

Oral delivery of semaglutide through multifunctional FcRn-peptide targeting nanoparticles for treatment of type 2 diabetes *mellitus*

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**ORAL DELIVERY OF SEMAGLUTIDE THROUGH
MULTIFUNCTIONAL FcRn-PEPTIDE TARGETING
NANOPARTICLES FOR THE TREATMENT OF TYPE 2 DIABETES
*MELLITUS***

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Porto, 8 de maio de 2024



Soraia Filipa Tavares Pinto

This thesis is dedicated to my parents, my sisters, my grandmother and my boyfriend

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- **Soraia Pinto**, Mahya Hosseini, Stephen T. Buckley, Wen Yin, Javad Garousi, Torbjörn Gräslund, Sven van Ijzendoorn, Hélder A. Santos, Bruno Sarmento, Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide, Journal of Controlled Release 2024, 366:621-636. doi: 10.1016/j.jconrel.2024.01.015.

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ABSTRACT

Type 2 diabetes *mellitus* (T2DM) is a widespread metabolic and chronic disorder mainly caused by insulin resistance and inadequate secretion of insulin from pancreatic β cells, leading to impaired glucose tolerance. Glucagon-like peptide-1 (GLP-1) analogs have arisen as an effective strategy for the treatment of T2DM, stimulating insulin secretion and inhibiting glucagon release in a glucose-dependent manner. However, GLP-1 analogs are mainly administered via invasive routes which act as a barrier for potential users and impairs patient compliance. Recently, the US Food and Drug Administration (FDA) approved the first oral GLP-1 analog, semaglutide (Rybelsus®). Rybelsus® is a tablet dosage form comprised of a co-formulation of semaglutide and the permeation enhancer, sodium N-(8-[2-hydroxybenzoyl]amino) caprylate (SNAC). Nevertheless, the modest oral bioavailability of Rybelsus® is still a concern. Drug delivery nanosystems have been designed to overcome the poor oral bioavailability of biopharmaceuticals by protecting them from the acidic pH and the enzymatic activity of the gastrointestinal tract (GIT), enabling a sustained release of the drug. Interestingly, nanoparticles (NPs) can be functionalized with mucodiffusive polymers, such as polyethyleneglycol (PEG), and ligands targeting intestinal epithelial receptors (e.g., neonatal Fc receptor (FcRn)) to facilitate their transport through the intestinal barriers. FcRn plays a key role in mediating the transcellular transport of albumin and immunoglobulin G (IgG), across polarized epithelial cells, protecting both ligands from lysosomal catabolism. Ligand-FcRn interaction is pH-dependent, meaning that the binding is favored at pH 6.0 and release occurs at pH 7.4. In the gut, the interaction occurs at the slightly acidic pH of the intestinal lumen, where FcRn is found on the luminal surface of the enterocytes. Afterwards, the complex is transported through the enterocytes inside acidic endosomes (pH 5.5 to 6.0). When the complex encounters the basolateral side of the enterocytes, it is exposed to the physiological pH of the lamina propria, where albumin and IgG are released. Although albumin and IgG have high binding-affinity to the FcRn and were used to be conjugated with biopharmaceuticals or NPs, both ligands can bind to other receptors, compromising their biodistribution across the human tissues. To surpass this limitation, alternative ligands (e.g., FcRn-binding peptides (FcBPs) and affibody molecules (Z_{FcRn})) with stronger acidic pH-dependent binding-affinity to FcRn have been exploited, demonstrating tremendous potential to improve the intestinal transcytosis and plasma half-life of fusion partners. These ligands have been demonstrated to bind specifically to the FcRn receptor, unlike albumin and IgG. Taking this in mind, it was hypothesized that FcBPs and Z_{FcRn} can be conjugated with nanomedicines to improve the oral delivery of antidiabetic peptides, such as semaglutide.

The main aim of this thesis was to improve the oral delivery of semaglutide by encapsulating into PEGylated NPs surface functionalized with FcRn-targeted ligands, FcBP₂ or Z_{FcRn}. For this purpose, poly(lactic-co-glycolic acid)-polyethyleneglycol-maleimide (PLGA-PEG-Mal) co-polymer was functionalized with either FcBP₂ or Z_{FcRn} that target the human FcRn (hFcRn). Both ligands were successfully conjugated with the PLGA-PEG-Mal via Mal-thiol chemistry, which was proved by X-ray photoelectron spectroscopy (XPS) and by determining the conjugation efficiency (CE) values, of approximately 80% and 30% for FcBP₂ and Z_{FcRn}, respectively. Thereafter, the functionalized polymers were used to produce semaglutide-loaded NPs. Monodisperse NPs with an average size of 170 nm, neutral surface charge, and 3% of semaglutide loading degree were obtained. Both FcRn-targeted NPs were able to interact with hFcRn at a pH-dependent manner, with a stronger interaction at pH 6.0 than at pH 7.4, as observed for the free ligands, showing that the chemical conjugation of the ligands with PLGA-PEG-Mal co-polymer and the manufacturing process used to produce the NPs did not interfere in the binding of FcBP₂ and Z_{FcRn} with FcRn. Additionally, FcBP₂-NPs and Z_{FcRn}-NPs exhibited improved interaction and association to Caco-2 cells that endogenously express hFcRn when compared to non-targeted NPs. The uptake of FcRn-targeted NPs was also observed to occur in human intestinal organoids (HIOs), expressing the hFcRn receptor, through the microinjection of the particles into the lumen of HIOs, resulting in an increase of NPs' internalization for both-functionalized nanocarriers. Furthermore, in a T2DM-induced hFcRn transgenic mice model, it was demonstrated that the incorporation of semaglutide into FcRn-targeted NPs was translated in an improved glucoregulatory effect when compared to oral free semaglutide or non-targeted NPs, with a 35% reduction in blood glucose levels, after a single oral administration and after daily oral administrations for 7 days. An enhancement of the pancreatic insulin content was also observed in animals treated with FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs. The biodistribution of fluorescently labeled NPs across the GIT demonstrated that the nanosystems targeting FcRn were retained longer in the distal sections of the small intestine (ileum) and large intestine (colorectum), where FcRn expression is more prevalent than non-targeted NPs. Consequently, FcRn-targeted nanocarriers proved to be an effective platform to improve the intestinal delivery of semaglutide.

Overall, this thesis demonstrated that the incorporation of semaglutide into FcRn-targeted PLGA-PEG NPs improved the oral delivery and the pharmacological effect of semaglutide, which could be a feasible therapeutic advance for managing T2DM.

Keywords

Type 2 diabetes *mellitus*; Neonatal Fc receptor-targeted ligands; Semaglutide; Oral delivery; Polymeric nanoparticles; Human intestinal organoids; Glucoregulatory effect; Gastrointestinal distribution.

RESUMO

A diabetes *mellitus* tipo 2 (DMT2) é uma doença metabólica e crônica à escala global, causada principalmente por resistência à insulina e secreção inadequada de insulina pelas células β do pâncreas, comprometendo a tolerância à glucose. Os análogos do péptido semelhante ao glucagon (GLP-1) surgiram como uma estratégia eficaz para o tratamento de DMT2, envolvendo a secreção de insulina e a inibição de glucagon de uma forma dependente de glucose. Contudo, os análogos de GLP-1 são administrados por vias invasivas, constituindo uma limitação para potenciais utilizadores e comprometendo a adesão dos pacientes. Recentemente, a *US Food and Drug Administration* (FDA) aprovou o primeiro análogo de GLP-1 para administração por via oral, semaglutide (Rybelsus®). Rybelsus® corresponde a uma forma farmacêutica em comprimido composto por semaglutide e o agente de permeação *sodium N-(8-[2-hydroxybenzoyl]amino) caprilate* (SNAC). No entanto, a reduzida biodisponibilidade oral de Rybelsus® é considerada uma limitação. As nanopartículas (NPs) são uma estratégia utilizada para melhorar a biodisponibilidade oral de fármacos, visando a sua proteção do pH ácido e da atividade enzimática do trato gastrointestinal (TGI), e permitindo a sua libertação controlada. Estes nanosistemas podem ser funcionalizados com polímeros com propriedades mucodifusivas, como por exemplo o polietileno glicol (PEG), e ligandos com afinidade para os recetores dos enterócitos, como é o caso do recetor Fc neonatal, com o intuito de promover o transporte eficiente de NPs através de barreiras intestinais. FcRn é responsável pelo transporte de albumina e de imunoglobulina G (IgG) em células epiteliais polarizadas, protegendo estes ligandos da degradação pelos lisossomas. A interação da albumina e da IgG com o recetor FcRn ocorre de uma forma dependente do pH, o que significa que a pH 6,0 a interação é favorecida, mas a pH 7,4 a interação não ocorre. No tecido intestinal, o pH ácido do lúmen favorece a ligação entre o recetor (localizado no lado apical dos enterócitos) e os seus ligandos endógenos. O complexo ternário é posteriormente transportado pelos enterócitos no interior de endossomas cujo pH ácido (pH 5,5 a 6,0) favorece a manutenção da interação. Quando o complexo atinge o lado basolateral dos enterócitos, este é exposto ao pH fisiológico da lâmina própria, permitindo a libertação de albumina e de IgG. Apesar da albumina e da IgG apresentarem uma elevada afinidade para o recetor e já terem sido conjugados com fármacos ou NPs, ambos podem ter afinidade para outros recetores biológicos, comprometendo a sua distribuição pelos tecidos. De forma a colmatar estas limitações, outros ligandos foram explorados com maior afinidade para o recetor FcRn, nomeadamente os péptidos de ligação ao FcRn (FcBPs) e as moléculas *affibody* (Z_{FcRn}), sendo a interação também favorecida a pH ácido. Estes ligandos demonstraram um enorme potencial para melhorar a transcitose intestinal e o

tempo de semivida de outras moléculas. Além disso, FcBPs e Z_{FcRn} ligam-se especificamente ao recetor FcRn, ao contrário da albumina e da IgG. Deste modo, FcBPs e Z_{FcRn} podem ser conjugados com NPs de forma a melhorar a biodisponibilidade oral de péptidos, como é o caso de semaglutide.

O principal objetivo desta tese foi encapsular semaglutide em NPs conjugadas com PEG e com ligandos com afinidade para o recetor FcRn, FcBP₂ e Z_{FcRn} , visando melhorar a biodisponibilidade oral do péptido. O co-polímero *poly(lactic-co-glycolic acid)-polyethyleneglycol-maleimide* (PLGA-PEG-Mal) foi funcionalizado com FcBP₂ ou Z_{FcRn} através da reacção química de Mal-tiol, com o intuito de promover a sua interação com o recetor FcRn humano (hFcRn). Atendendo aos resultados de espectroscopia de fotoelétrons de raios X e aos valores de eficiência de conjugação de aproximadamente 80% e 30% para FcBP₂ e Z_{FcRn} , respetivamente, comprovou-se que a funcionalização entre o co-polímero e os ligandos foi efetuada com sucesso. Posteriormente, ambos os polímeros funcionalizados foram utilizados para a produção de NPs de semaglutide, obtendo-se NPs monodispersas com tamanho de aproximadamente 170 nm, carga superficial neutra e 3% de capacidade de incorporação de semaglutide em NPs. Adicionalmente, comprovou-se que FcBP₂-NPs e Z_{FcRn} -NPs ligam-se a hFcRn de uma forma dependente do pH, com uma interação mais forte a pH 6,0 do que a pH 7,4, tal como verificado para os ligandos não conjugados, demonstrando que a conjugação do co-polímero PLGA-PEG-Mal com os ligandos, bem como a metodologia utilizada para a produção de NPs, não comprometeram a ligação de FcBP₂ e Z_{FcRn} ao recetor. Além disso, FcBP₂-NPs e Z_{FcRn} -NPs demonstraram maior capacidade de interação e internalização com células Caco-2, que expressam endogenamente hFcRn, comparativamente com NPs não funcionalizadas. A capacidade de interação e internalização de FcBP₂-NPs e Z_{FcRn} -NPs com um modelo 3D intestinal, organoides intestinais humanos que expressam hFcRn, foi avaliada. As NPs foram injetadas no lúmen dos organoides, o que resultou no aumento da capacidade de permeação de ambas as partículas funcionalizadas. De seguida, em modelos de camundongos transgénicos para o recetor hFcRn e com indução química de DMT2, observou-se que a incorporação de semaglutide em FcBP₂-NPs e Z_{FcRn} -NPs melhorou o seu efeito glucorregulatório quando comparado com o semaglutide administrado por via oral e com as NPs não funcionalizadas. Atendendo aos resultados, verificou-se uma redução de 35% dos níveis de glucose sanguíneos, após uma única administração oral e após administrações diárias durante 7 dias. Adicionalmente, os animais tratados com ambos os nanosistemas de interação a hFcRn apresentaram um aumento do conteúdo de insulina no pâncreas. A biodistribuição de NPs fluorescentes ao longo do TGI demonstrou que nanosistemas direcionados para o recetor FcRn foram retidos por um período mais extenso nas secções distais do intestino delgado (íleo) e do

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Assim sendo, esta tese de doutoramento demonstrou que a incorporação de semaglutide em NPs poliméricas direccionadas para o recetor FcRn teve o potencial de melhorar a entrega oral e o efeito farmacológico do péptido, desempenhando um papel importante para o tratamento de DMT2.

Palavras-chave

Diabetes *mellitus* tipo 2; Ligandos direccionados para o recetor Fc neonatal; Semaglutide; Administração oral; Nanopartículas poliméricas; Organoides intestinais humanos; Efeito glucorregulatório; Distribuição gastrointestinal.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABD	Albumin-binding domain
AE	Association efficiency
Ala	Alanine
APC	Allophycocyanin
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
BGD	Blood glucose decrease
B-NPs	Non-functionalized empty nanoparticles
B _R	Relative bioavailability
BSA	Bovine serum albumin
CA	Cholic acid
Calcein-AM	Calcein-acetoxymethyl
CD	Circular dichroism
CE	Conjugation efficiency
CEMUP	<i>Centro de Materiais da Universidade do Porto</i>
cFcRn	Cynomolgus monkey neonatal Fc receptor
CPP	Cell-penetrating peptide
CS	Chitosan
CSK	Goblet cell-target peptide
C-terminal	Carbon-terminal
Cys	Cysteine
Cy7.5	Cyanine 7.5
DAB	3,3'-Diaminobenzidine
DAPI	4'6-Diamidino-2-phenylindole
DGAV	<i>Direção-Geral de Alimentação e Veterinária</i>
DL	Drug loading
DLS	Dynamic light scattering
DM	Diabetes <i>mellitus</i>
DMEM	Dulbecco's Modified Eagle's Medium

DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Sodium docusate
DPP-4	Dipeptidyl peptidase 4
DPP-4i	Dipeptidyl peptidase 4 inhibitor
EDC/NHS	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/ N-hydroxysuccinimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELS	Electrophoretic light scattering
ER	Epitope recovery
Fab	Fragment antigen-binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable of immunoglobulin G
FcBPs	Neonatal Fc receptor-binding peptides
FcBP ₂ -semaglutide-NPs	FcRn-targeted peptide 2-semaglutide-loaded-nanoparticles
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
FGF-4	Fibroblast growth factor 4
GA	Glycolic acid
GFR	Growth Factor Reduced
GI	Gastrointestinal
GIP	Glucose-dependent insulintropic polypeptide
GIT	Gastrointestinal tract
Gln	Glutamine
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
Glu	Glutamic acid
GLUT2	Glucose transporter 2
Gly	Glycine
HA ₂	Hemagglutinin-2
HBS	HEPES-buffered saline
H&E	Hematoxylin and eosin
hFcRn	Human neonatal Fc receptor
HFD	High-fat diet

hIgG	Human Immunoglobulin G
HIOs	Human intestinal organoids
HIP	Hydrophobic-ion pairing
His	Histidine
HPMC	Hydroxypropyl methylcellulose
HRP	Horseradish peroxidase
HSA	Human serum albumin
HUES9	Human embryonic stem cells
IgG	Immunoglobulin G
IL	Interleukin
Ile	Isoleucin
<i>i.p.</i>	Intraperitoneal
i3S	<i>Instituto de Investigação e Inovação em Saúde</i>
K _D	Dissociation constant
LA	Lactic acid
LepR	Leptin receptor
Lys	Lysine
Mal	Maleimide
MASLD	Metabolic dysfunction-associated steatotic liver disease
M cells	Microfold cells
MDCK	Madin-Darby canine kidney
mFcRn	Mouse neonatal Fc receptor
MFI	Mean fluorescence intensity
MHC-I	Major histocompatibility class I
mIgG	Mouse immunoglobulin G
MPT	Multiple Particle Tracking
MSA	Mouse serum albumin
MUC	Mucin
NA	Nicotinamide
NAD ⁺	Nicotinamide adenine dinucleotide
NEAA	Non-essential amino acids
NIR	Near infrared
NLCs	Nanostructured lipid carriers
<i>NMeLeu</i>	<i>N</i> -methyl-Leucine
NPs	Nanoparticles
NTDD	Nanomedicines and Translational Drug Delivery

N-terminal	Nitrogen-terminal
O.C.T.	Optimal cutting temperature
o/w	Oil-in-water
o/w/o	Oil-in-water-in-oil
PARP-1	Poly-adenosine diphosphate(ADP)-ribose polymerase-1
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PD	Pharmacodynamic
PdI	Polydispersity index
PEG	Polyethyleneglycol
Pen	Penicillamine
PFA	Paraformaldehyde
Phe	Phenylalanine
pHPMA	Poly-N-(2-hydroxypropyl) methacrylamide
PI	Propidium iodide
PK	Pharmacokinetic
PLA	Poly(lactic) acid
PLGA	Poly(lactic-co-glycolic acid)
Pro	Proline
PSi NPs	Porous silicon nanoparticles
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene difluoride
RIPPA	Radioimmunoprecipitation assay
RLs	Rhamnolipids
RPMI1640	Roswell Park Memorial Institute 1640
RT	Room temperature
RU	Response units
Sar	Sarcosine
s.c.	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Semaglutide-NPs	Semaglutide-loaded nanoparticles
Ser	Serine
SLGT2	Sodium glucose co-transporter-2
SLNs	Solid lipid nanoparticles
SNAC	Sodium N-(8-[2-hydroxybenzoyl]amino) caprylate

SOS	N-octadecyl sulfate
SPR	Surface plasmon resonance
STZ	Streptozotocin
TCEP	Tris(2-carboxyethyl)phosphine
TEM	Transmission electron microscopy
Tf	Transferrin
THA	Tetraheptylammonium bromide
Thr	Threonine
TMB	5,5'-Tetramethylbenzadine
TNF- α	Tumor necrosis factor-alpha
Trp	Tryptophan
Tyr	Tyrosine
$t_{1/2}$	Half-life
T1DM	Type 1 diabetes <i>mellitus</i>
T2DM	Type 2 diabetes <i>mellitus</i>
UMCG	University Medical Center Groningen
Val	Valine
w/o/w	Water-in-oil-in-water
WT	Wild-type
XPS	X-ray photoelectron spectroscopy
Z-average	Average size
Z _{FcRn}	Affibody
Z _{FcRn} -NPs	Affibody-empty nanoparticles
Z _{FcRn} -Semaglutide-NPs	Affibody-semaglutide-loaded nanoparticles
ZO-1	Zonula occluden-1
α chain	Alpha chain
β 2m	Beta 2 microglobulin
ζ -potential	Zeta-potential

Chapter | 1

Overview, Aims and Structure

1.1. Overview

Type 2 diabetes *mellitus* (T2DM) is a metabolic and chronic disorder that comprises around 90% of the total cases of diabetes worldwide, characterized by insulin resistance and insufficient insulin production by the pancreas, resulting in sustained hyperglycemia. Glucagon-like peptide-1 (GLP-1) analogs are currently used for the treatment of T2DM, but their subcutaneous (s.c.) administration is considered a barrier for patient compliance. Recently, the first oral GLP-1 analog, semaglutide (Rybelsus®), was clinically approved, but its modest bioavailability is still a concern. Nanotechnology can be used as a tool to improve the oral delivery of peptides by protecting them from the harsh environment of the gastrointestinal tract (GIT) and promoting their transport across intestinal barriers (mucus and enterocytes) through the functionalization of the nanocarriers with mucodiffusive moieties and ligands targeting receptors of enterocytes. In this thesis, nanotechnology was used to develop semaglutide-loaded poly(lactic-co-glycolic acid)-polyethyleneglycol (PLGA-PEG) nanoparticles (NPs) targeting the intestinal neonatal Fc receptor (FcRn), found on the luminal surface of the enterocytes, for the management of T2DM.

In the framework of this thesis, a collaborative work between Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, Porto, Portugal, and the Department of Biomaterials and Biomedical Technology, University Medical Center Groningen (UMCG), Groningen, The Netherlands, was established. Data of this thesis were mainly obtained in the Nanomedicines and Translational Drug Delivery (NTDD) group at i3S, under the supervision of Professor Bruno Sarmento, while experiments regarding the human intestinal organoids (HIOs) study was entirely performed in the Department of Biomaterials and Biomedical Technology at UMCG, under the co-supervision of Professor Hélder Almeida Santos, and in collaboration with Professor Sven van Ijzendoorn's group from the Department of Biomedical Sciences at UMCG. Bruno Sarmento's group (NTDD group) at i3S is focused on the development of drug delivery systems, mainly on nanotechnology and their applicability to the pharmaceutical and biomedical fields. The group is also interested in engineering peptide delivery nanoplateforms by identifying biological targets, such as the intestinal FcRn receptor, to enhance their intestinal transcytosis, avoiding lysosomal catabolism, and further managing chronic and metabolic diseases, such as diabetes *mellitus* (DM). The group also specializes in mucosal engineering models for the validation of nanoplateforms and in correlating the *in vitro* and *in vivo* results of these systems. The group of Hélder Almeida Santos at UMCG specializes in the fields of biomedical nano/micro-technology, controlled drug delivery, nanomedicine, and innovative therapeutic modalities. He has several research collaborations inside and outside UMCG, and one of them is with Sven van Ijzendoorn's group at UMCG, whose research is focused on the molecular mechanisms that regulate the intracellular dynamic of proteins, lipids, and membranes in

relation to the functional cellular organization, and understanding how these mechanisms contribute to the health or disease. Additionally, his research is also focused on the development of HIOs derived from human embryonic stem cells or induced pluripotent stem cells.

1.2. Aim and specific objectives

The **main aim** of this thesis was to develop PLGA-PEG NPs, containing the GLP-1 analog semaglutide, and functionalized with ligands (FcRn-binding peptides, FcBPs, or affibody molecules, Z_{FcRn}) targeting the intestinal FcRn, envisioning further elucidation on the potential of oral semaglutide delivery and exploring its glucoregulatory ability in T2DM. The FcRn-targeted nanoplatforms were designed through the covalent linkage of FcBPs or Z_{FcRn} to PLGA-PEG-Maleimide (PLGA-PEG-Mal) co-polymer used to produce the nanosystems to explore their potential in targeting the human FcRn (hFcRn) at an acidic pH-dependent manner, improving the overall intestinal transcytosis of semaglutide, and simultaneously protecting the drug from the harsh conditions of the GIT. To achieve this aim, the **specific aims** proposed for this thesis were:

1. To select alternative FcRn-targeted ligands (e.g., FcBPs or Z_{FcRn}) over the endogenous ligands, albumin, or immunoglobulin G (IgG), to be anchored to the NPs in a site-oriented manner, maintaining the acidic pH-dependent binding-affinity for FcRn.
2. To functionalize the PLGA-PEG-Mal co-polymer with FcRn-targeted ligands via Mal-thiol chemistry and characterize the efficacy of conjugation.
3. To produce and characterize FcRn-targeted PEGylated PLGA NPs incorporating semaglutide.
4. To demonstrate the potential of functionalized NPs in targeting the hFcRn in intestinal Caco-2 cells endogenously expressing the receptor.
5. To evaluate the intestinal cellular trafficking of FcRn-targeted NPs in HIOs that suitable recapitulate the *in vivo* architecture and biological complexity of the human intestine.
6. To establish safe and effective FcRn-targeted NPs for improved oral delivery of semaglutide and reduced glycemia in T2DM-induced hFcRn transgenic mice model.

1.3. Thesis structure

This thesis is organized in six chapters. In **Chapter 1**, an overview of the theme of the thesis, and the specific objectives required to achieve the main goal are specified. Additionally, the structure and organization of the thesis are also mentioned. In **Chapter 2**,

a deep review of the literature regarding the main subjects of the theme is provided, including the epidemiology of DM and the current pharmacological treatment of T2DM, the physiological barriers that hinder the oral delivery of antidiabetic peptides, and the impact of nanotechnology in improving the transport of biopharmaceuticals through mucosal barriers. Moreover, a detailed background on the topic of the biological functions of the FcRn receptor, its target ligands, and the FcRn-targeted nanosystems incorporating antidiabetic peptides are discussed. **Chapter 3** is focused on the methods conducted to select the FcBPs and Z_{FcRn} that suitable bind to the receptor at an acidic pH-dependent manner, the conjugation of the selected FcRn-targeted ligands with the PLGA-PEG-Mal copolymer via Mal-thiol chemistry, the production and physicochemical characterization of semaglutide-loaded NPs functionalized with FcBPs or Z_{FcRn} , and the intestinal cellular trafficking of FcRn-targeted NPs in 2D and 3D *in vitro* intestinal models, Caco-2 cells and HIOs, respectively. Notwithstanding, the methods used to evaluate the glucoregulatory effect of FcRn-targeted semaglutide-loaded NPs and their biodistribution through the GIT of T2DM-induced hFcRn transgenic mice model are also described. **Chapter 4** is engaged in the results and discussion of the data regarding the FcRn-targeted ligands that suitable bind to the receptor, the successful conjugation of FcRn-targeted ligands with the copolymer, the production of monodisperse FcRn-targeted semaglutide-loaded NPs with an appropriate size for oral administration, and the effective incorporation of semaglutide into polymeric-based NPs. Furthermore, the higher ability of FcRn-targeted NPs in being internalized by Caco-2 cells and HIOs is also demonstrated, as well as their capacity to improve the glucoregulatory effect of semaglutide after a single oral administration and after daily oral administrations for 7 days. The capacity of these nanosystems to be retained longer in the GIT, particularly the distal sections of the small intestine and large intestine, when compared with non-targeted NPs, is also shown. **Chapter 5** includes the overall conclusions of the work and future studies to be considered. Finally, **Chapter 6**, includes the **Appendix**, which contains additional information about the review and research papers published regarding this PhD thesis and, the results obtained.

Chapter | 2

Literature Review

This chapter was written based in the following publications:

- **Soraia Pinto**, Hélder A. Santos, Bruno Sarmento, New insights into nanomedicines for oral delivery of glucagon-like peptide-1 analogs, *WIREs Nanomedicine and Nanobiotechnology Journal* 2024, 16(2):e1952. doi: 10.1002/wnan.1952.

In this review paper, I was responsible for the conceptualization, bibliographic research, and writing. The supervisors were responsible for reviewing, editing, and submitting the manuscript. This review was not previously included in another PhD thesis, and it is partially reproduced in this chapter. The first page of the review paper and the permission from the journal to reuse the manuscript in this thesis were included in **Appendix I**.

- Cláudia Azevedo*, **Soraia Pinto***, Sopisa Benjakul*, Jeannette Nilsen, Hélder A. Santos, Giovanni Traverso, Jan Terje Andersen, Bruno Sarmento, Prevention of diabetes-associated fibrosis: Strategies in FcRn-targeted nanosystems for oral drug delivery, *Advanced Drug Delivery Reviews* 2021, 175:113778. (*equal contribution). doi: 10.1016/j.addr.2021.04.016.

In this review paper, I was responsible for the conceptualization, bibliographic research, and writing of the manuscript as Cláudia Azevedo and Sopisa Benjakul. The supervisors were responsible for reviewing, editing, and submitting the manuscript. This review was partially mentioned in the PhD thesis of Cláudia Azevedo, and it is partially reproduced in this chapter. The first page of the review paper and the permission from the journal to reuse the manuscript in this thesis were included in **Appendix IV**.

2.1. Introduction

DM is a metabolic and chronic disorder and it is estimated that approximately 537 million people are living with diabetes, and this number will continue to rise up to 700 million by 2045 [1]. Specifically, T2DM comprises around 90% of the total cases of diabetes worldwide, and it is characterized by insulin resistance and insufficient insulin production by the pancreas, resulting in sustained hyperglycemia [1, 2]. Currently, the treatment of T2DM encompasses non-pharmacological actions (e.g., diet and exercise) and the exogenous administration of antidiabetic drugs, such as the GLP-1 analogs [3-6]. Regarding the function of GLP-1 analogs, these antidiabetic peptides reduce the hyperglycemia effect by stimulating insulin secretion and inhibiting glucagon release from pancreatic β and α cells, respectively, in a glucose-dependent manner [3, 5, 7]. Despite their substantial beneficial pharmacological actions, the mode of administration is likely a barrier for some potential users [3, 7].

In 2019, the US Food and Drug Administration (FDA) approved the first oral GLP-1 analog, semaglutide (Rybelsus®). Rybelsus® is a tablet-based dosage form, in which semaglutide is co-formulated with a permeation enhancer, sodium N-(8-[2-hydroxybenzoyl]amino) caprylate (SNAC) [8-10]. SNAC neutralizes the pH of the gastric lumen, avoiding the activation of gastric enzymes, such as pepsin, while also mediates semaglutide transcellular transport through the gastric epithelium. Nevertheless, the modest bioavailability of Rybelsus® is considered a limitation [8].

Nanotechnology has arisen as a promising strategy to overcome the poor oral bioavailability of peptides by protecting them from enzymatic degradation and the acidic pH of the GIT, enabling a tuneable release [11-13]. However, such nanosystems still need to overcome the intestinal mucus. Polyethyleneglycol (PEG) is a hydrophilic polymer, that can aid nanosystems transport through the intestinal mucus due to its neutral charge that reduces the interaction with the positively charged mucins. Additionally, the presence of PEG on NP's surface prevents particles aggregation, thereby contributing to their stability [11, 14-16]. Besides that, the nanosystems encounter the intestinal epithelial barrier (enterocytes). Several strategies have been applied to enable the intestinal transport of drugs, such as functionalizing the surface of PEGylated NPs with ligands that target a receptor expressed in intestinal epithelial cells, such as FcRn [17]. FcRn is a heterodimeric glycoprotein that plays a key role in mediating the transcellular transport of albumin and IgG through polarized epithelial cells, such as the enterocytes, and protecting albumin and IgG from lysosomal catabolism. Both ligands bind to FcRn in an acidic pH-dependent manner, with no interaction at physiological pH [18, 19]. When IgG and albumin encounter the intestinal lumen's acidic pH (pH 6.0), both ligands bind to FcRn, located on the apical side of the enterocytes. Subsequently, the ternary complex is internalized by receptor-mediated

endocytosis and transcytosed inside acidified endosomes (pH 5.5 to 6.0), escaping from lysosomal catabolism, and increasing their plasma half-life ($t_{1/2}$). The receptor-ligands complex is afterward directed to the basolateral side of the enterocytes, being exposed to the physiological pH of the lamina propria, which causes the release of the ligands [19, 20]. It was previously demonstrated the importance of intestinal FcRn in mediating the intestinal transport of surface-modified nanosystems employing the Fc fragment of IgG [21-23] and albumin [24, 25]. However, IgG and albumin can interact with other receptors (e.g., FcγRI, FcγRII, FcγRIII, Fcα/μR, FcαRI, FcβRI and FcμR for Fc fragment of IgG and gp60, gp18, megalin, cubilin, SPARC and CD36 for albumin), which could interfere with the biodistribution of the biopharmaceutical-loaded nanosystems [26-28]. To overcome the limitations associated with the endogenous ligands of FcRn, other molecules with small size and simple structures have been described in the literature, namely FcBPs and Z_{FcRn} . Those FcRn-targeted ligands exhibit stable affinity and specificity for FcRn in a pH-dependent manner when interacting with the receptor alone or associated with other molecules, such as proteins or fractions of antibodies [29-32]. Hereupon, it was hypothesized that FcBPs or Z_{FcRn} might be conjugated with nanosystems to improve the intestinal delivery of biopharmaceuticals via FcRn-mediated transcytosis. Based on this hypothesis, the present thesis focuses on the design and development of PEGylated PLGA NPs surface functionalized with FcBP₂ or Z_{FcRn} , to improve the oral delivery of semaglutide. PLGA is a biocompatible and biodegradable polymer approved by the US FDA with tremendous potential as a drug delivery carrier. Additionally, PLGA can be functionalized with specific ligands, before or after the production of NPs, therefore providing an accurate targeting to the biological site of interest [33, 34].

2.2. Epidemiology and physiology of Diabetes *mellitus*

DM is a widespread metabolic and chronic disorder. The estimated prevalence of DM worldwide is approximately 537 million people, and this number will continue to rise up to 700 million by 2045, if the current trends are maintained [1]. The full global costs of DM have grown from US \$232 billion in 2007 to US \$966 billion in 2021 and it is estimated that this value will reach US \$1.05 trillion by 2045 [1]. There are two main types of DM: type 1 diabetes *mellitus* (T1DM) and T2DM [1, 2]. T1DM is caused by an autoimmune response, in which the immune system attacks the insulin-producing β cells, and as a result, the secretion of insulin is impaired, leading to hyperglycemia episodes. This condition is mainly developed in childhood, and it is characterized by being insulin dependent. The patients need chronic daily insulin s.c. injections, which is invasive and inconvenient for patients since insulin needs to be maintained under sterile and chilled conditions, the syringes must be discarded in proper containers, and it requires patients education for the injection

procedure and regular blood glucose monitoring [1, 35, 36]. T2DM is the most common type of diabetes, comprising approximately 90% of the total cases of DM worldwide, and it is caused by insulin resistance and inadequate secretion of insulin from pancreatic β cells, leading to impaired glucose tolerance. The main causes for the development of T2DM include overweight or obesity, advancing age, ethnicity, and family history. Notwithstanding, T2DM is entirely associated with the onset of macrovascular complications (e.g., cardiovascular diseases) and microvascular complications (e.g., disorders affecting the nervous system, retina, and kidney) [1, 37, 38]. **Figure 1** represents the mechanism of entrance of glucose into target tissues mediated by insulin in a healthy, T1DM and T2DM individual.

