Peptide-loaded nanoparticle oral delivery strategies towards diabetes treatment

Yining Xu

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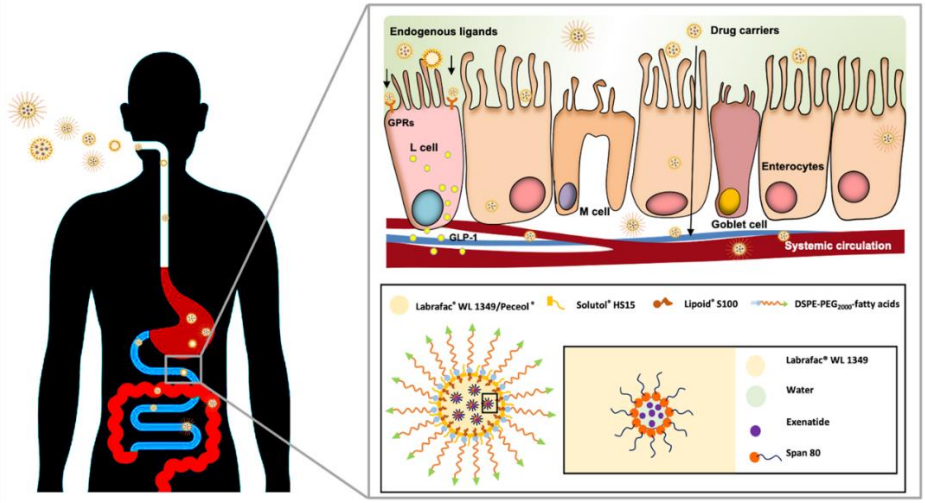

Directors : Professor Ana Beloqui
Professor Véronique Préat

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FOREWORD

The development of oral dosage forms that enable the absorption of therapeutic peptides into the systemic circulation is one of the greatest challenges for the pharmaceutical industry. Improved novel drug delivery systems are of utmost importance in order to fulfill the potential of this route of administration in the treatment of chronic diseases (e.g., type 2 diabetes mellitus (T2DM)), where daily injections are often required.

This work aims at exploiting the physiology of L cells, developing and modifying an innovative nanocapsule-based drug delivery system that synergizes its own biological effect (targeting enteroendocrine L cells to stimulate endogenous glucagon-like peptide-1 (GLP-1) release) and that of the encapsulated bioactive molecule (improved systemic circulation of GLP-1 receptor agonists (GLP-1RAs)) as an alternative strategy for the treatment of T2DM via oral route.

Chapter I – Introduction gives an historical background on diabetes and its challenges in oral drug delivery and a look into targeting approaches towards improved oral drug delivery systems.

Chapter II – Aim of this thesis exposes the objectives and potential impact of this work, which includes the discovery of the nanocarrier biological effect and the development a dual-action nanocapsule-based nanosystem for T2DM treatment via oral route.

Chapter III – Size effect on lipid nanocapsules-mediated GLP-1 secretion from enteroendocrine L cells reports the proof of concept that lipid nanocapsules with a proper size could induce GLP-1 secretion.

Chapter IV – Novel strategy for oral peptide delivery in incretin-based diabetes treatment describes the development of a novel lipid-based nanosystem compatible with human use that synergizes its own biological effect with an increased GLP-1RA bioavailability towards T2DM treatment via oral route.

Chapter V – Targeted nanoparticles towards increased L cell stimulation as a strategy to improve oral peptide delivery in incretin-based diabetes treatment investigates two potential contingency strategies towards increased GLP-1 secretion by tailoring the surface of the nanocarriers, including PEGylation and lipid ligand modification and examines their effect on the antidiabetic effect *in vivo* using different administration frequencies for the treatment of T2DM.

Chapter VI – Discussion, conclusions and future perspectives will explain how the present work can fit in the oral delivery of anti-diabetic peptides for incretin-based diabetes treatment, describing the advantages and disadvantages over current available strategies. The future perspectives of this work will be described, envisioning a future application of the formulation.

LIST OF ABBREVIATIONS

AAL	Aleuria aurantia lectin
ANGPTL	Angiopoietin-like protein
APCs	Antigen-presenting cells
APN	Anti-aminopeptidase N
ASBT	Apical sodium-dependent bile acid transporter
ASP	Acylation-stimulating protein
ATP	Adenosine triphosphate
AUC	Area under the curve
B12-hPYY	B12-human Peptide YY
B12-LHRH	B12-luteinizing-hormone-releasing hormone
BSA	Bovine serum albumin
BT	Botulinum toxoid
cAMP	Cyclic adenosine monophosphate
CCR5	C-C chemokine receptor 5
CKS9	CKSTHPLSC
Cpt1	Carnitine palmitoyltransferase 1
CPE	Clostridium perfringens enterotoxin
CRDs	C-type carbohydrate recognition domains
CS	Chitosan
CSK	CSKSSDYQC
C5aR	Complement C5a receptor
DCs	Dendritic cells
DDS	Targeted drug delivery systems
DiDDiIC18(5)	solid (1,1'-ioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt)
DLS	Dynamic light scattering
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPP-4	Dipeptidyl peptidase-4
DSPE	1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine
EAT	Epididymal adipose tissues
EDIII	Envelope domain III
EDTA	Ethylenediaminetetraacetic acid
EECs	Enteroendocrine cells
EXE RM	Exenatide-loaded reverse micelles
EXE RM LNC	Exenatide-loaded reverse micelle lipid nanocapsules
FAE	Follicle-associated epithelium
Fasn	Fatty acids synthase
FaSSGF	Fasted State Simulated Gastric Fluid
FaSSIF	Fasted State Simulated Intestinal Fluid

FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
FeSSIF	Fed State Simulated Intestinal Fluid
FFAR	Free fatty acid receptors
FQS	FQSIYPpIK
GALTs	Gut-associated lymphoid tissues
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitor peptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GLP-1RAs	GLP-1 receptor agonists
GRAS	Generally recognized as safe
GPCRs	G protein-coupled receptors
GP2	Glycoprotein 2
GRGDS	Glycine-arginine-glycine-aspartic acid-serine
HA	Hemagglutinin
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HDTA	Taurocholic acid-heparin-docetaxel
H&E	Hematoxylin and eosin
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IDF	International Diabetes Federation
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
KATP	Adenosine triphosphate-sensitive potassium
KPV	Lys-Pro-Val
LCFA	Long-chain fatty acids
LDL	Low-density lipoprotein
LDV	Laser Doppler velocimetry
LNC	Lipid nanocapsule
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
L-PTCs	L-progenitor toxin complexes
MAPKs	Mitogen-activated protein kinases
MCFA	Medium-chain fatty acids
MCP	Monocyte chemotactic protein
MCT	Monocarboxylate transporter

MDM2	Murine double minute 2
MTT	3-(4,5- dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAFLD	Nonalcoholic fatty liver disease
NLC	Nanostructured lipid carriers
NPs	Nanoparticles
OCTN	Organic cation/carnitine transporter
OGTT	Oral glucose tolerance test
OmpH	Outer membrane protein H
OVA	Ovalbumin
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PCL	Poly(ε-caprolactone)
PDI	Polydispersity index
PEDV	Porcine epidemic diarrhea virus
PEPT	Oligopeptide transporter
PEG	Poly(ethylene glycol)
PFA	Paraformaldehyde
PIT	Phase inversion temperature
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolide)
PPARs	Peroxisome proliferator-activated receptors
RPl19	Ribosomal protein L19
PRRs	Pattern recognition receptors
P/S	Penicillin-streptomycin
PYY	Peptide YY
qRT-PCR	Real-time quantitative PCR
RBP4	Retinol binding protein 4
RGD	Arginine–glycine–aspartic acid
RGDps	RGD peptidomimetics
RM	Reverse micelle
RPMI	Roswell Park Memorial Institute
SA	Streptavidin
SAA	Serum amyloid A
SAT	Subcutaneous adipose tissues
SCFAs	Short chain fatty acids
SFRP5	Secreted frizzled-related protein 5
SGLT2	Sodium/glucose co-transporter 2
SIgA	Secretory IgA
SLC	Solute carrier
SNAC	Sodium N-[8-(2-hydroxybenzoyl) aminocaprylate] caprylate
SMVT	Sodium-dependent multivitamin transporter
SVCT	Sodium-dependent vitamin transporter
TFA	Trifluoroacetic acid

TfR	Transferrin receptor
TGDK	Tetragalloyl-D-lysine dendrimer
TGF	Transforming growth factor
TLR	Toll-like receptor
TLs	Tomato lectins
TMC	Trimethyl chitosan
TNF	Tumor necrosis factor
TT	Tetanus toxoid
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UEA-1	Ulex europaeus agglutinin-1
VAT	Visceral adipose tissues
VLDL	Very low-density lipoprotein
WGA	Wheat germ agglutinin
WHO	World Health Organization
7pep	HAIYPRH

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CHAPTER I
INTRODUCTION

Adapted from:

Xu Y, Shrestha N, Préat V, Beloqui A. Overcoming the intestinal barrier: A look into targeting approaches for improved oral drug delivery systems. J Control Release, Accepted (2020).

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I. WHAT IS DIABETES AND ITS CHALLENGES ON THE TREATMENT?

Diabetes mellitus (DM), more simply called diabetes, is a metabolic disorder characterized by chronic hyperglycemia together with disturbances in the metabolism of carbohydrates, proteins and fat, which in general results from an insulin availability and/or need imbalance [1]. DM compromises the quality of life of patients, and its number is continuously increasing. The International Diabetes Federation (IDF) reports that there are staggering 463 million people (between the ages of 20 and 79) currently living with diabetes around the world (Fig. 1) [2]. A decade ago, in 2010, the global projection for diabetes in 2025 was 438 million. With over five years still to go, that prediction has already been surpassed by 25 million. IDF estimates that there will be 578 million adults with diabetes by 2030, and 700 million by 2045 (Fig. 1).

Diabetes belongs to the top ten leading causes of death globally. In 2019, IDF estimates that diabetes is responsible for approximately 4.2 million deaths, with almost half (46.2%) of them occurring prematurely before the age of 60 [2]. The World Health Organization (WHO) estimates that diabetes is the seventh leading cause of death worldwide. The main causes of death among diabetes patients result from cardiovascular and renal complications. Preventable deaths from diabetes and the loss of the workforce caused by diabetes-invoked complications are detrimental to economies worldwide. IDF estimates that total diabetes-related health expenditure will reach USD 760 billion in 2019, making diabetes one of the top most expensive diseases regarding health care [3]. The economic impact of diabetes is expected to continue to grow. It is projected that expenditure will reach USD 825 billion by 2030 and USD 845 billion by 2045 [2]. Another data collected from WHO reports that both the direct and indirect costs of diabetes will reach USD 1.7 trillion from 2011 to 2030. Moreover, WHO has emphasized that diabetes is one of four priority noncommunicable diseases targeted by world leaders [4]. Hence, there is an unquestionable unmet need for diabetes treatment.

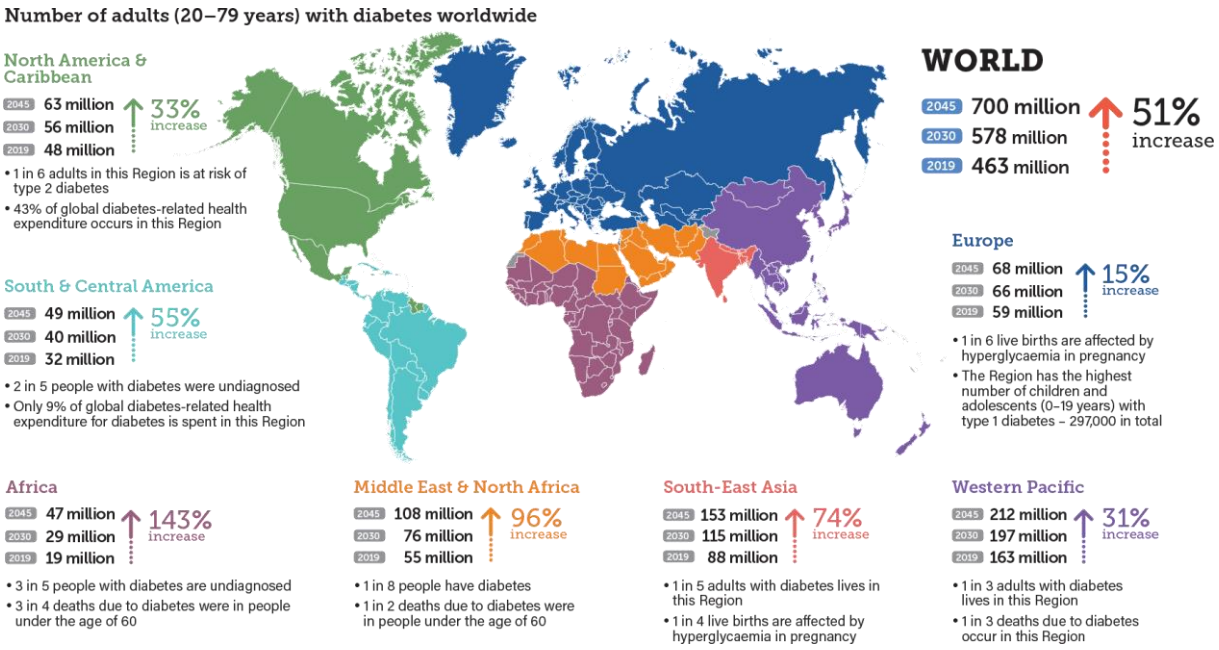

Figure 1. Number of people with diabetes worldwide and per region in 2019, 2030 and 2045 (20–79 years), including the percentage of increase in the incidence of the disease. Figure adapted from [2]. International Diabetes Federation Diabetes Atlas. IDF Diabetes Atlas, 9th edition Brussels, Belgium: International Diabetes Federation, 2019 http://www.diabetesatlas.org.

I.1. DIABETES CLASSIFICATIONS

Diabetes is due to an inadequate insulin production (pancreatic islet cells destruction), or a lack of sensitivity of host cells to endogenous insulin, mainly being classified into type 1 DM (T1DM), type 2 DM (T2DM), and gestational DM (GDM) [1, 5].

I.1.1. T1DM

T1DM, also known as insulin-dependent DM or juvenile-onset diabetes, is due to the insulin deficiency that occurs as the consequence of the loss of pancreatic islet β-cells [6-8], representing 5–10% of total DM cases [9]. Most cases of T1DM represent an immune, if not autoimmune-mediated disorder, meaning patients often show features of an immunological contribution to disease pathogenesis (e.g., autoantibodies or genetic associations with genes controlling immune responses) [8]. Although T1DM can occur at any age, it is the most common form of diabetes in children (<15 years of age), and >500,000 children are currently living with this condition globally [10]. The typical symptoms of T1DM includes unintended weight loss, polyuria, polydipsia, and polyphagia [11]. A cure in T1DM is not available, and patients currently depend on lifelong insulin injections to maintain normoglycemic levels and to prevent the occurrence of side effects, which if uncontrolled can lead to coma and death [12].

I.1.2. T2DM

T2DM, also known as non-insulin dependent DM or adult onset diabetes, is responsible for 90 % - 95 % of all DM cases [13]. It is characterized by insulin resistance, impaired insulin secretion by pancreatic islet β-cells, or a combination of both [14]. T2DM causes dysfunctions in multiple organs or tissues, including a defect in insulin-mediated glucose uptake in muscle, a dysfunction of the pancreatic β-cells, a disruption of secretory function of adipocytes, and an impaired insulin action in the liver [15]. The causes of T2DM are not completely understood but there is a strong link with overweight and obesity, and increasing age, as well as with ethnicity and family history [2]. T2DM is most commonly seen in older adults, but is increasingly seen in children and younger adults owing to rising levels of obesity, physical inactivity and inappropriate diet. The symptoms of T2DM may be similar to those of T1DM, but in general, the presentation of type 2 diabetes is much less dramatic and the condition may be completely symptomless, leading to a large number of undiagnosed cases [16]. Oral medication is usually initiated with metformin as the first-line medicine if changing lifestyle is not sufficient to control blood glucose levels [17]. If treatment with a single antidiabetic medication is not sufficient, a range of combination therapy options are now available such as sulphonylureas, dipeptidyl peptidase-IV (DPP-IV) inhibitors, glucagon-like peptide 1 (GLP-1) analogues) [18]. When those medications are unable to control hyperglycemia, insulin injections may be required [19].

I.1.3. GDM

GDM is currently the most common medical complication of pregnancy, and prevalence of undiagnosed hyperglycemia and even overt diabetes in young women is increasing [20]. Major GDM risk factors include advanced maternal age, ethnicity, previous history of gestational diabetes and family history of T2DM [21]. Such women are at greater risk of adverse pregnancy outcomes (e.g. high blood pressure or a large baby) [22]. The primary goal of GDM treatment is the prevention of fetal overgrowth and pregnancy complications. It is usually achieved by dietary modification [23], and promotion of physical activity to minimize postprandial glucose elevations [24] and pharmacotherapy (e.g. prescription of insulin) [25] is required only in a minority of women.

Among the three major types of diabetes as we described above, T2DM is far more common (accounting for more than 90% of all cases) than either T1DM or GDM [26]. Hence, this project is expected to make its main contribution to the advancement of the treatment of T2DM through oral delivery of antidiabetic peptides by the application of nanotechnology. In the following sections, we especially look at T2DM associated metabolic syndrome that could be a major risk

factor leading to multiple complications, and the current medications on the treatment of T2DM, as well as its challenges.

I.2. T2DM ASSOCIATED METABOLIC SYNDROME

T2DM was first described as a component of metabolic syndrome by Reaven in 1988 [27]. The metabolic syndrome associated with T2DM mainly includes insulin resistance, inflammation, dyslipidemia, hypertension, central obesity and nonalcoholic fatty liver disease (NAFLD) [28].

I.2.1. Insulin resistance

Insulin resistance, a component of the metabolic syndrome, refers to a decrease in a target cell's metabolic response to insulin, or, at the whole-organism level (e.g. adipose tissue, liver, and muscle), an impaired lowering effect of circulating or injected insulin on blood glucose [29]. In another word, deterioration of glucose tolerance can only be prevented if the pancreatic β-cell is able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia. When this goal cannot be achieved, gross decompensation of glucose homeostasis occurs [30]. Insulin resistance is typically present throughout the progression from prediabetes to the later stages of overt T2DM [31]. It should be noted that, although the exact etiology of metabolic syndrome is uncertain, insulin resistance is considered as a common mechanism underlying derangement associated with the syndrome, defined by the presence of hyperglycemia (fasting plasma glucose levels ≥130 mg/dL), central obesity (waist circumference ≥90 cm in men, and ≥80 cm in women), low high-density lipoprotein (HDL) cholesterol level (<1.03 mmol/L in men, <1.29 mmol/L in women), high total triglyceride level (≥1.7 mmol/L) and elevated blood pressure (≥130/85 mmHg) [32]. Namely, insulin resistance is tightly linked to the development of dyslipidemia and the increased release of inflammatory markers in the liver and adipose tissue. It also contributes to the elevated blood pressure, the impaired endothelial function and the effect of macrophages [33].

I.2.2. Inflammation

A chronic low-grade inflammation and an activation of the immune system are involved in the pathogenesis of insulin resistance, T2DM and its associated complications, such as heart attacks, strokes, kidney problems and others [34, 35]. Adipose tissue, liver, muscle and pancreas are sites of inflammation in the presence of obesity and T2DM. An infiltration of macrophages and other immune cells are observed in these tissues in animal models of obesity and diabetes as well as in obese human individuals with metabolic syndrome or T2DM [35]. These cells are crucial for the production of pro-inflammatory cytokines [36], including tumor necrosis factor (TNF)-α,

interleukin (IL)-6 and IL-1β, which act in an autocrine and paracrine manner to promote insulin resistance by interfering with insulin signaling in peripheral tissues.

Among these tissues, adipose tissues have a major endocrine function secreting multiple adipokines (including chemokines, cytokines and hormones) and play a central role in the induction of inflammation (Fig. 2) [28]. In the obese/T2DM state, the enlarged adipose tissues increase the release of free fatty acids and leads to dysregulated secretion of adipokines. In pathological conditions, adipocytes increasingly secrete various pro-inflammatory chemokines and cytokines, such as monocyte chemotactic protein (MCP)-1, TNF-α, IL-1, IL-6 and IL-1β. Moreover, macrophage content in adipose tissues increases, and the expression of some pro-inflammatory cytokines (e.g. TNF-α) is mostly derived from macrophages rather than adipocytes [37]. Along with the increased number of macrophages in adipose tissue, obesity/T2DM induces a phenotypic switch in these cells from an anti-inflammatory M2 polarization state to a pro-inflammatory M1 polarization state. The accumulation of M1 macrophages in adipose tissue alters adipokines secretion, in which the secretion of anti-inflammatory adipokines (e.g., adiponectin, secreted frizzled-related protein 5 (SFRP5) and IL 10) decreases, whereas the expression of pro-inflammatory adipokines as mentioned in Fig.2 increases, including MCP-1, TNF-α, TNF-β, IL-1, IL-6, IL-1β, leptin, resistin, acylation-stimulating protein (ASP), plasminogen activator inhibitor-1(PAI-1), visfatin, retinol binding protein 4 (RBP4), angiopoietin-like protein (ANGPTL), serum amyloid A (SAA) and angiotensin [38]. The increase of free fatty acids and pro-inflammatory adipokines further aggravates the development of metabolic syndrome (e.g., inflammation, insulin resistance, dyslipidemia and NAFLD). In addition, visceral adipose tissues have more macrophages [39-41], T-lymphocytes [40, 41], and inflammatory molecules compared with subcutaneous adipose tissues in obese and T2DM individuals. In the liver, inflammation plays a pivotal role in the progression of NAFLD that often accompanies abdominal obesity and T2DM, which increases expression and overproduction of inflammatory mediators (e.g. TNF-α, IL-6 and IL-1β) [42]. As per the adipose tissue, in obesity/T2DM state, skeletal muscle myotubes exhibit enhanced expression of pro-inflammatory cytokines (e.g., MCP-1, TNF-α, IL-1, IL-6 and IL-1β) accompanied with an increased accumulation of macrophages in muscle cells contributing to cellular inflammation and insulin resistance [43]. The content of macrophages in skeletal muscle in obesity and T2DM, however, is by far lower than in adipose tissue or liver [35]. In pancreas, the activation of inflammasome by high levels of glucose and fatty acids and a subsequent release of IL-1β lead to pancreatic β-cells dysfunction and apoptosis, insulin deficiency and progression to T2DM [44].

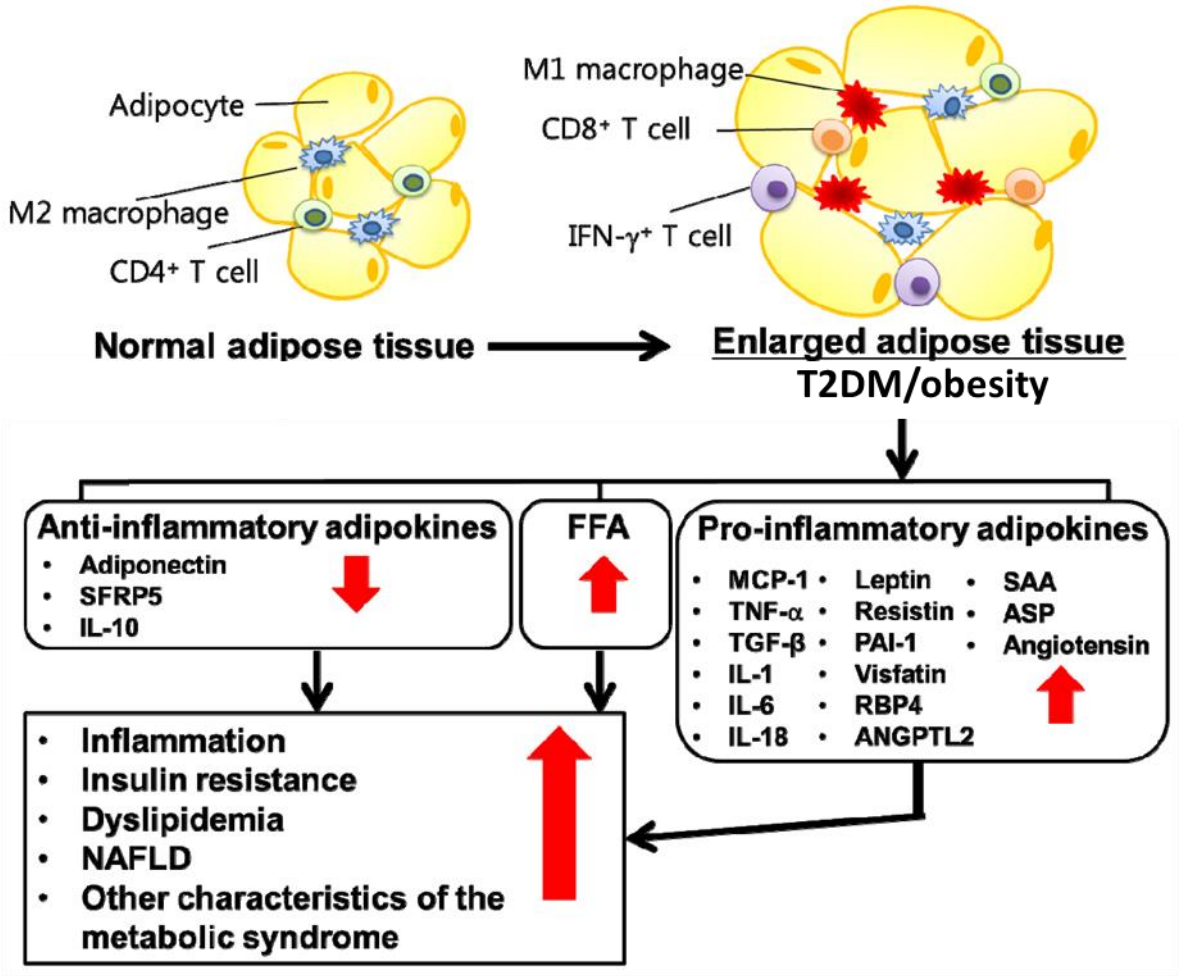

Figure 2. Secretion of inflammatory adipokines from adipose tissue in obese/T2DM state. The enlarged adipose tissue leads to dysregulated secretion of adipokines and increased release of free fatty acids. In addition, obesity/T2DM induces a phenotypic switch in adipose tissue from anti-inflammatory (M2) to pro-inflammatory (M1) macrophages. The red arrows indicate increased (when pointing upward) or decreased (when pointing downward) responses to obesity/T2DM. Reproduced from [28].

I.2.3. Dyslipidemia

Dyslipidemia is one of the major risk factors for cardiovascular disease in DM. Diabetic dyslipidemia is characterized by a high plasma triglyceride concentration, low HDL cholesterol concentration, and increased concentration of small dense low-density lipoprotein (LDL)-cholesterol particles [45]. These lipid changes associated with T2DM are attributed to increased free fatty acid flux secondary to insulin resistance [46]. Increased levels of free fatty acids can decrease mRNA expression or the activity of lipoprotein lipase in adipose tissue and skeletal muscle, and an increased synthesis of VLDL in the liver can inhibit lipolysis of chylomicrons, which promotes hypertriglyceridemia [47, 48]. Hypertriglyceridemia further triggers the action of the cholesterol ester transfer protein between triglyceride-rich lipoproteins (VLDL, immediate-density lipoprotein) and lipoproteins, which are relatively richer in cholesterol esters (LDL, HDL), which

leads to a decreased HDL-cholesterol concentration and a reduction in triglyceride content in LDL [49]. These triglyceride-rich LDL molecules are then hydrolyzed by hepatic or lipoprotein lipase leading to the production of small dense LDL that are associated with a higher risk of cardiovascular disease.

I.2.4. Hypertension

Hypertension, also known as high blood pressure, is defined as a blood pressure above 130 mmHg systolic or 85 mmHg diastolic in adult. In T2DM, hypertension is often present as part of the metabolic syndrome of insulin resistance also including central obesity and dyslipidemia, although the precise mechanisms that are involved remain partially unresolved [50]. It has been demonstrated that hypertension substantially increases the risk of both macrovascular and microvascular complications in diabetic patients, including stroke, coronary artery disease, and peripheral vascular disease, retinopathy, nephropathy, and possibly neuropathy [51].

I.2.5. Abdominal obesity

Abdominal obesity, also known as central obesity, is the key component of metabolic syndrome with a predominance of intra-abdominal visceral fat accumulation, indirectly measured by waist circumference (waist circumference ≥90 cm in men, and ≥80 cm in women) in clinical practice [52]. Despite the fact that abdominal obesity is a highly prevalent feature of metabolic syndrome, the mechanisms by which abdominal obesity is causally related to the metabolic syndrome are not fully understood [53]. An excess of visceral fat accumulation might be causally related to the features of insulin resistance, but might also be a marker of a dysfunctional adipose tissue being unable to appropriately store the energy excess [54].

I.2.6. NAFLD

T2DM is strongly associated with NAFLD; more than 50 % of T2DM patients (around 59.67 %) also suffer from NAFLD [55]. NAFLD is a continuum of liver abnormalities, from non-alcoholic fatty liver (NAFL, simple steatosis) to non-alcoholic steatohepatitis (NASH). Namely, it is a spectrum of liver damage that ranges from relatively benign hepatic steatosis (accumulation of triglycerides in the liver) to potentially fatal cirrhosis [56]. All forms of NAFLD tightly correlate with hepatic as well as peripheral insulin resistance [57, 58], which is aggravated during NAFLD progression [59]. In patients that are obese and have T2DM, the presence of NAFLD associates with more severe hyperinsulinemia, dyslipidemia and insulin resistance in hepatic and adipose tissue than in patients without NAFLD [60]. As described above, adipose tissue is a source of free fatty

acids and other factors entering the portal circulation. In obese/T2DM state, the free fatty acids from enlarged adipose tissue are then taken up by the hepatocytes, which lead to reduced hepatic insulin clearance with a further increase in circulating insulin levels (hyperinsulinemia) [61]. In the liver, free fatty acids promote increased glucose production and triglyceride synthesis (hepatic steatosis) and impair insulin suppression of hepatic glucose output [61]. After the development of steatosis, the liver becomes more vulnerable to the gut-derived bacterial toxins, adipokine/cytokine imbalance, mitochondrial dysfunction, oxidative damage, dysregulated hepatocyte apoptosis, release of pro-fibrogenic factors and pro-inflammatory mediators from impaired organelles and activation of hepatic stellate cells. Such multiple factors may collectively stimulate inflammation, apoptosis and fibrosis that ultimately lead to progressive liver disease (fatal cirrhosis) [28].

I.3. T2DM TREATMENT AND ITS CHALLENGES

Good glycemic control remains the main foundation of T2DM management, which could prevent and/or delay the onset and progression of diabetic complications [62, 63]. There are eight pathophysiological mechanisms underlying T2DM to lead hyperglycemia (Fig. 3), including (i) reduced insulin secretion from pancreatic β-cells, (ii) elevated glucagon secretion from pancreatic α cells, (iii) increased production of glucose in the liver, (iv) neurotransmitter dysfunction and insulin resistance in the brain, (v) enhanced lipolysis, (vi) increased renal glucose reabsorption, (vii) reduced incretin effect in the small intestine, and (viii) impaired or diminished glucose uptake in peripheral tissues such as skeletal muscle, liver, and adipose tissue [63]. The achievement of durable glycemic control requires antidiabetic medication that reverse the pathophysiological defects that are present in T2DM [14, 64]. There are several classes of antidiabetic medications (glucose-lowering pharmacotherapy) available currently, which target one or more of these pathophysiological pathways (summarized in Fig. 3) [63].

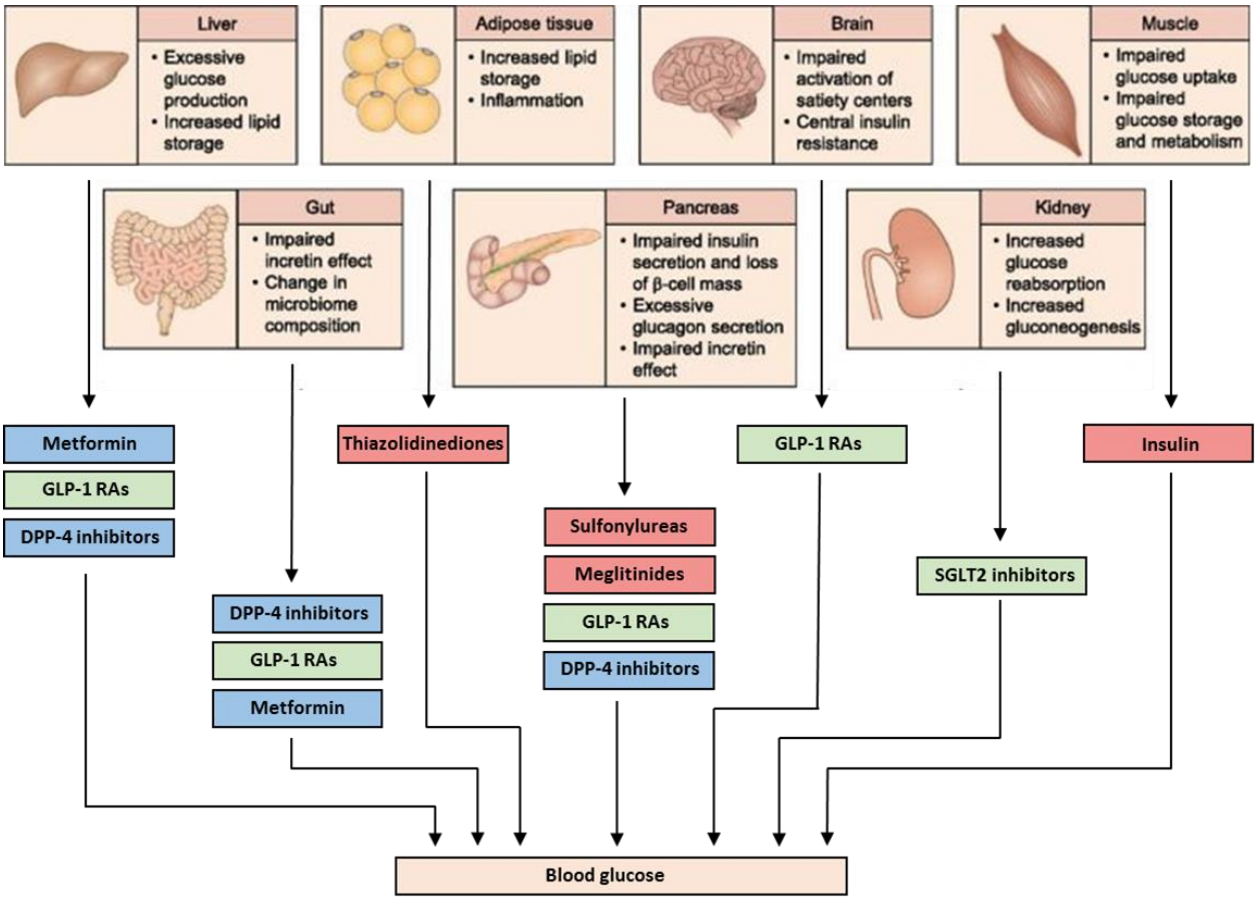

Figure 3. Pathophysiology and medication targets in T2DM. The hyperglycemia in T2DM results from the pathophysiological dysfunctions of various organs. The targeted pharmacologic interventions that decrease blood glucose levels are illustrated by the color of the boxes. Green indicates body weight loss, blue indicates body weight neutrality, and red indicates body weight gain. Reproduced from [65].

I.3.1. Metformin — the first line treatment in T2DM

Metformin, a biguanide derivative (1,1-dimethylbiguanide hydrochloride) [66], is the first line oral agent to treat T2DM, particularly in obese patients [67]. Metformin was discovered in 1922 [68]. It was approved in Europe in 1957, in Canada in 1972 and in the United States in 1995 as an anti-diabetic medication [69]. Metformin is prescribed as an anti-hyperglycemic medicine that suppresses hepatic glucose production by activating the adenosine monophosphate-activated protein kinase in the liver, improves insulin sensitivity by activating the insulin receptor expression and enhancing the tyrosine kinase activity, increases fatty acid oxidation, and decreases the absorption of glucose from the gastrointestinal tract [70, 71]. Metformin has shown to delay the progression of T2DM, reduce the risk of complications, and reduce mortality rates in patients by decreasing hepatic glucose synthesis (gluconeogenesis) and sensitizing peripheral tissues to insulin [71]. Metformin is highly efficient when there is enough insulin production [63]. However, it has no effect on β-cell function and, in the absence of weight loss, does not improve muscle insulin

sensitivity; thus, after an initial decrease, Hemoglobin A1c (HbA1c; glycated hemoglobin) rises progressively [67]. The most common adverse effect of metformin is gastrointestinal irritation, including diarrhea, cramps, nausea, vomiting, and increased flatulence; metformin is more commonly associated with gastrointestinal side effects than most other antidiabetic medications [17].

I.3.2. Sulfonylureas and meglitinides — stimulating endogenous insulin secretion

Sulfonylureas, well-established glucose-lowering drugs [72], are currently prescribed as second-line or add-on treatment options for the management of T2DM [63]. Sulfonylureas lower blood glucose levels by increasing insulin secretion in the pancreas by blocking adenosine triphosphate-sensitive potassium (K_{ATP}) channels on pancreatic β-cells [73]. They also limit gluconeogenesis in the liver. Sulfonylureas decrease the breakdown of lipids to fatty acids and reduce the clearance of insulin in the liver [74]. Since insulin secretion is non-glucose-mediated, conventional sulfonylureas have been associated with a higher risk of hypoglycemia, weight gain and cardiovascular events [75]. Sulfonylureas are divided into two groups: first-generation (conventional) agents, which include chlorpropamide, glycyclamide, tolazamide, and tolbutamide, and second-generation agents, which include glipizide, glimepiride, gliclazide and glyburide [63]. Compared with conventional agents (e.g. glibenclamide), the second-generation agents (e.g. gliclazide; a short-acting sulfonylurea) have been associated with a reduced risk of all-cause mortality and cardiovascular death and are less likely to cause weight gain and hypoglycemia [26].

Meglitinides (repaglinide and nateglinide), non-sulfonylurea secretagogues, act on the K_{ATP} channels in the pancreatic β-cells thereby stimulating the release of insulin from the β-cells, similar to sulfonylurea, though the binding site is different [76]. Moreover, the binding of meglitinides to the receptor in β-cells of the pancreas is weaker than sulfonylureas. Although less related to hypoglycemia than sulfonylureas, they do not prevent the progressive decline in β-cell function and rise in HbA1c that is associated with T2DM [26]. Meglitinides require administration before each meal and have a rapid onset and a short duration of action (4-6 h) with lowering risk of hypoglycemia [77].

I.3.3. Thiazolidinediones — insulin sensitizers

Thiazolidinediones (pioglitazone and rosiglitazone), the only insulin-sensitizing agents [26], are agonists of peroxisome proliferator-activated receptors (PPARs) [78] and facilitate increased glucose uptake in numerous tissues including the adipose tissue, muscle and liver [63, 79]. Their mechanisms of action include diminution of free fatty acid accumulation, reduction in

inflammatory cytokines, rising adiponectin levels, and preservation of β-cell integrity and function, all leading to improvement of insulin resistance and β-cell exhaustion [63, 80]. Weight gain is common with thiazolidinediones, but the greater the weight gain, the greater the decrease in HbA1c and the greater the improvement in insulin sensitivity and β-cell function [80, 81]. In addition, combined insulin-thiazolidinediones therapy causes heart failure [82, 83]. Thus, thiazolidinediones are not preferred as first-line or even step-up therapy [63].

I.3.4. Sodium/glucose co-transporter 2 (SGLT2) inhibitors — targeting renal glucose absorption

SGLT2 inhibitors are a new class of glucosuric agents: canagliflozin, dapagliflozin, empagliflozin, ertugliflozin, ipragliflozin, luseogliflozin and remogliflozin etabonate. They provide insulin-independent glucose lowering by blocking glucose reabsorption in the proximal renal tubule by inhibiting SGLT2 [84, 85], resulting in the amelioration of glucotoxicity, with improved β-cell function and enhanced insulin sensitivity as a consequence [86]. Because SGLT2 inhibitors also suppress sodium transport, they cause mild extracellular volume depletion and reduce blood pressure (~5–6 mmHg in systolic pressure and ~1–2 mmHg in diastolic pressure) [26]. Urinary tract infections leading to urosepsis and pyelonephritis, as well as genital mycosis, may occur with SGLT2 inhibitors. SGLT2 inhibitors rarely cause ketoacidosis [63]. SGLT2 inhibitors are currently prescribed to treat T2DM but are most often used as second- or third-line agents instead of first-line because there are other medications on the market that have much longer safety record and are less expensive than SGLT2 inhibitors [87, 88].

I.3.5. GLP-1 receptor agonists and DPP-IV Inhibitors — incretin therapy

The so-called 'incretin effect' is defined by an increased insulin secretion in response to the oral glucose administration compared with the same quantity of glucose administered intravenously [89]. This effect is attributed to the fact that oral glucose triggers the release of incretin hormones in the gut (coupled to the absorption of glucose), while intravenous glucose does not [90]. The incretin effect is responsible for 50–70% of total insulin secretion after oral glucose intake [90], while this effect is reduced or even lost in patients with T2DM [63]. The first incretin hormone to be discovered was the gastric inhibitor peptide (GIP, also referred to as glucose-dependent insulinotropic polypeptide). It is a 42-amino acid hormone synthesized in duodenal and jejunal enteroendocrine K cells in the proximal small intestine [18]. However, it has been suggested lately that GIP is the more important incretin hormone in healthy humans [91] since its insulinotropic actions are severely impaired in patients with T2DM [92]. Subsequently, the second incretin

hormone, GLP-1, was identified. It is produced and secreted by enteroendocrine L cells mainly in the distal ileum and colon [18]. The initial product GLP-1 (1–37), a 37 amino acid peptide, is susceptible to amidation and proteolytic cleavage, thereby existing in two circulating equipotent molecular forms, GLP-1 (7-37) and GLP-1 (7-36) amide [93]. Although both forms have equally potent insulinotropic effect, GLP-1 (7-36) amide is more abundant in the circulation after eating [18, 94]. In addition, GLP-1 — unlike GIP — retains its glucose-regulatory actions in patients with T2DM [95]. Consequently, there is an increasing interest in exploiting the glucose-lowering effects of the incretin hormones therapeutically (as anti-diabetic agents), which is mainly focused on GLP-1. This has led to the discovery and generation of structurally distinct GLP-1 receptor agonists (GLP-1RAs) that mimic the actions of GLP-1 *in vivo* in humans for the treatment of T2DM [18, 95]. Both native GIP and GLP-1 have a short half-life, as they are rapidly hydrolyzed by the dipeptidyl peptidase IV (DPP-IV) enzyme within ~ 2 min, thereby rapidly decreasing the circulating levels of intact GLP-1 and GIP [18, 96]. As a result of DPP-IV activity, intact, biologically active GLP-1 (GLP-1 (7-37) and GLP-1 (7-36 amide)) represents only 10–20% of total plasma GLP-1 [97]. The identification of the essential role of DPP-IV in the metabolic clearance of bioactive GLP-1 in human promoted the development of orally available DPP-IV inhibitors, administration of which stabilizes incretin hormones at physiologically active levels [98]. Although both forms of incretin-based therapy exert their glucose regulatory effects largely through potentiation of the actions of GLP-1, several key features distinguish the mechanisms of action of these agents. In the next sub-sections, we will review and discuss each class separately, with an emphasis on its mechanisms of action, market medications and emerging new delivery strategies.

I.3.5.1. GLP-1RAs

GLP-1 emerged as a target for type-2 diabetes treatment due to its unique mechanism of action. Firstly, one of the main advantages of GLP-1 as an antidiabetic agent is that it exerts its effect in a glucose-dependent manner; its effect is lost in the absence of elevated blood glucose concentrations, thereby reducing the risk of unwanted hypoglycemic events [99]. Furthermore, GLP-1 also exerts multiple physiological effects related to T2DM by activating GLP-1 receptors (G-protein-coupled receptors; GPCRs) distributed in various organs and tissues (e.g., pancreas, gastrointestinal (GI) tract, brain, liver and adipose tissues), such as (i) promoting insulin secretion/biosynthesis and decreasing glucagon secretion, (ii) enhancing insulin gene transcription, (iii) increasing pancreatic β-cell mass by promoting proliferation and neogenesis while inhibiting apoptosis, (iv) inhibiting gastric emptying, acid secretion and decreasing appetite, (v) reducing hepatic steatosis by improving hepatic lipid metabolism, (vi) suppressing inflammation in the immune system, (vii) improving insulin resistance of the adipose tissues (Fig. 4) [100]. All these favorable actions indicate that GLP-

1 is a highly promising antidiabetic agent. Despite its potent anti-diabetes effects, as abovementioned, the clinical application of native GLP-1 is hindered by its rapid clearance by DPP-IV *in vivo*, resulting in a half-life of only ~2 min [101]. To overcome this, GLP-1RAs have been developed and became a new class of injectable agents used in the management of T2DM.

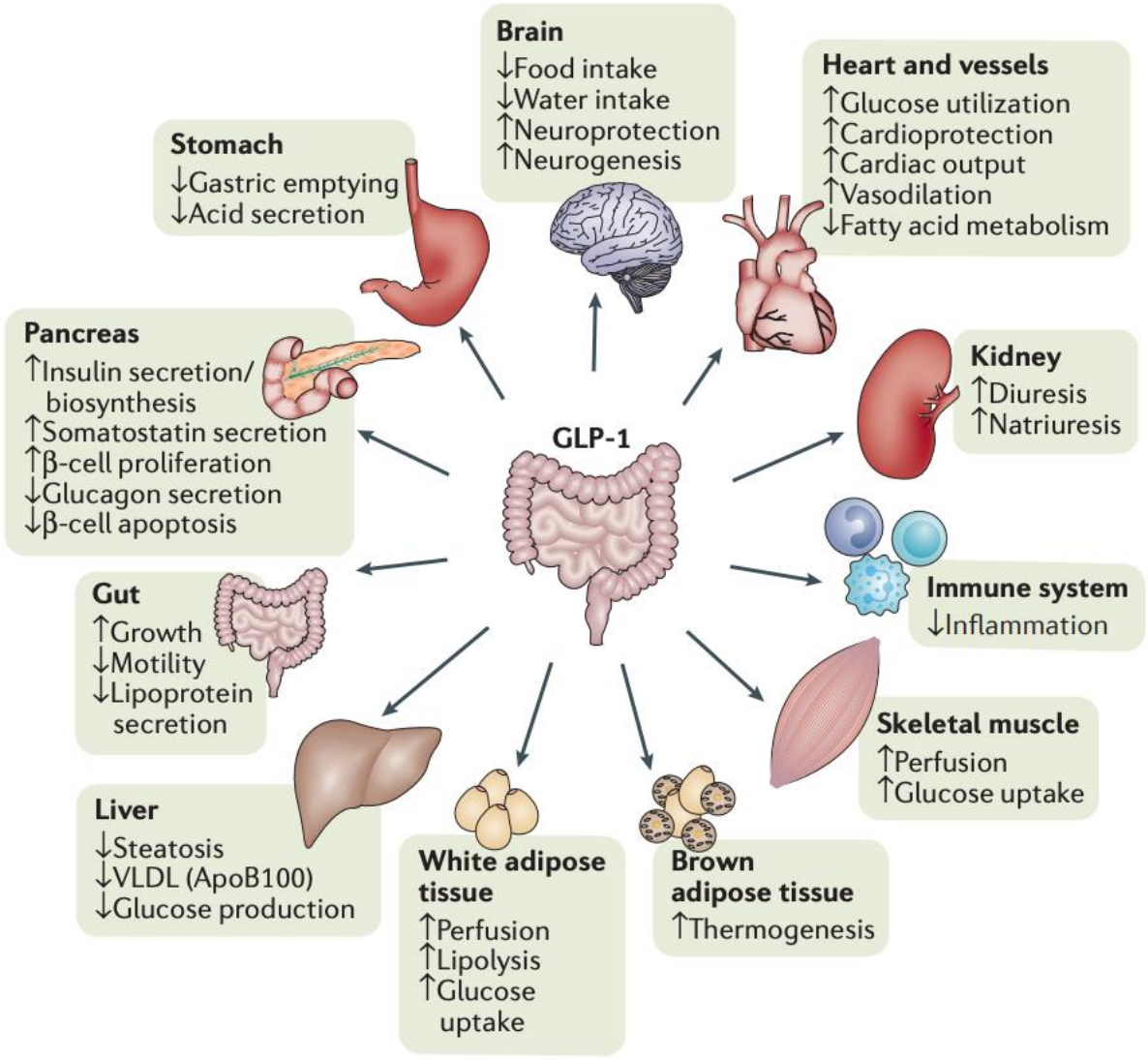

Figure 4. Putative actions of glucagon-like peptide 1 (GLP-1). GLP-1 exerts pleiotropic effects on various different tissues and organs, with various potential physiological, pathophysiological and pharmacological implications. VLDL, very low density lipoprotein. Reproduced from [100].

Six subcutaneous GLP-RAs solutions or powders (exenatide, liraglutide, semaglutide, lixisenatide, albiglutide and dulaglutide) are currently approved by the Food and Drug Administration (FDA) (Fig. 5), which are structurally similar to native GLP-1 with regard to their amino-acid sequences [102]. To fight against DPP-IV degradation and/or minimize their renal clearance, different half-life extension strategies have been exploited including sequential modification (exenatide and

lixisenatide), attachment of a fatty-acid (liraglutide and semaglutide), fusion with human serum albumin (albiglutide), and fusion with the fragment crystallizable (Fc) region of a monoclonal antibody (dulaglutide) [102]. All these strategies directly modify the sequences of GLP-1, which could prolong the half-life of GLP-1RAs varying from few hours (exenatide; 2.5 h) to few days (semaglutide; ~ 7 days) [103]. For instance, exenatide needs to be administered twice daily, whereas semaglutide and dulaglutide with a long half-life allow a once-weekly administration. Moreover, there is another injectable product of GLP-1RAs commercialized that exploits the advantage of a drug delivery system (exenatide-loaded PLGA microspheres), allowing a once-weekly subcutaneous administration, as the microspheres in the formulation release surface-bound exenatide initially, then gradually releasing exenatide from the microspheres to achieve sustained plasma levels [104].

Although there is a large number of GLP-1 RAs already approved in EU or USA, most of them are via subcutaneous injection. Exploring novel routes of administration for GLP-1 RAs has attracted increasing attention during the past decades. There are numerous ongoing efforts to develop oral GLP-1 RAs since oral products have better patient compliance relative to subcutaneous injectable products. It is noteworthy that the FDA approved oral semaglutide in 2019, to be sold as Rybelsus®, the first and only oral GLP-1RAs for adults with T2DM [105]. Oral semaglutide is approved in 2 doses (7 mg and 14 mg), co-formulated with the permeation enhancer sodium N-[8-(2-hydroxybenzoyl) aminocaprylate] (SNAC) in a tablet [106]. Additionally, there is another oral GLP-1RAs, ORMD-0901, that has been tested in Phase II clinical trials. In this product, exenatide is encapsulated into coated capsules with protease inhibitors and absorption enhancers [107]. However, there have been no recent updates nor published efficacy data regarding this investigational drug [102]. Therefore, the development of oral GLP-1RAs is still a hot area and faces a lot of challenges that merits further investigation.

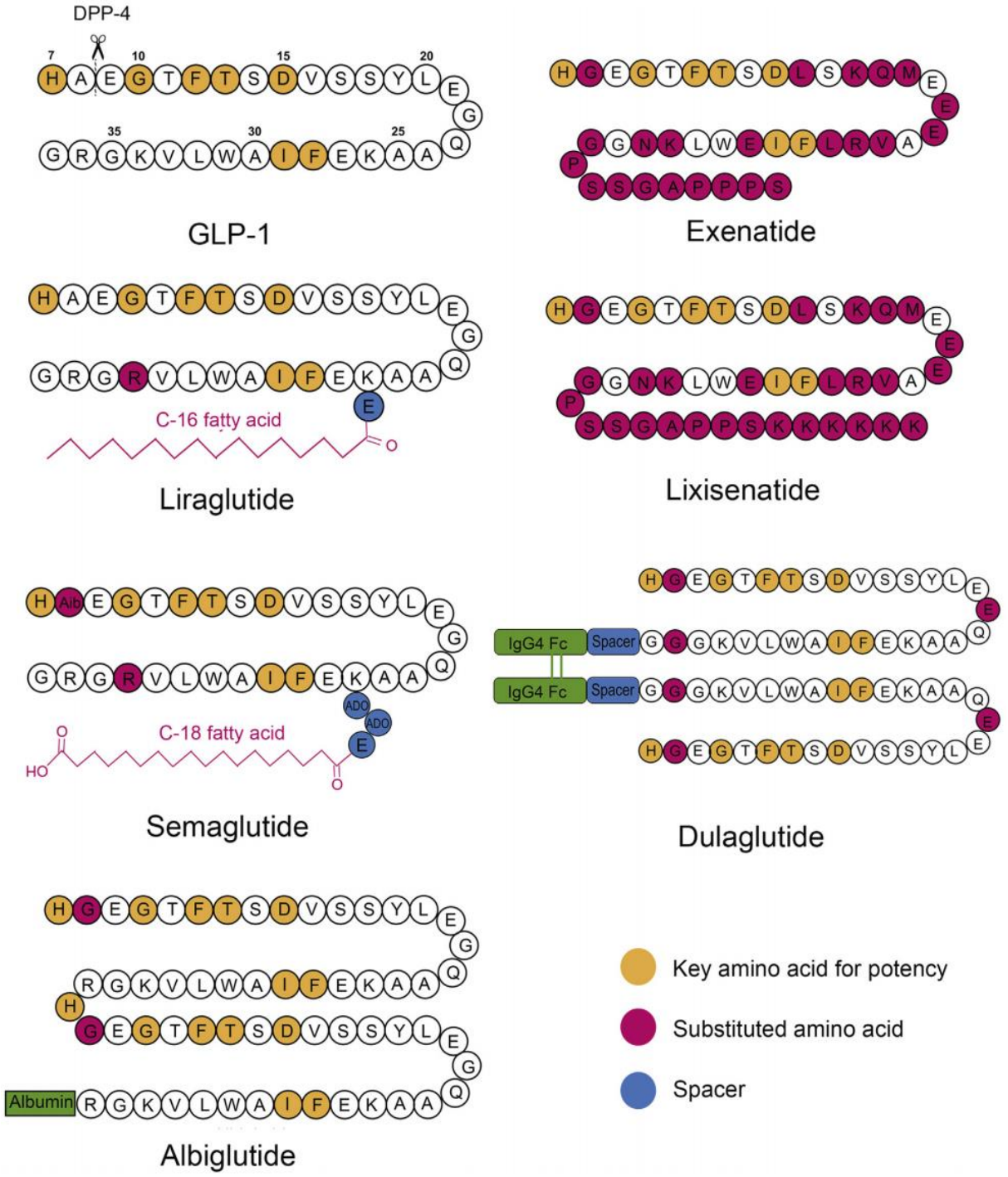

Figure 5. Peptide sequences and molecular structures of FDA approved GLP-1 RAs. Reproduced from [102].

I.3.5.2. DPP-IV inhibitors

DPP-IV, a homodimeric serine peptidase, consisting of approximately 700 amino acids in each subunit [108], is widely expressed in many tissues (e.g., liver, kidney, lungs, intestinal brush-border membranes, pancreas, spleen, adrenal gland, and the central nervous system) [18, 109, 110]. Both gastrointestinal hormones GLP-1 [111] and GIP [112] can be cleaved by DPP-IV peptidase at the

second amino terminal position with a proline or alanine residue. Thus, DPP-IV inhibition is also an interesting approach in the treatment of T2DM. With the fist compound of DPP-IV inhibitors (sitagliptin) launched in 2006, this new class of oral antidiabetic drugs has been approved to be on the market with more than ten medications, including vildagliptin, saxagliptin, linagliptin, gemigliptin, anagliptin, teneligliptin, alogliptin, trelagliptin, omarigliptin, evogliptin, and gosogliptin [113]. Typically, these agents reduce serum DPP-IV activity by more than 80%, with some inhibition maintained for 24 h after one dose or upon a once daily treatment [114].

