our research group (87).

Figure 5 – From left to right, physical appearance of 30, 50, 100, 150 and 220 nm EXE RM LNC

The LNC were characterized in terms of mean average diameter, PdI and surface charge (Table 4). The mean particle size of the LNC range between ~35 nm and ~221 nm. The small PdI index (PdI < 0.15) indicated the homogeneity of the LNC in terms of size distribution.

Table 4. Physicochemical Characterization of different LNC formulations (Mean ± SEM, *n*=3)

	30 nm	**50 nm**	**100 nm**	**150 nm**	**220 nm**
Mean size (z-average, nm)	34.38±1.4	52.97±1.3	97.91±2.6	141.63±1.5	221.30±1.1
Polidispersity index (PdI)	0.095±0.001	0.065±0.001	0.043±0.001	0.084±0.001	0.103±0.001
ζ potential (mV)	-3,46±0.74	-2.88±0.22	-2.06±0.28	-1.72±0.38	-3,83±0.32
EE (%)	58.35±0.93	65.00±3.46	71.64±1.45	80.87±6.78	84.95±3.76

3.2. Influence of simulated gastrointestinal fluids on the stability of 220 nm EXE RM LNC

In addition to the characterization of LNC physicochemical properties, the evaluation of their stability in GI conditions is essential to forecast the *in vivo* behaviour, assessing the potential effect on LNC interaction at the target site and the stability of encapsulated exenatide. Our research team had already evaluated the stability for the 30, 50, 100 and 150 nm EXE RM LNC, thus, herein only the stability for the 220 nm EXE LNC-PEG_{2k} and EXE LNC-PEG_{2k} -Pro was evaluated.

Accordingly, the influence of four different simulated fluids (FaSSGF, FaSSGF with pepsin, FaSSIF, and FeSSIF) was evaluated, mimicking the impact of gastric and intestinal conditions, before and after food intake, in LNC integrity. Based on the estimated intestinal transit time, 220 nm LNC were incubated in FaSSGF with and without pepsin for 2 h, and in FaSSIF and FeSSIF for 6 h, at 37 º C.

It was observed that the mean particle size of 220 nm LNC did not change during the incubation in the four different fluids, which is demonstrated in Figure 6. These results show a good colloidal stability maintained through the incubation time, which is

consistent with previous data showing that Solutol® influences the LNC stability by avoiding Lipoid® acid degradation (86). The same results were previously found while evaluating the stability of LNC of other diameters (data not shown). According to our results LNC present GI stability because of their PEG-coated surface.

Once 220 nm LNC were stable in acidic conditions at 2 h and in intestinal conditions at 6 h, regardless the fasted or fed state, we report 220 nm LNC as promising gastrorresistant nanocarriers for the treatment of T2DM.