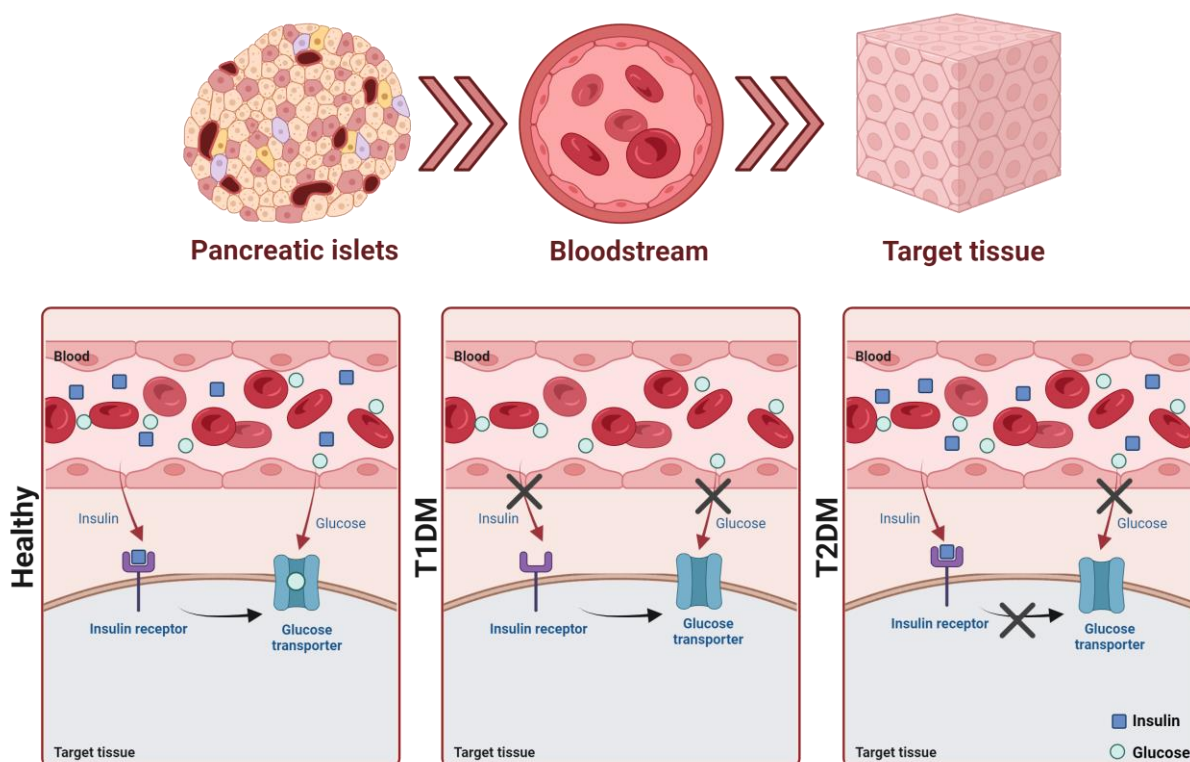


Figure 1. Schematic representation of the entrance of glucose into target tissues mediated by insulin in a healthy, T1DM and T2DM individual. In a healthy individual, insulin is secreted from pancreatic β cells to the bloodstream after meal intake, and further, this peptide binds to the insulin receptor found on the membrane surface of target tissues, leading to the absorption of glucose via a glucose transporter. In a T1DM individual, this mechanism is absent due to the complete destruction of pancreatic β cells and thus, the pancreas loses the capacity to produce and secrete insulin, which is crucial to control the blood glucose levels, causing hyperglycemia. In a T2DM individual, the target tissues cannot recognize the signal of insulin and the pancreatic β cells become unable to produce and secrete insulin into the bloodstream, causing an accumulation of glucose in the blood, and consequently a hyperglycemia condition. Created with BioRender.

2.3. Current treatment in type 2 Diabetes *mellitus*

The cornerstone of T2DM management encompasses non-pharmacological actions (e.g., diet and exercise) and the exogenous administration of antidiabetic drugs, such as

biguanides (e.g. metformin), insulin sensitizers (e.g., thiazolidinediones), insulin secretagogues (e.g., sulfonylureas), sodium glucose co-transporter-2 (SGLT2) inhibitors, insulin (only in the cases where the other antidiabetic drugs are not effective to regulate blood glucose levels), incretin hormones GLP-1 analogs and others [6, 39, 40]. Although metformin is the first-line pharmacologic treatment for T2DM, its oral administration is associated with several gastrointestinal (GI) side effects that cause low patient compliance and metformin discontinuation [41-43]. Currently, GLP-1 analogs have been employed to manage T2DM by regulating blood glucose levels in a glucose-dependent manner, stimulating insulin secretion, and suppressing glucagon release from pancreatic β and α cells, respectively [3, 44-46].

2.3.1. Role of glucagon-like peptide-1

The incretin hormone GLP-1 is a 37 amino acids peptide derived from the proglucagon gene [3, 46-50]. GLP-1 (1-37) is mainly secreted by neuroendocrine L-cells from ileum and colon in its inactive form, and afterwards, the first six amino acids from the nitrogen-terminal (N-terminal) of the GLP-1 (1-37) are cleaved, resulting in two biologically active forms, GLP-1 (1-36)-NH₂ and GLP-1 (7-37) [3, 46-51]. The secretion of GLP-1 from L-cells arises in hyperglycemia conditions, as noticed after nutrient intake [3, 46, 47, 50, 52]. Afterwards, GLP-1 binds to the GLP-1 receptors (GLP-1R), mainly located in the pancreatic islets, where GLP-1 stimulates insulin secretion and suppresses glucagon release from pancreatic β and α cells, respectively, as well as stimulates the neogenesis and the proliferation of β cells [3, 44-48, 50, 53]. Additionally, GLP-1 acts in the GLP-1R expressed in the brain, heart, GIT, lungs, liver, kidneys, adipose tissue, and muscle [3, 44, 45, 47, 48, 53].

Figure 2 illustrates the biological functions of GLP-1 in each tissue.

The bioactive forms of GLP-1 have a short plasma $t_{1/2}$ (< 2 min) since they are rapidly degraded by the dipeptidyl peptidase-4 (DPP-4) enzyme in circulation [47, 48]. DPP-4 cleaves the polypeptides that contain proline (Pro) or alanine (Ala) amino acid residues at position 2 of their backbone, as observed in human GLP-1. As a result, the peptide is inactivated, and the metabolites formed act as antagonists at the GLP-1R [48]. Since DPP-4 is mainly located on the luminal surface of the endothelial cells, when GLP-1 leaves the gut, a large portion of the peptide is already degraded to inactive metabolites, and only 25% of GLP-1 reaches the portal circulation [47, 49].

In healthy individuals, GLP-1 is involved in about two-thirds of insulin secretion after food intake, while in T2DM patients the secretion and activity of GLP-1 are diminished. However, GLP-1R is unaltered, and insulin response to GLP-1 is blunted [46, 47]. To overcome these limitations, the exogenous administration of GLP-1 can be an alternative to achieve physiological GLP-1 levels [46]. However, both endogenous and exogenous GLP-1 are

rapidly metabolized and inactivated by DPP-4 [46, 54]. Following this thought, GLP-1 agonists have been developed to surpass their counterparts [44, 47], which are deeply discussed in **Chapter 2, section 2.3.2**.

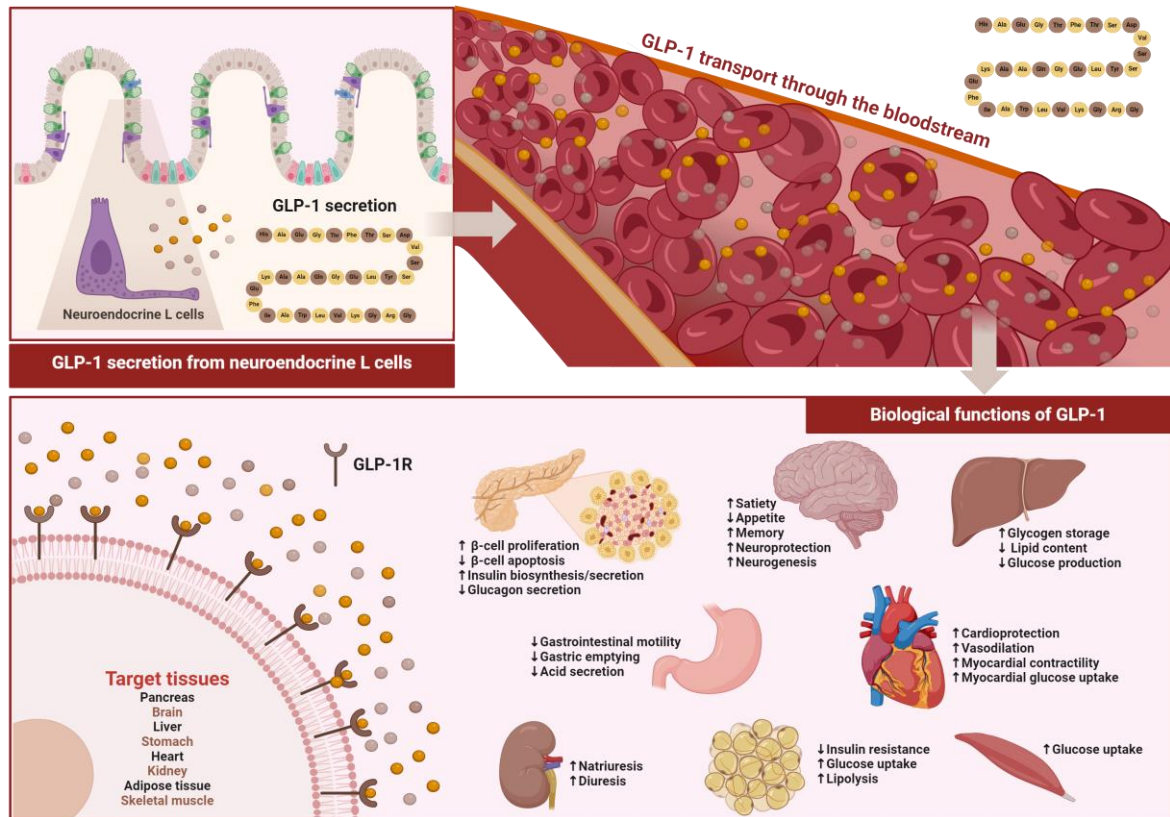


Figure 2. Biological functions of GLP-1 in the human body. GLP-1 is secreted by neuroendocrine L-cells of the gut in a post-prandial situation and then, transported through the bloodstream towards the target tissues (e.g., pancreas, brain, liver, stomach, heart, kidney, adipose tissue, and skeletal muscle), where the peptide binds to GLP-1R. In pancreatic islets, the binding of GLP-1 to GLP-1R stimulates insulin secretion and suppresses glucagon release, to control blood glucose levels. Created with BioRender.

2.3.2. Glucagon-like peptide-1 analogs

GLP-1 analogs are a class of antidiabetic peptides developed to mitigate the limitations associated with endogenous GLP-1, including the short plasma $t_{1/2}$ and the rapid enzymatic degradation by DPP-4. This class of antidiabetic peptides is divided into short- and long-acting GLP-1 analogs, based on the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of the peptides, and their ability to control glycemia [44, 46, 49, 53]. **Table 1** summarizes the properties of both short- and long-acting GLP-1 analogs.

Table 1. GLP-1 analogs developed for the management of T2DM.

GLP-1 analog	Commercial name	$t_{1/2}$	Route	Dose	Administration frequency	Clinical status	FcRn-ligand	References
Exenatide	Byetta®	2.4 h	s.c.	5 or 10 µg	Twice daily	Marketed	-	[3, 44, 48]
	Bydureon®	5–6 d	s.c.	2 mg	Once weekly	Marketed	-	[44, 55]
Lixisenatide	Lyxumia®	3–4 h	s.c.	10 or 20 µg	Once daily	Marketed	-	[44, 46, 56]
Liraglutide	Victoza®	13 h	s.c.	0.6, 1.2 or 1.8 mg	Once daily	Marketed	HSA	[45, 48, 57]
Albiglutide	Eperzan® Tanzeum®	6–8 d	s.c.	30 or 50 mg	Once weekly	Marketed	HSA	[3, 48, 56]
Albenatide	CJC-1134-PC	8 d	s.c.	2 mg	Once weekly	Phase III	HSA	[3, 48, 58]
Dulaglutide	Trulicity®	5–6 d	s.c.	0.75, 1.5, 3, or 4.5 mg	Once weekly	Marketed	hIgG4 Fc	[46, 48, 59]
Taspoglutide	R1538	2–3 d	s.c.	10 mg	Once weekly	Halted phase III	-	[60, 61]
Efpeglenatide	HM 11260C	6 d	s.c.	2, 4, or 6 mg	Once weekly	Phase III	hIgG4 Fc	[48, 62–64]
Semaglutide	Ozempic®	6–7 d	s.c.	0.25, 0.5, or 1.0 mg	Once weekly	Marketed	HSA	[3, 46, 65]
	Rybelsus®	7–8 d	oral	3, 7, or 14 mg	Once daily	Marketed	HSA	[46, 66, 67]
Tirzepatide	Mounjaro®	5 d	s.c.	5, 10, or 15 mg	Once weekly	Marketed	HSA	[68, 69]

GLP-1, glucagon-like peptide-1; hIgG4 Fc: human immunoglobulin G4 heavy Fc chain fragment; HSA, human serum albumin; s.c., subcutaneous; $t_{1/2}$, half-life.

Short-acting GLP-1 analogs (*e.g.*, exenatide short-release and lixisenatide) reduce *post*-prandial glucose response by enhancing insulin secretion and delaying gastric emptying. This effect is achieved due to their low plasma $t_{1/2}$ (**Table 1**), thereby requiring s.c. injections once or twice daily [3, 44, 46, 47, 53, 70]. On the other side, long-acting GLP-1 analogs (*e.g.*, exenatide extended-release, liraglutide, albiglutide, albenatide, dulaglutide, taspoglutide, efpeglenatide, semaglutide, and tirzepatide) control blood glucose levels in both *post*-prandial and fasting situations due to their long plasma $t_{1/2}$ (**Table 1**), allowing for once weekly s.c. injection, except for liraglutide, which still requires s.c. injections once daily due to its shorter plasma $t_{1/2}$ (~ 13 h) [44, 46, 53, 70, 71]. Regarding the long-acting GLP-1 analog, semaglutide (Ozempic®), it was approved for the treatment of T2DM in 2017. Semaglutide is a 31 amino acid peptide with higher resistance to DPP-4 degradation than the native GLP-1 due to the substitution of Ala and lysine (Lys) to α -aminoisobutyric acid and arginine at positions 8 and 34 of the peptide framework, respectively, reducing the

susceptibility to the enzymatic degradation by DPP-4 [46, 66, 72, 73]. Moreover, semaglutide is composed of a C18 fatty di-acid side chain attached via a L-γ-glutamic acid moiety and a hydrophilic spacer connected with Lys at position 26 that interacts with the circulating protein human serum albumin (HSA), which is responsible for the extended plasma $t_{1/2}$ of semaglutide (**Table 1**) [48, 56, 66, 67, 73]. Despite the substantial beneficial pharmacological actions of Ozempic®, the mode of administration is likely a barrier for some potential users [3, 47, 48]. An oral route is preferable for peptide administration since it is more convenient to handle and inexpensive, mimicking the endogenous secretion of GLP-1 [47, 49]. In 2019, the US FDA approved the tablet-based dosage form of semaglutide, Rybelsus®, which corresponds to the first oral GLP-1 analog to reach the market [48, 74]. Rybelsus® is composed of a co-formulation of semaglutide and the permeation enhancer, SNAC. SNAC serves to neutralize the pH of the gastric lumen in and around where the tablet resides and in doing so, precludes activation of gastric enzymes, such as pepsin, ultimately protecting semaglutide from the harsh conditions of the GIT and therefore, stimulating the transcellular transport of the peptide through the gastric epithelium (**Figure 3**) [48, 66, 75]. The starting dose of Rybelsus® is 3 mg once a day for 1 month, which should be readjusted to the maintenance dose of 7 mg or 14 mg once daily to control the blood glucose levels [10, 66]. Even though Rybelsus® demonstrated promising potential to control glycemia in *post*-prandial and fasting situations in diabetic patients, the management of T2DM with this SNAC-based formulation is still far from ideal. The modest bioavailability (~1%) achieved by Rybelsus® provides latitude for further innovations to expand the toolbox of drug delivery systems for delivering peptides orally and therefore, improve T2DM treatment [76].

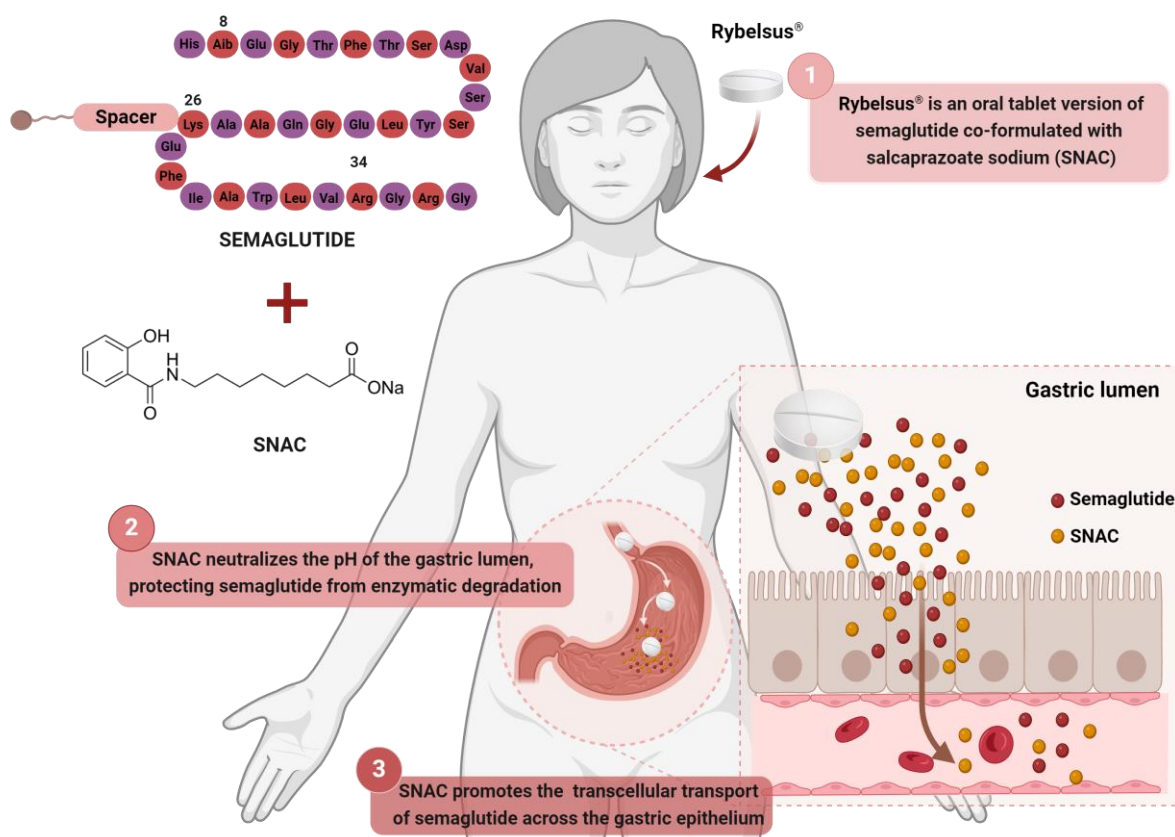


Figure 3. Mechanism of action of semaglutide tablet-dosage form, Rybelsus®. Created with BioRender.

2.4. Physiological barriers to the oral delivery of peptides

The oral administration is considered the preferred and most convenient route of drug delivery since it is simple to handle, less invasive, and allows for flexible dosing regimens, thereby being associated with higher patient compliance. However, oral peptides are exposed to the hostile environment of the GIT, which has several barriers that thwart the absorption of peptides, namely the acidic pH and enzymatic activity of the stomach, the intestinal mucus, and the small intestinal epithelium (**Figure 4**). The first impediments to the absorption of peptides are the acidic pH and enzymatic activity of the stomach [11, 77, 78]. The digestive fluids secreted by gastric glands are mainly composed of hydrochloric acid and proteins-digesting enzymes, such as pepsins [11, 78, 79]. Hydrochloric acid contributes to the acidic gastric environment (pH 1-2), which in turn, activates the pepsin involved in the cleavage of peptides with aromatic residues (e.g., phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr)) in their backbone, rendering them inactive [11, 77-80]. Although some peptides can be absorbed by the gastric mucosa, such as Rybelsus®, the systemic uptake of this GI portion is lower than that for the small intestine. The relevance of gastric absorption is restricted to biopharmaceuticals that remain stable at acidic pH and do not feature sites susceptible to pepsin cleavage [80]. After passing through the stomach, the

peptides must penetrate the intestinal mucus layer, which exhibits a thickness ranging from 100 to 150 μm [79, 80]. Mucus is a viscoelastic layer, secreted by goblet cells and composed of proteins, water, carbohydrates, lipids, electrolytes, immunoglobulins, cellular debris, and several other active proteins [14, 77, 78, 81]. Mucins represent the main protein components of the mucus [11, 14, 78, 79] and are secreted either by goblet cells (e.g., mucin 2 (MUC2), MUC5AC and MUC6) or cell-bound (e.g., MUC1, MUC3, MUC4, MUC12 and MUC13) [14, 81]. The main functions of mucus are to act as a lubricant and physical barrier, improving the transport of food matter and limiting the diffusion of foreign pathogens (e.g., bacteria) and xenobiotics (e.g., biopharmaceuticals) from the intestinal lumen to the underlying epithelium, while allowing the passaging of nutrients and signaling molecules [14, 78, 81]. The pore size of the mucus has been estimated to be approximately 100-200 nm, which would be wide enough for peptides to penetrate; however, ionic or hydrophobic interactions or hydrogen bonding can compromise the diffusion of peptides through the mucus [78, 80]. The epithelial lining below the mucus barrier creates another physical barrier between the intestinal lumen and the bloodstream [78, 79]. The small intestinal epithelium is mainly composed of enterocytes, containing microvilli on their apical side that increase the absorptive area for diffusion. Microvilli are also involved in nutrient absorption, mucus secretion, and other biological functions [11, 78, 79]. Besides the enterocytes, the intestine comprises mucus-secreting goblet cells, antimicrobial peptides-secreting Paneth cells, GLP-1-secreting enteroendocrine cells, microfold cells (M cells), and stem cells that renew the epithelium every 2–6 days [11, 78, 79]. Altogether, these cells form a continuous, polarized monolayer separating the gut lumen from the lamina propria, and also affecting the intestinal absorption of peptides [78, 79]. Biopharmaceuticals can enter the systemic circulation through the intestinal epithelium via transcellular or paracellular pathways. The transcellular route is commonly applied for hydrophobic drugs, in which the molecules are transported through the cellular barrier, involving endocytosis/transcytosis or carrier-mediated transport mechanisms. Conversely, the paracellular pathway is generally employed for hydrophilic drugs as the molecules are transported between adjacent intestinal cells, comprising tight junctions, adherens junctions, and desmosomes [77-80, 82]. However, foreign compounds that have access to the cellular barrier by transcellular transport can return to the intestinal lumen through active transport mechanisms, thereby reducing drug absorption [78]. Additionally, peptides that are internalized into enterocytic intestinal cells can be exposed to the enzymatic activity of lysosomes found in the cytosol and cellular organelles [11]. Nonetheless, the transport of molecules from the intestinal lumen to the lamina propria via the paracellular route can be compromised by the expression of tight junctions between the cells [77, 82].

Even though the GIT barrier directly interferes with the absorption of peptides, the molecular weight and the hydrophilic/hydrophobic nature of the drugs can also compromise their oral delivery. GLP-1 analogs have a hydrophilic nature, which limits their partition into lipid bilayers of epithelial cells, excluding them from the transcellular route of absorption through passive diffusion [49, 80, 82, 83]. Although the paracellular route might be an alternative for the intestinal transport of GLP-1 analogs due to a possible deficiency in proteolytic enzymes, their molecular weight makes them unable to fit in the spaces between the cells [49, 83]. Most therapeutic peptides cannot overcome all these barriers without auxiliary agents and smart formulations [80]. Nanotechnology has arisen as a promising strategy for the oral delivery of therapeutic peptides, which is deeply discussed in **Chapter 2, section 2.5**.

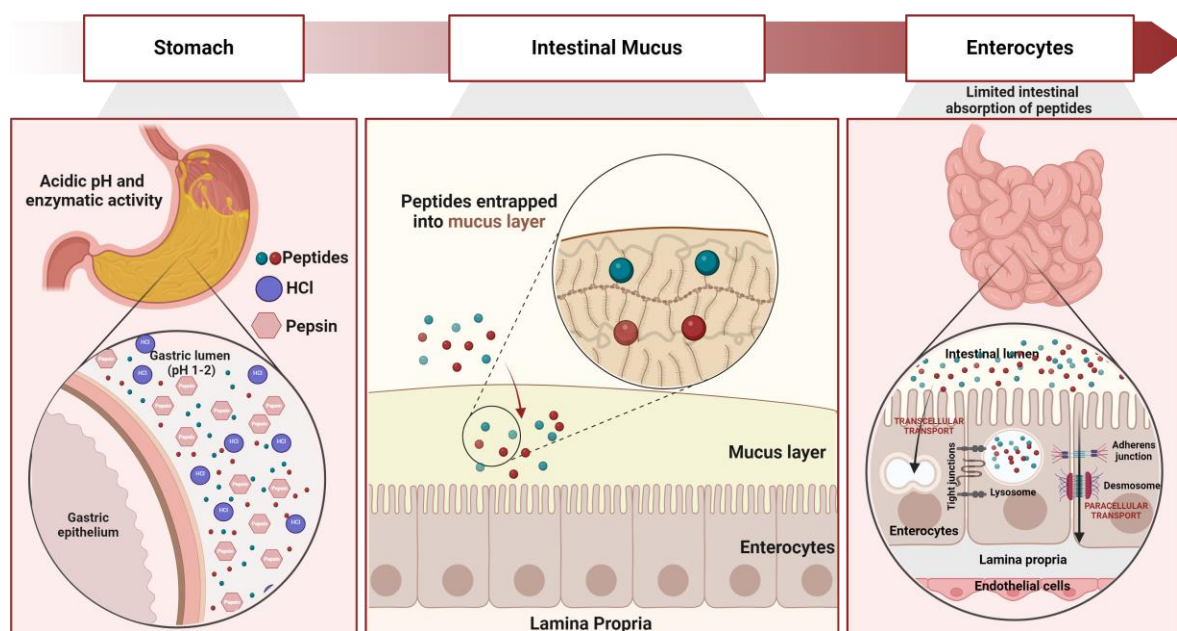


Figure 4. GI barriers that compromise the oral absorption of therapeutic peptides. The main physiological barriers to the absorption of oral peptides are the acidic pH and enzymatic activity of the stomach, the intestinal mucus, and the enterocytes. Created with BioRender.

2.5. Nanotechnology as drug delivery systems

Nanotechnology has demonstrated potential to render cargos with improved stability and solubility, to further enhance their transport across the GIT barriers and therefore, prolong their circulation time. Therapeutic agents can be encapsulated, adsorbed, embedded, conjugated onto nanocarriers, modified or associated with adjuvants for an optimized release profile and subsequent therapeutic action [11]. **Figure 5** represents the parameters to be considered to design a nanosystem for oral delivery.

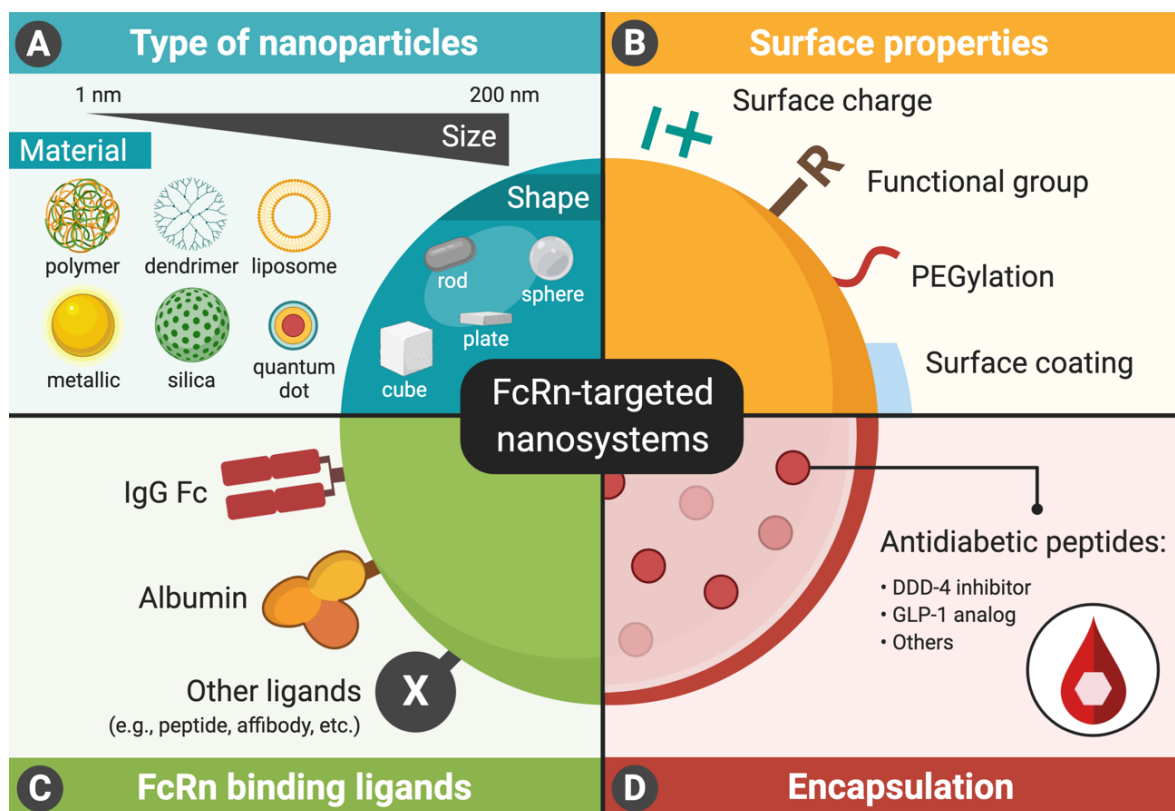


Figure 5. Design of nanosystems for oral delivery of antidiabetic peptides. The physicochemical properties of engineered NPs can be tailored by modifying (A) the particle size, shape, composition, and (B) their surface properties. (C) To ensure an efficient transport of the NPs through polarized epithelial barriers that endogenously express the FcRn receptor, the surface of the NPs may be functionalized with the endogenous ligands of the receptor or their variants (IgG Fc or albumin) and other engineered ligands (e.g., FcBPs or Z_{FcRn}). (D) Nanosystems may be used in the treatment of T2DM by encapsulating antidiabetic peptides in the internal core of the particles, protecting them from mucosal barriers and controlling the release upon oral administration. Created with BioRender.

2.5.1. Drug delivery nanoplateforms

NPs can be classified as lipid-, organic- or polymeric-based NPs. Lipid-based NPs, such as liposomes, solid-lipid NPs (SLNs), and nanostructured lipid carriers (NLCs), are considered attractive drug delivery systems due to their biocompatibility, simple processing, cost-effectiveness, similarity with biological membranes, and high drug loading (DL) capacity due to the high lipid content [84-87]. Regarding liposomes, this nanosystem is composed of an amphiphilic lipid bilayer that favors the loading of hydrophobic drugs and an aqueous core that enables the entrapment of hydrophilic drugs. For this reason, liposomes can entrap hydrophilic and hydrophobic compounds in the same system, thereby expanding their applicability [84, 85, 88]. Since liposomes can be rapidly taken up by the reticuloendothelial system, they usually include surface modifications to prolong their circulation time and improve drug delivery, such as to covalently-attach a hydrophilic polymer (e.g., PEG) to the

surface of liposomes [84, 88]. Lipidic-NPs (e.g., SLNs and NLCs) are composed of phospholipids for particle structure, cationic or ionizable lipids to complex with negatively charged biological material, cholesterol for stability and membrane fusion, and PEGylated lipids to improve the circulation time and the stability of the drug cargo [84]. In what concerns to SLNs, they are formulated with a monolayer phospholipid shell and a solid lipid core, being suitable for encapsulating water-insoluble drugs [85, 86, 89]. NLCs are considered the second-generation of SLNs, since they were developed to overcome the drawbacks associated with SLNs, including the drug quenching over time. The main structural difference is that NLCs are composed of a mixture of solid lipids and liquid lipids that creates a disorganized internal matrix and as a result, provide additional free spaces where the drugs can be entrapped. Therefore, NLCs are commonly associated with an increase in the DL capacity and a decrease in the drug diffusion during storage, when compared to SLNs [86, 90].