DPP-IV inhibitors, also known as incretin enhancers, show many similar therapeutic actions compared to GLP-1RAs, such as stimulating insulin secretion, inhibiting glucagon secretion, and preserving pancreatic β-cell mass through the stimulation of cell proliferation and inhibition of apoptosis [115, 116]. DPP-IV inhibitors also reduce the degradation of bioactive GIP [117]. Although the insulinotropic effects of GIP are impaired in patients with T2DM, they can be at least partially restored if glycaemic control is improved, thereby raising the possibility that DPP-IV inhibitors lower glucose levels, at least in part, through GIP-mediated stimulation of insulin secretion [118]. Compared with GLP-1RAs, DPP-IV inhibitors are generally not strongly associated with a deceleration of gastric emptying or weight loss [119]. Besides, it has been reported that DPP-IV inhibitors could cause some adverse effects, including gastrointestinal adverse effects (e.g., nausea, and vomiting), nasopharyngitis, upper respiratory tract infection, heart failure, headache, skin-related side effects and musculoskeletal disorders [63, 108]. It also increases the risk of hypoglycemia when the patients combine the treatment with sulfonylureas [120].

I.3.6. Insulin therapy

If non-insulin pharmacologic therapies at the maximum tolerated dose fail to achieve glycemic control, insulin therapy should be introduced in patients with T2DM [63]. During insulin treatment, close and frequent monitoring of the patient is needed for any dose titration to achieve target glycemic goals, as well as to prevent hypoglycemia. T2DM patients are usually reluctant to accept insulin therapy due to the fear of hypoglycemia, regimen complexity, and increased multiple daily injections. Although inhaled insulin is available [121], the use of inhaled insulin requires pulmonary function testing prior to and after starting therapy, and it is contraindicated in subjects with asthma or other lung diseases [63]. Furthermore, weight gain is a common side effect of taking insulin because it accelerates the uptake of glucose (sugar) into the cells [122]. Glucose that your cells don't use accumulates as fat. The weight gain can raise a barrier to the use of insulin in T2DM. In this circumstance, the combination with GLP-1RAs and/or DPP-IV inhibitors has been useful, promoting weight loss and reducing insulin dosage [123]. However, insulin injection might cause

lipohypertrophy that leads to poor insulin absorption and suboptimal glycemic control. In addition, insulin therapy aggravates the cardiovascular risk with T2DM [63].

I.3.7. The challenges in T2DM treatment

In 2019, more than 460 million adults were living with diabetes in which T2DM occupies above 90 % of those patients. The prevalence of T2DM still continues to increase; 374 million people are currently at increased risk of developing T2DM [2]. Patients with T2DM frequently have comorbidities that complicate the management of their disease. A key contributor to the remarkably high rates of morbidity and mortality is chronic poor metabolic control, especially poor glycemic control [124]. The treatment of T2DM is complex, and involves lifestyle modification and physical activity as well as the addition of pharmacologic therapy to provide needed physiologic support for insulin resistance [125]. A number of pharmacologic options that we mentioned earlier in this chapter can facilitate excellent glycemic control, with the potential to improve glucose homeostasis. Fig. 6 summarizes the main pharmacotherapies that are widely used for T2DM treatment in the clinics. Among these medications, although metformin (suppressing hepatic glucose production), sulfonylureas and meglitinides (stimulating endogenous insulin secretion), thiazolidinediones (insulin sensitizers), SGLT2 inhibitors (targeting renal glucose absorption) and insulin therapy are widely prescribed in the clinics improving glycemic control in patients with T2DM, these approaches fail to address the continuous loss of pancreatic β-cell function over time and the weight gain associated with the T2DM [126]. Furthermore, they also could cause some undesirable side effects (e.g., cardiovascular diseases) and increase the risk of hypoglycemia [63]. However, the discovery of incretin hormones and the use of incretin therapy have largely compensated the shortcomings of other anti-hyperglycemic medications that I mentioned above for the treatment of T2DM. The therapeutic agents based on incretin therapy work in a glucose-dependent manner avoiding the risk of hypoglycemia, and they also could reverse the continuous loss of pancreatic β-cell function that results in insulin resistance in patients suffering from T2DM.

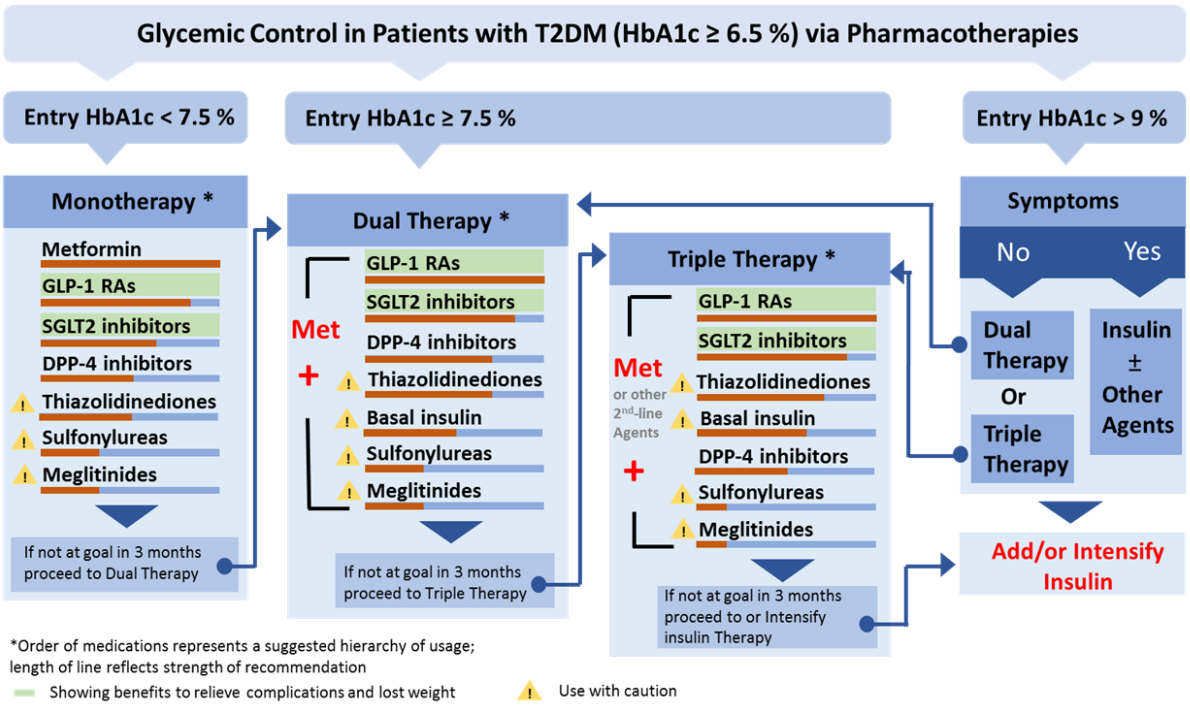

Figure 6. Anti-hyperglycemic therapy in T2DM: general recommendations. Figure modified from [127, 128].

Both GLP-1RAs and DPP-IV inhibitors can facilitate excellent glycemia control and have potentially advantageous effects on multiple components of the metabolic syndrome, such as insulin resistance and dyslipidemia [129]. However, compared with GLP-1RAs, DPP-IV inhibitors present some serious side effects, including upper respiratory tract infection, heart failure and headache [108], and they do not have an obvious effect on gastric emptying to help weight loss in T2DM patients. In contrast, GLP-1RAs, second-line anti-diabetic drug in clinical treatment (Fig. 6), not only provide with a significant reduction in HbA1c and body weight with little risk of hypoglycemia, several GLP-1RAs have also demonstrated they can improve cardiovascular outcomes, especially in patients with established atherosclerotic cardiovascular disease and chronic kidney disease [127, 128]. These agents also suggest an improvement in renal outcomes [130]. Nevertheless, most GLP-1RAs must be injected (except for oral semaglutide, Rybelsus®), remaining a big barrier for initiating therapy in T2DM patients [18].

The development of oral dosage forms that enable the absorption of therapeutic peptides into the systemic circulation is one of the greatest challenges for the pharmaceutical industry [131]. The oral delivery of GLP-1RAs is the holy grail of T2DM treatment. As we know, the benefits of oral peptide delivery over an intravenous or subcutaneous administration are remarkable. The oral administration of incretin mimetic peptides has the additional therapeutic advantage of simulating

the normal physiological pathway of the native peptide [132]. GLP-1 agonists target the liver, which can be accessed in much higher concentrations via the hepatic portal vein than via subcutaneous delivery, thereby reducing systemic exposure and its associated side effects [133]. Despite the numerous ongoing efforts to exploit the oral route of administration of incretin mimetic peptides, only one product (Rybelsus®; oral semaglutide) has been approved by the FDA few months ago, and another oral GLP-1 analogue is currently in clinical trials [102], and both of these require co-administration with functional excipients.

Oral semaglutide fills a gap in oral incretin peptides administration in the market. It is co-formulated with the absorption enhancer SNAC in a tablet [106]. The SNAC technology prevents destruction of semaglutide in the stomach and facilitates transcellular absorption through the gastric membrane enabling semaglutide to reach systemic circulation intact. The oral formulation of semaglutide indicates similar efficacy compared to the currently available GLP-1RAs with regard to HbA1c lowering and weight loss [134]. Oral semaglutide, however, has low bioavailability (0.4%-1%), and requires optimal conditions for absorption [135]. The drug should be administered in the fasting state to facilitate absorption and almost no plasma semaglutide was measurable in the fed state (Fig. 7) [106]. The oral form is dosed 7 to 14 times the subcutaneous dose on a mg basis (7 mg or 14 mg versus 0.5 mg or 1 mg) and given daily instead of weekly. The half-life of semaglutide is around 7 days, and thus we could not rule out serious side effects (e.g., thyroid cancer, pancreatitis and kidney damage) caused by excessive accumulation of semaglutide in the body upon daily administration. Besides, the common side effects of oral semaglutide include nausea, abdominal pain, diarrhea, increase in amylase, and increase in lipase [135].

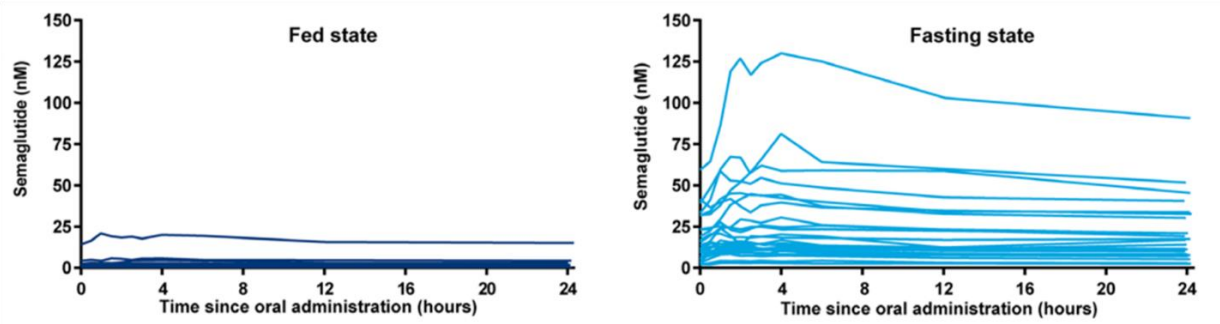

Figure 7. Food-effect study in healthy individuals receiving once-daily oral semaglutide in the fed state (n = 25) or fasted state (n = 26). Reproduced from [106].

Hence, despite oral semaglutide fills a gap in oral incretin mimetic peptides administration, it still presents various limitations and fails to remove the big obstacle in oral delivery of peptides — the low oral bioavailability. Over the past decades, various strategies, such as permeation enhancers, prodrugs and nanocarriers, have been developed to improve the oral bioavailability of therapeutics [136, 137]. Among current approaches to increase oral absorption, advanced formulations have received considerable attention, especially targeting-based strategies [138, 139]. In the next section, we will focus on current targeting approaches towards improved oral drug delivery systems. It summarizes different targeting strategies towards different targeting sites in the gastrointestinal tract to improve bioavailability of oral drug delivery systems. It includes, but it is not limited to, different strategies towards increased peptide bioavailability in T2DM treatment.

II. OVERCOMING THE INTESTINAL BARRIER: A LOOK INTO TARGETING APPROACHES FOR IMPROVED ORAL DRUG DELIVERY SYSTEMS

Oral drug administration is one of the most preferred and simplest routes among both patients and formulation scientists. Nevertheless, orally delivering some of the most widely used therapeutic agents (e.g., anticancer drugs, peptides, proteins and vaccines) is still a major challenge due to the limited oral bioavailability associated with them. The poor oral bioavailability of such drugs is attributed to one or many factors, such as poor aqueous solubility, poor permeability, and enzymatic degradation. Various technological strategies (such as permeation enhancers, prodrugs and nanocarriers) have been developed to enhance the bioavailability of these drugs after oral administration. Amongst the different approaches, advanced and innovative drug delivery systems, especially targeting-based strategies, have garnered tremendous attention. Furthermore, the presence of numerous types of cells and solute carrier transporters throughout the gastrointestinal tract represents numerous potential targeting sites for successful oral delivery that have not yet been exploited to their full potential. This review describes different targeting strategies towards different targeting sites in the gastrointestinal tract. Additionally, exciting improvements in oral drug delivery systems with different targeting strategies (e.g., M cells for oral vaccination, L cells for T2DM) are also discussed.

II.1. INTRODUCTION

For years, a wide variety of targeting-based formulations have been designed and developed to overcome the shortcomings of newly discovered drug molecules and conventional formulations. Targeted drug delivery systems (DDS) reduce the dose-related toxicity and improve the therapeutic efficacy by diminishing the minimum effective concentration of the drug. The targeted system achieves the aforementioned objectives by increasing the transport of the carrier/active compound to the targeted site and/or by enhancing target to non-target tissue ratio of the drug molecule [140]. Targeting is mainly classified as active and passive approach. Passive targeting is achieved by augmenting accumulation at the targeted site by exploiting pathophysiological features such as the temperature, pH, abnormal vasculature, or surface charge of the cells [141]. However, the random nature of targeting and its insufficient or unspecific characteristics seriously limits its application. To attain passive targeting, the properties of the system, such as charge, size, hydrophobicity and hydrophilicity, are altered [142]. On the other hand, in active targeting specific ligands are linked on the surface of the DDS, which directs the whole system to the specific tissue/site that expresses

ligand-specific biomarkers. Entire molecules or fragments of antibodies, vitamins, glycoproteins, folate, peptides and carbohydrates are some of the commonly used ligands for active targeting [143]. The ligands interact with the receptors present on the target site surface, thus resulting in the accumulation of the delivery system at the target site of action from where they either can act superficially or be internalized [144]. The necessity for targeted systems is remarkable in the case of anticancer drugs, where exposure to cytotoxic agents can damage healthy tissues. Other applications include targeting specific absorption sites, such as the small intestine or colon, or targeting specific organs, such as the brain [145].

Owing to the advantages such as, higher patient compliance, cheaper formulation and ease of administration, oral route is one of the most preferred routes of administration [139]. Oral administration is used for both local and systemic delivery of a wide range of drug molecules, from small molecule drugs to large biomacromolecules. Variations along the gastrointestinal tract (GIT) in combination with its broad application make targeting the GIT an appealing approach. Targeting different regions of the GIT can be achieved by exploiting its anatomic, histologic, physiologic, and biochemical features and combining the drug with advanced drug delivery systems or devices [146]. Some of the versatile physiological factors of the GIT include gastrointestinal pH differences, mucus thickness and GI transit time. Furthermore, the presence of numerous types of cells (e.g., enterocytes, Paneth cells, and L cells) and transporters interspersed throughout the GIT can also be exploited. Such unique variations and features allow for the design of a broad array of targeted delivery systems, both active and passive.

In humans, the GIT is a highly absorptive region that extends from the oral cavity to the anal canal, covering a total surface area of 250 to 400 m^2 [147]. There are four main types of gastrointestinal epithelial cells, which originate from stem cell populations in the crypt zone. These cells differentiate into absorptive (enterocyte/colonocyte), mucus secretory (goblet cells), hormone-secreting (enteroendocrine cells) or defensive peptide-secreting cells (Paneth cells). The specific physiological features of these gastrointestinal cells are described in detail in the next subsections, along with specific targeting strategies for each type of cell.

The present review overviews the latest and/or most commonly used strategies to target the different cell types and transporters present in the GIT.

II.2. TARGETED INTESTINAL CELL DRUG DELIVERY SYSTEMS

There are a wide variety of cells throughout the GI epithelia that present different characteristics and receptors, especially in the small intestine (Fig. 8). Enterocytes are the most common cells

found in the small intestine, and are polarized absorptive columnar epithelial cells. The apical side of the columnar epithelial cells is covered by microvilli brush border and a carbohydrate glycocalyx. The mucus-secreting goblet cells are the second most common cells (approximately 16%) in the small intestine. The mucin secreted from these cells comprise the protective mucus layer that envelopes and protects the intestinal epithelia. M cells only constitute less than 1% of total cells in the intestine, however, they play a vital role in the uptake of antigens and microorganisms. M cells are found in Peyer's patches where the mucus layer is absent, thus allowing a closer interaction of the antigens and microorganisms with the intestinal epithelia. Dendritic cells (DCs) are responsible for maintaining the immune environment homeostasis in the intestine, by linking the humoral and cellular immune responses [148]. Paneth cells are secretory cells and are found at the base of the crypts of Lieberkuhn. The main secretion of these cells are antimicrobial proteins and peptides [149]. The small intestine also contains enteroendocrine cells interspersed among the other epithelial cells. Although very low in number, the enteroendocrine cells are responsible for secreting hormones with very important digestive functions. Moreover, these cells are also involved in moderating communication between the central and enteric endocrine systems.

The targeting of intestinal cells is initiated by well-known ligand-receptor recognition. For this, surface functionalized DDS or ligand-grafted drugs (prodrugs) are required. Such targeted systems are designed to increase the uptake of the delivery system and encapsulated/grafted drugs at the target site. In the following subsections, the utilization of ligands for each type of epithelial cell will be discussed.

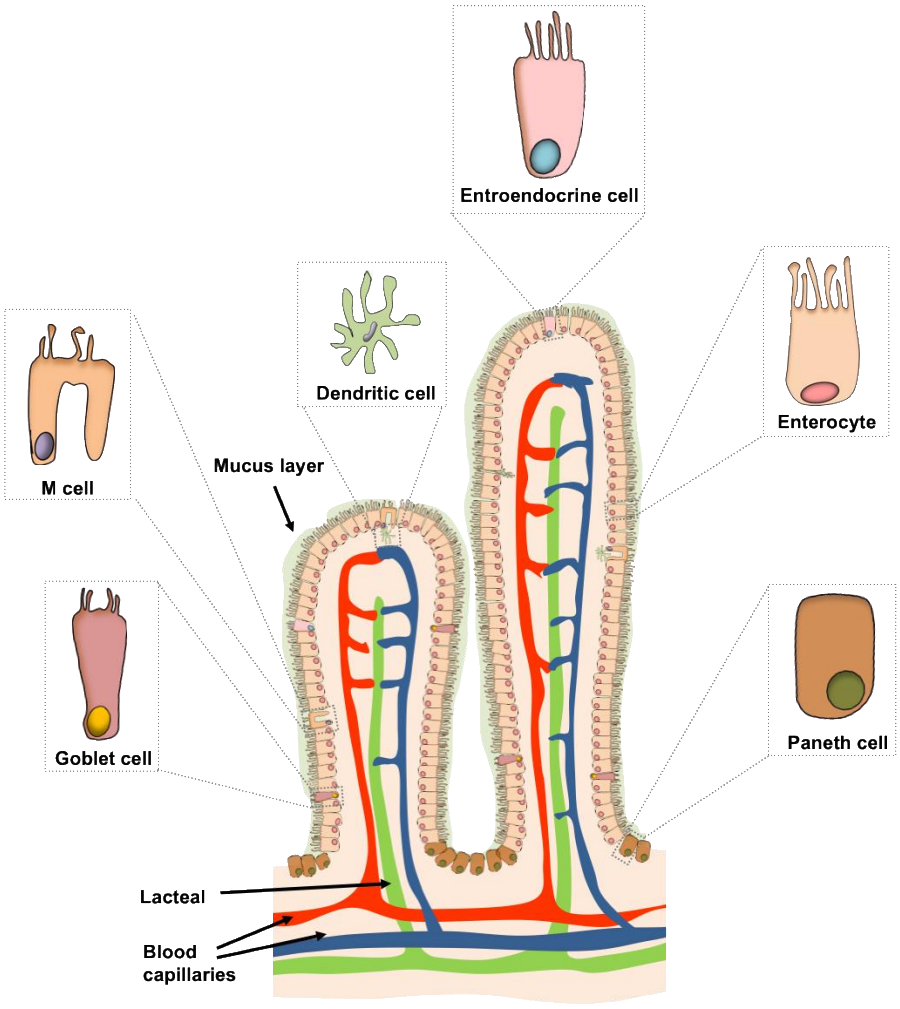

Figure 8. Schematic representation of different intestinal cells presents in the intestinal epithelium.

II.2.1. Enterocyte targeting

Enterocytes are the most prevalent type of cells in the intestinal epithelia, and thus are often targeted to maximize the delivery of drugs and their absorption into systemic circulation. The entry of targeted DDS into enterocytes normally occurs via receptor-mediated endocytosis. The endocytosis is initiated by the interaction between the ligand molecules on the surface of DDS and the receptor molecules present on the epithelial cell surface, which results in the internalization of the complex inside the cells [150]. Enterocytes are polarized cells with a columnar shape and have receptors for various compounds present on its apical surface (such as vitamins, transferrin and hormones) [151]. Therefore, taking advantage of such receptors is one of the most studied approach for enterocyte targeting for enhancing efficiency of oral drug delivery. These ligands generally include vitamins [152-154], transferrin [155], lectins [156, 157] and monoclonal antibody

fragments [158], as well as oligopeptides [159, 160]. The ligands that have been exploited to target the enterocytes are summarized in Table 1.

II.2.1.1. Vitamins

Vitamins are widely used as ligands to decorate functional systems owing to their good safety profile and stability and easy tunability [138]. The ligands of vitamins used in enterocyte targeting include vitamin B_{12} (B_{12}), biotin (vitamin B_7), folic acid (vitamin B_9) and thiamine, of which B_{12} and folic acid are the most studied ones for oral targeted delivery. B_{12}, a water-soluble vitamin, is a complex molecule which has a 'corrin ring' and a nucleotide [161]. B_{12} is orally absorbed after it binds to haptocorrin (a salivary enzyme), which protects and transports B_{12} to the small intestine. In the small intestine, B_{12} binds to an intrinsic factor forming the B_{12}-intrinsic factor complex, which interacts with the receptors mainly expressed on enterocytes in the iliac region [162]. Various poorly absorbed drugs, proteins and peptides, among others, such as insulin, have greatly increased their oral bioavailability by using B_{12} as a ligand for targeted delivery. B_{12}-decorated 70 kDa dextran nanoparticles (NPs) containing 4% w/w insulin demonstrated a significant increase in pharmacological availability (2.6-fold higher) as compared to the non-targeted NPs [154]. The effectiveness of B_{12}-NP conjugates was further improved by altering the different parameters of cross-linking [163]. The authors demonstrated that using amino alkyl B_{12} derivative resulted in significant hypoglycemic effect and extended (54 h) antidiabetic effects. Furthermore, the pharmacological availability of insulin was also increased up to 29.4 % [163]. Notably, the cellular internalization of the soluble B_{12}-intrinsic factor complex is mediated via clathrin-coated vesicles followed by an endosomal stage that results in the degradation of intrinsic factors and the recycling of B_{12}. However, B_{12} modified NPs disrupt the innate biological pathway of soluble B_{12}, changing it from clathrin-mediated endocytosis to the caveolae-mediated endocytic pathway (avoiding the endolysosomal pathway) [164]. Various B_{12}-protein/peptide bioconjugates, for example, B_{12}-insulin [165], B_{12}-human Peptide YY (B_{12}-hPYY) [166] and B_{12}-luteinizing-hormone-releasing hormone antagonists (B_{12}-LHRH) [167], have increased their intestinal absorption via the B_{12} pathway. B_{12}-coupled nanocarriers and bioconjugates have shown a significant increase in the absorption of drugs; however, the potential application of B_{12} is compromised by its relatively slower uptake compared with other vitamins and the limited availability of absorption sites (mainly in the distal ileum) [168].

Folic acid is absorbed via a saturable, pH- and sodium ion-dependent route and metabolic inhibitor-sensitive pathway [169]. Unlike B_{12}, folic acid receptors are present in sufficient quantity and fortify their response by improving the uptake and transport of bioactive molecules or vesicular

systems across the GIT. It has been reported that various poorly absorbed drugs, such as protein drugs (insulin), antibiotics (vancomycin), and anticancer drugs (docetaxel and paclitaxel), have shown significant enhancement in their bioavailability via specific folic acid receptor targeting [170-174]. Similarly, biotin is transported not only through sodium-dependent multivitamin transporter (SMVT) but also via the biotin receptor, which are both distributed throughout the small intestine [175]. Zhang *et al.* developed biotin modified liposomes for oral insulin delivery via specific clathrin-mediated endocytosis. The biotinylated liposomes demonstrated significant increase in oral insulin bioavailability (8.3 %), than classical liposomes (3.3 %) [176, 177]. Thiamine, as a vitamin ligand, has also been investigated for targeting the intestinal epithelia, owing to its outstanding features, such as high absorption in the human intestine, safety and affordability [153]. Salman *et al.* developed a Gantrez® AN NP (TNP), a thiamine-conjugated poly(methyl vinyl ether-co-maleic anhydride), for oral ovalbumin delivery (OVA, OVA-TNP) *in vivo* in male Wistar rats. The rats were orally immunized by OVA-TNP, which resulted in a stronger and more balanced immune response profiles compared to OVA-loaded non-coated nanoparticles. This improved efficacy was attributed to the higher bioadhesion of coated nanoparticles resulting in the colonization in the lower regions of the gastrointestinal tract [153]. This was the first time that thiamine was used as a ligand for orally delivery of an antigen for vaccination in an animal model.

II.2.1.2. Neonatal Fc receptors

Recently, the use of a membrane protein, neonatal Fc receptor (FcRn), to develop intestinal targeted delivery systems has gained momentum. The increasing use of these receptors to improve the oral efficacy of drug molecules is mainly due to its wide availability on the surface of different type of cells such as epithelial, endothelial, and myeloid lineages [178]. Furthermore, these FcRn receptors are expressed from neonatal stage to adulthood in human intestine. It functions as a receptor for both immunoglobulin G (IgG) and albumin, binding in the acidic intracellular compartment and protecting them from intracellular enzymatic breakdown [178]. These receptor-ligand complexes are then transcytosed by endosomes and released in the extracellular space at physiological pH, where the receptors are recycled [179] [180]. Thus, using Fc portion of IgG or albumin as a targeting ligand is a promising approach to augment the passage of drugs/nanocarriers across the duodenum and jejunum [181, 182]. Pridgen and co-workers were the first to demonstrate FcRn-targeted DDS for oral administration [183]. They developed poly(lactic acid)–poly(ethylene glycol) (PEG-PLA) block copolymers-based nanoparticles, with Fc portion of IgG chemically linked on the surface of the nanoparticles. Insulin was loaded into these nanoparticles (insNP-Fc). InsNP-Fc were able to demonstrate hypoglycemic effect *in vivo*, at dosage less than other insulin delivery systems. The efficacy of FcRn targeting was proven in FcRn knockout mice [183]. Similarly,

FcRn targeting was also used to enhance the oral delivery of SP141, a murine double minute 2 (MDM2) inhibitor [152]. Fc portion of IgG was also used in this study to modify the surface of maleimidyl-poly(ethylene glycol)-co-poly(ε-caprolactone) (Mal-PEG-PCL) nanoparticles. FcRn-targeted nanoparticles encapsulating SP141 (SP141FcNP) exhibited improved *in vitro* permeability across Caco-2 cell monolayers and were also able to enhance anti-tumor efficacy *in vivo* in preclinical models of human breast cancer. During a real-time biodistribution study, after oral administration of fluorescent NPs (DiD-NP and DiD-FcNP) and free DiD solution, the DiD-FcNP-treated group demonstrated significant fluorescent signal in the tumors, 2 h post-administration and was maintained up to 48 h (Fig. 9A). *Ex vivo* tumor accumulation studies exhibited the strongest fluorescence intensity in tumor tissues in the fluorescent FcNP treatment group (Fig. 9B). Therefore, these results demonstrated the ability of Fc-conjugated nanosystems to target and enhance the oral therapeutic efficacy of anticancer drugs for breast cancer treatment [152].

Similarly, formulation scientists have explored the possibility of using the second ligand of the FcRn receptor, albumin, to develop oral delivery systems for drugs with poor bioavailability. Owing to the targeting ability and its influence on pharmacokinetic parameters of encapsulated drug molecules, albumin has been studied tremendously as a drug delivery platform. Furthermore, albumin is non-immunogenic, abundantly available and is highly stable with inherent binding capacity [184]. Santo's group demonstrated the ability of albumin-decorated NPs to significantly improve insulin permeability *in vitro* across intestinal cell monolayers. However, these albumin-modified systems have not yet been evaluated *in vivo* [181, 185]. Despite numerous advantages, albumin-based oral delivery systems have not been exploited to its full potential. Albumin cannot only bind to the FcRn receptor but also interact with other cell surface receptors, such as gp18, cubilin and megalin [184]. Therefore, the structure of albumin must be maintained during formulations to avoid loss of affinity for FcRn and to also avoid any unnecessary recognition by other receptors (such as gp18).

FcRn receptors have the ability to bind both IgG and albumin simultaneously without any competitions [186]. This unique feature of the FcRn receptor can also be exploited to develop a novel dual-targeted system with both ligands (IgG and albumin) to further enhance the oral bioavailability.

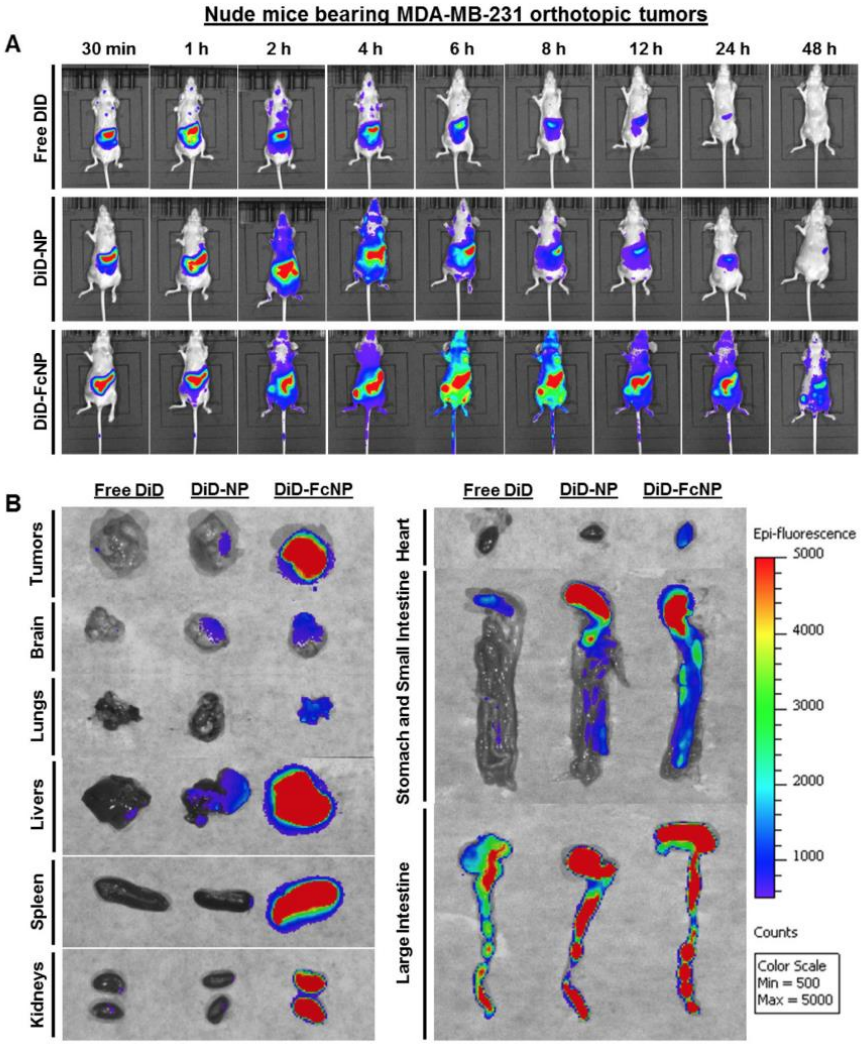

Figure 9. *In vivo real*-time tumor uptake and *ex vivo* tissue biodistribution of orally administered fluorescent NPs (DiD-NP and DiD-FcNP). (A) *In vivo* time-dependent whole body images of nude mice bearing MDA-MB-231 breast cancer cell orthotopic tumors at a dose of 0.5 mg/kg by oral gavage of free DiD and DiD-labeled NPs with or without ligand. (B) *Ex vivo* images of orthotopic tumors and various tissues from tumor-bearing nude mice that were sacrificed 6 h after oral gavage. The dose for DiD-NP and DiD-FcNP is the DiD equivalent in all experiments (n = 3 animals per group). Reproduced, with permission, from ref. [152].

II.2.1.3. Lectins

Lectins are naturally present proteins which can be derived from both plant or bacterial sources. Lectins have a strong binding affinity to the carbohydrates attached to proteins and lipids. The potential of using lectin-targeted systems arose from the abundance of glycosylated proteins and lipids found on cell membranes [187]. Naisbett and Woodley first demonstrated that tomato lectins (TLs) strongly bound to rat intestinal mucosa *in vitro* by targeting major glycoproteins that are present on the intestinal brush border [188]. Several studies have shown that lectins present a high affinity for carbohydrate residues on the glycocalyx of intestinal cells and the mucus layer.

Wheat germ agglutinin (WGA) is used to target the intestine as it specifically binds to N-acetyl-D-glucosamine and sialic acid residues present at the intestinal cell surface resulting in adhesion [189]. WGA has shown the highest binding constant to human intestinal cells and human colonocytes [190]. In addition to binding to the cell surface, WGA is also endocytosed by enterocytes via receptor-mediated endocytosis [191]. These features of WGA make it at attractive targeting ligand for the intestine. Furthermore, WGA has no oral toxicity and is resistant to the proteolytic enzymes present in the GIT [192]. Therefore, the use of WGA-grafting for the oral delivery of therapeutics has garnered considerable emphasis by formulation scientists. Gabor *et al.* demonstrated that bovine serum albumin (BSA)-WGA conjugates have a significantly higher binding affinity to the Caco-2 cell monolayer; 19-fold higher than BSA alone. The study confirmed the importance of lectin conjugation for bioadhesion [193]. WGA present on the surface of nanocarriers significantly augments the interactions with mucus and intestinal epithelial surface, thus greatly increasing the intestinal permeability. Yin *et al.* demonstrated that WGA modification of PLGA NPs resulted in significant increase in systemic uptake of thymopentin after oral administration [194]. The presence of WGA on the surface of the nanoparticles led to strong bioadhesion and augmented intestinal uptake [194, 195]. These studies emphasized that the endocytosis of WGA-NPs mainly contributed to enterocyte targeting rather than M cell targeting. In another study, WGA-decorated nanocages firmly adhered to the epithelial surface and subsequently entered into the lamina propria via goblet cells [196]. N-acetyl-D-glucosamine binding TLs has also demonstrated a high affinity for enterocytes [197, 198]. Unlike many other lectins, TL presents the advantage of being relatively non-toxic [199]. TL-modified NPs have demonstrated bioadhesion *in vivo,* and significantly higher absorption in the rat gut was observed for TL-decorated NPs compared to unmodified NPs. In the rat GIT, TL-decorated NPs mainly interacted with enterocytes [200]. Thus, both WGA and TL have specificity to enterocytes rather than to M cells and/or mucus.

II.2.1.4. Peptidic ligands

In addition, peptidic ligands that react specifically with surface receptors of enterocytes are being pursued as a potential strategy to improve intestinal drug permeability. Peptides are particularly well-suited for targeting because they are small (less than 30 amino acids on average), easy to synthesize and typically non-immunogenic. Moreover, the presence of multivalent sites of a peptide results in high specificity and avidity for the target [201]. Furthermore, the stability of peptides can be improved by using modified peptides, such N- and C-terminally blocked, using D-amino acids, and/or peptidomimetics are generally more resistant to proteolysis [202].

Integrins, heterodimeric glycoproteins, are cell receptors that are critical for binding to the extracellular matrix and other cells [203]. Arginine–glycine–aspartic acid (RGD) is one of the most commonly used ligand to target integrin receptors (e.g., $\alpha_v\beta_3$ receptor) [202]. $\alpha_v\beta_3$ is a member of the integrin family which is a transmembrane glycoprotein expressed on Caco-2 cells [204]. The FQSIYPpIK (FQS) peptide, not belonging to the RGD family, was identified as a ligand with high affinity for the $\alpha_v\beta_3$ receptor [205]. Liu *et al.* developed FQS-modified trimethyl chitosan chloride NPs for oral insulin delivery [159, 206]. FQS-modified NPs exhibited significantly accelerated intracellular uptake and transport *in vitro* due to active ligand–receptor mediation [159, 206]. FQS-modified NPs demonstrated 25-fold and 1.42-fold higher blood glucose lowering effect as compared to free insulin and unmodified NPs, respectively, in diabetic rats [159]. This newly discovered peptidic ligand has also been exploited in tumor-targeting systems recently by the same group [207, 208].

The transferrin receptor (TfR), an iron-transporting glycoprotein, can be found in the small intestinal epithelium substantially [209]. HAIYPRH (7pep) is a peptidic ligand that exhibits high affinity for the TfR. Du *et al.* developed 7pep-modified PEG-b-PCL micelles loaded with coumarin 6 (7pep-M-C6). 7pep-M-C6 exhibited higher intracellular uptake compared with the unmodified nanocarriers in Caco-2 cells and increased the *in vivo* intestinal distribution [160]. Although 7pep-modified micelles have shown efficient transcytosis across the Caco-2 cell monolayer, it should be noted that TfR receptors are found mainly on the basolateral side rather than apical side of the differentiated enterocytes, presenting a major hurdle for targeting [210], which is an important factor should be taken into account in future studies.

The number of peptides used to increase transport across the gastrointestinal tract is enormous. In the present review, we are only highlighting the most remarkable examples regarding their use as targets via drug delivery systems. The review by Sánchez-Navarro *et al.* [211] provides detailed information about peptides in oral drug delivery, as it is devoted specifically to the use of peptides for increased gastrointestinal absorption.

Table 1. Examples of active enterocyte targeting in oral drug delivery.

Ligands	Cargo	Drug delivery systems	References
Albumin	Insulin	Albumin-porous silicon NPs	[181]
	Insulin	Chitosan/albumin-alginate/dextran sulfate NPs	[185]
Biotin (Vitamin B7)	Insulin	Biotin-liposomes	[176, 177]
Fc	Exenatide	Fc-PEG-PLGA NPs	[212]
	Follicle stimulating hormone (FSH)	Fc-FSH prodrug	[213]
	Insulin	Fc-PEG-PLA NPs	[183]
	Glucagon-like peptide-1 (GLP-1)	Fc-porous silicon NPs	[214]
	GLP-1 gene	Human IgG1 (hIgG1)-Fc-Arg/pDNA complexes	[182]
	SP141	Fc-PEG-PCL NPs	[152]
Folic acid (Vitamin B9)	Cefotaxime	Folic acid-liposomes	[172]
	Docetaxel (DTX)	Folic acid-thiolated chitosan NPs	[173]
		Folic acid/bornel PLGA NPs	[215]
	Insulin	Folic acid-PEG-PLGA NPs	[216]
	Insulin	PLGA/folic acid-Chitosan NPs	[217]
	Insulin	Folic acid-liposomes	[170]
	Oxaprozin	Folic acid-PEG2k-NLCs	[218]
	Paclitaxel	Folic acid-PLGA NPs	[174]
	Paclitaxel	Folic acid-Pluronic F127-micelles	[219]
	Texas Red-Dextran 3000 (TR-dex)	Folic acid-PEO-liposomes	[169]
	Vancomycin	Folic acid-liposomes	[171]
FQSIYPpIK (FQS)	Insulin	FQS-TMC NPs	[159, 206]
HAIYPRH (7peptide)	coumarin-6 (C6)	7peptide-PEG-b-PCL micelles	[160, 220]
ICAM-1	-	ICMA-1-polystyrene nanocarriers	[221-223]
Thiamine	Ovalbumin	Thiamine-Gantrez® AN NPs	[153]
Tomato lectin (TL)	-	TL-polystyrene microspheres	[224, 225]
	-	TL-polystyrene NPs	[200]
	Insulin	TL-liposomes	[198]
Transferrin (Tf)	Insulin	Tf-Insulin prodrug	[155, 226-228]
Vitamin B12 (B_{12})	Cyclosporin A	B_{12}-polysaccharide-based polymeric micelles	[229]
	Erythropoietin (EPO)	B_{12}-EPO bioconjugates	[230]
	Granulocyte-colony stimulating factor (G-CSF)	B_{12}-G-CSF bioconjugates	[230]
	Human PYY (hPYY)	B_{12}-hPYY bioconjugates	[166]
	Insulin	B_{12}-Gel-Core-SLN	[231]
	Insulin	B_{12}-chitosan-calcium phosphate NPs	[232]
	Insulin	B_{12}-dextran NPs	[154, 163]

	Insulin	B_{12}-insulin bioconjugates	[233]
	Luteinizing-hormone-releasing hormone (LHRH)	B_{12}-LHRH bioconjugates	[167]
	Ovalbumin	B_{12}-Gantrez® AN NPs	[234]
	-	B_{12}-polystyrene NPs	[164]
Wheat germ agglutinin (WGA)	Birch pollen allergens	WGA-PLGA microspheres	[235]
	BSA	WGA-PLGA nanospheres	[236]
	BSA	WGA-BSA prodrug	[193]
	Bufalin	WGA-lipid NPs	[237, 238]
	[^{3}H]DPPC	WGA-liposomes	[239]
	Insulin	WGA-alginate microparticles	[190]
	Insulin	WGA-SLNs	[240]
	Oridonin	WGA-lipid-polymer hybrid NPs	[157]
	Paclitaxel (PTX)	WGA-SLN	[156]
	Thymopentin	WGA-PLGA NPs	[194, 195]
	-	WGA-biotin-avidin-biotin-PEG-PCL/PLA NPs	[241]
	-	WGA-PLAG microspheres	[242]
	-	WGA-Polysicences NPs	[197]
APN antibody	Protein G	APN antibody-β-glucan microparticles	[158]

II.2.2. Goblet cell targeting

Goblet cells represent ~16 % of total cells in the small intestine, and are responsible to produce, store and continuously release mucins, which are the main component of the mucus layer. They possess a protective barrier function, thereby strongly limiting the absorption of therapeutics [243]. Despite the abundance of this cell line, goblet cells are seldom used as a target. The limited targeting of these cell line can also be attributed to the presence of very few proven targeting ligands. Until now, the peptide CSKSSDYQC (CSK) has been the only proven potent ligand to target goblet cells for increasing the oral delivery of therapeutics [244-246]. Kang and co-workers were the first to identify CSK as a high affinity ligand for targeting goblet cells [247]. The authors demonstrated the ability of this ligand to increase the transport of M13 bacteriophages across the intestinal epithelium by targeting the goblet cells [247]. This study established CSK as a potential ligand for goblet cell targeting for oral formulations. Jin *et al.* developed CSK-functionalized trimethyl chitosan nanoparticles (CSK-TMC NPs) aimed at delivering insulin orally [244]. CSK-TMC enhanced the uptake of insulin across the Caco-2/HT29MTX cell monolayer model. It was also demonstrated that the nanoparticles were transcytosed via clathrin- and caveolae-mediated

endocytosis [244]. Furthermore, the presence of a mucus layer on the cell monolayers only partially hampered the uptake. Similar results were also seen in an *ex vivo* ligated ileum loop model, where FITC-labeled CSK-TMC NPs exhibited a higher fluorescence intensity in goblet cells [244]. CSK-modified NPs showed 1.5-fold higher relative bioavailability compared to unmodified NPs in diabetic rats [244]. Other studies further demonstrated that different drug delivery systems modified with the CSK peptide increased the oral bioavailability of other biomacromolecules, such as salmon calcitonin and exenatide [245, 248-250]. Gemcitabine-loaded TMC conjugates modified with the CSK peptide have been explored to improve the oral treatment of breast cancer [246]. CSK-TMC conjugates improved the cellular uptake of gemcitabine via goblet cell targeting *in vitro* in mucus producing cells. An *in vivo* efficacy study demonstrated that both drug-loaded CSK-TMC conjugates (60%) and drug-loaded TMC-NPs (54%) increased the oral bioavailability of gemcitabine over the drug solution and there was no significant difference between them [246].

Considering the positive results encountered in oral drug absorption through goblet cell targeting, it would be interesting to study a larger variety of ligands that target goblet cells. As an example, Nikitas *et al.* demonstrated *Listeria monocytogenes* possesses the ability to bind to E-cadherin which is expressed on the apical side of mucus-secreting goblet cells. Thus, the development of E-cadherin-targeting drug delivery systems could be a promising strategy for goblet cell targeting [251].

II.2.3. M cell targeting

Microfold (M) cells are specialized epithelial cells that are a part of the mucosal immune system. These cells are found primarily in the follicle-associated epithelium (FAE) of Peyer's patches or the gut-associated lymphoid tissues (GALTs) [252]. They have the ability to transport particulate matter, including antigens, bacteria and viruses [253]. Therefore, M cells act as an entrance for microorganism invasion and are involved in initiating antigen-specific mucosal immune responses and food tolerance [254]. In addition, they differ from columnar enterocytes due to their underdeveloped microvillus and glycocalyx structures and reduced levels of membrane hydrolase activity. Furthermore, a limited number of goblet cells in the FAE area results in little or no mucus, allowing for efficient uptake of particles via M cells [255]. Unlike DCs, M cells mainly transcytose antigens without passing through lysosomes, thus avoiding degradation [256]. Because of their special abovementioned features, these cells represent a highly promising target for oral drug delivery and oral immunization strategies [257, 258].

Although M cells only encompass < 1 % of intestinal cells (differing between rodents and humans), extensive research efforts have established M cells as a target for a wide array of orally administered

therapeutic agents such as peptides, proteins, nucleic acids, vaccines, etc. Various studies have demonstrated that specific M cell targeting could compensate for the limitation of the low number of M cells for oral drug delivery [138, 252, 259, 260]. Conjugating suitable M cell-targeting molecules (such as plant lectins, outer membrane bacterial and viral proteins, and monoclonal antibodies) with drug delivery systems has evolved as one of the main approaches to improve therapeutic efficacy of orally administered agents [261-264]. Some of the examples of M cell targeting are summarized in Table 2.

II.2.3.1. Lectins and lectin mimetics

Lectins are one of the most extensively studied ligands to target M cells. [265]. Lectins recognize specific carbohydrate residues present in the FAE glycocalyx, which has been exploited to develop M cell targeting systems. *Ulex europaeus agglutinin-1* (UEA-1) is one of the most used lectins for M cell targeting. It is a fucose-specific lectin with an affinity for glycoproteins having core fucα1-2Galβ located on the luminal side of M cells [266]. The conjugation of UEA-1 to the surface of liposomes, polymeric nanoparticles and microparticles has shown uptake by M cells after oral administration [267-269]. Potential proteolytic degradation, cytotoxicity and immunogenic effects limit the application of UEA-1 as a targeting agent to deliver therapeutics across the GIT. In addition, UEA-1 specifically targets M cells of mouse origin and not human origin, greatly limiting its application and clinical translation to humans. In addition to UEA-1 lectin, lectin mimetics have also been studied to target M cells [270]. The ability of the UEA-1 lectin mimetic to mediate M cell-specific delivery was tested *in situ* in a mouse gut loop model using dye-loaded polystyrene particles. UEA-1 lectin mimetics possess characteristics such as low molecular weight, stability, ease of synthesis and low cost, that make them ideal candidate ligands for M cell targeting [270]. The efficacy of UEA-1 lectin mimetics has only been demonstrated in mice; and its targeting ability in human M cells still remains unclear. Tetragalloyl-D-lysine dendrimer (TGDK), another UEA-1 lectin mimetic, was used for the targeted delivery of multiantigents, rhesus C-C chemokine receptor 5 (CCR5)-derived cyclopeptide and BSA. Its oral administration led to a significant increase in the therapeutic response against rhesus CCR5-derived cyclopeptide [271]. The specific binding affinity of TGDK for human M-like cells greatly widens its application in primates for M cell-targeted mucosal vaccines [271]. *Aleuria aurantia lectin* (AAL) is another fucose-binding lectin that has specificity for fucα1-6/3GlcNAc and targets murine M cells [266]. It includes the beneficial characteristics of UEA-1, i.e., targeting M cells, whilst eliminating the adverse toxicity with UEA-1. Roth-Walter *et al.* constructed a novel allergen oral delivery system based on PLGA microparticles conjugated with AAL [261]. An increase in vaccine absorption was also observed upon oral administration of the microparticles linked to AAL [272, 273]. Galectin 9 and sialyl Lewis

A antigen are lectins that are identified to be specific to the human FAE [138, 252]. However, until now, they have not been explored as targeting ligands to decorate drug delivery systems.

II.2.3.2. Pattern recognition receptor-mediated targeting

As mentioned above, M cells are involved in presenting and processing different types of pathogens and antigens. Therefore, the receptors that are involved in these interactions can be exploited as a strategy for M cell targeting [274]. Various pattern recognition receptors (PRRs) can be found expressed on the surface of M cells and other cells present in Peyer's patches. Toll-like receptor-4 or toll-like receptor-2 (TLR-4 or TLR-2), platelet-activating factor receptor (PAFR), and α5β1 integrin, are some of the interesting PRRs that can be used as a potential target. These receptors interact with pathogen-associated molecular patterns resulting in the translocation of bacteria across the intestinal lumen [252]. TLR-4 and TLR-2 are receptors for lipopolysaccharide (LPS) expressed by gram-negative bacteria [275-277], and for lipotechoic acids of gram-positive bacteria [278], respectively. They have binding affinity for the phosphorylcholine (PC) moiety of lipooligosaccharide (LOS) molecules present on bacterial surface [279], and α5β1 integrin has the affinity to bind to fibronectin-binding proteins expressed by many bacteria (such as *Yersinia*) [280]. *Salmonella enteritidis*-derived flagellin and monophosphoryl lipid A are TLR ligands have been used to modify the nanoparticle surface to target M cells [257, 281]. *Yersinia* interacts with invasin and the α5β1 integrins overexpressed on the apical surface of human M cells, resulting in internalization by M cells. As mentioned previously, RGD sequence (Section 2.1.4) has high affinity towards integrin receptors. Garinot *et al.* demonstrated that the grafting of RGD to the surface of PEGylated PLGA-based NPs, encapsulating ovalbumin, significantly improved the transport of NPs across a human M-like cell model *in vitro*. A slight improvement in the IgG immune response was also observed after oral immunization with RGD modified nanoparticles *in vivo* [282]. Lee *et al.* described the use of β-Glucan-functionalized glycine-arginine-glycine-aspartic acid-serine (GRGDS) carrier to oral delivery of PR8 (an inactivated antigen of influenza A). The resultant M cell-targeted β-glucan-GRGDS/PR8 carrier showed highest amount of Immunoglobulin A (IgA) antibody in the blood serum and mucus 21 days after the first oral dose [283]. RGD peptidomimetics (RGDps) have also been used as targeting ligands to functionalize nanoparticles [284]. Significant improvement in the transport of NPs *in vitro* across an M cell-like human model was observed after RGDp modification of nanoparticles. Furthermore, similar improved therapeutic efficacy was also observed in *in vivo* studies with RGD-NPs as compared to unmodified nanoparticles [284].

II.2.3.3. Glycoprotein 2-mediated targeting

Glycoprotein 2 (GP2) is a highly expressed transcytotic receptor found on the luminal membrane of M cells. It recognizes FimH which is a major component of the type I pili on the bacterial outer membrane of a subset of gram-negative enteric bacilli. *Escherichia coli*, *Salmonella enterica serovar Typhimurium* (S. Typhimurium) are some examples of $FimH^+$ bacteria [254, 285]. Shima *et al.* constructed a fusion protein, anti-GP2-streptavidin (SA), to deliver antigents. In the fusion protein, which comprised an antibody against GP2 fused with SA, to which biotinylated ovalbumin peptide (bOVA) was conjugated. The specific binding of anti-GP2-SA to M cells was demonstrated by an immunofluorescence study. Moreover, significant induction of OVA-specific fecal IgA secretion was observed after oral administration of the fusion protein in mice [286]. This study further presented anti-GP2-SA as an efficient mucosal vaccine by enduring the harsh gastrointestinal environment and efficiently delivering the antigen to M cells [286]. Matsumura *et al.* explored serotype A1 L-progenitor toxin complexes (L-PTCs), consisting of botulinum neurotoxin, non-toxic non-hemagglutinin and hemagglutinin (HA). HA interacts and bids to GP2, thus resulting in opening of the intestinal barrier (Fig. 10) [287]. Fig. 10A compares the interaction between HA and different binding molecules expressed on M cells that could be possible receptors for the toxin complexes. HA strongly binds to both mouse and human GP2 (mGP2 and hGP2). The extent interaction of HA with uromodulin is to a lesser amount, and to mouse cellular prion protein (PrP^C) is not strong. Furthermore, significant co-localization of L-PTCs with GP2 was also observed in M cells in intestinal loop assays (Fig. 10B). The involvement of GP2 receptors was proven as significantly less susceptible in GP2-deficient ($Gp2^{-/-}$) compared to wild-type ($Gp2^{+/+}$) mice, after oral administration of L-PTCs [287]. In addition to exploiting new GP2 ligands, researchers have also observed that the number of functional $GP2^+$ M cells can be significantly increased by systemic administration of a receptor activator of nuclear factor (NF)-κB ligand [288]. This increase in functional $GP2^+$ M cells could then induce mucosal and humoral immune responses upon oral delivery of the antigen via M cell-targeted delivery systems [288].

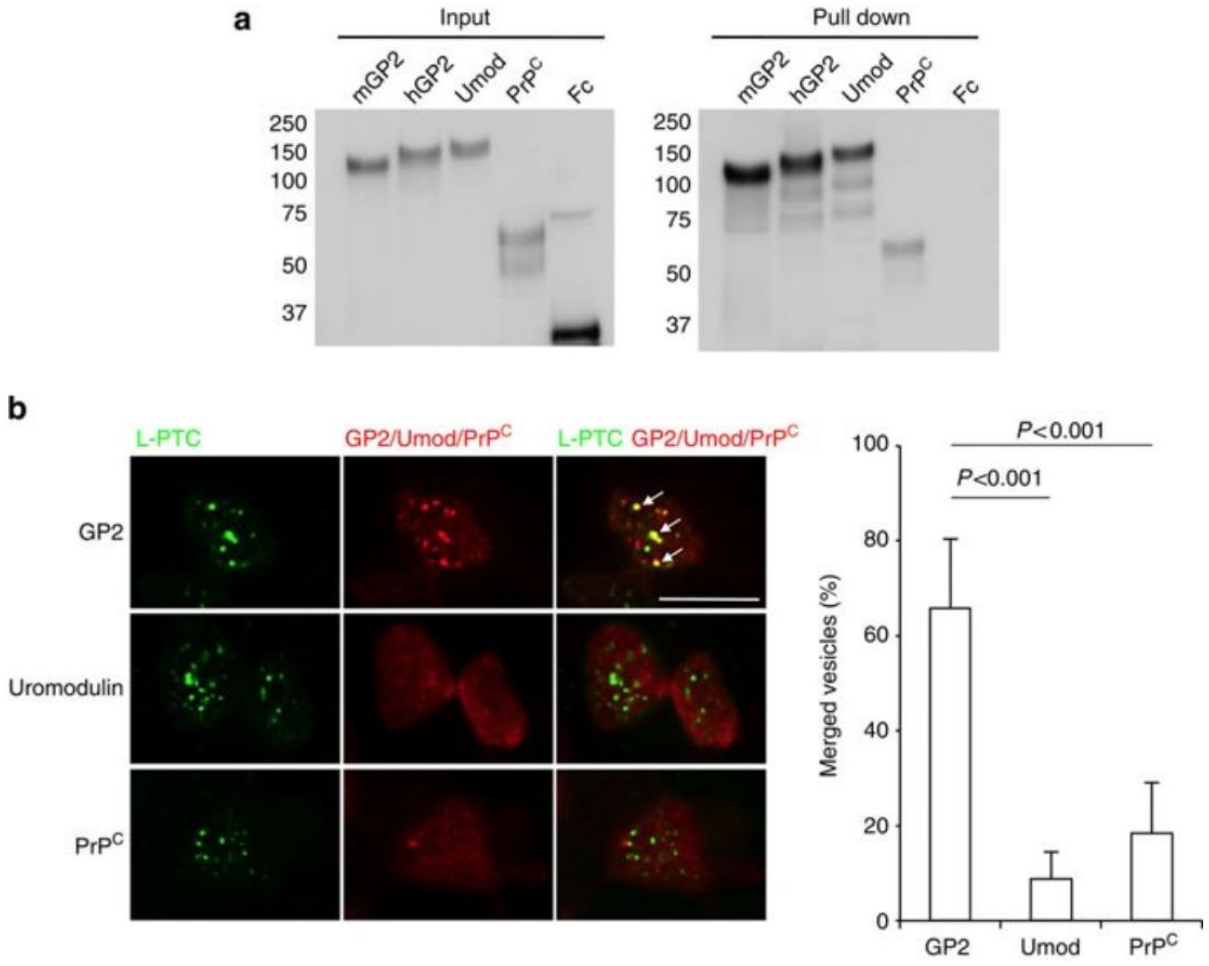

Figure 10. GP2 serves as a primary endocytotic receptor for the uptake of L-PTC in M cells. (a) HA pull-down assay. Recombinant Fc proteins, including mouse GP2-Fc (mGP2-Fc), human GP2-Fc (hGP2-Fc), mouse uromodulin-Fc (Umod-Fc), and mouse PrP^C-Fc, were incubated with Strep-Tactin Superflow agarose prebound to fully assemble HA (HA1/HA2/HA3). The HA-bound proteins were analyzed by immunoblotting using horseradish peroxidase (HRP)-labeled anti-human IgG antibody. (b) Colocalization of fluorescent L-PTC (green) and GP2, uromodulin and PrP^C (red) in M cells in *in situ* ligated intestinal loop studies. L-PTC predominantly colocalized with GP2 in M cells (arrows). Scale bar, 10 mm. Reproduced, with permission, from ref. [287].