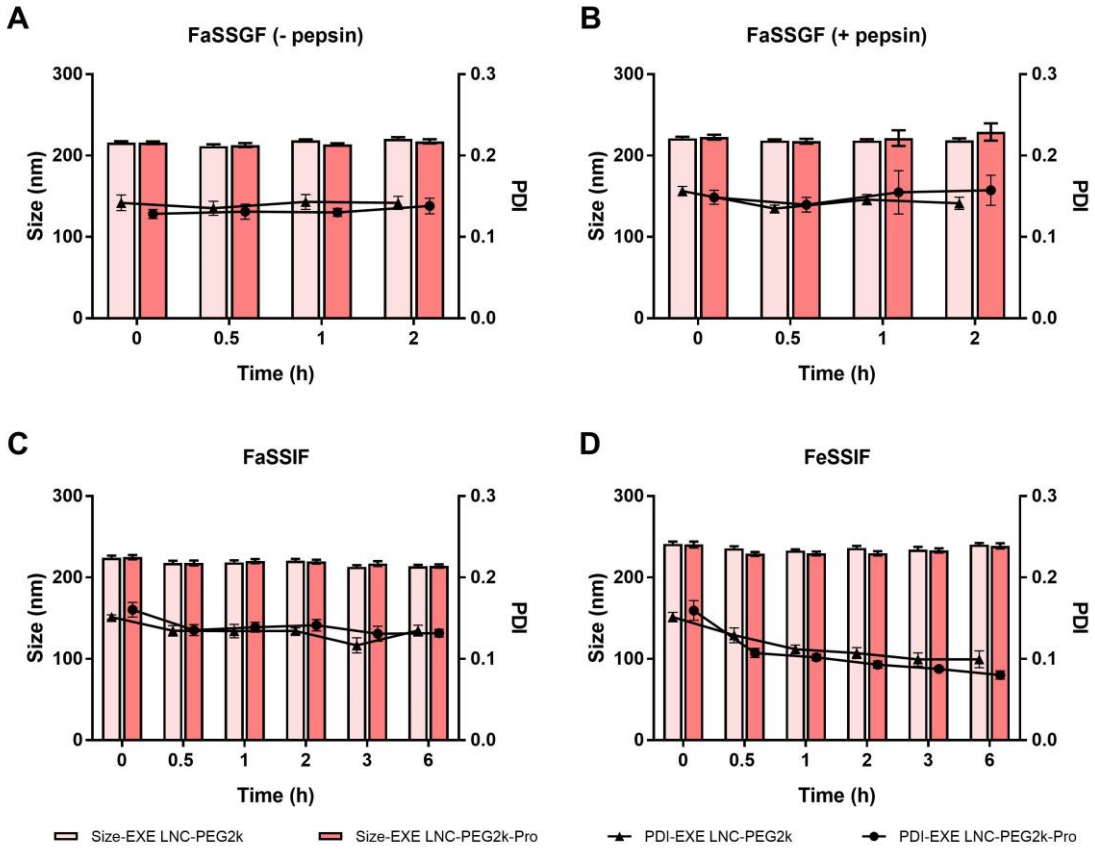

Figure 6 - Stability of 220 nm EXE LNC-PEG$_{2K}$ and EXE LNC-PEG$_{2K}$-Pro in simulated GI fluids. (A) Size (z-ave, nm) and PdI of LNC after incubation in FaSSGF for 2 h. (B) Size (z-ave, nm) and PdI of LNC after incubation in FaSSGF with pepsin for 2 h. (C) Size (z-ave, nm) and PdI of LNC after incubation in FaSSIF for 6 h. (D) Size (z-ave, nm) and PdI of LNC after incubation in FeSSIF for 6 h. Data shown as mean ± SEM (*N* = 3, *n* = 3). The particle size of 220 nm LNC was not significantly altered ($p > 0.05$), and it exhibited monodispersity (PdI < 0.2) after predetermined incubation time.

3.3. Exenatide *in vitro* release

The *in vitro* exenatide release profile obtained from 220 nm EXE RM LNC was evaluated under two different conditions, FaSSGF without pepsin for 2 h, and in FaSSIF for 6 h. The time gaps selected take into account the maximum transit time of LNC in the stomach and in the intestine following its oral administration (95).

The exenatide released from the RM LNC was quantified by HPLC, which has been used as the standard methodology for the preclinical assessment and quality control of the exenatide.

Regarding exenatide quantification, we chose a reversed-phase C_{18} column, with high porosity and surface, allowing for a fast elution with shorter run times (96,97). However, a fast elution may cause a decrease of the peak resolution and the interaction between exenatide and the stationary phase (96).

Concerning the mobile phase, it is essential to take into consideration the proportion of the organic phase and the final pH (96). The mobile phase suitable for exenatide elution must be constituted by a small percentage of water, since exenatide is hydrophilic, in order to enhance the interaction between exenatide and the stationary phase and the peak resolution. Among the several available solvents, water and acetonitrile (ACN) with formic acid (FA) or TFA are the most used mobile phase components, allowing symmetrical peaks shape, with residue peak tailing and high signal (98–100).

Exenatide has a high molecular weight and an isoelectric point of 4.96 (101). As a peptide, exenatide induces chromatographic peak tailing and carryover effect due to its hydrophobicity (102). The carryover effect may be avoided or decreased, through a wash run between analytical runs, in order to clean-up remaining residues from the sample previously injected (103). Concerning the peak tailing, it must also be considered pretreatment procedures of the samples, in order to obtain clean samples with higher concentrations (104,105). Considering the possible interferences of the matrix in exenatide, it is essential to proceed the samples pretreatment before its injection in the chromatographic system, in order to decrease the ion suppression and to increase the sensitivity of the method. The main sample pretreatment procedures developed for biological and biotechnology-based formulation matrices for incretins' quantification by HPLC are dilution, precipitation, protein precipitation, derivatization and solid phase extraction. The dilution of a biological sample comprises the addition of an organic

reagent to obtain a less concentrated sample, avoiding the obstruction of the chromatographic system or the column damage, because of the entrapment of exenatide or matrix endogenous constituents, and preventing the achievement of erroneous results (105). Concerning exenatide sample pre-treatment procedures, dilution is a usual procedure step (106).

The release profile of 220 nm EXE RM LNC is shown in Figure 7. EXE RM LNC with 220 nm demonstrated a burst release of 70% under intestinal conditions (FaSSIF), 6 h after incubation. However, the release profile of this LNC in gastric conditions (FaSSGF without pepsin) resulted in undetectable values (data not shown).

The differences observed in the release profile in FaSSGF and FaSSIF can be justified by the presence of bile salts, a natural wetting agent, in FaSSIF which enhances the dissolution and further release of the peptide (107,108). Exenatide tends to be totally released due to its higher affinity to bile salt present in FaSSIF (88).