In addition to lipid-based NPs, inorganic NPs, including gold, iron oxide, carbon nanotubes, graphene oxide, quantum dots, or mesoporous silica NPs, are extensively exploited for drug delivery, diagnostics, imaging applications and photothermal therapies due to their high stability in aqueous conditions, feasibility of functionalization and large surface area with high loading capacity [84, 85, 91]. These nanosystems can be engineered to own distinct sizes, geometries, and structures, and hold unique electrical, magnetic, physical, and optical properties, according to the material selected to produce the inorganic NPs. In the case of gold NPs, they are produced in different shapes, such as nanorods, nanospheres, nanoshells, nanostars, and nanocages [84]. Gold NPs are widely studied due to their biocompatibility, easy synthesis, and bioconjugation, as well as tunable optical and thermal properties, which make gold NPs of uppermost interest for photothermal therapy [84, 91]. Iron oxide NPs are another inorganic material composed of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) with superparamagnetic properties at certain sizes and have been exploited as contrast agents in magnetic resonance imaging, thermal-based therapeutics, and magnetically targeted drug delivery vehicles [84, 91]. Although inorganic NPs have several advantages as drug delivery nanosystems, their clinical applications are restricted due to their low solubility and toxicity concerns, mostly in formulations using heavy metals [84, 85]. The functionalization of inorganic NPs with biocompatible and biodegradable ligands can be performed during the preparation of the particles or after their production. For example, silica NPs are composed of silanol groups that facilitate their functionalization with target ligands [85].

Polymeric-based NPs have been developed as drug delivery systems to improve drug stability, solubility, efficacy, and safety through controlled release. These nanosystems can be produced using natural or synthetic polymers or a combination of them [84, 85, 87].

Among all the polymers available, PLGA is the most widely used for drug-loaded NPs, due to its US FDA approval biodegradability, biocompatibility, stability during storage, and sustained drug release [85, 87]. PLGA is a co-polymer of lactic acid (LA) and glycolic acid (GA), whose forms are generally identified by the ratio between these two monomers. Regarding the LA:GA ratio, the most frequently used type of PLGA is 50:50, which corresponds to 50% of LA and 50% of GA in the co-polymer structure. The ratio between LA and GA is directly related to the melting temperature of PLGA, which tends to decrease with the increasing mole fraction of GA in the composition of the polymer. The solubility of PLGA is also influenced by the ratio LA:GA, which decreases with the increasing content of LA in the structure of the co-polymer [33]. The degradation of PLGA into LA and GA occurs by hydrolysis of its ester linkages, through heterogenous or bulk erosion, in an aqueous medium. The cleavage of each ester linkage forms one carboxyl and one hydroxyl end groups [33, 85, 87]. One of the conditions that stimulates the hydrolysis of PLGA is the pH, where a strongly alkaline medium stimulates and accelerates the degradation of the polymer [33]. PLGA NPs can be produced by nanoprecipitation and microfluidics, generally applied for hydrophobic drugs encapsulation, or emulsification (single emulsions, such as oil-in-water (o/w), or double emulsions, such as water-in-oil-in-water (w/o/w) and oil-in-water-in-oil (o/w/o)), commonly applied for hydrophilic and hydrophobic drugs encapsulation [84, 87, 92, 93]. PLGA is a surface-functionalizable polymer that allows further attachment of several targeting ligands, which are key to enabling interactions with specific regions of the GIT or specific cell receptors, such as FcRn, as deeply discussed in **Chapter 2, section 2.6.**, facilitating transcytosis across mucosal surfaces and receptor-mediated intracellular drug delivery, and therefore reducing potential side effects [33]. The most common forms of polymeric-based NPs are nanospheres (solid matrix systems) or nanocapsules (cavities surrounded by a polymeric membrane or shell), which are further categorized according to their shapes in micelles, dendrimers, or polymersomes [84].

2.5.2. Physicochemical properties of nanosystems

The biological and therapeutic performance of NPs is dependent of an assortment of properties, namely architecture (size, shape, and surface charge), surface chemistry, and matrix composition [11]. **Particle size** dictates a pivotal role in the design of a suitable oral drug delivery system, as it directly affects the stability, loading capacity, and release profile of the therapeutic agents, and the cellular uptake, internalization, toxicity, and biodistribution of the NPs [94]. Nanoformulations with a size between 50 and 200 nm are considered ideal for the oral delivery of biopharmaceuticals, as they are more efficiently transported through the intestinal mucus and enterocytes than nanoformulations with larger sizes [95-97]. The diffusion of the particles through the intestinal mucus can be hindered because of its pore

size, which ranges from 100 to 200 nm. NPs with a size lower than 200 nm can penetrate faster the mucus layer, providing a prolonged retention of the biopharmaceuticals, while NPs with a size higher than that can be stocked or non-penetrated across the mucus layer, interfering with the overall drug delivery [97, 98]. Additionally, the size of NPs is a crucial feature in determining the mechanism involved in the intestinal uptake of the nanosystems. NPs with a diameter that ranges from 50 to 200 nm are mainly internalized via clathrin- or caveolae-mediated endocytosis or micropinocytosis, while larger NPs ($> 1 \mu\text{m}$) are mainly internalized via macropinocytosis [98-100]. In addition to the size, the **particle shape** also affects the uptake, internalization, toxicity, and diffusivity to the cells. Several studies demonstrated that spherical-shaped NPs are more efficiently internalized by intestinal epithelial cells and macrophages, since they are symmetric, compared to asymmetric-shaped NPs. Spherical-shaped NPs have a constant angle with the cells, while asymmetric-shaped NPs need to be in a specific orientation to interact with the cells, and their shape can also interfere with the integrity of the cell membrane, as in the case of needle-shaped NPs [96, 101-103]. According to the shape of the particles, the internalization pathway to the cells can be distinct. For this reason, spherical-shaped NPs are internalized via caveolae-mediated endocytosis or micropinocytosis, whereas asymmetric-shaped NPs are internalized via clathrin- or caveolae-mediated endocytosis or macropinocytosis [101, 102]. The NPs **surface charge** also interferes with the stability of NPs, DL, and mainly the interaction between the NPs and the biological systems. Either a strong positive or negative surface charge is ideal for the colloidal stability of the systems [11]. Neutrally charged NPs are the nanosystems with suitable ability to be transported through the intestinal mucus due to the limited interaction with the negatively charged mucins, whereas positively charged NPs are retained longer in the mucus than neutrally or negatively charged NPs owing to the electrostatic interactions established with mucins [97, 98, 104]. However, NPs with positive surface charge are more efficiently internalized and transported across the intestinal epithelium than NPs with neutral or negative surface charge due to their interaction with the negatively charged components of the cellular membrane, the phospholipids [104, 105]. Nevertheless, the uptake of positively charged NPs may disrupt the integrity of the cellular membrane, inducing cell death. Therefore, neutral, or negatively charged NPs are considered suitable for long-term circulation. Regarding the internalization pathway, neutrally and positively charged NPs use clathrin-mediated endocytosis or micropinocytosis pathways to be internalized by the cells, while negatively charged NPs use the caveolae-mediated endocytosis pathway [97, 98, 105]. The **surface composition** of NPs is also a critical step in increasing stability and modulating the cellular internalization of NPs, and it affects the surface charge of the nanosystems [105]. The coating or functionalization of the particles with neutral hydrophilic materials, such as polyethylene glycol (PEG), are ideal for

mucus diffusion [106, 107]. PEG is a hydrophilic polymer, that has been widely applied in the surface modification of NPs to increase their transport through the intestinal mucus due to the neutral charge that reduces the interaction with the negatively charged mucins. Additionally, the presence of PEG on NP's surface prevents particles aggregation, thereby contributing to their stability [11, 14-16, 108]. Particle coating with other neutral hydrophilic substances can also be considered, such as poloxamer 407 and polyethene oxide [106]. On the other hand, cationic materials, such as chitosan (CS), also called mucoadhesive materials, are retained longer in the intestinal mucus layer. This is explained due to the electrostatic interactions established between the positive amino groups of CS located on the surface of the particles and the negative-charged mucins. CS can also increase intestinal absorption of biopharmaceuticals by acting as a permeation enhancer, reversibly opening tight junctions to enhance paracellular pathway. However, most of the NPs cannot surpass the intestinal epithelium by paracellular mechanism due to their size [106, 107]. Additionally, the surface of the nanosystems can be functionalized with targeting ligands (e.g., peptides, proteins, antibodies, vitamins, cell-surface receptor, among others) to direct NPs towards a specific ou selective molecule expressed in the target cell or tissue, such as the enterocytes [17, 21, 22, 107]. One of the main strategies applied to conjugate molecules to the surface of NPs is the Mal-thiol reaction, which is based on the reaction between the thiol group of the cysteine (Cys) residues located on the targeting ligands framework, to the Mal ring linked to the surface of NPs at the pH range of 6.5 and 7.5, resulting in the formation of a thioether bond [24, 109-111]. At this pH range, the reaction rate of Mal with the thiol groups of the ligands is faster than the reaction rate of Mal with the primary amines of the ligands, favoring the formation of a stable thioether linkage. However, at pH > 7.5, the primary amines of the ligands react competitively with the thiol groups at the Mal C=C bound. For this reason, the Mal-thiol reaction must be performed at the pH range of 6.5 and 7.5. Additionally, under alkaline conditions (pH $\geq$ 8), Mal is susceptible to hydrolysis resulting in the opening of the ring and the formation of a non-reactive maleic acid amide derivative [109, 112]. The Mal-thiol reaction can also occur using organic solvents instead of buffer solutions, but in this case, the organic solvents must be anhydrous to avoid the hydrolysis of the Mal ring [113]. To boost the intestinal transport of peptides through polarized epithelial cells, a key strategy involves surface-functionalizing nanosystems with ligands that target the FcRn receptor found on the luminal surface of the enterocytes [17, 21, 22, 107]. The mechanism of action of FcRn is deeply discussed in **Chapter 2, section 2.6**.

2.5.3. Nanosystem platforms for the oral delivery of glucagon-like peptide-1 analogs

As previously discussed in **Chapter 2, section 2.3.2**, GLP-1 analogs have been applied for the management of T2DM. Despite the beneficial pharmacological actions of GLP-1 analogs, the mode of administration is considered a barrier for some patients. The administration of these peptides via oral route is preferable, but the hostile environment of the GIT is considered a challenge to the success of their absorption. According to this, several nanoplatforms have been developed over the years for enhancing oral delivery of GLP-1 analogs (e.g., GLP-1, exenatide, and liraglutide) by providing protection against the acidic pH and enzymatic activity of the GIT, which may improve uptake and transport across the intestinal cellular barriers. **Table 2** summarizes all the nanosystems developed in the literature incorporating GLP-1 analogs.

2.5.3.1. Glucagon-like peptide-1-loaded nanosystems

GLP-1 is rapidly degraded by the DPP-4 enzyme and therefore, the amount of peptide that reaches the pancreatic β cells and binds to GLP-1R is limited. The exogenous administration of GLP-1 has been considered to restore the physiological levels of GLP-1 [46, 54]. However, this peptide is currently administered via s.c. route, and therefore does not resemble the endogenous secretion of GLP-1 [47, 114]. Even considering oral delivery, the harsh conditions of GIT and the intestinal barriers (e.g., mucus and intestinal epithelial cells) compromise the bioavailability of GLP-1. Oral GLP-1-based delivery systems were developed to overcome these challenges [21, 115, 116]. Araújo and co-workers [116] investigated the effect of different types of NPs (polymeric NPs (PLGA NPs), SLNs and inorganic NPs (porous silica NPs, PSi NPs), all coated with CS, in the transport of GLP-1 across an *in vitro* intestinal co-culture model, composed of Caco-2 (mimicking enterocyte-like cells), HT29-MTX (mimicking mucus-producing goblet cells) and Raji B (stimulating the differentiation of Caco-2 cells in M cells) cells. The successful CS coating of all formulations was demonstrated by the significant increase ($p < 0.05$) in the zeta potential (ζ -potential) values from negative to positive and the augment ($p < 0.05$) in the average size (Z-average). Furthermore, the coating of the particles with CS guarantees a suitable internalization of the NPs into intestinal cells over non-coated particles. In addition, GLP-1 entrapped into PSi NPs was more efficiently transported across polarized epithelial cells than the peptide incorporated into PLGA NPs and SLNs [116]. Later, the same authors observed a similar effect on the intestinal delivery of GLP-1 by comparing the performance of PLGA NPs and PSi NPs, both coated with CS [117]. Here, the PLGA NPs and PSi NPs were conjugated with a cell-penetrating peptide (CPP) via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry and entrapped in a pH-sensitive polymeric

matrix composed of hydroxypropyl methylcellulose (HPMC) and containing a DPP-4 inhibitor (DPP-4i). This was the first work reporting the dual-delivery of GLP-1 and a DPP-4i in PLGA NPs and PSi NPs. The size of PLGA NPs and PSi NPs coated with CS and conjugated with CPP was 277 nm and 320 nm, respectively, and both systems were positively charged [117]. The particles entrapped into the pH-sensitive polymeric matrix were protected from the acidic pH of the stomach, with GLP-1 being mainly released at intestinal conditions. Nonetheless, the conjugation of both systems with CPP increased the interaction of the particles with intestinal cells when compared to non-conjugated NPs. Additionally, the presence of DPP-4i enhanced 5-fold and 1.5-fold the intestinal permeability of GLP-1 incorporated into PLGA NPs and PSi NPs, respectively, compared with nanosystems produced without DPP-4i. The synergistic effect on GLP-1 permeation in the presence of DPP-4i is probably due to the inhibition of DPP-4, which helped to protect GLP-1 from enzymatic degradation [117]. In 2016, Araújo and co-workers [118] evaluated the glucose-lowering effect of this dual-delivery PLGA NPs system in a T2DM-induced rat model [117]. The NPs were orally administered ($200 \mu\text{g.kg}^{-1}$ of GLP-1) to the animals, showing a reduction of 44% in blood glucose levels, after 8 h of the oral administration of the GLP-1 loaded PLGA-NPs entrapped into DPP-4i-loaded HPMC, where no reduction in blood glucose levels was visualized for the animals treated with the particles without DPP-4i, and the physical mixture of GLP-1 and DPP-4i. Furthermore, an enhancement in plasma insulin levels was also observed 6 h *post*-oral administration of the dual-drug composite system compared to the particles without DPP-4i and the physical mixture of GLP-1 and DPP-4i [118]. Shrestha *et al.* [119] followed the nanosystem designed by Araújo *et al.* in 2015 [117], and produced a similar carrier with GLP-1 loaded into PSi NPs and surface-coated with CS, and further entrapped into an enteric polymeric matrix containing DPP-4i [119]. The CS-coated nanosystem had a size of 208 nm and a positive surface charge. The oral administration of the nanocarrier ($250 \mu\text{g.kg}^{-1}$ of GLP-1) in T2DM-induced rats resulted in a 32% reduction in blood glucose levels and approximately a 6-fold enhancement in pancreatic insulin content compared to the physical mixture of GLP-1 and DPP-4i solution, after 8 h of the oral administration of the formulations [119]. Notwithstanding, the nanosystems developed by Araújo *et al.* [118] and Shrestha *et al.* [119] exhibited equivalent antidiabetic response, demonstrating to be promising platforms for improving the management of T2DM.

Overall, the GLP-1-loaded nanosystems showed tremendous potential in improving the transport of antidiabetic peptides across the intestinal epithelium and in controlling blood glucose levels in diabetic animal models after a single administration. However, other studies are required for their clinical translation, namely assessing the PK and PD profile of GLP-1, and evaluating the therapeutic efficacy and safety of the particles after oral chronic

administrations in diabetic animals and, ultimately, in humans. The principal *in vitro* and *in vivo* effects, and the limitations of each GLP-1-loaded nanosystem discussed in this section are summarized in **Table 2**.

2.5.3.2. Glucagon-like peptide-1 analogs-loaded nanosystems

Since the native GLP-1 is rapidly degraded by the DPP-4 enzyme, and its plasma $t_{1/2}$ is limited, which directly interferes with insulin secretion from pancreatic β cells, GLP-1 analogs have been explored for handling T2DM. Nevertheless, the prevalent use of invasive routes for administering GLP-1 analogs can compromise patient compliance. Although the oral administration of GLP-1 analogs can be considered an alternative, these peptides must overcome the GI barriers that hinder their absorption. To overcome these challenges, oral GLP-1 analogs-delivery systems have been designed, employing different scaffolds for peptide encapsulation. Most of the studies have concentrated on incorporating the short-acting GLP-1 analog, exenatide, and the long-acting GLP-1 analog, liraglutide, into these scaffolds, which are deeply discussed in **Chapter 2, sections 2.5.3.2.1. and 2.5.3.2.2.**

2.5.3.2.1. Exenatide-loaded nanosystems

Exenatide exhibits higher resistance to DPP-4 degradation than native GLP-1, yet its short plasma $t_{1/2}$ dictates a s.c. administration twice daily. Several studies have been conducted to overcome the hindrance of the current exenatide treatment by incorporating the peptide into nanocarriers for oral delivery. The first studies were focused on the development of nanoplatforms based on the surface-functionalization of the particles with peptides or proteins that target the intestinal epithelium. According to this, Li *et al.* [120] designed CS NPs coated with a goblet cell-target peptide (CSK) to interact with the mucus-producing goblet cells, resulting in NPs with a Z-average of 135 nm and a positive surface charge. The coating of the systems with CSK augmented 1.2-fold the permeability of exenatide compared to non-functionalized NPs across polarized intestinal epithelial cells (Caco-2/HT29-MTX cells), demonstrating that the transport of the particles is probably mediated via mucus-producing goblet cells. Further, *in vivo* studies were conducted in a T2DM mice model (db/db mice), where the nanoformulation was orally administered to the animals (50 $\mu\text{g.kg}^{-1}$ of exenatide), resulting in a 25% reduction in blood glucose levels 8 h after oral administration. Nonetheless, in the PK study performed in normoglycemic rats, the relative bioavailability (B_R) of CSK-coated NPs ($B_R \sim 7\%$) was 1.7-fold and 31-fold higher than non-coated NPs ($B_R \sim 4\%$) and free exenatide in solution ($B_R \sim 0.2\%$) [120]. Following this strategy, Song and co-workers [121] incorporated exenatide into a polymeric-based matrix of PLGA linked to the polysaccharide dextran and surface-conjugated with CSK peptide, resulting in a Z-average of around 100 nm and neutral surface charge, which facilitates the

passage through the intestinal mucus. Notwithstanding, the functionalized nanosystems were efficiently taken up and transported across intestinal polarized epithelial cells (Caco-2 cells) than non-targeted NPs [121], as also observed in the study of Li *et al.* [120]. In an *in vivo* study, the oral administration of a single dose of goblet cell-targeted NPs (100 $\mu\text{g.kg}^{-1}$ of exenatide) to db/db mice induced a 25% reduction in blood glucose levels after a single oral administration, which was similar to non-targeted NPs. A similar glucose-lowering effect was observed after daily oral administrations of the targeted NPs for 5 days, while non-targeted NPs only showed a 5% reduction in blood glucose levels. Regarding the PK study performed in normoglycemic rats, the B_R ($\sim 9\%$) value of CSK-targeted NPs was 1.6-fold and 3.5-fold higher than non-functionalized NPs ($B_R \sim 6\%$) and oral free exenatide ($B_R \sim 3\%$), respectively. In this sense, Song *et al.* developed goblet-cell target NPs that exhibited a prolonged effect in controlling glycemia than non-functionalized NPs and free exenatide in solution [121]. In summary, Li *et al.* [120] and Song *et al.* [121] showed that increasing the exenatide dose from 50 $\mu\text{g.kg}^{-1}$ [120] to 100 $\mu\text{g.kg}^{-1}$ [121], as well as loading the peptide in different NPs scaffolds induced a similar glucose-lowering response in diabetic mice and an equivalent oral bioavailability. Polymeric-based nanocarriers were also designed in the research of Zhang *et al.* [122], where exenatide was incorporated into PEGylated PLGA NPs surface-decorated with transferrin (Tf). By conjugating the nanosystems with Tf, the authors intended to improve the intestinal uptake and transcytosis of NPs due to the binding of Tf to the transferrin receptor found on enterocytes, which represent approximately 90% of the total intestinal cells. Upon conjugation of the particles with Tf, the particle size increased from 98 nm to 120 nm and the ζ -potential decreased from -6 mV to -12 mV. The intestinal permeability of Tf-targeted exenatide PEGylated NPs was 3-fold and 10-fold higher than non-targeted NPs and free exenatide in solution, suggesting that the functionalized NPs are being transported throughout the enterocytes via Tf receptor-mediated endocytosis. Tf-exenatide-loaded NPs (100 $\mu\text{g.kg}^{-1}$ of exenatide) induced a 40% reduction in blood glucose levels of db/db mice, 8 h after oral administration, and a 37% glycemia reduction after daily oral administrations for 5 days. In a PK study carried out in normoglycemic rats, the B_R value of Tf-exenatide-loaded NPs ($\sim 6\%$) was 1.6-times and 28-times higher than that in non-targeted NPs ($B_R \sim 4\%$) and oral free exenatide in solution ($B_R \sim 0.2\%$), respectively [122].

Xu *et al.* [123] explored the impact of lipid-based nanocapsules in ameliorating the oral delivery of exenatide. The authors reported that exenatide loaded-nanocapsules had a mean size of 224 nm and a neutral surface charge (-3 mV). Notably, these lipid nanocapsules were the first exenatide-loaded nanosystems exhibiting the potential to trigger the endogenous secretion of GLP-1 *in vitro* in enteroendocrine L-cells (humans and murine) and *in vivo* in normoglycemic mice [123]. Moreover, daily oral administrations of

exenatide-loaded nanocapsules (500 $\mu\text{g.kg}^{-1}$ of exenatide) in a high-fat diet (HFD)-diabetes-induced mice model for 5 weeks were at least as efficient as s.c. exenatide in solution (50 $\mu\text{g.kg}^{-1}$ of exenatide) and the marketed exenatide, Byetta[®], showing a 25% reduction in blood glucose levels. A PK study measuring exenatide levels in normoglycemic mice confirmed that the blood levels of exenatide were significantly higher ($p < 0.05$) when orally administered within nanocapsules compared to free exenatide in solution, with B_R ($\sim 4\%$) value 3-times greater than oral free exenatide in solution ($B_R \sim 1\%$). Overall, the authors developed a dual-action approach that synergizes the biological effect of nanocarriers (inducing endogenous GLP-1 secretion) with that of the incorporated peptide (increasing absorption of exenatide) [123]. Despite the striking effects observed, it is still essential to develop strategies to strengthen the GLP-1 secretory effect of the nanocarrier and/or prolong the *in vivo* antidiabetic effect by reducing the frequency of administrations to facilitate the translation to the clinic. Based on their previous study [123], the same researchers [124] designed exenatide-loaded nanocapsules, but surface coated with PEG, demonstrating a Z-average of 213 nm and a negative surface charge. The PEGylated nanocarriers increased 2.5-fold and 8-fold the endogenous secretion of GLP-1 *in vitro* in murine L cells and *in vivo* in normoglycemic mice, respectively [124], a notable improvement over their previous study [123]. Moreover, the PEGylated nanosystems effectively prolonged the *in vivo* antidiabetic effect of exenatide in an HFD-diabetes-induced mice model over 5 weeks, resulting in a 25% decrease in blood glucose levels. The key advancement in this system was its ability to induce a similar glucoregulatory response with the oral administration being performed to T2DM-animals once every two days [124], whereas in the previous study conducted with non-PEGylated nanocapsules, a daily oral administration of the system was required [123].

The hydrophobic ion pairing (HIP) is another technique currently applied to improve the entrapment of peptides into nanocarriers. This strategy consists of temporarily modifying the hydrophilic properties of the peptide through non-covalent interactions with compounds that retain an opposite charge of the drug to increase its hydrophobicity and facilitate the encapsulation into nanoplateforms [125]. Several studies have been conducted to ion-pair exenatide with other molecules, such as zinc (Zn^{2+}), tetraheptylammonium bromide (THA), sodium docusate (DOC), *n*-octadecyl sulfate (SOS), cholic acid (CA) and phosphatidylcholine (PC). Zhang *et al.* [126] designed PEGylated PLGA NPs to incorporate exenatide ions paired with Zn^{2+} . The polymeric NPs produced with the Zn^{2+} -exenatide complex exhibited a particle size of 98 nm and a negative surface charge. The oral administration of this nanoformulation (100 $\mu\text{g.kg}^{-1}$ of exenatide) to db/db mice reduced 40% of the blood glucose levels after 8 h of experiment [126]. In contrast, the free exenatide in solution and the NPs produced without the Zn^{2+} -exenatide complex only induced a decrease

of 5% in blood glucose levels. Moreover, the B_R values of Zn^{2+} -exenatide-PEGylated PLGA NPs ($\sim 4\%$) were approximately 4-times greater compared to exenatide-PEGylated PLGA NPs ($B_R \sim 1\%$) and 17-times greater than free exenatide in solution ($B_R \sim 0.2\%$) in normoglycemic rats. In another study, Phan *et al.* [127] paired exenatide with SOS and DOC, and the resulting complexes were incorporated into lipid-based nanocarriers, yielding particles with a size of approximately 20 nm and a negative surface charge. An *ex vivo* permeability study conducted on rat small intestine samples revealed a 3.5-fold and 6-fold improvement in the permeability of exenatide-SOS nanosystem than exenatide-DOC loaded nanosystem and free exenatide in solution, respectively. Nonetheless, the B_R value of exenatide-SOS NPs ($20 \pm 2\%$) and exenatide-DOC NPs ($15 \pm 5\%$) remained quite similar following their oral administration in normoglycemic animals at a dose of $300 \mu\text{g.kg}^{-1}$ of exenatide [127]. Ismail *et al.* [128] adopted the approach outlined by Phan *et al.* [127] to produce lipid-based nanocarriers. In this study, exenatide was pre-complexed with THA, resulting in NPs with a size of 20 nm and a positive surface charge. Exenatide-THA lipid nanocarriers exhibited a 10-fold enhancement in intestinal permeability compared to free exenatide in an *ex vivo* permeability study conducted in rat small intestine samples [128]. Additionally, exenatide-THA NPs demonstrated higher potential to improve the oral delivery of exenatide [128] over the nanosystems developed by Phan *et al.* [127], with B_R value of 28% following oral administration of the systems to normoglycemic rats ($300 \mu\text{g.kg}^{-1}$ of exenatide) [128]. Even though the HIP-nanosystems developed by Phan *et al.* [127] and Ismail *et al.* [128] exhibited the ability to ameliorate the oral delivery of exenatide, being the first studies reporting B_R values of exenatide $\geq 15\%$, their antidiabetic effect was not explored. Considering this, Bao *et al.* [129] formed a complex of exenatide with CA and PC and further, incorporated the HIP into zein NPs coated with the enteric polymer, hypromellose phthalate. The formulation had a particle size of 241 nm and a negative surface charge, resulting in a 2.5-fold higher intestinal permeability across Caco-2 monolayers than the free exenatide in solution. Exenatide-CA-PC complex-NPs showed potential in glycemia control in db/db mice, with a blood glucose reduction of 50% over 7 weeks of daily oral administrations ($600 \mu\text{g.kg}^{-1}$ of exenatide). However, no significant differences were observed compared to NPs produced without exenatide-CA-PC complex. Additionally, this nanosystem exhibited a B_R ($\sim 11\%$) value significantly higher ($p < 0.05$) than the NPs produced without exenatide-CS-PC complex ($\sim 6\%$) in normoglycemic animals [129].

In summary, exenatide-loaded nanosystems developed in the literature have demonstrated efficacy in ameliorating the intestinal delivery of exenatide and in improving the *in vivo* antidiabetic effect, whether administered as a single dose or through multiple administrations, when compared to oral free exenatide in solution. Additionally, the B_R

values of exenatide significantly increased upon entrapment into the NPs, regardless of the dose and the scaffold designed. Despite these promising results, the translation of the nanosystems to the clinic remains a distant prospect. Currently, the only commercially available exenatide delivery system is Bydureon®, where exenatide is incorporated into polymeric-based microspheres [44, 48, 56]. However, Bydureon® is administered via s.c. route, requiring larger needles for its administration than for Byetta® (commercially available s.c. free exenatide form) due to the viscosity of the polymeric suspension, which can be uncomfortable for patients (**Table 1**). Additionally, these microspheres can display irregular drug release and instability of their peptide cargos following administration [48, 53, 130-132]. Therefore, further studies are needed to demonstrate the remarkable potential of oral delivery exenatide-loaded NPs developed in the literature in managing T2DM, evaluating the sustained glucoregulatory response of the nanoformulations over longer treatment periods, and assessing their safety and efficacy in both animals and humans. **Table 2** provides a comprehensive review of the main *in vitro* and *in vivo* results, along with the challenges associated with each exenatide-loaded nanosystem discussed in this section.

2.5.3.2.2. Liraglutide-loaded nanosystems

Liraglutide, a long-acting GLP-1 analog, exhibits higher endurance to DPP-4 catabolism and longer plasma $t_{1/2}$ than the native GLP-1 and exenatide. However, the main inconvenience of liraglutide is the need for daily s.c. administrations. Even when considering oral delivery as a more convenient alternative, liraglutide faces rapid degradation in the hostile conditions of GIT. To overcome this challenge, liraglutide-loaded nanosystems have been designed. The initial efforts to enhance the intestinal permeability of liraglutide focused on polymeric-based nanocarriers. In the study of Uhl *et al.*, liraglutide was incorporated into polylactide acid (PLA)-NPs coated with a CPP peptide designed to target the enterocytes [133]. The resulting CPP-conjugated PLA NPs exhibited a size of 350 nm and a neutral surface charge. Importantly, these nanosystems demonstrated a 1.5-fold improvement in intestinal permeability compared to the free peptide in solution [133]. In another study, liraglutide was loaded within PLGA NPs surface-coated with a pH-sensitive polymer (Eudragit® L30D). This formulation yielded NPs with a Z-average of 159 nm and a positive surface charge [134]. The coating of PLGA NPs with Eudragit® L30D protected the peptide from the acidic environment of the stomach, being liraglutide mainly released at physiological pH, where Eudragit® L30D disintegrated, exposing the NPs to the physiological conditions. Upon oral administration of liraglutide-loaded NPs (150 µg.kg⁻¹ of liraglutide) to HFD-diabetes rats a 1.4-fold reduction in blood glucose levels (around 30%) and a 1.2-fold increase in insulin secretion were observed compared to the animals receiving oral free liraglutide in solution during 8 weeks of treatment. Despite the promising

results in controlling blood glucose levels, further studies are required to evaluate the PK profile of liraglutide delivered through the nanosystem [134]. Bearing this in mind, Liang *et al.* [135] devised nanosystems with a core of PLGA and a NP target-ligand of hemagglutinin-2 (HA₂), a viral membrane peptide known to stimulate the disruption of lysosomal membranes, safeguarding the particles from lysosomal catabolism. In terms of the physicochemical properties of HA₂-liraglutide-loaded PLGA NPs, the Z-average (150 nm) and ζ -potential (−2 mV) remained consistent upon HA₂ conjugation. The HA₂-NPs were efficiently internalized and transported across intestinal polarized epithelial cells (Caco-2 cells) than non-targeted NPs. This nanoformulation was also able to escape from lysosomal catabolism in Caco-2 cells, revealing that NPs conjugated with HA₂ exhibited less co-localization with lysosomes than non-targeted NPs, indicating that the peptide facilitated the escape of the NPs from the lysosomal pathway. Regarding the *in vivo* studies, the oral administration of HA₂-liraglutide-loaded PLGA NPs (540 $\mu\text{g} \cdot \text{kg}^{-1}$ of liraglutide) to db/db mice led to a 35% reduction in blood glucose levels after 8 h and 5 days of treatment, inducing a more pronounced glucose-lowering response than the animals receiving oral free liraglutide in solution (15% reduction in blood glucose levels). Nonetheless, the B_R value of HA₂-liraglutide-PLGA NPs (~ 10%) was approximately 2-fold higher than that in oral free liraglutide in solution (~ 5%) in a normoglycemic rat model [135]. Following the strategy of conjugating liraglutide-loaded NPs with the HA₂ peptide, Shi *et al.* [136] designed a pH-sensitive nanosystem consisting of CS and CSK peptide as the backbone structure of the NPs, with liraglutide and HA₂ entrapped into the system. The entire nanoplateform was coated with a pH-sensitive polymer, poly-N-(2-hydroxypropyl) methacrylamide (pHPMA). Coating the particles with pHPMA resulted in an increase in the Z-average from 113 nm to 140 nm. The NPs maintained a neutral surface charge before and after coating with pHPMA, which is ideal to avoid the interaction of the particles with mucus. The authors also showed that the nanosystem had a 2.5-fold higher liraglutide B_R (~ 10%) values, than oral free liraglutide in solution (~ 4%) in normoglycemic mice [136]. In summary, regardless of the NP scaffolds used to incorporate liraglutide, the B_R values obtained were equivalent. However, the prolonged glucose-lowering response of both nanosystems was not effectively explored [135, 136]. Another strategy to improve the oral delivery of liraglutide involves ion-pairing the peptide with other molecules to temporarily increase their hydrophobicity, without compromising their functionality and bioactivity, as mentioned in **section 2.5.3.2.1**. In the study of Bao *et al.* [137], liraglutide was non-covalently bound to CA and further, the HIP was incorporated into a protein-lipid-based matrix of zein and rhamnolipids (RLs), responsible for promoting the transcellular transport of the particles. Regarding the physicochemical properties, the NPs exhibited a mean size of 160 nm and a negative surface charge. Furthermore, the NPs produced with only free liraglutide had an

intestinal permeability 1.3-fold lower than the NPs produced with the complex, since CA is responsible for improving the intestinal transcytosis of the particles by binding to the bile acid transporter found in the apical membrane of the intestinal epithelium. The antidiabetic effect and the oral bioavailability of liraglutide entrapped into Zein/RLs NPs were evaluated *in vivo* through the oral administration of the formulation to a diabetic mice model (4 mg.kg⁻¹ of liraglutide). Liraglutide-CA complex-Zein-RLs NPs were able to induce an antidiabetic response, with a 35% decrease in blood glucose levels in T2DM-induced mice, 8 h *post*-treatment, which was similar to the NPs produced without the liraglutide-CA complex, but slightly higher than the mice that received oral free liraglutide in solution. Also, the oral B_R of liraglutide-CA complex Zein-RLs NPs (~ 10%) was 1.5-fold and 6-fold greater than the nanosystems produced without liraglutide-CA complex (~ 6%) and the free liraglutide in solution, respectively [137]. Even though the dose of liraglutide administered to diabetic animals in the study by Bao *et al.* (4 mg.kg⁻¹) [137] was 7-fold higher than that in the study by Liang *et al.* (540 µg.kg⁻¹) [135], both nanosystems were able to improve the oral delivery of liraglutide and reduce blood glucose levels in a comparable manner.