II.2.3.4. Claudin 4-mediated targeting

Claudin 4, a transmembrane protein, is located in tight junctions between intestinal cells as well as on the surface of M cells (human and murine origin). In M cells, claudin 4 acts as a receptor for *Clostridium perfringens* enterotoxin (CPE) by directly binding to its C-terminal 30 amino acids (CPE30) [263, 289, 290]. Ye *et al.* designed CPE30 peptide modified chitosan-based delivery systems (CPE30-CS-pVP1) for M cell targeting. The CPE30-CS-pVP1 vaccine demonstrated *in vivo* oral efficacy after four immunization doses by significantly increasing the specific fecal SIgA levels and augmenting the mucosal T cell immune [291]. Exploiting tight junction proteins to deliver bioactive compounds to the FAE through M cells has also been exploited in other studies [263, 289]. Ling *et al.* demonstrated that conjugating CPE30 to the C terminus of influenza hemagglutinin (HA) does not significantly change its binding ability to Claudin 4 [289]. Thejani *et al.* produced

submicron size PLGA particles to incorporate this recombinant protein (HA-CPE) to protect against the harsh gastrointestinal conditions while largely retaining its targeting function. Besides, the targeted systems also showed a considerable increase in the uptake of HA-CPE in Peyer's patches *in vivo* after oral administration [263].

II.2.3.5. Complement C5a receptor-mediated targeting

The complement C5a receptor (C5aR) can be found on the apical surface of human M-like cells and this receptor plays a major role during antigen uptake [292]. The outer membrane protein H (OmpH) of *Yersinia enterocolitica* is one of the identified ligands of C5aR. Kim and co-workers used this ligand to modify the envelope domain III (EDIII) (EDIII-OmpH), the pathogenic antigen of the dengue virus, to develop M cell-targeted antigen delivering systems. An enhancement in the EDIII-specific mucosal and systemic immune responses without systemic tolerance against EDIII was observed after orally administering EDIII-OmpH [293], demonstrating it is an effective oral mucosal vaccine against DENV infection. Co1 is another C5aR-targeting peptidic ligand [294], which was used by the same group to successfully deliver EDIII antigen into Peyer's patches and demonstrate virus-neutralizing activity after its oral administration [295].

II.2.3.6. Peptidic ligands

A number of peptide sequences have been discovered that interact with unknown ligands present on the M cells' surface. For instance, CTGKSC and LRVG are two peptides that showed enhanced phage transport across M-like cells. LRVG and CTGKSC peptide decorated PEGylated PLGA-based nanoparticles exhibited an eight- and four-fold increase in their transport in *in vitro* coculture cell lines (M cell-like model [253]), respectively, when compared to nonmodified nanoparticles [296]. Another M cell-homing peptide (CKSTHPLSC (CKS9)) was reported by Cho *et al.*, which was selected for grafting onto chitosan nanoparticles (CSK9-CNs). CKS9-CNs demonstrated increased *in vitro* transport and accumulation in PP regions in *ex vivo* closed ileal loop assay [297]. Moreover, CKS9-water-soluble chitosan (WSC)-PLGA microparticles were developed encapsulating a membrane protein B of *Brachyspira hyodysenteriae* (BmpB) (BmpB-CKS9-WSC-PLGA MPs). After oral administration, the microparticles were able to elevate the systemic IgG antibody response, which was accredited to M cell targeting [298]. Li *et al.* reported a novel mucosal adjuvant, IL-6-CKS9, which is a recombinant cytokine. IL-6-CKS9 is formed by chemically crosslinking CKS9 with the murine interleukin 6 (IL-6). In this study, a recombinant lactic acid bacterium secreting IL-6-CKS9 was orally administered with a CKS9-conjugated antigen protein to BALB/c mice for mucosal immunization. After oral administration, an increase in the oral bioavailability was observed along with increase in the induction of immune responses (Th1- and

Th2-type) [299]. Thus, such hybrid delivery system represents an interesting approach to achieve efficient oral vaccine delivery.

II.2.3.7. Antibodies

The use of M cell specific antibodies has also been utilized in developing targeted systems. In addition to anti-GP2-SA (as previously described), NKM 16-2-4 mAb is another monoclonal antibody against M cells. Nochi *et al.* demonstrated the use of NKM 16-2-4 mAb for M cell-targeted oral mucosal vaccines delivery. When NKM 16-2-4 was conjugated to tetanus toxoid (TT) or botulinum toxoid (BT) and administered together with an adjuvant, the resultant oral vaccination resulted in the induction of high-level, antigen-specific serum and mucosal immunoglobulin responses [300]. Another interesting study was performed by Rochereau *et al.* where they demonstrated M cell targeting ability of secretory IgA (SIgA) via the dectin-1 receptor. The interaction resulted in the transportation of SIgA in the GALT [301]. This specific targeting approach was explored for oral delivery of p24HIV antigen, by forming a complex with the SIgA (p24-SIgA). Oral administration of this complex resulted in the induction of immunity, humoral and cellular, against p24 at both systemic and mucosal levels [302].

II.2.3.8. Other ligands

In addition to the abovementioned commonly used ligands for M cell targeting, several studies have found other novel ligands that present specific binding affinity to M cells. A novel ligand was discovered from the outer capsid protein of retrovirus, protein σ1 (pσ1). Pσ1 has the ability to specifically interact with the α-(2, 3)-linked sialic acid-containing glycoconjugates present on the luminal side of the M cells [303-305]. However, no further studies have exploited it as a ligand to orally deliver antigens and/or drugs into Peyer's patches.

Caveolin-1 is a scaffolding protein which is a main part of caveolae expressed on M cells. A possible role of caveolin-1 in enhancing the susceptibility of M cells towards *Salmonella* infection has been identified *in vitro* in cocultured intestinal cell lines (Caco-2 and Raji B cell cocultures) [306]. Thus, suggesting that caveolin-1 is involved in the M cell-mediated entrance of microbial pathogens, and establishing it as a promising new approach for achieving mucosal immunity via M cell targeting.

Table 2. Examples of different ligands used in active M cell targeting for oral drug delivery.

Ligands	Cargo	Drug delivery systems	References
Aleuria aurantia lectin (AAL)	S-91 cell antigens	AAL-albumin microparticles	[272]
	Birch pollen allergens	AAL-PLGA microspheres	[261]
	ID 8 cell lysate	AAL-microparticles	[307]
	Mycobacterium tuberculosis antigens	AAL-microspheres	[273]
	Melanoma vaccine	AAL-microparticles	[272]
	Whole cell lysate (WCL)	AAL-microparticles	[308]
	-	AAL-microparticles	[309]
Arachis hypogaea (PNA, lectin)	Hepatitis B surface antigen (HBsAg)	PNA-PLGA NPs	[310]
Cholera toxin B subunit (CTB)	Hepatitis B surface antigen (HBsAg)	CTB-bilosomes	[311]
CKSTHPLSC (CKS9)	Brachyspira hyodysenteriae (BmpB)	CKS9-chitosan-PLGA microparticles	[298]
	Interleukin 6 (IL-6)	IL-6-CKS9	[299]
	-	CKS9-chitosan NPs	[297]
Clostridium perfringens enterotoxin (CPE 30)	Coxsackievirus B3 predominant antigen VP1 (pVP1)	CPE30-chitisan-pVP1 NPs	[291]
	Recombinant influenza hemagglutinin (HA)	CPE 30-PLGA sub-micron particles	[263]
	Recombinant influenza hemagglutinin (HA)	CPE30-HA	[290]
Col	Envelope domain III (EDIII)	EDIII-Col complex	[295]
	Core neutralizing epitope (COE)	pPG-COE-Col-DCpep/L393 (*Lactobacillus casei* 393)	[312]
CTGKSC	-	CTGKSC-PEG-PCL/PLGA NPs	[296]
GRGDS	PR8 antigen	β-Glucan-GRGDS NPs	[283]
Glycoprotein 2 (GP2) Ligands	Biotinylated ovalbumin peptide (bOVA)	Anti-GP2-streptavidin-bOVA	[286]
LDV peptidomimetic (LDVp)	Ovalbumin (OVA)	LDVp-PEG-PCL/PLGA NPs	[284]
LRVG	-	LRVG-PEG-PCL/PLGA NPs	[296]
Neuraminidase (NA)	Allergen	NA-PLGA microparticles	[313]
mAb NKM 16-2-4	tetanus toxoid (TT)/ botulinum toxoid (BT)	mAb NKM 16-2-4-TT/BT	[300]
Outer membrane protein H (OmpH)	Envelope domain III (EDIII)	EDIII-OmpH complex	[293]
RGD peptide	Antigen heat shock protein 65-6×P277 (H6P)	RGD- and mannose-modified chitosan (RMCS) NPs	[314]
	Doxorubicin	RGD-conjugated triglyceride-based nanocarrier	[315]
	Ovalbumin (OVA)	RGD-PEG-PCL/PLGA NPs	[282]
	-	RGD- polystyrene particles	[316]
RGD peptidomimetic (RGDp)	Ovalbumin (OVA)	RGDp-PEG-PCL/PLGA NPs	[284]
Salmonella enteritidis extract (SE)	Fluorescein isothiocyanate (FITC)	SE-Gantrez® AN NPs	[317]
Salmonella Enteritidis derived flagellin	Ovalbumin (OVA)	Flagellin-Gantrez® AN NPs	[281]

Secretory IgA (SIgA)	p24HIV antigen	p24-SIgA complexes	[302]
Tetragalloyl-D-lysine dendrimer (TGDK)	rhesus CCR5-derived cyclopeptide/BSA	TGDK-cyclopeptide	[271]
Ulex europaeus agglutinin-1 (UEA-1)	Bovine serum albumin, (BSA)	UEA-1-chitosan NPs	[267]
	BSA	UEA-1- polystyrene microspheres	[318]
	Dextran rhodamine	UEA-1-polymerised liposome	[269]
	HIV peptides	UEA-1-PLG microparticle	[268]
	H. pylori or C. jejuni	UEA-1-agglutinated H. pylori or C. jejuni	[319]
	Ovalbumin (OVA)	UEA-1-PLGA-lipid NPs	[320]
	Hepatitis B surface antigen (HBsAg)	UEA-1-liposomes	[321]
	Hepatitis B surface antigen (HBsAg)	UEA-1-PLGA NPs	[322]
	-	UEA-1-microspheres	[323]
UEA-1 mimetics	-	UEA-1 mimetics- polystyrene particles	[270]
Wheat germ agglutinin (WGA)	Thymopentin	WGA-PLGA NPs	[194]

II.2.4. Dendritic cell targeting

Dendritic cells (DCs), also known as antigen-presenting cells (APCs), play a key role in protective immunity against pathogens by synchronizing the innate and adaptive immunity. Furthermore, they are also responsible for maintaining the commensal flora's tolerance against bacteria, food antigens and self-antigens [324]. These cells have the ability to accurately recognize different signals in their surrounding area and responding to these signals to induce appropriate immune response. Intestinal DCs are a small subset that includes a large network of the intestinal immune system. These cells are found distributed throughout the gut, including the lamina propria of the intestine, Peyer's patches and the mesenteric lymph node [325]. As shown in Fig. 11A, DC's underlying M cells can extend their dendrites between the intestinal cells to recognize the antigens that are present in the intestinal lumen. The highly efficient interaction of DC surface receptors and administered vaccines has opened the possibility of using DCs to enhance the immunogenicity of oral vaccines [314, 326]. A number of receptors are present on the DCs' surface (Fig. 11B) that are involved in the interaction and transport of foreign molecules and the induction of immune responses. TLRs, FcRs, C-type lectins (CLRs), mannose, integrins and scavenger receptors are some of the receptors that are present on the surface of DCs [327]. Mannose receptors are well-known receptors expressed on DCs that offer the potential to target vaccines to DCs. However, depending on the specific receptor being targeted, the outcome of the resulting immune response may vary. Moreover, the expression of these receptors can vary between their location and DC subsets, as

each of the subsets have their unique patterns for antigen recognition, capture, processing and presentation. For example, TLRs are distributed on the cell membrane surface [328], enabling them to deliver a strong activating signal to DCs, which forms the basis for their potent adjuvant properties. CLRs are key receptors for stimulating intracellular signaling cascades. They facilitate receptor-mediated endocytosis by binding to carbohydrate ligands [329]. FcγR is a receptor that binds to the Fc portion of IgG antibodies. These receptors are involved in several immunological processes such as antigen presenting, DC maturation and activation of natural effector cells [330].

Owing to such interesting properties of DCs, the targeting of these cells has been widely studied for systemically delivered vaccines. The targeting of DCs is achieved by conjugating the appropriate antigen to a specific antibody or ligands, and with immunostimulatory adjuvants. This method is not suitable for the oral administration of vaccines since antigens are susceptibly degraded in harsh gastrointestinal milieu. Thus, developing delivery systems for vaccines is crucial; as such, delivery systems can improve immune responses by enhancing the endocytosis of antigen-loaded carriers. However, there are only a handful of studies (as mentioned Table 3) that have developed proper systems to specifically target the intestinal DC subsets.

Recently, antibodies or ligands have been used to decorate the surface of oral vaccine delivery systems aimed towards DC targeting (Table 3). Beart *et al.* reported DC-targeting functional β-glucan microparticles (GPs) decorated with anti-aminopeptidase N (APN)-specific antibodies for the oral delivery of the antigen FedF (the tipadhesin of F18 fimbriae) [331]. APN is an epithelial receptor present on different cells, such as intestinal enterocytes and DCs. *In vitro* studies in DCs demonstrated more efficient internalization and DC maturation induction. Such promising effect of the modified GPs was attributed to the combined effects of APN targeting, FcγR employment and β-glucan recognition [331]. In addition, this study further evaluated intestinal DC targeting *in vivo* in piglets. After oral administration, APN-targeted FedF-GPs prompted a significantly higher systemic antibody response against FedF as compared to control microparticles [331]. Thus, the APN-targeted FedF-GPs were internalized by intestinal cells and induced DC maturation. Both of these activities are critical is stimulating the desired protective immunity.

Mannose has been used as a ligand for the enhanced targeting of vaccines to intestinal DCs. The mannose receptor (MR/CD206) can be found highly expressed on DCs [332], and has eight C-type carbohydrate recognition domains (CRDs) per polypeptide [333]. The receptor recognizes mannose presenting antigens and mediates its endocytosis, processing and presentation [334]. Chen *et al.* employed mannose as a ligand to develop DC targeting chitosan (CS) NPs, encapsulating the antigen heat shock protein 65-6×P277 (H6P) [314]. The mannose-decorated nanoparticles

demonstrated higher uptake in DCs in the Peyer's patches. Furthermore, oral vaccination with mannose decorated antigen containing nanoparticles induced antigen-specific T cell tolerance and completely inhibited the induction of diabetes in NOD mice [314].

Lactobacillus strains are also used for the development of mucosal vaccines aimed at efficient oral antigen delivery [335, 336]. However, the number of antigens reaching the targeted immune sites via these recombinant systems is very limited. A DC-targeting peptide modified oral recombinant *Lactobacillus casei* 393 vaccine was reported to orally deliver the core neutralizing epitope against porcine epidemic diarrhea virus (PEDV) [312]. The DC-targeted recombinant lactobacillus strain was able to induce anti-PEDV mucosal, humoral, and cellular immune responses. The effective induction of immune response achieved was because *Lactobacillus casei* 393 is resistant to the harsh gastrointestinal milieu and has the ability to form colonies in the GIT of swine and mice [337]. The promising results from this study unveiled new opportunities for PEDV vaccine development.

Although antigen-loaded drug delivery systems modified towards DC targeting have significantly increased the endocytosis of antigens by specifically interacting with DC subsets, most studies of intestinal DCs are targeted to only one DC subset. Therefore, one feasible strategy would be combinatorial targeting of multiple DC subsets at the same time. A deep understanding of the uptake mechanism after targeting multiple intestinal DC subsets is required to elucidate whether this method could be more efficient than the current oral vaccines.

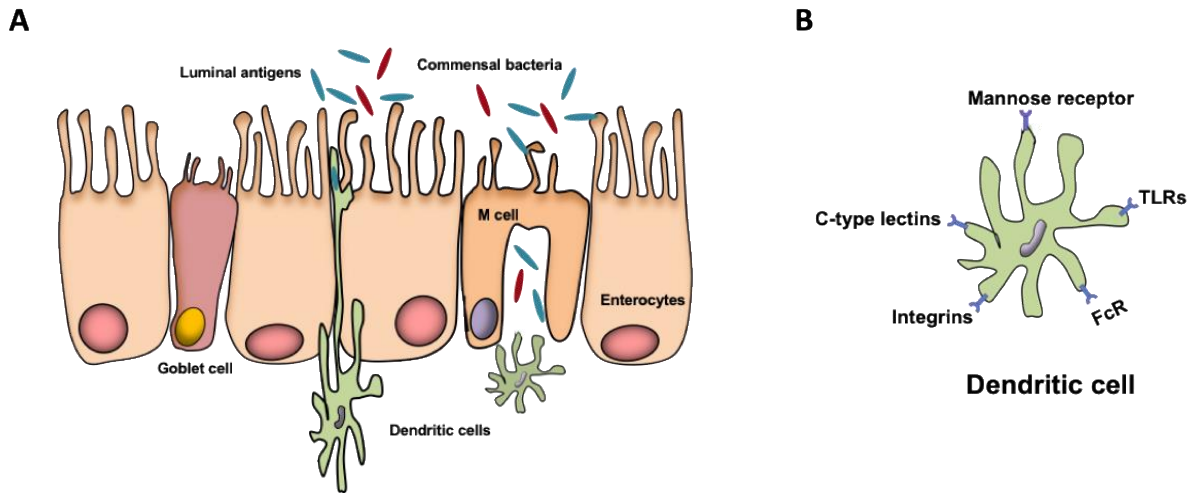

Figure 11. (A) Schematic representation of the uptake mechanisms of luminal antigens by DCs. (B) Examples of well-known receptors on DCs, which may be potential targeting receptors for oral delivery.

Table 3. Examples of preclinical studies of active dendritic cell-targeting strategies in oral drug delivery.

Ligands	Cargo	Drug delivery systems	References
Antibody (mAB) IMM013	Antigen FedF	Anti-aminopeptidase N(APN) FedF-β-glucan microparticles (GPs)	[331]
DCpep	Core neutralizing epitope (COE)	pPG-COE-Col-DCpep/L393 (*Lactobacillus casei* 393)	[312]
Mannose	Antigen heat shock protein 65-6×P277 (H6P)	RGD- and mannose-modified chitosan (RMCS) NPs	[314]
	Ovalbumin (OVA)	Mannosylated PLGA NPs	[284]
	Ovalbumin (OVA)	Mannosylated Gantrez® AN NPs	[281]
	Rhodamine B isothiocyanate (RBITC)	Mannosylated Gantrez® AN NPs	[338]

II.2.5. Enteroendocrine cell targeting

Enteroendocrine cells (EECs) are found interspersed throughout the whole GIT, from the stomach to the rectum, and account for approximately 1 % of the total intestinal cells [339]. EECs constitute the largest endocrine system in the human body; more than twenty different hormones secreted from 12 types of intestinal EECs. These enteroendocrine hormones moderate their effects via auto-, neuro-, and paracrine mechanisms [340, 341]. Gut hormones physiologically regulate multiple biological effects, such as food intake, gastric emptying, intestinal motility, glucose metabolism and intestinal barrier function [138].

L and K cells are the most interesting enteroendocrine cells. They are responsible for secreting different gut hormones that have been proven therapeutic efficacy in different prevalent diseases such as inflammatory bowel disease (IBD) and T2DM. The gut hormones produced by K and L cells and their physiological functions are summarized in Table 4. GLP-1 and GIP have established roles in gastrointestinal function, glucose homeostasis and satiety. Owing to their insulinotropic effects, GIP and GLP-1 are responsible for more than a half of the total insulin secretion after oral administration of glucose, which is also known as *"the incretin effect"* [342]. These incretin peptides have an extremely short half-life because of its rapid degradation by the DPP-IV within the first few minutes [95]. Until now, there are two classes of drugs based on the incretin effect that are available on the market for the treatment of obesity/T2DM, including orally administrated DPP-IV inhibitors (e.g., linagliptin, saxagliptin and sitagliptin) and GLP-1 mimetics (e.g., liraglutide, semaglutide and exenatide). In contrast, GIP demonstrated lower therapeutic effectiveness in T2DM treatment than GLP-1. GLP-2 and peptide YY (PYY) are also released from intestinal L cells after ingestion of nutrients, including carbohydrates and fat. Intestinal GLP-2 is co-secreted along with GLP-1, stimulating cell proliferation and inhibiting apoptosis. PYY reduces food intake

and induces food aversion, and in combination with GLP-1, it plays a major role in the ileal brake, a physiological mechanism that delays intestinal motility and transit to enhance nutrient (mainly lipid) absorption [339]. Targeting L and/or K cells, therefore, represents an exciting new therapeutic opportunity in some major gastrointestinal diseases.

The apical membrane of enteroendocrine L and K cells expresses a wide variety of receptors and transporters (Fig. 12). They could be activated by different dietary nutrients (e.g., carbohydrates, proteins, and lipids) and regulate the gut hormones release from EECs. Some G protein-coupled receptors (GPCRs) are lipid-sensing receptors, such as free fatty acid receptors (FFAR) 1-4. The FFAR2 (GPR43) and FFAR3 (GPR41) are highly expressed in the colonic L cells and activated by short-chain fatty acids (including acetate (C2), propionate (C3), and butyrate (C4)), thereby stimulating GLP-1 secretion into blood circulation [343]. FFAR1 (GPR40) and FFAR4 (GPR120) are responsive to medium- chain fatty acids (C7 to C12) and long-chain fatty acids (C12 to C18) [344]. GPR119 is a lipid derivative receptor activated by fatty acid amide (e.g., oleoylethanolamide). This receptor is involved in regulating the secretion of GLP-1, GLP-2 and PYY [345]. EECs also respond to luminal stimuli other than nutrients, such as bile acids. The G protein–coupled bile acid receptor GPBAR1 (TGR5) is highly expressed in distal gut L cells [346]. Apart from these GPCR sensors, some nutrient transporters, including the sodium-coupled glucose transporter SGLT1 and peptide transporter PEPT1 also participate in the modulation of gut hormone secretion from EECs.

To our knowledge, to date, only a few studies have illustrated enteroendocrine cell targeting in oral drug delivery, which were all reported by the same research group [347, 348]. Rather than grafting one of these certain ligands on the surface of a drug delivery system, Beloqui *et al.* first introduced the concept that lipid-based nanoparticles act as endogenous ligands stimulating higher GLP-1 release via lipid-sensing pathways in L cells. The researchers quantified the abilities of different lipid-based nanoparticles on triggering GLP-1 secretion *in vitro* in L cells. Among the different tested lipid-based and polymeric nanoparticles, nanostructured lipid carrier (NLC) was the only one that could significantly trigger more endogenous GLP-1 from murine and human L cells, owing to its interaction with the receptors GPR41 and GPR84 on the surface of L cells [347]. This study describes a potential novel targeting strategy by exploiting the inherent targeting potential of nanocarriers without external ligand modification. Two GLP-1 analogs (exenatide and liraglutide) were encapsulated within these NLCs to provide a dual-action nanocarrier in which the nanocarriers would induce endogenous GLP-1 secretion while providing increased systemic absorption of the encapsulated GLP-1 analog. However, NLCs mainly adhered to the mucus layer and did not come in close proximity to L cells and failed to trigger GLP-1 secretion *in vivo* [348]. Thus, in future studies, to successfully exploit the biological effect of nanocarriers, we need more

efforts on the development of nanosystems that could trigger endogenous GLP-1 secretion from L cells *in vivo* not just *in vitro*. We believe that it would be interesting to develop a nanosystem with biological effect since it could both encapsulate GLP-1 analogs, acting as drug carries, and serve as an endogenous ligand to induce endogenous GLP-1 secretion for the treatment of T2DM via oral route.

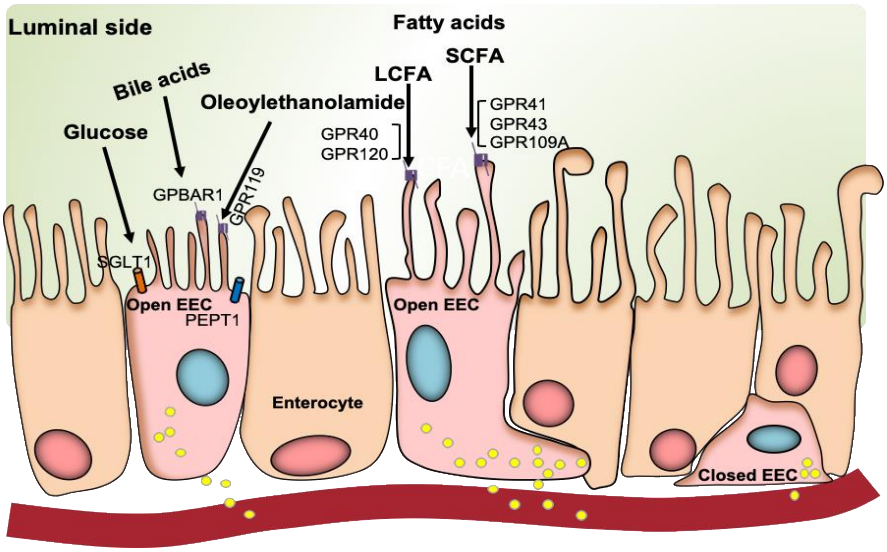

Figure 12. Graphic overview of the morphology of enteroendocrine cells (EECs) and the well-known receptors on the surface of open EECs. The open type EECs are covered by microvilli and directly reach the luminal surface, whereas the closed type EECs lack microvilli and are located close to the basal side in epithelia, not reaching the gut lumen. Open EECs (such as enteroendocrine L cells and K cells) express various nutrient-sensing proteins, including G protein-coupled receptors (GPCRs), such as GPR40, GPR41, GPR43, GPR119, GPR120, and nutrient transporters (SGLT1 and PEPT1). They are activated by different dietary nutrients in the intestinal lumen (e.g., carbohydrates, proteins, and lipids), and thus modulate the secretion of EECs-hormones.

Table 4. Summary of gut hormones secreted by enteroendocrine L cells and K cells and their physiological functions.

Hormones	Site of secretion	Physiological functions	Physiopathological situations
GIP	K cells; proximal small intestine	Induces insulin secretion; inhibits GI motility, secretion of gastric acid and apoptosis of the pancreatic beta cells; reduces lipoprotein lipase activity in adipose tissue.	T2DM; Alzheimer's disease
GLP-1	L cells; duodenum, jejunum, distal ileum, colon	Incretin effect on insulin secretion; promotes pancreatic β cells proliferation and neogenesis; delays gastric emptying; postprandial satiety; inhibits energy intake	Obesity; T2DM; Insulin resistance
GLP-2	L cells; distal small intestine, colon	Stimulates cell proliferation and inhibits apoptosis; promotes mucosal repair; reduces inflammatory responses to bowel injury; maintains the mucosal permeability barrier	Inflammatory bowel diseases (Crohn's disease); short bowel syndrome; osteoporosis
PYY	L cells; ileum, colon	Slows gastric emptying and inhibits intestinal motility; inhibits gastric acid secretion and pancreatic exocrine function; suppresses appetite; stimulates mucosal enterocytes proliferation	Obesity
Oxyntomodulin	L cells; colon	Inhibits gastric acid; reduces gastric mobility; suppresses appetites	Obesity

Although EEC targeting in oral delivery is still in its infancy, the studies published so far on L cell targeting have demonstrated great potential for the treatment of T2DM. Future research will not only explore other alternative L cell-targeting approaches but also develop other EEC-targeting (e.g., K cells) strategies for oral therapy. Moreover, as potential pharmacotherapies in the future, multiple agonists (e.g., GIP/GLP-1 co-agonist, glucagon/GLP-1 co-agonist and GIP/GLP-1/glucogon tri-agonists) and combination therapy of PYY/GLP-1 oxyntomodulin are being investigated and show more effectiveness than GLP-1 alone [349, 350]. Thus, a gut hormone agonist-loaded oral drug delivery system with multiple ligands targeting GPCRs and/or nutrient transporters on L/K cells simulating at least two types of functional hormones secreted in the intestine would be a new vista in the EEC-targeting field for a better clinical therapeutic effect.

II.2.6. Paneth cell targeting

Paneth cells are highly specialized cells that are strongly involved in assisting in the maintenance of the microbiome and can be found at the foot of the crypts of small intestinal villi. Moreover, they are also responsible for establishing stem cell niche, promoting cell renewal and mucosal morphogenesis [149]. Paneth cells have a turnover of around 60 days [351]; they might be a good target compared with enterocytes that only survive several days. The biological injuries of Paneth cells are closely associated with the pathogenesis of different intestinal diseases [149] such as Crohn's disease and enterocolitis [352]. Wang *et al.* recently identified improved Paneth cell function through activation of the interleukin 22 (IL-22)/phosphorylated Stat3 (p-Stat3) pathway. This study indicated that the IL-22/Stat3 pathway could be a potential target for repairing the total parenteral nutrition-related intestinal barrier damages [353]. Furthermore, Paneth cells are also associated with the release of growth factors and antimicrobial peptides, thus playing a vital function in protecting the small intestine [354]. Toll-like receptor 9 (TLR9), expressed on the surface of Paneth cells, recognizes bacterial DNA containing the sequences of unmethylated cytidine–phosphate–guanosine (CpG) dinucleotides. A study has demonstrated that the oral delivery of oligonucleotides containing a CpG sequence (CpG-ODNs) led to a rapid Paneth cell degranulation. Moreover, mice pre-treated with CpG-ODNs increased their resistance to oral challenges with virulent *Salmonella typhimurium* [355]. Rumio *et al.* further investigated the different TLRs expressed on Paneth cells by orally administering TLR agonists. This study demonstrated that in addition to the CpG-ODNs (TLR9 agonist), the TLR3 agonist of polyinosinic-polycytidylic acid also produced a rapid Paneth cell degranulation [356]. TLR9 and TLR3 agonist-induced Paneth cell degranulation provides with a pathway towards preventing infection and treating inflammatory bowel diseases. Unfortunately, there is no specific example that has applied for the

treatment of related diseases by using Paneth cell targeting since the mechanisms underlying Paneth cell function remain unknown.

II.3. TRANSPORTER TARGETING

One possibility towards an increased drug delivery system-cell interaction is the targeting of the transporters expressed on the intestinal epithelial cell surface. Membrane transporters expressed across the GIT are classified into adenosine triphosphate (ATP)-binding cassette (ABC) transporters and the solute carrier (SLC) transporter superfamily [357]. ABC transporters mainly include the efflux transporters (e.g., P-glycoprotein and multidrug resistance-associated protein 2) that block the absorption of drug molecules [358]. In contrast, SLC transporters are involved in vital physiological activity (e.g., nutrient absorption) and the uptake and transport of several important drug molecules (such as acyclovir, saquinavir and docetaxel) [359]. The majority of SLC transporters (especially influx/uptake transporters) are expressed on the intestinal epithelia (both on the apical and basolateral sides), thus making it an attractive targeting site to improve the oral bioavailability of drugs. These transporters mainly include the oligopeptide transporter PEPT1 [360, 361], the apical sodium-dependent bile acid transporter (ASBT) [362], the sodium-dependent vitamin transporter (SVCT1), the sodium-dependent multivitamin transporter (SMVT), the monocarboxylate transporter (MCT1) [363, 364], amino acid transporters (LAT1 [365, 366] and $ATB^{0,+}$ [367, 368]), and the organic cation/carnitine transporter (OCTN2). The in-depth understanding of the substrate specificity of these transporters allows for the modification of drug moieties (prodrugs) or the surface of the nanocarrier to achieve optimal intestinal targeting and an enhanced therapeutic outcome. This part of the review will provide with an overview of the targeting strategies selective for specific transporters. Examples are shown in Table 5.

II.3.1. Oligopeptide transporter PEPT1

PEPT1 is a low-affinity and high-capacity transporter with the unique ability to not easily be saturated by a high concentration of orally administered drugs in the GI tract. Owing to this peculiar function, PEPT1 has captured the attention of the oral drug delivery community. PEPT1 is expressed on the apical membrane of the small intestine (predominantly in duodenum), and only limited or not expressed in the normal colon [369, 370]. PEPT1 is involved in the absorption of nitrogen as well as in the transport of peptide-like therapeutic agents, such as angiotensin-converting enzyme inhibitors, β-lactam antibiotics, and bestatin [371]. Considering its extensive substrate specificity, the targeting to the PEPT1 transporter has emerged as a promising approach to increase the orally systematic absorption of poorly permeable drugs [372].

PEPT1-targeting prodrugs have been widely exploited, in which parent drugs linked to a natural amino acid to become dipeptide analogs (e.g., acyclovir [373], didanosine [374] and L-dopa [375]) or conjugated with a dipeptide to become tripeptide analogs with higher binding affinity (e.g., acyclovir [376, 377], glucosamine [378], lopinavir [379], oleanolic acid [380] and saquinavir [381]). In addition to prodrug strategies, some researchers have used functionalized nanocarriers to load poorly absorbed drugs to target the PEPT1 transporter. Gourdon *et al.* formulated a PEPT1-targeting nanoparticle by nanoprecipitation for the oral delivery of acyclovir. Valine was used to modify the surface of PLA-PEG-based NPs, which resulted in improved drug permeability in Caco-2 cells by interaction with intestinal PEPT1 [382]. The same group also encapsulated the peptide oxytocin into different PEPT1-targeting PLA-PEG-based NPs using a number of techniques. Among these PEPT1 transporter-targeted formulations, oxytocin encapsulated by a double emulsion exhibited a drug load of approximately 4 % (wt/wt) and significantly increased the drug plasma concentration after oral administration as compared to the free drug in solution [383]. Recently, researchers further demonstrated that PEPT1-targeted micelles and polymeric NPs were promising nanocarriers to enhance the intestinal absorption of hydrophobic molecules (e.g., docetaxel and curcumin) [384, 385]. Of note, various drugs (e.g., anti-inflammatory therapeutics, Lys-Pro-Val, KPV), essentially dipeptides and tripeptides, are mainly mediated via the PEPT1 transporter [386]. PEPT1 is overexpressed in the chronically inflamed colon, although human PEPT1 is not expressed in normal colonic epithelium [387]. This unique relationship indicates that PEPT1 might be a novel and promising target for IBD treatment. Recently, Zeng *et al.* developed a fluorescent peptide receptor-targeted probe (dicyanomethylene-4H-pyran-KPV, DCM-KPV). This fluorescent probe DCM-KPV lead a significant breakthrough in recognition and diagnosis of the colonic inflammatory regions through a direct non-invasive observation. This fluorescent probe allowed successful differentiation between the chronic, acute ulcerative colitis and normal groups [388]. This is the first report on a PEPT1-targeted fluorescent probe to differentiate inflammatory activity in ulcerative colitis models *in vivo*. Although dipeptides and tripeptides such as KPV, have PEPT1-targeting specificity, their therapeutic efficacy is still very low after oral administration because they hardly accumulate in colitis tissues and are rapidly eliminated by diarrhea. Tailoring nanocarrier-based drug delivery systems with these dipeptide or tripeptide drugs could greatly improve their orally therapeutic efficacy via not only protecting the dipeptides and tripeptides from the harsh GI tract but also allowing further modification on the surface of the nanocarrier (such as decorating ligands that could target specific cells or tissues).

II.3.2. Apical sodium-dependent bile acid transporter (ASBT)

ASBT, mainly expressed in the apical region of ileal enterocytes, contributes to the enterohepatic recirculation of bile acids. It is expressed more abundantly in the distal ileum than in the proximal ileum [362]. Bile acid-drug conjugates (prodrugs) could effectively improve the intestinal absorption of poorly soluble drugs. One of the most interesting strategies studied so far is the development of acyclovir-bile acid conjugates linked with valine. Among the four bile acid prodrugs, the prodrug acyclovir valylchenodeoxycholate showed the highest affinity for human ASBT (hASBT) [389]. The cellular uptake of the prodrug showed a greatly higher accumulation (16-fold) *in vitro* in hASBT-COS cells; COS-7 cells were transfected with human ASBT. However, the oral bioavailability of acyclovir valylchenodeoxycholate only exhibited a 2-fold increase, probably due to hydrolysis of the prodrug in the proximal GIT before reaching the absorption site in the intestine [389]. Khatun *et al.* developed hydrophilic bile acid-conjugated self-assembly nanoparticles for ameliorating the oral bioavailability of docetaxel. Hydrophilic taurocholic acid was used to modify the outer surface of the nanoparticles, which increased the possibility of an interaction between docetaxel-loaded nanoparticles and the intestinal ASBT transporter. The results obtained from *in vivo* studies indicated that taurocholic acid-heparin-docetaxel (HDTA) nanoparticles not only improved the oral bioavailability of docetaxel (from 0.8 % to 9.08 %) but also increased its antitumor activity; a significant reduction in tumor volume was observed in animals treated with HDTA nanoparticles compared with the mice treated with saline [390]. The same group further developed two taurocholic acid-modified drug delivery systems (a taurocholic acid-chitosan-coated siRNA-gold nanoparticle complex and a hyaluronic acid-taurocholic acid conjugated siRNA/protamine nanocomplex) for the oral delivery of siRNA to efficiently treat colorectal liver metastasis [391, 392]. Glycocholic acid, the most abundant component in human bile salts [393], was also selected as a ligand to modify the surface of the nanoparticulate system targeting ASBT. Kim *et al.* were the first to demonstrate that glycocholic acid can conjugate to the surface of solid fluorescent probe nanoparticles. The modified nanoparticles increased the oral bioavailability up to 47 %, which was achieved by combining the ASBT-mediated cellular uptake and chylomicron transport pathways [394]. The same group further developed glycocholic acid-modified liposomes for oral delivery of exendin-4 via ASBT targeting, showing a striking oral bioavailability (approximately 19.5 %) in rats. After a 4-weeks treatment with daily administrations, exendin-4-loaded ASBT targeting-liposomes via oral route were found be at least as efficient as the subcutaneous injection of an exendin-4 solution *in vivo* in T2DM rats [395]. These studies demonstrated that the components (e.g., taurocholic acid and glycocholic acid) present in human bile salts could be successfully decorated on the surface of DDS as ligands via a specific ASBT

route to increase the oral bioavailability of low permeable and fragile drugs since ASBT targeting prodrugs are not stable in the harsh GI environment.

II.3.3. Sodium-dependent vitamin transporters (SMVT and SVCT1)

Sodium-dependent vitamin transporters (SMVT) are involved mainly in the absorption of vitamins (e.g., lipoic acid, pantothenic acid and biotin) and can be found in large numbers in the proximal intestinal epithelia [396]. The interaction of biotin with SMVTs highly depends on the pH of the surrounding environment. Biotin has been exploited as a targeting ligand to develop SMVT-targeted prodrugs of poorly permeable therapeutics. Ramanathan *et al.* conjugated biotin with a nanopeptide (N-acetyl-L-Arg-L-Lys-L-Lys-L-Arg-L-Arg-L-Gln-L-Arg-L-Arg-L-Arg-L-Cys-NH_2, R.I.-K-Tat9) for exploiting the intestinal SMVT transporter. R.I.-K-Tat9-biotin conjugates significantly increased the transport *in vitro* in Caco-2 cell monolayer compared to R.I.-K-Tat9 alone [397]. PEG–biotin was synthesized and these conjugates could also target the SMVT transporter [398]. Other peptide drugs (GLP-1 and sCT), antiviral drugs (acyclovir), anticonvulsant drugs (gabapentin) and antiretroviral drugs (saquinavir) in the form of biotin conjugates also exhibited enhanced permeability owing to their interaction with the SMVT transporter [399-403].

SVCT is predominantly responsible for the intestinal absorption of vitamin C (ascorbic acid, AA). Luo *et al.* demonstrated the advantage of using SVCT1-targeting prodrug for the oral delivery of saquinavir. The transport of SVCT1-targeted saquinavir was increased by approximately 5-fold *in vitro* in intestinal cell monolayers [399]. Ascorbic acid was also coated on the outer surface of paclitaxel-loaded PLGA nanoparticles (As-PLGA NPs) [404]. As-PLGA NPs with 20 % ascorbate conjugation showed a higher uptake primarily through the caveolae-mediated pathway *in vitro* in Caco-2 cells. *In situ* perfusion and *ex vivo* biodistribution studies showed that, after passing through the mucus layer, As-PLGA NPs interacted with the SVCT1 transporter and were further uptaken by enterocytes, finally entering the systemic circulation [404]. This is the only study exploiting the SVCT1-targeting approach for developing nanoparticulate systems to enhance the oral bioavailability of encapsulated drugs.

II.3.4. Monocarboxylate transporter MCT1

The monocarboxylate transporter (MCT1), present on both apical and basolateral regions of intestinal epithelial cells, has a predominant expression in the distal segment of the large bowel [405]. MCT1 mainly moderates the transport of unbranched monocarboxylates (such as SCFAs), and its substrates include β-hydroxybutyrate, acetoacetate, lactate and pyruvate [364]. The exploitation of MCT1 targeting has become an effective strategy to improve the oral absorption of

some pharmacological therapeutics. Gabapentin, an anticonvulsant and analgesic drug, is primarily absorbed by the L-type amino acid transporter that is a low-capacity solute transporter in the intestine, resulting in its limited oral absorption [406]. Cundy *et al.* produced a gabapentin prodrug (XP13512) by reversible modification of its amine group with an acyloxyalkylcarbamate moiety [407]. The targeting of MCT1 by ligand-decorated nanocarriers has also shown improved oral bioavailability of insulin [408]. Butyrate-decorated NPs, via high affinity to the MCT transporter, elevated the transport of insulin up to 3.5-fold in Caco-2 cell monolayers, and increased oral bioavailability of insulin up to 9.28 % in diabetic rats compared with undecorated nanoparticles [408]. Then, the authors further disclosed the comprehensive transport mechanisms of these butyrate-modified NPs (Bu NPs) [409]. The results showed that Bu NPs actively bound to MCT1, and then most of the endocytosed Bu NPs were exocytosed both from the apical side back to the intestinal lumen and from the basal side into the bloodstream. Based on the mechanistic studies of Bu NPs *in vitro*, the researches further discovered that increasing the number of MCT1 in the basolateral membrane of cells, or increasing the surface hydrophobicity of the delivery system, could facilitate the transport of Bu NPs.

II.3.5. Amino acid transporters (LAT1 and $ATB^{0,+}$)

Two amino acid transporters, $ATB^{0,+}$ and LAT1, have been studied as targets in oral drug delivery. They are predominately located on the luminal surface of the colon, and their expression levels are dramatically decreased in the jejunum and ileum. $ATB^{0,+}$, being driven by Na^+/ Cl^- gradients, has extensive substrates; it can transport most of neutral and cationic amino acids. On the other hand, LAT1, unlike $ATB^{0,+}$, does not have such large substrate specificity; it is merely responsible for the transport of L-type large neutral amino acids presenting branched and aromatic side chains (e.g., valine) [368, 410]. Amino acid-based prodrugs exhibit a great potential as targeted delivery systems to improve the cellular absorption of an antiarrhythmic agent (quinidine [411]) and an antiviral drug (ganciclovir [412]). Both $ATB^{0,+}$ and LAT1 are highly expressed in cancer cells. As an example, the expression levels of $ATB^{0,+}$ significantly increased in colon cancer cells [413], and LAT1 is highly expressed in gastric tumors [414]. Only one study has been able to demonstrate that $ATB^{0,+}$-targeted PLGA NPs could increase cellular uptake *in vitro* in Caco-2 cells compared with non-targeted nanoparticles [413]. There is no example for orally improved nanoparticulate systems via LAT1. Addressing the potential amino acid transporters ($ATB^{0,+}$ and LAT1) towards enhanced orally drug delivery systems still needs to be further studied.

II.3.6. Sodium-coupled glucose transporter SGLT1 and organic cation/carnitine transporter OCTN2

SGLT1 predominately locates in the small bowel rather than in the large bowel [415]. Glycosylated molecules and glucose- and galactose-conjugated compounds have been suggested to enhance the intestinal absorption via the SGLT1 transporter [416-419].

OCTN2, largely expressed in the apical membrane of enterocytes, transports carnitine and other organic cations across the epithelium [420]. Recently, it has attracted attention as a target for increasing intestinal absorption. Wang *et al.* reported the development of gemcitabine prodrugs conjugated with L-carnitine via different lipophilic linkages. All the developed prodrugs could significantly increase the cellular permeability and oral bioavailability of gemcitabine, among which the hexane diacid-linked prodrug dramatically increased its uptake by 15-fold across Caco-2 cells and its plasma absorption 5-fold in rats. This study was the first to demonstrate that OCTN2 may represent a novel target for oral prodrug delivery [421]. They further exploited L-carnitine as a ligand to conjugate PLGA NPs for the oral delivery of paclitaxel [422]. PLGA NPs with a 10 % modification of L-carnitine exhibited the highest systemic absorption of paclitaxel *in vivo* in rats, and its intestinal absorption was predominantly via the lymphatic system [422]. Apart from OCTN2, $ATB^{0,+}$ is another transporter that is responsible for the transportation of carnitine, but shows a relatively lower affinity compared to OCTN2 [423]. Both OCTN2 and $ATB^{0,+}$ exhibit a significantly increased expression in colon cancer cells compared to normal colonocytes. Kou *et al.* conjugated L-carnitine with 5-fluorouracil-loaded PLGA NPs to develop a dual targeting nanoparticle, which could deliver 5-fluorouracil to overexpressed OCTN2- and $ATB^{0,+}$in colon cancer cells [413]. In addition, OCTN2 is associated with other pathologies, such as IBD and diabetes [420]. Thus, they might represent a novel and promising pharmacological target in oral drug delivery.

To date, over 400 membrane transporter proteins have been identified in the human genome, and they are classified as two major superfamilies: 49 ABC transporters and 395 SLC transporters [424], while their targeting role in oral drug delivery has only been exploited for approximately 10 proteins (belonging to the SLC transporter superfamily), as reviewed above. These key transporters, located on both the apical and basolateral membrane of intestinal epithelium cells, represent excellent targets in oral drug delivery because they mediate nutrient–drug interactions. In recent years, the targeted-prodrug approach has been well developed in transporter-based oral delivery research, by which moieties are covalently conjugated to the drug molecules, thereby selectively targeting certain transporters in the intestine. The developed targeted-prodrugs, recognized by one or more well-

known transporters in GIT, offer a distinguished feasibility for increasing selectivity and oral bioavailability of poorly permeable therapeutics. However, these transporter-targeted prodrugs still face large challenges, presenting limitations in the harsh GI environment and/or the expression and localization of transporters. Various transporter-targeting prodrugs might be hydrolyzed in the stomach and proximal intestine, resulting in the inadequate absorption of the parent drugs. Some transporters are expressed throughout the whole intestinal tract, but are expressed in high levels in certain intestinal segments, disease areas and specific cancer cells. Although it provides a long oral absorption window, the risk of toxicity for some therapeutics (such as anticancer drugs) will be largely increased because of the difficult accumulation of these prodrugs at specific sites and/or cells in the intestine. In recent years, nanoparticulate delivery systems have also accomplished some achievements in the intestinal transporter-targeting field. NPs enhance efficacy of oral administration of drugs not only by increasing solubility, avoiding acidolysis and hydrolysis and controlling release in GIT, but also by further modifying specific substrates with drug-carriers as ligands to target the transporters expressed in certain segments of the intestine. The examples we reviewed showed that nanoparticulate delivery systems conjugated with ligands of one or more specific substrates represent a promising potential towards orally administered therapeutics. Nevertheless, the data on how relevant transporters mediate the intestinal absorption of targeted NPs are still limited, and more studies are required to unveil the behavior of NPs, such as the comprehensive transport mechanism of transporter-targeting NPs. This would provide with more information for the rational design of transporter-targeting NPs.

Table 5. Examples of targeting strategies towards intestinal transporters in oral drug delivery.

Transporter	Cargo	Targeting strategies	References
LAT1	Quindine	Val, Ile-quindine; prodrug	[411, 425]
$ATB^{0,+}$	Acyclovir (ACV)	Valacyclovir	[368]
	Ganciclovir	Valganciclovir	[412]
	5-Fluorouracil	L-carnitine-PLGA NPs	[413]
ASTB	Acyclovir (ACV)	ACV-Val-CDCA; prodrug	[389]
	Akt2-siRNA	Taurocholic acid- chitosan coated Akt2-siRNA- gold nanoparticles complex	[391]
	Akt-siRNA	Hyaluronic acid-taurocholic acid conjugated siRNA/protamine nano-complex	[392]
	Curcumin (Cur)	Taurocholic acid-Cur loaded nanostructured lipid carriers (NLCs)	[426]
	Exendin-4 (Ex-4)	Chondroitin sulfate-g-glycocholic acid-coated and Ex-4-loaded liposomes	[395]
	Gabapentin	CDCA-alpha-benzyl-glu-gapapentin, CDCA-glu-gapapentin; prodrug	[427]
	Insulin	Deoxycholic acid-modified and insulin-loaded chitosan nanoparticles	[428]
	Ketoprofen	CDCA-Lys-ketoprofen; prodrug	[429]

	Docetaxel	Taurocholic acid-nanoparticles	[390, 430]
	-	Glycocholic acid-solid fluorescent probe nanoparticles	[394]
	-	Dexoycholic acid-nanocomplex	[431]
MCT1	Carbenicillin	Carindacillin; prodrug	[432, 433]
	Gabapentin	XP13512; prodrug	[407, 434]
	Insulin	Butyrate-lipid-polymer hybrid nanoparticles	[408, 409]
OATP	Tebipenem	Tebipenem pivoxil	[435]
OCTN2	Butyrate	Butyryl-L-carnitine	[436]
	Gemitabine	L-carnitine-succinic-gemcitabine (GSC), L-carnitine-hexylic-gemcitabine (GHC), L-carnitine-octanedioic-gemcitabine (GOC), L-carnitine-decanedioic-gemcitabine (GDC) ; prodrugs	[421]
	5-Fluorouracil	L-carnitine-PLGA NPs	[413]
	Paclitaxel	L-carnitine-PLGA NPs	[422]
SGLT1	Acyclovir (ACV)	beta-glucoside-ACV; prodrug	[437]
	p-nitrophenol (p-NP)	p-nitrophenyl beta-D-glucopyranoside (p-NPglc); prodrug	[416, 417]
		p-nitrophenyl beta-disaccharide; prodrug	[438]
	Gly-Gly-Tyr-Arg, GGYR	alpha,beta-SAPG-GGYR; prodrug	[418]
	Quercetin	Quercetin 4'-beta-glucoside (Q4G) ; prodrug	[419]
SMVT	Acyclovir (ACV)	Biotin-ACV; prodrug	[403]
	Gabapentin	Gabapentin enacarbil (XP13512); prodrug	[401, 407]
	Glucagon-like peptide-1 (GLP-1)	Biotin-GLP-1; prodrug	[402]
	Glucagon-like peptide-1 (GLP-1)	Biotin-PEG-GLP-1; prodrug	[439]
	R.I-K-Tat9	PEG:(R.I-Cys-K(biotin)-Tat9)$_8$; prodrug	[398]
	R.I-K-Tat9	R.I.-K (biotin)-Tat9	[397]
	Saquinavir (SQV)	Biotin-SQV; prodrug	[396]
	Salmon calcitonin (sCT)	Biotin-sCT; prodrug	[400]
SVCT1	Saquinavir (Saq)	Ascorbyl-succinic-Saq; prodrug	[399]
	Paclitaxel	Ascorbate-conjugated PLGA NP	[404]
PepT1	Acyclovir (ACV)	L-val-ACV; prodrug	[373, 440-445]
		L-Glu-Sar-ACV; prodrug	[376, 377]
		Val-PLA-PEG nanoparticles	[382]
	Cytarabine	5'-L-Val-cytarabine; prodrug	[446]
	Curcumin	Val-, Phe-polymeric micelles	[384]
	d-Amphetamine	lisdexamfetamine dimesylate (LDX)	[447]
	Decitabine (DAC)	5'-O-L-val-DAC, 5'-O-L-trp-DAC and 5'-O-L-phe-DAC; prodrug	[448, 449]
	Dicyanomethylene-4H-pyran (DCM)	DCM-KPV	[388]
	Didanosine (DDI)	5'-O-L-val-DDI; prodrug	[374]
	Docetaxel (DTX)	L-Val-Val-; L-Val-Phe-PLGA-PEG nanoparticles	[385]

1-(2',5' dimethoxyphenyl)-2-aminoethanol) (DMAE)	Gly-DMAE; prodrug	[450]
Floxuridine	D-Val-floxuridine; produg	[451, 452]
	5'-L-isoleucyl or 5'-L-val- floxuridine; prodrug	[453]
Ganciclovir (GCV)	Tyrosine-val-GCV; prodrug	[454]
Glucosamine	Gly-Val-glucosamine; prodrug	[378]
Guanidine oseltamivir carboxylate (GOCarb)	L-val-GOCarb; prodrug	[372, 455]
L-alpha-methyldopa	L-Phe-L-alpha-methyldopa; prodrug	[456]
L-dopa	L-Phe-L-dopa; prodrug	[375]
	D-Phe-L-dopa; prodrug	[457]
Levovirin	5'-(L)-val-levovirin; prodrug	[458]
Lopinavir (LVR)	Val-Val-LVR and Gly-Val-LVR; prodrug	[379]
LY354740	Alanyl-LY354740 (LY544344); prodrug	[459, 460]
Oleanolic acid (OA)	Amino acid/dipeptide-OA; prodrug	[380, 461]
Oxytocin (OXY)	Val-PEG-PLA/PLGA NPs	[383]
Pterostilbene (Pt)	Ile-Pt; prodrug	[462]
Saquinavir (SQV)	Val-Val-SQV and Gly-Val-SQV; prodrug	[381]
Zanamivir	L-val-zanamivir; prodrug	[463]

II.4. CONCLUSION

Drug delivery systems targeted to the gastrointestinal epithelium are one of the most exploited strategies in oral delivery of drugs. This strategy has been greatly exploited to increase the bioavailability of poorly water-soluble drugs or biologics or to increase the accumulation of the drug delivery system at the site of interest, as mentioned in the sections above. Advanced formulations, via unique characteristics, promising strategies and potential ligands, can be used to target different sites, including different cells, transporters and specific disease sites, throughout the whole GIT. However, many receptors/cells/strategies remain unexplored and represent promising therapeutic targets for successful oral drug delivery systems. There are important gaps in knowledge regarding the interaction of the drug delivery systems with the gastrointestinal barrier that hinder the evolution of oral targeted drug delivery. As most of the studies described herein, the mechanisms by which the drug delivery system interacts with a cell/transporter are still unknown. This is a key limitation in the evolution of oral drug delivery.