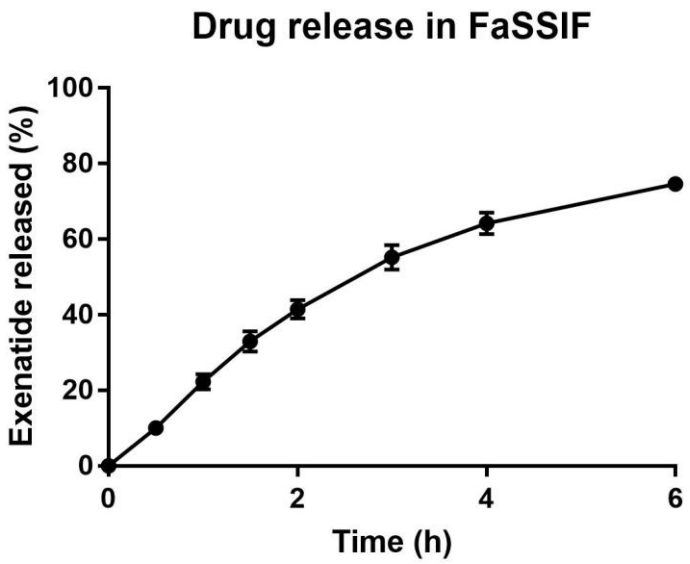

Figure 7 - *In vitro* release profile of exenatide in FaSSIF at 37 ºC. Data shown as mean ± SEM (*N* = 3, *n* = 3)

4. Conclusions and Future Prospects

During this research work developed in the Louvain Drug Research Institute, we formulated EXE RM LNC presenting five different average mean diameters (30, 50, 100, 150 and 220 nm) in order to develop viable LNC that potentially work as dual-action oral delivery systems, since we believe they will be capable of targeting and stimulating the enteroendocrine L cells to secrete the endogenous GLP-1 and were also able to act as an oral nanocarrier for GLP-1 RA (exenatide).

The obtained LNC shown suitable colloidal stabilities. Moreover, 220 nm EXE RM LNC released an approximately 70% of the entrapped exenatide in intestinal conditions (FaSSIF) after 6 h of incubation. Therefore, these data indicate that the 220 nm EXE RM LNC constitute promising gastrorresistant nanocarriers, able to encapsulate and release exenatide, offering an alternative to the current available therapies for the treatment of T2DM, allowing for the oral delivery of these bioactive agents.

Regarding the approval of Rybelsus® last September, the first oral GLP-1 RA for the treatment of T2DM, Lisa Yanoff, MD, acting director of the division of metabolism and endocrinology products of the FDA, said in a release that “Patients want effective treatment options for diabetes that are as minimally intrusive on their lives as possible, and the FDA welcomes the advancement of new therapeutic options that can make it easier for patients to control their condition,”. Yanoff also said “Before this approval, patients did not have an oral GLP-1 option to treat their type 2 diabetes, and now patients will have a new option for treating type 2 diabetes without injections.”.

In Novo Nordisk’s press release announcing Rybelsus® approval, Vanita R. Aroda, MD, director of diabetes clinical research at Brigham and Women's Hospital in Boston and a PIONEER clinical trial investigator, said that “The availability of an oral GLP-1 receptor agonist represents a significant development, and primary care providers, specialists and patients alike may now be more receptive to the use of a GLP-1 therapy to help them achieve their blood sugar goals,”. Moreover, Todd Hobbs, vice president and U.S. chief medical officer of Novo Nordisk said, "People living with type 2 diabetes deserve more innovation, research and support to help them achieve their individual A1C goals," and "With Rybelsus®, we have the opportunity to expand use of effective GLP-1 receptor agonist therapy by providing adults with type 2 diabetes an oral medication which was previously only available as an injection to help with managing their blood sugar.”.

According to these statements and, as a health professional, I think that it is of utmost importance that pharmaceutical industries and research institutes continue to invest and focus on the improvement of these type of innovative therapies as the strategy we developed herein.

From my point of view, the future of nanomedicine applied to T2DM treatments must continue to provide better and distinct therapies that could be adapted to each T2DM patient's profile. Therefore, it could be achieved better patient compliance to anti-diabetic therapies, increased therapeutic efficacy and, thereby better quality of life for T2DM patients.