Overall, the entrapment of liraglutide into nanosystems, regardless of the composition of the NPs matrix, improved the oral delivery and the antidiabetic effect over the free liraglutide in solution (**Table 2**). However, all these studies are not yet ready for clinical translation, as additional aspects need to be explored, including the ability to prolong the glucoregulatory response and stimulate insulin secretion, as well as to evaluate the safety of these nanoformulations in diabetic animals for prolonged treatment.

Table 2. Drug delivery nanosystems designed to improve the intestinal delivery and glucoregulatory response of GLP-1 and GLP-1 analogs (exenatide and liraglutide).

Antidiabetic peptide	Oral drug delivery system	Conjugation chemistry	T2DM <i>in vivo</i> aspects	<i>In vitro</i> and <i>in vivo</i> effects	Limitations	References
GLP-1	Polysaccharide-coated NPs: CS-PLGA NPs CS-SLN CS-PSi NPs	n.a.	n.a.	- Ability to open the tight junctions ↑<i>in vitro</i> GLP-1 intestinal permeability	- Unexplored glucoregulatory response - Untested PK profile	[116]
	Dual-action polymeric NPs: DPP-4i-HPMC-CPP-CS-PLGA NPs	Target molecule: CPP Conjugation reaction: carbodiimide Target site: intestinal cells (non-specific target)	Animal model: Wistar rats T2DM induction: NA:120mg.kg ⁻¹ STZ:60mg.kg ⁻¹ GLP-1 dose: 200µg.kg ⁻¹	- Ability to open the tight junctions ↑5-fold <i>in vitro</i> GLP-1 intestinal permeability - Induce glucoregulatory response 8 h treatment: ↓44% glycemia - Improve PK profile over oral mixture GLP-1/DPP-4i	- Unexplored prolonged glucoregulatory response - Untested ability to stimulate insulin secretion	[117, 118]
	Dual-action porous silicon NPs: DPP-4i-HPMC-CS-PSi NPs	n.a.	Animal model: Wistar rats T2DM induction: NA:120mg.kg ⁻¹ STZ:60mg.kg ⁻¹ GLP-1 dose: 250µg.kg ⁻¹	- Induce glucoregulatory response 8 h treatment: ↓32% glycemia ↑6-fold pancreatic insulin content over oral mixture GLP-1/DPP-4i	- Similar PK profile to the oral mixture GLP-1/DPP-4i - Unexplored prolonged glucoregulatory response	[119]
Exenatide	Intestinal-targeted polysaccharide NPs: CSK-CS NPs	Target molecule: CSK Target site: goblet cells	Animal model: db/db mice Exenatide dose: 50µg.kg ⁻¹	↑1.2-fold <i>in vitro</i> exenatide intestinal permeability - Induce glucoregulatory response 8 h treatment: ↓25% glycemia ↑B_{R (oral)} (~ 7%) over oral free exenatide (B_{R (oral)} ~ 0.2%)	- Unexplored prolonged glucoregulatory response - Untested ability to stimulate insulin secretion	[120]
	Intestinal-targeted polymeric NPs: CSK-dextran-PLGA NPs	Target molecule: CSK Conjugation reaction: carbodiimide Target site: goblet cells	Animal model: db/db mice Exenatide dose: 100µg.kg ⁻¹	- Induce glucoregulatory response 8 h treatment: ↓25% glycemia - Induce prolonged glucoregulatory response 5 days treatment: ↓25% glycemia ↑B_{R (oral)} (~ 9%) over oral free	- Unexplored glucoregulatory response for more than 5 days - Untested ability to stimulate insulin secretion	[121]

			exenatide ($B_{R(oral)} \sim 3\%$).	
Intestinal-targeted PEGylated polymeric NPs: Tf-PEG-PLGA NPs	Target molecule: Tf Conjugation reaction: thioether Target site: Enterocytes (Tf-receptor)	Animal model: db/db mice Exenatide dose: 50 $\mu\text{g} \cdot \text{kg}^{-1}$	• $\uparrow$ 10-fold <i>in vitro</i> exenatide intestinal permeability • Induce glucoregulatory response 8 h treatment: $\downarrow$ 40% glycemia • Induce prolonged glucoregulatory response 5 days treatment: $\downarrow$ 37% glycemia • $\uparrow B_{R(oral)} (\sim 6\%)$ over oral free exenatide ($B_{R(oral)} \sim 0.2\%$).	• Unexplored glucoregulatory response for more than 5 days • Untested ability to stimulate insulin secretion [122]
Lipid nanocapsules	n.a.	Animal model: C57BL/6J mice T2DM induction: HFD Exenatide dose: 500 $\mu\text{g} \cdot \text{kg}^{-1}$	• Ability to trigger the secretion of endogenous GLP-1 <i>in vitro</i> and <i>in vivo</i>; • Induce prolonged glucoregulatory response for 5 weeks treatment: $\downarrow$ 25% glycemia • $\uparrow B_{R(oral)} (\sim 4\%)$ over oral free exenatide ($B_{R(oral)} \sim 1\%$).	• Unexplored short-glucoregulatory response • Untested ability to stimulate insulin secretion [123]
PEGylated lipid nanocapsules	n.a.	Animal model: C57BL/6J mice T2DM induction: HFD Exenatide dose: 150 $\mu\text{g} \cdot \text{kg}^{-1}$	• Ability to trigger secretion of endogenous GLP-1 <i>in vitro</i> and <i>in vivo</i>; • Induce prolonged glucoregulatory response for 5 weeks treatment: $\downarrow$ 25% glycemia	• Untested ability to stimulate insulin secretion • Untested PK profile [124]
Exenatide-hydrophobic-ion-pairing polymeric NPs: Exenatide- Zn^{2+} complex-PEG-PLGA NPs	n.a.	Animal model: db/db mice Exenatide dose: 100 $\mu\text{g} \cdot \text{kg}^{-1}$	• Induce glucoregulatory response 8 h treatment: $\downarrow$ 40% glycemia • $\uparrow B_{R(oral)} (\sim 4\%)$ over oral free exenatide ($B_{R(oral)} \sim 0.2\%$)	• Unexplored prolonged glucoregulatory response • Untested ability to stimulate insulin secretion [126]
Exenatide-hydrophobic-ion-pairing lipid NPs: Exenatide-SOS complex-Lipid NPs/Exenatide-	n.a.	Animal model: no T2DM model Exenatide dose: 300 $\mu\text{g} \cdot \text{kg}^{-1}$	• $\uparrow$ 3.5-fold (Exenatide-SOS complex NPs) and $\uparrow$ 6-fold (Exenatide-DOC NPs) exenatide <i>ex vivo</i>	• Unexplored glucoregulatory response • Not compared PK profile exenatide [127]

	DOC complex-Lipid NPs		intestinal permeability	entrapped in both systems with oral free exenatide	
			• B_R (oral) Exenatide-SOS complex NPs ~ 20% • B_R (oral) Exenatide-DOC complex NPs ~ 15%		
	Exenatide-hydrophobic-ion-pairing lipid NPs: Exenatide-THA complex-Lipid NPs	n.a.	Animal model: no T2DM model Exenatide dose: 300µg.kg ⁻¹	• ↑10-fold <i>ex vivo</i> exenatide intestinal permeability • B_R (oral) ~ 28%	• Unexplored [128] glucoregulatory response • Not compared PK profile exenatide-loaded NPs with oral free exenatide
	Exenatide-hydrophobic-ion-pairing protein-based NPs: Exenatide-CA-PC complex-hypromellose phthalate-Zein NPs	n.a.	Animal model: db/db mice Exenatide dose: 600µg.kg ⁻¹	• ↑2.5-fold <i>in vitro</i> exenatide intestinal permeability • Induce prolonged glucoregulatory response after 7 weeks treatment: ↓50% glycemia • B_R (oral) ~ 11%	• Untested ability [129] to stimulate insulin secretion • Not compared PK profile of exenatide entrapped into system with oral free exenatide
	Intestinal-targeted polymeric NPs: Fc-PEGylated-PLGA-NPs	Target molecule: Fc Conjugation reaction: thioether Target site: Enterocytes (FcRn)	Animal model: db/db mice Exenatide dose: 100µg.kg ⁻¹	• Induce glucoregulatory response 8 h treatment: ↓20% glycemia	• Unexplored [138] prolonged glucoregulatory response • Untested ability to stimulate insulin secretion • Untested PK profile
	Intestinal targeted porous silica NPs: HPMC-Fc-CS-PSi NPs	Target molecule: Fc Conjugation reaction: carbodiimide Target site: Enterocytes (FcRn)	n.a.	• Ability to open the tight junctions • ↑1.7-fold <i>in vitro</i> exenatide intestinal permeability	• Unexplored [21] glucoregulatory response • Untested PK profile
Liraglutide	Intestinal-targeted peptide polymeric NPs: CPP-PLA NPs	Target molecule: CPP Conjugation reaction: thioether Target site: enterocytes	n.a.	• ↑1.5-fold <i>in vitro</i> liraglutide intestinal permeability	• Unexplored [133] glucoregulatory response • Untested PK profile
	Eudragit®-PLGA NPs	n.a.	Animal model: Wistar rats	• Induce glucoregulatory response 8 weeks	• Unexplored [134] prolonged

		T2DM induction: HFD STZ:35mg.kg ⁻¹ Liraglutide dose: 150µg.kg ⁻¹	<i>post-treatment:</i> ↓30% glycemia	glucoregulatory response • Untested ability to stimulate insulin secretion • Untested PK profile	
Intestinal-targeted polymeric NPs HA ₂ -PLGA NPs	Target molecule: HA ₂ (encapsulated) Target site: Enterocytes (lysosomes)	Animal model: db/db mice Liraglutide dose: 540µg.kg ⁻¹	• Improve lysosomal escape • Induce glucoregulatory response 8 h treatment: ↓35% glycemia • Induce prolonged glucoregulatory response for over 5 days treatment: ↓35% glycemia • ↑B_{R (oral)} (~ 10%) over oral free liraglutide (B_{R (oral)} ~ 5%)	• Untested ability to stimulate insulin secretion	[135]
pH-sensitive intestinal-targeted polysaccharide NPs: pHPMA-HA ₂ -CSK-CS NPs	Target molecule: CPP and HA ₂ (encapsulated) Target site: enterocytes	n.a.	• ↑B_{R (oral)} (~ 10%) over oral free liraglutide (B_{R (oral)} ~ 4%).	• Untested <i>in vitro</i> liraglutide intestinal permeability • Unexplored glucoregulatory response	[136]
Liraglutide-hydrophobic-ion-pairing protein-based NPs: Liraglutide-CA complex-Zein-RLs NPs	Target molecule: CA Target site: Enterocytes (bile acid transporter)	Animal model: Not mentioned T2DM induction: STZ:35mg.kg ⁻¹ Liraglutide dose: 4mg.kg ⁻¹	• ↑ <i>in vitro</i> liraglutide intestinal permeability • Induce glucoregulatory response 8 h treatment: ↓35% glycemia • ↑B_{R (oral)} (~ 10%) over oral free liraglutide (B_{R (oral)} ~ 2%).	• Unexplored prolonged glucoregulatory response • Untested ability to stimulate insulin secretion	[137]

B_R, relative bioavailability; CA, cholic acid; CPP, cell-penetrating peptide; CSK, cell-target peptide; CS, chitosan; DOC, sodium docusate; DPP-4i, dipeptidyl peptidase 4 inhibitor; Fc, fragment crystallizable of immunoglobulin G; FcRn, neonatal Fc receptor; GLP-1, glucagon-like peptide-1; HA₂, hemagglutinin-2; HFD, high-fat diet; HPMC, hydroxypropyl methylcellulose; i.p., intraperitoneal; n.a., not applicable; NA, nicotinamide; NPs, nanoparticles; PC, phosphatidylcholine; PEG, polyethylene glycol; pHPMA, poly-N-(2-hydroxypropyl) methacrylamide; PK, Pharmacokinetic; PLA, polylactide acid; PLGA, poly(lactic-co-glycolic acid); PSi, porous silicon; RLs, rhamnolipids; SLN, solid lipid nanoparticles; SOS, *n*-octadecyl sulfate; STZ, streptozotocin; T2DM, type 2 diabetes mellitus; Tf, transferrin; THA, tetraheptylammonium bromide; Zn²⁺, zinc.

2.6. Biology and molecular mechanism of FcRn

2.6.1. FcRn structure and pH-dependent binding with ligands

FcRn, encoded by the *Fcgrt* gene, is a heterodimeric glycoprotein composed of a unique transmembrane alpha (α) heavy chain (40-46 kDa) that is non-covalently associated with the common beta 2 microglobulin (β 2m) light chain (12 kDa) [139, 140]. The heavy chain consists of three α domains (α 1, α 2 and α 3), a transmembrane region and a cytoplasmic tail. The β 2m unit is tightly bound to residues located below the α 1 and α 2 units and to the α 3 domain [141, 142]. FcRn is structurally similar to the major histocompatibility class I (MHC-I) family; however, the peptide-binding groove located between the α 1 and α 2 domains of the MHC-I molecules is occluded in FcRn and therefore, this receptor is not involved in the presentation of antigens to T cells [141, 143-146]. Instead, FcRn is a dual-binding receptor that binds to albumin and IgG in a pH-dependent manner, binding at acidic pH (5.0-6.5) and no binding or release at physiological pH (7.4), which in turn protects both ligands from the intracellular catabolism and therefore, prolongs their plasma $t_{1/2}$ for around 3 weeks [147-150]. This strict pH-dependent mode of action is mediated by the protonation of the imidazole group of the histidine (His) residues found on the amino acid framework of the receptor, IgG, and albumin, since this is the only amino acid that changes its charge between pH 5.5 and 7.4. At acidic pH, the imidazole group of the His residues becomes protonated favoring the interaction between FcRn and both ligands, while at physiological pH, this amino acid loses its protonation, and the ligands are released, or no interaction occurs with the receptor [147, 149, 151]. It is important to highlight that the binding sites of IgG and albumin to FcRn are distinct and non-overlapping, and no significant conformational changes are visualized after the formation of the ternary complex [148, 150]. IgG is the most prevalent antibody in the blood, protecting the human body against foreign substances, such as toxins and pathogens. It is composed of two identical polypeptide chains called fragment antigen-binding (Fab) domain, each of which is linked to a N-glycosylated fragment crystallizable (Fc) heavy chain. Fab domains are responsible for antigen recognition, while the Fc domains are involved in the binding to the FcRn [152-154]. The interaction between FcRn and the Fc portion of IgG occurs at the CH₂-CH₃ domains of the Fc heavy chain, where His310, His435, and isoleucine (Ile)253 are the main binding sites for FcRn. However, His433 and Tyr436 residues present in the Fc domain are also involved in IgG-FcRn binding, but with less relevance [141, 155-160]. In FcRn, the α 2 and β 2m are the main domains involved in the interaction with IgG. At acidic pH, His310 and Ile253 interact with glutamic acid (Glu)115 and Trp131 residues of FcRn, respectively [141, 142, 159, 161, 162]. Additionally, the His310 and His435 form a salt bridge with the Glu117, Glu132, and/or Glu135 and aspartic acid (Asp)137 of the α 2 domain of FcRn. The Ile1 of β 2m domain of FcRn also contributes to IgG binding through van der Waals interactions

with valine (Val)309 and Pro307 of the Fc domain [142, 161]. The stoichiometry of IgG-FcRn interaction is 1:2, with one molecule of IgG per two molecules of FcRn since the antibody contained two Fc domains in its structure [163, 164].

Regarding the other endogenous ligand of FcRn, albumin is the most prevalent protein in the blood and its main biological function is to transport small insoluble cargos, such as metal ions, fatty acids, hormones, as well as poorly soluble drugs for delivery to the tissues [165, 166]. It is composed of three homologous domains (DI, DII, and DIII), each one subdivided into two subdomains (A and B). The three domains are linked by loops and form a heart-shaped structure [149, 167]. The domain DI is responsible for modulating the binding of albumin to FcRn, while the domain DIII contains the principal binding pockets for FcRn [149, 162, 168]. The His residues involved in the pH-dependent binding of albumin to FcRn are located in the DIII domain of albumin, corresponding to His464, His540, and His535, and within the $\alpha 2$ domain of the receptor, corresponding to His166. At acidic pH, His166 becomes protonated, being able to form hydrogen bonds with Tyr60 and Glu54 located in the $\alpha 1$ domain loop, which in turn stabilizes the loop orientation, enabling an efficient binding of albumin to FcRn. Additionally, Lys536, Lys538, and Pro537 close to the His535 residue were also important for albumin-binding to the receptor since mutation of these residues for Ala residues decreased the interaction [149, 150, 162, 168, 169]. The hydrophobic residues Trp51, Trp53, Trp59, and Trp61 present within the $\alpha 1$ domain loop of FcRn are also important for the interaction with the hydrophobic pockets of DIIIA and DIIIB of albumin [168, 169]. Additionally, Lys63, serine (Ser)58, and Threonine (Thr)153 of FcRn contacts with asparagine (Asn)109, Asn11, and arginine (Arg)81-Asp89 of the DI domain of albumin, respectively [162, 168, 170]. The FcRn-target sites of albumin are located on the opposite structural face of the receptor, where IgG binds [150, 151]. The stoichiometry of albumin-FcRn interaction is 1:1, with one molecule of albumin per one molecule of FcRn [142, 150]. The nature of the FcRn-IgG binding is mainly pH-dependent, while the nature of the FcRn-albumin binding is pH- and hydrophobic-dependent [27]. The interactions between both endogenous ligands with the hFcRn are summarized in **Figure 6**.

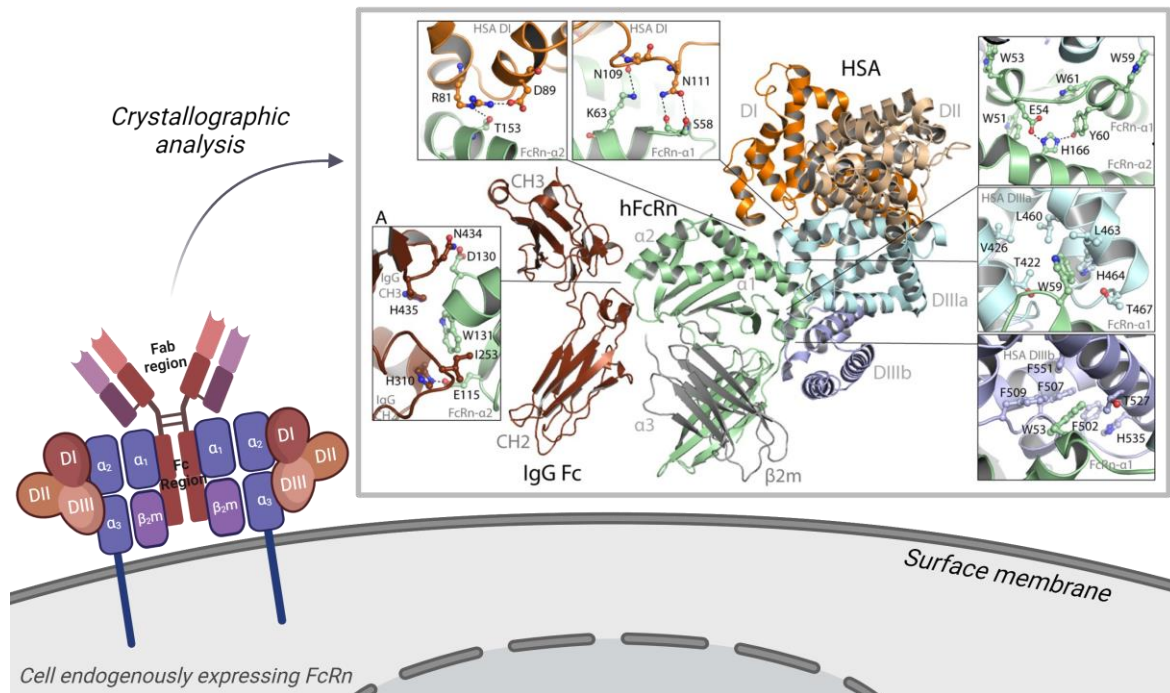


Figure 6. Crystallographic representation of the amino acid residues involved in the interaction of albumin and IgG with the FcRn receptor. Created with BioRender.

2.6.2. Recycling and transcytosis mediated by FcRn

Albumin and IgG are the most abundant blood proteins in humans, which is directly associated to the binding to the FcRn receptor that rescues them from intracellular degradation [147-150, 162, 169]. FcRn is mainly expressed within acidified endosomes (pH 6.0 to 6.5) of endothelial cells lining the inner surface of blood vessels and in the luminal surface of polarized epithelial cells [18, 149, 171-177]. The intracellular transport of albumin and IgG occurs via two FcRn-mediated pathways, recycling, and transcytosis (**Figure 7**). Both processes are regulated by strict pH-dependent binding-and-release interaction between the receptor and the ligands, with the binding being favored at acidic pH and the release at physiological pH [172, 173, 178, 179].

Regarding the recycling pathway, the endogenous ligands are internalized into cells expressing FcRn, namely the vascular endothelial cells, via fluid-phase pinocytosis at pH 7.4, and further bind to the receptor located within acidic endosomes. The acidic pH of the endosomal compartments facilitates the protonation of the His residues present in the albumin, IgG, and FcRn frameworks, and consequently, the interaction between ligands and the receptor. The ligands are further protected from the lysosomal catabolism by exporting the FcRn-ligands complex to the cell surface, where the exposure to the physiological pH of the bloodstream promotes the release of the ligands from FcRn. Albumin and IgG that do not bind to the receptor progress to the lysosomal pathway for proteolytic degradation. The recycling mechanism is involved in the extension of the $t_{1/2}$ of albumin and IgG [172, 178-

181]. The recycling of both ligands can be quantified by the human endothelial-cell based recycling assay, in which it is used the human microvascular endothelial cell line modified to stably express $\beta 2m$ and the full-length of hFcRn with an N-terminal HA-tag and EGFP-fused carbon-terminal (C-terminal) [178].

FcRn also plays a key role in mediating the transcytosis of albumin and IgG through polarized epithelial cells, such as the enterocytes. In these cells, FcRn is expressed in the luminal surface of the enterocytes and within acidified endosomal compartments. Briefly, when IgG and albumin encounter the intestinal lumen milieu's acidic pH (pH 6.0), both ligands bind to FcRn, located on the apical side of the enterocytes. Subsequently, the ternary complex is internalized by receptor-mediated endocytosis and transcytosed inside acidified endosomes (pH 5.5 to 6.0), escaping from lysosomal catabolism, and increasing their plasma $t_{1/2}$. The receptor-ligands complex is afterward directed to the basolateral side of the enterocytes, the lamina propria, where the exposure to the physiological pH causes the release of the ligands, which can enter the systemic circulation via the thoracic lymph nodes. The ligands that do not bind to the receptor suffer lysosomal catabolism [18, 20, 142, 150, 173-177]. The FcRn-mediated transcytosis is being studied *in vitro* by using Transwell® inserts with cells endogenously expressing FcRn, such as Caco-2 cells or Madin-Darby canine kidney (MDCK) cells [18, 175, 176, 182-185].

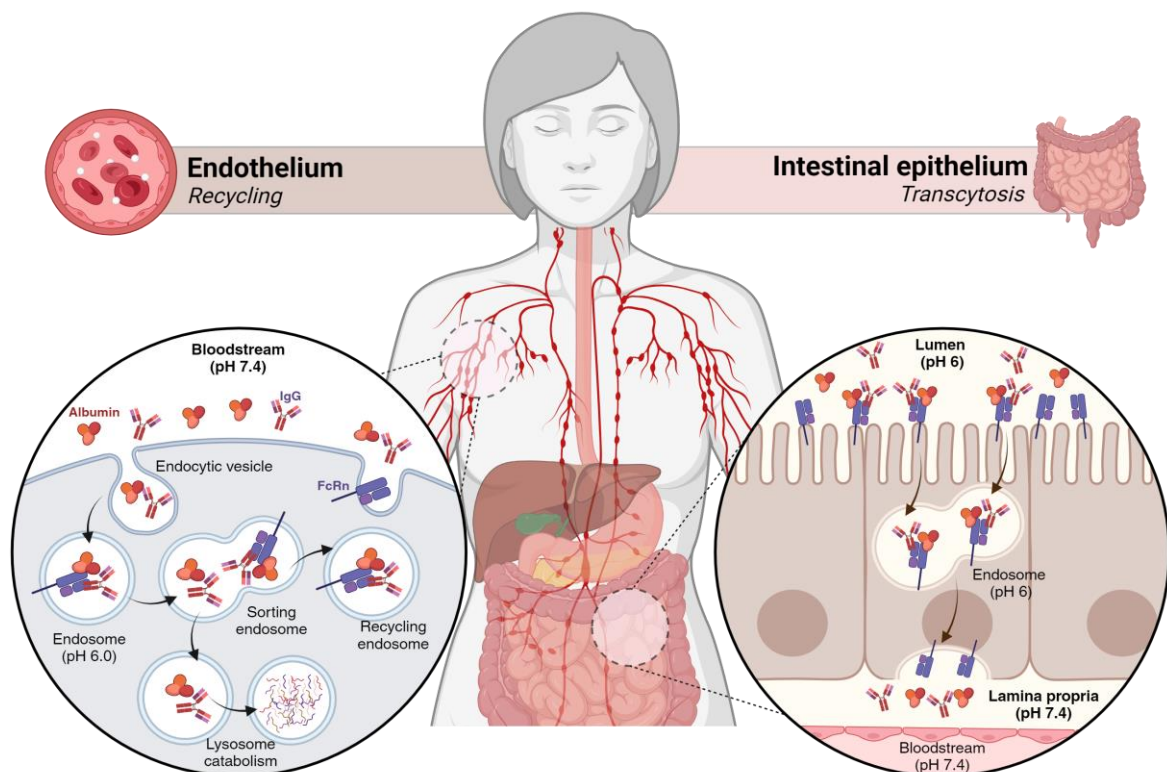


Figure 7. Schematic representation of the intracellular transport of albumin and IgG via FcRn-mediated pathways, recycling in endothelial cells and transcytosis in intestinal polarized epithelial cells. Created with BioRender.

2.6.3. Albumin and IgG-based drug delivery of glucagon-like peptide-1 analogs

The aim of T2DM is to control blood glucose levels with antidiabetic medication, which can be achieved through the administration of GLP-1 analogs, as discussed in **Chapter 2, section 2.3.2**. Engineered GLP-1 analogs based on the interaction or conjugation with HSA and human IgG4 (hIgG4) Fc have been designed to improve the efficacy and safety of GLP-1 in T2DM patients (**Table 1**).

Liraglutide (Victoza®) was the first long-acting GLP-1 analog to reach the market, in which the ϵ -amino group of Lys26 is acetylated with the γ -carboxyl group of N-palmitoyl-L-glutamic-acid (fatty acid chain) that reversibly binds to the hydrophobic pockets of HSA [3, 46, 47, 186]. As such, it associates non-covalently with endogenous HSA in the s.c. tissues and bloodstream. This strategy extends $t_{1/2}$ to 13 h and allows once-daily dosing [45, 48, 56, 57]. Another example of non-covalent association with HSA is the dual analog GLP-1 and glucose-dependent insulintropic polypeptide (GIP) **tirzepatide** (Mounjaro®) [68, 187, 188]. Structurally, tirzepatide combined the bioactive N-terminal GIP sequence with the exenatide-like C-terminal sequence, conjugated with a fatty di-acid (arachidic di-acid) that non-covalently binds to HSA. As a result, the circulation time is prolonged to 5 days with a once-daily administration [68, 69, 187, 189, 190]. Despite the interaction of these peptides with HSA provides a prolonged blood persistence compared to the endogenous GLP-1, the treatment relies on s.c. injection. To allow oral administration, **semaglutide** (Rybelsus®) was developed, as deeply discussed in **Chapter 2, section 2.3.2**. The Lys26 of semaglutide is engineered to contain a spacer and a fatty acid that non-covalently bind to the endogenous HSA. Rybelsus® is administered once daily, having a plasma $t_{1/2}$ of approximately 7 days [48, 49, 56, 66, 67, 73]. In the context of FcRn, it has been demonstrated that binding fatty acids to the hydrophobic pockets of HSA can negatively affect the binding to the receptor, and the effect depends on the length of the fatty acid. Therefore, the $t_{1/2}$ prolongation upon the interaction of HSA with fatty acids can have limitations [168]. Regarding oral delivery, this means that fatty acid-based strategies may not benefit from albumin binding to FcRn expressed at epithelial cell barriers in the GIT. Alternative strategies to the non-covalent interaction between GLP-1 analogs and HSA is the site-specific conjugation of the endogenous protein to the antidiabetic peptides. One example is the HSA-fused GLP-1 receptor agonist **albenatide** (CJC-1134-PC), where exendin-4 is linked to albumin via the PC-DAC™ technology, which consists in the attachment of Mal to the Lys40 residue of the peptide that is responsible to support the covalent binding of the peptide to the thiol group of the free Cys34 located at DI domain of HSA. This strategy has been shown to gain a $t_{1/2}$ of approximately 8 days with a frequency of administration of once-a-week dosing, in a phase III clinical trial, while the exendin-4 molecule injected in the absence of HSA exhibits a $t_{1/2}$ of 2.4 h [48, 56, 58, 191-193]. Another approach is to genetically fuse the peptide of

interest to the C- or N-terminal end of HSA. **Albiglutide** (Eperzan® in Europe or Tanzeum® in the USA) is a di-peptide of GLP-1 (7-36) fused to the N-terminal end of HSA, which rises the $t_{1/2}$ to 6-8 days, rendering it suitable for once weekly s.c. injection [3, 48, 53, 194]. The fusion of the GLP-1 dimer to the N-terminal of HSA leaves the C-terminal end of the protein free, which is composed of a Leu residue that can be enzymatically degraded in the blood, reducing the binding-affinity of HSA to hFcRn, and therefore reduce its $t_{1/2}$ [195]. Albiglutide was withdrawn from the market due to economic reasons [46].

The fusion of hIgG Fc with a GLP-1 receptor agonist, as **dulaglutide** (Trulicity®) and **efpeglenatide** (HM 11260C), is also a distinct approach applied to improve the overall circulation time of antidiabetic peptides. Regarding dulaglutide, it is composed of two hIgG4 Fc heavy chain fragments linked to each other through disulfide bonds and with a GLP-1(7-34-NH₂) attached to the N-terminal end of each hIgG4 molecules [48, 56, 196]. In this case, the Fc region of hIgG4 was modified to reduce its binding with other Fc receptors and the cytotoxicity and immunogenicity of the peptide [48]. As a result, the plasma $t_{1/2}$ is extended to 5-6 days, and the product is approved for once weekly s.c. administration [3, 48, 56, 197]. Lastly, efpeglenatide is derived from exendin-4 as albenatide, but the Lys27 of this molecule is linked to the N-terminal end of the Fc region of hIgG4 via a PEG linker. PEG is present in efpeglenatide structure to preserve the biological activity of GLP-1, which can be lost due to steric hindrance [48, 62]. In a phase III clinical trial, efpeglenatide demonstrated to be safe and effective in controlling blood glucose levels in T2DM patients with a $t_{1/2}$ of around 6 days a once-a-week s.c. dosing [63, 64].

Overall, among all the GLP-1 receptor agonists designed to improve the management of T2DM, only semaglutide was clinically approved for oral delivery. Nevertheless, these antidiabetic peptides can be incorporated into nanosystems tailored for oral administration to enable an efficient transport across the intestinal epithelium. This may be achieved by functionalizing the surface of the nanocarriers with ligands targeting intestinal receptors, such as the FcRn. Taking advantage of the transcytosis mechanism of FcRn, the nanosystems are protected from lysosomal degradation therefore, being able to achieve systemic circulation. The drug delivery nanosystems can be decorated with the endogenous ligands of FcRn or its variants, but also with other FcRn-engineered molecules with higher binding-affinity to the receptor, as discussed in **Chapter 2, section 2.6.4**.