The examples we reviewed showed that cell receptors/transporters-mediated NPs with the further modification of one or more ligands represent potential formulations for multiple applications towards orally administered drugs. However, it should be taken fully into consideration that the

examples described within this review correspond to preclinical studies and that the real clinical value of these strategies remains uncertain. Current studies in oral targeting strategies mostly are proof-of-concept, obtaining promising results in preclinical phases but not reaching the clinics. Although these studies have shown good experimental results by targeting different intestinal cells and segments via specific receptors or transporters, it is essential to consider the real interest in the targeting of those cells and sites in a foreseen clinical translation. From the physiological point of view, enterocytes have an absolute advantage in improving the oral bioavailability of poorly absorbed drugs since they represent the most abundant cell type in the gut. They are mainly responsible for the absorption of nutrients from the lumen, and express various vital receptors and transporters. This does not mean that the targeting of other intestinal cells has no clinical value. On the contrary, the studies regarding different cell targeting strategies provide a deep understanding on how to overcome the intestinal barriers via oral route. In addition to the improved bioavailability via enterocytes-targeting, current studies have also demonstrated the contribution of M cells to oral vaccine development and the potential use of L cells-targeting for the treatment of diabetes. Furthermore, once the formulations are administered, it is impossible to only deliver the administered cargoes into specific cells due to the direct interplay with different types of intestinal cells. The studies regarding different cell targeting summarized here highlight the advantages of those targeting approaches. Depending on the characteristics of the disease to be treated and the type of drug to be selected, we can select single or combined strategies towards cell targeting in future applications [464]. Moreover, considering a foreseen clinical application, one must also be realistic regarding the manufacture of these drug delivery systems. Most of these systems are available in small scale laboratory batches but its manufacturing on a large scale would be too expensive and complicated.

Another important conclusion that could be drawn from all these examples is that we might not need to engineer the surface of the drug delivery system in each case for it to be effective, and we might be capable to exert biological effects and/or targeting capacities simply by exploiting the physicochemical properties of the drug delivery system. However, the optimal physicochemical properties (e.g., surface charge, particle size) would vary depending on the cell type or the transporter being targeted, and these properties should be established for each and every example described herein.

In any case, recent studies on the targeting of L cells and the targeting of specific cell receptors/transporters prove that there is still room for development and improvement in the oral drug delivery field towards more effective targeted DDS.

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CHAPTER II
AIM OF THE THESIS

AIM OF THE THESIS

The development of oral dosage forms that allows absorption of therapeutic peptides to the systemic circulation is one of the greatest challenges for the pharmaceutical industry. Improved novel drug delivery systems are of utmost importance in order to fulfill the potential of this route of administration in the treatment of chronic diseases (e.g., T2DM), where daily injections are often required. The benefit of oral peptide delivery is remarkable especially in the case of anti-diabetic drugs (e.g., GLP-1RAs), within an additional therapeutic advantage of simulating the normal physiological pathway. Compared with subcutaneous delivery of GLP-1RAs, it passes through the liver in oral delivery manner, which has much higher concentrations via the hepatic portal vein, thereby reducing systemic exposure and its associated side-effects. However, current advanced drug delivery systems on oral peptide delivery are not fulfilling their potential merely as a vehicle, not reaching the clinics yet, and none of them has exploited the potential physiological effect of the carrier on the ultimate therapeutic effect of the formulation. Moreover, the gut physiology offers a stimulating environment, which has attracted particular interest because of the pleiotropic effects of the peptides secreted at the intestinal level, such as GLP-1 secreted from enteroendocrine L cells. L cells, expressing various G protein-coupled receptors (GPCR) that can be activated by different fatty acids in lumen, are attractive targets for the treatment of T2DM. Thus, the development of improved alternative drug delivery systems is of utmost importance in order to fulfill the potential of the oral route of administration.

The aim of this thesis is to develop a nanoparticle-based drug delivery system for the oral delivery of GLP-1 analogs in order to overcome the limitations of marketed injectable drug delivery systems and oral semaglutide. This project is based on the hypothesis that nanoparticles (NP) targeting L cells encapsulating antidiabetic peptides (GLP-1RAs) may present synergized antidiabetic effects both by L cell stimulation (increase endogenous GLP-1 secretion) and peptide delivery via oral route (Fig. 1). To assess this hypothesis, the thesis is divided in the following aims:

(i) To develop a nanocarrier exerting a biological effect represents a main goal in this study. Lipid-based nanocarriers are commonly used drug delivery systems in the drug delivery field. It is important to note that different types of oils are frequently used in the preparation of lipid-based formulations. Normally, the oils consist of various fatty acids, which could potentially activate the GPCR expressed on L cells. Along this line, lipid nanocapsules presenting different sizes (from 25 nm to 200 nm) were evaluated *in vitro* in murine L cells and *in vivo* in normoglycemic mice.

(ii) To develop a nanocarrier-based alternative for the oral delivery of peptides by exploiting the own biological effect of the carrier. To achieve this goal, we need to encapsulate a hydrophilic

molecule within the liquid lipid inner core of the nanocapsule. Therefore, we developed a novel exenatide-loaded lipid nanocapsule by introducing reverse micelles and a mixed oil phase for the oral delivery of peptides for the treatment of T2DM.

(iii) To prolong the *in vivo* antidiabetic effect exerted by the nanosystem towards less frequent peptide administrations, targeted nanocapsules towards increased L cell stimulation were further exploited towards improved oral peptide delivery in incretin-based diabetes treatment.

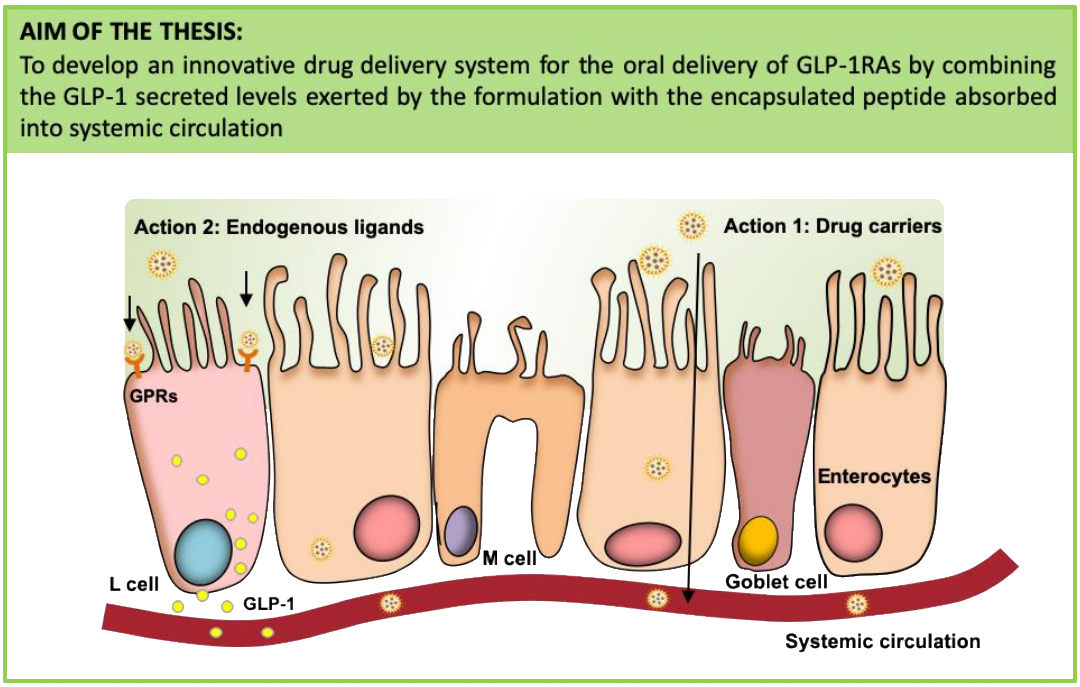

Figure1. Schematic image representing the aim of this PhD thesis.

The innovative aspects of this work were: (i) exploiting the therapeutic physiological ability of drug-carriers by increasing endogenous GLP-1 secretion *in vivo*; (ii) the development of an innovative nanocapsule-based drug delivery system that synergizes its own biological effect (stimulation of GLP-1 release) and that of the encapsulated bioactive molecule (exenatide) as an alternative strategy for the treatment of T2DM via an oral route; (iii) the use of contingency strategies able to greatly increase endogenous GLP-1 triggered by the nanosystem *per se*.

The specific objectives of this project were:

1. To investigate the size effect on lipid nanocapsule-mediated GLP-1 secretion *in vitro* in enteroendocrine L cells and *in vivo* normoglycemic mice. (Chapter III)
2. To fulfil an unmet therapeutic need for treating T2DM by developing an innovative oral drug delivery nanosystem increasing the production of GLP-1 and the absorption of peptides into the circulation. (Chapter IV)
3. To further modify the newly developed nanosystem increasing the endogenous GLP-1 secretion, prolonging the *in vivo* antidiabetic effect and minimizing the oral administration frequency of peptides in the treatment of T2DM. (Chapter V)

CHAPTER III
SIZE EFFECT ON LIPID NANOCAPSULE-MEDIATED GLP-1 SECRETION FROM ENTEROENDOCRINE L CELLS

Adapted from:

Xu Y, Carradori D, Alhouayek M, Muccioli G G, Cani P D, Préat V, Beloqui A. Size effect on lipid nanocapsule-mediated GLP-1 secretion from enteroendocrine L cells. Mol. Pharmaceutics 15: 108-115 (2018).

ABSTRACT

L cells are enteroendocrine cells located throughout the gastrointestinal tract that secrete physiologically important peptides. The most characterized peptides secreted by L cells are the peptide YY (PYY) and the glucagon-like peptide (GLP) -1 and -2. These peptides are released rapidly into the circulation after oral nutrient ingestion. Recently, lipid-based nanoparticles (NP) have been described as triggers for GLP-1 secretion by L cells. NP physicochemical properties play a key role in the NP-cell interaction, and drive NP cell internalization. We herein hypothesize that lipid-based NP with appropriate size would not only be able to deliver drugs into blood circulation, but also act like endogenous ligands to stimulate GLP-1 secretion. We tested five different size (25, 50, 100, 150 and 200 nm) lipid nanocapsules (LNC) on murine L cells *in vitro* to confirm this hypothesis. Our study showed that GLP-1 secretion was induced only by the 200 nm size LNC, highlighting the importance of LNC particle size on the secretion of GLP-1 by L cells. The different formulations did not affect proglucagon mRNA expression, suggesting there was not an increased GLP-1 synthesis. As a proof of concept, we further demonstrated in normoglycemic mice that 200 nm LNC administration increases GLP-1 levels by 4- and 3-fold compared to untreated control mice 60 and 180 min after the administration, respectively. Our study suggests that 200 nm LNC as a nanocarrier to encapsulate drug candidates and as a ligand to induce endogenous GLP-1 secretion might represent a promising strategy for type 2 diabetes mellitus treatment.

KEYWORDS

Enteroendocrine L cells; lipid nanocapsules; GLP-1; type 2 diabetes mellitus; size effect; incretin

TABLE OF CONTENTS

I. INTRODUCTION

Enteroendocrine cells are located throughout the epithelium of the gastrointestinal (GI) tract, which represents the largest endocrine organ [1]. Among the different types of enteroendocrine cells, L cells have attracted particular interest [2]. The L cell directly contacts luminal nutrients and neural as well as vascular tissue through its apical surface and basolateral surface [3, 4]. L cells produce several gut peptides; however, the most characterized peptides are the peptide YY (PYY) and the glucagon-like peptide (GLP) -1 and -2. These peptides are released rapidly into the circulation after oral nutrient ingestion, i.e. glucose and other sugars, dietary fiber or fatty acids [5]. These secreted peptides play an important role in the control of both glucose and energy homeostasis, where GLP-1 and PYY reduce food intake, and GLP-1 also potentiate insulin secretion (i.e., the so-called incretin effect). In addition, GLP-2 is involved in the maintenance of the gut barrier. It is worth noting that both GLP-1 and GLP-2 are degraded within a couple of seconds in inactive peptides after cleavage by the enzyme dipeptidyl peptidase IV (DPP-IV). Nowadays, inhibitors of the DPP-IV activity and GLP-1 analogs are currently used for the treatment of type 2 diabetes mellitus (T2DM). DPP-IV inhibitors are ingested orally whereas GLP-1 analogs must be injected [6]. This incretin effect accounts for approximately 50% to 70% of the total insulin secreted after oral glucose administration [3]. The number of studies including GLP-1 loaded drug delivery systems have substantially increased lately [7, 8].

Several G-protein-coupled receptors (GPCRs) are expressed by L cells [9, 10]. They can be activated by nutrients (including lipids) found in the gut lumen. Therefore, any strategies able to promote the endogenous secretion of these peptides via a safe and physiological route constitute a key strategy for several prevalent diseases such as T2DM, inflammatory bowel diseases (IBD) and obesity [11-13]. Along this line, we have recently reported that nanostructured lipid carriers (NLC) were able to induce the secretion of GLP-1 by L cells by mimicking endogenous lipid ligands [14]. A novel and more physiological alternative for T2DM treatment could be enhancing endogenous GLP-1 secretion [14-17].

Nanoparticles (NP) are generally defined as particulate dispersions or solid particles in size from 10 nm to 1000 nm. Within the NP, the drug can be found dissolved, entrapped, encapsulated or attached to the NP matrix which is made of biocompatible and biodegradable materials [18]. NP physicochemical properties play a key role in the NP-cell interaction, and drive NP cell internalization [19]. In concrete, NP size is often an essential factor to take into account when designing NP [20].

Therefore, lipid-based NP with appropriate size would not only be able to deliver drugs into blood circulation, but also act like endogenous ligands actively targeting L cells thus inducing a combined incretin effect towards T2DM treatment. In the present study, we evaluated the ability of five different size LNC (25, 50, 100, 150 and 200 nm) to induce the secretion of GLP-1 by L cells. This formulation was chosen based on altering the proportion of the different components of the formulation to obtain different NP sizes [21]. We hypothesize that the size of LNC may affect their behavior towards GLP-1 secretion by L cells [22-24].

II. MATERIALS AND METHODS

II.1. MATERIALS

Solutol® HS15 (mixture of free PEG 660 and PEG 660 12-hydroxystearate, Mw 870 Da) was purchased from Sigma-Aldrich (St. Louis, USA). Labrafac® WL 1349 (caprylic/capric acid triglycerides) was kindly provided by Gattefossé (Saint-Priest, France). Lipoid® S75-3 (soybean lecithin at 69% of phosphatidylcholines and 10% of phosphatidylethanolamines) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Sodium chloride (NaCl), lecithin, sodium taurocholate, pepsin, 3-(4,5- dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Triton-X 100 were bought from Sigma-Aldrich (St. Louis, USA). VECTASHIELD® Hard Set™ Mounting Medium with DAPI was purchased from Vector Laboratories (Burlingame, USA). The total GLP-1 ELISA kit was purchased from Meso Scale Discovery (USA). Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX, penicillin-streptomycin (P/S), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin (0.25%) containing ethylenediaminetetraacetic acid (EDTA, 0.02%), Alexa Fluor 488 phalloidin and DiIC18(5) solid (1,1'-ioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) (DiD) were purchased from Thermo Fisher Scientific (Invitrogen, UK). Dipeptidyl peptidase IV (DPP-IV) inhibitor was obtained from Millipore (St. Charles, USA). Basement membrane matrix (Matrigel™) was purchased from BD Bioscience (Belgium). All chemical regents used in this study were of analytical grade.

II.2. FORMULATION AND CHARACTERIZATION OF LIPID NANOCAPSULES

II.2.1. Preparation of lipid nanocapsules differing in particle size

LNC were prepared following a phase inversion process previously described by Heurtault et al.[21] Briefly, all components, including lipophilic Labrafac® WL 1349, Lipoid® S75-3, Solutol® HS15, NaCl and MilliQ water, were mixed together at 40 °C. Three temperature cycles of progressive heating/cooling were conducted (from 60 °C to 90 °C). Within the last cycle, 12.5 mL of cold water (4 °C) was added during the cooling step at 74 °C under high speed stirring. The LNC were filtered using a 0.45 µm filter and stored at 4 °C until use. Different sizes of LNC, ranging from 25 to 200 nm, were obtained using different amounts of water, surfactant and oil. In addition, DiD-labeled LNC were prepared by adding DiD in the last cycle, as previously described by Carradori *et al.* [25]. Namely, during the cooling of the last cycle, at 80 °C, 27.5 µL of DiD (1 mg/mL in

absolute ethanol) was added to the solution and once at 74 °C, 12.5 mL of cold water was added, under high speed stirring.

The concentration of LNC (C_{LNC}) was calculated by the following equation: the total weight of Labrafac® WL 1349, Solutol® HS15 and Lipoid® S75-3 divided by the final volume of each formulation.

II.2.2. Characterization of lipid nanocapsules

The LNC particle size and the polydispersity index (PDI) were characterized by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was measured by laser Doppler velocimetry (LDV) also using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Each measurement was performed in triplicate.

II.3. *IN VITRO* STABILITY STUDIES

The stability of the different batches of LNC was evaluated *in vitro* in biomimetic gastrointestinal fluids containing enzymes to assess the foreseen *in vivo* stability of the formulation. The stability was tested in three different biomimetic media: Fasted State Simulated Gastric Fluid (FaSSGF), Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF). The composition of FaSSGF, FaSSIF and FeSSIF is presented in Table S1[26, 27]. The LNC were incubated in FaSSGF, FaSSIF and FeSSIF at 37 °C (100 µL of nanoparticles in 10 mL media). At predetermined time intervals (0, 0.5, 1, 2 h for FaSSGF and 0, 0.5, 1, 3, 6 h for FaSSIF and FeSSIF), samples were withdrawn. At each time point, the particle size, as well as the PDI, were measured.

The size stability of 25, 50, 100, 150 and 200 nm LNC in the DMEM GlutaMAX medium was also assessed at 37 °C for 2 h. Samples were withdrawn after 2 h, and the particle size and PDI were measured. Each formulation was analyzed in triplicate.

II.4. GLP-1 SECRETION BY MURINE L CELLS

II.4.1. GLUTag cell culture

GLUTag cells (intestinal murine L cells, passage between 17 to 28) were donated by Dr. Daniel Drucker (University of Toronto, Canada). L cells were grown in DMEM-GlutaMAX medium supplemented with 1% (v/v) P/S and 10% (v/v) inactivated FBS, at 37 °C with 5% CO_2 supply. Cells were sub-cultured using trypsin (0.25%) containing EDTA (0.02%) every 4-5 days [28].

II.4.2. Cytocoxicity studies by the MTT assay

For the assessment of cell viability, 5×10^4 GLUTag cells per well were co-incubated for 2 h on Matrigel™-coated 96-well plates (Costar Corning CellBIND Surface, USA) (10 µL/mL of medium) with the LNC (concentrations ranging from 1 mg/mL to 10 mg/mL) dispersed in DMEM-GlutaMAX medium without FBS. Then, the supernatants were replaced by 100 µL of 0.5 mg/mL MTT for 3 h. After this incubation period, the MTT solution was retrieved and 200 µL of DMSO was used per well. Absorbance was measured at 560 nm using a MultiSksan EX plate reader (Thermo Fisher Scientific, USA). Each test was performed in triplicate.

The absence of cytotoxicity was confirmed by cell counting, using Trypan Blue as staining reagent. No differences among groups were observed unless otherwise stated.

II.4.3. GLP-1 secretion assay

GLUTag cells (1.8×10^5 cells/well) seeded onto Matrigel™-coated 24-well plates were allowed to adhere for 24 h. After, the medium was removed and the cells were cultured with the different size LNC at 2 mg/mL (37 °C for 2 h). To corroborate that the secretory effect was nanoparticle-mediated and not due to a different surfactant concentration, equivalent amounts of Solutol® HS15 for each formulation were also assayed. Within the different LNC formulations, both the amounts of Solutol® HS15 and Labrafac® WL 1349 were altered. However, due to the insolubility of the latest (HLB = 1), only Solutol® HS15 could be tested. The experiment was conducted in DMEM GlutaMAX medium, without FBS, with DPP-IV inhibitor (20 µL per mL of culture medium). After incubation with LNCs or Solutol® HS15, the collected supernatants were centrifuged at 250 *g* for 5 min at 4 °C (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) and preserved until further analysis. Cells were then washed three times with PBS. Cell extracts containing GLP-1 were obtained after three freeze-thaw cycles followed by centrifugation (250 *g*, 5 min at 4 °C). Total GLP-1 concentration were determined by ELISA [29]. The GLP-1 secretion was calculated by the following equation:

$$\text{GLP-1 secretion} = C_{\text{extracellular}} / (C_{\text{intracellular}} + C_{\text{extracellular}})$$

Where $C_{extracellular}$ is the concentration of GLP-1 tested in the supernatants, and $C_{intracellular}$ is the concentration of GLP-1 tested in GLUTag cells.

II.5. MECHANISM OF SIZE-MEDIATED GLP-1 SECRETION BY GLUTAG CELLS

II.5.1. Expression of Proglucagon

GLUTag cells (1.8 x 10^5 cells/well) seeded onto Matrigel™-coated 24-well plates were treated with LNC for 2h, after which time we removed the medium and added TriPure (Roche, Basel, Switzerland) to each well. The total RNA was extracted based on the manufacturer's protocol. Reverse transcription was performed with the Promega GoScript Reverse Transcription system (Promega, Madison, WI, USA) on 1 μg of RNA. qPCR reactions were run using the GoTaq qPCR Master Mix from Promega on a StepOne Plus instrument (Applied Biosystems, Foster City, CA, USA) as previously described [30]. RPL19 was used as a reference gene and its expression was not affected by the different experimental conditions. Primers for proglucagon: forward primer (5' to 3') ATGAAGACCATTACTTTG and reverse primer (5' to 3') CGGTTCCTCTTGGTGTTCATCAAC. Primers for RPL19: forward primer (5' to 3') GAAGGTCAAAGGGAATGTGTTCA and reverse primer (5' to 3') CCTTGTCTGCCTTCAGCTTGT [31].

II.5.2. Nanocapsules localization

The localization of LNC within GLUTag cells was visualized by confocal laser scanning microscopy (CLSM). Briefly, cells were seeded onto Matrigel™-coated coverslips for 24 h at 37 °C prior to the study. The day after, the cells were co-incubated with DiD-labeled LNC of different sizes for 2 h at 37 °C (2 mg/mL LNC concentration). Negative controls were incubated without LNC. At the end of the 2 h incubation period, the supernatants were removed and cells were fixed with 4 % paraformaldehyde. After washing with PBS, cell cytoskeletons were stained with Alexa Fluor 488 phalloidin (200 μL, 1:1000 in PBS for 1 h). After washing the coverslips with PBS, these were mounted on glass slides with VECTASHIELD® Hard Set™ Mounting Medium containing DAPI (staining cell nuclei in blue). The samples were tested by CLSM using a Zeiss™ confocal microscope (LSM 150) to capture serial pictures along the x–z axis. The Axio Vision software (version 4.8) was used to analyze the data.

II.6. *IN VIVO* STUDIES

In vivo studies were carried out in normoglycemic mice (C57BL/6J male mice, 20-25 g, 11 weeks; Janvier Laboratories, France). The experimental protocols were approved by and performed in accordance with the local animal committee (2014/UCL/MD/033) and as specified by the Belgian

Law of 29 May 2013, on the protection of laboratory animals. Animals were fasted overnight before the experiments with free access to water. The animals were divided in two groups (8 mice per group): untreated control mice and 200 nm LNC-treated mice. LNC-treated mice were administered with 100-150 µl of a 285 mg/mL nanoparticle suspension, corresponding to a 1.62 mg/g nanoparticle dose by oral gavage. Untreated control mice received drinking water instead. Blood samples were withdrawn at times 60 and 180 min after oral administration from the tip of the tail vein. Samples were collected in the presence of DPP-IV inhibitor (20 µL per mL of blood) and maintained in ice. Immediately after the studies, blood samples were centrifuged (3,000 rpm, 10 min at 4° C) and the plasma was kept frozen at - 80° C until analysis. The total GLP-1 levels were quantified by ELISA (Meso Scale Delivery, USA).

II.7. STATISTICAL ANALYSIS

The GraphPad Prism program (version 7) (CA, USA) was used to complete the statistical analysis. The Shapiro−Wilk test was applied to confirm the normal distribution of the samples. Differences among groups were calculated by ANOVA followed by Tukey's post-hoc test. Student's t-test or Mann−Whitney test were conducted for other statistical analyses. A difference of $*p < 0.05$ was considered statistically significant. Results are expressed as mean ± standard error of the mean (SEM).

III. RESULTS AND DISCUSSION

III.1. PREPARATION AND CHARACTERIZATION OF LIPID NANOCAPSULES

The phase inversion process was used to prepare LNC whose sizes were strongly dependent on the proportion among the different excipients [32]. The particle size increases by increasing the oil phase (Labrafac®) while it decreases by increasing the nonionic surfactant (Solutol®) [33, 34]. The heating/cooling cycle process crossing the phase-inversion zone is essential in the preparation of LNC [35]. When reducing the quantity of surfactant, the number of temperature cycles needs to be higher to stabilize the system and improve the nanoparticle dispersion [32, 36]. Among all components, the percentage of water seems to have no significant effect on particle diameter [32, 37]. Based on these observations, the formulation and preparation processes were optimized to produce LNC with different particle sizes ranging from 25 nm to 200 nm. The final composition for each nanocapsule formulation is summarized in Table 1. The mean particle sizes of the LNC were in the range of ~25 nm to ~200 nm (Table 2). The small PDI index (PDI<0.15) indicated the homogeneity of the LNC in terms of size distribution.

Table 1. Composition of the lipid nanocapsules presenting different particle sizes.

LNC Size (nm)	25	50	100	150	200
Solutol® HS15 (g)	1.934	0.846	0.580	0.532	0.290
Lipoid® S75-3 (g)	0.075	0.075	0.075	0.075	0.075
Labrafac®WL1349 (g)	0.846	1.028	1.112	1.257	1.789
NaCl (g)	0.089	0.089	0.089	0.089	0.089
Water (mL)	2.055	2.962	3.143	3.047	2.756
Water at 0 °C (mL)	12.5	12.5	12.5	12.5	12.5
Temperature Cycles	3	3	3	3	5

Table 2. Physicochemical characterization of the different lipid nanocapsules (mean ± SEM, n=3).

LNC	25	50	100	150	200
Size (nm)	27.99±0.19	55.42±0.46	107.47±0.54	148.67±1.25	200.50±0.96
PDI	0.159±0.01	0.105±0.02	0.109±0.01	0.177±0.01	0.145±0.03
Zeta (mV)	-4.76±0.54	-6.18±0.55	-10.53±0.28	-8.96±0.16	-19.97±0.49

III.2. *IN VITRO STABILITY STUDIES*

The simulation of gastrointestinal conditions represents a valuable tool to predict the foreseen *in vivo* behavior of the formulations [38, 39]. Therefore a stability study of different LNC particle sizes was performed in three biomimetic media (FaSSGF, FaSSIF and FeSSIF) simulating the composition of the gastric and intestinal conditions, before and after food intake, respectively [26]. Meanwhile, in this study, we based on the estimated intestinal transit time, all sizes of LNC were incubated with FaSSGF for 2 h, and in intestinal fluids FaSSIF and FeSSIF for 6 h.

Freshly prepared LNC (size ranging between 25 and 200 nm) were incubated in various simulated media for 2 h and 6 h at 37 °C. It could be observed that the mean particle diameter of all LNC remained unaltered during the incubation in the different fluids (Fig. 1), indicating no significant aggregation or disintegration (count data not shown) and good colloid stability whatever the incubation times. Solutol® has been suggested to play an important role in LNC stability by preventing Lipoid® acid degradation [40]. This is consistent with our results on LNC presenting a good gastrointestinal stability, whatever the mean particle size. All formulations are stable in acidic medium at 2 h and in intestinal medium at 6 h whatever fasted and fed state, we suppose that LNC could be used as a gastro-resistant device successfully in the pharmaceutical industry.

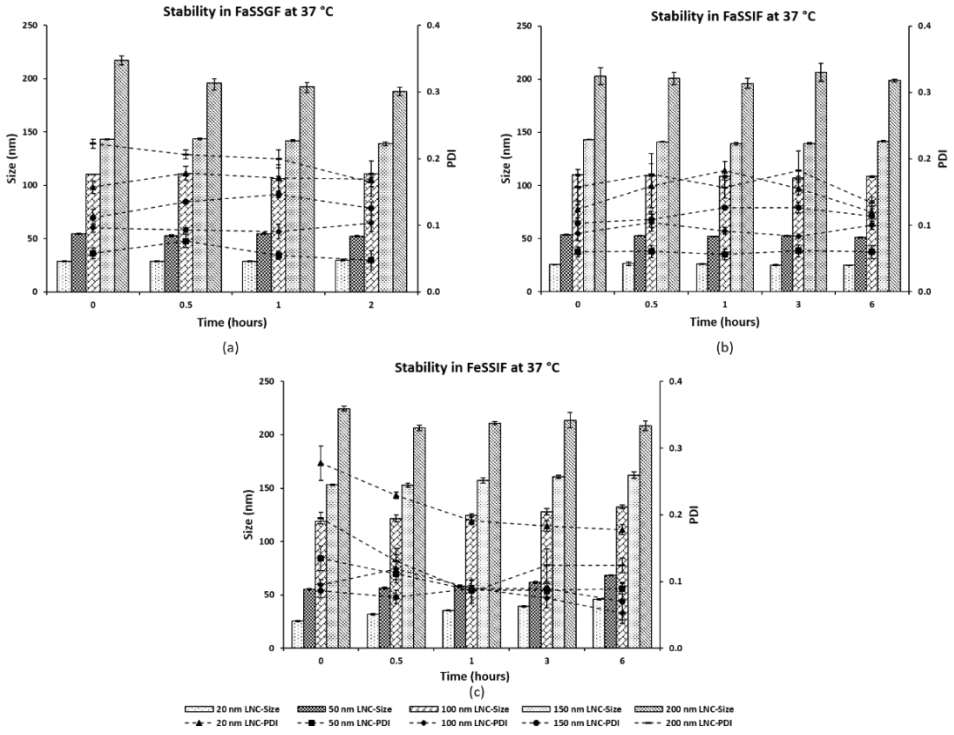

Figure 1. Variation in particle size and PDI of LNC with different size from 25 nm to 200 nm following incubation in FaSSGF (a), FaSSIF (b) and FeSSIF (c) at predetermined time intervals (mean ± SEM; N=3, n=3). The particle size of all nanocapsules was not significantly altered (p>0.05) and all LNC exhibited monodispersity (PDI<0.3) after predetermined incubation time.

To assess the stability of the LNC in culture medium, we evaluated the size stability of the LNC in DMEM-GlutaMAX medium (37 °C, 2 h). After the 2 h incubation time, the mean particle size was not significantly altered and presented monodispersity (PDI <0.15), whatever the formulation (Fig. S1).

III.3. GLUTAG CELLS VIABILITY STUDIES

To assess the *in vitro* safety and cytocompatibility of the LNC, a cell viability study was performed using the MTT assay in GLUTag cells (1 mg/mL to 10 mg/mL LNC concentrations). As shown in Fig. 2, the cytotoxicity of LNC was size-dependent. Small particle diameter nanocapsules (<150 nm) appeared to be more toxic than large ones. Smaller particle sizes require a higher amount of surfactant (Solutol® HS15) in order to stabilize their large surface area. This is consistent with the composition of the formulations described in Table 1. The amounts of Solutol® HS15 decreased with increased 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 [41, 42]. Consequently, small LNC have more chances to interact with the cell membrane, thus triggering adverse responses [20]. Overall, the size of LNC appeared to affect their cytotoxicity, being dependent on the size and the surfactant concentration. All formulations exhibited no significant cytotoxicity (cell viability over 80 %) at 2 mg/mL.

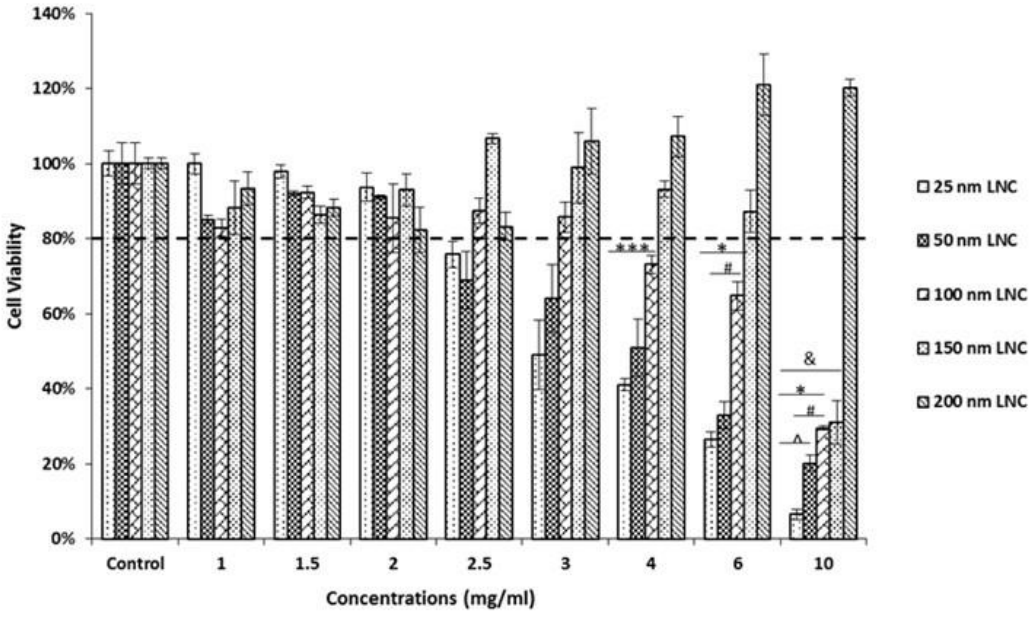

Figure 2. Cell viability of 25, 50, 100, 150 and 200 nm LNC on GLUTag cells at tested different concentrations (1 mg/mL to 10 mg/mL) (mean ± SEM; N=3, n=3). $^{\wedge}p<0.05$ compared to 25 nm and 50 nm groups; $^{*}p<0.05$ and $^{***}p<0.001$ compared to 25 nm and 100 nm LNC groups; $^{\&}p<0.05$ compared to 25 nm and 150 nm groups; $^{\#}p<0.05$ compared to 50 nm and 100 nm groups.

III.4. SIZE EFFET ON GLP-1 SECRETION

To elucidate whether the size of LNC exerted an effect on the secretion of GLP-1 by L cells, LNC (range 20 nm to 200 nm) were co-incubated with GLUTag cells for 2 h and the GLP-1 levels were measured using an ELISA kit. As shown in figure 3, among all the tested formulations, only the 200 nm size LNC increased significantly the secretion of GLP-1 compared to untreated cells (*p<0.05). This result is in line with our previous study, where we described that 50 nm size LNC failed to increase GLP-1 secretion by GLUTag cells [14]. However, 200 nm size LNC seemed to be efficient at inducing the activation of L cells and the secretion of GLP-1. This result suggests that, in the case of LNC, the particle size does play an important role in the ability of the nanocapsules to trigger GLP-1 secretion. Interestingly, both the extracellular and the intracellular concentrations appeared to be also size-dependent (Fig. 3). The extracellular GLP-1 concentrations were significantly increased after the incubation with LNC presenting a size of 100, 150 and 200 nm (***p<0.001). Moreover, the intracellular concentrations of GLP-1 were significantly increased after the incubation with 50 and 100 nm LNC, when compared to the untreated group (*p<0.05). Furthermore, we found that the larger particle size was associated with a higher secretion of GLP-1 (i.e., extracellular GLP-1). Conversely, the intracellular GLP-1 levels were proportionally decreased according to the particle size.

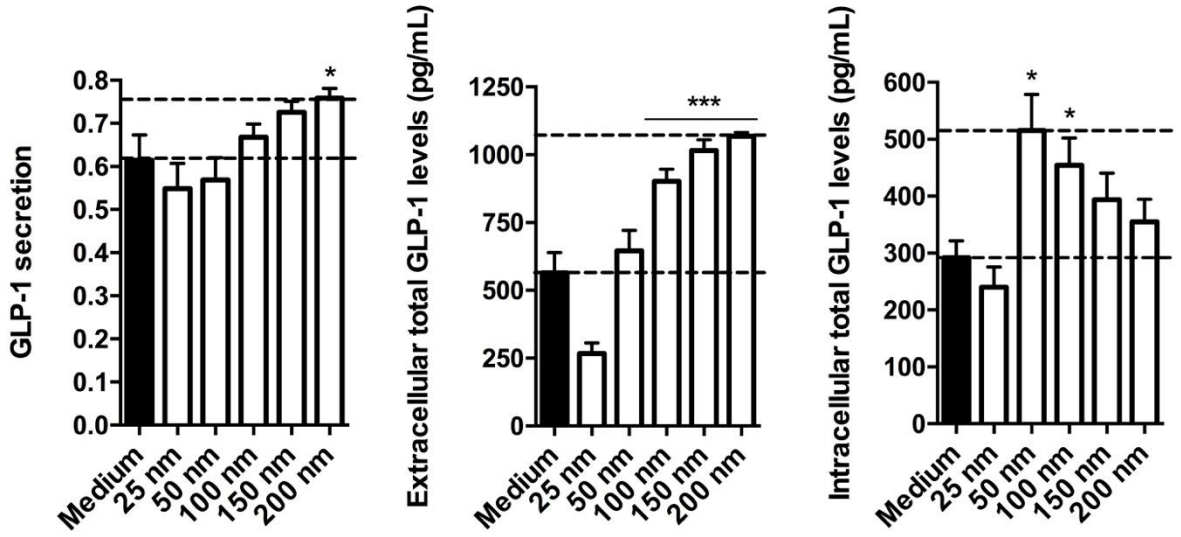

Figure 3. Effect of LNC (a range of size between 25 nm and 200 nm) on GLP-1 secretion after a 2 h co-incubation period (N=3, n=4; mean ± SEM). *p<0.05 and ***p<0.001 compared to the untreated control group.

To further confirm that LNC-mediated GLP-1 secretion was a nanoparticle-mediated effect and not a surfactant-mediated, we evaluated the influence of Solutol® HS15 concentrations on GLP-1 secretion. Solutol® HS15 final concentrations for each nanocapsule formulation are expressed in Table S2. Interestingly, the total GLP-1 secretions with the different Solutol® HS15 (HS15) concentrations were not significantly different (p>0.05) (Fig. 4). Although it was not possible to test Labrafac® alone due to solubility issues, our previous studies evaluating the effect of a caprylic/capric acid triglycerides mixture (Miglyol®) on GLP-1 secretion showed no effect on GLP-1 secretory levels [14]. Based on these data, one might conclude that GLP-1 secretion by LNC is size-mediated and is not a surfactant-mediated effect.

According to the above results, we found that only 200 nm LNC could induce GLP-1 secretion. Therefore, on the one hand, these results emphasize the importance of size on LNC-induced GLP-1 secretion. In addition, we cannot rule out the effect of the amount of lipids present in each nanocarrier, as the bigger the nanoparticle size, the bigger the core, loading in theory more lipids inside. On the other hand, they offer a chance that 200 nm LNC can be part of a larger gastro-resistant nanocarrier (Fig. 1), which liberates the content (in this case LNC) in order to have a permanent stimulation of GLP-1 secretion.

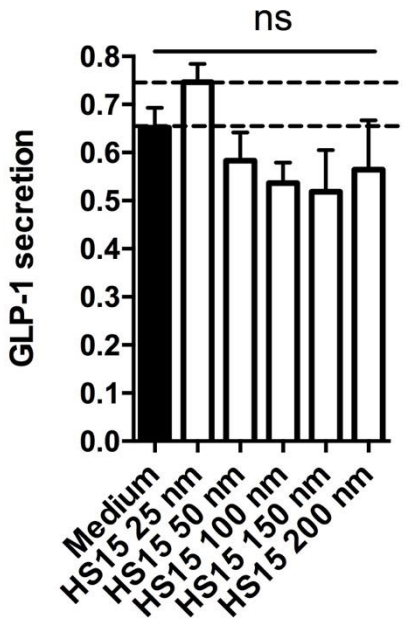

Figure 4. Effect of Solutol® HS15 (HS15) concentrations on the GLP-1 secretion (mean ± SEM; N=3, n=4).

III.5. MECHANISMS OF SIZE-MEDIATED GLP-1 SECRETION

III.5.1. Proglucacon expression in GLUTag cells

To elucidate whether the effect of LNC on the secretion of GLP-1 was due to an increased proglucagon synthesis, we quantified the proglucagon mRNA expression in GLUTag cells. As shown in Fig. 5, none of the different formulations did affect proglucagon mRNA expression. An increase in GLP-1 secretion in the absence of an increase in GLP-1 synthesis was previously reported [14, 43].

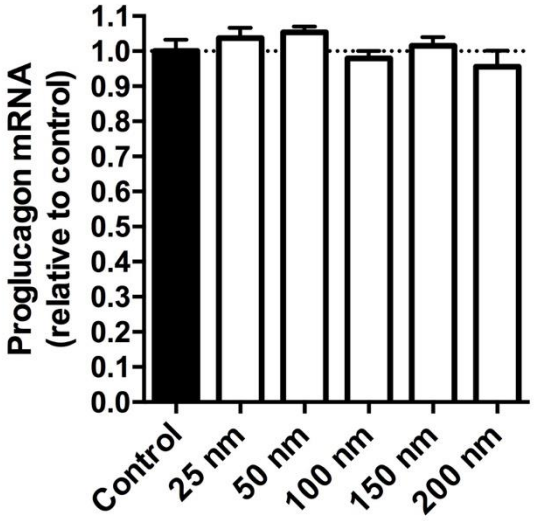

Figure 5. mRNA expression of proglucagon in control and LNC-treated cells (mean ± SEM; N=3, n=4).

III.5.2. Localization of nanocapsules within GLUTag cells

To unravel whether the LNC were located extracellularly or intracellularly, we visualized the association of DiD-labeled LNC of different sizes with GLUTag cells qualitatively by CLSM. The red fluorescence co-localized with cells, which confirmed that LNC entered into the GLUTag cells, increased with the size increasing compared with CLSM images corresponding to the location of DiD-labeled 100 nm LNC (Fig. S3), 150 nm LNC (Fig. S4) and 200 nm LNC (Fig. 6). Numerous 200 nm LNC were observed inside of GLUtag cells, while it was difficult to observe 50 nm LNC in the cells (data not shown). The co-localization results showed excellent correlation with the impact of different sizes on the secretion of GLP-1 by GLUTag cells (Fig. 3). After the incubation time, 200 nm DiD-labeled LNC were mainly located inside of the cells. Thus, one might hypothesize that the effect of 200 nm LNC in GLUTag cells after 2 h is due to LNC internalization rather than an interaction with the cell membrane. The influence of LNC on GLP-1 secretion could

be due to an increased fluidification of the membrane due to Solutol® HS15 molecules causing changes in membrane structure fluidity [44]. Solutol® HS15 can increase the flexibility of nanoparticles while inducing an effect on cell membrane increasing its permeability simultaneously [45]. Considering a single nanocarrier, the bigger the LNC size, the bigger the nanoparticle might present a higher density of PEG 660 and PEG 660 12-hydroxystearate around the surface of LNC. Thus, 200 nm LNC present the biggest surface area and the highest amount of Solutol® HS15. This increased membrane fluidification could explain the increased internalization of 200 nm LNC into L cells. This strongly argue that the mechanism of activation of GLP-1 secretion by LNC (intracellular) is different from the mechanisms followed by NLC (extracellular [14]), and therefore constitute a new and interesting mode of stimulation of GLP-1 secretion.

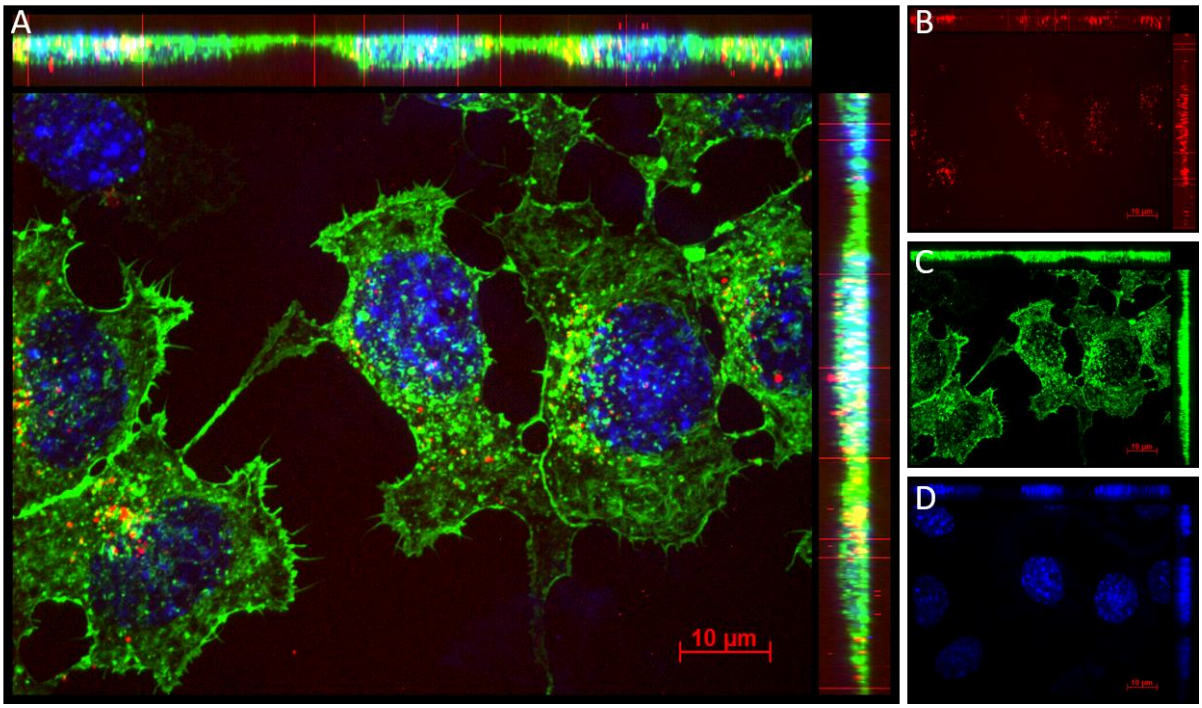

Figure 6. Localization of 200 nm DiD-labeled LNC within GLUTag cells. (A) Merged image of *y-z*, *x-z* and *z-y* sections; (B) DiD-labeled LNC in red; (C) cell cytoskeletons stained in green; and (D) cell nuclei stained in blue (N=3, n=4).

III.6. *IN VIVO* STUDIES

To evaluate the *in vivo* efficacy of 200 nm LNC at inducing endogenous GLP-1 secretion, we administered the nanocapsules to normoglycemic mice. As shown in Fig. 7, the total GLP-1 levels in LNC-treated mice were found to be 4- and 3-fold higher compared to untreated mice 60 min and 180 min after the administration, respectively. This result confirmed the efficacy of LNC at inducing endogenous GLP-1 *in vivo*.

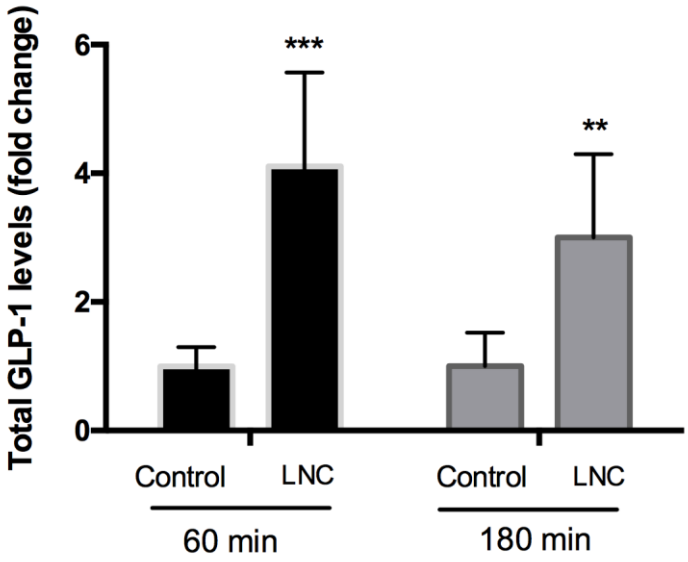

Figure 7. Total GLP-1 levels in normoglycemic mice 60 and 180 min after the oral administration of LNC (n=8; mean ± SD). ** $p<0.01$ and *** $p<0.001$ compared to the untreated control group.

Although further *in vivo* experiments regarding mechanism studies (e.g. the different concentrations of 200 nm LNC and the mixtures of components in formulation), and in diseases models (e.g. acute and chronic diabetic mice,) are crucial to deeply understand the efficacy of this nanocapsule, this proof-of-concept study was enough to demonstrate that it is possible to induce GLP-1 secretion *in vivo* using lipid-based nanocarriers.

IV.CONCLUSION

In summary, we rationally developed LNC presenting five different particle sizes (25, 50, 100, 150 and 200 nm) by altering the proportion of the different components of the formulation. The resulting LNC presented adequate colloidal stabilities. Moreover, the cytotoxicity of LNC was found to be size-dependent. Among all formulations, we found that 200 nm LNC appeared as the most potent and successful LNC able to increase GLP-1 secretion, thus confirming our hypothesis that particle size plays an important role on LNC-induced GLP-1 secretion by L cells. *In vivo*, 200 nm LNC significantly increased endogenous GLP-1 secretion in normoglycemic mice (4-fold higher levels compared to untreated mice). Although further *in vivo* experiments in disease animal models are essential to evaluate the efficacy of this nanocapsules, our work demonstrates that these nanocapsules may represent an innovative alternative approach in the context of T2DM treatment where GLP-1 must be administered via injection.

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VI.SUPPLEMENTARY MATERIALS

Table S1: Composition of the biomimetic media FaSSGF, FaSSIF and FeSSIF.

Composition	FaSSGF Concentration (mM)	FaSSIF Concentration (mM)	FeSSIF Concentration (mM)
Sodium taurocholate	0.08	3	15
Lecithin	0.02	0.75	3.75
Sodium chloride	34.2	34.2	203.18
Sodium monobasic phosphate anhydrous	/	28.65	/
Sodium hydroxyde	/	8.71	101.02
Acetic acid	/	/	144.04
Pepsin	0.1 mg/ml	/	/
pH	1.6	6.5	5

Table S2: Solutol® HS15 concentrations for each LNC formulation.

LNCs Size (nm)	25	50	100	150	200
Concentration of Solutol® HS15 per nanocapsule formulation (mg/ml)	113.00	49.44	33.91	31.15	17.08
Concentration of Solutol® HS15 in 2 mg/ml LNCs dispersed in DMEM	1.36	0.89	0.64	0.58	0.27

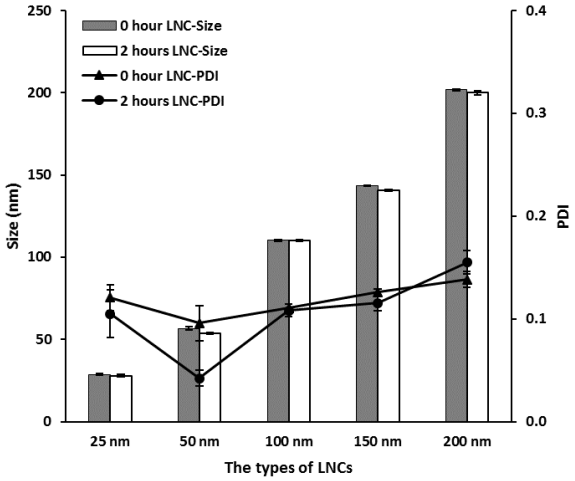

Figure S1. Variation in particle size and PDI of LNC with different sizes, from 20 nm to 200 nm, following a 2 h co-incubation time in DMEM GlutaMAX medium (mean ± SEM; n=3).

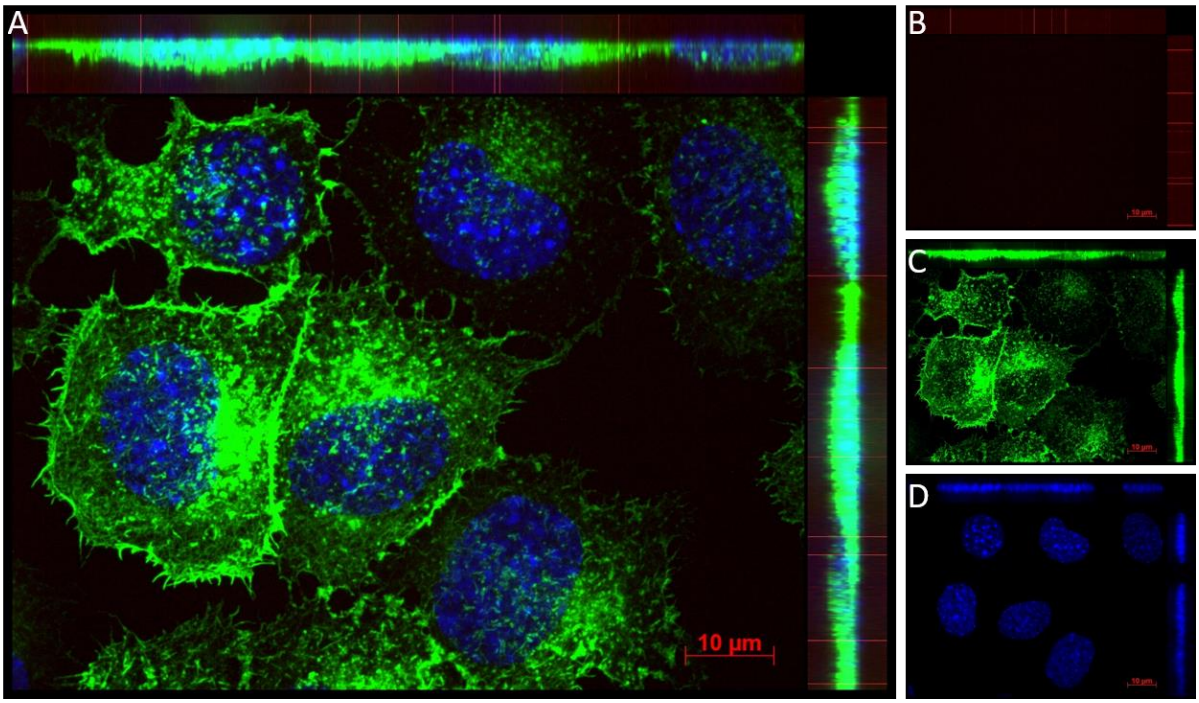

Figure S2. CLSM images of untreated GLUTag cells. (A) Merged image of *y-z*, *x-z* and *z-y* sections; (B) red filter; (C) cell cytoskeletons stained in green; and (D) cell nuclei stained in blue (n=4).