The approach herein describes an oral therapeutic option to achieve better patient compliance to GLP-1 therapies once it avoids the administration by painful injections, but also an effective strategy that allows the reduction of exenatide dose once we are providing already the endogenous GLP-1. Therefore, it represents a cheaper and safer therapeutic alternative, as the endogenous peptide is cleaved by DPP-IV and the synthetic one is not recognized, so it might accumulate. Besides this, we chose exenatide because it would be safer to use a short-life peptide, from a safety point of view regarding an oral administration, where we are administering the drug daily and several times in some cases. If we have a peptide like semaglutide with a half-life of 165 h, that is administered daily, it might accumulate in the body. Therefore, it would be better and safer to use a short-life peptide as exenatide (half-life of 2,5 h) at least for a daily treatment. Additionally, our strategy differentiates from Rybelsus® because this last one uses functional excipients, such as permeation enhancers, to improve the absorption of the active ingredient of this formulation. Thus, the efficacy relies on the absorption. In our case, we are using a dual-action strategy that enable us to have the same effect using a shorter half-life peptide (safer), at the same time we are providing with endogenous GLP-1 (less dose needed which means cheaper therapeutic alternative).

As future perspectives, further *in vitro* studies and *in vivo* experiments in disease animal models are crucial to assess the efficacy of these 220 nm LNC. It would be interesting to test the other sizes of LNC as platforms for oral delivery of other therapeutic peptides.

5. References

1. Chan M. Global Report on Diabetes. Vol. 58, World Health Organization. 2016.

2. Tahrani AA, Barnett AH, Bailey CJ. Pharmacology and therapeutic implications of current drugs for type 2 diabetes mellitus. Nat Rev Endocrinol. 2016 Oct 24;12(10):566–92.

3. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. Nat Rev Dis Prim. 2015 Jul 23;1:15019.

4. Szoke E, Gerich JE. Role of impaired insulin secretion and insulin resistance in the pathogenesis of type 2 diabetes mellitus. Compr Ther. 2005 Jun 1;31(2):106–12.

5. Stolar MW. Insulin resistance, diabetes, and the adipocyte. Am J Heal Pharm. 2002 Dec 1;59:S3–8.

6. Rehman K, Akash MSH. Mechanisms of inflammatory responses and development of insulin resistance: how are they interlinked? J Biomed Sci. 2016 Dec 3;23(1):87.

7. Akash MSH, Shen Q, Rehman K, Chen S. Interleukin-1 Receptor Antagonist: A New Therapy for Type 2 Diabetes Mellitus. J Pharm Sci. 2012 May;101(5):1647–58.

8. Defronzo RA. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. Diabetes. 2009 Apr 1;58(4):773–95.

9. International Diabetes Federation - Home [Internet]. [cited 2019 Sep 11]. Available from: https://www.idf.org/

10. Yu M, Benjamin MM, Srinivasan S, Morin EE, Shishatskaya EI. Battle of GLP-1 delivery technologies. Adv Drug Deliv Rev. 2018 May 1;130:113–30.

11. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract. 2014 Feb;103(2):137–49.

12. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 2006 Nov 11;368(9548):1696–705.

13. Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM. Glucose sensing in L cells: a primary cell study. Cell Metab. 2008 Dec;8(6):532–9.

14. Talukdar S, Olefsky JM, Osborn O. Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. Trends Pharmacol Sci. 2011 Sep;32(9):543–50.

15. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature. 2003 Jun 12;423(6941):762–9.

16. des Rieux A, Pourcelle V, Cani PD, Marchand-Brynaert J, Préat V. Targeted nanoparticles with novel non-peptidic ligands for oral delivery. Adv Drug Deliv Rev. 2013 Jun 15;65(6):833–44.

17. Russell S. Incretin-based therapies for type 2 diabetes mellitus: a review of direct comparisons of efficacy, safety and patient satisfaction. Int J Clin Pharm. 2013 Apr 22;35(2):159–72.

18. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009 Aug 1;58(8):1091–103.

19. Baggio LL, Drucker DJ. Biology of Incretins: GLP-1 and GIP. Gastroenterology. 2007 May;132(6):2131–57.

20. Manandhar B, Ahn J-M. Glucagon-like Peptide-1 (GLP-1) Analogs: Recent Advances, New Possibilities, and Therapeutic Implications. J Med Chem. 2015 Feb 12;58(3):1020–37.

21. Chae SY, Jin C-H, Shin JH, Son S, Kim TH, Lee S, et al. Biochemical, pharmaceutical and therapeutic properties of long-acting lithocholic acid derivatized exendin-4 analogs. J Control Release. 2010 Mar 3;142(2):206–13.

22. Näslund E, Barkeling B, King N, Gutniak M, Blundell JE, Holst JJ, et al. Energy intake and appetite are suppressed by glucagon-like peptide-1 (GLP-1) in obese men. Int J Obes Relat Metab Disord. 1999 Mar;23(3):304–11.

23. De Marinis YZ, Salehi A, Ward CE, Zhang Q, Abdulkader F, Bengtsson M, et al. GLP-1 Inhibits and Adrenaline Stimulates Glucagon Release by Differential Modulation of N- and L-Type Ca2+ Channel-Dependent Exocytosis. Cell Metab. 2010 Jun 9;11(6):543–53.