2.6.4. Ligand engineering for improving FcRn binding

Besides engineering Fc and HSA, alternative scaffolds with FcRn-binding properties have been developed (**Table 3**). One example is the selection of FcBPs by phage display technology that bind in the nanomolar and micromolar affinity range to hFcRn, as studied by Mezo *et al.* [32]. FcBPs share a consensus motif of Gly-His-Phe-Gly-Gly-Leu-Tyr that

includes the major amino acids involved in the interaction with FcRn, namely His, Phe, and Tyr. The His residue is responsible for the pH-dependent binding of FcBPs to the receptor due to its property to become positively charged at acidic pH, promoting the interaction with the negatively charged amino acids of the receptor. In fact, at pH 6.0, His residue interacts with the side chain of Trp131 and forms a hydrogen bond with Glu115 of FcRn. Additionally, the Phe residue establishes a hydrophobic interaction with Leu112 and Trp131 of FcRn, while the phenolic hydroxyl group of Tyr makes an end-on-end hydrogen bond with the FcRn-Tyr88. The consensus amino acid motif of these peptides bears no homology with HSA or the Fc domain of IgG. SYN746 (glutamine(Gln)-Arg-Phe-Cys-Thr-Gly-His-Phe-Gly-Gly-Leu-Tyr-Pro-Cys-Asn-Gly) was the first amino acid designed by Mezo and co-workers with a cyclic conformation due to the presence of two Cys residues that form a disulfide bond. The addition of four methyl groups to the peptide SYN746, due to the substitution of Cys4, Gly10, and Leu11 to penicillamine (Pen), sarcosine (Sar) and *N*-methyl-Leu (NMeLeu), respectively, creates the SYN1327 peptide (Arg-Phe-Pen-Thr-Gly-His-Phe-Gly-Sar-NMeLeu-Tyr-Pro-Cys), which was further chemically homodimerized forming SYN1436 (Dimer of Arg-Phe-Pen-Thr-Gly-His-Phe-Gly-Sar-NMeLeu-Tyr-Pro-Cys). Even though the structural modifications in SYN746 practically maintained the interaction of the peptides to hFcRn at acidic pH since the dissociation constants (K_D) were quite similar, a stronger interaction to the receptor was visualized at pH 7.4, meaning that the binding of the peptides to the receptor does not occur in a pH-dependent manner [32]. As such, the peptides developed by Mezo *et al.* [32] are not directly attractive for $t_{1/2}$ extension since only minor differences in binding under acidic and physiological conditions were visualized. Inspired by the work on SYN746 peptide, Sockolosky and colleagues [29] created engineered version of FcBPs by introducing the substitution of Cys at positions 4 and 14 to Val and Ala, respectively, to avoid the formation of disulfide bonds and therefore, converting the cyclic structure of the peptide in a linear structure (Gln-Arg-Phe-Val-Thr-Gly-His-Phe-Gly-Gly-Leu-Tyr-Pro-Ala-Asn-Gly). Additionally, the Tyr12 residue of SYN746 was substituted by His residue (Gln-Arg-Phe-Cys-Thr-Gly-His-Phe-Gly-Gly-Leu-His-Pro-Cys-Asn-Gly) to make the binding more pH-dependently to the receptor. In this case, His12 creates a pH-dependent salt bridge near to Glu133 of FcRn. When the cyclic and linear FcBPs were fused to the N- or C-terminal end of a model fusion partner (fluorescent protein mKate), or both, it bound to hFcRn without affecting the biological function of the fusion partner. Also, the interaction of the FcBPs to the hFcRn was similar when the FcBPs were genetically fused to the N- or C-terminal end of the protein model, suggesting that FcBPs bind to hFcRn independently of their orientation in the protein model. Notwithstanding, the genetic fusion of the model protein at both terminal ends with cyclic and/or linear FcBPs resulted in at least a 10-fold increase in the binding-affinity to the receptor at acidic pH when compared to the fusion in

only one of the terminal ends, which was consistent with the role of avidity caused by a bivalent interaction with hFcRn, while at physiological pH the interaction was maintained or drastically reduced. Notably, the fusion protein harboring an FcBP on each end, bound to hFcRn with an apparent K_D comparable to that of hIgG at pH 6.0. Nonetheless, the FcBP fused with a model protein bound to hFcRn at pH 7.4, unlike hIgG, but the interaction strength was significantly weaker than that observed at pH 6.0. Furthermore, one of the fusion proteins was also shown to be transcytosed 14-fold more efficiently than the non-fused counterpart across hFcRn-expressing polarized epithelial cell monolayers (MDCK cells). The ability of the fused FcBPs to extend the $t_{1/2}$ or deliver the fusion partner across the mucosal barrier *in vivo* was not addressed in this study [29]. However, in the study of Datta-Mannan *et al.* [30], FcBPs developed by Mezo *et al.* [32] and further optimized by Sockolosky *et al.* [29] were used to build a panel of antibody Fab fusions. Here, linear, and cyclic versions of FcBPs were fused to the light chain and/or heavy chain of the Fab, a domain of IgG, at either the N- or C-terminal end, or both, via a Gly₄-Ser linker. The fusion Fab was shown to have varying degrees of receptor binding activity and ability to be transported across polarized human epithelial cells (Caco-2 cells) *in vitro*. The binding-affinity to hFcRn at pH 6.0 increased with the increased number of peptides fused to Fab, independently of the position of the FcBPs linked to the light and/or heavy chains of Fab. A PK study in cynomolgus monkeys revealed that four linear FcBPs fused to the N- and C-terminal ends of both light and heavy chains of the Fab, gave the greatest systemic exposure. These data are in line with the *in vitro* intestinal transcytosis assay, where the Fab fused with four linear FcBPs had the highest intestinal permeability. While the $t_{1/2}$ of the Fab fragment was measured to be about 3-5 h, the fused FcBPs could extend the $t_{1/2}$ to 67 h. Despite this, the study pinpoints the importance of avidity for efficient receptor engagement and transport [30], as already outlined by Sockolosky and co-workers [29].

Another scaffold is the selection of affibody molecules by phage display technique. Affibody molecules are a three-helix bundle based on a domain (6 kDa) derived from IgG-binding protein A from *Staphylococcus aureus*. These ligands are composed of 58 amino acids with generally no Cys residues in their framework, being fused with peptides, proteins, or antibodies by chemical conjugation or genetic recombination, with minimal or no effect on the activity of the fusion partner [198]. Seijsing *et al.* [31] developed Z_{FcRn} (Z_{FcRn_2}, Z_{FcRn_4}, and Z_{FcRn_16}) that showed to bind to the receptor in a pH-dependent manner, with a 20- to 42-fold stronger interaction at pH 6 than at pH 7.4 to hFcRn. The binding of the affibody molecules to the receptor was mediated by the presence of two His residues; however, it is not completely discerned the other amino acid residues involved in the Z_{FcRn}-hFcRn interaction. Interestingly, the fusion of the affibody molecules to an albumin-binding domain (ABD), which will hijack albumin *in vivo* resulted in a $t_{1/2}$ extension of 61-91 h, which was

1.8- to 2.7-fold longer than that of ABD alone ($t_{1/2} \sim 33$ h) [31]. Notably, ABD interacts with albumin across species without affecting FcRn binding [199]. The genetic fusion of Z_{FcRn} -ABD with therapeutic peptides can extend their plasma $t_{1/2}$ because on one side this strategy increases the efficiency of FcRn-mediated recycling in endothelium due to the Z_{FcRn} high binding-affinity to the receptor and on the other side avoids the rapid glomerular filtration via ABD-mediated albumin binding [31]. Recently, Lee *et al.* [200] studied this hypothesis through the fusion of Z_{FcRn} -ABD to an antidiabetic peptide, exendin-4, which resulted in a 241-fold longer plasma $t_{1/2}$ (~ 166 h) than the non-fused exendin-4 ($t_{1/2} \sim 0.7$ h) in mice. The fusion of the exendin-4 with Z_{FcRn} -ABD also did not interfere with the binding of the peptide with the GLP-1R. Notwithstanding, this strategy could provide a significant glucose-lowering effect and a reduction in body weight of an HFD-fed obese mice model for over 12 days with a single s.c. administration, while non-fused exendin-4 only showed a glucose-lowering effect for 6 h [200].

Overall, FcBPs and Z_{FcRn} demonstrated to be promising alternative ligands over albumin and IgG. One of the main drawbacks related to albumin and IgG is the fact that both ligands can interact with other receptors (*e.g.*, Fc γ RI, Fc γ RII, Fc γ RIII, Fc α/μ R, Fc α RI, Fc μ RI and Fc μ R for Fc fragment of IgG and gp60, gp18, megalin, cubilin, SPARC and CD36 for albumin), which could interfere with the biodistribution of the biopharmaceuticals [27, 28, 201], while FcBPs and Z_{FcRn} exhibit stable affinity and specificity for hFcRn in a pH-dependent manner, when interacting with the receptor alone or associated with other fusion partners. Therefore, it is hypothesized that these FcRn-engineered ligands can be applied to improve the oral delivery of biopharmaceuticals, as GLP-1 analogs, by targeting the intestinal FcRn through the entrapment of the peptides into nanocarriers.

Table 3. Examples of FcRn-engineered ligands.

FcRn-ligand	Amino acid sequence	K _D , pH 6.0 (nM)	K _D , pH 7.4 (nM)	Genetic fusion	t _{1/2}	References
FcBPs	SYN746 or Cyclic FcBP: Gln-Arg-Phe-Cys-Thr-Gly-His-Phe-Gly-Gly-Leu-Tyr-Pro-Cys-Asn-Gly	5,100	30,000	-	-	[29, 30, 32]
		14,625	207	C-terminal mKate	-	
		11,290	252	N-terminal mKate	-	
		1,023	78	N-and-C-terminal mKate	-	
		5,600	25,000	C-terminal Fab heavy chain	-	
		770	9,770	C-terminal Fab heavy and light chains	~ 60 h (t _{1/2} ~4 h, free Fab)	[32]
	SYN1327: Arg-Phe-Pen-Thr-Gly-His-Phe-Gly-Sar-NMeLeu-Tyr-Pro-Cys	31	170	-	-	
	SYN1436: Dimer of Arg-Phe-Pen-Thr-Gly-His-Phe-Gly-Sar-NMeLeu-Tyr-Pro-Cys	< 0.5	<0.8	-	-	
	Cyclic FcBP (Tyr286His mutation): Gln-Arg-Phe-Cys-Thr-Gly-His-Phe-Gly-Gly-Leu-His-Pro-Cys-Asn-Gly	1,480	<1,000	N-and-C-terminal mKate	-	
	Linear FcBP: Gln-Arg-Phe-Val-Thr-Gly-His-Phe-Gly-Gly-Leu-Tyr-Pro-Ala-Asn-Gly	9,253	154	C-terminal mKate	-	[29, 30]
ZFcRn		398	6,330	C-terminal Fab heavy and light chains	-	
		4,333	21,600	C-terminal Fab heavy chain	-	
		398	6,330	C-terminal Fab heavy and light chains	~ 49 h (t _{1/2} ~4 h, free Fab)	
		31	833	N-and-C-terminal Fab heavy and light chains	~ 15 h (t _{1/2} ~4 h, free Fab)	
	Linear FcBP (Tyr286His mutation): Gln-Arg-Phe-Val-Thr-Gly-His-Phe-Gly-Gly-Leu-His-Pro-Ala-Asn-Gly	63,000	n.b.	C-terminal Fab heavy chain	-	
	ZFcRn₂: Val-Asp-Ala-Lys-Tyr-Ala-Lys-Glu- Gln-Asp-Ala -Ala-Ala-His-Glu-Ile-Arg-Trp-Leu-Pro-Asn-Leu-Thr- Phe -Asp-Gln-Arg-Val-Ala-Phe-Ile-His-Lys-Leu- Ala -Asp-Asp-Pro-Ser-Gln-Ser-Ser-Glu-Leu-Leu-Ser-Glu-Ala-Lys-Lys-Leu-Asn-Asp-Ser-Gln-Ala-Pro-Lys	12	>500	ABD	61 h (t _{1/2} 33h, free ABD)	[31]
	ZFcRn₄: Val-Asp-Ala-Lys-Tyr-Ala-Lys-Glu- Phe-Glu-Ser -Ala-Ala-His-Glu-Ile-Arg-Trp-Leu-Pro-Asn-Leu-Thr- Tyr -Asp-Gln-Arg-Val-Ala-Phe-Ile-His-Lys-Leu- Ser -Asp-Asp-Pro-Ser-Gln-Ser-Ser-Glu-Leu-Leu-Ser-Glu-Ala-	50	>1,000	ABD	73 h (t _{1/2} 33h, free ABD)	

Lys-Lys-Leu-Asn-Asp-Ser-Gln-Ala-Pro-Lys						
Z _{FcRn_16} :	Val-Asp-Ala-Lys-Tyr-Ala-Lys-Glu-Trp-Met-Arg-Ala-Ala-His-Glu-Ile-Arg-Trp-Leu-Pro-Asn-Leu-Thr-Phe-Asp-Gln-Arg-Val-Ala-Phe-Ile-His-Lys-Leu-Glu-Asp-Asp-Pro-Ser-Gln-Ser-Ser-Glu-Leu-Leu-Ser-Glu-Ala-Lys-Lys-Leu-Asn-Asp-Ser-Gln-Ala-Pro-Lys	14	>500	ABD	91 h	[31, 200]
					(t _{1/2} 33h, free ABD)	
		83	n.b.	Exendin-4-ABD	166 h	
					(0.7 h, free exendin-4)	

FcRn, neonatal Fc receptor; K_D, dissociation constant; t_{1/2}, half-life; FcBPs, FcRn-binding peptides; Z_{FcRn}, affibody; Fab, fragment antigen-binding domain of IgG; ABD, albumin-binding domain.

2.6.5. FcRn-targeted nanoplateforms for the oral delivery of GLP-1 analogs

FcRn-targeted nanosystems have been exploited for improving the oral delivery of antidiabetic peptides, enabling the active transport across polarized intestinal epithelium to ultimately achieve systemic circulation [21, 23, 24, 138]. Relevant parameters need to be considered for the design of FcRn-targeted NPs, namely how the pH-dependent binding kinetics of the FcRn-binding molecules affect the ability of the NPs to be transported, and in this context, how the number and density of the targeting molecules, and such, avidity and flexibility are affecting the outcome [202].

NPs can be decorated with FcRn-targeted ligands in a non-covalent manner by adsorption, but preferably covalently to secure stability [203]. Nanocarriers can be site-specifically conjugated via Mal-thiol or carbodiimide chemistry. Regarding the Mal-thiol reaction, it required coupling of sulfhydryl reactive Mal on the surface of NPs to the thiol groups present on the FcRn-targeted ligands, resulting in an irreversible thioether bond. The Fc domain of hIgG does not contain free Cys residues in its structure, so functional groups containing primary amine reactive reagents or crosslinkers, also called thiolating reagents, are required to form sulfhydryl groups. As hIgG, the engineered FcBPs, and Z_{FcRn} do not contain free Cys residues in the amino acid sequence, and therefore, Cys residue can be included during the synthesis, although in this case, it is required to confirm if the addition of this amino acid does not affect the binding affinity of the ligands to the receptor, or uses thiolating reagents. On the other side, HSA contains a free Cys residue at position 34 in DI, which can be used as an attractive site for NPs conjugation [23, 24, 138, 204]. Conversely, the carbodiimide reaction can be achieved by using the EDC/NHS reaction to form an amide between the carboxylic groups of the FcRn-targeted ligands and the functionalized amidated NPs [21, 25, 205].

FcRn-targeted polymeric-based NPs have been explored to improve the oral efficacy of the GLP-1 analog exenatide, as conducted by Shi *et al.* [138]. In this study, thiolated hIgG Fc was covalently conjugated to exenatide-loaded PLGA NPs using the Mal-thiol chemistry.

The particle size of native PLGA NPs was about 109 nm, which was smaller than that of the Fc-decorated PLGA NPs (140 nm). Notwithstanding, Fc-NPs were found to be more efficiently taken up and transcytosed across polarized epithelial cells endogenously expressing hFcRn (Caco-2 cells) than non-targeted NPs. A mouse model of human T2DM (db/db mice) was used to study the glucoregulatory response of exenatide-loaded NPs. Upon oral administration ($100 \mu\text{g}\cdot\text{kg}^{-1}$ of exenatide), the Fc-NPs showed a 20% reduction in blood glucose levels and higher particle retention in the GIT compared with non-decorated NPs and oral free exenatide, which showed no glycemic response. Furthermore, Fc-NPs exhibited a prolonged glucose-lowering effect when compared to injected exenatide, although the reduction effect of the free drug solution was shown to be more efficient in the first few hours following s.c. injection [138]. Although the nanosystems developed by Shi and co-workers demonstrated promising potential for improving the oral delivery and the glucose-lowering effect of exenatide in a diabetic mice model, further studies are needed to investigate the effect of the nanosystems in prolonging the antidiabetic response for long-term treatment and their safety and efficacy in animals and humans. In addition to polymer-based NPs, inorganic nanosystems were explored for the oral delivery of GLP-1, where the peptide was loaded into undecylenic acid-coated silicon NPs (PSi NPs) functionalized with amine-modified Fc via EDC/NHS reaction [21]. The nanosystems were further coated with a mucoadhesive polysaccharide, CS, and entrapped into a pH-responsive polymeric matrix of HPMC to improve drug absorption in the intestinal epithelium and avoid the premature release of the encapsulated drugs under the strong acidic conditions of the GIT. The successful coating of Fc-conjugated PSi NPs with CS was demonstrated by the increase in the Z-average from 250 nm to 500 nm, as well as surface charge exchanged from -30 mV to +30 mV. The entrapment of the CS-coated NPs into HPMC resulted in slightly larger particles (around 600 nm). Regarding the intestinal delivery of the nanosystems, the Fc-targeted NPs showed a 1.7-fold enhanced transcytosis *in vitro* in polarized epithelial cell lines (Caco-2/HT29-MTX cells) compared with non-targeted NPs [21]. Overall, these findings demonstrated that the decoration of NPs with hIgG Fc may enable the oral delivery of antidiabetic peptide-loaded NPs across intestinal epithelial barriers. Other studies using hIgG Fc to decorate the surface of nanocarriers were performed but in the scope of T1DM [22, 23]. However, further studies are required to explore the potential of FcRn-targeted NPs in improving the glucoregulatory effect of antidiabetic peptides for a prolonged therapeutic window. Moreover, it is recommended to perform the *in vivo* studies in animal models expressing hFcRn, instead of mouse FcRn (mFcRn), such as the hFcRn transgenic mice model, to better translate the outcomes for humans.

HSA can also be used for the decoration of NPs and unlike hIgG Fc, this ligand already has a free Cys residue that enables the conjugation with NPs via a Mal-thiol chemistry. In fact, HSA was already combined with polymeric-based NPs and HPMC-treated undecylenic acid-coated PSi NPs via Mal-thiol and EDC/NHS reactions, respectively, but only in the context of T1DM, specifically to improve the oral delivery of insulin [24, 25]. Even considering the engineered FcRn-targeted ligands abovementioned in **Chapter 2, section 2.6.4.**, no studies were conducted regarding the conjugation of these molecules with NPs incorporating peptides for the treatment of T2DM, opening a toolbox of opportunities.

2.7. *In vivo* models for pharmacokinetic and pharmacodynamic studies

2.7.1. *In vivo* type 2 diabetes-induced mice model

Rodents are considered the most appropriate animal models to explore the mechanism of insulin resistance, and the pathogenesis and potential treatments for DM, owing to their close resemblance with the human disorder [206-208]. Regarding T2DM, several models have been described in the literature to induce this disease in rodents, including low doses streptozotocin (STZ), HFD, HFD-STZ, nicotinamide (NA)-STZ, genetical or spontaneous induced T2DM, or surgical T2DM models [206, 207, 209-211].

STZ model can be employed to induce both T1DM and T2DM in rodents. High doses of STZ induce insulin deficiency with extensive β cell apoptosis and ketosis, which is characteristic of T1DM. On the other side, low doses of STZ cause a mild impairment in insulin secretion without a tendency to develop ketosis, which is associated with T2DM [208, 209, 212]. STZ was discovered in 1960 as a natural antibiotic isolated from the bacteria *Streptomyces achromogenes*; however, its diabetogenic properties (toxicity towards pancreatic β cells) were only described in 1963 by Rakieta [207, 213, 214]. STZ is considered a nitrosourea derived analog composed of deoxyglucose and methyl-nitrosourea moieties [207, 208, 215, 216]. The uptake of STZ by pancreatic β cells is mediated by the deoxyglucose moiety, which interacts with the glucose transporter 2 (GLUT2) found on the plasma membrane of β cells. Only cells expressing GLUT2 in the plasma membrane are vulnerable to the toxicity of STZ, as observed for pancreatic β cells, but also for renal tubular cells and hepatocytes [208, 216-218]. The methyl-nitrosourea is responsible for the cytotoxicity to β cells since it stimulates the formation of reactive methyl carbonium ions that causes deoxyribonucleic acid (DNA) damage *via* methylation, and poly-adenosine diphosphate(ADP)-ribose polymerase-1 (PARP-1, nuclear enzyme that catalyzes the synthesis of poly(ADP-ribose) activation, leading to the depletion of nicotinamide adenine dinucleotide (NAD⁺) and adenosine triphosphate (ATP) in β cells and the reduction in protein synthesis, thereby causing β cell death. Moreover, methyl-

nitrosourea also causes β cell death *via* oxidative stress and the production of nitric oxide (NO), which in turn decreases the consumption of oxygen by mitochondria, also contributing to the limited production of ATP [207, 216, 218-220]. The induction of T2DM *via* STZ model is associated with the development of hyperglycemia, insulin deficiency, weight loss, and partial destruction of Langerhans islets cell mass, without causing ketosis, which is directly dependent on the STZ dose administered to the animals [212, 214, 219, 220]. However, the dosage of STZ is difficult to determine to lead to a stable T2DM without either causing a gradual recovery or a complete destruction of β cells, which is characteristic of T1DM. Moreover, the STZ model by itself does not address the insulin resistance, characteristic of a T2DM scenario [208, 220]. When STZ is combined with NA, HFD, or used within genetically susceptible animals, these agents induce conditions that more accurately reflect T2DM.

Regarding the HFD model, it is associated with insulin resistance, hyperinsulinemia, obesity, and glucose intolerance [206, 208, 221]. To induce the disease, the rodents are fed with HFD for at least 8 to 10 weeks, leading to metabolic disorders, hypertension, obesity, and production of pro-inflammatory cytokines [123, 124, 208, 221-224]. However, feeding the animals with HFD might not cause frank hyperglycemia [206, 208]. Therefore, Reed *et al.* [225] developed a T2DM model based on the combination of HFD that causes insulin resistance and hyperinsulinemia, followed by the injection of a low dose of STZ, which is associated with the reduction of both β cell mass and insulin production, suitable resembling the pathophysiology of T2DM in humans [208, 209, 214, 225]. For induction of T2DM through HFD-STZ model, the rodents must be fed with HFD for a specific period (3 to 8 weeks) to cause insulin resistance, followed by a low dose injection of STZ (30-40 mg.kg⁻¹), resulting in an increase in blood glucose levels within 3 to 10 days after STZ injection. The severity of the disease depends on the doses of STZ administered, the composition of the HFD, and the animal strain used [134, 208, 212, 214, 226].

The NA-STZ model was first developed in 1998 by Masiello *et al.* [227], and it is based on the protective effect of NA against the β cell cytotoxic activity of STZ, creating a state of partial insulin deficiency, which is similar to what happens in T2DM [207, 216, 220, 228, 229]. NA is a vitamin B₃ derivative with antioxidant capacity, being responsible for reducing the cytotoxic effect of STZ by acting as an inhibitor of PARP-1 expression, scavenger of NO and oxygen-free radicals, and precursor of NAD⁺ and ATP. Additionally, NA can act as a methyl group acceptor, reducing DNA methylation, and in doing so, it can inhibit apoptosis and enhance β cell regeneration and Langerhans islet cell growth. NA must be administered before STZ administration to play a protective function for pancreatic β cells [207, 216, 218, 227, 230]. In the literature, the doses of NA and STZ employed to induce T2DM in rodents are not consensual. According to several studies, both drugs can be administered in rats at

the doses of 120 mg.kg⁻¹ NA and 60 mg.kg⁻¹ STZ or 230 mg.kg⁻¹ NA and 65 mg.kg⁻¹ STZ, while in mice, the doses range from 120 to 240 mg.kg⁻¹ NA and from 65 to 180 mg.kg⁻¹ in STZ. The NA and STZ doses selected are dependent on the animal strain selected to induce T2DM since different strains have different sensibilities to the activity of STZ. Moreover, both drugs can be administered more than once during the period of T2DM induction [118, 119, 214, 216, 228, 229, 231-233]. The combination of STZ with NA produces a model characterized by a 40% reduction in pancreatic β cells mass, a 60% decrease in pancreatic insulin stores, a stable and moderate hyperglycemia without requiring exogenous insulin administration, glucose intolerance due to impaired insulin secretion, polyphagia and polydipsia [207, 214, 229, 230]. This model has been widely exploited for biochemical and pharmacological applications, including to evaluate the beneficial potential of antidiabetic drugs (e.g. GLP-1 and exenatide) on the course of blood-glucose lowering effect and insulin secretory properties, as well as the regenerative capacity of the endocrine pancreas [118, 119, 207, 216, 229, 231]. However, the NA-STZ model is limited for not being insulin-resistant, which is a major feature of most human cases [214]. To overcome this challenge, other studies have been reported using HFD followed by NA and STZ administrations as described by Wu *et al.* [234] and Yu *et al.* [235]. One drawback related to the combinations of HFD and NA-STZ models is the long time required to induce T2DM [234, 235].

Regarding the genetically or spontaneously induced T2DM models, the db/db mouse model is the most widely used in T2DM research. The db/db mouse is formed through an autosomal recessive mutation in the leptin receptor (LepR) gene, in which Gly residue is modified to Thr residue on chromosome 4 of LepR [209, 236-238]. The mice are obese due to hyperphagia, and display polydipsia, polyuria, insulin resistance, hyperinsulinemia, and hyperglycemia, within 4 weeks of age. The hyperinsulinemic events start within 2 weeks of age, while the hyperglycemia condition is observed during 4 to 8 weeks of age. [236, 237]. The db/db mice possess certain aspects of human T2DM that are absent in the NA-STZ and HFD-STZ models, namely adipose tissue inflammation and metabolic flexibility modifications [239]. This model has been exploited to investigate the pathogenesis of human T2DM complications, including wound healing impairment and susceptibility to infections, as well as to evaluate the pharmacological effect of antidiabetic peptides (e.g., exenatide and liraglutide) [102, 120-122, 126, 129, 130, 135, 138, 240-243]. Although this model can resemble the features of a human T2DM condition, at an age of 12 to 16 weeks, db/db mice achieve a severe hyperglycemic condition combined with a drastic depletion of pancreatic β cells, a scenario similar to T1DM, and this is why this model cannot live longer than 32 to 40 weeks. Moreover, the T2DM model derived from spontaneous or genetic mutations is expensive and does not mimic so well the disease compared with NA-STZ and HFD-STZ models [236, 237].

2.7.2. Human FcRn transgenic mouse models

FcRn is expressed in almost all cell types and tissues of the human body, including the intestine, vascular endothelium, placenta, lung, myeloid-derived antigen-presenting cells (e.g., macrophages, monocytes, B cells, and dendritic cells), blood brain barrier, kidney, liver, breast, genitals, eye, skin, and bone [17, 244]. Interestingly, in the intestinal tissue of humans, FcRn has been shown to be expressed throughout the life, while in rodents is downregulated immediately after weaning [182, 245-250]. For this reason, it is required caution when FcRn-targeted molecules or nanosystems are being displayed in conventional mice. In humans, FcRn is expressed in different sections of the intestine, but somewhat more in the ileum and colorectum, and less in the duodenum, jejunum, and cecum. On the contrary, in rodents, FcRn is expressed at high levels in the duodenum and jejunum during the neonatal life [176, 183, 244-247, 251]. Although the FcRn expression of intestinal tissues is downregulated in mice, it is maintained in other tissues throughout life, still regulating the biodistribution and $t_{1/2}$ of albumin and IgG. Despite the mouse and human forms of the FcRn heavy chain sharing nearly 70% similarity in the amino acid composition, and that the key interacting residues responsible for the pH-dependent binding are conserved, there are distinct variations that result in large differences in cross-species binding [143, 252]. Specifically, HSA binds very poorly to mFcRn, with more than 20-fold weaker binding than mouse serum albumin (MSA), while MSA binds slightly stronger than HSA to hFcRn. Therefore, when HSA is administered to the mice, it cannot be protected from lysosomal catabolism, since HSA competes for binding to mFcRn in the presence of a high amount of endogenous MSA, which is continuously produced by the liver. In contrast to HSA, hIgG binds more strongly to the mFcRn than to hFcRn, whereas mouse IgG (mIgG) binds very poorly, or no binding is detected to the human receptor [253-256]. Regarding the FcBPs mentioned in **section 2.6.4.**, it was not evaluated the interaction strength of the peptides to the mFcRn, only for the cynomolgus monkey FcRn (cFcRn), where the authors observed that the binding-affinity of the peptides to cFcRn was similar to the hFcRn [30]. However, in the case of Z_{FcRn} molecules, it was detected a 7-fold weaker acidic pH-binding-affinity of the affibody molecules to mFcRn than to hFcRn [31]. Therefore, there is an urgent need to develop transgenic mice expressing hFcRn to be used as a preclinical PK and PD model for hFcRn-targeted systems, as well as to evaluate the delivery of these carriers through the GIT mucosal barriers, mimicking the behavior in humans.

Several hFcRn transgenic mouse models have been described in the literature, including Tg32, Tg276, Tg32-Albumin^{-/-}, and double transgenic mice (also called AlbumusTM) (**Figure 8**) [148, 249, 256-266]. Tg32 and Tg276 mice are the most commonly hFcRn transgenic mouse models used for evaluating the $t_{1/2}$ of the endogenous ligands of hFcRn. Both strains are generated from the conventional C57BL/6J mice from Jackson laboratory through the

substitution of the mouse heavy chain of the receptor with the human heavy chain, which in turn can be non-covalently associated with the mouse $\beta 2m$. The main difference between both mouse strains is that in the Tg276 strain, the expression of hFcRn α -chain is generated containing the human cytomegalovirus and the immediate early promoter/enhancer with the chicken β -actin/rabbit β -globin hybrid promoter (CAG), whereas the expression in Tg32 strain occurs under the control of its human promoter. Consistent with the promoters employed, the expression pattern of hFcRn in the tissues of Tg32 mice is similar to humans, while Tg276 mice express higher levels of hFcRn in several tissues [248, 251, 257-262]. Since endogenous mIgG binds to hFcRn with weaker affinity relative to mFcRn, the Tg32 and Tg276 strains have decreased plasma concentrations of endogenous mIgG compared to the wild-type (WT) C57BL/6J strain. Regarding the endogenous plasma MSA concentrations, they are similar in the Tg32 and Tg276 strains, when compared to the WT C57BL/6J strain [262]. Additionally, the Tg32 transgenic strain was already used in the scope of nanotechnology and T1DM fields, where the authors evaluated the glucoregulatory effect of insulin-loaded PLGA PEG NPs surface-functionalized with WT HSA and HSA variants in T1DM-induced hFcRn transgenic mice (T1DM chemically induced through the *i.p.* injection of 150 mg/kg of STZ) [24]. This study opens the door for the applicability of the hFcRn transgenic mouse model being tested to evaluate the drug delivery of FcRn-targeted nanosystems. Proetzel *et al.* [257] developed a Tg32 mice expressing both hFcRn α -chain and $\beta 2m$, but the authors did not observe differences regarding the $t_{1/2}$ of the ligands when using the Tg32 mice transgenic for hFcRn α chain or the Tg32 mice transgenic for hFcRn α chain and $\beta 2m$. Therefore, these findings led to the understanding that Tg32 mice are suitable for studies evaluating the mucosal delivery of FcRn-targeted ligands or nanosystems and even their PK and PD behavior when compared to Tg276 mice since it more closely resembles the expression profile of the receptor to that of humans. Tg32 and Tg276 have been generated for determining the PK of monoclonal antibodies, and hIgG Fc and HSA-based therapeutics [256-258, 263].

A version of Tg32 mice without expression of MSA (Tg32-Albumin^{-/-} mice) has been exploited for studies in the absence of competition and in the presence of HSA following preloading. Roopenian *et al.* [265] observed that the injection of HSA to Tg32-Albumin^{-/-} mice resulted in an extended HSA $t_{1/2}$ (around 24 days), comparable to what happens in humans, than to C57BL/6J and Tg32 strains, which was explained by the absence of competition between HSA and MSA, and by the expression of hFcRn in the mouse. This model demonstrated to be appropriate for evaluating the PK of HSA and HSA-based molecules [265]. Alternatively, to the Tg32-Albumin^{-/-} mice, another model has been explored to genetically express both HSA and hFcRn, the double transgenic mice also called AlbumusTM, being already used to evaluate the PK profile of WT HSA and engineered

HSA [266, 267]. One drawback of this strain is that the α -chain of hFcRn is not under the control of the human promoter as observed for the Tg32 and Tg32-Albumin^{-/-} strains, and therefore, the similarity with that in humans is limited. However, studies performed with engineered HSA variants in Tg32-Albumin^{-/-} and AlbumusTM strains have similar $t_{1/2}$ [256, 266, 268]. Since both strains have a different construction, the expression of the receptor in the mouse tissues can be different; however, this subject was not deeply explored [173, 266, 268].

In summary, the hFcRn transgenic mouse models can be used for evaluating the pharmacological effect of FcRn-targeted nanoplatforms in several diseases, including DM. However, few studies were conducted with these genetically modified animals to generate DM models. In fact, Tg32 mouse model was the only model used to induce T1DM for evaluating the glucoregulatory response of FcRn-targeted insulin-loaded NPs [24]. No studies regarding the applicability of these models in the scope of T2DM were conducted, which also highlight the novelty of this thesis.