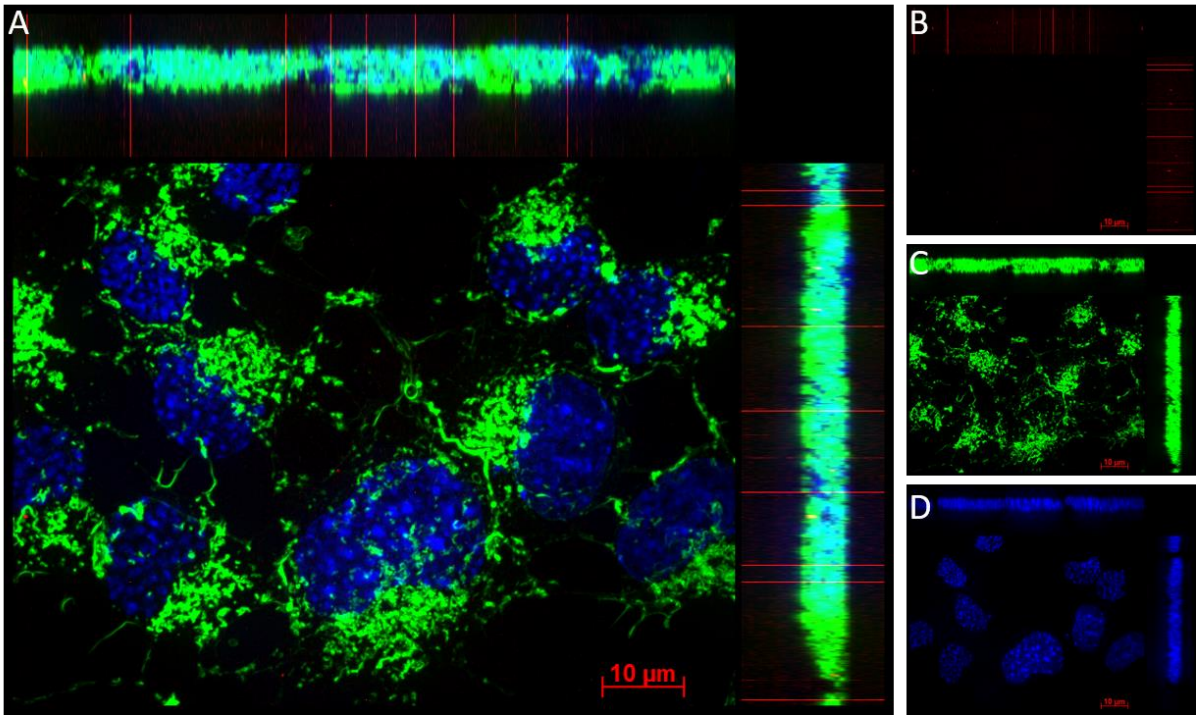

Figure S3. CLSM images of GLUTag cells after 2 h of co-incubation with 100 nm DiD-labeled LNCs.. (A) Merged image of y-z, x-z and z-y sections; (B) DiD-labeled LNCs in red; (C) cell cytoskeletons stained in green; and (D) cell nuclei stained in blue (N=3, n=4).

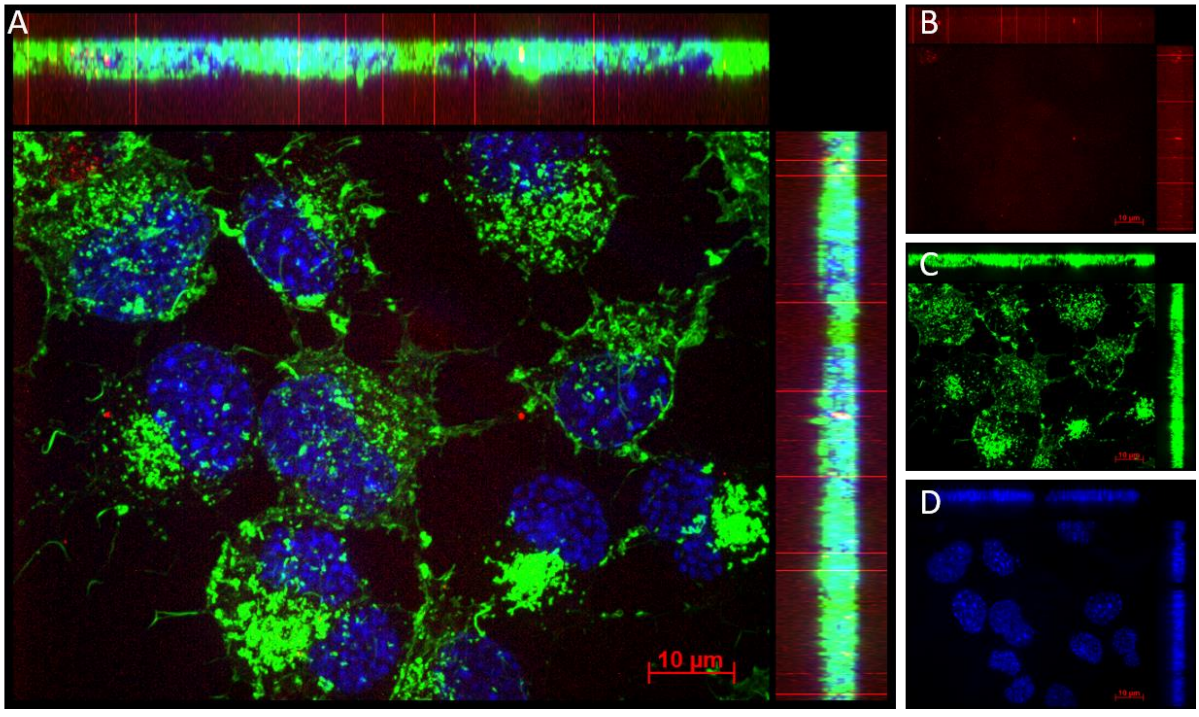

Figure S4. CLSM images of GLUTag cells after 2 h of co-incubation with 150 nm DiD-labeled LNCs. (A) Merged image of y-z, x-z and z-y sections; (B) DiD-labeled LNCs in red; (C) cell cytoskeletons stained in green; and (D) cell nuclei stained in blue (N=3, n=4)

CHAPTER IV

NOVEL STRATEGY FOR ORAL PEPTIDE DELIVERY IN INCRETIN-BASED DIABETES TREATMENT

Adapted from:

Xu Y, Van Hul M, Suriano F, Préat V, Cani P D, Beloqui A. Novel strategy for oral peptide delivery in incretin-based diabetes treatment. Gut 69: 911-919 (2020).

ABSTRACT

Objective: To fulfil an unmet therapeutic need for treating type 2 diabetes by developing an innovative oral drug delivery nanosystem increasing the production of Glucagon-Like Peptide-1 (GLP-1) and the absorption of peptides into the circulation.

Design: We developed a nanocarrier for the oral delivery of peptides using lipid-based nanocapsules. We encapsulated the GLP-1 analog exenatide within nanocapsules and investigated *in vitro* in human L-cells (NCl-H716) and murine L-cells (GLUTag cells) the ability of the nanosystem to trigger GLP-1 secretion. The therapeutic relevance of the nanosystem *in vivo* was tested in high-fat diet (HFD)-induced diabetic mice following acute (one administration) or chronic treatment (5 weeks) in obese and diabetic mice.

Results: We demonstrated that this innovative nanosystem triggers GLP-1 secretion in both human and murine cells models as well as *in vivo* in mice. This strategy increases the endogenous secretion of GLP-1 and the oral bioavailability of the GLP-1 analog exenatide (4% bioavailability with our nanosystem).

The nanosystem synergizes its own biological effect with the encapsulated GLP-1 analog leading to a marked improvement of glucose tolerance and insulin resistance (acute and chronic). The chronic treatment decreased diet-induced obesity, fat mass, hepatic steatosis, together with lower infiltration and recruitment of immune cell populations and inflammation.

Conclusion: We developed a novel nanosystem compatible with human use that synergizes its own biological effect with the effects of increasing the bioavailability of a GLP-1 analog. The effects of the formulation were comparable to the results observed for the marketed subcutaneous formulation. This nanocarrier-based strategy represents a novel promising approach for oral peptide delivery in incretin-based diabetes treatment.

KEYWORDS

Nanocarriers; diabetes; GLP-1; steatosis; bioavailability; oral peptide delivery

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I. INTRODUCTION

The development of oral dosage forms that enable the absorption of therapeutic peptides into the systemic circulation is one of the greatest challenges for the pharmaceutical industry [1]. Selected diabetes peptides have transitioned to the late phase of development despite their low oral bioavailability (estimated to be between 0.5-1.0%) [2]. Nevertheless, the benefits of oral peptide delivery over an intravenous or subcutaneous administration are remarkable, and especially in the case of anti-diabetic drugs, e.g., glucagon-like peptide-1 (GLP-1) [3]. The oral administration of incretin mimetic peptides has the additional therapeutic advantage of simulating the normal physiological pathway of the native peptide [4]. GLP-1 agonists target the liver, which can be accessed in much higher concentrations via the hepatic portal vein than via subcutaneous delivery, thereby reducing systemic exposure and its associated side effects [5]. Despite the numerous ongoing efforts to exploit the oral route of administration of incretin mimetic peptides, only two GLP-1 analogs are currently administered orally in clinical trials [1, 3], and both of these require coadministration with functional excipients [3].

The gut physiology offers a stimulating environment, which has not yet been fully exploited to its full potential in the drug delivery field. Enteroendocrine L-cells have attracted particular interest because of the pleiotropic effects of their secreted peptides (e.g., GLP-1 and GLP-2) [6]. These cells are attractive targets for the treatment of diseases such as type 2 diabetes (T2DM) and inflammatory bowel diseases (IBD) [7]. In the context of T2DM, GLP-1 secreted from intestinal L-cells stimulates postprandial insulin secretion and is quickly hydrolyzed by dipeptidyl peptidase-IV (DPP-IV) [8]. Thus, several GLP-1 analogs with improved plasma half-life (e.g., exenatide, semaglutide) have been developed and proven to be successful for treating T2DM. Recently, researchers have turned their attention from the secreted peptides towards the L-cells themselves. Indeed, enhancing endogenous GLP-1 secretion would represent a novel alternative in incretin-based diabetes therapy [9-11]. Here we hypothesized that using nanocarriers targeting intestinal L-cells could represent an alternative therapeutic strategy to stimulate the production of gut peptides. Nanocarriers could be engineered to simulate certain ligands and can be designed to have increased gastrointestinal retention, thereby evoking long-term activation of L-cells [6]. In this context, a major breakthrough of our research was proving the ability of lipid-based nanocapsules to trigger endogenous GLP-1 secretion *in vivo* after oral administration in mice [12-14]. However, despite these encouraging results and the promising properties attributed to nanomedicines, we need to prove that these novel formulations perform better than standard formulations incorporating functional excipients in oral peptide delivery.

Here, we report an innovative nanocapsule-based drug delivery system that synergizes its own biological effect (stimulation of GLP-1 release) and that of the encapsulated bioactive molecule (exenatide) as an alternative strategy for the treatment of T2DM and hepatic steatosis via an oral route.

II. MATERIALS AND METHODS

II.1. MATERIALS

Labrafac® WL 1349 (caprylic/capric acid triglycerides) and Peceol® (oleic acid mono-, di- and triglycerides) were gifts from Gattefossé (Saint-Priest, France). Lipoid® S 100 (soybean lecithin at 94% of phosphatidylcholines) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Solutol® HS15 (mixture of free PEG 660 and PEG 660 12-hydroxystearate, Mw 870 Da) and Span® 80 (sorbitan oleate) were purchased from Sigma-Aldrich (St. Louis, USA). Sodium chloride (NaCl), lecithin, sodium taurocholate, pepsin, 3-(4,5- dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Triton-X 100 were purchased from Sigma-Aldrich (St. Louis, USA). Exenatide was purchased from Bachem (Bubendorf, Switzerland). Total GLP-1 (ver.2) and active GLP-1 assay kits were purchased from Meso Scale Discovery (Maryland, USA). An Exendin-4 enzyme immunoassay kit was purchased from Phoenix Europe GmbH (Karlsruhe, Germany). An Ultrasensitive Mouse Insulin ELISA Kit was purchased from Mercodia AB (Uppsala, Sweden). Matrigel™ was obtained from BD Bioscience (Belgium). Dipeptidyl peptidase IV (DPP-IV) inhibitor was purchased from Millipore (St. Charles, USA). Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX (5.5 Mm glucose), Roswell Park Memorial Institute (RPMI)-1640 medium, penicillin-streptomycin (P/S), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin (0.25%) containing ethylenediaminetetraacetic acid (EDTA, 0.02%) were also used and purchased from Thermo Fisher Scientific (Invitrogen, Belgium). All chemical regents utilized in this study were of analytical grade.

II.2. PREPARATION AND CHARACTERIZATION OF REVERSE MICELLE-LOADED LIPID NANOCAPSULES

II.2.1. Preparation of reverse micelle-loaded lipid nanocapsules

Reverse micelle-loaded lipid nanocapsules (RM-LNC) were formulated in two steps, in which the drug was first encapsulated within reverse micelles and then further encapsulated within LNC. First, exenatide-loaded reverse micelles (EXE-RM) were prepared by high-speed stirring of a surfactant (Span® 80) and an oil (Labrafac® WL 1349) mixture (1:5 weight ratio). Then, 50 μL of EXE (30 mg/mL in MilliQ water) was dripped into the mixture and maintained under stirring. Exenatide-loaded reverse micelle lipid nanocapsules (EXE-RM-LNC) were prepared following a modified phase inversion process described by Heurtault *et al* [15]. Briefly, all components (shown in Table 1, Fig. 1C), including lipophilic Labrafac®WL 1349, Peceol®, Lipoid®S100, Solutol®HS15, sodium

chloride (NaCl) and MilliQ water, were mixed together under magnetic stirring at 40 °C at 200 rpm for 5 min. Temperature cycles of progressive heating/cooling were conducted between 50 °C and 67 °C. During the last cycle, 500 µL of preheated drug loaded RM was added to the mixture at ~3 °C above the phase inversion zone (PIZ; 59 to 61.5 °C). The solution was cooled to reach the phase inversion zone (PIZ) temperature, and 2.5 mL of cold water (4 °C) was added and stirred at high speed for 2 min. Blank RM-LNC were prepared following the same protocol in the absence of exenatide.

II.2.2. 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. [13] Briefly, a Kinetex® EVO C18 column (100 Å, 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 0.05% (v/v) trifluoroacetic acid (TFA) in water, and the organic mobile phase consisted of 0.05% (v/v) in acetonitrile. A gradient system was developed with an initial ratio of 10:90 (v/v, aqueous:organic phase) at a flow rate of 1 mL/min, which was linearly changed to 90:10 (v/v) over 10 min and kept constant for the next minute. Then, the ratio was linearly changed to the initial composition in the next 1.5 min 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 were 1.1 ± 0.4 µg/mL and 3.3 ± 1.1 µg/mL, respectively.

II.2.3. Characterization of EXE RM LNC

EXE RM LNC were characterized by measuring their particle size and polydispersity index (PDI) by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was determined by laser Doppler velocimetry (LDV) using a Zetasizer Nano ZS. For the measurement, 5 µL of nanocapsule suspension was dispersed in 995 µL of ultrapure water. All measurements were performed in triplicate.

EXE RM LNC were also characterized based on their drug encapsulation efficiency (EE, %). 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 min) (Millipore). Filtrates were further diluted using a 1:2 dilution factor. The exenatide in the filtrate and the exenatide dissolved

in methanol were quantified using the above-described HPLC method. The EE was calculated using the following equation:

EE (%) = (total amount of exenatide – free exenatide) / (total amount of exenatide) ×100

II.3. NANOCAPSULE STABILITY AND DRUG RELEASE IN SIMULATED GASTROINTESTINAL FLUIDS

II.3.1. In vitro stability studies

The *in vitro* stability of EXE LNC was tested in five different biomimetic media: Fasted State-Simulated Gastric Fluid (FaSSGF) with and without pepsin, Fasted State-Simulated Intestinal Fluid (FaSSIF), Fed State-Simulated Intestinal Fluid (FeSSIF) and FeSSIF version 2 (FeSSIF-V2) (biorelevant.com, UK). A detailed description of the composition of the simulated fluids used is presented in the Supplementary Material in Table S1. The influence of gastric and intestinal conditions on nanocapsule stability was evaluated based on the nanocapsule size and the PDI. EXE RM LNC were incubated in FaSSGF with and without pepsin, FaSSIF, FeSSIF and FeSSIF-V2 at 37 °C (100 µL of nanocapsules in 10 mL of media) under gentle stirring. At predetermined time intervals (0, 0.5, 1 and 2 h for stimulated gastric media and 0, 0.5, 1, 3 and 6 h for stimulated intestinal media and FeSSIF), samples were withdrawn and then analyzed by DLS.

II.3.2. In vitro drug release studies

The drug release from EXE RM LNC was evaluated in FaSSGF in the absence of pepsin and in FaSSIF media for 2 h and 6 h, respectively. 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 35 mL of medium at 37 °C under magnetic stirring. At predetermined times, 50 µL of sample was withdrawn and dissolved in 950 µL of methanol. The concentration of exenatide was determined by the HPLC, as described above.

II.4. *IN VITRO* CELL STUDIES

II.4.1. Cell cultures

The human NCI-H716 L cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and was used from passages 15 to 20. The completed media was composed of Roswell Park Memorial Institute (RPMI) 1640 medium with 1% (v/v) penicillin−streptomycin

(P/S) and 10% (v/v) fetal bovine serum (FBS). Cells were suspended growth in 75 cm^2 flasks (Corning, Lowell, MA, USA) in an atmosphere of 5% CO_2/95% air (v/v) at 37°C. Some fresh medium was added every other day. After replacement of the medium, cultured were centrifuged and subsequently resuspended at a proper density.

The intestinal murine L cell line GLUTag was kindly provided by Dr. Daniel Drucker (University of Toronto, Canada). GLUTag cells were used from passages 16 to 29. Cells were grown in DMEM GlutaMAX (5.5 mM glucose) supplemented with 10% (v/v) inactivated FBS and 1% (v/v) P/S (complete DMEM medium) at 37 °C with 5% CO_2 supply. Cells were subcultured using trypsin (0.25%) containing EDTA (0.02%) every 4-5 days.

Caco-2 cells (clone-1) were kindly provided by Dr. Maria Rescigno (University of Milano-Bicocca, Milano, Italy) and used from passages 25 to 30. The Caco-2 cell line was maintained in medium consisting of DMEM supplemented with 10% (v/v), HyClone™ FBS, 1% (v/v) L-Glutamine, 1% (v/v) nonessential amino acids and 1% (v/v) P/S at 37 °C in an atmosphere of 10% CO_2/95% air (v/v). The medium was replaced every other day.

II.4.2. Cytotoxicity studies

In vitro cytotoxicity studies of EXE RM LNC were performed in Caco-2 cells based on the drug and nanocapsule concentration calculated as previously described using 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay [16]. The influence of unloaded RM LNC on cell viability was also tested in GLUTag and NCI-H716 cells. Caco-2 cells (5×10^4 cells per well) were seeded in 96-well tissue culture plates (Costar Corning CellBIND Surface, USA) and were allowed to adhere overnight. For cytotoxicity studies in GLUTag and NCI-H716, 5×10^4 cells per well were seeded on Matrigel™-coated (10 µL/mL of medium) 96-well plates. After washing the plates with prewarmed PBS buffer (x3), 100 µL of EXE RM LNC at increasing drug concentrations (0.5-10 mg/mL), corresponding to increasing nanocapsule concentrations (2-18 mg/mL), were dispersed in DMEM (without FBS) and coincubated with Caco-2 cells for 2 h at 37 °C. Increasing unloaded RM LNC concentrations (from 1 mg/mL to 10 mg/mL) were dispersed in DMEM GlutaMAX or RPMI-1640 medium (without FBS) and coincubated with GLUTag or NCI-H716 cells, respectively, at 37 °C for 2 h. After incubation, the supernatants were replaced by 100 µL of 0.5 mg/mL MTT for 3 h. The purple formazan crystals were dissolved in 200 µL of DMSO for absorbance determination at 560 nm using a MultiSksan EX plate reader (Thermo Fisher Scientific, USA). Cells with Triton-X 100 (100% dead) and cells with culture

medium (100% alive) were considered positive and negative controls, respectively. Tests were performed in triplicate.

II.4.3. In vitro GLP-1 secretion

GLUTag and NCI-H716 cells (1.8×10^5 cells per well) were seeded onto Matrigel™-coated 24-well cell culture plates and allowed to adhere for 24 h. The day after, the plate was gently washed using prewarmed PBS. Then, the GLUTag cells were coincubated with DMEM GlutaMAX without FBS or unloaded RM LNC. On the other hand, NCI-H716 cells were incubated with RPMI-1640 medium without FBS and unloaded RM LNC. Both media contained DPP-IV inhibitor at a final concentration of 50 μM (Millipore, St. Charles, MO, USA). To confirm the efficacy of our nanocapsules compared with previously tested nanocapsules [14], we used a 2 mg/mL nanocapsule concentration. After 2 h of incubation at 37 °C, supernatants were collected, centrifuged at 250 g for 5 min at 4 °C (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) and preserved at – 80 °C until further analysis. Cells were collected in PBS in the presence of DPP-IV inhibitor. Cell extracts containing GLP-1 were obtained after three freeze-thaw cycles followed by centrifugation at 250 g for 5 min at 4 °C. Total GLP-1 concentration was determined using a Total GLP-1 ELISA kit (Meso Scale Delivery, Gaithersburg, USA). GLP-1 secretion is expressed as the amount of GLP-1 detected in the supernatants plus cells. GLP-1 secretion was calculated by the following equation:

$$\text{GLP-1 secretion} = C_{extracellular} / (C_{intracellular} + C_{extracellular})$$

where $C_{extracellular}$ is the concentration of GLP-1 tested in the supernatants, and $C_{intracellular}$ is the concentration of GLP-1 tested in cells.

II.5. *IN VIVO* STUDIES

II.5.1. Animals

All animal experiments were approved by and performed in accordance with the local animal committee (2014/UCL/MD/033 and 2017/UCL/MD/005) and as specified by the Belgian Law of 29 May 2013 on the protection of laboratory animals.

II.5.2. Total GLP-1 secretion in normoglycemic mice

Normoglycemic mice (C57BL/6J male mice, 20–25 g, 10 weeks; Janvier Laboratories, France) were randomly divided into two groups with 8 mice each. Animals were fasted overnight before the experiments with free access to water. Mice were treated with blank RM LNC corresponding to

~1.62 mg/g nanoparticle dose. Control mice were treated by oral gavage with an equivalent volume of MilliQ water. Blood samples were withdrawn from the tip of the tail vein at 60 and 180 min after oral administration. Samples were collected in the presence of DPP-4 inhibitor (20 µL per mL of blood) and maintained on ice. Immediately after the study, blood samples were centrifuged (3,000 rpm, 10 min at 4 °C), and the plasma was kept frozen at −80 °C until analysis. The total GLP-1 levels were quantified using a Total GLP-1 ELISA kit. (Meso Scale Delivery, USA). The total GLP-1 plasma values are expressed as the fold-change compared with the untreated control group.

II.5.3. Oral glucose tolerance test in high-fat diet induced obese/diabetic mice

Eight-week-old male mice were housed 5 per cage and divided into 5 groups (10 mice per group). After 2 weeks of acclimation, mice were fed a high-fat diet (60% fat and 20% carbohydrates (kcal per 100 g), D12492i, Research Diets, USA) (HFD and exenatide-treated groups) or a normal chow diet (control, AIN93Mi, Research Diets, USA) for 3, 8 or 10 weeks before experiments and underwent a period of fasting overnight before being treated with an oral exenatide solution (EXE, 500 µg of exenatide/kg body weight), exenatide-reverse micelle-loaded lipid nanocapsules (EXE RM LNC, 500 µg of exenatide/kg body weight) or unloaded micelle-loaded lipid nanocapsules (RM-LNC, equivalent concentrations as per EXE-RM-LNC) 1 h before being challenged with an oral glucose gavage. Control groups (control diet and HFD groups) were treated with an oral gavage of an equivalent volume of sterile MilliQ water. After 1 h, mice were challenged with an oral glucose gavage (2g/kg glucose dose), as previously described by Everard et al [17]. Blood samples were collected at -30 min and 15 min to test total GLP-1 and insulin plasma concentration by using ELISA kits (Meso Scale Delivery, USA and Mercodia, Uppsala, Sweden, respectively). The insulin resistance index was determined by multiplying the area under the curve of both blood glucose and insulin in the plasma obtained by the oral glucose tolerance test (OGTT).

II.5.4. Pharmacokinetics study in normoglycemic and obese/diabetic mice

Eight-week-old male mice were randomly divided into three groups (10 mice per time point) and housed in a controlled environment (room temperature of 23 ± 2 °C, 12 h daylight cycle) with free access to food and sterile water. After two weeks of acclimation, mice underwent 3 weeks of HFD (60% fat). Prior to the experiments, mice were fasted overnight with free access to sterile Milli-Q water. Exenatide in solution and EXE-RM-LNC were orally administered at a 500 µg/kg dose. Exenatide was also subcutaneously administered at a 50 µg/kg dose. At different time points (0, 0.5, 1, 1.5, 2, 4, 6 and 8 h), blood samples were collected from the tip of the tail vein. Blood samples

were then centrifuged (1,500 *g*, 10 min at 4 °C), and exenatide plasma concentration was quantified using an ELISA kit (EK-070-94, Phoenix Europe GmbH, Karlsruhe, Germany). The relative bioavailability (FR %) of exenatide was calculated using the following equation:

$$FR(\%) = \frac{\mathrm{AUC_{oral}} \times \mathrm{Dose_{s.c.}}}{\mathrm{AUC_{s.c.}} \times \mathrm{Dose_{oral}}} \times 100$$

The pharmacokinetic parameters were analyzed using PKSolver [18]. In the case of normoglycemic mice, 4 mice per time point were used instead.

II.5.5. Chronic exenatide long-term treatment study in obese/diabetic mice

Eight-week-old male mice were randomly divided into 7 groups (10 mice per group) and housed 5 per cage in a controlled environment (room temperature of 23 ± 2 °C, 12 h daylight cycle) with free access to sterile food (AIN93Mi; Research diet) and sterile water. After a two-week acclimation period, mice underwent 8 weeks of HFD or a normal chow diet (Control). After this period, mice were treated daily at 4 pm (i) orally with exenatide in solution or encapsulated within RM-LNC (500 µg/kg dose) (EXE-RM-LNC) or the corresponding concentration of unloaded RM-LNC or (ii) subcutaneously with exenatide in solution (10 µg/kg) (EXE s.c.) or as Byetta™ (10 µg/kg). Control groups (healthy and HFD) were treated orally daily with an equivalent volume of sterile Milli-Q water. At the end of the treatment period, animals were anesthetized with isoflurane (Forene, Abbott, England), and blood was sampled from both the portal and the cava veins. After exsanguination, mice were euthanized by cervical dislocation. Subcutaneous adipose tissue, liver and spleen were precisely dissected, weighed, and immediately immersed in liquid nitrogen followed by storage at -80°C for further analysis or preserved in 4% paraformaldehyde (PFA) for histological analysis (liver). Total lipids were measured after extraction with chloroform-methanol according to a modified Folch method [19], as previously described [17]. Triglyceride and cholesterol concentrations were measured using a kit coupling an enzymatic reaction and spectrophotometric detection of the final product (Diasys Diagnostic and System, Holzheim, Germany). All samples were run in duplicate.

Effects on body composition and adipose tissue were evaluated by the weights of subcutaneous (SAT), epididymal (EAT), visceral adipose tissues (VAT) and brown adipose tissues (BAT) (mg). Levels of glucose and gastrointestinal hormones involved in food intake and body weight, including total GLP-1 (ELISA kit, Meso Scale Delivery, Gaithersburg, USA) and insulin (Ultrasensitive insulin ELISA, Mercodia, Uppsala, Sweden), were measured in peripheral blood and portal vein blood. Liver steatosis was visualized by oil red O staining. Liver tissue was embedded in Tissue-

Tek Optimal Cutting Temperature compound (Sakura Europe, Leiden, Netherlands) and flash-frozen in cold isopentane. Five-micrometer-thick tissue sections were stained with oil red O staining for lipid content analysis. Five high-magnification fields (20x) were analyzed per mouse. Quantification of the mean droplet area was performed using ImageJ software (Version 2.0.0-rc-69/1.52i, National Institutes of Health, Bethesda, Maryland, USA). The general morphology of the liver was assessed by hematoxylin and eosin (H&E)-stained sections. For the real-time quantitative PCR (qRT-PCR) analysis, the total RNA was isolated from tissues using TriPure reagent (Roche). Complementary DNA was prepared by reverse transcription of 1 μg total RNA using a Reverse Transcription System kit (Promega, Madison, Wisconsin, USA). Real-time PCR was performed with a CFX96 real-time PCR system and CFX Manager 3.1 software (Bio-Rad, Hercules, California, USA) using GoTaq® qPCR Master Mix (Promega, Madison, USA) for detection according to the manufacturer's instructions. Ribosomal protein L19 (*Rpl19*) was chosen as the housekeeping gene. All samples were run in duplicate in two 96-well reaction plates, and data were analyzed according the $2-\Delta\Delta CT$ method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of the amplification. Primer sequences for the targeted mouse genes are presented in supplementary table 5.

II.6. STATISTICAL ANALYSIS

GraphPad Prism 7 program (CA, USA) was used to perform the statistical analyses. For all analyses and for each group, any exclusions were supported by the use of the Grubbs test for outlier detection. Values were normalized by log-transformation when variances were significantly different between groups before conducting the analysis. Two-way or one-way ANOVA followed by Tukey's post hoc test was applied for comparisons among multiple groups. If the variance differed significantly between groups even after normalization, a nonparametric test was performed. The results are expressed as the mean ± standard error of the mean (SEM). A difference of $P<0.05$ was considered statistically significant.

III. RESULTS

III.1. EXENATIDE IS SUCCESSFULLY ENCAPSULATED AND PRESERVED WITHIN LIPID BASED NANOCAPSULES

We selected lipid-based nanocapsules (LNC) as nanocarriers for the oral delivery of peptides. We recently discovered that these nanocapsules triggered endogenous GLP-1 secretion *in vivo* in mice when presenting a size of ~ 200 nm [14]. Therefore, we hypothesized that synergizing the biological effect of LNC together with the pharmacological effects of an encapsulated GLP-1 analog could be highly innovative. As a proof of concept, we selected the GLP-1 analog exenatide (EXE) as the incretin mimetic to be encapsulated within LNC containing reverse micelles (RM-LNC). The final composition of the formulation is described online in supplementary table 1, and the physico-chemical characterization is detailed online in table 2 and a schematic representation of EXE RM LNC is depicted in Fig. S1. Notably, EXE-RM-LNC exhibited an entrapment efficiency of ~ 85%. It is worth noting that we already considered the potential translational approach; therefore, we have conceptually chosen scalable preparation procedures and *generally recognized as safe* (GRAS) excipients that could be directly transferred to the pharmaceutical market.

Lipid nanocapsules remained stable and prevented exenatide degradation *in vitro* in biomimetic gastrointestinal fluids (Fig. S2). The stability of the nanocapsules was confirmed in five biomimetic media, including fasted- and fed-state simulated gastric or intestinal fluids (FaSSGF with or without pepsin, FaSSIF, FeSSIF and FeSSIF-v2, respectively) (Fig. S2a-e). A detailed description of the composition of the biomimetic media used is described online in the supplementary table 3.

These results confirmed that the newly developed nanocapsules retained the same gastro-resistant properties as previously described conventional LNC [14, 20]. The *in vitro* exenatide release profile was further evaluated in gastric medium (FaSSGF without pepsin, pH 1.6) and intestinal medium (FaSSIF, pH 6.5). Exenatide was progressively released from the RM-LNC over a 6-h period, reaching a cumulative exenatide release of 60% after this time in FaSSIF, which was undetectable in FaSSGF (Fig. S2f).

III.2. RM-LNC INDUCE GLP-1 SECRETION BOTH IN VITRO AND IN VIVO AND INCREASE EXE BLOOD LEVELS IN NORMOGLYCEMIC MICE

First, we investigated the ability of RM-LNC on their ability to trigger GLP-1 secretion *in vitro* in both murine L-cells (GLUTag cells) and in human L-cells (NCl-H716) (Fig. 1A). We found that

RM LNC of particle size ~ 220 nm were able to trigger endogenous GLP-1 secretion in both *in vitro* models (Fig. 1A).

Second, we investigated whether RM-LNC could trigger GLP-1 secretion *in vivo* in normoglycemic mice (Fig. 1B). Notably, the pharmacological effect was preserved *in vivo* because following the oral administration of RM-LNC, we found that the GLP-1 levels were increased up to ~ 3-fold. Therefore, the pharmacological effect of the nanocarriers was reproduced *in vitro* in both cell lines irrespective of the nature of the cells (murine or human) and *in vivo* in normoglycemic mice.

To validate our hypothesis that RM-LNC not only trigger the endogenous secretion of GLP-1 but also act as a dual-action nanocarrier for the oral delivery of incretin mimetic peptides, we needed to prove the ability of the nanocarriers to increase their absorption. For this purpose, we measured exenatide plasma concentration upon oral administration as a solution in water or encapsulated within RM-LNC (500 μg/kg exenatide dose) (Fig. 1C). Exenatide plasma levels were higher when encapsulated within RM-LNC than when delivered as a solution. Altogether, these data confirmed our hypothesis regarding the efficacy of RM-LNC at both enhancing endogenous GLP-1 levels and increasing EXE plasmatic levels.

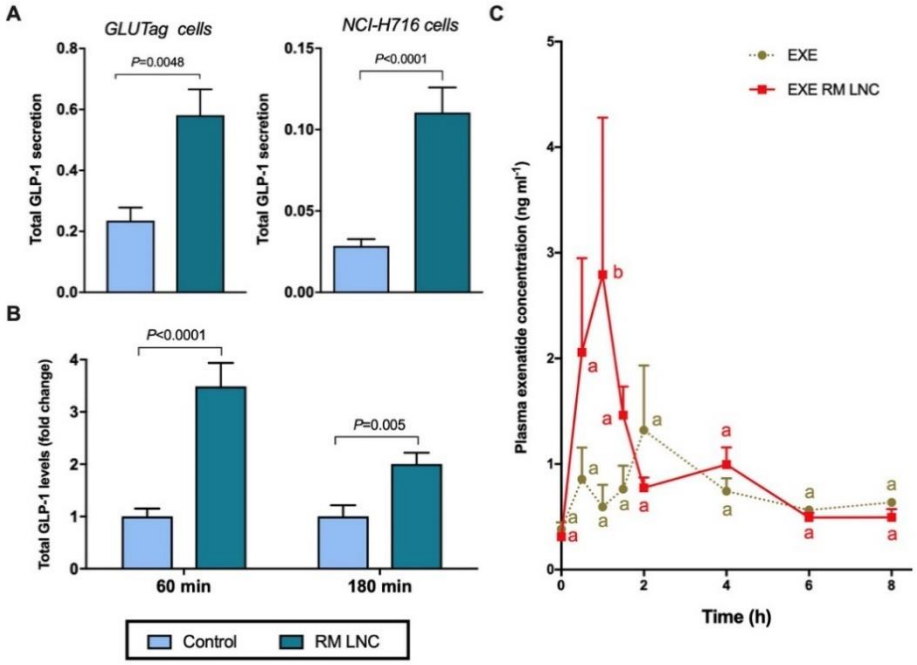

Figure 1. RM-LNC-mediated GLP-1 secretion *in vitro* and *in vivo* and exenatide plasma levels in normoglycemic mice. (A) RM-LNC-mediated *in vitro* GLP-1 secretion (2 mg/mL) in GLUTag (left) and NCI-H716 (right) cells (murine and human L-cells, respectively) after a 2-h coincubation period (mean ± SEM; n=6-10). (B) *In vivo* total GLP-1 secretion in normoglycemic mice 60 and 180 min after RM LN oral administration (mean ± SEM; n=7-8). (C) Exenatide blood profile after oral administration to normoglycemic mice in solution in water (EXE) or encapsulation within RM LNC (EXE RM LNC) (500 μg/kg exenatide dose) (mean ± SEM; n=4). *P* values in (A, B) were determined by Student's *t* test or Mann-Whitney test. Data with different superscript letters (C) are significantly different *(P*<0.05) according to two-way ANOVA followed by Tukey's post hoc test.

Complementary data on GLP-1 extracellular and intracellular levels measured in murine and human L-cells *in vitro* are supplied in supplementary figure 3. Of note, we found no evidence of cytotoxicity in human intestinal Caco-2 cells or NCI-H716 cells (FIG. S4a-b and Fig. S4c and S4d).

III.3. THE COMBINATION OF ENDOGENOUS GLP-1 RELEASE WITH INCREASED EXENATIDE PLASMA LEVERS IMPROVES GLYCEMIA IN DIABETIC MICE

The therapeutic relevance regarding the combination of nanocarrier-mediated endogenous GLP-1 levels with increased exenatide plasma concentration was evaluated in a diet-induced obese/diabetic mouse model. As a proof-of-concept study, we tested EXE-RM-LNC in a murine high-fat diet (HFD)-induced T2DM model (Fig. 2) following acute treatment (one single administration). First, we confirmed that the HFD mice were markedly hyperglycemic and hyperinsulinemic in fasted state (Fig. 2A and 2D) and displayed a strong degree of insulin resistance (e.g., index of insulin resistance) (Fig. 2F).

We administered once orally a 500 µg/kg exenatide dose (free and encapsulated within RM-LNC) and equivalent concentrations of RM-LNC or water 60 min prior to an oral glucose administration (2 g/kg) (corresponding to the time point of -60 min in Fig. 2A), this time point is based on the increased secreted GLP-1 levels measured in normoglycemic mice (Fig. 1B).

Strikingly, we found that EXE-RM-LNC treatment completely normalized the glycemia, as glycemia in these mice followed the same profile throughout the overall oral glucose challenge as that observed in lean normoglycemic control mice (Fig. 2A).

Conversely, the glucose levels measured in the EXE-treated mice remained similar to those in the HFD-fed mice at 30 min and then remained higher than those in the EX-RM-LNC-treated mice until 90 min (Fig. 2A). EXE-RM-LNC were able to significantly decrease plasma glucose levels and glucose area under the curve (AUC) (Fig. 2A). Importantly, we found that total GLP-1 levels were significantly increased in both RM-LNC and EXE-RM-LNC-treated groups compared with the control groups (Control and HFD), confirming the ability of the nanosystem *per se* to stimulate GLP-1 release under pathological conditions (Fig. 2B).

Importantly, only EXE-RM-LNC significantly reduced the insulin resistance index compared with the HFD group (Fig. 2C-E).

Interestingly, RM-LNC alone had an effect on lowering blood glucose levels compared with untreated HFD mice. However, as we anticipated, this effect was not sufficient as per reducing the hyperglycemia. A pharmacokinetic study measuring exenatide levels in HFD mice confirmed that

exenatide blood levels were significantly increased when orally administered within RM-LNC (EXE RM-LNC) compared with exenatide in solution (relative bioavailability 4.32% with LNC, $P<0.001$). This relative bioavailability can be considered a high value and a great improvement, considering that peptides presenting between a 0.5-1% bioavailability have progressed to the late phase of development [2]. The calculated pharmacokinetic parameters are summarized online in supplementary table 4. We observed a different exenatide pharmacokinetic profile (e.g., different C_{max}, AUC, T_{max}) in normoglycemic (Fig. 1C) versus HFD mice (Fig. 2F).

Taking together the increased exenatide blood levels and the increased endogenous GLP-1 levels, these data serve as a strong proof of concept regarding the effectiveness of our nanosystem in ameliorating T2DM symptoms.

Based on the present data, we decided to further expand our findings by investigating the impact of EXE versus EXE-RM-LNC in mice treated for 8 weeks and 10 weeks with HFD. This model is a stronger model of diet-induced diabetes and metabolic disorders in mice. It is important to highlight that we obtained equivalent results regarding the efficacy of EXE-RM-LNC on the reduction of blood glucose levels during OGTT, regardless of the chronicity of the disease (Fig. S5).

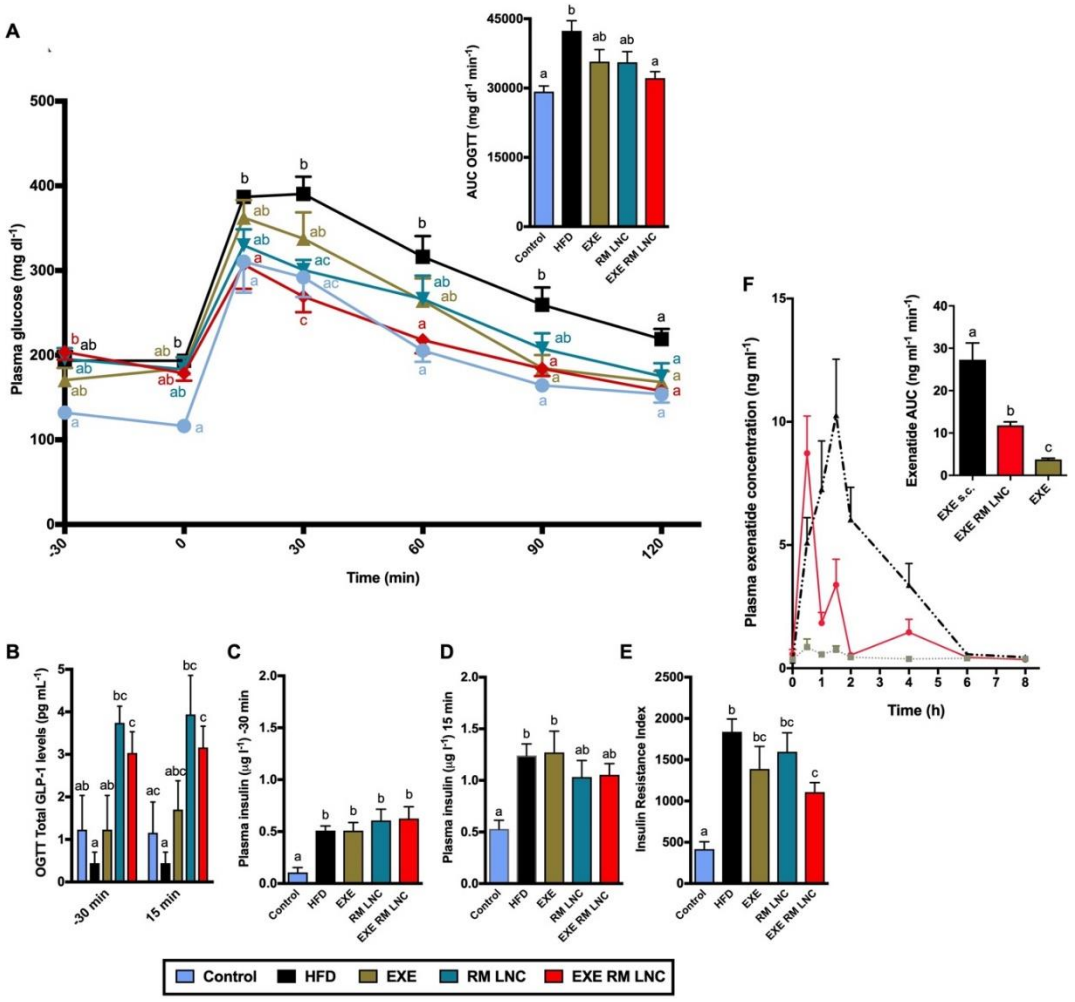

Figure 2. Pharmacodynamic and pharmacokinetic evaluation of EXE-RM-LNC in a HFD-induced obese/diabetic mouse model following acute treatment. (A) Plasma glucose levels (mg dl^{-1}) measured 30 min before and 120 after glucose challenge (n= 8-9) and mean area under the curve (AUC, mg dl^{-1} min^{-1}) measured 30 min before and 120 after glucose challenge (n= 8-9). (B) Plasma total GLP-1 levels and (C, D) plasma insulin levels measured 30 min before and 15 min after glucose challenge (n= 8-10). (E) Insulin resistance index (n= 8-9). (F) Concentration-time profile and AUC of exenatide after subcutaneous administration (EXE s.c.) (50 µg/kg exenatide dose) and oral administration, in solution and within RM-LNC (EXE and EXE-RMLNC, respectively) (500 µg/kg exenatide dose). Data are presented as the mean ± SEM (n=8-10). Data with different superscript letters (A, B) are significantly different ($P<0.05$) according to a two-way ANOVA followed by Tukey's post hoc test or (C-F) according to a one-way ANOVA followed by Tukey's post hoc test.

III.4. CHRONIC EXE-RM-LNC LONG TERM TREATMENT IMPROVES GLUCOSE METABOLISM IN OBESE AND DIABETIC MICE

To evaluate the impact of chronic and long-term treatment with EXE-RM-LNC on glucose metabolism, we induced obesity and type 2 diabetes in mice by treating them for 8 weeks with a HFD, then we continued the HFD for 5 weeks and treated the mice with daily administration of 500 µg/kg exenatide (oral) (EXE-RM-LNC) or equivalent amounts of unloaded nanocapsules (RM-LNC) or free exenatide (EXE) or water. To evaluated the impact of our experimental

treatment with existing treatment strategies including a group treated with a marketed subcutaneous form of exenatide, Byetta®. The oral administration of exenatide was compared to the subcutaneous injection (s.c.) of 10 μg/kg exenatide in solution or as Byetta®.

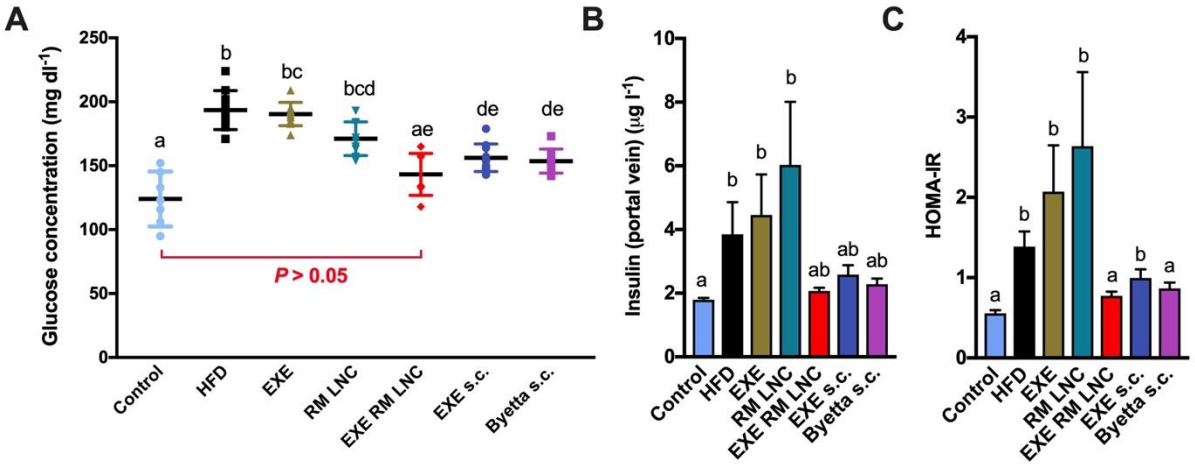

Figure 3. Effect of EXE-RM-LNC on glucose homeostasis and hyperinsulinemia in obese/diabetic mice. (A) Plasma glucose levels (mg dl^{-1}) after 5 weeks of treatment (13 weeks of HFD feeding). Data are presented as the mean ± SEM (n=10). (B) Insulin plasma levels measured from the portal vein (n=8-10). Data are presented as the mean ± SEM. Data with different superscript letters are significantly different *(P*<0.05). (C) HOMA-IR was calculated using the equation [fasting glucose (mg/dL) x fasting insulin (ng/mL)]/405 as previously described.[21] Data are presented as the mean ± SEM (n=8-10). *P* values in (A) were determined by a two-way ANOVA followed by Tukey's post hoc test. *P* values in (B, C) were determined by Kruskal-Wallis followed by Dunn's post hoc test.

After 5 weeks of daily treatment, mice were sacrificed, and blood was drawn from both the portal and the cava vein. Plasma glucose and insulin levels are depicted in Fig.3A and 3B, respectively. Interestingly, after 5 weeks of treatment, only the EXE-RM-LNC were able to decrease plasma glucose levels to reach levels comparable to that of the control group (Fig. 3A). It is noteworthy that EXE-RM LNC-treated mice had significantly lower plasma glucose levels than the RM-LNC-treated mice. Plasma glucose levels were also significantly lower in the RM-LNC group than in the EXE-treated. Therefore, these data demonstrate the synergistic effect provided by EXE-RM-LNC on glucose homeostasis. This effect is likely due to the combination of the endogenous GLP-1 secretion triggered by RM-LNC and elevated exenatide plasma levels. Additionally, EXE-RM-LNC-treated and subcutaneously treated mice exhibited comparable insulin levels as the control group. Although this reduction compared with the HFD group did not reach statistical significance when the data were analyzed by the Kruskal-Wallis followed by Dunn's post hoc test (Fig. 3B), a significant difference was found when analyzed by the Mann-Whitney test (*P*=0.04 for EXE-RMLNC versus HFD). HFD induce a massive insulin resistance (measured by HOMA-IR) which was completely normalized with the EXE-RM-LNC, an effect which was therapeutically equivalent to the marketed drug (Byetta®) based on GLP-1 (Fig. 3C).

III.5. CHRONIC TREATMENT WITH EXE-RM-LNC DECREASES DIET-INDUCED STEATOSIS IN OBESE/DIABETIC MICE

We found that the liver weight was only significantly lower in the EXE-RM-LNC-treated group after 5 weeks of treatment (13 weeks of HFD feeding) compared with the HFD-fed group (Fig. 4A). Histological analysis by H&E and oil red O staining revealed a marked decrease in hepatic steatosis, as evidenced by lower hepatic lipid accumulation and by fewer and smaller lipid droplets in EXE-RM-LNC-treated mice than in HFD mice (Fig. 4B and 4C). Despite the minor impact on triglyceride levels, hepatic total lipid content and cholesterol levels were comparable between EXE-RM-LNC-treated and subcutaneously treated mice and were significantly lower than those in the HFD group (Fig. 4D-F).

It is of the utmost importance to note that our innovative approach using EXE-RM-LNC was more efficient in decreasing liver weight than all the other treatments and was efficient as the marketed drug by the typical administration route (s.c.) (Byetta®), thereby clearly showing the better effects on glucose parameters and a noninferiority on liver markers compared with the subcutaneous approach.

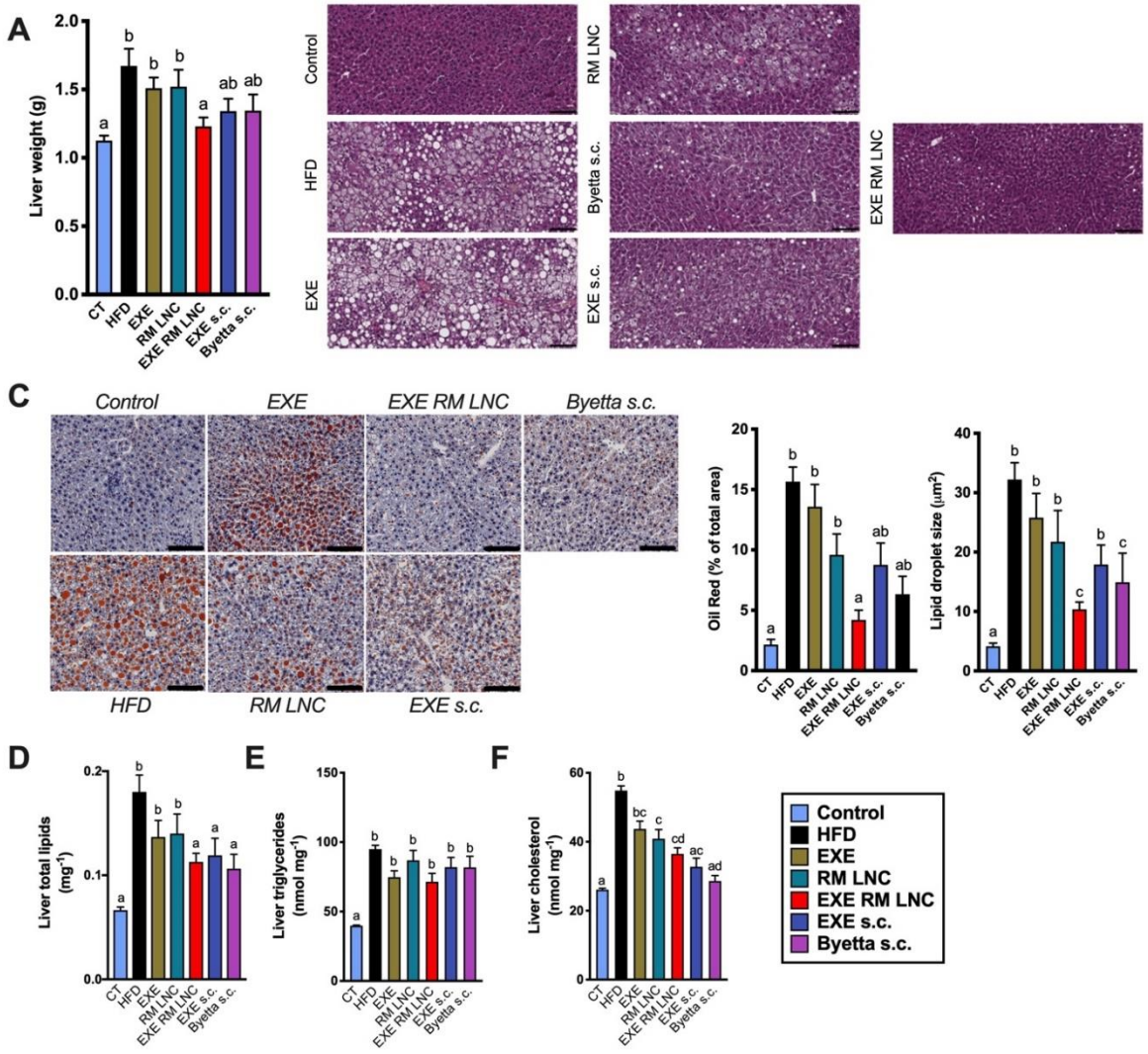

Figure 4. EXE-RM-LNC treatment impacts lipid homeostasis. (A) Liver weight (g). (B) Morphology of the liver in H&E-stained sections following a chronic/long-term EXE treatment in diabetic and obese mice. Representative liver H&E staining (scale bar: 100 µm). (C) Representative liver oil red O staining (scale bar: 100 µm), oil red O staining expressed in % of area stained and mean lipid droplet size (µm², n = 5–7). (D) Liver total lipid content (mg^{-1} per 100 mg of tissue). (E) Liver triglycerides (nmol mg^{-1}). (G) Liver cholesterol (nmol mg^{-1}). Data with different superscript letters (A-F) are significantly different ($P<0.05$). *P* values in (A) were determined following one-way ANOVA with Tukey's post hoc test. *P* values in (C-F) were determined by the Kruskal-Wallis test followed by Dunn's post hoc test.

In addition to the biochemical and histological analysis, key markers associated with infiltration/recruitment of immune cell populations (*F4/80, Cd11c, Mcp1*), inflammation (*Tnfa*) and lipid metabolism (*Fasn, Pparg, Cpt1a*) were analyzed by quantitative PCR both in the liver and in visceral adipose tissue (VAT). Although we encountered no significant differences in the mRNA expression of *F4/80, Cd11c, Mcp1, Tnfa, Fasn* and *Pparg* in the liver by ANOVA (Fig. 5B) (large number of groups being compared), we found significant differences by the Mann-Whitney test. In the liver, *Cd11c* and *Mcp1* mRNA expression in EXE-RM-LNC-treated mice was significantly lower than that in the HFD group ($P=0.05$ and $P=0.0355$, respectively), and the same effect was observed in the Byetta®-treated group ($P=0.0015$ and $P=0.0007$, respectively).

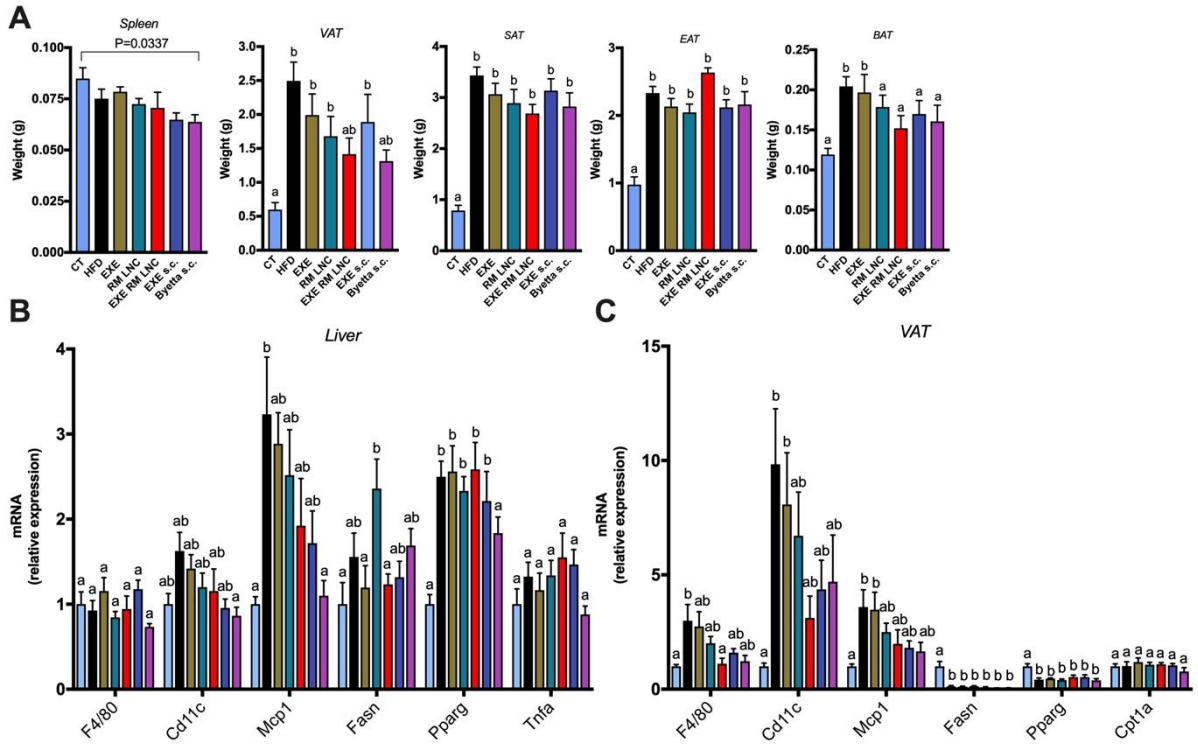

Figure 5. EXE-RM-LNC treatment impacts infiltration/recruitment of immune cell populations in the visceral adipose tissue. (A) Weights of the spleen, different white adipose tissues and brown adipose tissue (g). Data are presented as the mean ± SEM (n=9-10). (B) mRNA expression of *F4/80, Cd11c*, *Mcp1*, *Tnf*a, *Fasn*, and *Pparg* in the liver (n=9-10). (C) mRNA expression of *F4/80, Cd11c*, *Mcp1*, *Fasn*, *Pparg* and *Cpt1*a in visceral adipose tissue (n=9-10). Data with different superscript letters (A-C) are significantly different *(P*<0.05). *P* values in (A,C) were determined following one-way ANOVA with Tukey's post hoc test. *P* values in (B) were determined by a one-way ANOVA with Tukey's post hoc test or the Kruskal-Wallis test followed by Dunn's post hoc test.