24. Consoli A, Formoso G. Potential side effects to GLP-1 agonists: understanding their safety and tolerability. Expert Opin Drug Saf. 2015 Feb 12;14(2):207–18.

25. Zhang H, Xin B, Caporuscio C, Olah T V. Bioanalytical strategies for developing highly sensitive liquid chromatography/tandem mass spectrometry based methods for the peptide GLP-1 agonists in support of discovery PK/PD studies. Rapid Commun Mass Spectrom. 2011 Nov 30;25(22):3427–35.

26. Gallwitz B. Novel Therapeutic Approaches in Diabetes. In: Endocrine development. 2016. p. 43–56.

27. Meier JJ. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. Nat Rev Endocrinol. 2012 Dec 4;8(12):728–42.

28. Guo X-H. The value of short- and long-acting glucagon-like peptide-1 agonists in the management of type 2 diabetes mellitus: experience with exenatide. Curr Med Res Opin. 2016 Jan 2;32(1):61–76.

29. Parkes DG, Mace KF, Trautmann ME. Discovery and development of exenatide: the first antidiabetic agent to leverage the multiple benefits of the incretin hormone, GLP-1. Expert Opin Drug Discov. 2013 Feb 12;8(2):219–44.

30. Bachhav YG, Kalia YN. Development and validation of a rapid high-performance liquid chromatography method for the quantification of exenatide. Biomed Chromatogr. 2011 Jul;25(7):838–42.

31. Syed YY, McCormack PL. Exenatide Extended-Release: An Updated Review of Its Use in Type 2 Diabetes Mellitus. Drugs. 2015 Jul 13;75(10):1141–52.

32. Karagiannis T, Liakos A, Bekiari E, Athanasiadou E, Paschos P, Vasilakou D, et

al. Efficacy and safety of once-weekly glucagon-like peptide 1 receptor agonists for the management of type 2 diabetes: a systematic review and meta-analysis of randomized controlled trials. Diabetes, Obes Metab. 2015 Nov;17(11):1065–74.

33. Lovshin JA, Drucker DJ. Incretin-based therapies for type 2 diabetes mellitus. Nat Rev Endocrinol. 2009 May;5(5):262–9.

34. Blevins T, Pullman J, Malloy J, Yan P, Taylor K, Schulteis C, et al. DURATION-5: Exenatide Once Weekly Resulted in Greater Improvements in Glycemic Control Compared with Exenatide Twice Daily in Patients with Type 2 Diabetes. J Clin Endocrinol Metab. 2011 May;96(5):1301–10.

35. Baggio LL, Huang Q, Brown TJ, Drucker DJ. A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. Diabetes. 2004 Sep;53(9):2492–500.

36. Østergaard L, Frandsen CS, Madsbad S. Treatment potential of the GLP-1 receptor agonists in type 2 diabetes mellitus: a review. Expert Rev Clin Pharmacol. 2016 Feb 8;9(2):241–65.

37. ITCA 650 (exenatide implant) [Internet]. [cited 2019 Sep 24]. Available from: https://www.intarcia.com/pipeline-technology/itca-650.html

38. FDA approves Rybelsus® (semaglutide), the first GLP-1 analog treatment available in a pill for adults with type 2 diabetes [Internet]. [cited 2019 Sep 25]. Available from: https://www.novonordisk-us.com/media/news-releases.html?122973

39. Malhaire H, Gimel J-C, Roger E, Benoît J-P, Lagarce F. How to design the surface of peptide-loaded nanoparticles for efficient oral bioavailability? Adv Drug Deliv Rev. 2016 Nov 15;106:320–36.

40. Jenkins K. II. Needle phobia: a psychological perspective. Br J Anaesth. 2014 Jul;113(1):4–6.

41. Aguirre TAS, Teijeiro-Osorio D, Rosa M, Coulter IS, Alonso MJ, Brayden DJ. Current status of selected oral peptide technologies in advanced preclinical

development and in clinical trials. Adv Drug Deliv Rev. 2016 Nov 15;106:223–41.

42. Bastiancich C, Vanvarenberg K, Ucakar B, Pitorre M, Bastiat G, Lagarce F, et al. Lauroyl-gemcitabine-loaded lipid nanocapsule hydrogel for the treatment of glioblastoma. J Control Release. 2016 Mar 10;225:283–93.

43. Huynh NT, Passirani C, Saulnier P, Benoit JP. Lipid nanocapsules: A new platform for nanomedicine. Int J Pharm. 2009 Sep 11;379(2):201–9.

44. Lagarce F, Passirani C. Nucleic-Acid Delivery Using Lipid Nanocapsules. Curr Pharm Biotechnol. 2016 May 17;17(8):723–7.