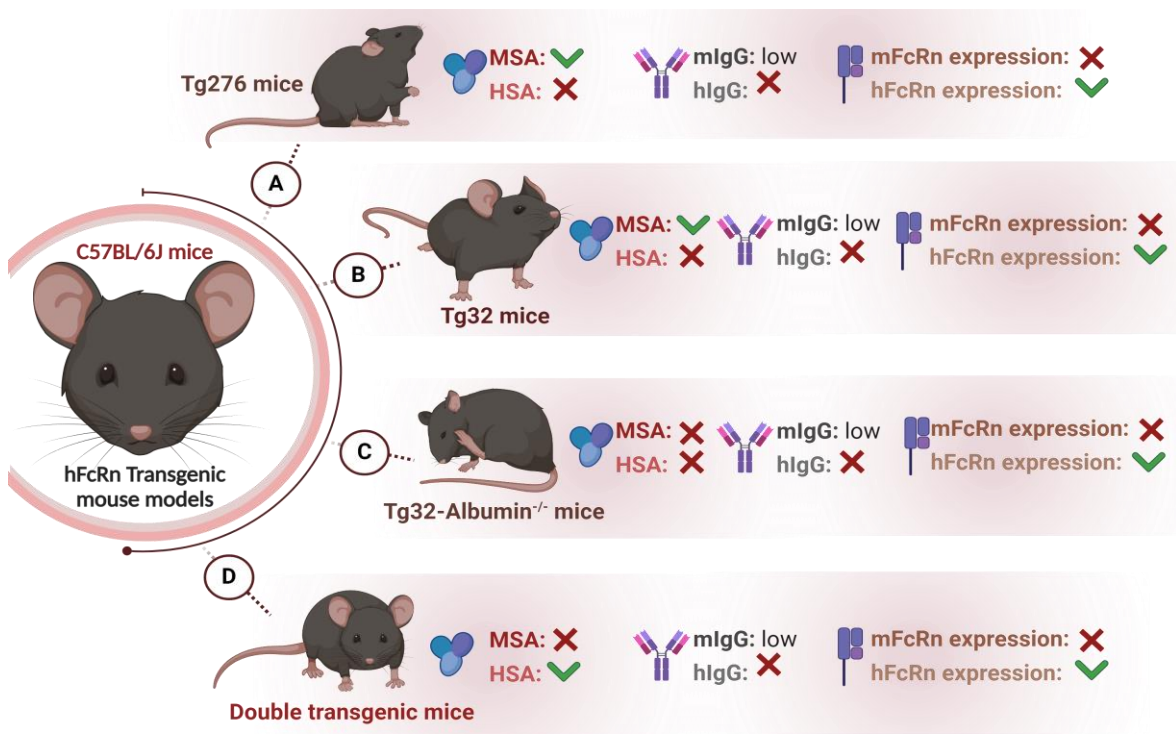


Figure 8. hFcRn transgenic mouse model generated from C57BL/6J mice. (A–B) Tg276 and Tg32 mice that express MSA and mIgG (lower extension). Tg32 mice were the hFcRn transgenic mouse model selected to induce T2DM and to evaluate the glucoregulatory effect of FcRn-targeted semaglutide-loaded NPs in this thesis, instead of Tg276 mice since the expression of hFcRn α -chain occurs under the control of a human promoter, while in Tg276 mice, this expression is under the control of a chicken β -actin promoter. (C) Albumin knockout Tg32 mice that do not express MSA and HSA, but express mIgG (lower extension), Tg32-Albumin^{-/-}. (D) Double transgenic mice expressing HSA instead of the MSA and expressing hIgG (lower extension), AlbumusTM. Created with Biorender.

Chapter | 3

Materials and Methods

This chapter was written based in the following publications:

- **Soraia Pinto**, Juliana Viegas, Cecília Cristelo, Catarina Pacheco, Sofia Barros, Stephen T. Buckley, Javad Garousi, Torbjörn Gräslund, Hélder A. Santos, Bruno Sarmiento, Multifunctional nanomedicines targeting intestinal Fc receptor achieve improved glucoregulatory effect of semaglutide in a type 2 diabetic mice model, under review.

In this scientific paper, I was responsible for the writing (review, editing and original draft), methodology, conceptualization, and bibliographic research. The supervisors were responsible for reviewing, editing, conceptualization, funding acquisition, and submitting the manuscript. These data were not previously included in another PhD thesis, and it is fully reproduced in this chapter.

- **Soraia Pinto**, Mahya Hosseini, Stephen T. Buckley, Wen Yin, Javad Garousi, Torbjörn Gräslund, Sven van Ijzendoorn, Hélder A. Santos, Bruno Sarmiento, Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide, *Journal of Controlled Release* 2024, 366:621-636. doi: 10.1016/j.jconrel.2024.01.015

In this scientific paper, I was responsible for the writing (review, editing and original draft), methodology, conceptualization, and bibliographic research. The supervisors were responsible for reviewing, editing, conceptualization, funding acquisition, and submitting the manuscript. These data were not previously included in another PhD thesis, and it is fully reproduced in this chapter. The first page and the graphical abstract of the review paper, and the permission from the journal to reuse the manuscript in this thesis were included in **Appendix II**. I was also responsible for designing an inside back cover page for the edition of the *Journal of Controlled Release*, where this research paper was published, being cited in **Appendix III**.

3.1. Materials

For surface plasmon resonance (SPR) analysis of FcRn-targeted ligands, CM5 chip and hFcRn were purchased from Cytiva (Massachusetts, USA) and Acrobiosystems Inc. (Newark, USA), respectively. FcBPs (FcBP₁: QRFVTGHFGGLHPANG; FcBP₂: QRFVTGHFGGLYPANG; FcBP₃: QRFCTGHFGGLYPC NG; and FcBP₄: RF-Pen-TGHFG-Sar-NMeLeu-YPC), were supplied by Genscript Biotech (Leiden, The Netherlands). The affibody Z_{FcRn} (VDAKYAKEWMRAAHEIRWLPNLTFDQRVAFIHKLEDDPSQSSELLSEAKK LNSQAPKC), previously described as Z_{FcRn_16} [31], was expressed with a N-terminal hexahistidine tag and a C-terminal Cys and purified with immobilized metal-ion affinity chromatography under native conditions. HSA was purchased from Sigma-Aldrich (Massachusetts, USA). Regarding the production of NPs, PLGA (50:50 Lactic Acid:Glycolic Acid; 44 kDa; Purasorb® PDLG 5004A) and semaglutide were kindly provided by Corbion Purac (Gorinchem, The Netherlands) and Novo Nordisk (Bagsværd, Denmark), respectively; PLGA(30 kDa)-PEG(5 kDa)-Mal was purchased from Ruixibiotech (Xian, China); PLGA(45 kDa)-PEG(5 kDa)-methoxy and PLGA(30 kDa)-FKR648 were supplied by Polysciotech/Akina® (Indiana, USA). Spermidine, polyvinyl alcohol (PVA), tris(2-carboxyethyl)phosphine (TCEP), diethyl ether, and dimethylformamide (DMF) were purchased from Sigma-Aldrich (Missouri, USA). Dichloromethane and dimethyl sulfoxide (DMSO) were acquired from Fisher Scientific (New Hampshire, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Biowest (Nuaille, France), while non-essential amino acids (NEAA), Roswell Park Memorial Institute 1640 (RPMI1640) medium and advanced DMEM/F-12 1× were purchased from ThermoFisher Scientific (Massachusetts, USA). Fetal bovine serum (FBS) was acquired from Biochrom GmbH (Berlin, Germany). Penicillin and streptomycin were acquired from Invitrogen (Massachusetts, USA). Micro BCA™ protein assay kit was supplied by Honeywell (North Carolina, USA). Flash Phalloidin™ Green 488 was purchased from BioLegend (California, USA), while 4'6-diamidino-2-phenylindole (DAPI) was acquired from Merck Millipore (Massachusetts, USA). Caco-2 and HT29-MTX cell lines were purchased from American Type Culture Collection (ATCC) (Virginia, USA). Human IntestiCult™ Organoid Growth Medium and mTeSR™ were purchased from StemCell Technologies (Cologne, Germany). Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, and Cell Recovery Solution were supplied by Corning® (Massachusetts, USA). Zonula occluden-1 (ZO-1) monoclonal antibody, Alexa Fluor™ 647 donkey anti-mouse IgG (H + L) and Alexa Fluor™ 488 donkey anti-goat IgG (H + L) were purchased from ThermoFisher Scientific (Massachusetts, USA). Villin monoclonal antibody was obtained from Santa Cruz Biotechnology (Texas, USA). Human/mouse E-cadherin antibody and hFcRn antibody were purchased from R&D Systems (Minnesota, USA). Anti- MUC2 antibody was supplied by Abcam (Cambridge, UK).

IRDye® 680RD goat anti-mouse IgG secondary antibody was purchased from LICOR (Nebraska, USA). Calcein-acetoxymethyl (calcein-AM) and propidium iodide (PI) were purchased from Sigma-Aldrich (Missouri, USA). Protease inhibitor cocktail was acquired from Roche (Basileia, Switzerland). Y-27632 was acquired from Tocris Bioscience (Bristol, UK). Regarding the *in vivo* studies, STZ and NA were supplied by Sigma-Aldrich (Missouri, USA). Insulin plus proinsulin antibody and mouse anti-insulin/proinsulin monoclonal antibody, horseradish peroxidase (HRP) conjugated were purchased from abcam (Cambridge, UK). 1-Step™ Ultra 5,5'-tetramethylbenzidine (TMB)-enzyme-linked immunosorbent assay (ELISA) substrate was acquired from Thermo Fisher Scientific (Massachusetts, USA). Mouse anti-insulin monoclonal antibody and rabbit anti-glucagon polyclonal antibody were supplied by Sigma-Aldrich (Missouri, USA) and Alfacene (Carcavelos, Lisbon), respectively. UltraVision Protein Block (Epredia®) was purchased from Fisher Scientific (New Hampshire, USA). Poliview® Plus AP (anti-mouse), Poliview® Plus HRP (anti-rabbit), HIGHDEF® red IHQ chromoagen (AP, plus) and HIGHDEF® hematoxylin were supplied by Enzo Life Sciences (New York, USA). Optimal cutting temperature (O.C.T.) was purchased from WVR International (Vila Nova de Gaia, Portugal). Bio-Plex Pro™ Mouse Diabetes assay kit was supplied by Bio-rad (Alges, Portugal). ProcartaPlex™ Mouse Interleukins assay kit and Alexa Fluor Phalloidin™ 546 was purchased from Alfacene (Carcavelos, Lisbon), whereas cyanine 7.5 (Cy7.5) was acquired from Lumiprobe (Hannover, Germany). Fluoromount® aqueous mounting medium was supplied by Sigma-Aldrich (Missouri, USA).

3.2. Surface Plasmon Resonance analysis

The binding-affinity of FcBPs and Z_{FcRn} to hFcRn was evaluated by SPR analysis, using a BIACORE X100 instrument (Cytiva, Massachusetts, USA). The hFcRn was coated to the dextran surface of a CM5 sensor chip at pH 5.0 by amine coupling using EDC/NHS, resulting in a final density of approximately 400 response units (RU). The binding-affinity analysis of FcBPs (FcBP₁, FcBP₂, FcBP₃ and FcBP₄) was evaluated as previously described by Sockolosky *et al.* [29] with some modifications. A concentrations range of FcBPs (0.16 µM – 100 µM) was prepared in running buffer (phosphate (50 mM), NaCl (100 mM) and Tween® 20 (0.01% (v/v))) at pH 6.0 and 7.4 and injected over the hFcRn-CM5 chip at 30 µL.min⁻¹ for 180 sec. The peptides were then dissociated from the surface of the chip for 600 sec. After the dissociation step, HEPES-buffered saline (HBS)-buffer (HEPES (10 mM), NaCl (150 mM) and Tween® 20 (0.005% (v/v))) at pH 7.4 was used to remove the remaining peptides from the chip. For Z_{FcRn} binding-affinity analysis, the experiments were performed according to the protocol described by Seijnsing *et al.* [31] with some alterations. The concentrations range of the affibody, 1.4 nM – 875 nM, was prepared in running buffer

(McIlvaine's phosphate-citrate buffer) at pH 6.0 and 7.4 and injected over the hFcRn-CM5 chip at $30 \mu\text{L}\cdot\text{min}^{-1}$ for 180 sec. The affibody was afterwards dissociated from the surface of the chip for 600 sec and further, the remaining affibody was removed from the chip using a regeneration buffer Tris-buffer (Tris-HCl (50 mM), NaCl (100 mM) and Tween[®] 20 (0.01%(v/v))) at pH 9.0. As a proof of concept, the binding-affinity of HSA to hFcRn was also tested since this molecule is an endogenous ligand of hFcRn [17]. The receptor was coated to the surface of CM5 sensor chip at a final density of approximately 1,300 RU. The experiment was conducted using as running buffer, phosphate (50 mM), NaCl (100 mM) and Tween[®] 20 (0.01% (v/v)). A range of concentrations of HSA (0.13 μM -80 μM) was tested. The solutions were prepared in running buffer at pH 6.0 and 7.4 and injected over the hFcRn-CM5 chip at $30 \mu\text{L}\cdot\text{min}^{-1}$ for 120 sec. HSA was then dissociated from the surface of the chip for 120 sec. After the dissociation step, HBS-buffer at pH 7.4 was used to remove the remaining HSA from the chip. The RU values obtained for each injection were plotted against the concentration of FcBPs, affibody, and HSA. The K_D values of FcBPs, Z_{FcRn} and HSA were determined by applying the steady-state affinity model. Before SPR analysis, the FcRn-targeted ligands were purified by size exclusion chromatography.

3.3. Conjugation of FcRn-targeted ligands with PLGA-PEG-Mal co-polymer

According to the previous results of SPR analysis, the FcRn-targeted ligands (FcBP₂ and Z_{FcRn}) with better pH-dependent binding-affinity to hFcRn, were covalently linked to the PLGA-PEG-Mal co-polymer through Mal-thiol chemistry, following the protocol previously performed [113]. The thiol group of the Cys residue located on the N-terminal and C-terminal of FcBP₂ and Z_{FcRn} , respectively, was conjugated to the Mal ring of PLGA-PEG-Mal. Briefly, 15 mg of PLGA-PEG-Mal was dissolved in 1 mL of anhydrous DMF in an inert atmosphere (N₂) for 1 h at room temperature (RT). TCEP was added to both FcRn-targeted ligands in a molar ratio of 1:2 (FcRn-targeted ligands:TCEP) and the mixture was incubated for 1 h at RT and inert atmosphere to ensure the thiol group of the Cys residue is accessible to be conjugated to the polymer. For this, 0.369 mg of TCEP in 1 mL of anhydrous DMF was added to 1.2 mg of FcBP₂ and 5 mg of Z_{FcRn} in 1 mL of anhydrous DMF under inert atmosphere. Afterwards, the FcBP₂:TCEP solution and Z_{FcRn} :TCEP solution were separately added to the PLGA-PEG-Mal solution, in a molar ratio of 1:1.5 (PLGA-PEG-Mal:FcRn-targeted ligands) to allow the covalent linkage between the free thiol group of FcRn-targeted ligands and the Mal ring of PLGA-PEG-Mal. The mixture was incubated at 4 °C for 24 h under inert atmosphere and magnetic stirring. Nonetheless, PLGA-PEG-Mal was also subjected to the same procedure mentioned above, but without incubation with FcRn-targeted ligands, to be used as control of the reaction. Additionally, a *mock* reaction for both ligands was performed. In the case of FcBP₂, PLGA-PEG-Mal was incubated with

the peptide without the Cys residue in the N-terminal of the peptide framework, while in the case of Z_{FcRn}, PLGA-PEG was incubated with affibody. To remove the non-conjugated molecules, PLGA-PEG-Mal:FcBP₂ and PLGA-PEG-Mal:Z_{FcRn} were precipitated with 35 mL of cold diethyl ether and centrifuged at 3,200 g for 30 min at 4 °C. The supernatants were discarded, and PLGA-PEG-Mal:FcBP₂ and PLGA-PEG-Mal:Z_{FcRn} were washed twice with 10 mL of MilliQ® ultrapure water and centrifuged at 3,200 g for 10 min at 4 °C. The supernatants from both washing steps were collected and freeze-dried to quantify the CE of both ligands through indirect method. The conjugate was precipitated again with 5 mL of cold diethyl ether followed by centrifugation at 3,200 g for 15 min at 4 °C. Finally, the conjugates were dried in a vacuum oven overnight at RT and stored at -20 °C under inert atmosphere until the production of NPs.

3.3.1. Conjugation efficiency of FcRn-targeted ligands and PLGA-PEG-Mal copolymer

The conjugation efficiency (CE) of both FcRn-targeted ligands (FcBP₂ and Z_{FcRn}) was calculated by the difference between the total amount of ligand used in the Mal-thiol reaction and the amount of ligand detected in the supernatants, after washing the precipitated polymers twice with MilliQ® ultrapure water, as described in **Equation 1**:

$$CE (\%) = \frac{\text{Total amount of FcRn ligand} - \text{Free FcRn ligand in the supernatant}}{\text{Total amount of FcRn ligand}} \times 100 \text{ (Equation 1)}$$

The amount of FcBP₂ and Z_{FcRn} non-conjugated with the polymer was quantified by Micro BCA™ assay. The absorbance was measured at 562 nm with the BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). All the measurements were performed in triplicates in at least 3 independent experiments.

3.3.2. X-ray photoelectron spectroscopy analysis

The successful conjugation of FcBP₂ and Z_{FcRn} with PLGA-PEG-Mal was assessed by X-ray photoelectron spectroscopy (XPS) analysis. The samples were prepared by compressing the conjugated polymers into thin pellets in a hydraulic press. The measurements were conducted on a Kratos Axis Ultra HAS spectrometer (Kratos Analytical Ltd., Manchester, UK) at *Centro de Materiais da Universidade do Porto* (CEMUP), using aluminum (15 kV) as a radiation source. The survey spectra were obtained in the 0–1350 eV range with an analyzer pass energy of 160 eV. The high-resolution spectra of C1s, N1s, O1s and S2p were collected with analyzer pass energy of 40 eV. The spectra were fitted using the CasaXPS (version 2.3.17PR 1.1) software and peak intensities of high-resolution spectra (C1s, N1s, O1s and S2p) were normalized using C1s peaks as reference (285 eV).

3.4. Semaglutide solubility assay

The solubility of semaglutide in DMSO, MilliQ® ultrapure water, buffer mimicking the intestinal environment (0.68% (w/v) KH_2PO_4 and 7.7% (w/v) 2 N NaOH) at pH 6.8, and buffer mimicking the gastric environment (0.2% (w/v) NaCl and 8% (v/v) 1M HCl) at pH 1.2, was evaluated. Additionally, it was tested the solubility capacity of semaglutide in simulated gastric fluid supplemented with 0.5% (w/v) and 1% (w/v) Tween® 80. Briefly, semaglutide was added to 1 mL of each solvent until visualize the saturation of the system (peptide undissolved). Further, all the samples were maintained under stirring at 120 rpm, 37 °C overnight. In the next day, the samples were centrifuged at 1,200 rpm for 5 min at RT and the supernatants were collected. The amount of semaglutide was quantified from the supernatants by fluorescence method, since the peptide framework is composed of aromatic amino acids, such as Trp and Tyr residues, that ascribe fluorescence to the drug [269]. Each sample (200 μL) was transferred to a black 96-well plate, with a black bottom, and measured at excitation and emission wavelengths of 295 nm and 350 nm, respectively, in a BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). A calibration curve of semaglutide (5 $\mu\text{g}.\text{mL}^{-1}$ –100 $\mu\text{g}.\text{mL}^{-1}$) dissolved in MilliQ® ultrapure water was outlined. The blank of the calibration curve corresponded to MilliQ® ultrapure water, while the blank of each sample corresponded the solvents tested in the solubility assay (DMSO, MilliQ® ultrapure water, intestinal buffer at pH 6.8, gastric buffer at pH 1.2 with or without 0.5% (w/v) and 1% (v/v) Tween® 80). All samples were evaluated in triplicates.

3.5. Production of FcRn-targeted semaglutide-loaded nanoparticles

FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs were produced by w/o/w double emulsion technique [24, 270]. Briefly, the organic phase of FcBP₂-semaglutide-NPs, composed of 20 mg of polymer, 50% (w/v) of PLGA-COOH (PURASORB® PDLG 5004A in a 50/50 molar ratio of DL-lactide and Glycolide), 10% (w/v) of PLGA-PEG-methoxy, and 40 % (w/v) of PLGA-PEG-Mal:FcBP₂, was dissolved in 1 mL of dichloromethane; while the organic phase of Z_{FcRn}-semaglutide-NPs contained 20 mg of polymer, 80% (w/v) of PLGA-COOH, 10% (w/v) of PLGA-PEG-methoxy, and 10% (w/v) of PLGA-PEG-Mal:Z_{FcRn}, was dissolved in 1 mL of dichloromethane. The aqueous phase of both FcRn-targeted semaglutide-NPs was composed of semaglutide complexed with a polyamine, spermidine. Briefly, semaglutide was solubilized in 0.1 M NaOH solution and further, 12.5 mg. mL^{-1} of spermidine was added to 10 mg. mL^{-1} of semaglutide solution in a semaglutide:spermidine charge ratio of 4:9. Then, the mixture of semaglutide and spermidine was incubated overnight at 4 °C and 120 rpm. In the next day, 100 μL of semaglutide:spermidine complex, corresponding to the aqueous phase, was added to the polymeric solution, and homogenized using a Bioblock Vibracell™ sonicator (Sonics & Materials, Connecticut, USA)

for 2 min at 70% of amplitude, forming the first emulsion (w/o). Further, the w/o emulsion was added to 4 mL of 2% (w/v) of PVA aqueous solution and homogenized for 2 min at 70% amplitude, using the same equipment, forming the second emulsion (w/o/w). The w/o/w emulsion was then poured to 7.5 mL of 2% (w/v) of PVA and left for 3 h under magnetic stirring (300 rpm) to evaporate the organic solvent. After 1.5 h of magnetic stirring, NPs were concentrated by centrifugation using a high-speed centrifuge Beckman Avanti™ J-25 (Marshall Scientific, New Hampshire, USA) at 30,000 *g* for 20 min at 4 °C, to remove the unloaded semaglutide and the excess of PVA. Then, the supernatant was collected, and the NPs *pellet* was washed once with 10 mL of MilliQ® ultrapure water (30,000 *g*, 20 min, 4 °C), followed by resuspending the *pellet* obtained in 1 mL of MilliQ® ultrapure water and storing at 4 °C for further experiments. The fluorescent NPs produced for the *in vitro* uptake studies were composed by the same components of organic phase previously mentioned but containing PLGA-FK648 (10% (w/v)), adjusting the final composition of the NPs to 20 mg of polymer (100% (w/v)). For the *in vivo* biodistribution study, the organic phase of the NPs contained the same elements earlier indicated, and also Cy7.5 in a concentration of 50 µg.mL⁻¹.

3.5.1. Physicochemical characterization of FcRn-targeted nanoparticles

3.5.1.1. Average particle size, size distribution and zeta potential

The PLGA-NPs formulations were characterized in terms of Z-average and polydispersity index (Pdl) by dynamic light scattering (DLS), and ζ-potential by electrophoretic light scattering (ELS), using a Malvern Zetasizer Nano ZS instrument (Malvern Panalytical, Malvern, UK). All the samples were dispersed (1:50) in a 10 mM NaCl solution. Three measurements were performed for each formulation and three different formulation batches were analysed.

3.5.1.2. Semaglutide association efficiency and loading capacity

The association efficiency (AE) and DL of semaglutide were determined by direct method. AE was calculated by the ratio between the amount of peptide loaded to NPs and the total amount of peptide used to produce semaglutide-NPs (**Equation 2**), while DL was calculated by the ratio between the amount of peptide loaded to NPs and the total amount of peptide and NPs used to produce the formulations (**Equation 3**):

$$AE (\%) = \frac{\text{Amount of semaglutide loaded to NPs}}{\text{Total amount of semaglutide}} \times 100 \text{ (Equation 2)}$$

$$DL (\%) = \frac{\text{Amount of semaglutide loaded to NPs}}{\text{Total amount of semaglutide} + \text{Total amount of NPs}} \times 100 \text{ (Equation 3)}$$

The amount of semaglutide was quantified by fluorescence method, as described in **Chapter 3, section 3.4**. Briefly, after washing the NPs, the formulations were stored overnight at -80°C , and then, freeze-dried at -80°C for 12 h. Further, the freeze-dried was resuspended in DMSO for 2 h at 120 rpm. Each sample (200 μL) was transferred to a black 96-well plate, with a black bottom, and measured at excitation and emission wavelengths of 295 nm and 350 nm, respectively, in a BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). A calibration curve of semaglutide ($5\text{ }\mu\text{g.mL}^{-1}$ – $100\text{ }\mu\text{g.mL}^{-1}$) dissolved in the freeze-dried empty NPs resuspended in DMSO was outlined. All samples were evaluated in triplicates, and the fluorescence intensity was applied to determine the AE and DL of semaglutide.

3.5.1.3. Morphological characterization

The morphology of the formulations was analyzed by transmission electron microscopy (TEM) with a JEOL JEM 1400 microscope (JEOL, Ltd, Tokyo, Japan) at an accelerating voltage of 80 kV. The samples were prepared by transferring them (5 μL) onto a 300-mesh nickel grid. The excess of liquid was removed with a filter paper and then, the samples on the grid were stained with uranyl acetate.

3.5.1.4. Stability study

The stability of semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs in phosphate-buffered saline (PBS) at pH 7.4 was evaluated for 2 months at 4°C of storage conditions. The Z-average, Pdl and ζ -potential of each nanoformulation were measured every week for two months, using a Malvern Zetasizer Nano ZS instrument (Malvern Panalytical, Malvern, UK). All the samples were dispersed (1:50) in a 10 mM NaCl solution and three measurements were performed for each formulation.

3.5.2. Secondary structure of semaglutide

The secondary structure of semaglutide was evaluated through circular dichroism (CD), after incubating the peptide with or without spermidine in a basic pH solution (0.1 M NaOH) overnight at 4°C and 120 rpm. Semaglutide dissolved in MilliQ® ultrapure water was used as control. After incubation, the peptide samples ($150\text{ }\mu\text{g.mL}^{-1}$) and the peptide control ($150\text{ }\mu\text{g.mL}^{-1}$) were diluted in MilliQ® ultrapure, and then, evaluated by CD. The experiments were carried out in a JASCO J-815 CD spectrometer (Jasco International Co., Tokyo, Japan), and the spectra of the samples and the controls were obtained in the wavelength range from 200 nm to 260 nm at RT. Measurements were performed in a 0.1 cm quartz cuvette using a scanning speed of 50 nm.min^{-1} , a bandwidth of 1 nm and a data integration time of 2 sec. Each spectrum was obtained as the average of 16 repeated scans. The volume of

sample required was 350 μL . CD spectrum of the MilliQ[®] ultrapure water, the 0.1 M NaOH solution and the spermidine solubilized in 0.1 M NaOH solution, were subtracted from CD spectrum of the corresponding samples.

3.5.3. Semaglutide *in vitro* release study

Semaglutide-NPs (1 mL) were dispersed in a pH 1.2 buffer (3 mL) to mimic the gastric environment (0.2% (w/v) NaCl, 8% (w/v) 1 M HCl and 0.5% (w/v) Tween[®] 80) and incubated at 37 °C under magnetic stirring at 120 rpm for 120 min. Then, the suspension (4 mL) was centrifuged at 30,000 g for 30 min at RT to remove the buffer, and the NPs were resuspended in 4 mL of pH 6.8 buffer to simulate the intestinal environment (0.68% (w/v) KH_2PO_4 and 7.7% (v/v) 0.2 N NaOH) and incubated at 37 °C for 360 min under magnetic stirring at 120 rpm. Aliquots (200 μL) were removed at predetermined time-points (15, 30, 45, 60, 90, 120, 150, 180, 240, 360 and 480 min) and the withdrawn volume was replaced with fresh pre-heated buffer to maintain the volume constant. Each aliquot was centrifuged at 21,000 g for 15 min at RT and the supernatant was collected to calculate the amount of semaglutide released from the NPs over time by fluorescence method, as described in **Chapter 3, section 3.4**.

3.6. Binding-affinity of FcRn-targeted nanoparticles to human FcRn

The pH-dependent binding-affinity of FcRn-targeted NPs (FcBP_2 -NPs and Z_{FcRn} -NPs) to hFcRn was evaluated by SPR analysis, using a BIACORE X100 instrument (Cytiva, Massachusetts, USA). Firstly, hFcRn was coated to the dextran surface of a CM5 sensor chip at pH 5.0 by amine coupling using EDC/NHS at a final density of approximately 400 RU. Further, FcRn-targeted nanosystems (2 $\text{mg}\cdot\text{mL}^{-1}$) were diluted in the running buffer, McIlvaine's phosphate-citrate buffer at pH 6.0 and 7.4, and injected over the hFcRn-CM5 chip at 30 $\mu\text{L}\cdot\text{min}^{-1}$ for 120 sec. Afterwards, the NPs were dissociated from the surface of the chip for 600 sec. Tris-buffer at pH 9.0 was used to remove the remaining particles from the chip. Non-functionalized NPs (B-NPs) were also tested. The sensorgram profile and the RU values obtained for FcBP_2 -NPs and Z_{FcRn} -NPs were compared with B-NPs. Each condition was tested in five replicates.

3.7. Cell culturing maintenance

Caco-2 (human colorectal adenocarcinoma cell line), HT29-MTX (human colorectal adenocarcinoma cell line) and HeLa (human cervical adenocarcinoma cell line) cells were cultured separately in 75 cm^2 T-flasks in DMEM supplemented with FBS (10% (v/v)), NEAA (1% (v/v)) and antibiotic/antimycotic (1% (v/v) mixture (penicillin (100 $\text{U}\cdot\text{mL}^{-1}$) and streptomycin (100 $\text{U}\cdot\text{mL}^{-1}$)), inside an incubator with humidified atmosphere at 37 °C and

5% CO₂. The medium was changed three times per week. When confluence reached about 70% to 80%, cells were detached by treating with trypsin-ethylenediaminetetraacetic acid (EDTA) at 37 °C and seeded in other 75 cm² T-flask.

3.7.1. Metabolic activity assay

Caco-2 and HT29-MTX cells were seeded in 96-well plates at a density of 2 x 10⁴ cells and 1 x 10⁴ cells per well, respectively, and incubated for 24 h in a humidified 5% CO₂ incubator at 37 °C. Then, the medium was collected, and the cells were treated with free semaglutide, semaglutide:spermidine, non-functionalized empty PLGA-NPs produced without spermidine (B-NPs w/ spermidine), non-functionalized empty PLGA-NPs produced with spermidine (B-NPs), FcBP₂-NPs, Z_{FcRn}-NPs, semaglutide-NPs produced without spermidine (semaglutide-NPs w/ spermidine), semaglutide-NPs produced with spermidine (semaglutide-NPs), FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs, in a range of semaglutide concentrations between 0.16 µg.mL⁻¹ and 500 µg.mL⁻¹, which corresponds to a range of NPs concentrations between 0.0048 mg.mL⁻¹ and 15 mg.mL⁻¹. The samples were prepared in serum free-DMEM medium. A negative control, consisting in cells incubated with 1% (v/v) of Triton™ X-100 in serum-free medium, a positive control, consisting in cells incubated with serum-free medium, and the blank, consisting of serum-free medium without cells, were also prepared and treated similarly to the sample wells. The cells were incubated with the samples for 4 h and 24 h at 37 °C inside an incubator with humidified atmosphere and 5% CO₂. After this period, the formulations were discarded and 20% (v/v) of resazurin reagent in serum-free medium was added to each well and incubated at 37 °C for 2 h. The fluorescence levels were measured at an excitation and emission wavelengths of 530 nm and 590 nm, respectively, using the BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). The cellular metabolic activity was calculated based on

Equation 4:

$$\text{Metabolic Activity (\%)} = \frac{\text{Fluorescence signal treated cells} - \text{Fluorescence signal blank}}{\text{Fluorescence signal Positive Control} - \text{Fluorescence signal blank}} \times 100 \text{ (Equation 4)}$$

A similar experiment was conducted for HeLa cells, in which the cells were seeded in 96-well plates at a density of 1 x 10⁴ cells per well, respectively, and incubated for 24 h in a humidified 5% CO₂ incubator at 37 °C. Then, the medium was collected, and HeLa cells were treated with B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs, in a range of NPs concentrations between 4.8 µg.mL⁻¹ and 3000 µg.mL⁻¹, and incubated for 4 h at 37 °C and 5% CO₂. Further, similar steps were performed as described above.

3.7.2. Nanoparticles cellular uptake

To evaluate the cell-NPs interaction, flow cytometry and confocal fluorescence microscopy were conducted as quantitative and qualitative analysis, respectively.

For flow cytometry analysis, Caco-2 cells were plated in 6 well-plates with 5×10^5 cells per well. After 48 h, the cells were treated with 1 mg.mL^{-1} of B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs with 10% (w/v) of PLGA-FKR648 in serum-free medium for 4 h at 37 °C. Afterwards, the cells were centrifuged at 2,000 g for 15 min at 4 °C and re-dispersed in 400 µL of PBS at pH 7.4. A similar experiment was conducted in HeLa cells. The quantification of cell-NPs interaction was done using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, New Jersey, USA). The NPs were detected through allophycocyanin (APC) channel (FL4 detector, 675/700 nm). All measurements were carried out in triplicate in three independent experiments and 15,000 events were acquired in the gated region of the forward-scatter/side scatter plot per sample. Controls based only on cells, and cells incubated with non-fluorescent NPs were also performed to exclude cellular autofluorescence and interference of NPs components, respectively. The results were analyzed using the software FlowJo v10 (BD Biosciences, New Jersey, USA) by gating out cellular debris and doublets and expressed as the mean fluorescence intensity (MFI). A competition assay was also conducted by incubating Caco-2 cells with 100 µg.mL^{-1} of free FcBP₂ and free Z_{FcRn}, 1 h before the incubation with the nanoformulations. Afterwards, a similar experiment was performed as described above.

For confocal fluorescence microscopy analysis, 2×10^5 Caco-2 cells per well were plated on glass coverslips (13 mm of diameter) of a 24-well plate. After 24 h, the cells were treated with 1 mg.mL^{-1} of B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs in serum-free medium and incubated for 4 h. Afterwards, the cells were fixed with 2% (v/v) of paraformaldehyde (PFA) in PBS at pH 7.4 for 10 min at RT and then, PFA was discarded, and each well was washed twice with PBS at pH 7.4. The cells were permeabilized with 0.5% (v/v) of Triton™ X-100 for 10 min at RT, washed twice with PBS at pH 7.4, and the unspecific sites were blocked by incubating the cells with 5% (v/v) of FBS for 30 min at RT. Afterwards, the FBS was discarded and Flash Phalloidin™ Green 488 (1:100) in 5% (v/v) of FBS in PBS at pH 7.4 was added to each well to stain the actin filaments, followed by 40 min of incubation at RT, protected from the light. After 40 min, Flash Phalloidin™ Green 488 was removed, and the cells were washed thrice with PBS at pH 7.4. The glass coverslips were removed from the wells and mount on a microscope slide using DAKO® mounting medium. The interaction between NPs and intestinal cells was observed in a Spectral Confocal Microscope Leica TCS-SP5 AOBs (Leica Microsystems, Wetzlar, Germany) with a 63× water-immersion apochromatic objective. Caco-2 cells without any treatment was also performed to remove autofluorescence. The images were obtained with projections of 40 Z-stacks. The brightness

and the contrast of each image was adjusted using the ImageJ software (Laboratory for Optical and Computational Instrumentation, Wisconsin, USA).