Visceral fat mass is considered a risk factor for developing liver diseases and insulin resistance [22, 23]. Hence, we found that both the EXE-RM-LNC- and Byetta®-treated groups exhibited less fat than in HFD-fed mice, and there was not significant difference in the amount of fat in these groups from that in the untreated control group (Fig. 5A). Although their weights were not significantly different from the HFD group according to the Kruskal-Wallis analysis (large number of groups being compared), they were highly significantly different when analyzed by the Mann-Whitney test (*P*=0.0057 for EXE-RM-LNC versus HFD and *P*=0.0004 for Byetta® versus HFD). Interestingly, higher expression of *F4/80* observed in the HFD group was significantly downregulated only in EXE-RM-LNC-treated mice following ANOVA. No significant effects were observed for the other markers when compared with the HFD group (Fig. 4C).

IV.DISCUSSION

Despite numerous ongoing efforts, the transformation of injectable therapies for T2DM into oral drug delivery strategies remains a challenge. As a result, current treatments with GLP-1 analogs on the market are still administered exclusively subcutaneously. Current state-of-the-art strategies for oral peptide delivery use the delivery system merely as a vehicle, and none of them have explored the possibility that the carrier could have additional therapeutic effects on the final formulation. In the context of incretin-based diabetes disease therapy, the enhancement of endogenous GLP-1 secretion represents a novel alternative treatment that more closely resembles the physiology of the peptide [24, 25]. Here, we demonstrated the therapeutic effect of secreted endogenous peptides with encapsulated synthetic analogs within a lipid-based drug delivery system as an innovative approach for the oral delivery of peptides. Translational considerations have been conceptually integrated by choosing scalable preparation procedures and GRAS excipients that could be directly transferred into the pharmaceutical market.

We confirmed *in vitro*, both in murine and human L-cells, and *in vivo* in normoglycemic mice, the ability of reverse micelles-loaded lipid nanocapsules (RM-LNC) to induce GLP-1 secretion. We also found that drug-loaded nanosystems (EXE-RM-LNC) could stimulate GLP-1 secretion in *vitro* in murine L cells and *in vivo* in normal C57BL/6J mice (10 weeks-old) (data not shown). Additionally, we conducted a pharmacokinetic study confirming the absorption of exenatide into systemic circulation. After proving the stability of the formulation, the ability of the nanosystem to preserve peptide integrity, and its ability to increase GLP-1 levels *in vivo* while enabling the absorption of the peptide into systemic circulation, we demonstrated the efficacy of the formulation in the pathological context in a HFD-induced obese/diabetic mouse model.

The dual-action of the nanosystem for improving glycemia was observed *in vivo* in obese/diabetic mice following acute or chronic treatment. A single acute administration of the nanosystem normalized blood glucose levels, which were comparable to those of the control group. Although both empty and EXE-loaded nanocapsules presented increased GLP-1 levels compared with untreated groups, only EXE-RM-LNC-treated mice exhibited a significant decrease in the insulin resistance index. Therefore, suggesting an additional mechanism of action. A pharmacokinetic analysis in obese/diabetic mice confirmed that EXE-RM-LNC increased exenatide bioavailability to more than 4%. These data served as a strong proof-of-concept regarding the effectiveness of a dual-action drug delivery nanosystem in ameliorating glycemia by combining both increased endogenous GLP-1 levels and increased peptide bioavailability.

To demonstrate that this nanosystem represents a plausible alternative to current strategies for the oral delivery of incretin peptides in the treatment of T2DM, we conducted a chronic/long-term treatment consisting of a five-week daily administration protocol. After 5 weeks of treatment, EXE-RM-LNC-treated mice exhibited normalized plasma glucose levels comparable to those of untreated control mice, along with decreased insulin levels. Therefore, the beneficial effects observed are not limited to the acute effect of the nanocarriers but are also translated to a therapeutic effect on oral glucose tolerance.

It is worth noting that our approach led to comparable results regarding glucose homeostasis to those observed for the current marketed drug that is administered subcutaneously. Therefore, our results demonstrate the noninferiority of our approach together with the benefit of administration by the oral route for chronic treatments.

However, unlike subcutaneously treated mice, the metabolic effect observed for EXE-RM-LNC was decoupled from any effect on the total body weight (Fig. S6). However, as both EXE-RM-LNC and the subcutaneously administered EXE decreased the visceral adipose fat mass and, to some extent, the liver lipid content, we cannot rule out the possibility that the metabolic effects were due to an impact on these organs (e.g., visceral fat and liver lipid) rather than an overall change in body weight.

Our data point to a strong trend towards lower key markers associated with infiltration-recruitment of immune cell populations (macrophages, dendritic cells) and inflammatory markers in both the liver and visceral adipose tissue, again highlighting the beneficial effects of our approach on such markers. *F4/80* is a marker of inflammatory cell infiltration (mature macrophages), whereas *Cd11c*, *Mcp1* and *Tnfa* are known to reflect the M1 macrophage phenotype during obesity-associated inflammation. It is important to note that EXE-RM-LNC were the only treatment that significantly reduced macrophage infiltration in the visceral adipose tissue, a hallmark of chronic inflammation also considered as triggering factor for insulin resistance and diabetes[26, 27] [28]. From a mechanistic point of view, we previously showed that RM-LNC increase the secretion of GLP-1 and, likely, the co-peptide GLP-2, which has been shown to reinforce the gut barrier function, thereby reducing bacterial compound translocation, inflammation and steatosis in obese rodents [29]. Whether the lower inflammation observed here could depend on a similar mechanism is plausible but remains to be demonstrated.

The mechanisms by which GLP-1 levels are increased via lipid nanocapsules remain unclear; however, we may not rule out that the daily oral gavage stimulates the production of the gut peptides throughout the day and, therefore, contributes to maintaining a better metabolic profile

in the HFD-fed mice. Indeed, a daily chronic administration was sufficient to improve metabolism and even normalize other markers. Finally, we cannot exclude the possibility that lipid nanocapsules could modulate the activity of DPP-IV, thus preserving increased circulating total GLP-1 levels in the body, which warrants further investigation. Another important matter that will need further investigation is the potential toxicological impact of the formulation in the intestine upon the accumulation of the formulation following repeated doses. It would be interesting to compare the potential toxicological effect of our formulation (providing with GLP-1 levels cleaved by DPP-IV enzyme, and reduced GLP-1 analog doses) with the potential toxicological effect of the synthetic peptide administered alone, which has been modified not to be recognized by DPP-IV and therefore could be potentially accumulated in the body.

V. CONCLUSION

In conclusion, we developed an innovative approach using incretin mimetics via the oral route. We discovered that combining nanocarriers with GLP-1 analogs is sufficient to normalize the glycemia of obese/diabetic mice after either acute or chronic treatment. Interestingly, in addition to the strong advantage of using the oral route, this approach is at least as efficient as the current marketed drug as a comparison and could even be more potent for improving oral glucose tolerance, insulin resistance and hepatic steatosis. Thus, our strategy offers an additional advantage over current approaches for oral incretin mimetic peptide delivery and by increasing endogenous GLP-1 levels. All these developments could lead to enhanced clinical translation of nanomedicines in oral incretin-based T2DM treatment.

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VII. SUPPLEMENTARY MATERIALS

Table 1. Composition of 220 nm EXE RM LNC.

Composition	Reverse micelles (RM)	Lipid nanocapsules (LNC)
Drug solution (µL)	50	/
Span® 80 (mg)	100	/
Labrafac® WL 1349 (mg)	500	769.5
Peceol® (mg)	/	85.5
Lipoid® S100 (mg)	/	13.4
Solutol® HS15 (mg)	/	120
Sodium chloride (mg)	/	50
MilliQ water (µL)	/	1025
Cold MilliQ water (µL)	/	2500

Table 2. Physicochemical characterization of EXE-loaded or unloaded RM LNC (PDI: polydispersity index; EE: encapsulation efficiency).

Formulations	Mean Size (nm)	PDI	Zeta potential (mV)	EE (%)
EXE RM LNC	224.4 ± 3.2	0.185 ± 0.013	-3.06 ± 0.40	84.95±3.76
RM LNC	215.7 ± 3.3	0.165 ± 0.012	-2.993 ± 0.29	-

Table 3. Composition of biomimetic intestinal fluids.

Composition	FaSSGF	FaSSIF	FeSSIF	FeSSIF-V2
Sodium taurocholate	0.08	3	15	10
Lecithin	0.02	0.75	3.75	2
Glycerol monooleate	/	/	/	5
Sodium oleate	/	/	/	0.8
Sodium chloride	34.2	34.2	203.18	126
Sodium monobasic	/	28.65	/	/
Sodium hydroxide	/	8.71	101.02	82
Acetic acid	/	/	144.04	/
Maleic acid	/	/	/	55
Pepsin	0.1 mg/ml	/	/	/
pH	1.6	6.5	5	5.8

Table 4. Pharmacokinetic parameters of the *in vivo* study in obese/diabetic mice for s.c. exenatide (50 μg/kg), oral exenatide (500 μg/kg) and oral EXE RM LNC (500 μg/kg). *P* values were significantly different *(***P*<0.01 and ***P*<0.001) according to a one-way ANOVA followed by Tukey's post hoc test. Significance is expressed compared to the exenatide oral group.

Formulation	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-8} (ng · h/mL)	$t_{1/2}$	FR (%)
Exenatide s.c.	10.27 ± 6.82**	1.5	27.30 ± 12.46***	1.38 ± 0.36	100***
EXE LNC oral	8.73 ± 4.53**	0.5	11.79 ± 2.73***	1.68 ± 0.35	4.32 ± 0.95***
Exenatide oral	0.86 ± 0.93	0.5	3.71 ± 0.93	---	1.36 ± 0.34

Table 5. Primer sequences for gene expression analyses by RT-qPCR

	Forward primer	Reverse primer
Rpl19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
F4/80	TGACAACCAGACGGCTTGTG	GCAGGCGAGGAAAAGATAGTGT
Cd11c	ACGTCAGTACAAGGAGATGTTGGA	ATCCTATTGCAGAATGCTTCTTTACC
Mcp1	GCAGTTAACGCCCCACTCA	TCCAGCCTACTCATTGGGATCA
Tnfa	TCGAGTGACAAGCCTGTAGCC	TTGAGATCCATGCCGTTGG
Fasn	CAGGCCCCTCTGTTAATTGG	TCCAGGGATAACAGCACCTT
Pparg	CTGCTCAAGTATGGTGTCCATGA	TGAGATGAGGACTCCATCTTTATTCA
Cpt1a	AGACCGTGAGGAACTCAAACCTAT	TGAAGAGTCGCTCCCACT

Rpl19, ribosomal protein L19; *F4/80* (also known as Adgre1), egf-like module-containing, mucin-like, hormone receptor-like1; *Cd11c* (also known as Itgax), clusters of differentiation; *Mcp1* (also known as Ccl2), monocyte chemotactic protein 1; *Tnfa*, tumor necrosis factor, alpha; *Fasn*, fatty acids synthase; *Pparg*, peroxisome proliferative activated receptor, gamma; *Cpt1a*, carnitine palmitoyltransferase 1, alpha.

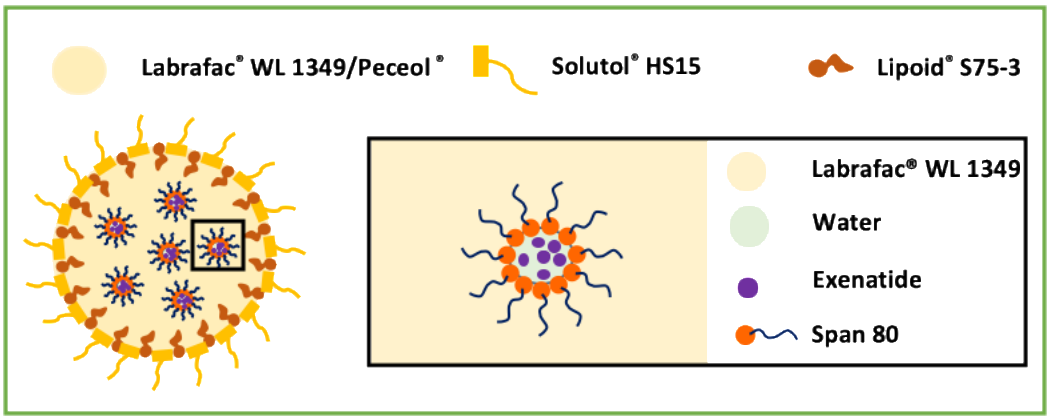

Figure S1. A schematic representation of EXE RM LNC.

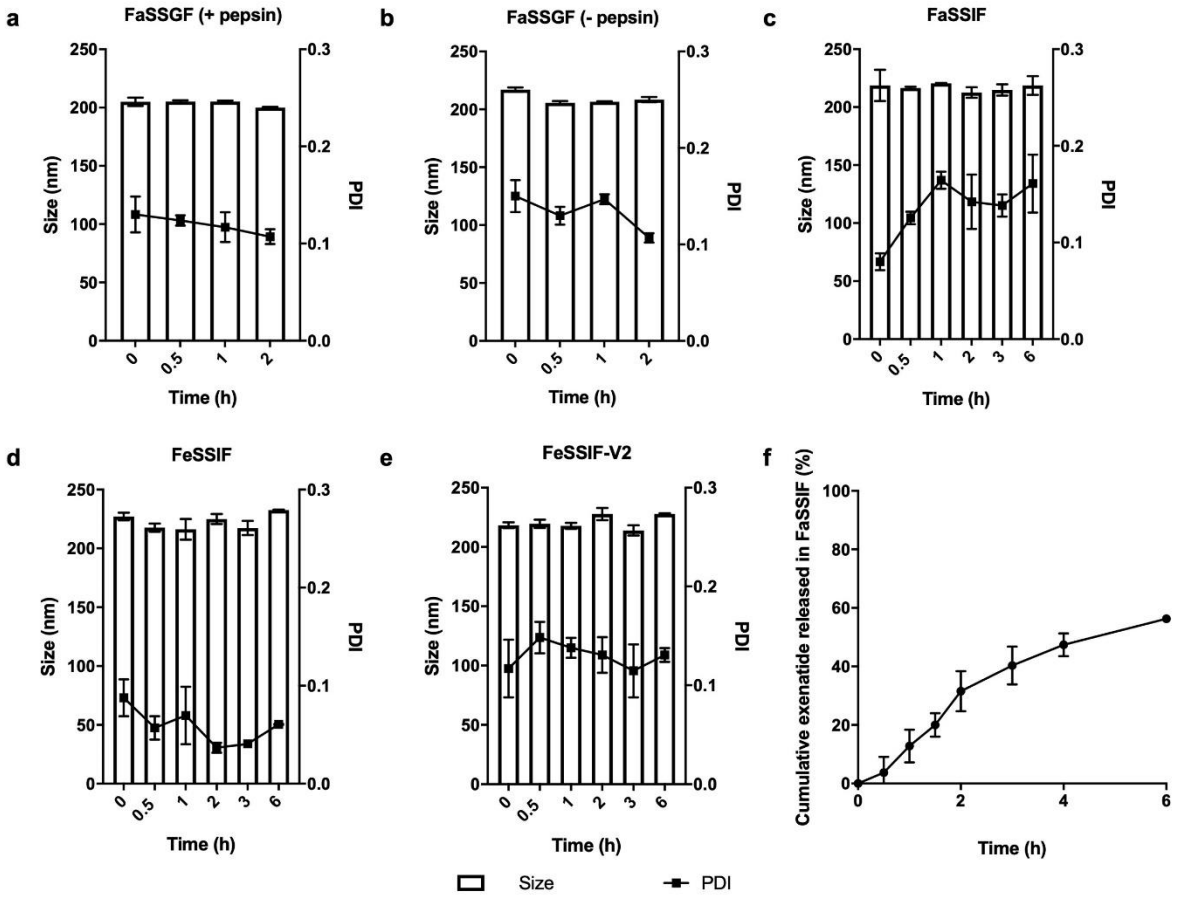

Figure S2. Nanocapsule stability and exenatide release in biomimetic intestinal fluids. Particle size and PDI of EXE RM LNC variation following incubation in FaSSGF in the absence **a** and in the presence of pepsin **b**, in FaSSIF **c**, in FeSSIF **d** and in FeSSIF-V2 **e** at predetermined time intervals (mean ± SEM; n=9). The particle size of the EXE RM LNC was not significantly altered in any case ($P>0.05$) and exhibited monodispersity in the assayed media (PDI<0.2). **f** Cumulative exenatide release profile in FaSSIF (pH 6.5) at 37 °C over 6 h as measured by HPLC (mean ± SEM; n=9).

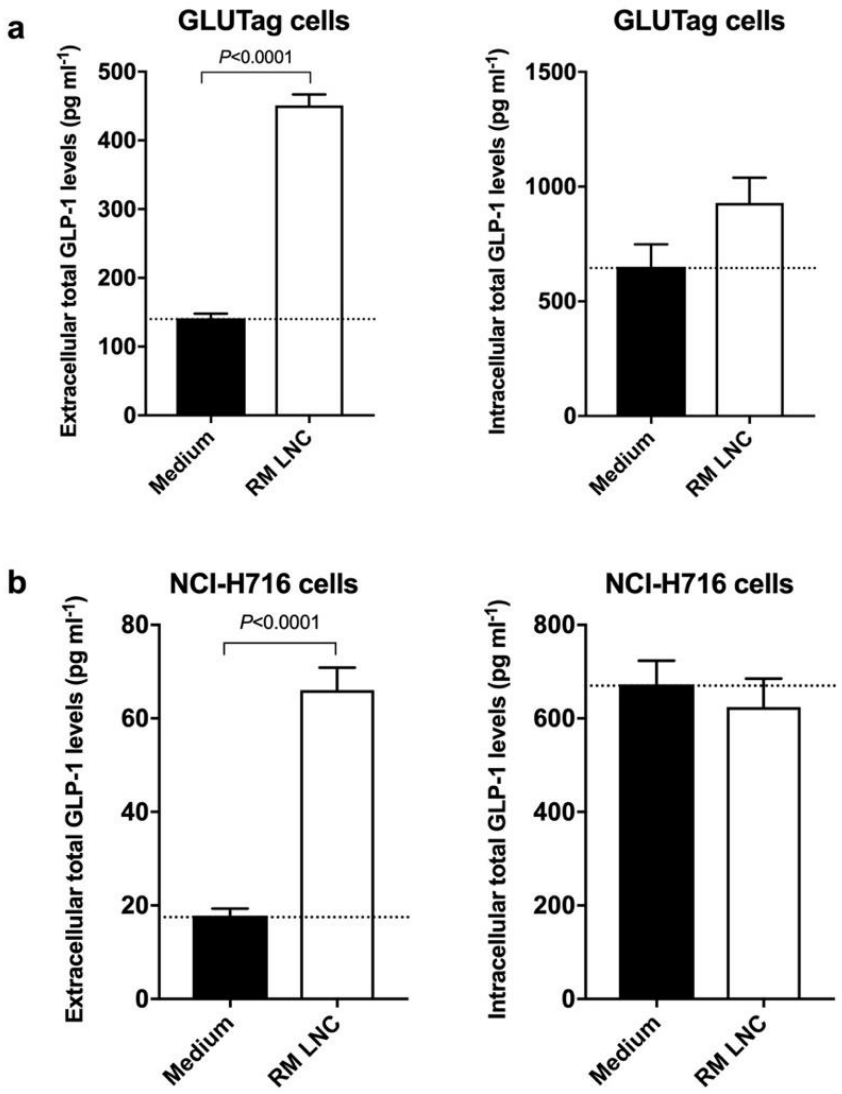

Figure S3. GLP-1 secretion *in vitro* in GLUTag and NCl-H716 cells. **a** Extracellular (left) and intracellular (right) total GLP-1 levels measured by ELISA after 2 h of coincubation with RM LNC in GLUTag cells (murine L-cells). **b** Extracellular (left) and intracellular (right) total GLP-1 levels measured by ELISA after 2 h of coincubation with RM LNC in NCl-H716 cells (human L-cells). Data are presented as the mean ± SEM (n=12). *P* values were determined by Student's *t* test. The data correspond to three independent experiments.

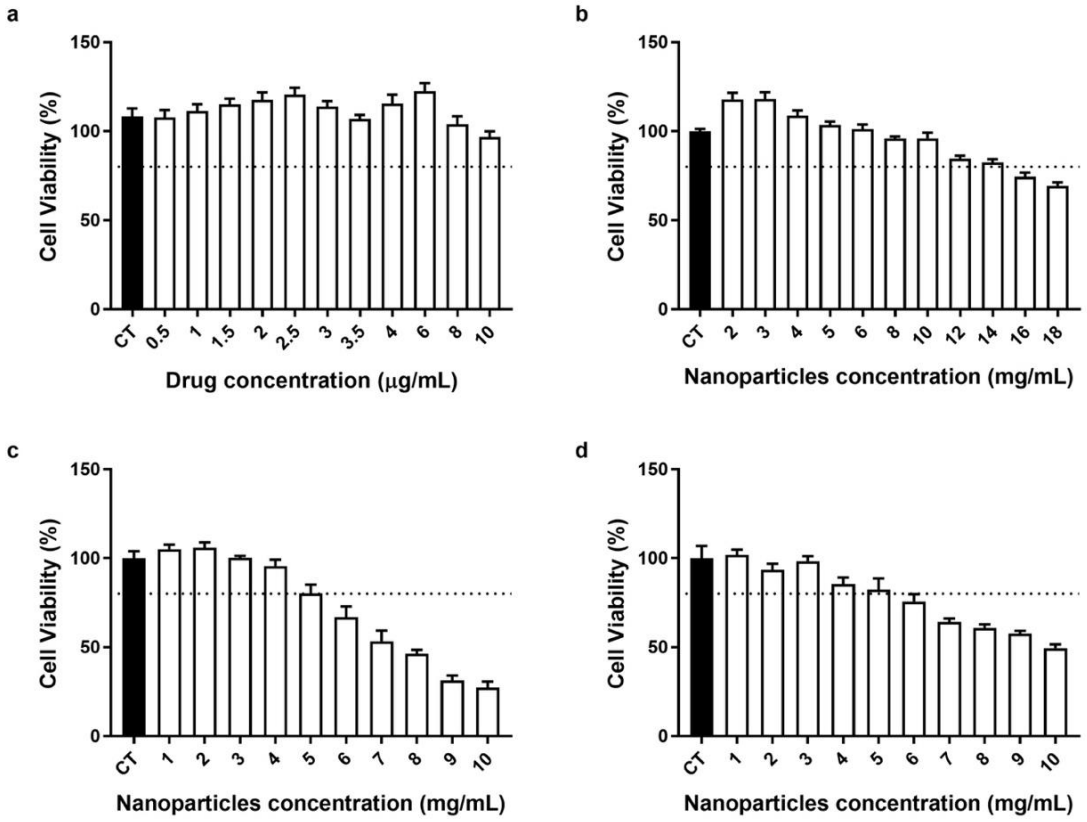

Figure S4. *In vitro* cytotoxicity studies in L-cells and enterocyte-like cells. Cell viability of EXE RM LNC on Caco-2 cells after incubation for 2 h at 37°C expressed as cell viability with respect to drug concentration **a** and to nanoparticle concentration **b**. Cell viability of RM LNC on GLUTag cells **c** and human NCI-H716 cells **d** after coincubation with increasing nanocapsule concentrations (1 mg/mL to 10 mg/mL) for 2 h. Data are shown as the mean ± SEM (n=9). The dotted line corresponds to 80% availability. The data correspond to three independent experiments.

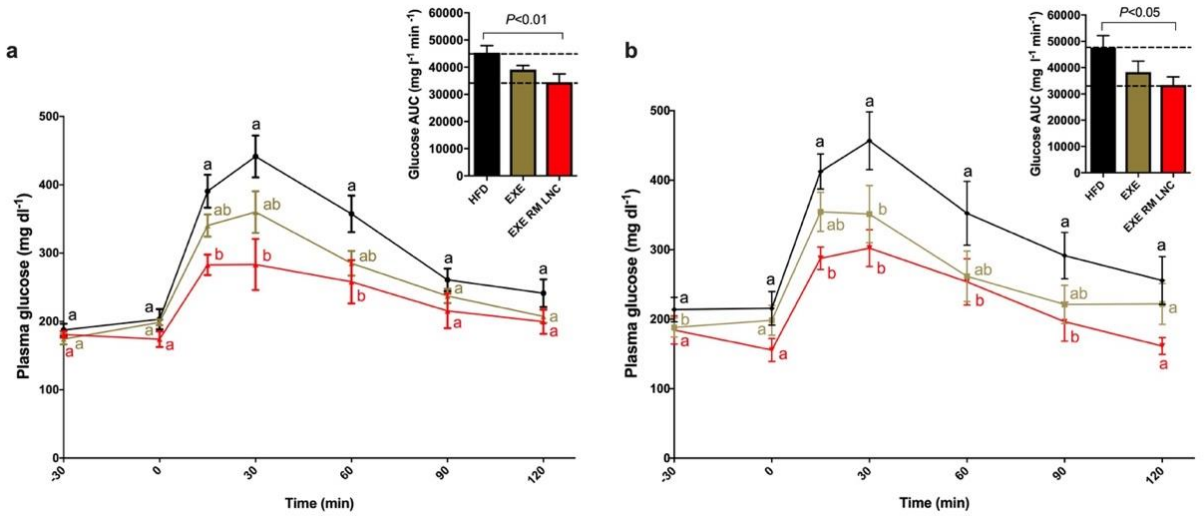

Figure S5. OGTT evaluation of EXE RM LNC in 8- and 10-week HFD-induced diabetic mice. **a** Plasma glucose levels and AUC following a 2 g/kg glucose oral challenge measured in mice (C57BL6/J mice) fed a control diet and a HFD (8 weeks) and **b** fed a control diet and a HFD (10 weeks). Data are presented as the mean ± SEM (n= 7-8). Values with different superscript letters are significantly different *(P*<0.05). *P* values were determined by two-way ANOVA for the OGTT and by one-way ANOVA followed by Tukey's post hoc test for comparing the AUC between groups.

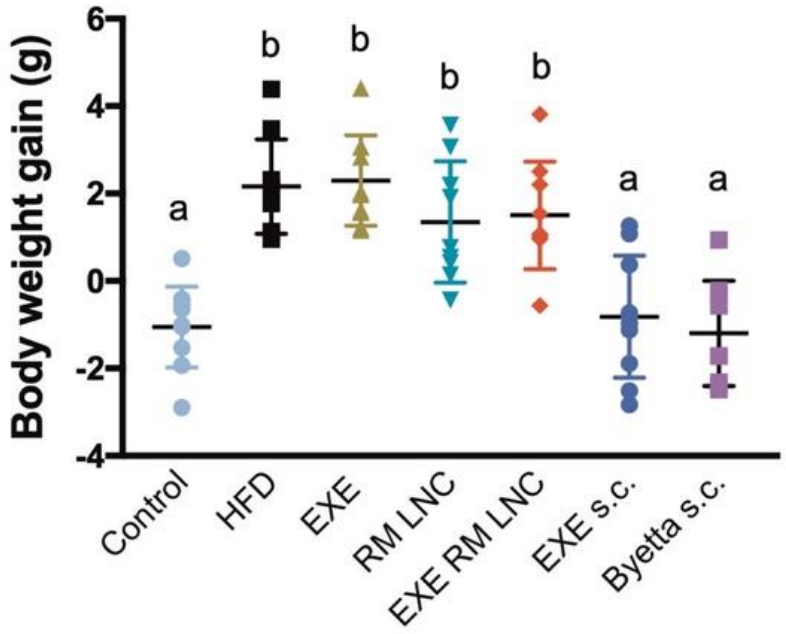

Figure S6. Body weight gain following chronic/long-term administration of exenatide in obese and diabetic mice. Body weight gain (g). Data are presented as the mean ± SEM (n=10). *P* values were determined following by a one-way ANOVA followed by Tukey's post hoc test.

CHAPTER V

TARGETED NANOPARTICLES TOWARDS INCREASED L CELL STIMULATION AS A STRATEGY TO IMPROVE ORAL PEPTIDE DELIVERY IN INCRETIN-BASED DIABETES TREATMENT

Adapted from:

Xu Y, Keersmaecker H D, Braeckmans K, De Smedt S C, Cani P D, Préat V, Beloqui A. Targeted nanoparticles towards increased L cell stimulation as a strategy to improve oral peptide delivery in incretin-based diabetes treatment. Biomaterials, Submitted (2020).

ABSTRACT

The delivery of therapeutic peptides via the oral route remains one of biggest challenges in the pharmaceutical industry. Recently, we have described an alternative improved drug delivery system for the oral delivery of peptides, consisting on a lipid-based nanocapsule. Despite the striking effects observed, it is still essential to develop strategies to strengthen the nanocarriers' GLP-1 secretory effect and/or prolong its antidiabetic effect in *vivo* towards its translation into the clinics. For this purpose, we have developed and compared different fatty acid-targeted lipid and polymeric nanoparticles, and evaluated the L cell stimulation induced by the nanocarriers both in *vitro* in murine L cells, and *in vivo* in normoglycemic mice. We further examined their antidiabetic effect *in vivo* in a high fat diet-induced obese/diabetic mouse model, examining the effect of the oral administration frequency. Among the tested nanocarriers, only lipid-based nanocarriers modified with DSPE-PEG_{2000}-CH_3 on the surface were able to significantly strengthen the biological effect of the nanocarriers. They were able to increase endogenous GLP-1 levels up to 8-fold *in vivo* in normoglycemic mice. Moreover, they were effective at prolonging the *in vivo* antidiabetic effect normalizing plasma glucose levels in obese/diabetic mice following a long-term treatment (one month). Ultimately, the targeted nanocarriers were as effective when reducing the administration frequency from once daily to once every other day.

KEYWORDS

Enteroendocrine L cells; GLP-1; targeted nanocarriers; diabetes; oral peptide delivery

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I. INTRODUCTION

Therapeutic peptides (more than 60 peptide drugs [1]) are now commercialized via parenteral administration [2]. The delivery of therapeutic peptides via the oral route remains a challenge, especially regarding the low oral bioavailability (less than 1 %) of these peptides. Despite the challenges, the advantages of the oral route are remarkable, ameliorating the shortcomings of the parenteral route (e.g., needle phobia, non-patient convenience) [3, 4]. The development of oral dosage forms that allows absorption of therapeutic peptides to the systemic circulation still is one of the greatest challenges for the pharmaceutical industry [5]. This is especially true in the case of antidiabetic peptides, e.g., glucagon-like peptide (GLP)-1. The oral administration of these gut hormone mimetic peptides could simulate the normal physiological pathway of the native incretin peptides [6], which can access the liver in much higher concentrations through the hepatic portal vein avoiding large systemic exposure and its associated side effects [7].

The first oral GLP-1 analog has reached the market (semaglutide, Rybelsus®, NovoNordisk), representing a major breakthrough in the oral delivery of peptides [8]. The formulation is based on the co-administration of the peptide with a permeation enhancer (sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC). The formulation presents two main limitations: 1) small differences in the peptide or the SNAC substantially affect the absorption efficacy (variable bioavailability) and 2) according to the authors, the co-administration of SNAC with liraglutide failed to provide circulating levels of liraglutide (the efficacy is limited to the administration of the GLP-1 analog semaglutide (half-life of 165h)). We have recently described what we believe would represent an alternative improved drug delivery system for the oral delivery of peptides, consisting on a lipid-based nanocapsule [9]. Regardless of the encouraging properties of nanomedicines [9-11], they still need to prove that they can do better than standard formulations incorporating functional excipients in oral peptide delivery [12]. We have developed an innovative dual-action approach that synergizes the biological effect of the nanocarriers (inducing endogenous GLP-1 secretion) with that of the encapsulated molecule (increased absorption of the encapsulated peptide). In our proof of concept studies, we encapsulated exenatide (half-life of 2.5 h) as model GLP-1 analog to prove

that following our strategy we can reach a therapeutic effect with a short half-life peptide [9]. We conducted a long-term treatment study (one month) in a chronic model of type 2 diabetes mellitus (T2DM) in mice. Our results demonstrated that this approach was at least as efficient as the current marketed drug (Byetta®, subcutaneous injection) and could even be more potent for improving oral glucose tolerance, insulin resistance and hepatic steatosis. In addition, our strategy offers an additional advantage over current approaches for oral incretin mimetic peptide delivery providing with increased endogenous GLP-1 levels. This allows us to potentially use a lower dose in a foreseen application and proves to be effective with a short half-life peptide, thus avoiding a potential associated toxicity regarding the accumulation of a long half-life peptide upon daily administrations.

Despite the striking effects observed, it is still essential to develop strategies to strengthen the nanocarriers' GLP-1 secretory effect and/or prolong its antidiabetic effect *in vivo* towards its translation into the clinics. We hypothesized that this could be attained modulating the effect of our lipid-based drug delivery system by tailoring the surface of the nanocarriers.

Enteroendocrine L cells express various G protein-coupled receptors (GPCRs) that are activated by different dietary nutrients present in the lumen to modulate the secretion of hormones (e.g., GLP-1) [13]. Each GPCRs has its own ligands, thereby correspondingly modulating the different gut hormones secreted from L cells. For instance, free fatty acid receptors FFAR2 and FFAR3, also known as GPR43 and GPR41, are activated by short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, stimulating GLP-1 secretion [14]. SCFA propionate displays similar agonism both on FFAR2 and FFAR3, whereas acetate and butyrate only have high affinity for FFAR2 and FFAR3, respectively [15, 16]. In addition, propionate is an end-product of fermentation and thus is not cross-metabolized by the microbiota unlike acetate and butyrate [17]. We hypothesized that grafting propionate on the surface of our lipid-based nanosystem could lead to an increased GLP-1 secretory effect by directly targeting L cells.

In the present study, we have developed targeted nanoparticles towards increased L cell stimulation to improve oral peptide delivery in incretin-based diabetes treatment. We have compared the

strategies in both lipid (lipid nanocapsules) and polymeric (PLGA) nanoparticles *in vitro* in murine L cells, and *in vivo* in normoglycemic mice. We also examined their antidiabetic effect *in vivo* in a high fat diet-induced obese/diabetic mouse model, examining the effect of the oral administration frequency.

II. MATERIALS AND METHODS

II.1. MATERIALS

Exenatide (exenatide acetate) (EXE) was purchased from Bachem (Bubendorf, Switzerland). Labrafac® WL 1349 (caprylic/capric acid triglycerides) and Peceol® (oleic acid mono-, di- and triglycerides) were kindly provided by Gattefossé (Saint-Priest, France). Lipoid® S 100 (soybean lecithin at 94% of phosphatidylcholines) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Solutol® HS15 (mixture of free PEG 660 and PEG 660 12-hydroxystearate, Mw 870 Da) and Span® 80 (sorbitan oleate) were purchased from Sigma-Aldrich (St. Louis, USA). 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-methoxylpoly(ethylene glycol)2000 (DSPE-PEG_{2000}-CH_3), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[poly(ethylene glycol)-2000]-propionate (DSPE-PEG_{2000}-CH_2-CH_2-COOH), Poly(lactide-co-glycolide) (10k, d,l-LA/GA: 50:50)-block-[poly(ethylene glycol)-2000]-propionate (PLGA-PEG_{2000}-CH_2-CH_2-COOH), Poly(lactide-co-glycolide) (10k, d,l-LA/GA: 50:50)-block-[poly(ethylene glycol)-2000] methyl ether (PLGA-PEG_{2000}-CH_3) were obtained from Nanosoft Polymers (Winston-Salem, USA). Sodium chloride (NaCl), saponin, lecithin, sodium taurocholate, pepsin, 3-(4,5- dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Triton-X 100 were purchased from Sigma-Aldrich (St. Louis, USA). Total GLP-1 (ver.2) and active GLP-1 assay kits were purchased from Meso Scale Discovery (Maryland, USA). The Exendin-4 enzyme immunoassay kit was purchased from Phoenix Europe GmbH (Karlsruhe, Germany). the Ultrasensitive Mouse Insulin ELISA Kit was purchased from Mercodia AB (Uppsala, Sweden). Matrigel™ was obtained from BD Bioscience (Belgium). Dipeptidyl peptidase 4 (DPP-4) inhibitor was purchased from Millipore (St. Charles, USA). Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX (5.5 Mm glucose), penicillin-streptomycin (P/S), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin (0.25%) containing ethylenediaminetetraacetic acid (EDTA, 0.02%) were also used and purchased from Thermo Fisher Scientific (Invitrogen, Belgium). DiIC18(5) solid (1,1'-ioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) (DiD) was purchased from

Thermo Fisher Scientific (Invitrogen, UK). All chemical reagents utilized in this study were of analytical grade.

II.2. PREPARATION OF CHARACTERIZATION OF NON-TARGETED AND TARGETED NANOPARTICLES

II.2.1. Preparation of non-targeted and targeted lipid-based nanosystems

Reverse micelle-loaded lipid nanocapsules (RM LNC) were prepared in two steps as described by Xu *et al.* [9]. First, exenatide-loaded reverse micelles (EXE RM) were prepared by high-speed stirring of a surfactant (Span® 80) and an oil (Labrafac® WL 1349) mixture (1:5 weight ratio). Then, 50 µL of EXE (30 mg/mL in MilliQ water) was dripped into the mixture and maintained under stirring. Secondly, 769.5 mg Labrafac® WL 1349, 85.5 mg Peceol®, 13.4 mg Lipoid® S100, 120 mg Solutol® HS15, 50 mg sodium chloride (NaCl) and 1.025 mL of MilliQ water, were mixed together under magnetic stirring at 40 °C at 200 rpm for 5 min. Temperature cycles (three) of progressive heating/cooling were conducted between 50 °C and 67 °C. During the last cycle, 500 µL of pre-heated drug loaded RM was added to the mixture at ~3 °C above the phase inversion zone (PIZ; 59 to 61.5 °C). The solution was cooled down to reach the PIZ temperature, and 2.5 mL of cold water (4 °C) was added and stirred at high speed for 2 min. Blank RM LNC were prepared following the same protocol without EXE.

II.2.2. Preparation of DiD-labeled reverse micelle-loaded lipid nanocapsules

DiD-labeled RM LNC were prepared by adding 225 µg of DiD to the mixture. Namely, 45 µL of DiD (5 mg/mL in ethanol) was added into the vial and then heated in a water bath until the organic solvent was completely evaporated. After that, Labrafac® WL 1349 was added into the same vial and mixed with DiD in the water bath until DiD was totally dissolved in the oil phase. The mixture was then cooled down to room temperature, other components were added and continued the preparation process as above described.

II.2.3. Preparation of PEGylated reverse micelle-loaded lipid nanocapsules with or without propionate

PEGylated reverse micelle-loaded lipid nanocapsules with or without propionate (RM LNC PEG or RM LNC PEG-PRO, respectively) were produced by incubating DSPE-PEG_{2000}-CH_3 or DSPE-PEG_{2000}-CH_2-CH_2-COOH, respectively, with RM LNC at the concentration of 5 mg/mL, under gentle stirring at 37 °C for 4 h. During the incubation, the suspension was vortexed every 15 min and then quenched in an ice bath for 1 min [18].

II.2.4. Preparation of PEGylated PLGA nanoparticles with or without propionate

PLGA nanoparticles were prepared by the double emulsification method [19]. Typically, 25 mg of PLGA-PEG_{2000} or PLGA-PEG_{2000}-propionate was dissolved in 1 mL dichloromethane (DCM). For PLGA-PEG_{2000} NPs, 50 µL of an aqueous (drug) solution (in MilliQ water) was dropped into the oil phase under sonication (Digital sonifier 450, Branson, USA) at 30% amplitude for 30 seconds to form the primary water-in-oil emulsion. This primary emulsion was poured into 2 mL of a second aqueous phase containing 2 % (w/v) saponin under sonication at 30% amplitude for 30 seconds to form the water-in-oil-in-water emulsion. This step was repeated twice. The emulsion was added to another 10 mL of 2 % saponin solution under magnetic stirring to evaporate the organic solvent. The final nanoparticle suspension was centrifuged at 15,000 rpm for 30 min at 4 °C (Avanti® J-E centrifuge, Beckman coulter, USA) and thoroughly washed with MilliQ water. PLGA-PEG_{2000}-PRO NPs were prepared using 1 % saponin and sonicating at 30% amplitude for 15 seconds instead.

II.2.5. Quantification of exenatide

The exenatide encapsulated within PEGylated RM LNC was quantified by high-performance liquid chromatography (HPLC, Shimadzu, Japan) using a gradient method as previously described by Xu *et al.* [9]. Briefly, a Kinetex® EVO C18 column (100 Å, 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 0.05% (v/v) trifluoroacetic acid (TFA) in water, and the organic mobile

phase consisted of 0.05% (v/v) in acetonitrile. A gradient system was developed with an initial ratio of 10:90 (v/v, aqueous : organic phase) at a flow rate of 1 mL/min, which was linearly changed to 90:10 (v/v) over 10 min and kept constant for the next minute. Then, the ratio was linearly changed to the initial composition in the next 1.5 min 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 were 1.1 ± 0.4 μg/mL and 3.3 ± 1.1 μg/mL, respectively.

II.2.6. Characterization of nanoparticles

PEGylated RM LNC and PLGA NPs were characterized by measuring their particle size and polydispersity index (PDI) by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was determined by laser Doppler velocimetry (LDV) using a Zetasizer Nano ZS. For the measurement, 5 μL of nanocapsule suspension was dispersed in 995 μL of ultrapure water. All measurements were performed in triplicate.

EXE RM LNC and PEGylated EXE RM LNC were also characterized based on their drug encapsulation efficiency (EE, %). To calculate the total drug content, 50 μL of LNC suspension 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 min) (Millipore). Filtrates were further diluted using a 1:2 dilution factor. The exenatide in the filtrate and the exenatide dissolved in methanol were quantified using the above-described HPLC method. The EE was calculated using the following equation:

EE (%) = (total amount of exenatide – free exenatide) / (total amount of exenatide) ×100

II.3. NANOCAPSULE STABILITY AND DRUG RELEASE IN STIMULATED GASTROINTESTINAL FLUIDS

II.3.1. PEGylated nanocapsules' stability in stimulated gastrointestinal fluids

The *in vitro* stability of PEGylated EXE RM LNC, with or without propionate as a ligand, was tested in four different biomimetic media: Fasted State-Simulated Gastric Fluid (FaSSGF) with and without pepsin, Fasted State-Simulated Intestinal Fluid (FaSSIF) and Fed State-Simulated Intestinal Fluid (FeSSIF). A detailed description of the composition of the simulated fluids used was previously described by Xu *et al.* [9]. The influence of gastric and intestinal conditions on nanocapsule stability was evaluated based on the nanocapsule size and the PDI. PEGylated EXE RM LNC were incubated in FaSSGF with and without pepsin, FaSSIF, and FeSSIF at 37 °C (100 µL of nanocapsules in 10 mL of media) under gentle stirring. At predetermined time intervals (0, 0.5, 1 and 2 h for stimulated gastric media and 0, 0.5, 1, 3 and 6 h for stimulated intestinal media and FeSSIF), samples were withdrawn and then analyzed by DLS.

II.3.2. In vitro drug release studies

The drug release from PEGylated EXE RM LNC was evaluated in FaSSGF in the absence of pepsin and in FaSSIF media for 2 h and 6 h, respectively. Studies were performed using the dialysis method. Briefly, 1 mL of PEGylated 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 35 mL of medium at 37 °C under magnetic stirring. At predetermined times, 50 µL of sample was withdrawn and dissolved in 950 µL of methanol. The concentration of exenatide was determined by HPLC, as described above.

II.4. IN VITRO CELL STUDIES

II.4.1. Cell culture

The intestinal murine L cell line GLUTag was kindly provided by Dr. Daniel Drucker (University of Toronto, Canada). GLUTag cells were used from passages 17 to 25. Cells were grown in DMEM

GlutaMAX (5.5 mM glucose) supplemented with 10% (v/v) inactivated FBS and 1% (v/v) P/S (complete DMEM medium) at 37 °C with 5% CO_2 supply. Cells were sub-cultured using trypsin (0.25%) containing EDTA (0.02%) every 4-5 days.

II.4.2. Cytotoxicity studies

In vitro cytotoxicity studies of unloaded PEGylated RM LNC and PEGylated PLGA NPs were performed in GLUTag cells using nanoparticle concentrations calculated previously by the 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay [20]. GLUTag cells (5×10^4 cells per well) were seeded on Matrigel™-coated (10 µL/mL of medium) 96-well plates. After washing the plates with prewarmed PBS buffer (x3), 100 µL of nanoparticle suspension at increasing nanocapsule concentrations (0.5-10 mg/mL) and at increasing PLGA nanoparticle concentration (0.1-8 mg/mL) were dispersed in DMEM GlutaMAX medium (without FBS) and co-incubated with GLUTag cells at 37 °C for 2 h. After incubation, the supernatants were replaced by 100 µL of 0.5 mg/mL MTT for 3 h. The purple formazan crystals were dissolved in 200 µL of DMSO for absorbance determination at 560 nm using a MultiSksan EX plate reader (Thermo Fisher Scientific, USA). Cells with Triton-X 100 (100% dead) and cells with culture medium (100% alive) were considered positive and negative controls, respectively. Tests were performed in triplicate.

II.4.3. In vitro GLP-1 secretion

GLUTag (1.8×10^5 cells per well) were seeded onto Matrigel™-coated 24-well cell culture plates and allowed to adhere for 24 h. The day after, the plate was gently washed using prewarmed PBS. Then, the GLUTag cells were co-incubated with DMEM GlutaMAX without FBS or unloaded PEGylated NPs (RM LNC and PLGA NPs with or without ligand). Media contained DPP-IV inhibitor at a final concentration of 50 µM (Millipore, St. Charles, MO, USA). To test the effect of different nanoparticles' concentration on GLP-1 secretion, we used 0.5-2 mg/mL nanocapsule concentrations and 0.1-3 mg/mL nanoparticles concentrations, respectively. After 2 h of incubation at 37 °C, supernatants were collected, centrifuged at 250 g for 5 min at 4 °C (Centrifuge

5804 R, Eppendorf AG, Hamburg, Germany) and preserved at –80 °C until further analysis. Cells were collected in PBS in the presence of DPP-IV inhibitor. Cell extracts containing GLP-1 were obtained after three freeze-thaw cycles followed by centrifugation at 250 g for 5 min at 4 °C. Total GLP-1 concentration was determined using a Total GLP-1 ELISA kit (Meso Scale Delivery, Gaithersburg, USA). GLP-1 secretion is expressed as the amount of GLP-1 detected in the supernatants plus cells. GLP-1 secretion was calculated by the following equation:

$$\text{GLP-1 secretion} = C_{extracellular} / (C_{intracellular} + C_{extracellular})$$

where $C_{extracellular}$ is the concentration of GLP-1 tested in the supernatants, and $C_{intracellular}$ is the concentration of GLP-1 tested in cells.

II.5. IN VIVO STUDIES

II.5.1. Animals

All animal experiments were approved by and performed in accordance with the local animal committee (2018/UCL/MD/45) and as specified by the Belgian Law of 29 May 2013 on the protection of laboratory animals.

II.5.2. Total GLP-1 secretion of ligand-conjugated and non-conjugated PEGylated NPs in normoglycemic mice

To test PEGylated RM LNC (with or without propionate), normoglycemic mice (C57BL/6J male mice, 20–25 g, 10 weeks; Janvier Laboratories, France) were randomly divided into four groups (8 mice each). Animals were fasted overnight before the experiments with free access to water. Mice were treated with blank RM LNC, blank PEGylated RM LNC and blank PEGylated RM LNC-PRO corresponding to ~1.62 mg/g nanoparticle dose. Control mice were treated by oral gavage with an equivalent volume of MilliQ water. Blood samples were withdrawn from the tip of the tail vein at 60 and 180 min after oral administration. Samples were collected in the presence of DPP-IV inhibitor (20 µL per mL of blood) and maintained on ice. Immediately after the study, blood samples were centrifuged (3,000 rpm, 10 min at 4 °C), and the plasma was kept frozen at −80 °C

until analysis. The total GLP-1 levels were quantified using a Total GLP-1 ELISA kit (Meso Scale Delivery, USA).

In case of PEGylated PLGA NPs (with or without conjugated propionate), 4 mice per group and a 200 mg/kg nanoparticle dose were used instead. The blood collection and total GLP-1 secretion analysis was conducted following the same protocol as described above. Total GLP-1 levels were measured under the same conditions.

II.5.3. Nanocapsules distribution in obese/diabetic mouse small intestine and colon

Eight-week-old male mice were randomly divided into four groups (4 mice each). After two weeks of acclimation, mice underwent 10 weeks of HFD (60% fat and 20% carbohydrates (kcal per 100 g), D12492i, Research Diets, USA). Before the experiments, the mice were fasted overnight with free access to water. For nanocapsule distribution in small intestine and colon, DiD-labeled nanocapsules, including RM LNC, PEGylated RM LNC and PEGylated RM LNC-PRO, were administered via oral gavage corresponding to ~1.62 mg/g nanoparticle dose. Control mice were orally administered with an equivalent volume of MilliQ water. Sections of duodenum, jejunum, ileum and colon were excised 1 h post administration and subsequently flash frozen in Optimal Cutting Temperature compound (OCT). For all tissues, sections were cut 6 μm thick using a Leica CM-3050-S cryostat. The tissue sections were briefly fixed in 4% paraformaldehyde for 5 min. After washing the PBS containing 0.02 % polysorbate 20, slides were mounted with VECTASHIELD Hard Set Mounting Medium containing DAPI (staining cell nuclei in blue). The samples were examined by CLSM using a Zeiss confocal microscope (LSM 150) to capture serial pictures. The Axio Vision software (version 4.8) was used to analyze the data.

II.5.4. Mucus extraction from obese/diabetic mouse small intestine

Untreated 10 weeks-HFD induced diabetic mice were fasted overnight with free access to water. Prior to mucus extraction, mice were sacrificed by cervical dislocation. Briefly, the duodenum and apical jejunum were dissected and placed in a Petri dish, following a very gently flush with PBS to remove fecal matter using a syringe with a 19.5-gauge needle through one open end of intestinal

segments. The intestinal segments were cut longitudinally using surgical scissors. Then, the mucus was scraped off from the intestinal segments using a cell scraper and was transferred to a clean tube. The isolated mucus was stored at -20 degrees until further conducting mucus diffusion and mucus stability studies.

II.5.5. Single particle tracking and confocal microscopy

DID RM LNC, DID RM LNC PEG and DID RM LNC PEG-PRO were diluted in water or small intestine mucus of mice and mixed by gentle stirring. A volume of 5 µl was placed on a microscopy glass slide sealed with an adhesive spacer (S24737, Secure-Seal™ Spacer, Thermofisher) and a cover glass (#1.5).

For single particle tracking, fluorescent signals were recorded using a spinning disk confocal microscope (Nikon Eclipse Ti, Tokyo, Japan) equipped with an MLC 400 B laser box (Agilent Technologies, Santa Clara, CA, USA), a Yokogawa CSU-X1 confocal spinning disk device (Andor, Belfast, UK), an iXon ultra EMCCD camera (Andor Technology, Belfast, UK) and NIS Elements software (Nikon, Japan). A 100x oil immersion objective lens (Plan Apo VC 100x oil, 1.4 NA, Nikon, Japan) was used. A stage-top incubator (37°C, Tokai Hit) in combination with an objective lens heater (Biotechs) was used during imaging. Movies of 100 frames with a temporal resolution of 43.9 ms were recorded 5-10 µm above the coverslip. No further analysis could be performed due to either NP accumulation in distinct parts of the mucus or leakage of the fluorescent label so that individual NPs could no longer be seen or analyzed.

The samples prepared for single particle tracking were also studied by laser scanning confocal microscopy. Images were recorded on a Nikon A1R HD Confocal laser scanning microscope with a 60x/1.27 Plan Apo IR Water immersion objective (SR Plan Apo IR AC, Nikon) using a galvano scanner. A 637 nm (LU-N4 Laser Unit) was used for excitation of DiD. Fluorescence signals were detected with a Multi-Alkali PMT (A1-DUG-2 GaAsP Multi Detector Unit). A stack of 19.5 µm above the glass surface with a step size of 0.5 µm at Nyquist resolution was recorded and a maximum intensity projection was made for visualization. All imaging was performed at RT.

II.5.6. Oral glucose tolerance test in high-fat diet induced obese/diabetic mice

Eight-week-old male mice were housed 4 per cage and divided into 5 groups (8 mice per group). After 2 weeks of acclimation, mice were fed a high-fat diet (60% fat and 20% carbohydrates (kcal per 100 g), D12492i, Research Diets, USA) (HFD) or a normal chow diet (control, AIN93Mi, Research Diets, USA) for 10 weeks before experiments and underwent a period of fasting overnight before being treated with an oral exenatide solution (EXE, 500 µg of exenatide/kg body weight), exenatide-reverse micelle-loaded lipid nanocapsules (EXE RM LNC, 500 µg of exenatide/kg body weight), exenatide-reverse micelle-loaded PEGylated lipid nanocapsules (EXE RM LNC PEG) or unloaded reverse micelle-loaded PEGylated lipid nanocapsules (RM LNC PEG, equivalent concentrations as per EXE RM LNC and EXE RM LNC PEG) 1 h before receiving an oral glucose dose. Control groups (control diet and HFD groups) were treated with an oral gavage of an equivalent volume of sterile MilliQ water. After 1 h, mice were challenged with an oral glucose gavage (2 g/kg glucose dose), as previously described by Xu *et al.* [9]. Blood glucose was measured 30 min before oral glucose (-30 min) and at 0, 15, 30, 90 and 120 min after oral glucose load. Blood glucose levels were collected determined with a glucose meter (Accu Check, Roche, Switzerland) from blood samples collected from the tip of the tail vein. Blood samples were collected at -30 min and 15 min from tail vein to test total GLP-1 and insulin plasma concentration by using ELISA kits (Meso Scale Delivery, USA and Mercodia, Uppsala, Sweden, respectively). The insulin resistance index was determined by multiplying the area under the curve of both blood glucose and insulin in the plasma obtained by the oral glucose tolerance test (OGTT). At the end of OGTT test, animals were anesthetized with isoflurane (Forene, Abbott, England), and blood was sampled from the portal vein. The levels of active GLP-1 were tested by ELISA (Meso Scale Delivery, Gaithersburg, USA).

II.5.7. Pharmacokinetics study in obese/diabetic mice

Eight-week-old male mice were randomly divided into two groups (10 mice per time point) and housed in a controlled environment (room temperature of 23 ± 2 °C, 12 h daylight cycle) with free access to food and sterile water. After two weeks of acclimation, mice underwent 10 weeks of HFD

(60% fat and 20% carbohydrates (kcal per 100 g), D12492i, Research Diets, USA). Prior to the experiments, mice were fasted overnight with free access to sterile Milli-Q water. Exenatide in solution and EXE RM LNC PEG were orally administered at a 500 µg/kg dose. At different time points (0, 0.5, 1, 1.5, 2, 4, 6 and 8 h), blood samples were collected from the tip of the tail vein. Blood samples were then centrifuged (1,500 *g*, 10 min at 4 °C) and the plasma was stored at -80 degrees until further analysis. Exenatide plasma concentration was quantified using an ELISA kit (EK-070-94, Phoenix Europe GmbH, Karlsruhe, Germany).