45. Minkov I, Ivanova T, Panaiotov I, Proust J, Saulnier P. Reorganization of lipid nanocapsules at air–water interface: Part 2. Properties of the formed surface film. Colloids Surfaces B Biointerfaces. 2005 Sep 1;44(4):197–203.

46. Heurtault B, Saulnier P, Pech B, Proust J., Benoît J. Properties of polyethylene glycol 660 12-hydroxy stearate at a triglyceride/water interface. Int J Pharm. 2002 Aug 21;242(1–2):167–70.

47. Anton N, Gayet P, Benoit J-P, Saulnier P. Nano-emulsions and nanocapsules by the PIT method: An investigation on the role of the temperature cycling on the emulsion phase inversion. Int J Pharm. 2007 Nov 1;344(1–2):44–52.

48. Anton N, Benoit J-P, Saulnier P. Design and production of nanoparticles formulated from nano-emulsion templates—A review. J Control Release. 2008 Jun 24;128(3):185–99.

49. Anton N, Gayet P, Benoit J-P, Saulnier P. Nano-emulsions and nanocapsules by the PIT method: An investigation on the role of the temperature cycling on the emulsion phase inversion. Int J Pharm. 2007 Nov;344(1–2):44–52.

50. Heurtault B, Saulnier P, Pech B, Venier-Julienne M-C, Proust J-E, Phan-Tan-Luu R, et al. The influence of lipid nanocapsule composition on their size distribution. Eur J Pharm Sci. 2003 Jan;18(1):55–61.

51. Heurtault B, Saulnier P, Pech B, Proust J, Benoit J. A Novel Phase Inversion-Based Process for the Preparation of Lipid Nanocarriers. Pharm Res.

2002;19(6):875–80.

52. Anton N, Saulnier P, Béduneau A, Benoit J-P. Salting-Out Effect Induced by Temperature Cycling on a Water/Nonionic Surfactant/Oil System. J Phys Chem B. 2007 Apr 12;111(14):3651–7.

53. Dulieu C, Bazile D. Influence of lipid nanocapsules composition on their aptness to freeze-drying. Pharm Res. 2005 Feb;22(2):285–92.

54. Vonarbourg A, Saulnier P, Passirani C, Benoit J-P. Electrokinetic properties of noncharged lipid nanocapsules: Influence of the dipolar distribution at the interface. Electrophoresis. 2005 Jun;26(11):2066–75.

55. Heurtault, B., Saulnier, P.,Proust, J.E.,Benoit JP. Lipid nanocapsules: preparation process and use as Drug Delivery Systems. 2000 Mar 2;

56. Heurtault B, Saulnier P, Pech B, Proust J., Benoît J. Properties of polyethylene glycol 660 12-hydroxy stearate at a triglyceride/water interface. Int J Pharm. 2002 Aug;242(1–2):167–70.

57. Heurtault B, Saulnier P, Pech B, Proust J-E, Benoit J-P. Physico-chemical stability of colloidal lipid particles. Biomaterials. 2003 Oct 1;24(23):4283–300.

58. Manconi M, Aparicio J, Vila A., Pendás J, Figueruelo J, Molina F. Viscoelastic properties of concentrated dispersions in water of soy lecithin. Colloids Surfaces A Physicochem Eng Asp. 2003 Jul 30;222(1–3):141–5.

59. Vonarbourg A, Saulnier P, Passirani C, Benoit J-P. Electrokinetic properties of noncharged lipid nanocapsules: Influence of the dipolar distribution at the interface. Electrophoresis. 2005 Jun 1;26(11):2066–75.

60. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest. 2002 Mar;109(5):571–7.

61. Gruber P, Longer MA, Robinson JR. Some biological issues in oral, controlled drug delivery. Adv Drug Deliv Rev. 1988 Sep 1;1(3):268.

62. Lai SK, Wang Y-Y, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev. 2009 Feb 27;61(2):158–71.

63. Medina-Kauwe LK, Xie J, Hamm-Alvarez S. Intracellular trafficking of nonviral vectors. Gene Ther. 2005 Dec 4;12(24):1734–51.

64. FAROKHZAD O, LANGER R. Nanomedicine: Developing smarter therapeutic and diagnostic modalities☆. Adv Drug Deliv Rev. 2006 Dec 1;58(14):1456–9.

65. Allémann, Leroux, Gurny. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. Adv Drug Deliv Rev. 1998 Dec 1;34(2–3):171–89.

66. Huckaby JT, Lai SK. PEGylation for enhancing nanoparticle diffusion in mucus. Adv Drug Deliv Rev. 2018 Jan 15;124:125–39.

67. Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang Y-Y, Cone R, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. Proc Natl Acad Sci. 2007 Jan 30;104(5):1482–7.