3.8. Generation of human intestinal organoids

The generation of human embryonic stem cells (HUES9)-derived HIOs from HUES9 involves a multistep technique, whereby HUES9 cells were directed to form definitive endoderm, mid/hindgut endoderm and ultimately organoids, as previously described [271]. Briefly, the HUES9 cells were dissociated into single cells with Accutase®. A density of 1×10^5 HUES9 cells was plated on a Nunc 24-well plate and treated with mTeSR1™ medium, containing 10 μ M of Y-27632. The cells were refreshed with mTeSR1™ medium until 80% of confluence was achieved. Afterwards, HUES9 cells were treated with RPMI 1640 medium containing NEAA, 100 I.U.mL⁻¹ penicillin-streptomycin and increasing concentrations of defined FBS (0% (v/v), 0.2% (v/v), and 2% (v/v)) for 3 consecutive days to obtain the definitive endoderm. To induce mid/hindgut endoderm, the definitive endoderm was daily refreshed up to 4 days with 500 μ L mid/hindgut induction medium containing RPMI 1640, 2% (v/v) of FBS, NEAA, 100 I.U.mL⁻¹ of penicillin-streptomycin, 500 ng.mL⁻¹ of fibroblast growth factor 4 (FGF-4) and 3 μ M of CHIR99021. The detached spheroids from days 3 and 4 were collected and embedded in Matrigel®. After Matrigel® polymerization, the organoids were incubated with Human IntestiCult™ Organoid Growth medium. The medium was changed for the first 14 days considering the phenol-red indicator. After 14 days, the organoids were passaged. Briefly, Matrigel® was disrupted by pipetting with ice-cold advanced DMEM/F12 medium and removed from organoids by two times centrifugation at 450 g at 4 °C for 5 min. The retrieved triturated organoids were resuspended in Matrigel® and droplets (50 μ L) were plated in the center of a pre-warmed Nunc 24-well plate and overlaid with intestinal growth medium (1 mL). The medium was changed every 5–6 days and the organoids were maintained in culture at 37 °C and 5% CO₂ for 10–12 days.

3.8.1. Immunofluorescence analysis of human intestinal organoids in sections

The expression of hFcRn, E-cadherin (epithelial cell marker), ZO-1 (tight junction), MUC2 (goblet cells marker) and villin (enterocytes marker) in HIOs was evaluated through immunofluorescence analysis. The organoids were retrieved from Matrigel® using ice-cold Corning® Cell Recovery solution, followed by fixation for 1 h at RT in 4% (v/v) of PFA. Further, organoids were embedded in Eprelia™ Histogel™ HG-4000-012 and were processed for paraffin embedding. After dewaxing and rehydrating, the 4 μ m sections were blocked with 1% (w/v) of bovine serum albumin (BSA) and incubated with the primary antibodies (hFcRn mouse antibody (25 μ g.mL⁻¹), anti-h/mE E-cadherin goat IgG (10 μ g.mL⁻¹), mouse MUC2 antibody (10 μ g.mL⁻¹), mouse ZO-1 antibody (5 μ g.mL⁻¹) and mouse villin

antibody ($4 \mu\text{g.mL}^{-1}$) diluted in 1% (w/v) of BSA, overnight at 4 °C. The secondary antibodies (Alexa Fluor™ 488 donkey anti-goat IgG (H+L) for E-cadherin detection and Alexa Fluor™ 647 donkey anti-mouse IgG (H+L) for hFcRn, MUC2, ZO-1 and villin detection) were diluted (1:750) in 1% (w/v) of BSA solution containing DAPI (1:750) and added to the sections for 1 h at RT. Lastly, sections were mounted using DAKO® mounting medium and analyzed using a Spectral Confocal Microscope Leica SP8 (Leica Microsystems, Wetzlar, Germany) with a 40× water-immersion apochromatic objective. The brightness and the contrast of each image was adjusted using the ImageJ software (Laboratory for Optical and Computational Instrumentation, Wisconsin, USA).

3.8.2. Immunofluorescence analysis of human intestinal organoids in suspension

The expression of hFcRn, E-cadherin, MUC2, villin and ZO-1 in HUES9-derived HIOs was evaluated through immunofluorescence analysis. After 10 days of culture, HIOs were retrieved from the Matrigel® using an ice-cold Corning® Cell Recovery solution and then, fixed with 4% (v/v) of PFA in PBS at pH 7.4 for 2 h in an ice bath at 25 rpm. PFA was discarded and HIOs were then, permeabilized with 0.5% (v/v) of Triton™ X-100 and 1% (w/v) of BSA in PBS (1 h, 25 rpm, RT) and blocked with 0.05% (v/v) of Tween® 20 and 1% (w/v) of BSA in PBS (1 h, 25 rpm, RT). The organoids were subsequently incubated with the primary antibodies of hFcRn ($25 \mu\text{g.mL}^{-1}$), E-cadherin ($10 \mu\text{g.mL}^{-1}$), MUC2 ($10 \mu\text{g.mL}^{-1}$), ZO-1 ($5 \mu\text{g.mL}^{-1}$) and villin ($4 \mu\text{g.mL}^{-1}$), previously diluted in blocking solution, overnight at 4 °C. Afterwards, HIOs were rinsed with PBS and incubated with the secondary antibodies (Alexa Fluor™ 488 donkey anti-goat IgG (H+L) for E-cadherin marker and Alexa Fluor™ 647 donkey anti-mouse IgG (H+L) for hFcRn, MUC2, ZO-1 and villin markers), also diluted in blocking solution, for 2 h at RT. The HIOs were then, rinsed with PBS, transferred to a Nunc Lab-Tek 8-well chambered cover glass, and analyzed in ZEISS Celldiscoverer 7 microscope (Oberkochen, Germany). The brightness and the contrast of each image was adjusted using the ImageJ software (Laboratory for Optical and Computational Instrumentation, Wisconsin, USA).

3.8.3. Western blot analysis of human FcRn expression in human intestinal organoids

The expression of hFcRn in HIOs was evaluated by western blot analysis. HeLa and Caco-2 cell lines were used as negative and positive controls, respectively. To solubilize the Matrigel®, HIOs with passage number 6–9 were incubated with ice-cold Corning® Cell Recovery Solution for 1 h in an ice bath at 25 rpm and then, centrifuged at 3,000 rpm and 4 °C for 30 sec. The cell *pellet* was lysed in radioimmunoprecipitation assay (RIPPA) lysis buffer supplemented with protease inhibitor cocktail for 30 min in an ice bath. Protein

concentrations of the lysates were determined using the bicinchoninic acid (BCA) method. The total proteins (60 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-10% gel under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with Odyssey Fc solution diluted in PBS at pH 7.4 containing 0.1% (v/v) of Tween® 20, the membrane was incubated for 48 h at 4 °C with the primary antibody (1:100 in blocking solution). Afterwards, the bound antibody was detected with goat anti-mouse IRDye® 680 RD (1:5000 in blocking solution, 1 h) by using the Odyssey Fc equipment (LI-COR Biosciences, Nebraska, USA). The band intensity was normalized with a monoclonal mouse anti-β-tubulin.

3.8.4. Microinjection of FcRn-targeted nanoparticles into the lumen of human intestinal organoids

The formulations of B-NPs, FcBP₂-NPs and Z_{FcRn}-NPs diluted in the intestinal growth medium were injected into the lumen of the 10 days intestinal organoids, using the FemtoJet® 4i microinjector equipment (Eppendorf, Hamburg, Germany). Glass capillaries with a tip diameter of at least 20 µm were used for NPs injection. The parameters applied for NPs microinjection were 250 hPa of injection pressure, 0.1 sec of injection time, 10 hPa of compensation pressure and 20 µL of injection volume. HIOs with a diameter equal or higher than 100 µm were considered for the experiments. After injection, HIOs were incubated for 4 h at 37 °C and 5% CO₂.

To ensure the NPs are being injected into the HIOs lumen, organoids were incubated with 4 µM of Calcein-AM for 3 h at 37 °C and 5% CO₂ in a Nunc 24-well plate. Then, fluorescent B-NPs were injected into the lumen, using the microinjection parameters mentioned above. The detection of B-NPs inside the organoids was visualized through the ZEISS CellDiscoverer 7 microscope (ZEISS Group, Oberkochen, Germany). Then, metabolic activity assays and *in vitro* uptake studies were performed.

3.8.4.1. Metabolic activity assays

3.8.4.1.1. AlamarBlue® assay

The formulations B-NPs, FcBP₂-NPs and Z_{FcRn}-NPs diluted in intestinal growth medium were injected to the lumen of HIOs, as described in **Chapter 3, section 3.8.4.**, and incubated for 4 h at 37 °C and 5% CO₂. Three concentrations of NPs (3, 8 and 15 mg.mL⁻¹) were tested. A negative control, corresponding to organoids treated with 1% (v/v) of Triton™ X-100, a positive control consisting of HIOs without treatment and the blank composed of Matrigel® without HIOs were also prepared. Additionally, on the lumen of HIOs was only injected intestinal growth medium to evaluate if the microinjection procedure can

interfere with the metabolic activity of the organoids. After 4 h, the wells were washed twice with PBS at pH 7.4 followed by addition of AlamarBlue® reagent (20% (v/v)) in intestinal growth medium and incubation at 37 °C for 6 h. The fluorescence levels were measured at an excitation and emission wavelengths of 530 nm and 590 nm, respectively, using the BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). The cellular metabolic activity was calculated based on **Equation 5**:

$$\text{Metabolic activity (\%)} = \frac{\text{Fluorescence signal treated HIOs} - \text{Fluorescence signal blank}}{\text{Fluorescence signal positive control} - \text{Fluorescence signal blank}} \times 100 \text{ (Equation 5)}$$

3.8.4.1.2. Live-dead assay

The nanoformulations B-NPs, FcBP₂-NPs and Z_{FcRn}-NPs were diluted in intestinal growth medium and then, injected to the lumen of HIOs at three different NPs concentrations (3, 8 and 15 mg.mL⁻¹). Negative control and positive control corresponded the same controls used in the AlamarBlue® assay. Nonetheless, HIOs were only injected with intestinal growth medium to assess the impact of the microinjection procedure on the metabolic activity of the organoids. The microinjected organoids were incubated for 4 h at 37 °C, inside an incubator with humidified atmosphere and 5% CO₂. After rinsing the wells with PBS, HIOs were retrieved from the Matrigel®. The organoids were incubated for 30 min with 4 µM of calcein-AM, representing the live cells, and then, 15 min with 6 µM of PI, representing the dead cells. To qualitatively determined the proportion of live-dead cells, HIOs were transferred to a Nunc Lab-Tek 8-well chambered cover glass and imaged with ZEISS Celldiscoverer 7 microscope (ZEISS Group, Oberkochen, Germany). The brightness and the contrast of each image was adjusted using the ImageJ software (Laboratory for Optical and Computational Instrumentation, Wisconsin, USA).

3.8.4.2. Nanoparticles cellular uptake

The interaction of HIOs and NPs was quantitatively and qualitatively evaluated through flow cytometry and confocal microscopy analysis, respectively.

For flow cytometry analysis, 4 h of *post*-microinjection of 15 mg.mL⁻¹ of B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs, the intestinal growth medium was discarded and the Matrigel® was dissolved with ice-cold Advanced DMEM/F12 medium, containing 10 µM of Y-27632. Retrieved organoids were converted into single cells by incubating with TrpLE™ containing 10 µM of Y-27632 in a 37 °C water bath, followed by mechanical dissociation. Single cells were resuspended in cold advanced DMEM/F12 medium and centrifuged for 5 min at 550 *g* and 4 °C. Afterwards, the samples were washed once with cold advanced DMEM/F12 and re-dispersed in 400 µL of this medium, containing 10 µM of Y-27632. The interaction between

HIOs cells and the formulations was quantitatively determined using a QuantumTM Flow Cytometer (Quantum Analysis GmbH, Münster, Germany), where NPs were detected through the APC channel (675/700 nm). All measurements were carried out in triplicate and 20,000 events were acquired in the gated region of the forward-scatter/side scatter plot per sample. Control based only on HIOs without treatment and HIOs treated with non-fluorescent NPs were also performed to exclude cellular autofluorescence and interference of NPs components, respectively. The data were analyzed using the software FlowJo v10 (BD Biosciences, New Jersey, USA) by gating out cellular debris and doublets and expressed as MFI. A competition assay was also conducted by injecting 500 µg.mL⁻¹ of FcBP₂ and Z_{FcRn} into the intestinal lumen of the organoids, 1 h before NPs injection. Afterwards, a similar experiment was performed as described above.

For confocal analysis, after 4 h of the microinjection of 15 mg.mL⁻¹ of B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs to the organoid's lumen, the intestinal growth medium was discarded, the Matrigel[®] was retrieved with ice-cold advanced DMEM/F12 medium and the organoids were fixed with 4% (v/v) of PFA in PBS at pH 7.4 for 2 h in an ice bath at 25 rpm. PFA was discarded and HIOs were permeabilized with 0.5% (v/v) of TritonTM X-100 and 1% (w/v) of BSA in PBS (1 h, 25 rpm, RT) and blocked with 0.05% (w/v) of Tween[®] 20 and 1% (w/v) of BSA in PBS (1 h, 25 rpm, RT). HIOs were subsequently incubated with the anti-h/mE E-cadherin goat IgG (10 µg.mL⁻¹), previously diluted in blocking solution, overnight at 4 °C to stain the epithelial cells of the organoids. Then, HIOs were rinsed with PBS and incubated with the secondary antibody, Alexa FluorTM 488 donkey anti-goat IgG (H + L) (1:750), also diluted in blocking solution, for 2 h at RT. The HIOs were then, rinsed with PBS, transferred to a Nunc Lab-Tek 8-well chambered cover glass, and analyzed in ZEISS Celldiscoverer 7 microscope (ZEISS Group, Oberkochen, Germany). The images were obtained with projections of 80 Z-stacks. The brightness and the contrast of each image was adjusted using the ImageJ software (Laboratory for Optical and Computational Instrumentation, Wisconsin, USA).

3.9. *In vivo* experiment

3.9.1. Induction of type 2 diabetes in human FcRn transgenic mice model

Animal experiments were conducted under the guidelines and recommendations of FELASA and the European Directive 2010/63/EU, after approval by the Animal Welfare at i3S, *Universidade do Porto* (reference BSm_2017_10). Male homozygous hFcRn 32 transgenic mice (The Jackson Laboratory, B6.Cg-Fcgrt^{tm1Dcr}Tg(FCGRT)32Dcr/DcrJ), age 8–12 weeks and weighing between 20 g and 28 g, were used to evaluate the effect of the FcRn-targeted semaglutide-NPs on glycemia. The mice were maintained under standard

laboratory conditions (12 h light/dark cycles, temperature of 21 ± 2 °C and relative humidity of 35% to 60%) and fed with standard *pellet* food and water *ad libitum*.

The induction of T2DM in hFcRn transgenic mice was optimized. Two different approaches were tested to select the one that suitably induced the disease in the animals. In the first approach (**Approach A**), an intraperitoneal (*i.p.*) injection of 210 mg.kg^{-1} NA followed by an *i.p.* injection of 180 mg.kg^{-1} STZ, was administered to mice, as previously performed by Novelli *et al.* [231]. In the second approach (**Approach B**), mice received the same doses of NA and STZ by *i.p.* injection as mentioned in **Approach A**, but a second dose was administered 2 days after the first administration. In both studies, the animals were fasted for 4 h before the administration of the drugs. NA (40 mg.mL^{-1}) was previously dissolved in 0.9% (w/v) of NaCl at pH 7.4 and STZ (20 mg.mL^{-1}) was dissolved in 50 mM sodium citrate buffer at pH 4.5. In both approaches, the levels of glucose were measured for 16 days (days 7, 9, 12, 14 and 16), regarding the first administration of NA and STZ, and the animals were weighed every two days. The approach that induced T2DM, where the blood glucose levels of the animals were equal or higher than 200 mg.dL^{-1} [232, 272], was selected for further experiments.

3.9.2. Biodistribution study in type 2 diabetic-induce human FcRn transgenic mice

Diabetic mice were fasted for 4 h with free access to water and afterwards, fluorescent (Cy7.5-loaded) B-NPs, FcBP₂-NPs and Z_{FCRn}-NPs were orally administered to the animals in a NPs dose of 90 mg.kg^{-1} , which corresponds to a semaglutide dose of 3 mg.kg^{-1} . All the particles were resuspended in PBS at pH 7.4 prior to the administration. Each group was composed of 4 animals. Whole body near infrared (NIR) imaging of animals treated with Cy7.5-loaded NPs was performed at specific time-points (0.5, 2, 4 and 6 h) using IVIS Lumina LT system (Perking Elmer, Waltham, MA, USA). Before imaging, the abdominal hair of the mice was removed with a hair clipper and a brief anesthetization of the mice with inhalational isoflurane was performed. Background signal from non-treated animals was used to establish relevant radiance signals. Animals were sacrificed at 0.5, 2, 4 and 6 h by cardiac puncture followed by cervical dislocation. GIT was collected and placed in a black dish and analyzed using the IVIS system. Background signal of the GIT of non-treated animals was used to establish the relevant radiance signal. Quantification of the radiant efficiency from the same sized regions including complete excised tissues was performed using Living Image® software (PerkinElmer, Massachusetts, USA). Afterwards, the GIT was divided into 5 segments: stomach, duodenum (roughly 3 cm), jejunum, ileum (roughly 3 cm), cecum and colorectum. The segments were individually frozen at -80 °C in O.C.T. and processed for fluorescence imaging to evaluate the internalization of the particles. Briefly, $10 \mu\text{m}$ cryosections were stained with DAPI and Phalloidin™ 546 and mounted using

Fluoromount® aqueous mounting medium. Images were obtained with a Confocal Microscope Leica TCS-SP5 AOBS (Leica Microsystems, Wetzlar, Germany) with a 40× water-immersion apochromatic objective. Regarding the quantitative analysis, the segments after being collected were stored at –80 °C until further processing. After thawing, weighing, and washing the fluids, the tissues were mixed with DMSO, where Cy7.5-loaded NPs were soluble, homogenized by using an Ultra-turrax processor (IKA®-Werke, Staufen, Germany), and incubated at 180 rpm for 1 h at RT. The homogenates were then centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatants were collected and transferred to a black 96-well plate, with a black bottom, and measured at excitation and emission wavelengths of 745 nm and 815 nm, respectively, in a BioTek Synergy Mx microplate reader (BioTek Instruments Inc., Vermont, USA). A calibration curve of Cy7.5-loaded NPs (0.002 µg.mL⁻¹–0.5 µg.mL⁻¹) dissolved in DMSO was outlined.

3.9.3. Pharmacodynamic studies in type 2 diabetic induced human FcRn transgenic mice

3.9.3.1. Single administration of nanoparticles targeting human FcRn

T2DM was induced to hFcRn transgenic mice following **Approach B** described in **Chapter 3, section 3.9.1**. After 9 days of the first administration of NA and STZ, the diabetic mice were randomly divided into 7 groups, with 8 animals per group: Group 1 – normoglycemic mice treated with PBS at pH 7.4, Group 2 – diabetic mice treated with PBS at pH 7.4, Group 3 – semaglutide dissolved in PBS at pH 7.4, Group 4 – semaglutide-NPs, Group 5 – FcBP₂-semaglutide-NPs, and Group 6 – Z_{FcRn}-semaglutide-NPs, all formulations resuspended in PBS at pH 7.4 and administered by oral gavage in a peptide dose of 3 mg.kg⁻¹; and lastly, Group 7 – semaglutide dissolved in PBS and administered via s.c. route in a peptide dose of 60 µg.kg⁻¹. Only animals with blood glucose levels equal or higher than 200 mg.dL⁻¹ were treated with the samples. The s.c. dose of semaglutide was selected based on the previous studies [273, 274], while the oral dose of semaglutide was selected, considering the dose of the peptide administered to the humans (233 µg.kg⁻¹), and converting to the mice dose following **Equation 6** [275]:

$$\text{Mouse dose (mg.kg}^{-1}\text{)} = \text{Human dose (mg.kg}^{-1}\text{)} \times 12.3 \text{ (Equation 6)}$$

Blood glucose levels were monitored for 8 h (0, 0.5, 1, 2, 4, 6 and 8 h), after withdrawing blood from the tail vein of the animals, using a glucometer. To evaluate the glucoregulatory effect of the formulations, the blood glucose levels were plotted against time. The area under the curve (AUC) over 8 h was calculated for each group, using the trapezoidal method. Blood glucose decrease (BGD) (%) parameter was calculated based on **Equation 7**:

$$BGD (\%) = \frac{AUC (Control\ 0-8\ h) - AUC (Group\ 0-8\ h)}{AUC (Control\ 0-8\ h)} \times 100 \text{ (Equation 7)}$$

where, AUC (Control 0 – 8 h) is the AUC of blood glucose levels of Group 2, and AUC (Group 0 – 8 h) corresponds to the AUC of blood glucose levels of the Groups 1, 3, 4, 5, 6 and 7. At the end of the study, the animals were sacrificed by cardiac puncture followed by cervical dislocation by trained personnel certified by Portuguese *Direção-Geral de Alimentação e Veterinária* (DGAV).

3.9.3.2. Multiple administrations of nanoparticles targeting human FcRn

T2DM was induced to hFcRn transgenic mice following **Approach B** described in **Chapter 3, section 3.9.1**. After 9 days of the first administration of NA and STZ, the diabetic mice were randomly divided into 7 groups with 8 animals per condition, which corresponds to the same groups tested in **Chapter 3, section 3.9.3.1**. Only animals with blood glucose levels equal or higher than 200 mg.dL⁻¹ were considered for the study. Each group was orally or subcutaneously treated with the samples once a day for 7 days and on day 8, the animals were sacrificed by cardiac puncture followed by cervical dislocation by trained personnel certified by DGAV. A blood drop was collected from the tail vein on days 0, 4 and 8 to measure the blood glucose levels using a glucometer. The blood glucose levels were plotted against time to evaluate the glucose-lowering effect of each formulation. The AUC over 8 days was calculated for each group, using the trapezoidal method.

3.9.3.3. Plasmatic insulin levels

The withdrawn blood collected during the cardiac puncture of the mice subjected to the experiments described in **Chapter 3, section 3.9.3.1** and **3.9.3.2** was transferred to EDTA K3 tubes to prevent the blood clotting. The samples were centrifuged at 3,000 g for 10 min at 4 °C to separate the plasma and stored at –80 °C for further analysis. The levels of insulin in plasma were quantified using a sandwich ELISA. Nunc MaxiSorp™ plates were coated with 50 µL of anti-insulin antibody diluted 1:1,000 in PBS, and incubated overnight at 4 °C. Afterwards, each well was washed three times for 1 min/wash at RT, and 4% (w/v) of skimmed milk (100 µL per well) was added to the wells and incubated for 1 h at RT to block nonspecific sites. Further, 50 µL of human insulin standards and plasma samples were added to the wells and incubated for 2 h at RT, under gentle shaking, after each well being washed once (1 min/wash) at RT. The detection antibody anti-insulin HRP diluted 1:2,000 in PBS at pH 7.4 was added to each well at the volume of 50 µL/well, and incubated for 1 h at RT, under gentle shaking. Afterwards, the wells were washed five times (1 min/wash, RT) and TMB was used as a substrate for HRP (50 µL/well). After 5 min of incubation with TMB, the reaction was stopped with 1 M H₂SO₄ (50 µL/well), and the absorbance of each

well was measured at the wavelength of 450 nm in a BioTek Synergy Mx microplate reader (BioTex Instruments Inc., Vermont, USA). The wash buffer used along the experiment was 0.05% (v/v) Tween 20 in PBS at pH 7.4.

3.9.3.4. Pancreatic content evaluation

After euthanizing the animals, the pancreas was extracted and isolated by removing the fat and the connective tissue and then weighed. The pancreas was placed into 1 mL of ice-cold acid ethanol (0.18 M HCl in 70% (v/v) ethanol) and kept on ice. The tissue was homogenized with an Ultra-turrax processor (IKA®-Werke, Staufen, Germany), and then, 0.5 mL of acid ethanol were added into the tube of the tissues and stored for 24 h at 4 °C, under magnetic stirring (180 rpm). Afterwards, the samples were centrifuged at 3,500 rpm for 15 min at 4 °C and the supernatant was transferred to another tube to be stored at –20 °C. These steps were repeated once and the supernatants from the different centrifugations were mixed in the same tube. The amount of insulin in the pancreas was quantified using the Bio-Plex Pro™ Mouse Diabetes assay kit. Samples were left at RT, mixed by vortexing, and diluted 50 times, before quantification.

3.9.3.5. Plasma levels of cytokines

The withdrawn blood collected during the cardiac puncture was transferred to EDTA K3 tubes to prevent the blood clotting. The samples were centrifuged at 3,000g for 10 min at 4 °C to separate the plasma and stored at –80 °C for further analysis. The levels of the following cytokines, interleukin (IL)-1 β , IL-6, IL-18, and tumor necrosis factor-alpha (TNF- α), were quantified using the ProcartaPlex™ Mouse Interleukins assay kit.

3.9.3.6. Hematoxylin and eosin staining

Pancreas, heart, lung, liver, kidneys, spleen, stomach, small intestine, and large intestine were collected, after euthanizing the animals. The tissues were fixed in 10% (v/v) buffered formalin for at least 24 h and then, routinely processed in an automatic tissue processor for paraffin embedding. Serial 4 μ m thick sections were cut for hematoxylin and eosin (H&E) staining. Slides were observed through a light microscopy (Leica DM2000 LED, Wetzlar, Germany).

3.9.3.7. Immunohistochemistry

Double chromogenic immunohistochemical assays were performed with mouse monoclonal anti-insulin antibody (1:10000 dilution) and rabbit polyclonal anti-glucagon antibody (1:25 dilution) in 4 μ m tissue sections. All immunohistochemistry protocol, dewaxing included, was performed automatically in a Ventana Discovery Ultra Platform

(Roche Diagnostics, Tucson, USA) using CC2 as epitope recovery (ER). The downstream immunohistochemical protocol was performed as follows: tissue sections containing two sequential sections of mouse pancreatic tissue were blocked using UltraVision Protein Block. Tissues were simultaneously incubated for 1 h at 37 °C with a mixture of the antibodies at the abovementioned dilutions. Tissues were previously blocked for endogenous peroxidase activity for 12 min. Detection was performed with Poliview® Plus AP (anti-mouse) and Poliview® Plus HRP (anti-rabbit) for insulin and glucagon, over a 32 min and 1 h of incubation, respectively. Reactions were detected with HIGHDEF® red IHQ chromogen (AP, plus) which converts the product of the reaction in red and with 3,3'-Diaminobenzidine (DAB) in a brown product, for insulin and glucagon tissues, respectively. Tissues were then counterstained with HIGHDEF® hematoxylin, dehydrated and coverslipped using a permanent mounting solution (Entellan®). Positive and negative controls were included in every set of reactions for each antibody used. Slides were observed through light microscopy (Leica DM2000 LED) and all slides were scanned using Phenolmager HT (formerly known as Vectra Polaris) microscopes (Akoya Biosciences, Marlborough, MA, USA). QuPath software was used to analysis the samples digitalized in Phenolmager HT microscopes to evaluate the area occupied of β and α cells in the tissue sections and the ratio between β and α cells.

3.10. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 8 Software (GraphPad Software Inc., San Diego, California). The statistical differences between more than two groups were compared using the one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. The results were reported as mean $\pm$ standard deviation (SD) and geometric mean with 95% confidence intervals. The levels of significance were considered for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Chapter | 4

Results and Discussion

This chapter was written based in the following publications:

- **Soraia Pinto**, Juliana Viegas, Cecília Cristelo, Catarina Pacheco, Sofia Barros, Stephen T. Buckley, Javad Garousi, Torbjörn Gräslund, Hélder A. Santos, Bruno Sarmiento, Multifunctional nanomedicines targeting intestinal Fc receptor achieve improved glucoregulatory effect of semaglutide in a type 2 diabetic mice model, under review.

In this scientific paper, I was responsible for the writing (review, editing and original draft), methodology, conceptualization, and bibliographic research. The supervisors were responsible for reviewing, editing, conceptualization, funding acquisition, and submitting the manuscript. These data were not previously included in another PhD thesis, and it is fully reproduced in this chapter.

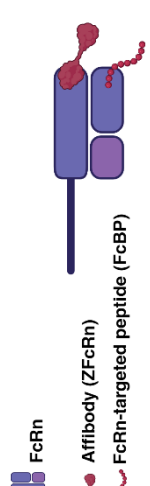
- **Soraia Pinto**, Mahya Hosseini, Stephen T. Buckley, Wen Yin, Javad Garousi, Torbjörn Gräslund, Sven van Ijzendoorn, Hélder A. Santos, Bruno Sarmiento, Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide, *Journal of Controlled Release* 2024, 366:621-636. doi: 10.1016/j.jconrel.2024.01.015

In this scientific paper, I was responsible for the writing (review, editing and original draft), methodology, conceptualization, and bibliographic research. The supervisors were responsible for reviewing, editing, conceptualization, funding acquisition, and submitting the manuscript. These data were not previously included in another PhD thesis, and it is fully reproduced in this chapter. The first page and the graphical abstract of the review paper, and the permission from the journal to reuse the manuscript in this thesis were included in **Appendix II**. I was also responsible for designing an inside back cover page for the edition of the *Journal of Controlled Release*, where this research paper was published, being cited in **Appendix III**.

4.1. Selection of FcRn-targeted ligands with affinity for human FcRn

FcRn-binding peptides, FcBPs (FcBP₁, FcBP₂, FcBP₃ and FcBP₄), and affibody Z_{FcRn}, were selected from the literature based on their pH-dependent binding-affinity to hFcRn [29-32, 276]. The affibody Z_{FcRn} was expressed in *Escherichia coli* and purified by immobilized metal-ion affinity chromatography. The peptides FcBP₁, FcBP₂, and FcBP₃ were supplied with a Cys residue in the N-terminal of their framework, while in Z_{FcRn}, the Cys residue was included in the C-terminal during its acquisition, in order to be covalently linked to the Mal ring of PLGA-PEG-Mal co-polymer. The binding-affinity of FcBPs and Z_{FcRn} to the receptor was evaluated by SPR analysis at pH 6.0 and 7.4. The K_D values were determined by applying the steady-state affinity model (**Table 4** and **Appendix V**). The results showed that the peptides FcBP₁, FcBP₃ and FcBP₄, did not interacted with the receptor at acidic and physiological pH. Notwithstanding, the FcRn-targeted ligands, FcBP₂ and Z_{FcRn}, demonstrated micromolar ($K_D = 16000 \pm 3200$ nM) and nanomolar ($K_D = 72 \pm 32$ nM) affinities for hFcRn at acidic pH, respectively. The interaction between those ligands and the receptor occurred in a pH-dependent manner since the peptide and the affibody bound to hFcRn at pH 6.0 and no binding was observed at pH 7.4 (**Table 4** and **Appendix V**), which is in accordance with the data reported by Sockolosky *et al.*[29] and Seijnsing *et al.* [31]. The strict pH-dependent mode of action is likely mediated by protonation of the imidazole group of His residues found on the receptor and the ligands frameworks. At physiological pH, the protonation of the His residues is lost, and the ligands are released from the receptor [29, 31]. Importantly, the K_D value of Z_{FcRn} was approximately 200-fold lower than FcBP₂ at pH 6.0, which means the binding between Z_{FcRn} and hFcRn is stronger than the binding between FcBP₂ and hFcRn ($p < 0.001$). A positive molecule control, corresponding to HSA, an endogenous ligand of hFcRn [17], was also tested. As expected, HSA bound to hFcRn at pH 6.0 ($K_D = 7300 \pm 1300$ nM), while no binding was visualized at pH 7.4 (**Table 4** and **Appendix V**), validating the methodology. The K_D value of HSA was approximately 2-fold lower and 100-fold higher than the K_D values of FcBP₂ and Z_{FcRn}, respectively. These results suggest the affibody and the peptide had, respectively, significantly higher ($p < 0.05$) and lower ($p < 0.01$) interactions to hFcRn at pH 6.0 than HSA, which was already reported in the literature [29, 31]. Hereupon, both FcRn-targeted ligands, FcBP₂ and Z_{FcRn}, were covalently linked to PLGA-PEG-Mal co-polymer.

Table 4. Binding-affinity of FcRn-targeted ligands to hFcRn at pH 6.0 and 7.4.



FcRn-targeted ligand	Code	K_D (pH 6.0) [nM]	K_D (pH 7.4) [nM]
HSA	HSA	$7300 \pm 1300^{*,**}$	No binding
C QRFVTGHFGGL H PANG	FcBP ₁	No binding	No binding
C QRFVTGHFGGL Y PANG	FcBP ₂	$16000 \pm 3200^{*,***}$	No binding
C QRF C TGHFGGL Y P C NG	FcBP ₃	No binding	No binding
RF- Pen -TGHFG- Sar -NMeLeu- YPC	FcBP ₄	No binding	No binding
VDKYAKEWMRAA H EIRWLPNLTF			
DQRVAFIHKLEDDPSQSSELLSEAK	Z _{FcRn}	$72 \pm 32^{*,***}$	No binding
KLNDSQAPK C			

K_D obtained by applying the steady-state affinity model.