II.5.8. Chronic PEGylated nanocapsules long-term treatment study in obese/diabetic mice

Eight-week-old male mice were randomly divided into 7 groups (10 mice per group) and housed 5 per cage in a controlled environment (room temperature of 23 ± 2 °C, 12 h daylight cycle) with free access to sterile food (AIN93Mi; Research diet) and sterile water. After a two-week acclimation period, mice underwent 8 weeks of HFD (60% fat and 20% carbohydrates (kcal per 100 g), D12492i, Research Diets, USA) (nanocapsules-treated groups) or a normal chow diet (Control). After this period, within the following 4-week treatment period, mouse body weight was recorded daily, and the glycemia was monitored once per week. Mice were treated daily (D) or once every two days (T) at 4 pm (i) orally with exenatide encapsulated within RM LNC PEG (500 µg/kg dose) (EXE RM LNC PEG) or the corresponding concentration of unloaded RM LNC PEG every day or (ii) orally with exenatide encapsulated within RM LNC (500 µg/kg dose) (EXE RM LNC), within RM LNC PEG (500 µg/kg dose) (EXE RM LNC PEG), or the corresponding concentration of unloaded RM LNC PEG every two days. Control groups (healthy and HFD) were treated orally daily with an equivalent volume of sterile Milli-Q water. Mice were fasted once per week for 6 h prior to undergo a glucose test.

II.6. STATISTICAL ANALYSIS

GraphPad Prism 8 program (CA, USA) was used to perform the statistical analyses. For all analyses and for each group, any exclusions were supported by the use of the Grubbs test for outlier

detection. Values were normalized by log-transformation when variances were significantly different between groups before conducting the analysis. Two-way or one-way ANOVA followed by Tukey's post hoc test was applied for comparisons among multiple groups. If the variance differed significantly between groups even after normalization, a nonparametric test was performed. The results are expressed as the mean ± standard error of the mean (SEM). A difference of $p<0.05$ was considered statistically significant.

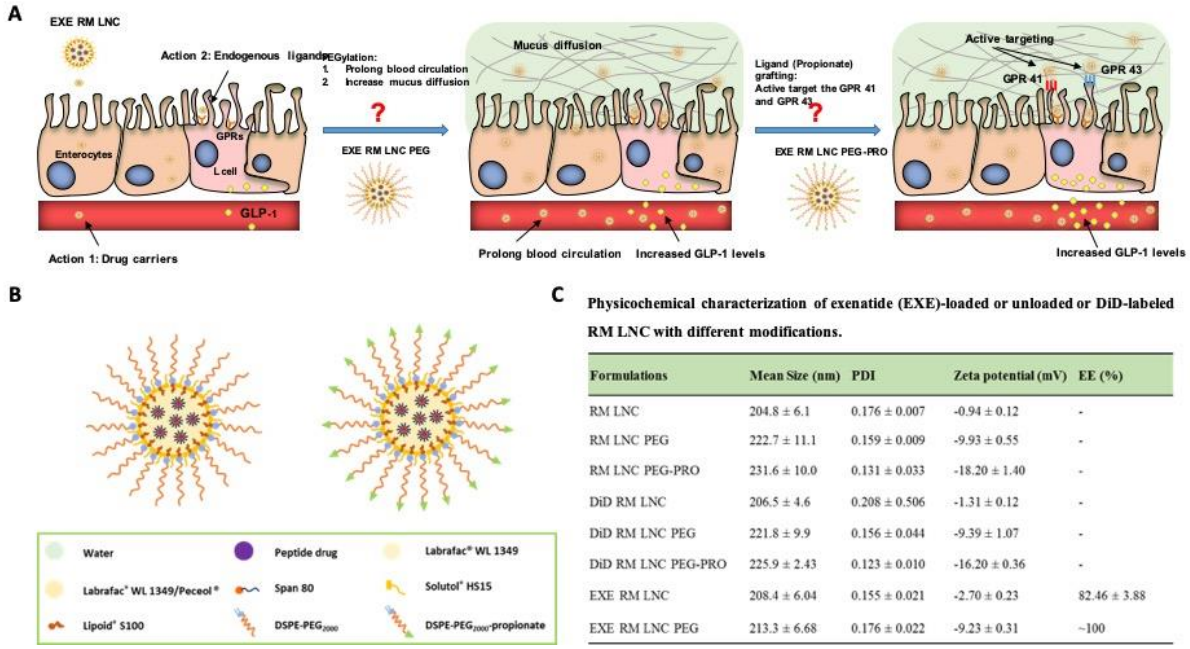
III. RESULTS

III.1. PREPARATION AND CHARACTERIZATION OF PEGYLATED NANOPARTICLES WITH PROPIONATE AS TARGETING MOIETY

We firstly encapsulated a GLP-1 analogue (exenatide) within lipid nanocapsules (LNC) following a previously described procedure, to obtain 200 nm particle size nanocarriers [9]. This nanoparticle size was selected based on our previous studies demonstrating the effect of lipid nanocapsules presenting this nanoparticle size on GLP-1 secretion [9, 20]. To develop L cells-targeted lipid-based nanocapsules, we selected DSPE-PEG_{2000} as a linker to PEGylate to provide the nanocapsules with enhanced capacity of mucus diffusion (Fig. 1 A-B) [21]. The post-insertion method was selected to ensure the PEG chains are localized only on the particle surface rather than retained within the particle core. We used both DSPE-PEG_{2000}-CH_3 and DSPE-PEG_{2000}-CH_2-CH_2-COOH, the latter to provide with a specific targeting to L cell GPCRs present on their surface. The physicochemical properties of empty lipid nanocapsules were assessed before (RM LNC) and after the post-insertion of both DSPE-PEG_{2000}-CH_3 and DSPE-PEG_{2000}-CH_2-CH_2-COOH (RM LNC PEG and RM LNC PEG-PRO, respectively) (Fig. 1C). Blank LNC presented a ~ 200 nm particle size and a narrow size distribution (PDI=0.18). These data are in accordance with our previous results [9]. After the incubation of LNC with the biofunctional polymers, we observed an increase on size of ~ 18 nm and ~ 27 nm for RM LNC PEG and RM LNC PEG-PRO, respectively. The zeta potential values decreased significantly from -0.94 mV (RM LNC) to -9.93 mV (RM LNC PEG). The surface charge of RM LNC PEG-PRO decreased even more reaching -18.20 mV in the presence of the fatty acid. We obtained similar particle size and zeta potential after labeling the nanocarriers with DiD when compared to the respective empty nanocapsules (Fig. 1C). Besides, there was also no significant influence on the size and the surface charge of the nanocapsules when encapsulating exenatide within PEGylated nanocapsules (EXE RM LNC PEG) (Fig. 1C).

In order to quantify the targeting capacity of propionate, we used empty PEGylated polymeric PLGA nanoparticles (PLGA NPs) with or without propionate (PLGA-PEG NPs and PLGA-

PEG-PRO NPs, respectively) (Supplementary Fig. S1A). We selected these polymneric nanocarriers for this purpose because they exerted no effect on the stimulation of GLP-1 secretion by L cells when non-targeted [22]. We prepared PLGA-PEG NPs and PLGA-PEG-PRO NPs presenting a particle size ~ 220 nm with a narrow size distribution (PDI=0.1) (Fig. S1B) to compare targeted polymeric nanoparticles with targeted lipid nanocapsules. The zeta potential of PLGA-PEG-PRO NPs decreased from -11.30 mV (PLGA-PEG NPs) to -23.50 mV after the grafting of propionate (Supplementary Fig. S1B).



C

Physicochemical characterization of exenatide (EXE)-loaded or unloaded or DiD-labeled RM LNC with different modifications.

Formulations	Mean Size (nm)	PDI	Zeta potential (mV)	EE (%)
RM LNC	204.8 ± 6.1	0.176 ± 0.007	-0.94 ± 0.12	-
RM LNC PEG	222.7 ± 11.1	0.159 ± 0.009	-9.93 ± 0.55	-
RM LNC PEG-PRO	231.6 ± 10.0	0.131 ± 0.033	-18.20 ± 1.40	-
DiD RM LNC	206.5 ± 4.6	0.208 ± 0.506	-1.31 ± 0.12	-
DiD RM LNC PEG	221.8 ± 9.9	0.156 ± 0.044	-9.39 ± 1.07	-
DiD RM LNC PEG-PRO	225.9 ± 2.43	0.123 ± 0.010	-16.20 ± 0.36	-
EXE RM LNC	208.4 ± 6.04	0.155 ± 0.021	-2.70 ± 0.23	82.46 ± 3.88
EXE RM LNC PEG	213.3 ± 6.68	0.176 ± 0.022	-9.23 ± 0.31	~100

Figure 1. Targeting strategies towards increased L cell stimulation to reach increased endogenous GLP-1 secretion. (A) Graphic description of the two steps followed to prepare L cell-targeted nanocarriers. Step one: provision of the nanosystem with stealth properties (PEGylation) aiming at (i) increasing the mucopenetration, (ii) thus further enhancing GLP-1 secretion with increasing number of nanocarriers able to get access to L cells, and ultimately (iii) prolonging the blood circulation of the nanosystems upon intestinal absorption. Step two: grafting of propionate on the surface of the nanosystems to improve L cell active targeting and strengthen the GLP-1 secretory effect. (B) Schematic representation of exenatide-loaded RM LNC PEG and RM LNC PEG-PRO. (C) Physicochemical characterization of empty, DiD-labeled or exenatide-loaded RM LNC after including the different surface modifications (mean ± SEM; n=9). For the schematic representation of EXE RM LNC please refer to [9].

III.2. (TARGETED) PEGYLATED NPS INDUCE GLP-1 SECRETION BOTH *IN VITRO* AND *IN VIVO*

We first investigated the ability of unloaded, PEGylated and propionate-grafted RM LNC to trigger GLP-1 secretion *in vitro* in murine L cells (GLUTag cells). We used a nanoparticle concentration ranging from 0.5 mg/mL to 2 mg/mL, after observing no evidence of cytotoxicity at concentrations below 4 mg/mL (supplementary Fig.S2). As shown in Fig. 2A, all lipid-based formulations were able to significantly trigger endogenous GLP-1 secretion *in vitro* in L cells whatever the nanoparticle concentration (**$p<0.05$). However, the stimulation of GLP-1 secretion was no significantly different among the tested groups, regardless of the incorporation of DSPE-PEG_{2000} or DSPE-PEG_{2000}-propionate on the surface of the nanocapsules. Thus, these strategies were not able to increase the ability of the nanocarrier to induce GLP-1 secretion *in vitro*. This observation of PEGylation not directly leading to an increased nanoparticle cell uptake has been also reported previously by others [23]. To further confirm the capacity of the grafted ligand (propionate) to stimulate L cells, we also examined the L cell stimulation exerted by targeted and non-targeted PEGylated PLGA NPs. Plain PLGA NPs were found to be inert on L cell stimulation [22]. Hence, if an increased GLP-1 secretion was to be observed using propionate-grafted PLGA NPs, this effect could be attributed to the ligand. PEGylated PLGA NPs failed to induce GLP-1 secretion when compared to the control group with all the nanoparticle concentrations tested ($p>0.05$) (Fig. 2B). The results were comparable to those observed for plain PLGA NPs [22]. Moreover, PLGA-PEG NPs grafted with propionate also did not induce GLP-1 secretion in GLUTag cells *in vitro* ($p>0.05$), further demonstrating that introducing propionate as a ligand on the surface of nanoparticles was not sufficient to activate the corresponding GPCRs on the surface of L cells, thus inducing GLP-1 secretion. We also tested the GLP-1 secretion induced on murine L cells by targeting PEGylated PLGA NPs with capric acid (medium/long-chain fatty acid that binds to GPR84 (PLGA-PEG-CAP NPs)), as a comparison. This ligand also failed to induce GLP-1 secretion *in vitro* in murine L cells (Supplementary Fig. S3).

Despite the discouraging results at increasing GLP-1 secretion by PEGylated lipid nanocapsules *in vitro*, and none effect at all by PLGA NPs, we investigated whether these nanocarriers could trigger GLP-1 secretion *in vivo* in normoglycemic mice. When orally administered to normoglycemic mice,

both PEGylated lipid nanocapsules (RM LNC PEG and RM LNC PEG-PRO, respectively) and non-PEGylated lipid nanocapsules (RM LNC), significantly increased GLP-1 levels 60 min post-administration (*p<0.05) (Fig. 2C). Notably, RM LNC PEG increased GLP-1 levels up to ~ 8-fold at 60 min, whereas RM LNC PEG-PRO kept the same effect as the original unmodified RM LNC, that increased up to ~ 4-fold GLP-1 secretion as compared to the untreated control group. Furthermore, only RM LNC PEG prolonged this effect at 180 min (*p<0.05) when compared to the control group. Although PEGylated NPs did not exert a significant effect *in vitro* when compared to non-PEGylated NPs (Fig. 2A), PEGylation could significantly improve and prolong the ability of the nanocarriers to induce GLP-1 secretion *in vivo*. However, further grafting propionate on PEGylated RM LNC not only could not activate the GPCRs on the surface of L cells to trigger more GLP-1 secretion, but also failed to reach the same effect as PEGylated RM LNC. Moreover, when orally administering PEGylated polymeric PLGA NPs (with or without propionate) both PLGA NPs failed to increase the GLP-1 secretion in normoglycemic mice (values were too low to be detectable, below 0.98 pg/mL), which further confirmed that decorating propionate on the surface of the nanocarriers had no L cell-targeting effect. However, it should be taken into account that the concentration of propionate at the surface of the nanocarriers might be too low to attain the effective concentration of propionate needed as per exerting an effect on L cells (140 mmol/L in mice [24]). In other words, the dose of propionate within the nanocapsules could be too low as per inducing the effect we were looking at.

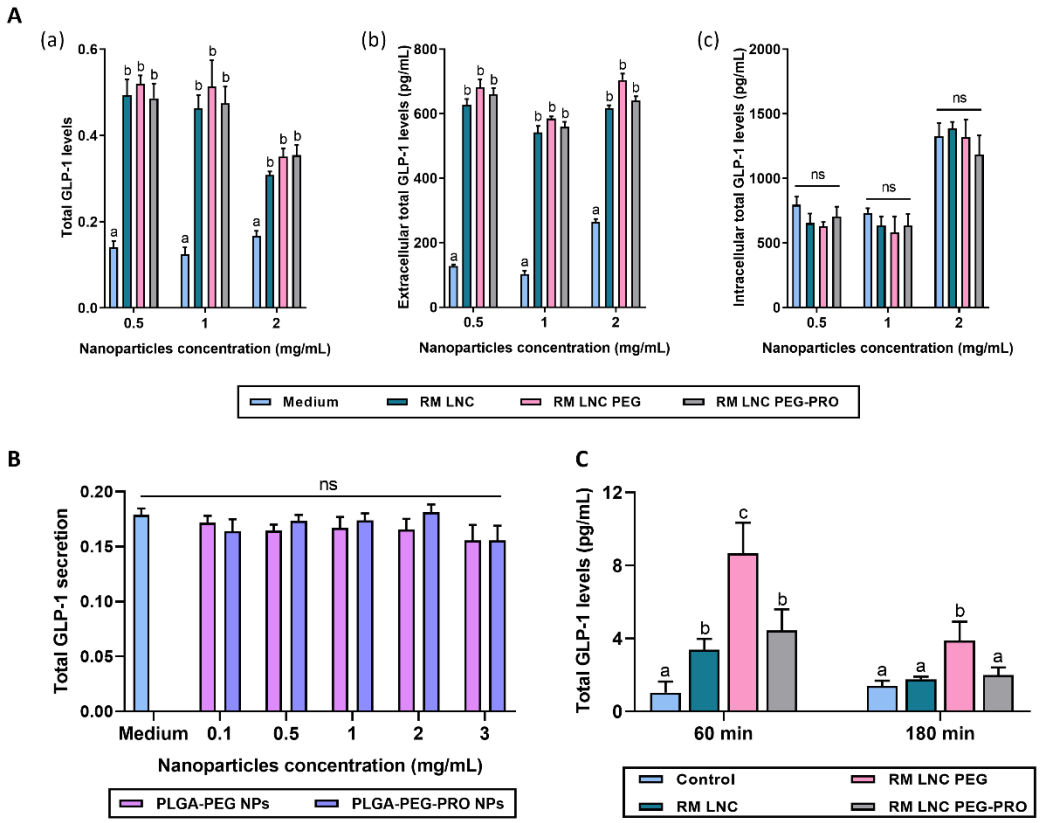


Figure 2. Effect of PEGylated NPs (with or without propionate grafted as a ligand) on GLP-1 secretion both *in vitro* and *in vivo*. (A) Effect of PEGylated RM LNC (with or without propionate grafted as a ligand) on GLP-1 secretion in murine GLUTag cells (L cells) after 2 h of co-incubation with increasing nanocapsule concentrations from 0.5 to 2 mg/mL) (mean ± SEM; n=4; N=3). (B) Effect of polymeric PLGA-PEG NPs (with or without propionate grafted as a ligand) on GLP-1 secretion from GLUTag cells after 2 h co-incubation with an increasing nanoparticle concentration from 0.1 to 3 mg/mL (mean ± SEM; n=4; N=3). (C) *In vivo* total GLP-1 secretion in normoglycemic mice 60 and 180 min after oral gavage with RM LNC, RM LNC PEG and RM LNC PEG-PRO, using the same nanocapsule dose (1.62 mg/g) (mean ± SEM; n=8). Data with different superscript letters are significantly different (*$p<0.05$) according to two-way analysis of variance followed by Tukey's post hoc test (A), by Mann-Whitney test (B) or by one-way ANOVA followed by Tukey's post hoc test (C).

III.3. NANOPARTICLE DISTRIBUTION IN THE SMALL INTESTINE AND COLON OF OBESE/DIABETIC MICE

We tracked the distribution of the nanocapsules in different segments of gut after oral gavage *in vivo* in 10 weeks HFD-fed mice. The accumulation of DiD-labeled nanocapsules within the small intestine and the colon upon oral administration to HFD mice is depicted in Fig. 3. All the fluorescent particles (DiD RM LNC, DiD RM LNC PEG and DiD RM LNC PEG-PRO) could

be found in the duodenum, in which DiD RM LNC PEG group showed the strongest red fluorescence (DiD) compared to other groups. RM LNC PEG-PRO seemed to fail reaching the surface of L cells and thus failed to reach the targeting goal we were searching for when grafting fatty acids (propionate) on the surface of the lipid nanocapsules.

To investigate a potential difference in mucus diffusion we performed single particle tracking. Therefore particles were mixed with small intestinal mucus, placed in a custom made glass chamber and visualized on a spinning disk microscope. However, it was not possible to determine particle diffusion *in vitro* in small intestinal mucus of mice due to a strongly reduced amount of observed particles as compared to the particles in water (Supporting Fig. S4 A). With confocal microscopy large structures with increased fluorescence intensity compared with mucus alone were observed (Supporting Fig. S4 B) for all formulations, indicating aggregation and/or binding to mucus constituents. Since the mobility of individual NPs could not be investigated, it was not possible to conclude if there are any differences in net mobility between the different formulations.

DiD labeled RM LNC were observed mainly in the duodenum and barely in the jejunum 1 h post-administration. Interestingly, one hour after the treatment, only RM LNC PEG were able to pass through the intestinal epithelia in the jejunum, with a high amount of red fluorescence observed in the epithelia basal layer (arrows) compared to other groups. Additionally, only RM LNC PEG were able to pass through the whole small intestine (duodenum, jejunum and ileum) reaching the colon. These data could explain why PEGylated RM LNC considerably increased GLP-1 secretion whereas RM LNC PEG-PRO exhibited no improvements on GLP-1 secretion. Complementary images on nanoparticle distribution are depicted in Supplementary Fig. S5.

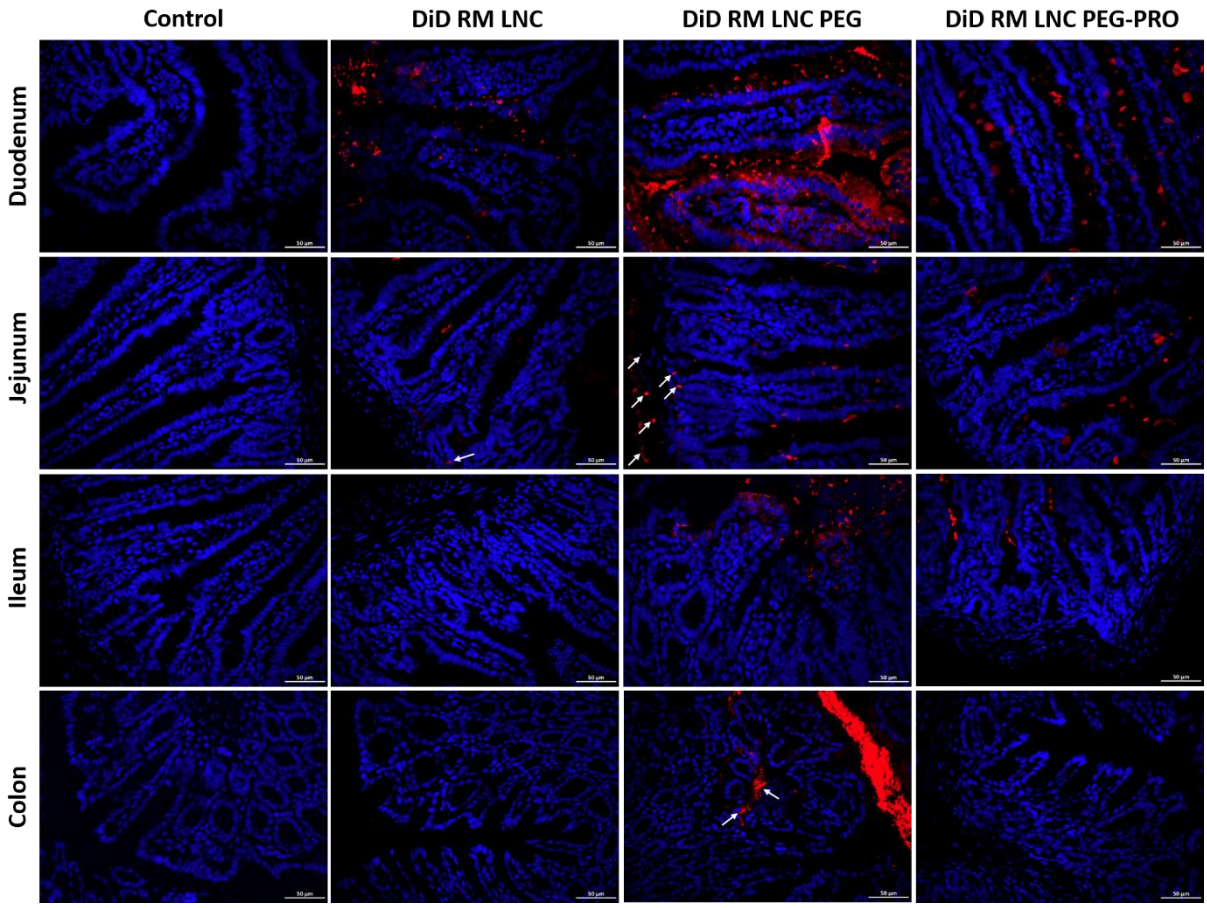


Figure 3. I*n vivo* distribution within the small intestine and the colon of mice. Representative images of fluorescent nanocapsules (red) distribution in duodenum, jejunum, ileum and colon in 10 weeks-HFD obese/diabetic mice 1 h after the oral gavage of MilliQ water, DiD RM LNC, DiD RM LNC PEG and DiD RM LNC PEG-PRO (1.62 mg/g nanoparticle dose), respectively (n = 4). The images show merged fluorescent nanocapsules (red)/ DAPI (blue) staining cell nucleus. White arrows point to lipid-based nanocapsules. Scale bar = 50 µm.

III.4. PHARMACOLOGICAL AND PHARMACOKINETICS STUDIES IN OBESE/DIABETIC MICE

As we observed no benefit on grafting propionate on the surface of PEGylated lipid nanocapsules with regard to L cell stimulation, we pursued pharmacological and pharmacokinetic studies with PEGylated nanocapsules only. Prior to the evaluation of the efficacy of the formulation *in vivo*, we demonstrated the stability of the formulation *in vitro* in simulated gastric and intestinal fluids, and evaluated the release profile of exenatide (Supplementary Fig. S6).

To determine the therapeutic efficacy of exenatide-loaded RM LNC PEG (EXE RM LNC PEG) on controlling the post-prandial hyperglycemia in type 2 diabetic mice, the pharmacodynamics

profile was performed in 10 weeks HFD-fed mice. We administered one single dose of the nanocapsules prior to an oral glucose tolerance test (OGTT). A 500 μg/kg EXE dose (EXE in solution, EXE RM LNC and EXE RM LNC PEG, respectively), empty RM LNC PEG (equal volume as per drug-loaded nanocapsules), or equivalent water volumes, were orally administered 60 min prior to an oral glucose challenge (2 g/kg). The time point of glucose administration corresponds to 0 h (Fig. 4A). The plasma glucose profile of drug solution-treated mice showed a similar trend as the HFD-fed mice receiving water. Conversely, all the nanocapsules-treated groups, including EXE RM LNC, EXE RM LNC PEG and empty RM LNC PEG, were able to significantly decrease plasma glucose levels and the glucose area under the curve (AUC) (Fig. 4A). It is remarkable that empty RM LNC PEG had a similar effect on lowering blood glucose levels and a similar AUC compared with exenatide-loaded RM LNC, indicating that the hypoglycemic effect achieved by the nanocarrier alone after PEGylation is comparable to non-PEGylated nanocarriers encapsulating the drug. Hence, the GLP-1 levels secreted by PEGylated nanocapsules (Fig. 2C) were found to be therapeutically relevant regarding a glucose lowering effect. It is important to note that only EXE RM LNC PEG-treated mice significantly decreased plasma glucose levels throughout the overall OGTT test compared to untreated HFD mice.

Additionally, we found out that total GLP-1 levels (Fig. 4B) were significantly increased in all nanocapsules-treated groups (*p<0.05). Although the increase of total GLP-1 levels in EXE RM LNC group did not reach statistical significance compared with HFD group when the data were analyzed by Kruskal-Wallis followed by Dunn's post hoc test, significant differences were observed when analyzed by the Mann-Whitney test (p=0.008) (Fig. 4B). The active GLP-1 concentration measured in the portal vein (3 h post-administration of the formulation) was also significantly increased in EXE RM LNC PEG-treated mice after OGTT test (*p<0.05) when compared with untreated HFD mice (Fig. 4C). Although we encountered no significant differences between the RM LNC PEG and the HFD when analyzing the data by one-way ANOVA, we encountered significant differences between these groups when analyzed by the Student t-test (p=0.028). These data would confirm that the PEGylation was able to improve the ability of the nanosystems to stimulate GLP-1 release and prolong this effect under pathological conditions. We encountered no

differences among groups regarding insulin levels (Fig. 4D). However, EXE RM LNC PEG were able to significantly reduce the insulin resistance index compared with the untreated 10 weeks HFD-fed group (Fig. 4E). Although we encountered no significant differences between the EXE RM LNC and RM LNC PEG when analyzing the data by one-way ANOVA, we encountered significant differences between these groups when analyzed by Student *t*-test (p=0.035 and p=0.023, respectively). Strikingly, we obtained a comparable result with unloaded PEGylated RM LNC when compared to the EXE-loaded nanocapsules, further proving the therapeutic relevance of the increased GLP-1 levels obtained through PEGylation.

To note, it is our understanding that the most suitable statistical test regarding the size of our study (groups and number of animals per group) is an ANOVA (parametric) or Kruskal-Wallis analysis (non-parametric). However, increasing the number of groups also increases the variability. We wanted to point out the fact that the large number of groups, and the variability of the study, could be hindering the identification of more significant differences among the groups. We have used the appropriate statistical test in the figure, however we wanted to highlight the differences encountered among groups, pointing to a strong physiological effect. This is highlighted by the differences encountered when conducting the Student *t*-test or Mann-Whitney test (parametric and non-parametric tests comparing two groups only).

To investigate the oral delivery of exenatide via PEGylated RM LNC, we conducted a pharmacokinetic study measuring the absorption of exenatide in HFD-induced obese/diabetic mice (10 weeks of HFD; n=10 per point) after oral administration of a single dose of 500 μg/kg exenatide via drug solution or within RM LNC PEG (EXE RM LNC PEG) (Fig. 4F). A different pattern of exenatide absorption was observed in obese/diabetic mice after oral gavage of drug solution alone or EXE RM LNC PEG. When orally administrated as a solution, the plasma concentration of exenatide remained unchanged through all the testing period. In comparison, EXE RM LNC PEG elicited a higher systemic absorption of the peptide after oral administration of for 8 h, with T_{max} and C_{max} of 1 h and 27.91 ± 1.22 ng/mL, respectively. The calculated pharmacokinetic parameters are summarized in Supplementary Table S1. One should note that we observed a different exenatide pharmacokinetic profile (e.g., different C_{max}, AUC, T_{max}) after oral

administration of EXE RM LNC PEG versus EXE RM LNC [9], suggesting that PEGylation extended the circulation time of the formulation ($t_{1/2}$ of drug-loaded formulation prolonged from 1.68 ± 0.35 to 5.69 ± 1.02 h) and then increased the systemic absorption of the encapsulated peptide (AUC increased from 11.79 ± 2.73 to 27.91 ± 1.22 ng·h/mL).

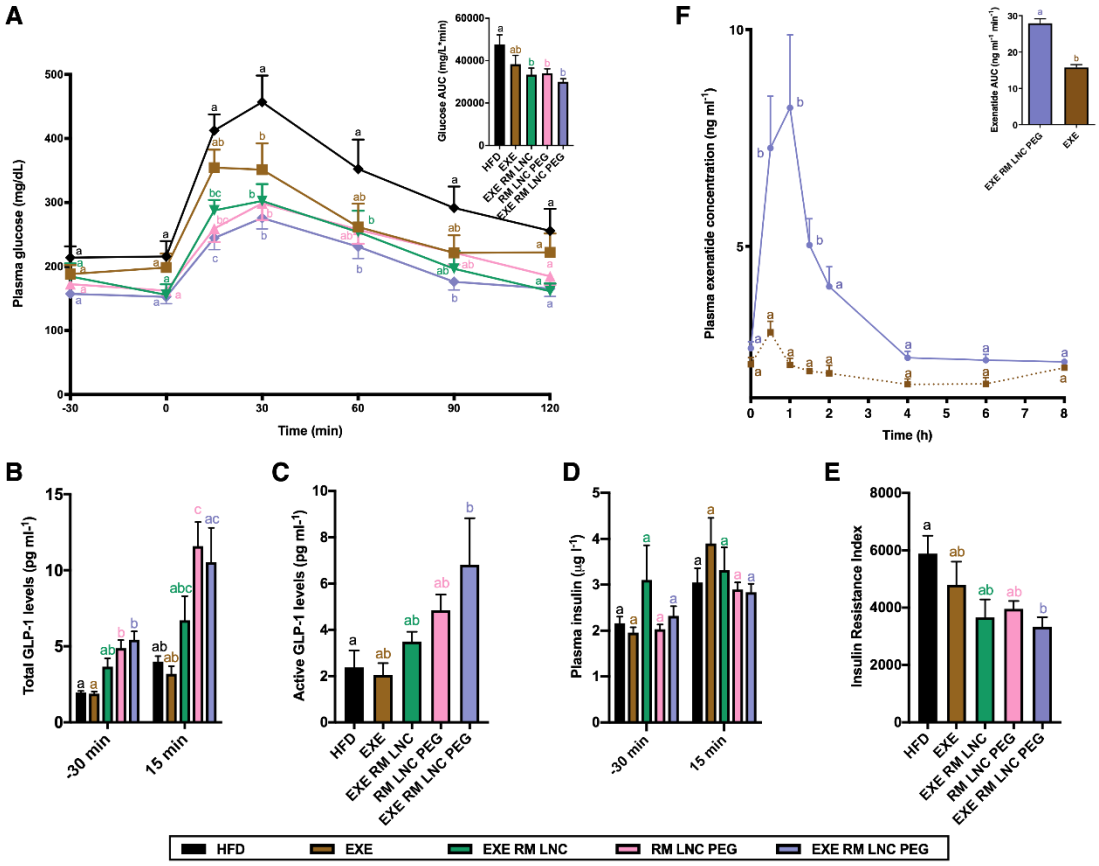


Figure 4. Pharmacological and pharmacokinetics studies in obese/diabetic mice after one single oral administered dose. (A) Plasma glucose levels (mg·dL^{-1}) and mean area under the curve (AUC, mg dL^{-1}·min) measured 30 min before and 120 after glucose challenge (n=7–8). (B) Plasma total glucagon-like peptide-1 (GLP-1) levels measured 30 min before and 15 min after glucose challenge (n=6–8). (C) Active GLP-1 levels measured in the portal vein of obese/diabetic mouse after OGTT (3 h post-administration of the formulation) (n=6–8). (D) Plasma insulin levels measured 30 min before and 15 min after oral glucose challenge (n=6–8). (E) Insulin resistance index (n=7–8). (F) Concentration-time profile and AUC of exenatide after the oral administration of EXE in solution or loaded within PEGylated nanocapsules (EXE and EXE RM LNC PEG, respectively) (500 µg/kg exenatide dose) (n=9-10). Data are presented as the mean ± SEM. Data with different superscript letters are significantly different (*p<0.05) according to two-way analysis of variance (ANOVA) followed by Tukey's post hoc test (A, F), or to Kruskal-Wallis followed by Dunn's post hoc test (B) or to one-way ANOVA followed by Tukey's post hoc test (C-E).

III.5. LONG-TERM TREATMENT IN OBESE/DIABETIC MICE WITH PEGYLATED LIPID NANOCAPSULES

To investigate the impact of the increased GLP-1 secretion observed after PEGylation on the glucose metabolism, we conducted a chronic and long-term treatment (one month) in obese/diabetic mice, administering exenatide-loaded or unloaded RM LNC PEG (500 μg/kg) with different administration frequencies (daily or once every other day) (14 weeks of HFD in total, daily or every other day administrations for 4 weeks from week 10). The mice treated less frequently (once every other day) with 500 μg/kg exenatide (oral) (EXE RM LNC PEG), or equivalent amounts of unloaded PEGylated nanocapsules (RM LNC PEG), were also compared with non-PEGylated EXE-loaded RM LNC (500 μg/kg dose) (EXE RM LNC) following the same regime of administration.

The plasma glucose levels were monitored weekly within the 4-weeks of treatment and are depicted in Fig. S7. The plasma glucose and insulin levels and the calculated HOMA-IR after a 4 weeks-treatment are depicted in Fig. 5. The PEGylated groups (loaded or unloaded) administered in a daily basis and the EXE-loaded PEGylated group administered every other day exhibited comparable glucose plasma levels. These levels were in turn comparable to the non-obese/diabetic untreated control mice (p>0.05, Fig. 5A). It is noteworthy that it was only when PEGylated nanocapsules were encapsulating EXE that they were effective at lowering glucose plasma levels following a long-term treatment. More importantly, it was efficient even under a less frequent administration, that was the main goal of the present study (Fig. 5A). Additionally, these groups exhibited an equivalent insulin resistance analyzed by the homeostatic model assessment of insulin resistance (HOMA-IR) comparable to the control values obtained for the healthy control group (p>0.05) (Fig. 5C). Compared to the HFD group, the reduction among these groups did not reach statistical significance when the data were analyzed by the Kruskal-Wallis followed by Dunn's post hoc test (Fig. 5C). However, the significant differences were observed when analyzed by the Mann-Whitney test (p=0.0012 for EXE RM LNC PEG-D versus HFD, p=0.0003 for RM LNC PEG-D treatment versus HFD, and p=0.05 for EXE RM LNC PEG-T treatment versus HFD, respectively).

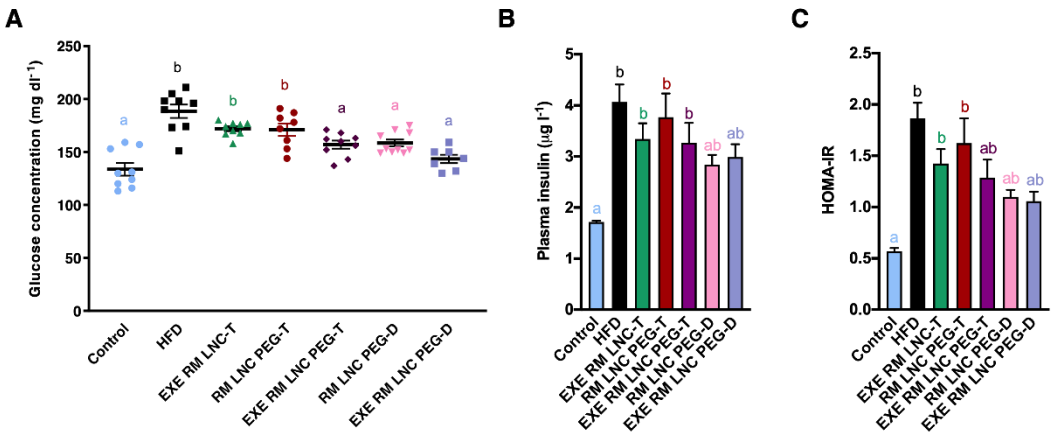


Figure 5. Effect of PEGylated and non-PEGylated EXE-loaded lipid nanocapsules on glucose homeostasis and hyperinsulinemia in obese/diabetic mice in a long-term treatment. (A) Plasma glucose levels (mg·dL^{-1}) after 4 weeks of treatment (14 weeks HFD feeding in total). Data are presented as the mean ± SEM (n=7-10). (B) Insulin plasma levels measured from blood retrieved from the tail vein (mean ± SEM; n=6-9). (C) HOMA-IR was calculated using the equation [fasting glucose (mg/dL) × fasting insulin (ng/mL)]/405 as previously described [25] (mean ± SEM; n=8-9). Different superscript letters are significantly different (*p<0.05). P values in (A) were determined by a two-way analysis of variance followed by Tukey's post hoc test. P values in (B, C) were determined by Kruskal-Wallis followed by Dunn's post hoc test.

IV. DISCUSSION

The aim of the present study was to increase the L cell stimulation exerted by our nanocarriers, thus increasing the induced secreted GLP-1 levels. This would allow us to prolong the *in vivo* antidiabetic effect exerted by our nanosystem towards less frequent peptide administrations. To that purpose, the aim of this study was to develop targeted-nanosystems towards an increased L cell stimulation. Based on the literature [26], we chose propionate as fatty acid to be grafted on the surface of our lipid-based nanocapsules, as it is known to be a potent highly effective ligand for GPCRs located on the surface of L cells.

We first evaluated the ability of propionate-targeted lipid-based formulations to stimulate GLP-1 secretion by L cells *in vitro* in murine GLUTag cells. All tested lipid-based nanocarriers effectively increased the secretion of GLP-1 by L cells, regardless of being grafted with propionate or not (Fig. 2A). Indeed, grafting the surface of lipid nanocapsules with propionate did not provide with any additional advantage compared to plain nanocapsules. However, as we pointed out, the propionate dose administered within the nanocapsules might be insufficient as per inducing an effect. The same applied when PLGA-based nanoparticles were grafted with both propionic and capric acid (Fig. 2B). As PLGA nanoparticles failed at increasing GLP-1 *in vitro*, we tested the effect of lipid-based nanocapsules (propionate-targeted or not) *in vivo* in normoglycemic mice. We surprisingly found out that RM LNC with PEG alone (the linker used to graft propionate on the surface of LNC) (RM LNC PEG) significantly increased the secreted GLP-1 levels from ~ 4-fold to ~ 8-fold at 60 min, prolonging the effect observed for RM LNC and increasing the effect at least up to 180 min (Fig 2C). Propionate-grafted nanocapsules (RM LNC PEG-PRO) significantly increased GLP-1 secretion compared to the control untreated group at 60 min. However, the effect was significantly lower than the effect exerted by PEGylated lipid nanocapsules alone and they did not prolong this effect (p>0.05 at 180 min) (Fig. 2C). We further investigated the localization of nanocapsules (fluorescence) in 10 weeks HFD-treated obese/diabetic mice 1 h after the oral gavage of the formulation (Fig. 3). We chose to evaluate the effect directly in a diabetic model and not in normoglycemic mice because the number of L cells is known to be increased in the pathological

context [27]. After 1 h of treatment, DiD-RM LNC PEG showed the strongest red fluorescence (DiD) in the intestinal lumen, and they were able to pass through the whole small intestine (duodenum, jejunum and ileum) to finally reach the colon. It should be noted that the colon harbors the highest amount of enteroendocrine L cells in the whole gut [28].

The ultimate goal of our study was to prolong the *in vivo* antidiabetic effect exerted by our nanosystem towards less frequent peptide administrations. For this purpose, once confirmed propionate-targeted nanoparticles were not exerting the effect we were looking at, we pursued our studies with PEGylated lipid nanocapsules. We evaluated the ability of PEGylated nanocapsules, and their non-PEGylated counterparts, EXE-loaded or unloaded, at improving glycaemia *in vivo* in obese/diabetic mice (fed 10 weeks HFD) following acute or chronic treatments. For the acute treatment with one single dose, all tested nanoparticles, PEGylated or not, were able to normalize blood glucose levels, significantly increasing the GLP-1 secretion. However, only PEGylated nanoparticles were able to significantly increase the secretion of active GLP-1, and the secreted circulating GLP-1 levels were higher than the ones observed for the non-PEGylated nanoparticles. We observed a different exenatide pharmacokinetic profile for EXE RM LNC PEG when compared to our previous studies with EXE RM LNC [9]. The PEGylation extended the circulation time of the formulation ($t_{1/2}$ of drug-loaded formulation prolonged from 1.7 ± 0.4 to 5.7 ± 1.0 h) and then increased the systemic absorption of the encapsulated peptide (AUC increased from 11.8 ± 2.7 to 27.9 ± 1.2 ng·h/mL).

To further demonstrate that the PEGylated nanosystem could be efficient at prolonging the *in vivo* antidiabetic effect and help minimize the administration frequency, we performed a chronic/long-term treatment consisting of a 4-weeks treatment with a protocol including two different administration regimes: once daily (D) or once every other day (T). After a 4-week treatment, EXE RM LNC PEG-T (once every other day treatment), RM LNC PEG-D and EXE RM LNC PEG-D (daily treatments) mice were able to normalize plasma glucose levels (Fig. 5A), significantly reducing the insulin resistance (measured by HOMA-IR, Fig. 5C). We were thus able to get comparable results among PEGylated nanocapsules, reaching basal glucose levels even with a less frequent administration when encapsulating EXE. Interestingly, administered once daily, the

unloaded PEGylated nanocapsules were able also to reach basal glucose levels and significantly reduce the insulin resistance. This confirms that the increase in GLP-1 secretion (~ 8-fold) was inducing sufficient stimulus as per lowering glucose levels. Moreover, this effect was prolonged in time, thus allowing us to reduce the administration frequency.

V. CONCLUSION

In conclusion, we reached our goal of increasing GLP-1 secretion and prolonging the antidiabetic effect of our formulation via PEGylation. Although many questions remain (e.g. exact mechanism of action, potential toxicity on the gastrointestinal tract upon frequent administrations, anti-PEG antibodies in the clinic), we have demonstrated our proof of concept that we can decorate the surface of our nanocapsules to modulate their effect on GLP-1 secretion. This brings hope for the treatment not only of type 2 diabetes mellitus, but also of other gastrointestinal diseases that require the secretion of other gastrointestinal peptides (e.g. inflammatory bowel diseases), and also on the use of nanomedicine towards oral peptide delivery. All these developments will help foster the translation of this approach to the clinics.

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VII. SUPPLEMENTARY MATERIALS

VII.1. RESULTS

VII.1.1. Cytotoxicity studies in murine GLUTag cells

The viability studies of different unloaded PEGylated lipid-based and polymeric nanoparticles were evaluated in murine enteroendocrine L cell line (GLUTag cells). The cytotoxicity of PEGylated lipid-based nanocapsules (both RM LNC PEG and RM LNC PEG-PRO) was nanoparticle concentration-dependent (Fig. S2A), which correlated with the data retrieved for RM LNC that we reported previously [9]. Moreover, there was no sign of cytotoxicity for both RM LNC PEG and RM LNC PEG-PRO when the nanoparticle concentration was lower than 4 mg/mL. The viability of PEGylated polymeric PLGA NPs (PLGA-PEG NPs and PLGA-PEG-PRO NPs) was nanoparticle concentration-dependent at tested nanoparticle concentrations ranging from 0.5 mg/mL to 10 mg/mL (Fig. S2B).

VII.1.2. Stability and drug release in stimulated gastrointestinal fluids in vitro

Evaluating the stability of the nanocarriers is of utmost importance to predict the *in vivo* behavior of the formulation. We performed a stability study of PEGylated exenatide-loaded RM LNC (EXE RM LNC PEG) in four biomimetic media, including FaSSGF (with and without pepsin), FaSSIF, and FeSSIF, representing different gastric and intestinal conditions contents before and after food intake [29]. Because of the different transit times in the gastrointestinal tract, EXE RM LNC PEG were incubated with gastric fluids for 2h, and with intestinal fluids for 6 h. As shown in Fig. S5A the mean particle diameter of EXE RM LNC PEG remained unchanged all through the incubation period, demonstrating that PEGylated nanocarriers exhibited no significant aggregation or disintegration and good colloid stability in all tested media.

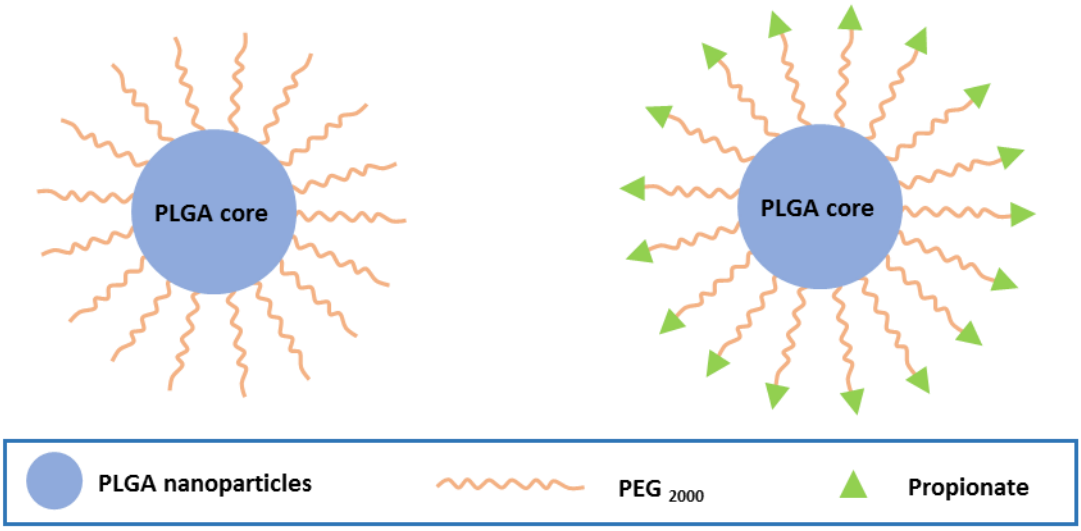
The *in vitro* exenatide release profile was further evaluated in gastric medium (FaSSGF without pepsin, pH 1.6) and in intestinal medium (FaSSIF, pH 6.5), respectively. The amount of exenatide released in gastric medium was undetectable, indicating the encapsulated peptide was protected

from degradation. Then, 70% of cumulative exenatide was progressively released from RM LNC PEG over a 6-h period in FaSSIF (Fig. S5B).

Table S1. Pharmacokinetic parameters of EXE in solution (500 µg/kg) and in EXE RM LNC PEG (500 µg/kg) after oral administration (n=9-10). Data are presented as the mean ± SEM. P values were significantly different (***p<0.001 and ****p<0.0001) according to Mann-Whitney test.

Formulations	**C_{max} (ng/mL)**	**T_{max} (h)**	**AUC_{0-8} (ng·h/mL)**	**$t_{1/2}$ (h)**
EXE RM LNC PEG	8.19 ± 1.68***	1	27.91 ± 1.22****	5.69 ± 1.02
EXE	3.01 ± 0.25	0.5	15.73 ± 0.80	-

A



B

Physicochemical characterization of blank PLGA-PEG NPs with or without ligand of propionate grafting.

Formulations	Mean Size (nm)	PDI	Zeta potential (mV)	EE (%)
PLGA-PEG NPs	224.5 ± 5.48	0.115 ± 0.009	-11.3 ± 0.231	-
PLGA-PEG-PRO NPs	212.6 ± 2.86	0.110 ± 0.038	-23.5 ± 0.146	-

Figure S1. PLGA PEG nanoparticles prepared by the double emulsification method. (A) Schematic representations of PLGA PEG NPs and PLGA PEG-PRO NPs. (B) Physicochemical characterization of blank PLGA-PEG NPs with or without propionate (mean ± SEM; n=3).

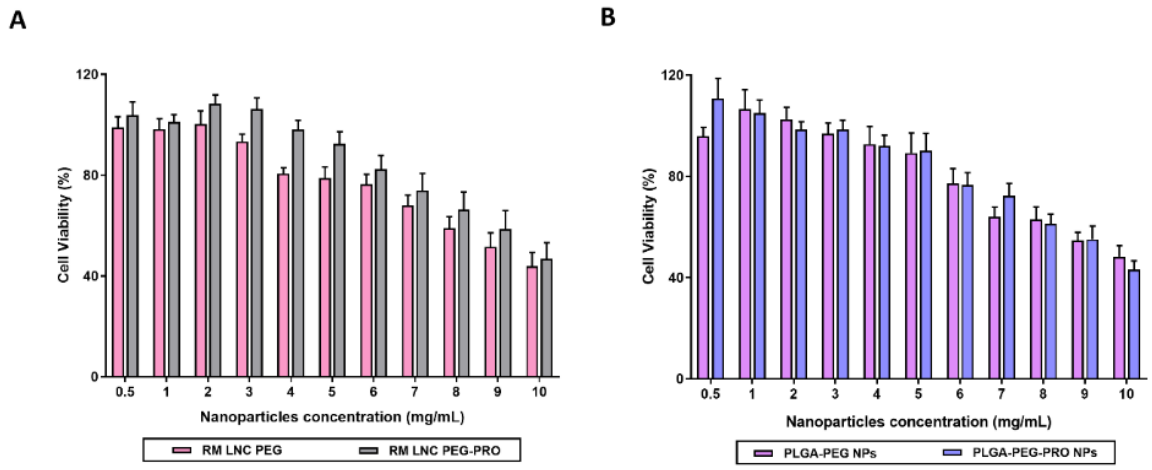


Figure S2. ***In vitro*** **cytotoxicity studies in murine L cells.** Cell viability of lipid nanocapsules (RM LNC PEG and RM LNC PEG-PRO) (A) and polymeric PLGA nanoparticles (PLGA-PEG NPs and PLGA-PEG-PRO NPs) (B) on murine GLUTag cells after incubation for 2 h at 37°C with increasing nanoparticle concentrations from 0.5 mg/mL to 10 mg/mL. Data are shown as the mean ± SEM (n=12). The data correspond to three independent experiments.

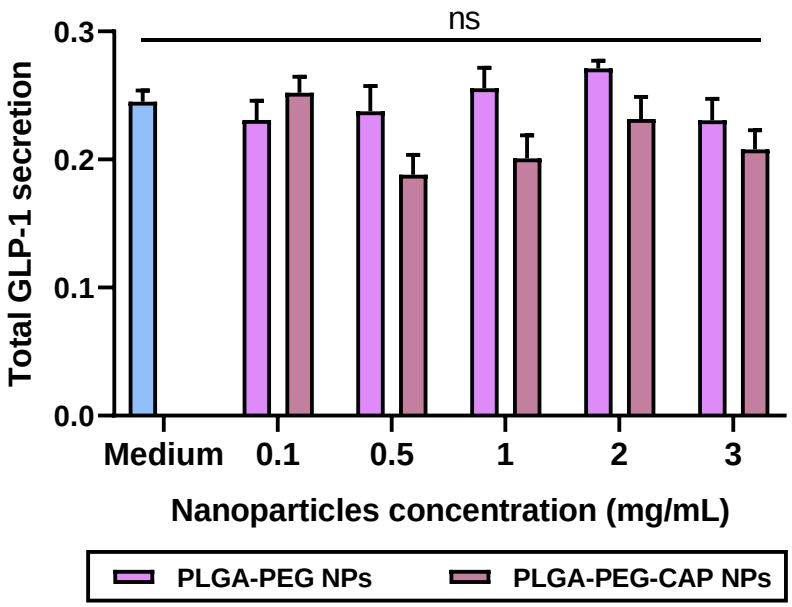


Figure S3. Effect of polymeric PLGA-PEG NPs, with or without caprate grafting, on GLP-1 secretion from GLUTag cells after 2 hours coincubation with an increasing nanoparticle concentration from 0.1 to 3 mg/mL (mean ± SEM; n=4; N=3).

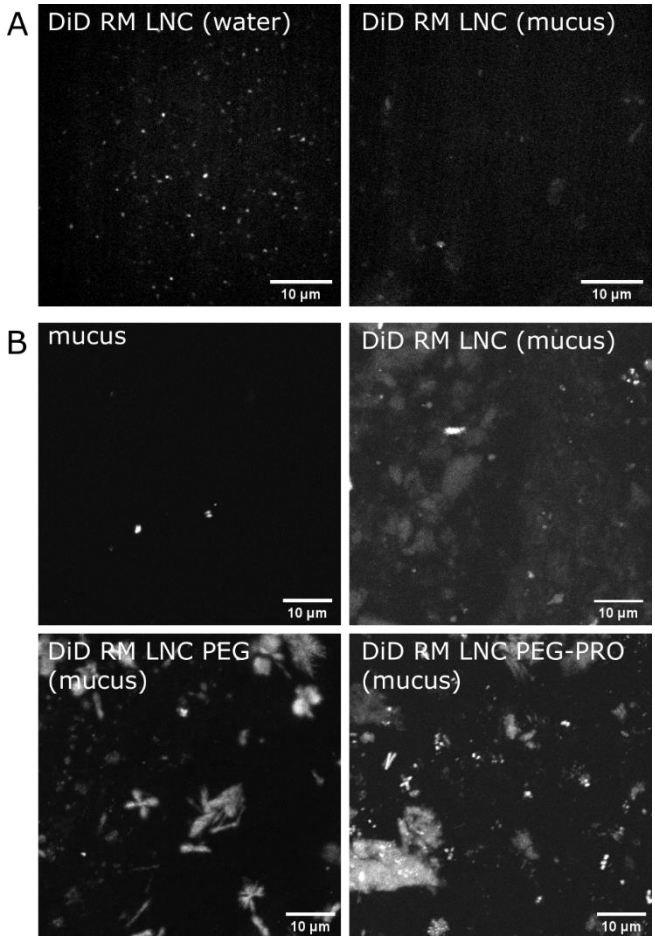


Figure S4. Imaging of particle behavior *ex vivo* in small intestinal mucus of mice. (A) Representative spinning disk confocal images used for single particle tracking of DiD RM LNC particles in water and in small intestinal mucus of mice. (B) Maximum intensity projections of laser scanning confocal images of small intestinal mucus of mice, DiD RM LNC, DiD RM LNC PEG, DiD RM LNC PEG-PRO in small intestinal mucus of mice *ex vivo*.