68. Wang Y-Y, Lai SK, Suk JS, Pace A, Cone R, Hanes J. Addressing the PEG Mucoadhesivity Paradox to Engineer Nanoparticles that "Slip" through the Human Mucus Barrier. Angew Chemie Int Ed. 2008 Dec 1;47(50):9726–9.

69. Vonarbourg A, Passirani C, Saulnier P, Simard P, Leroux JC, Benoit JP. Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J Biomed Mater Res Part A. 2006 Sep 1

70. Vonarbourg A, Passirani C, Saulnier P, Benoit J-P. Parameters influencing the stealthiness of colloidal drug delivery systems. Biomaterials. 2006 Aug;27(24):4356–73.

71. Hervella P, Alonso-Sande M, Ledo F, Lucero M, Alonso M, Garcia-Fuentes M. PEGylated Lipid Nanocapsules with Improved Drug Encapsulation and Controlled Release Properties. Curr Top Med Chem. 2014 Mar 29;14(9):1115–23.

72. Resnier P, Lepeltier E, Emina AL, Galopin N, Bejaud J, David S, et al. Model Affitin and PEG modifications onto siRNA lipid nanocapsules: cell uptake and *in vivo* biodistribution improvements. RSC Adv. 2019 Aug 29;9(47):27264–78.

73. Kim J, Ramasamy T, Choi JY, Kim ST, Youn YS, Choi H-G, et al. PEGylated

polypeptide lipid nanocapsules to enhance the anticancer efficacy of erlotinib in non-small cell lung cancer. Colloids Surfaces B Biointerfaces. 2017 Feb 1;150:393–401.

74. Tirosh A, Calay ES, Tuncman G, Claiborn KC, Inouye KE, Eguchi K, et al. The short-chain fatty acid propionate increases glucagon and FABP4 production, impairing insulin action in mice and humans. Sci Transl Med. 2019 Apr 24;11(489):eaav0120.

75. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta - Mol Cell Biol Lipids. 2010 Nov 1;1801(11):1175–83.

76. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987 Oct;28(10):1221–7.

77. Karuppiah V, Kannappan N, Manavalan R, Pharma Lt A. In-vitro and siumlated in-vivo dissolution of dipyridamole extended release capsules. Int J Pharm Sci Rev Res. 13(1).

78. Kostewicz ES, Brauns U, Becker R, Dressman JB. Forecasting the oral absorption behavior of poorly soluble weak bases using solubility and dissolution studies in biorelevant media. Pharm Res. 2002 Mar;19(3):345–9.

79. Jantratid E, Janssen N, Reppas C, Dressman JB. Dissolution Media Simulating Conditions in the Proximal Human Gastrointestinal Tract: An Update. Pharm Res. 2008 Jul 11;25(7):1663–76.

80. Dressman JB, Reppas C. In vitro-in vivo correlations for lipophilic, poorly water-soluble drugs. Eur J Pharm Sci. 2000 Oct;11 Suppl 2:S73-80.

81. Nicolaides E, Symillides M, Dressman JB, Reppas C. Biorelevant Dissolution Testing to Predict the Plasma Profile of Lipophilic Drugs After Oral Administration. Pharm Res. 2001;18(3):380–8.

82. Hörter D, Dressman JB. Influence of physicochemical properties on dissolution

of drugs in the gastrointestinal tract. Adv Drug Deliv Rev. 2001 Mar 1;46(1–3):75–87.

83. Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. Pharm Res. 1998 Jan;15(1):11–22.

84. Marques M. Dissolution Media Simulating Fasted and Fed States. Dissolution Technol. 2004;11(2):16–16.

85. VERTZONI M, DRESSMAN J, BUTLER J, HEMPENSTALL J, REPPAS C. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. Eur J Pharm Biopharm. 2005 Aug;60(3):413–7.

86. Roger E, Lagarce F, Benoit J-P. The gastrointestinal stability of lipid nanocapsules. Int J Pharm. 2009 Sep 11;379(2):260–5.

87. Xu Y, Carradori D, Alhouayek M, Muccioli GG, Cani PD, Préat V, et al. Size Effect on Lipid Nanocapsule-Mediated GLP-1 Secretion from Enteroendocrine L Cells. Mol Pharm. 2018 Jan 2;15(1):108–15.

88. Shrestha N, Bouttefeux O, Vanvarenberg K, Lundquist P, Cunarro J, Tovar S, et al. The stimulation of GLP-1 secretion and delivery of GLP-1 agonists via nanostructured lipid carriers. Nanoscale. 2018 Jan 3;10(2):603–13.

89. Maupas C, Moulari B, Béduneau A, Lamprecht A, Pellequer Y. Surfactant dependent toxicity of lipid nanocapsules in HaCaT cells. Int J Pharm. 2011 Jun 15;411(1–2):136–41.

90. Aránzazu Partearroyo M, Ostolaza H, Goñi FM, Barberá-Guillem E. Surfactant-induced cell toxicity and cell lysis. A study using B16 melanoma cells. Biochem Pharmacol. 1990 Sep 15;40(6):1323–8.

91. Hoarau D, Delmas P, David S, Roux E, Leroux JC. Novel long-circulating lipid nanocapsules. Pharm Res. 2004 Oct;21(10):1783–9.

92. Zhang L, Shi Y, Song Y, Sun X, Zhang X, Sun K, et al. The use of low molecular weight protamine to enhance oral absorption of exenatide. Int J Pharm. 2018 Aug

25;547(1–2):265–73.

93. Huynh NT, Passirani C, Saulnier P, Benoit JP. Lipid nanocapsules: A new platform for nanomedicine. Int J Pharm. 2009 Sep;379(2):201–9.

94. Anton N, Saulnier P, Béduneau A, Benoit J-P. Salting-Out Effect Induced by Temperature Cycling on a Water/Nonionic Surfactant/Oil System. J Phys Chem B. 2007 Apr;111(14):3651–7.

95. Araújo F, Shrestha N, Shahbazi M-A, Liu D, Herranz-Blanco B, Mäkilä EM, et al. Microfluidic Assembly of a Multifunctional Tailorable Composite System Designed for Site Specific Combined Oral Delivery of Peptide Drugs. ACS Nano. 2015 Aug 25;9(8):8291–302.

96. Bicker J, Fortuna A, Alves G, Falcão A. Liquid chromatographic methods for the quantification of catecholamines and their metabolites in several biological samples—A review. Anal Chim Acta. 2013 Mar 20;768:12–34.

97. Howard JW, Kay RG, Tan T, Minnion J, Ghatei M, Bloom S, et al. Development of a high-throughput UHPLC–MS/MS (SRM) method for the quantitation of endogenous glucagon from human plasma. Bioanalysis. 2014 Dec;6(24):3295–309.

98. Morin L-P, Mess J-N, Garofolo F. Large-molecule quantification: sensitivity and selectivity head-to-head comparison of triple quadrupole with Q-TOF. Bioanalysis. 2013 May;5(10):1181–93.

99. Sila A, Alvarez OM, Haddar A, Frikha F, Dhulster P, Nedjar-Arroume N, et al. Purification, identification and structural modelling of DPP-IV inhibiting peptides from barbel protein hydrolysate. J Chromatogr B. 2016 Jan 1;1008:260–9.

100. Silveira ST, Martínez-Maqueda D, Recio I, Hernández-Ledesma B. Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. Food Chem. 2013 Nov 15;141(2):1072–7.

101. Kim JY, Lee H, Oh KS, Kweon S, Jeon O, Byun Y, et al. Multilayer

nanoparticles for sustained delivery of exenatide to treat type 2 diabetes mellitus. Biomaterials. 2013 Nov;34(33):8444–9.

102. Chambers EE, Legido-Quigley C, Smith N, Fountain KJ. Development of a fast method for direct analysis of intact synthetic insulins in human plasma: the large peptide challenge. Bioanalysis. 2013 Jan;5(1):65–81.

103. Xu Y, Sun L, Anderson M, Bélanger P, Trinh V, Lavallée P, et al. Insulin glargine and its two active metabolites: A sensitive (16 pM) and robust simultaneous hybrid assay coupling immunoaffinity purification with LC–MS/MS to support biosimilar clinical studies. J Chromatogr B. 2017 Sep 15;1063:50–9.

104. Karpievitch Y V., Polpitiya AD, Anderson GA, Smith RD, Dabney AR. Liquid chromatography mass spectrometry-based proteomics: Biological and technological aspects. Ann Appl Stat. 2010 Dec;4(4):1797–823.

105. Kim C, Ryu H-D, Chung EG, Kim Y, Lee J. A review of analytical procedures for the simultaneous determination of medically important veterinary antibiotics in environmental water: Sample preparation, liquid chromatography, and mass spectrometry. J Environ Manage 2018 Jul

106. Carmical J, Brown S. The impact of phospholipids and phospholipid removal on bioanalytical method performance. Biomed Chromatogr. 2016 May;30(5):710–20.

107. Shrestha N, Araújo F, Shahbazi M-A, Mäkilä E, Gomes MJ, Herranz-Blanco B, et al. Thiolation and Cell-Penetrating Peptide Surface Functionalization of Porous Silicon Nanoparticles for Oral Delivery of Insulin. Adv Funct Mater. 2016 May 1;26(20):3405–16.

108. Klein S. The Use of Biorelevant Dissolution Media to Forecast the In Vivo Performance of a Drug. AAPS J. 2010 Sep 11;12(3):397–406.