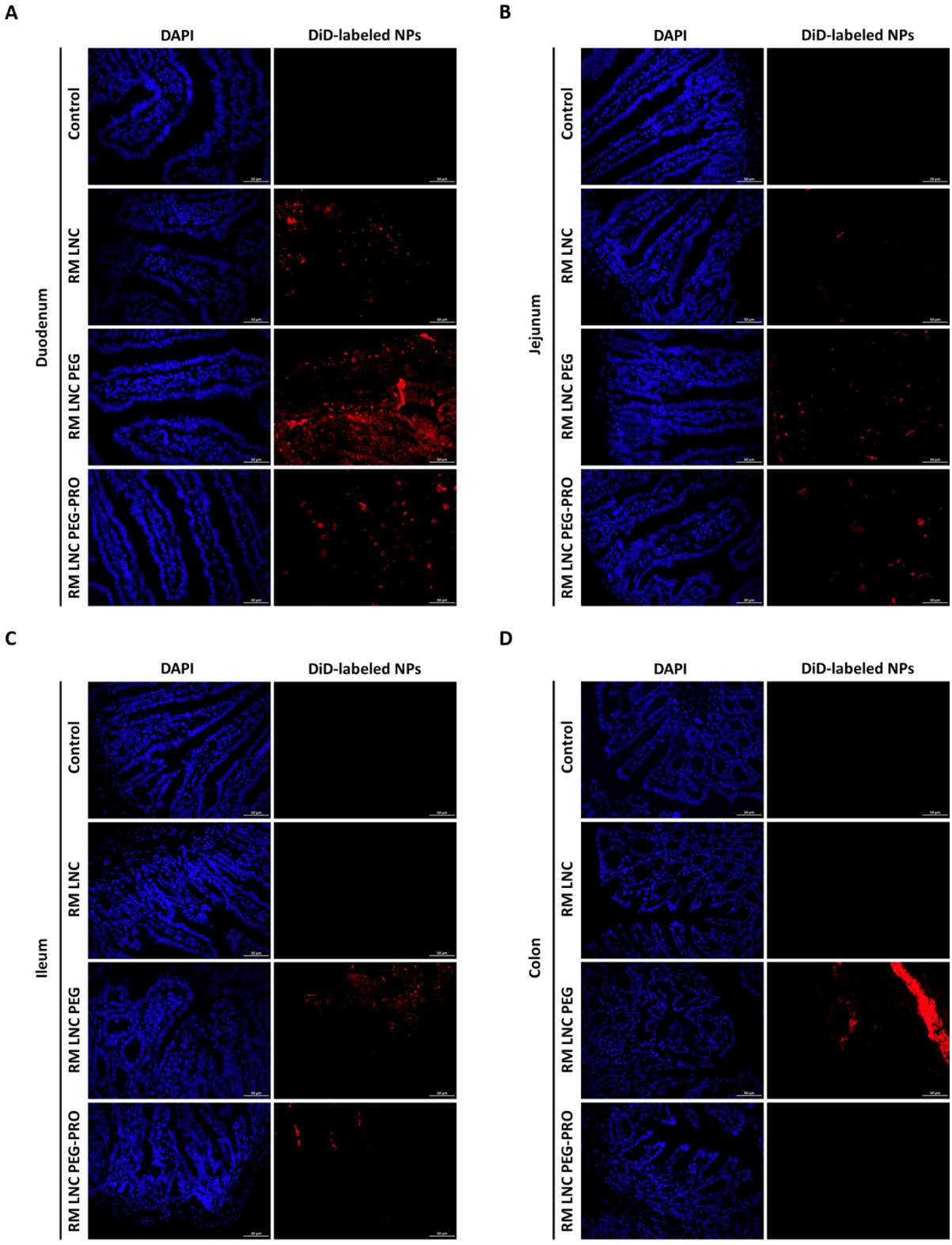


Figure S5. Distribution of nanocapsules in obese/diabetic mouse small intestine and colon. Representative confocal laser scanning microscopy images of nanocapsule distribution (DiD-labeled nanocapsules (red) DAPI (blue)) in duodenum (A), jejunum (B), ileum (C) and colon (D) in 10 weeks-HFD mice after 1 h oral gavage of MilliQ water, RM LNC, RM LNC PEG and RM LNC PEG-PRO (1.62 mg/g nanoparticle dose), respectively (n = 4). Scale bars: 50 μm.

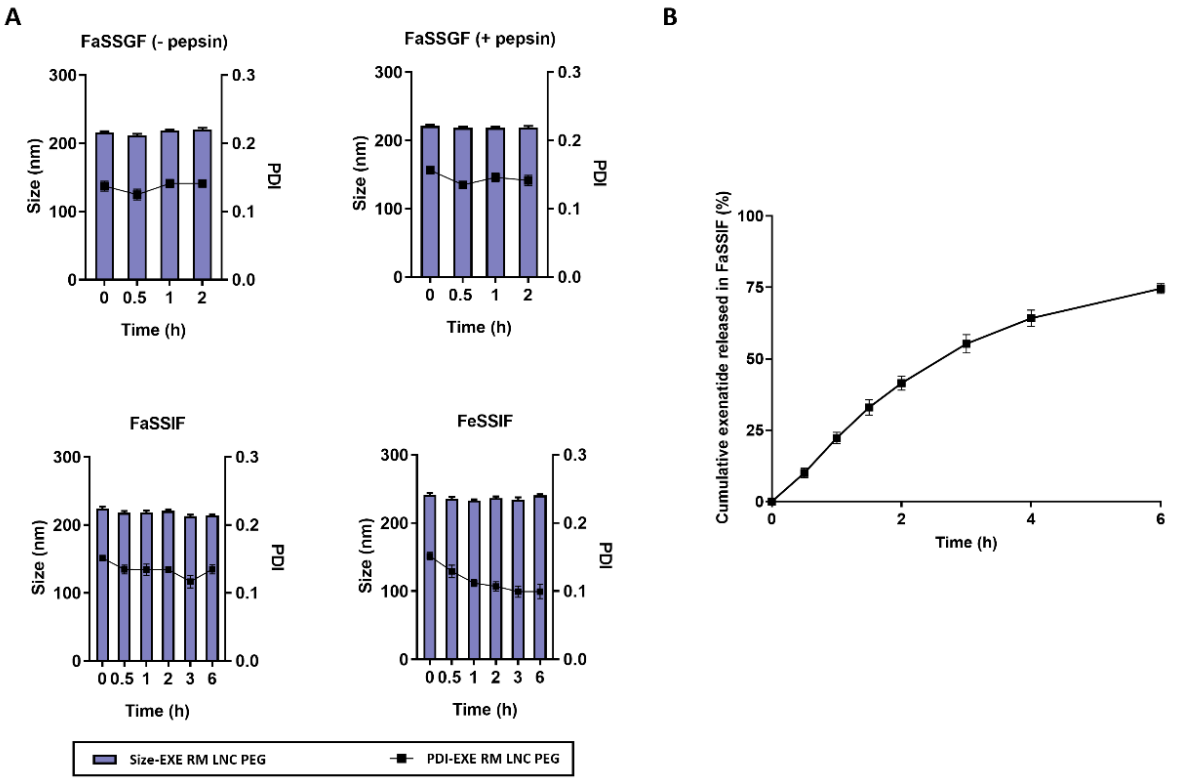


Figure S6. PEGylated nanocapsules' stability and exenatide release in biomimetic intestinal fluids. (A) Variation of particle size and PDI of EXE RM LNC PEG following incubation in FaSSGF (in the absence and in the presence of pepsin), in FaSSIF, and in FeSSIF, respectively, at predetermined time intervals (mean ± SEM; n=3; N=3). (B) Cumulative exenatide release profile in FaSSIF (pH 6.5) at 37 °C over 6 h as measured by HPLC (mean ± SEM; n=3; N=3).

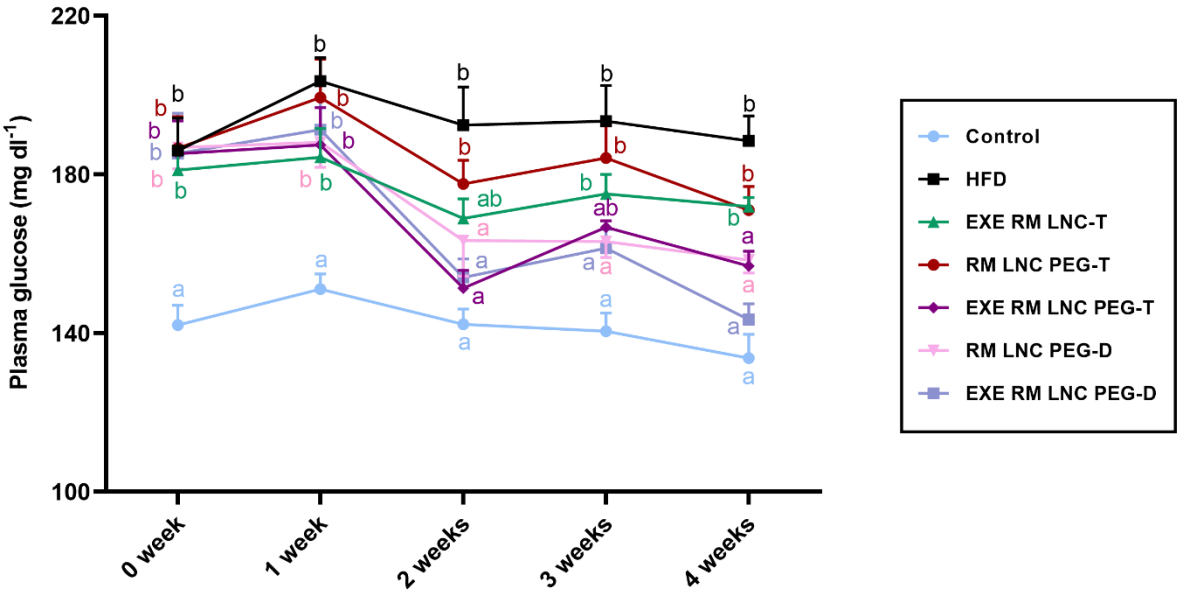


Figure S7. Plasma glucose levels over a 4-weeks exenatide treatment. Data are mean ± SEM (n=7-10). Data with different superscript letters are significantly different (*p<0.05). P values were determined following a two-way ANOVA followed by Tukey's post-hoc test.

CHAPTER VI

DISCUSSION, CONCLUSION & PERSPECTIVES

TABLE OF CONTENTS

I. MAIN CONTRIBUTIONS

The development of new oral drug delivery systems that will enable the absorption of therapeutic peptides to the systemic circulation is one of the greatest current challenges for the pharmaceutical industry [1]. Over the past decades, various strategies (e.g., permeation enhancers, prodrugs, nanocarriers) have been exploited to improve the oral bioavailability of these peptide drugs. Among them, advanced formulations have received considerable attention, especially targeting-based strategies (Chapter I). Although a number of improved targeting drug delivery systems for oral peptide delivery have been developed offering an enhanced bioavailability and greater therapeutic efficacy, they are not fulfilling their potential and they are not reaching the clinics yet [2].

The gut pathophysiology offers a stimulating environment, which is yet to be exploited in the drug delivery field. There is a wide variety of cells scattered through the gastrointestinal epithelium. Among these, enteroendocrine L cells have attracted particular interest because of the pleiotropic effects of their secreted peptides (e.g. glucagon-like peptide-1 (GLP-1)). These cells are attractive targets for the treatment of diseases such as obesity and type 2 diabetes mellitus (T2DM) [3]. The secreted GLP-1 from L cells possess an extremely short half-life due to its rapid degradation by the dipeptidyl peptidase (DPP)-IV enzyme within the first few minutes [4]. Several GLP-1 receptor agonists (GLP-1RAs) with improved plasma half-life (e.g. exenatide, semaglutide) have been developed (Chapter I). Apart from oral semaglutide (Rybelsus®, NovoNordisk, approved in September, 2019), most GLP-1RAs are approved for the treatment of T2DM only via subcutaneous injection. The formulation of Rybelsus® is based on the co-administration of the peptide with a permeation enhancer (sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC). The formulation presents two main limitations: 1) small differences in the peptide or the SNAC amounts substantially affect the absorption efficacy (variable bioavailability) and 2) the efficacy is limited to the administration of the GLP-1 analog semaglutide (half-life of 165h). We hypothesized that these two major limitations could be overcome using a nanoparticle-based drug delivery system.

The main contribution of this PhD project was to exploit the physiology of L cells, developing an innovative nanocapsule-based drug delivery system that synergizes its own biological effect (targeting enteroendocrine L cells to stimulate endogenous GLP-1 release) and that of the encapsulated bioactive molecule (improved systemic circulation of GLP-1RAs) as an alternative strategy for the treatment of T2DM via oral route (Chapter II). This innovative approach could maximize the potential of the nanocarriers in the oral delivery of an anti-diabetic peptide towards an improved incretin-based diabetic treatment, including (i) increased GLP-1 secretion from L cells triggered by a lipid-based nanocarrier with an adequate size (Chapter III; Fig. 1A), (ii) combined

increased endogenous GLP-1 secretion and improved blood circulation of GLP-1RAs towards T2DM treatment via oral route (Chapter IV; Fig. 1B), and (iii) enhanced nanocarrier's biological effect towards increased endogenous GLP-1 stimulation from through further PEGylation (Chapter V; Fig. 1C). They would offer additional advantages over current approaches for oral incretin mimetic peptide delivery by increasing endogenous GLP-1 levels.

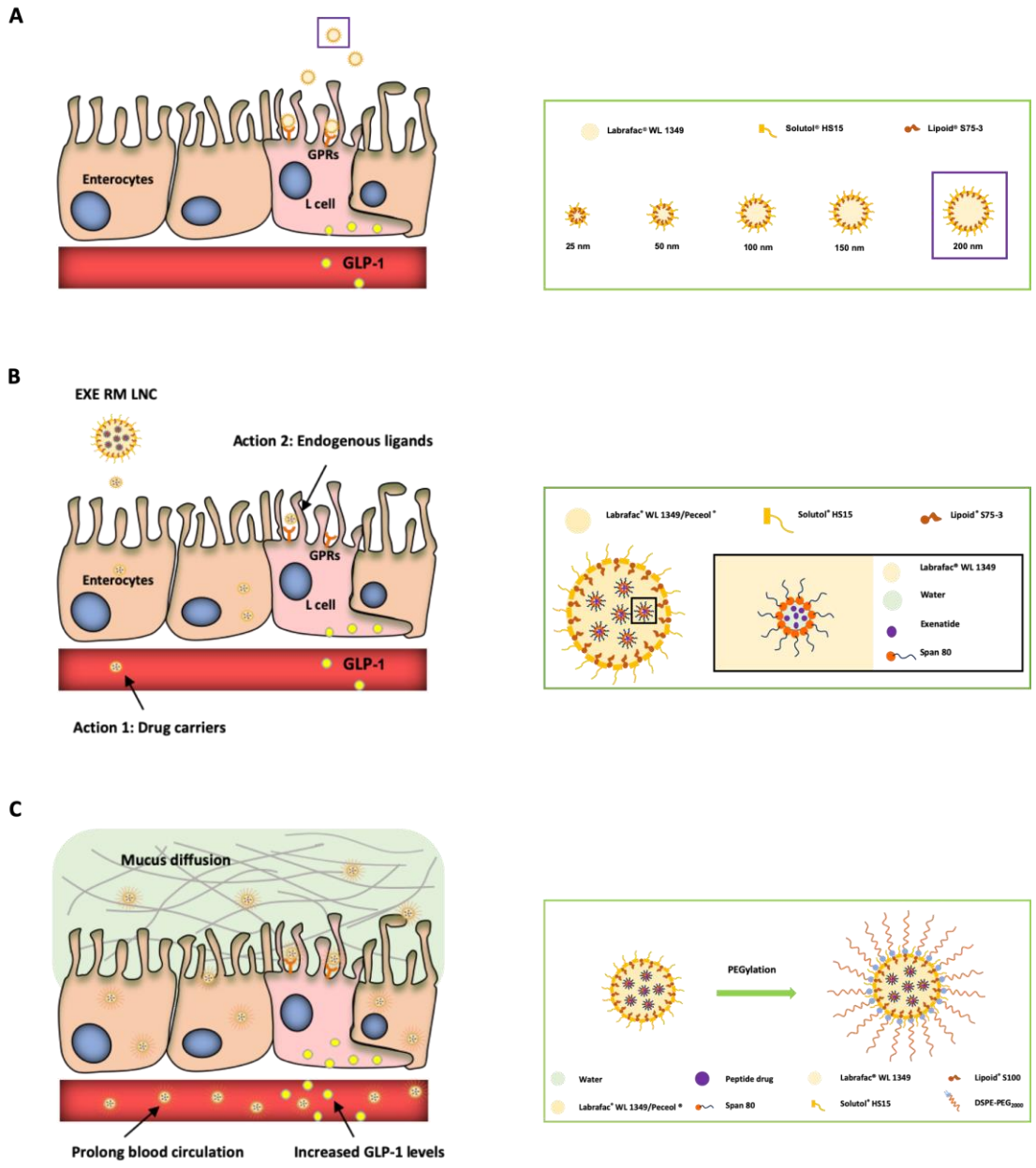


Figure. 1. Schematic overview of the approaches exploited in the project for the development of our nanocarriers. (A) Discovery of lipid nanocapsule-mediated GLP-1 secretion from enteroendocrine L cells when presenting a specific size; (B) Combination of increased endogenous secretion of GLP-1 induced by the nanocapsule and the increased oral bioavailability of the encapsulated GLP-1RA (exenatide) towards T2DM treatment; (C) Improvement of endogenous GLP-1 secretion with plain nanocapsules achieved via PEGylation.

II. MAIN FINDINGS DISCUSSION

In this PhD project, we developed, and further modified, a dual-action drug delivery system targeting enteroendocrine L cells for the oral delivery of peptides. The main objective of this project was to develop a lipid-based nanocarrier that could mimick the lipids found in the gut lumen that induce physiologically the secretion of GLP-1, thus stimulating GLP-1 secretion from enteroendocrine L cells. Based on previous studies conducted in our laboratory, we knew that lipid-based nanoparticles were able to induce GLP-1 secretion *in vitro*, but not polymeric nanoparticles [5]. We focused our attention on lipid-based nanoparticles, as a versatile platform for the oral delivery of drugs. Lipid nanocapsules (LNC), biomimetic carriers, present generally an oily core, corresponding to medium-chain triglycerides of caprylic (C_8) and capric (C_{10}) acids [6]. These lipids may activate L cell receptors mimicking endogenous ligands that trigger GLP-1 secretion. Bearing in mind that the nanoparticle size is often an essential factor to consider when designing nanocarriers, we firstly designed LNC presenting different sizes, from 25 nm to 200 nm, and evaluated their effect on GLP-1 secretion from enteroendocrine L cells *in vitro* and *in vivo* [7]. We demonstrated that only 200 nm size LNC increased significantly the secretion of GLP-1 in murine L cells when compared to smaller sizes, emphasizing the importance of size on LNC-induced GLP-1 secretion [7]. The efficacy of LNC at inducing endogenous GLP-1 was also confirmed *in vivo* in normoglycemic mice, being able to increase the endogenous GLP-1 secreted levels up to 4-fold compared to untreated mice [7]. This proof-of-concept study was enough to demonstrate that it is possible to induce GLP-1 secretion *in vivo* using lipid-based nanocarriers when presenting an appropriate size. However, the comprehensive mechanism of how 200 nm lipid nanocapsules induce GLP-1 secretion remains unknown. Further studies would be necessary for instance to investigate the contribution of a concrete receptor or second messenger, such as calcium (Ca^{2+}), cyclic adenosine monophosphate (cAMP), or mitogen-activated protein kinases (MAPKs) to GLP-1 secretion [8, 9], and the contribution of excipients and lipid concentrations of the formulation on the stimulation of GLP-1secretion. These studies would eventually lead to a new sight on the nanocarriers' physiological effect.

Considering that increasing endogenous GLP-1 secretion alone might not be sufficient to induce a therapeutic effect in the pathological context, we needed to encapsulate a hydrophilic molecule (an GLP-1RA) within the liquid lipid inner core of the nanocapsule, while preserving the physico-chemical characteristics of the nanocarrier, responsible for the *in vivo* biological effect observed with the nanocapsule *per se*. Although there are many GLP-1RAs approved on the market, we chose exenatide (half-life 2.4 h) in this project since it was easier to work from a technical point of view.

As an example, liraglutide (a long-acting GLP-1RA; 12 h) sticks to the Transwell inserts and hampers the quantification of the transported peptide across Caco-2 monolayers [10]. Moreover, there are available Elisa kits specific for exenatide quantification using a minimum plasma volume and not being responsive to endogenous GLP-1 [11, 12]. Hence, from a proof-of-concept point of view, it was easier to work with exenatide. Moreover, regarding a foreseen *in vivo* application, considering that T2DM is a chronic disease needing of a daily treatment, it would be safer to use a short half-life peptide, thus avoiding a potential associated toxicity regarding the accumulation of a long half-life peptide upon daily administrations.

LNC are produced by the phase inversion temperature (PIT) process, which needs of a high temperature cycle (from 60 °C to 90 °C) reached within the inversion of the two phases (O/W-W/O), thus resulting unsuitable to incorporate numerous therapeutic principles with hydrophility and heating instability (e.g. peptides) [6] (Fig. 2A). It has been demonstrated that reverse micelles (RM) could encapsulate hydrophilic drugs into lipid-based formulations [13]. Labrafac® WL 1349, consisting of medium-chain triglycerides of caprylic (C_8) and capric (C_{10}) acids, is the conventional oil phase in LNC. Span® 80, sorbitan monoleate, has a pseudo C_9 chain that could enhance its interaction with the capric and caprylic chains of Labrafac® WL 1349 to form RM [14]. Thus, to protect and stabilize exenatide within the nanocapsules, we firstly entrapped exenatide within reverse micelles (RM) and further encapsulated them within the nanosystem, as depicted in Fig. 2A. Moreover, by adding Peceol® within the oily core, we are able to modify this temperature, decreasing the PIT from 60 to 90 °C to 50 to 67 °C. Peceol® is approved by FDA as an oily carrier in oral delivery and consists of mono-, di- and triglycerides of oleic acid (long chain fatty acids). Modifying the length of the fatty acid would affect its interaction with the non-ionic surfactant (Solutol® HS15), thus changing the temperature of PIZ. These modifications allowed us to obtain exenatide-loaded reverse micelles lipid nanocapsules (EXE RM LNC) with the size of ~ 220 nm [15]. A schematic representation of EXE RM LNC is depicted in Fig. 2B.

It is worth noting that we already considered the potential translational approach; therefore, we conceptually chose scalable preparation procedures and *generally recognized as safe* (GRAS) excipients that could be easy to transfer to the pharmaceutical market. We have filled US and European patents on this formulation. Nevertheless, for a foreseen translation of this nanosystem into the clinics still some challenges remain due to its low loading capacity. In theory, a loading capacity in the order of 10% or better will be desirable in order to achieve acceptable oral dosage forms. We would expect the drug loading to be higher in our nanocarriers if we replace exenatide by a more lipophilic peptide (e.g. liraglutide).

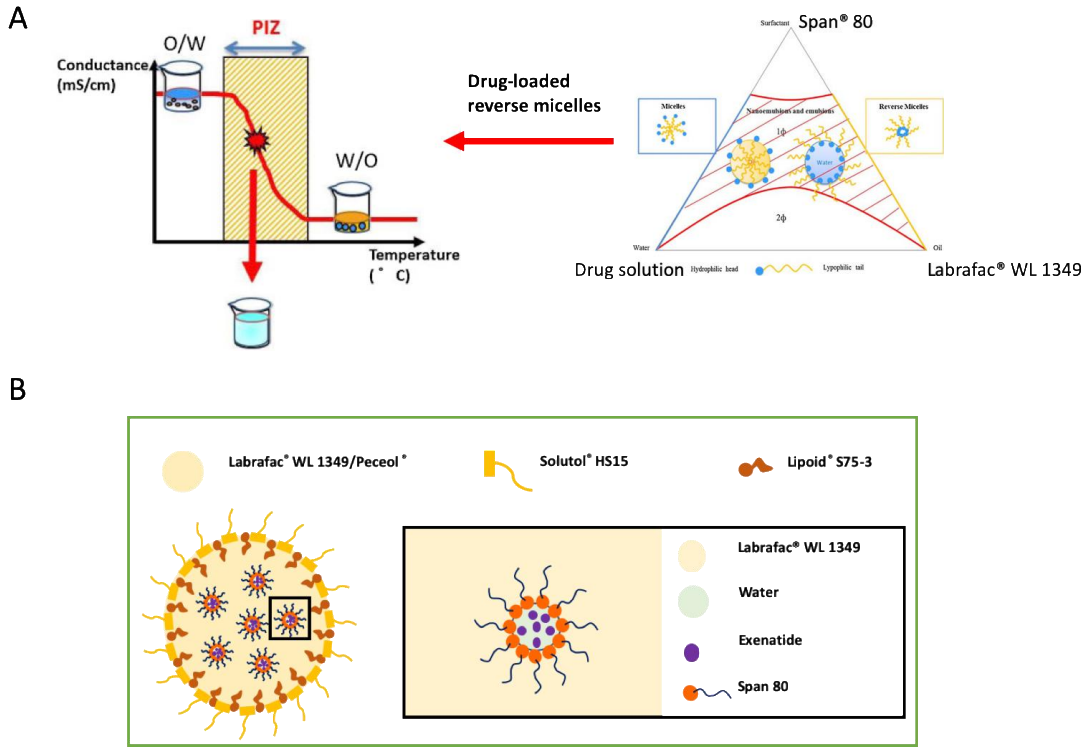


Figure 2. (A) Scheme describing a modified phase inversion process for the preparation of exenatide-loaded reverese micelles lipid nanocapsules. (B) A schematic representation of EXE RM LNC. Figure 2A modified from [16].

Although LNC are not new, and are well-known in the literature, this was the first time that 200 nm LNC were described. This was due to the difficulty on stabilizing the system, that we reached by using a different lipid core (including Peceol®) and reverse micelles to retain the peptide on the core.

To verify that this newly developed drug delivery system retained the biological ability of stimulating endogenous GLP-1 secretion, we first confirmed that the ability of reverse micelles-lipid nanocapsules (RM LNC) alone (unloaded) to induce GLP-1 secretion was preserved *in vitro* both in murine and human L-cells, and *in vivo* in normoglycemic mice [15]. A pharmacokinetic study confirmed the absorption of exenatide into systemic circulation *in vivo* in normoglycemic mice [15]. Taking altogether the increased exenatide blood levels and the increased endogenous GLP-1 levels, this nanosystem combined the two features that we were looking for.

Good glycaemia control is the main foundation of the management of T2DM treatment. In this project, we demonstrated that a single acute administration of EXE RM LNC normalized blood glucose levels in a HFD-induced obese/diabetic mouse model (3 weeks of HFD feeding), which were comparable to those of the healthy control group [15]. We also discovered that although empty nanocapsules exerted a therapeutic effect on lowering the blood glucose levels due to its

increased GLP-1 secretion, however, only EXE RM LNC-treated mice exhibited a significant decrease in the insulin resistance index compared to the HFD group [15]. We further confirmed this effect in chronic models (8 weeks or 10 weeks of HFD feeding), obtaining the similar results regarding the efficacy of EXE RM LNC on reducing blood glucose levels during OGTT, regardless of the chronicity of the disease. We also confirmed that EXE RM LNC increased exenatide bioavailability to more than 4% [15].

T2DM usually associates with metabolic syndrome, which is defined as a cluster of glucose intolerance, hypertension, dyslipidemia and central obesity with insulin resistance as the source of pathogenesis [17]. We therefore evaluated the effect of a long-term treatment following daily administrations on T2DM-associated hyperinsulinemia, dyslipidemia and obesity in an obese/diabetic mouse model, also comparing the effect to a marketed subcutaneous treatment (e.g. Byetta®). We firstly demonstrated that EXE RM LNC-treated mice exhibited normalized plasma glucose levels comparable to those of untreated control mice after 5 weeks of treatment (14 weeks of HFD in total), along with decreased insulin levels and insulin resistance. It is worth noting that our approach via the oral route was found to be at least as efficient as the current marketed drug (subcutaneous; Byetta®) as a comparison and could even be more potent on obesity/T2DM-associated metabolic dysfunction including oral glucose tolerance, insulin resistance, hepatic steatosis and inflammatory sites. These results demonstrated the noninferiority of our approach together with the benefit of administration by the oral route for chronic treatments. We do believe these data served as a strong proof of concept regarding the effectiveness of a dual-action drug delivery nanosystem at ameliorating glycaemia by combining both increased endogenous GLP-1 levels and increased peptide bioavailability.

Compared to previously described strategies for oral incretin peptide delivery [12, 18, 19], we were able to provide to endogenous GLP-1 levels, and that is the main advantage of our formulation. Previously described formulations for exenatide oral delivery, e.g. self-emulsifying drug delivery systems, reported a 14.62% ± 3.07% oral bioavailability in normoglycemic rats [12]. It would be expected to be higher in the diabetic context. However, the fact that we were able to induce GLP-1 secretion preserved us from needing an extraordinary systemic absorption of our peptide. The greatest limitation in oral peptide delivery is the poor bioavailability of the peptide. This is also true for the recently marketed formulation Rybelsus®. This is especially true for nanoparticles, and it is also true for our formulation. To our knowledge, none of the previously described nanoparticle-based strategies have been exploited their biological effect (increased endogenous GLP-1 secretion) to component the insufficient oral bioavailability of peptide drugs for T2DM treatment. Thus,

although we were not able to overcome the poor bioavailability limitation in this work, providing with a peptide absorption higher that previously described studies, we provided with additional endogenous GLP-1 secretion and that was what made the difference and allowed us to reach significant therapeutic levels of the peptide that was comparable to the effect of the market subcutaneous injection. In that sense, our results were ground-breaking.

Despite the striking effects observed, we believed it was still essential to develop strategies to strengthen the nanocarriers' GLP-1 secretory effect and/or prolong its antidiabetic effect *in vivo* towards its translation into the clinics. We hypothesized that this could be attained modulating the effect of our lipid-based drug delivery system by tailoring the surface of the nanocarriers. To strengthen the GLP-1 secretory effect, we exploited two strategies: (i) post-inserting a 2 kDa PEG on the surface of the nanosystem (EXE RM LNC PEG) to provide the nanocapsule with increased mucopenetration properties while prolonging the blood circulation once absorbed; (ii) grafting propionate on the surface of this lipid-based nanosystem towards the development of targeted nanosystems, using PEG as linker between the ligand and the nanocarrier (EXE RM LNC PEG-PRO). In this work, in order to elucidate the mechanisms and functions of the fatty acid ligands, we also prepared corresponding targeted PLGA nanoparticles as controls. We first observed that, among all the tested formulations, only RM LNC with PEGylation alone (RM LNC PEG) significantly increased GLP-1 levels from ~4-fold to ~8-fold at 60 min *in vivo*. After grafting with the ligand of propionate, nanocapsules seemed to fail reaching the surface of L cells and thus failed to achieve its targeting goal regarding the images on nanoparticle localization in the gut. The propionate-decorated PLGA nanoparticles also showed no improvement on GLP-1 secretion, which might be due to the low concentration of ligands on the surface of nanoparticles.

Based on above results, we further evaluated the anti-diabetic effect of the increased GLP-1 levels triggered by the PEGylated formulation with PEG alone *in vivo* in diabetic mice. In this part of the work, we demonstrated that RM LNC PEG, enhancing GLP-1 release, did significantly lower the post-prandial hyperglycemia in a strong diabetic mouse model (10 weeks of HFD-fed), which was even comparable to those of exenatide-loaded unmodified nanosystems (EXE RM LNC). Additionally, the pharmacokinetic study in HFD mice confirmed that exenatide blood levels were significantly increased when orally administered within EXE RM LNC PEG, and further PEGylation did extend the absorption period of the drug. To further investigate whether the increased GLP-1 secretory effect was sufficient to prolong the *in vivo* antidiabetic effect and minimize the administration frequency for the oral delivery of incretin peptides in the treatment of T2DM, we performed a chronic/long-term treatment consisting of a 4-weeks treatment with a protocol including different administration frequencies via oral route (once daily (D) or once every

other day (T)). After a 4-week treatment, the PEGylated groups (loaded or unloaded) administered in a daily basis and the EXE-loaded PEGylated group administered every other day exhibited comparable glucose plasma levels. These data confirmed that increased endogenous GLP-1 triggered by PEGylated nanosystems not only could reduce the dosing frequency of GLP-1RAs, but could also achieve a therapeutic efficacy in the treatment of T2DM. However, we were not able to achieve increased GLP-1 secreted levels when targeting the nanocapsules with fatty acids. The dose of these fatty acids might be too low as per exerting an *in vivo* effect. In our opinion, it still represents an interesting targeting strategy to be further investigated in the future.

The most important question remaining from this thesis is how the nanocarriers are exerting their biological effect *in vivo*, inducing GLP-1 secretion. In the following section, we discuss the alternatives for the mechanisms of action of the nanocarriers and we propose experiments that could be carried out to discard and/or confirm each of the potential mechanism.

III. UNRAVELLING THE MECHANISM BEHIND THE BIOLOGICAL EFFECT EXERTED BY THE NANOSYSTEM

In this PhD dissertation, we have exploited the biological effect of lipid-based nanosystems as oral delivery systems for GLP-1RAs (exenatide). The oral bioavailability improvement of those lipid-based novel nanomedicines was subjected to the combination of pharmacodynamics (e.g. glucose homeostasis regulation), pharmacokinetics (e.g. peptide plasma concentration) and drug delivery (e.g. targeting the site of interest). The promising efficacy of those nanosystems in preclinical animal models following a long-term treatment showed that these nanomedicines could represent an alternative approach for oral peptide delivery in incretin-based diabetes treatment. However, the mechanisms by which these nanocarriers exert a biological effect increasing endogenous GLP-1 secretion remain unclear. Identifying the mechanisms of the biological effect exerted by the developed nanosystem would represent a major break-through in the drug delivery field, opening new paves in the development of new therapeutic strategies to intervene in the pathogenesis of T2DM.

In this section, I propose a series of studies to unravel the mechanisms underlying the biological effect of this lipid-based nanocapsule. I am highlighting the potential technical problems that we may encounter and providing with feasible solutions to resolve them. All the key problems and feasible approaches are evaluated and discussed in this section.

III.1. EVALUATING THE COMPOSITION OF THE FORMULATION

The biological effect triggered by the developed nanosystems might be determined by one component of the formulation, a combination, or the whole nanocarrier *per se*. Although the first studies carried out in our lab pointed out that it might be the nanocarrier exerting the effect, we cannot rule out that one or more component(s) of the formulation might be responsible for the *in vivo* effect. Further studies could be undertaken in primary L cells (closer to the *in vivo* situation; see section 3.3). We are aware that most of the lipids present within the formulation (or the raw lipid material from the different commercial products) are immiscible in water, and that the use of (co) surfactants and or (co) solvents might lead e.g. the formation of micelles. Hence, the effect observed would not correspond to that of the lipid alone. For that reason, (i) we could alter different lipid components within the formulation (e.g. amounts) and/or (ii) substitute them by others (e.g. medium chain fatty acids by long chain fatty acids). (iii) We could increase the concentration of the formulation, to increase the number of particles per mL (without altering the

final volume administered, to avoid changes on the gastric emptying) to modify the dose of particles administered to the mice. These parameters can be evaluated first *in vitro* studies and further confirmed *in vivo* in different animal models.

In addition, we could not exclude the effect of surfactants present in our formulation (e.g., Span 80) on the gut microbiota, for example. Chassaing *et al* reported that Tween 80 with extremely high concentration (1% v/v via drinking water) exhibited a worsening of metabolic syndrome (such as glucose intolerance) [20]. However, in our study, we used extremely low concentrations of surfactant (Span 80), and obtained exact opposite results, ameliorating their metabolic syndrome in T2DM mice. Although we cannot rule out that this very small amount of Span 80 might have an impact on the effect exerted by our nanocarrier, we find it unlikely. However, this could be further study to confirm our assumptions.

III.2. *IN VITRO* LYPOLISIS STUDIES

Our nanosystem presents generally an oily core, corresponding to medium-chain triglycerides of caprylic (C_8) and capric (C_{10}) acids (Labrafac®) and long-chain triglycerides of oleic (C_{18}) acid (Peceol®). Four free fatty acid receptors (FFAR) 1-4, expressed on the surface of L cells have recently attracted attention as potential activators of endogenous GLP-1 release [21]. Among them, FFAR1 (GPR40) and FFAR1 (GPR120) might interact with our formulation and then increase the release of GLP-1 from L cells since these receptors respond to medium-chain fatty acids to long-chain fatty acids. Thus, we cannot exclude neither the possibility that sub-products resulting from the lipid digestion of the formulation exert an effect on the overall effect involving endogenous GLP-1 secretion. *In vitro* lipolysis by pancreatic lipases is the most common technique for the evaluation of the digestibility of orally administered lipid-based formulations. The parameters to standardize a method to evaluate digestion of lipid-based formulations has been validated by a consortium of academic and industrial scientists, *Lipid Formulation Classification System consortium* [22-27]. The intestinal lipolysis is simulated in a pH-stat apparatus consisting of a reaction vessel equipped with a glass electrode linked with a pH-meter that is connected to an adjustable controlling device. This device operates a motor-driven burette dipping into the reaction system and supplying the system with sodium hydroxide to maintain the desired pH value. Digestion is initiated by adding porcine pancreatic extracts to the reaction medium containing the dispersed lipid-based formulations [28]. Aliquots are withdrawn at given times and the lipase-induced digestion process is immediately stopped, either by addition of an inhibitor or freezing in liquid nitrogen. The resulted supernatants can be directly analyzed and tested for an *in vitro* and *in vivo* effect on GLP-1 secretion.

Once we find which supernatants could simulate GLP-1 secretion, further studies separating the different components in these digestive supernatants and evaluating them on the impact of GLP-1 secretion could be performed. We are aware that there is a big challenge in separating those components in the digestive supernatants. For this, the digestive supernatants could be analyzed to identify their specific composition. Then, the pure commercialized products that have the same components could be used as substitutes for further evaluating *in vitro* in primary L cells (section 3.3) and *in vivo* in mice (secetion 3.4) their effect. In addition, lipids are substrates of the gut microbiome. It would be interesting to evaluate the correlations of an acute or chronic exposure to these lipid-digestive components and/or a lipid-based formulation on the different gut microbiota.

III.3. *IN VITRO* PRIMARY L CELL STUDY

There are multiple cell lines used as models for GLP-1-producing L cell research, including GLUTag (a murine endocrine tumor-adherent cell line), STC-1 (a rat adherent cell line) and NCI-H716 (a human-derived suspension cell line) [29, 30]. Such cell lines are useful and suitable models for cell biological studies since they can be routinely passaged and maintained and scaled up [30, 31]. However, they are incompletely validated as accurate models of the native L cell because multiple limitations are associated with the use of cell lines in research, mainly including (i) all three cell lines are derived from tumors and therefore have genetic and morphological differences from their *in vivo* L cells; (ii) they lack many physiological traits of their native L cells with regards to cell morphology and polarity; and (iii) they exhibit different nutrient sensitivities [29].

Primary L cells are thus more relevant to investigate the mechanisms of GLP-1 secretion triggered by nutrients and/or drugs [29]. Primary L cells are directly isolated from the intestine and maintain their morphological and functional characteristics [32]. Compared with immortalized cell lines, the key advantages of primary cells are their functional and genetic fidelity. To unravel GLP-1 secretory mechanisms on primary L cells triggered by nutrients and/or drugs, several advanced experimental tools can be applied to assess primary L cells, such as flow cytometry (e.g., fluorescence-activated cell sorting), transcriptomics (e.g., RNA-sequencing methods), electrophysiology (e.g., patch clamp studies) and live-cell imaging (e.g., calcium imaging techniques), the measurement of gut hormones secretion [29, 32]. The combined application of these techniques could further facilitate the identification of the activated receptors on the surface of primary L cells, the triggered sensors in cells, and activated pathways of hormones secretion.

III.4. *IN VIVO* TRANSGENIC AND GENE KNOCKOUT MOUSE MODELS

A transgenic mouse is a mouse that has exogenous genome sequences through the use of genetic engineering techniques [33]. A gene knockout mouse defines an animal model in which, by means of gene targeting, one or more genes of interest are disrupted or inactivated [34]. Over past years, both of them have provided a powerful means to elucidate the biological mechanisms of gut hormones secreted from enteroendocrine cells [35-37]. There are many types of transgenic and knockout mouse models that have been created for this purpose, e.g., fluorescence-based specific sensors-transgenic mice, specific receptors-knockout mice and specific enzymes-knockout mice.

The *in vitro* studies abovementioned could provide with information regarding the mechanisms of the biological effect exerted by our nanosystems, (i) identifying the potential lipid component(s) and/or combinations within the formulation responsible for this effect; (ii) identifying the activated receptors, sensors and pathways in primary L cells. All these data should be further demonstrated *in vivo* in corresponding transgenic and gene knockout mouse models. There are some limitations on primary L cells-based studies: (i) the short-live nature of the culture; (ii) the isolation of enteroendocrine L cells from some of the surrounding cell types, loss of apico-basal polarity, and lack of the epithelial barrier; (iii) they lack some important enzymes for secreted hormones (e.g., DDP-IV). These limitations may hinder the complete picture on how the nanocapsules exert their biological effect. For instance, to study the mechanisms by which GLP-1 levels are increased via our lipid-based nanosystems, we may not rule out the possibility that lipid nanocapsules could modulate the activity of DPP-IV, thus preserving increased circulating total GLP-1 levels in the body, which warrants further investigation. For this, DPP-IV knockout (DPP-IV$^{-/-}$) mice [38] could be used to provide an answer for our hypothesis. In addition, other hormones might be also altered when administering our nanocapsules, e.g. GIP or proglucagon. It would be interesting to measure the levels of these peptides and further evaluate their role on the secretion of GLP-1. In the event the levels of both GIP and DPP-IV were elevated, we could use knockout animal models to confirm their contribution to the overall effect.

IV. FUTURE PERSPECTIVES

This PhD thesis provides with a huge amount of data demonstrating that a more physiological alternative for T2DM treatment is feasible. We have developed an oral peptide delivery system that exploits the biological effect of the nanocarrier (stimulating GLP-1 secretion from enteroendocrine L cells) combined with the absorbed encapsulated peptide (increased plasma levels). In this PhD we have focused our attention on GLP-1 for T2DM treatment. However, to exploit the full potential of the nanosystem, further studies can be conducted in the near future. These are according to the following facts:

(i) Enteroendocrine L cells not only produce GLP-1, but also control the secretion of other hormones with therapeutic abilities, such as GLP-2 (involved in inflammatory bowel diseases (IBD)) and PYY (involved in obesity).

(ii) Enteroendocrine K cells produce another incretin hormone called GIP that plays an important role in controlling insulin release from pancreas.

(iii) The innovative oral drug delivery nanosystem could also encapsulate other peptide drugs, not just exenatide.

Based on these basic theories, this section aims at evaluating some other alternative perspectives of this PhD project and shows some preliminary results that were have obtained to establish future studies.

IV.1. TO EXPLOIT THE BIOLOGICAL EFFECT OF NANOCARRIERS AT AMELIORATING INFLAMMATORY BOWEL DISEASE

In this doctoral dissertation, we have demonstrated that the developed nanosystem can simulate endogenous GLP-1 *in vitro* both in murine and human L cells and *in vivo* in normoglycemic and diabetic mice. L cells produces several gut hormones, not just GLP-1 [39]. Intestinal GLP-2, as an example, is co-secreted along with GLP-1, which is involved in the maintenance of the physiological gut barrier function and regulates the stimulation of intestinal epithelial cell proliferation [40]. Although there is a degradation resistant analogue of GLP-2, teduglutide, on the market, supraphysiological injection doses are required to elicit its growth promoting effects. The therapeutic potential of GLP-2 has been studied in mice with trinitrobenzene sulfonic acid (TNBS)-induced or dextran sulphate sodium (DSS)-induced colitis [41]. The protective effects of GLP-2 in both models of intestinal inflammation and damage were indicated by increases in crypt depth, villus height and a reduced hyperproliferative activity of crypt cells. Hence, increasing physiological

concentrations of biologically active GLP-2 offers an alternative therapeutic strategy to direct peptide administration. It would be interesting (i) to exploit the therapeutic effect of nanoparticle-mediated endogenous GLP-2 levels at restoring the intestinal barrier and at reducing inflammation, (ii) to encapsulate GLP-2 analogs and/or other anti-inflammatory drugs in our dual-action nanocarrier to ameliorate IBD *in vivo* following a long-term treatment.

Higher endogenous GLP-2 production has been associated with improved mucosal barrier function via the restoration of tight junction protein expression and distribution [42]. To evaluate whether our nanosystem induced the simulation of GLP-2 secretion, we firstly performed GLP-2 secretion studies on GLUTag cells upon incubation with 200 nm lipid nanocapsules. The preliminary results of this experiment are presented in Fig. 3 (unpublished data) and show that 200 nm lipid nanocapsules could significantly increase GLP-2 levels *in vitro* in murine L cells. We further demonstrated *in vivo* that empty lipid nanocapsules significantly increased GLP-2 levels compared to untreated control mice 60 and 180 min after their oral administration in a DSS-induced acute colitis model (see unpublished preliminary data in Fig.4).

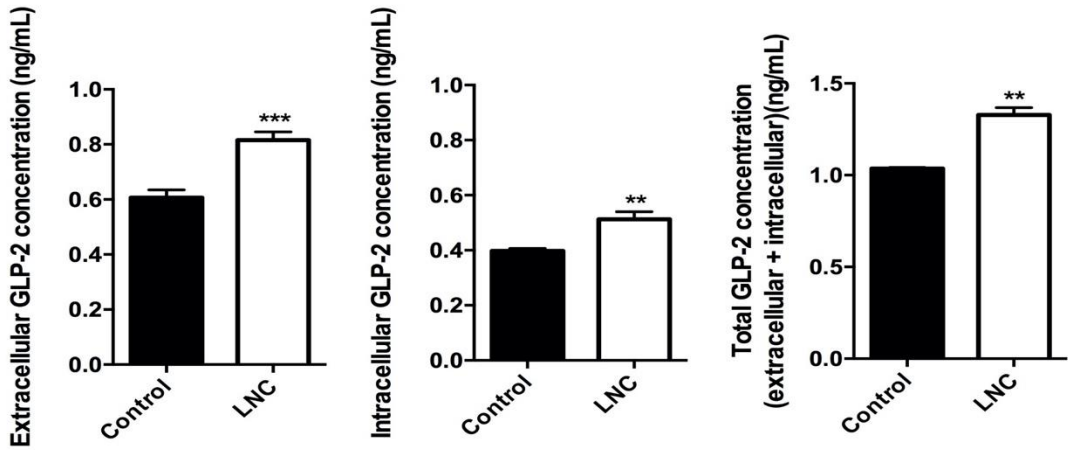


Figure 3. Preliminary data on the effect of 200 nm (LNC) on GLP-2 secretion by GLUTag cells after a 2 h co-incubation period (mean ± SEM; N=3, n=4). $^{**}p<0.01$ and $^{***}p<0.001$ compared to the untreated control group.

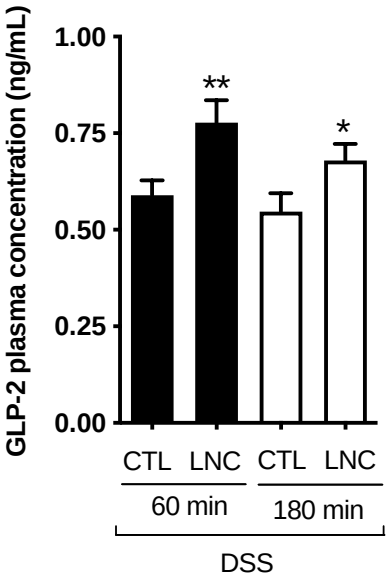


Figure 4. Preliminary data on the effect of 200 nm lipid-based nanocapsules on GLP-2 levels in an acute murine DSS-induced model 60 and 180 min post-oral administration of a 1.62 mg/g nanoparticles dose. GLP-2 levels were significantly higher in the empty lipid nanocapsules-treated DSS group (LNC) compared to the untreated DSS group (CTL) (n=8; mean ± SD; $^{**}p<0.01$ and $^{*}p< 0.05$ vs CTL).

Then, we investigated whether these increased GLP-2 levels triggered by our nanosystems can achieve a therapeutic efficacy in a pathological context. In this study, C57BL/6J mice were treated with 5 days-dextran sodium sulfate (DSS) to induce an acute colitis model, and then they were orally administered with 200 nm empty lipid nanocapsules for 3 days. After a 3-days treatment, we quantified plasma citrulline levels. Plasma citrulline is mainly produced by enterocytes in the small bowel and its concentrations are decreased in IBD patients. Citrulline level is a marker for mucosal healing (the higher the citrulline, the higher the intestinal mucosal mass) [43]. Strikingly, we found that the empty nanocapsules-treated mice significantly increase plasma citrulline levels compared with both untreated healthy and DSS groups, as shown in unpublished preliminary data in Fig.5.

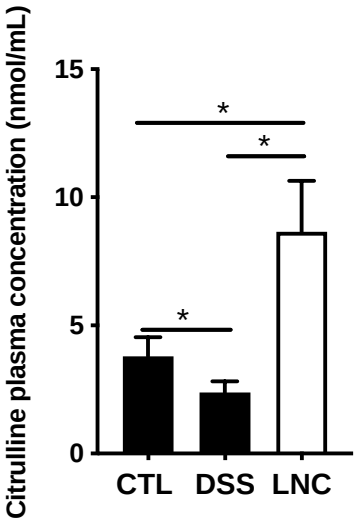


Figure 5. Preliminary results of plasma citrulline levels in health or DSS-induced colitis mice after the oral administration of 200 nm empty lipid nanocapsules (LNC) for 3 days (n = 8; mean ± SEM). *$p < 0.05$ shows significant differences between the tested groups.

All these data could serve as strong proof-of-concept that lipid-based nanosystems can induce endogenous GLP-2 secretion *in vitro* in murine L cells and *in vivo* in an acute colitis model, and confirm the effectiveness of LNC at increasing GLP-2 levels in the IBD pathological condition. Following the same principle as per GLP-1, we hypothesize that combining endogenous GLP-2 levels with that of the encapsulated peptide can provide with therapeutically relevant plasma levels for its use in the treatment of IBD.

The GLP-2 analog teduglutide is only approved for the treatment of short bowel disease, not for IBD. In future studies, an anti-inflammatory molecule could be encapsulated within our nanosystems instead of teduglutide. In this perspective project, we would evaluate the effect of combining increased GLP-2 levels (exerted by our nanosystems, Fig. 4) with the anti-inflammatory properties of the encapsulated drug. Most of the anti-inflammatory drugs used in IBD treatment (e.g. budesonide) are likely to cause systemic adverse effects. Taking into account the increased bioavailability observed for exenatide (Chapter IV and V), we need an anti-inflammatory drug exhibiting non-associated systemic side effects. On this regard, the PeptT1-mediated tripeptide Lys-Pro-Val (KPV) can been selected as anti-inflammatory drug [44, 45]. This is a very potent anti-inflammatory peptide, but it has not been yet fully exploited to its full potential in the treatment of IBD.

IV.2. TO COMBINE THE PHYSIOLOGICAL EFFECTS OF MULTI-GUT HORMONES VIA NANOCARRIERS

In this thesis we have exploited the biological effect of GLP-1 triggered by nanocarriers to treat T2DM, and also introduced a perspective project of exploiting the physiological functions of GLP-2 at ameliorating IBD. However, there still exist other important gut hormones (e.g., PYY and GIP), which also regulate several beneficial effects in the body. PYY, secreted by L cells, reduces food intake and induces food aversion, and in combination with GLP-1, plays a major role in the ileal brake, a physiological mechanism delaying gut motility and transit in order to enhance nutrient (mainly lipids) absorption [46]. GIP is another incretin peptide, produced by K cells, also involved in gastrointestinal function, glucose homeostasis and satiety [47]. Unlike GLP-1 and GLP-2, PYY and GIP have not received much commercial interest from the industry because of their limitations when applied alone. For example, PYY alone was not enough to achieve a therapeutic purpose. The insulinotropic actions of GIP are severely impaired in the pathological context, being less effective than GLP-1 in T2DM treatment [48]. To overcome these limitations, recent studies have demonstrated that combination therapies, such as GLP-1–GIP agonists and GLP-1–oxyntomodulin–PYY tri-agonists, exerted a higher efficacy compared with GLP-1 alone in the treatment of obesity and diabetes. Furthermore, our nanosystem might also be able to trigger PYY and GIP secretion from EECs, not just increasing GLP-1 and GLP-2 levels, which should be further confirmed.

Our nanocarriers are not limited to the delivery of a single specific molecule. Our nanosystems could also encapsulate other peptides or molecules (e,g, PYY/GLP-1/GIP agonists). Our formulation also presents the advantage of being possibly grafted at their surface with one or more ligands to target specific cells and tissues. Enteroendocrine cells (EECs) express various GPCRs and nutrient transporters, which participate in the modulation of EECs-hormones secretion. In chapter V, compared with plain nanosystems, the active L cell-targeted nanosystem that we developed failed to increase GLP-1 secretion due to its instability *in vivo*. Even so, we could not rule out the great potential of EECs active targeting in oral delivery. In future studies, to obtain active L cell-targeting or other enteroendocrine cell-targeting drug delivery systems, there are many strategies that we could exploited, such as 1) developing stable hybridized lipid/polymer nanocarriers with modified ligands, or 2) directly fabricating, post-inserting or coating with enough ligands on the surface of nanocarriers.

Considering the superior efficacy of combination therapies and the effect of our nanocarriers, we believe that gut hormone agonists-loaded nanocarriers including the modification of one or more

ligands, to trigger at least two types of functional hormone secretions from EECs, would be able to achieve a better therapeutic effect in gut hormone-based therapy via oral route. Despite we do not have any direct preliminary data about PYY and GIP for this future perspective, the data retrieved when exploiting GLP-1 and GLP-2 secretion suggest this could be feasible as a future perspective of this thesis.

V. IMPACT ON FUTURE ORAL PEPTIDE DELIVERY

Therapeutic peptides have achieved a great success in the treatment of many prevalent diseases (such as diabetes) in the world, due to their potency, specificity, and low toxicity [49]. However, the currently marketed peptide drugs are predominantly under the form of injection, and they still face a lot of challenges in the development of oral forms [50]. Based on current achievements on oral peptide delivery, the most successful approaches for systemic delivery often involve a combination of enteric coating, protease inhibitors and permeation enhancers in relatively high amounts [51, 52]. It should be noted that some of these excipients have shown local or systemic adverse reactions in preclinical and clinical studies, and long-term studies are often missing. Up to day, there is only one product approved by FDA (oral semaglutide), and current clinic trails in oral peptide and protein delivery include insulin, calcitonin, desmopressin and octreotide. However, all of those products that have moved into clinic or are under clinical trials still do not fix the biggest problem in oral peptide delivery: the low oral bioavailability (0.4%-1%) [53, 54]. Therefore, strategies aimed at increasing the oral absorption of peptide drugs should carefully take into account the benefit–risk ratio. The development of improved alternative drug delivery systems is of utmost importance in order to fulfill the oral route in peptide delivery.

Distinct peptide delivery approaches (chapter IV and V) were developed in this thesis for the oral delivery of antidiabetic peptides in T2DM treatment. The peptide drug (exenatide) showed a relatively high oral bioavailability (~4 %) via our nanosystems, and its oral absorption was not affected by food *in vivo* following a chronic treatment. It should be highlighted here that our nanosystems exhibited a striking therapeutic efficiency on T2DM treatment following the oral delivery of exenatide (a GLP-1RA), via the combination of the increased endogenous GLP-1 secretion and encapsulated GLP-1RA. Although the nanosystems are still in early pre-clinical investigation, they have the potential to impact the future of oral peptide delivery.

First and foremost, unlike conventional drug delivery systems that act merely as a carrier preserving the encapsulated cargo, we have exploited the physiological effect of the carrier on the ultimate therapeutic effect of the formulation. It was the first time that this approach was described and achieved in the drug delivery field. Apart from the biological effect triggering endogenous GLP-1 secretion in diabetic mice, our preliminary data have demonstrated that the developed nanosystem could also increase endogenous GLP-2 secretion *in vivo* in an acute colitis model, demonstrating the potential towards IBD treatment. We believe that elucidating the biological mechanisms of

action followed by the drug delivery system on GLP-1/GLP-2 secretion will open new paves on the way we design future lipid-based drug delivery systems for oral peptide delivery.

Regarding a foreseen translation into the clinics, our approach is solvent-free, easily scalable and low-priced, characteristics that make it highly attractive for the pharmaceutical industry. We have submitted European and US patents on the oral nanosystems described in this work. We strongly believe that our dual-action system has a great potential to become a commercial product eventually.

In conclusion, the main contribution of this project is in the advancement of the treatment T2DM via oral route through the rational design of innovative oral peptide delivery systems exploiting the gut pathophysiology. The nanosystems developed and further modified in this thesis resulted incredibly effective in T2DM treatment, representing a novel promising approach for oral peptide delivery in incretin-based diabetes treatment.

VI. REFERENCES

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