One-way analysis of variance (ANOVA) Tukey's multiple comparison test was used for the statistical analysis of the K_D values. *, ** and *** denote significant differences of $p < 0.05$, $p < 0.01$, $p < 0.001$ between Z_{FcRn} and HSA; FcBP₂ and HSA; FcBP₂ and Z_{FcRn}, respectively. The results are represented as mean $\pm$ SD ($n = 3$).

4.2. FcRn-targeted ligands covalently linked to the PLGA-PEG-Mal co-polymer

The FcRn-targeted ligands, FcBP₂ and Z_{FcRn}, were conjugated with PLGA-PEG-Mal through Mal-thiol chemistry, where the free SH group of the Cys residue located in the N-terminal and C-terminal of FcBP₂ and Z_{FcRn}, respectively, was covalently linked to the Mal ring of PLGA-PEG-Mal co-polymer. PLGA-PEG-Mal combined with FcBP₂, without Cys in the N-terminal of the peptide, and PLGA-PEG without the Mal ring combined with the Z_{FcRn}, were also tested as *mock* reactions. The CE values of FcBP₂ with PLGA-PEG-Mal and Z_{FcRn} with PLGA-PEG-Mal were around 83 ± 4 % and 33 ± 7 %, respectively. Regarding the results of the peptide *mock* reaction, the efficiency was approximately 29 ± 13 % for FcBP₂, suggesting that a portion of the peptide also bound nonspecifically to the polymer. However, in the affibody *mock* reaction, no interaction was detected between the polymer and the affibody. Additionally, the successful conjugation of FcBP₂ and Z_{FcRn} with the PLGA-PEG-Mal was confirmed by XPS analysis. In **Table 5** is represented the relative atomic percentages of carbon, oxygen, nitrogen, and sulfur of non-functionalized and functionalized PLGA-PEG-Mal. In non-functionalized PLGA-PEG-Mal co-polymer, the elemental composition percentages of carbon, oxygen and nitrogen were 70.8%, 29.1% and 0.1%, respectively. Regarding the PLGA-PEG-Mal:FcBP₂, the elemental composition percentages of carbon, oxygen and nitrogen were 67.5%, 31.5% and 0.9%, respectively; and those elements in PLGA-PEG-Mal:Z_{FcRn} were 66.4%, 33.0% and 0.6%, respectively. As expected, the PLGA-PEG-Mal:FcBP₂ and PLGA-PEG-Mal:Z_{FcRn} had an increase in the

atomic percentage of oxygen and nitrogen, comparatively with the non-functionalized polymer. Sulfur was also detected in both conjugated polymers. Hence, the results obtained through XPS analysis combined with the CE values of those functionalized polymers, suggest the successful covalent conjugation of FcBP₂ and Z_{FcRn} with PLGA-PEG-Mal.

Table 5. Relative atomic percentages of carbon, oxygen, nitrogen, and sulfur in non-functionalized PLGA-PEG-Mal and functionalized PLGA-PEG-Mal (PLGA-PEG-Mal:FcBP₂ and PLGA-PEG-Mal:Z_{FcRn}) determined by XPS analysis.

Polymer	Atomic composition [%]			
	C1s	O1s	N1s	S2p
PLGA-PEG-Mal	70.8	29.1	0.1	n.a.
PLGA-PEG-Mal:FcBP ₂	67.5	31.5	0.9	0.09
PLGA-PEG-Mal:Z _{FcRn}	66.4	33.0	0.6	0.04

n.a. – non-applicable

4.3. Solubility of semaglutide in organic and aqueous solvents

The solubility of semaglutide in organic solvents (DMSO) and aqueous solutions (MilliQ[®] ultrapure water, intestinal buffer at pH 6.8, and gastric buffer at pH 1.2) was tested (**Table 6**). The data showed that semaglutide was solubilized in DMSO (organic solvent used to resuspend the freeze-dried semaglutide-NPs to determine the AE and DL of the peptide, as described in **Chapter 3, section 3.5.1.2.**) in a concentration of 14 mg.mL⁻¹. Additionally, the solubility of semaglutide in MilliQ[®] ultrapure water (aqueous phase used to produce the semaglutide-NPs described in **Chapter 3, section 3.5.**) was at least 100 mg.mL⁻¹. Moreover, the gastric and intestinal buffers applied to evaluate the GI release of semaglutide from the PLGA NPs (**Chapter 3, section 3.5.3.**) were also tested. In the intestinal buffer, semaglutide had a solubility of at least 32 mg.mL⁻¹, while in the gastric buffer, the antidiabetic peptide was not solubilized, which can be attributed by the low solubility of the peptide in solutions at pH below 6 [277]. Tween[®] 80 (0.5% (w/v) or 1% (w/v)) was added to the previous gastric buffer to stimulate the dissolution of semaglutide in the buffer. In **Table 6** is showed that the addition of Tween[®] 80 to the gastric buffer was able to solubilize semaglutide (gastric buffer containing 0.5% (w/v) or 1% (w/v) of Tween[®] 80 had a solubility of 5 mg.mL⁻¹ and 8 mg.mL⁻¹, respectively). According to this, the *in vitro* release assay was conducted using the gastric buffer, containing 0.5% (w/v) of Tween[®] 80.

Table 6. Solubility of semaglutide in organic and aqueous solvents.

Solvent	Solubility (mg.mL ⁻¹)
DMSO	14
MilliQ® ultrapure water	≥ 100
Intestinal buffer at pH 6.8	≥ 32
Gastric buffer at pH 1.2	Not soluble
Gastric buffer (0.5% (w/v) Tween® 80) at pH 1.2	5
Gastric buffer (1% (w/v) Tween® 80) at pH 1.2	8

4.4. Physicochemical properties of FcRn-targeted semaglutide-loaded nanoparticles

Functionalized semaglutide-NPs (FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs) were produced by w/o/w double emulsion technique, using PLGA-PEG-Mal conjugated with the FcRn-targeted ligands, FcBP₂ (PLGA-PEG-Mal:FcBP₂) or Z_{FcRn} (PLGA-PEG-Mal:Z_{FcRn}). FcRn is a heterodimeric glycoprotein receptor widely expressed in several cells, including the intestinal polarized epithelial cells, allowing the intestinal transcytosis of serum molecules, such as albumin and IgG, through the binding to the receptor [17]. It was previously demonstrated the potential of hFcRn in mediating the intestinal transcytosis of surface-decorated nanosystems with the Fc fragment of IgG [21, 22] or albumin [24, 25]. Based on these data, it was hypothesized that the PLGA-NPs can also be functionalized with other ligands that target the hFcRn and therefore, favor intestinal transcytosis of semaglutide-loaded nanosystems.

Following production of the functionalized semaglutide-NPs, they were concentrated by centrifugation and subsequently, physicochemically characterized based on their Z-average, Pdl, ζ-potential (**Figure 9A**), AE and DL (**Figure 9B**). The formulations had a Z-average lower than 200 nm, which is ideal for oral drug delivery. Previous studies demonstrated that NPs with lower size (≤ 200 nm) were more efficiently internalized by intestinal epithelial cells and had a higher intestinal permeability than larger sized NPs (> 200 nm) [96, 278]. Regarding the Pdl value, it was below 0.100 for all the produced formulations, demonstrating the homogenous size distribution of the nanosystems, being considered a monodisperse dispersion [94]. Furthermore, NPs presented a neutral surface charge, which is likely attributed to the presence of PEG on the surface of the particles. PEG has mucodiffusive properties, due to its hydrophilic nature and neutral charge that reduce the interaction with mucins, promoting the unhindered transport of PEGylated NPs throughout the intestinal mucus [11, 14, 15]. No differences in the Z-average, Pdl and ζ-

potential were observed between B-NPs and semaglutide-NPs as well as between B-NPs and FcRn-targeted NPs. Notwithstanding, TEM technique was performed to ascertain the morphology of the formulations, revealing a spherical shape for the nanoformulations produced (**Figure 9C**). The shape of the particles has influence in the interaction with the cells, and NPs with a spherical shape presented an easier and faster cell internalization when compared to irregular shaped NPs [279].

The AE and DL of semaglutide were directly determined through fluorescence (**Figure 9B**). Firstly, semaglutide-NPs were produced using as internal aqueous phase, semaglutide solubilized in MilliQ® ultrapure water, corresponding a peptide concentration of 10 mg.mL⁻¹, and the values of AE and DL were approximately 48 ± 1 % and 2.30 ± 0.03 %, respectively. Further, the effect of spermidine in increasing the amount of semaglutide incorporated into PLGA-NPs was studied. Spermidine is an endogenous polyamine present in umpteen organisms, being involved in biological functions that are important for the growth and survival of the cells [280, 281]. In the literature, it was already demonstrated the impact of polyamines in improving the AE of drugs in nanotechnology field [282-284]. Thus, it was hypothesized that the amount of semaglutide loaded into PLGA-NPs could be improved by forming a HIP between semaglutide and spermidine [125, 128]. The formation of HIP was conducted at basic pH, in which semaglutide and spermidine were negatively and positively charged (4 negative charges for semaglutide and 3 positive charges for spermidine), respectively, favoring the non-covalent interaction between the peptide and the polyamine. The AE (66 ± 2 %) and loading (3.0 ± 0.1 %) of semaglutide incorporated into PLGA-NPs produced with the semaglutide:spermidine complex as internal aqueous phase were higher than semaglutide-NPs produced in the absence of this complex (AE = 48 ± 1 % and DL = 2.30 ± 0.03 %). These results suggest that the non-covalent interaction between semaglutide and spermidine favored the entrapment of the peptide into the hydrophobic matrix. The AE and DL values of semaglutide loaded to functionalized NPs (FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs) were similar to the values obtained for non-functionalized semaglutide-NPs, as described in **Figure 9B**.

To confirm the electrostatic interaction between semaglutide and spermidine at basic conditions, did not modify the secondary structure of semaglutide, a CD spectroscopy analysis was performed. Control groups were included in the analysis, corresponding to semaglutide dissolved in MilliQ® ultrapure water and semaglutide dissolved in 0.1 M NaOH solution. As depicted in **Figure 9D**, the CD spectrum of semaglutide:spermidine complex was similar to free semaglutide in MilliQ® ultrapure water and in 0.1 M of NaOH solution, which corresponds to a mixture of random coil and α-helical conformations, characteristic of semaglutide, as previously described by Venanzi *et al.* [269]. Hereupon, the secondary

structure of semaglutide was maintained after formation of semaglutide:spermidine complex at basic pH.

The colloidal stability of the nanoformulations in PBS at pH 7.4 was evaluated for over 2 months at 4 °C. The results depicted in **Figure 10** showed that the Z-average of non-targeted NPs and FcRn-targeted NPs were preserved throughout the experiment, with values of around 180 nm, 160 nm, and 170 nm for semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs, respectively. The Pdl was also consistent for over 2 months, being below 0.200, suggesting that the nanosystems had a narrow size distribution throughout the experiment. Notwithstanding, the formulations had a neutral surface charge that was also maintained for over 2 months.

An *in vitro* release study was conducted to evaluate the release profile of semaglutide from PLGA-NPs in conditions that simulate the gastric and intestinal environment. The release profile of semaglutide from PLGA-NPs produced without semaglutide:spermidine complex (semaglutide-NPs w/ spermidine) and in the presence of this complex (semaglutide-NPs) was evaluated (**Figure 11**). In semaglutide-NPs w/ spermidine, around 70% of semaglutide was released, after 120 min of incubation in the gastric medium, which was maintained under the intestinal medium. This result suggests that semaglutide was not effectively incorporated into PLGA-NPs but adsorbed on the NPs surface. Regarding semaglutide-NPs produced with spermidine, it was visualized that only 5% of the peptide was released after 120 min in the gastric buffer, and this profile was maintained in the intestinal buffer for 360 min. The 5% release of semaglutide can be explained by some adsorption of the peptide on the surface of the particles. Overall, the PLGA-NPs produced with semaglutide:spermidine complex was considered the most suitable formulation to be used for further experiments since it had the highest AE and DL of semaglutide and the lowest release of the peptide under GI conditions, suggesting that the drug might be protected from the harsh conditions of the GIT.

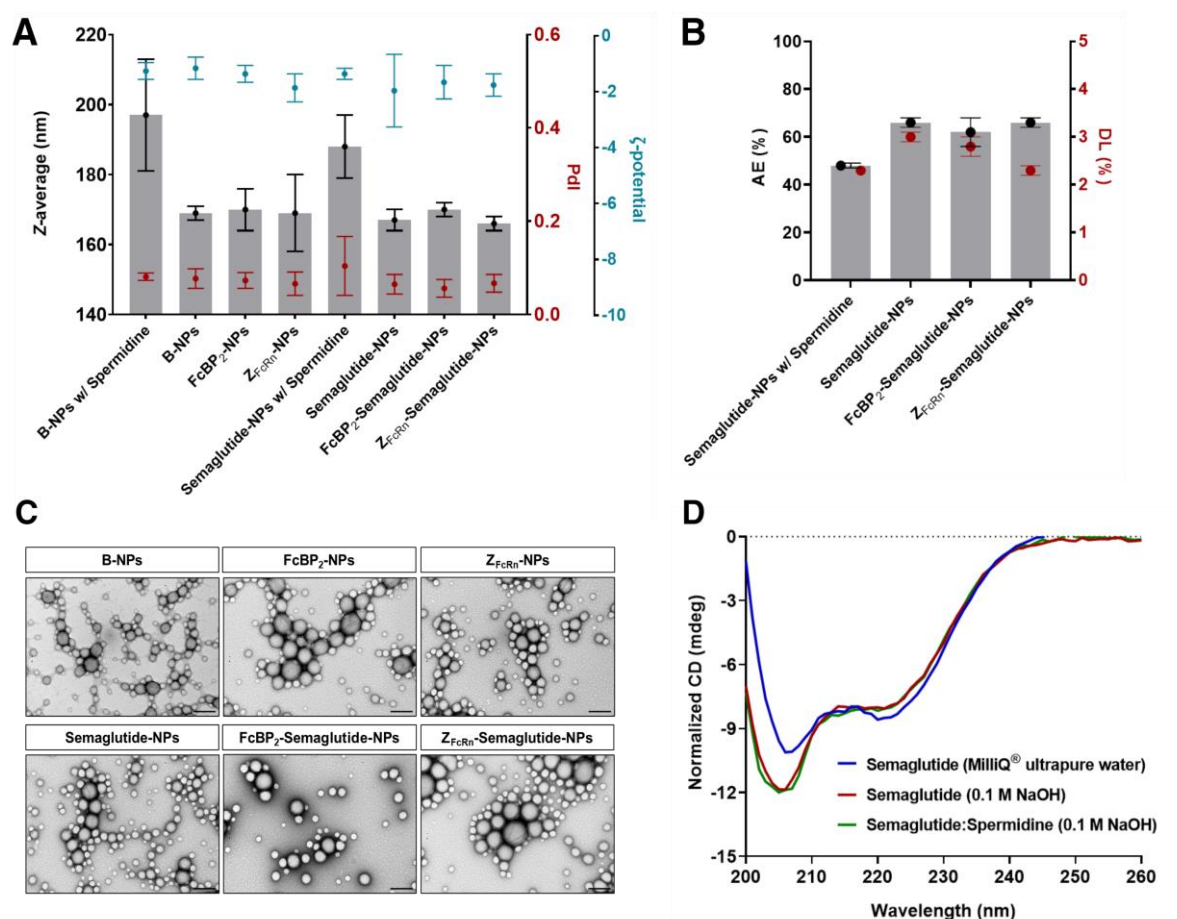


Figure 9. (A) Z-average, PDI and ζ -potential of B-NPs w/ spermidine, B-NPs, FcBP₂-NPs, Z_{FcRn}-NPs, semaglutide-NPs w/ spermidine, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs determined through DLS and ELS analysis. (B) AE and DL of semaglutide-NPs w/ spermidine, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs. Values are represented as mean $\pm$ SD ($n = 3$). (C) TEM images of B-NPs, functionalized unloaded NPs (FcBP₂-NPs and Z_{FcRn}-NPs), semaglutide-NPs and functionalized semaglutide-NPs (FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs). Scale bars represent 200 nm. (D) CD spectra of semaglutide dissolved in MilliQ[®] ultrapure water and 0.1 M NaOH solution, and semaglutide:spermidine complex dissolved in 0.1 M NaOH solution.

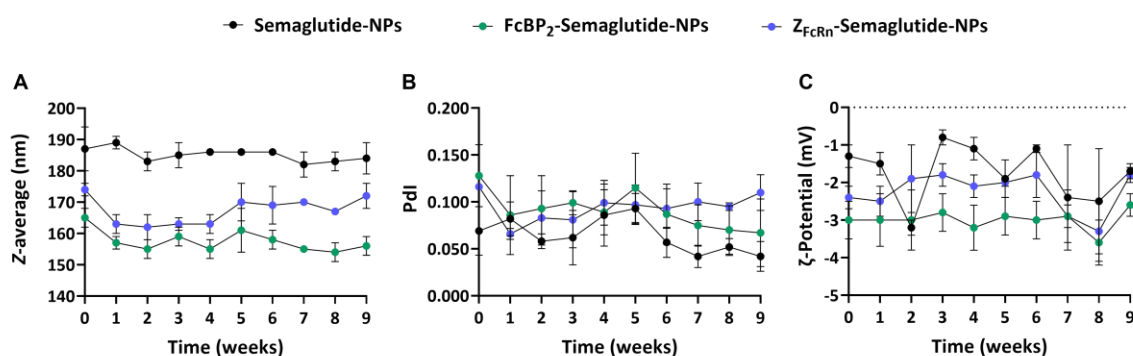


Figure 10. Stability of semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs in PBS at pH 7.4 and stored at 4 °C. The physicochemical properties of the formulations, namely (A) Z-average, (B) PDI, and (C) ζ -potential, were measured every week for over 2 months. The results are represented as mean $\pm$ SD ($n = 3$).

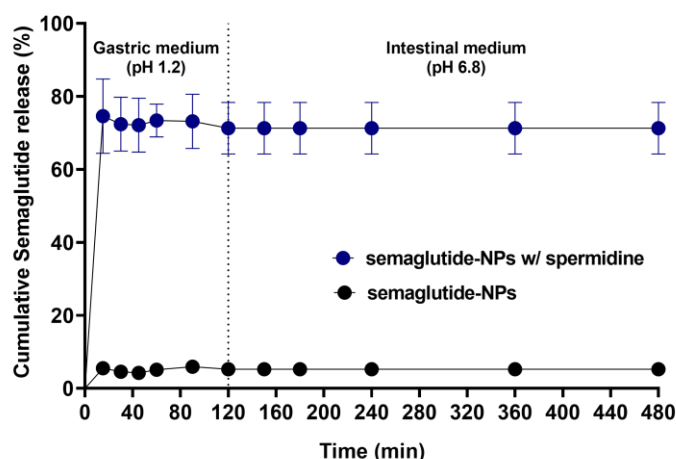


Figure 11. *In vitro* release profile of semaglutide from PLGA-NPs produced with an internal aqueous phase composed of semaglutide solubilized in MilliQ® ultrapure water (semaglutide-NPs w/ SPM) or semaglutide:spermidine complex solubilized in 0.1 M of NaOH solution (semaglutide-NPs), under gastric medium at pH 1.2 and intestinal medium at pH 6.8 for 120 min and between 120 min and 480 min, respectively. The results are represented as mean $\pm$ SD ($n = 3$).

4.5. FcRn-targeted nanoparticles bind to human FcRn receptor

As previously mentioned in **Chapter 4, section 4.1.**, FcBP₂ and Z_{FcRn} demonstrated a stronger interaction with hFcRn at acidic pH than at physiological pH. Taking this into consideration, PLGA-NPs, surface-conjugated with FcBP₂ or Z_{FcRn} were designed to interact with hFcRn. The binding-affinity of those FcRn-targeted nanosystems to the receptor was tested through SPR analysis at pH 6.0 and 7.4, as performed for the free ligands, to ensure the conjugation of FcBP₂ and Z_{FcRn} to PLGA-PEG-Mal, and the manufacturing process applied to produce the polymeric NPs do not compromise the interaction between those ligands and the receptor. B-NPs were used as control. At acidic pH, it was shown the interaction of both functionalized nanosystems to the receptor was significantly higher than B-NPs ($p < 0.0001$), as evidenced by the sensorgram profiles and RU values represented in **Figure 12A**. In fact, 2-fold and 6-fold increase in the RU values was obtained for FcBP₂-NPs (5.5 ± 0.1 RU) and Z_{FcRn}-NPs (14.5 ± 0.8 RU), respectively, comparatively with B-NPs (2.3 ± 0.3 RU). Moreover, Z_{FcRn}-NPs had a stronger binding-affinity to the receptor than FcBP₂-NPs at pH 6.0, which is in accordance with the results obtained for the free ligands. Notwithstanding, the binding-affinity of FcRn-targeted nanosystems to the receptor was also evaluated at pH 7.4 (**Figure 12B**). It was visualized the B-NPs and FcBP₂-NPs did not interact with hFcRn. However, the Z_{FcRn}-NPs had a response of approximately 5.7 ± 0.7 RU at pH 7.4, but this was lower than the response obtained at pH 6.0 (14.5 ± 0.8 RU). In conclusion, the functionalized NPs had a stronger interaction with the hFcRn at acidic pH than at physiological pH, highlighting that the binding-affinity of those nanosystems with

hFcRn occurs in a pH-dependent manner. These results are in line with the previous outcomes obtained for the free FcRn-targeted ligands, FcBP₂ and Z_{FcRn} (**Chapter 4, section 4.1.**). Moreover, these findings also demonstrated the covalent conjugation of both FcRn-targeted ligands to the PLGA-PEG-Mal co-polymer and further, the manufacturing process involved in the production of functionalized NPs through w/o/w double emulsion technique did not interfere with the pH-dependent binding-affinity of FcBP₂ and Z_{FcRn} to hFcRn receptor.

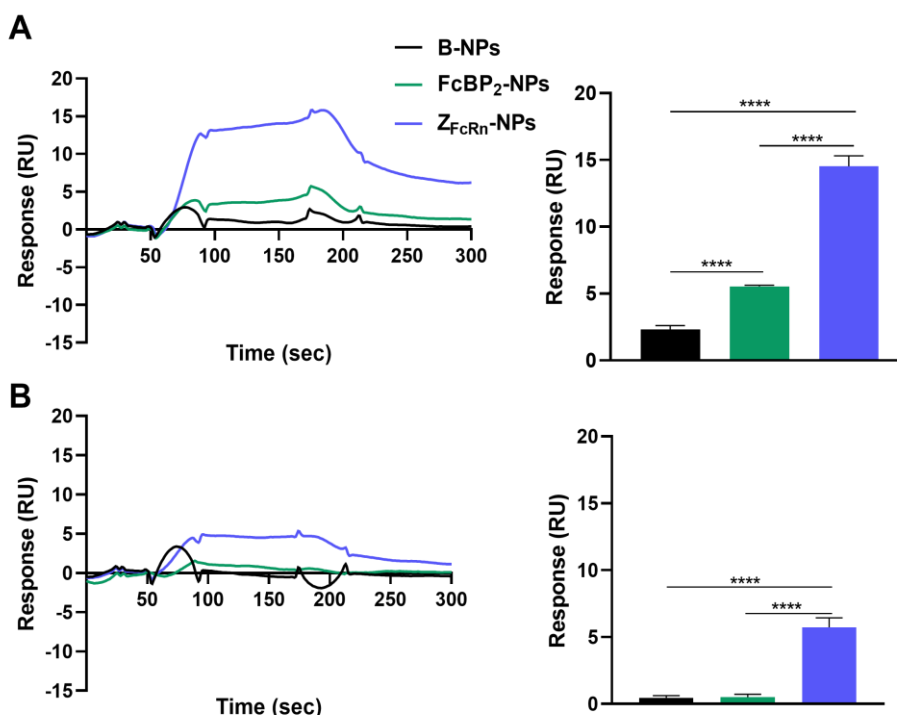


Figure 12. SPR analysis of the binding-affinity of both FcRn-functionalized nanosystems (FcBP₂-NPs or Z_{FcRn}-NPs) to the hFcRn coated to the CM5 sensor chip at (A) acidic pH and (B) at physiological pH. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the RU values of non-functionalized and functionalized nanosystems. (****) denotes significant differences of $p < 0.0001$. The results are represented as mean $\pm$ SD ($n = 3$).

4.6. Effect of nanoparticles in the metabolic activity of intestinal cells

The developed NPs aim to improve the intestinal delivery of semaglutide, and ultimately increase its oral bioavailability. For this purpose, the compatibility of semaglutide-NPs with cells mimicking the intestinal epithelium was assessed via a resazurin assay. Caco-2 cells were used to resemble the enterocytes, whereas HT29-MTX were used to resemble the intestinal goblet cells involved in the secretion of mucus [285-288]. The viability of both intestinal cell lines was measured after incubation with the samples (semaglutide, semaglutide:spermidine complex, B-NPs w/ spermidine, B-NPs, FcBP₂-NPs, Z_{FcRn}-NPs, semaglutide-NPs, FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs) for 4 h and 24 h, in

a range of concentrations, in relation to semaglutide, from 0.16 $\mu\text{g.mL}^{-1}$ to 500 $\mu\text{g.mL}^{-1}$ (**Figure 13**). After 4 h of incubation, the results shown in **Figure 13A** and **Figure 13C** demonstrated that all the concentrations tested for each formulation were safe for Caco-2 and HT29-MTX cells, since the cellular viability was higher than 70%, which is in accordance with ISO 10993-5 [289]. On the other hand, it was demonstrated that Caco-2 (**Figure 13B**) and HT29-MTX (**Figure 13D**) cells, after 24 h of incubation with the semaglutide:spermidine complex, in regard to the semaglutide concentrations of 100 and 500 $\mu\text{g.mL}^{-1}$, had a cellular viability lower than 70%, while the other formulations had a cellular viability higher than those value, which might be assigned to the influence of spermidine that already demonstrated in other studies to be cytotoxic for intestinal cells [281]. Additionally, the viability of HeLa cells was also evaluated after 4 h of incubation with B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs, in a range of NPs concentrations between 4.8 $\mu\text{g.mL}^{-1}$ and 3000 $\mu\text{g.mL}^{-1}$. As depicted in **Figure 14**, none of the treatments interfere with the viability of HeLa cells, since the cell viability was higher than 70% for all the conditions tested. Overall, all the concentrations evaluated of the nanoformulations were safe to the intestinal epithelial cells.

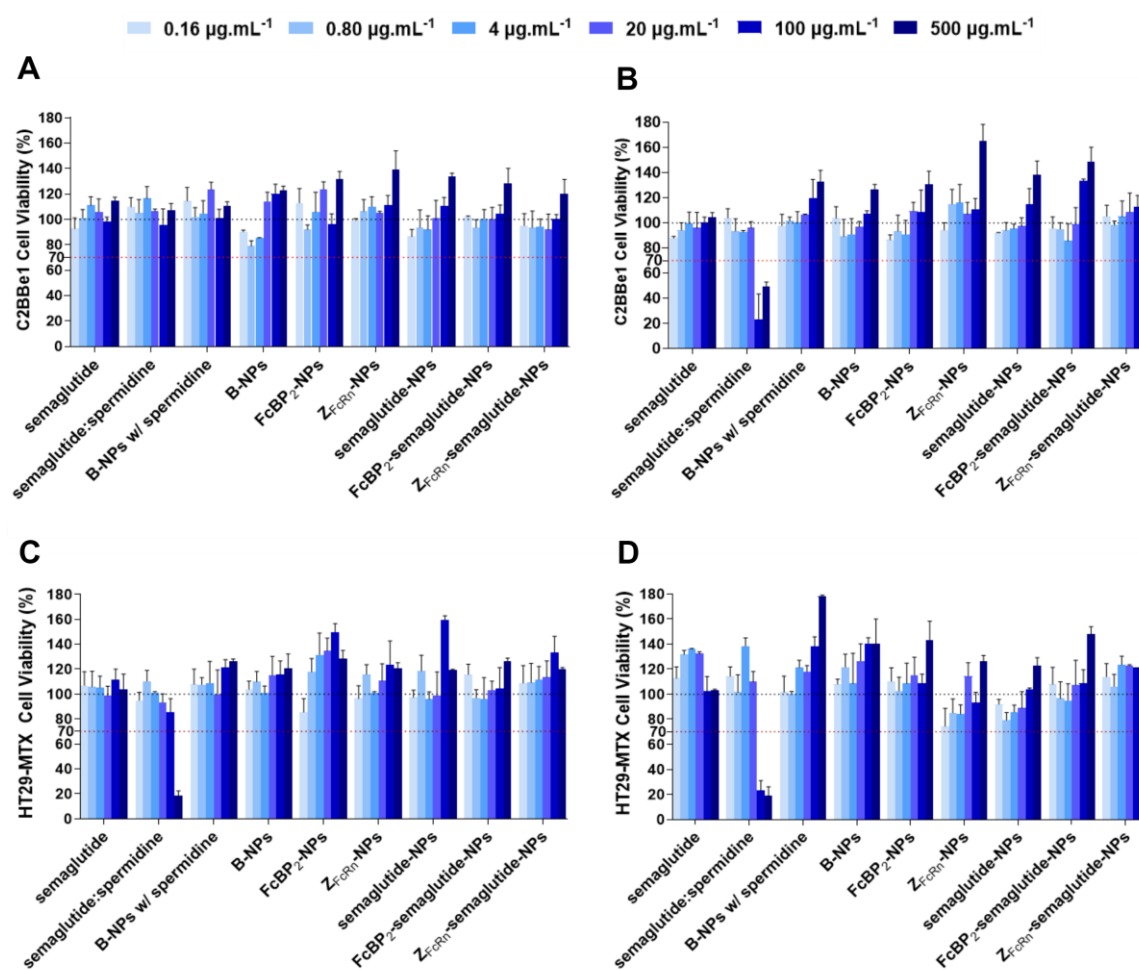


Figure 13. Metabolic activity of Caco-2 cells, when treated for 4 h (**A**) and 24 h (**B**) with the samples. Metabolic activity of HT29-MTX cells after treatment for 4 h (**C**) and 24 h (**D**) with the formulations. Values represent the mean $\pm$ SD. Each condition was tested in 6 replicates ($n = 3$).

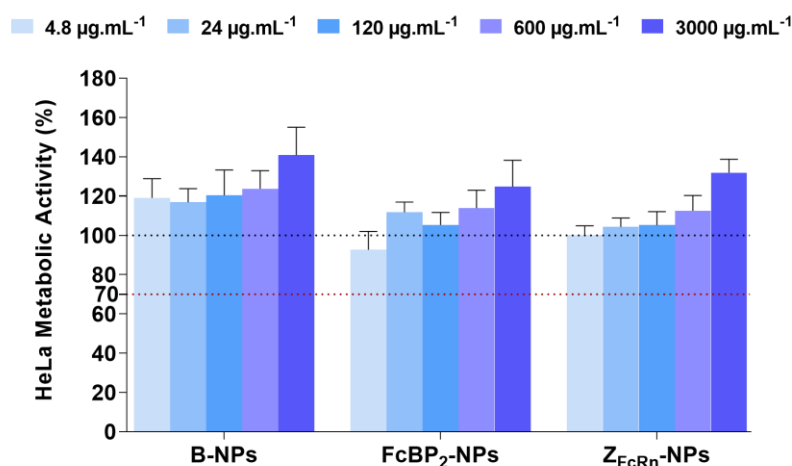


Figure 14. Metabolic activity of HeLa cells, when treated with B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs and further, incubated for 4 h at 37 °C and 5 % CO₂. Values are represented as the mean ± SD. Each condition was tested in 6 replicates ($n = 1$).

4.7. FcRn-targeted nanoparticles interact with intestinal cells

The interaction between B-NPs and FcBP₂-NPs or Z_{FcRn}-NPs with Caco-2 cells was quantitatively and qualitatively studied through flow cytometry and confocal microscopy analysis, respectively. The main purpose was to evaluate the effect of FcBP₂ and Z_{FcRn} in mediating the internalization of nanosystems into Caco-2 cells, a cell line that endogenously express the hFcRn [18, 22, 25, 176]. Additionally, as a control, the interaction of the nanosystems was also evaluated with a cell line without expression of the hFcRn, namely HeLa cells [290]. Both cell lines (Caco-2 and HeLa cells) were incubated with a concentration of 1 mg.mL⁻¹ of B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs for 4 h. The concentration of NPs tested was established from Caco-2 (**Figure 13**) and HeLa (**Figure 14**) metabolic activity assays, in which a NPs concentration of at least 3 mg.mL⁻¹ (corresponding to a semaglutide concentration of 100 µg.mL⁻¹) did not interfere with the viability of both cell lines. The results of cell-NPs interaction studies through flow cytometry were expressed as MFI values, as represented in **Figure 15A**. All the formulations interacted with Caco-2 cells, but the cells incubated with FcBP₂-NPs and Z_{FcRn}-NPs had significantly higher MFI values ($p < 0.0001$) than B-NPs (**Figure 15A**). Although in **Chapter 4, section 4.1.**, it was shown that the free Z_{FcRn} had 200-fold higher interaction to the hFcRn receptor than the free FcBP₂, the NPs functionalized with both FcRn-targeted ligands had similar interaction with Caco-2 cells (**Figure 15A**). This outcome could be attributed by the different ligand density on the surface of the NPs, since the CE of the FcBP₂ with the PLGA-PEG-Mal co-polymer (83 ± 4 %) was 2.5-fold greater than that of Z_{FcRn} with the PLGA-PEG-Mal co-polymer (33 ± 7 %), which may potentially influence the binding-affinity degree to the hFcRn. Therefore, the functionalization of the particles with the FcRn-targeted ligands increased the interaction of

the nanoformulations with Caco-2 cells. Confocal microscopy analysis also evidenced the B-NPs and FcRn-targeted NPs interacted with the intestinal cells, since all the nanosystems were located intracellularly (**Figure 15B**). However, an increased accumulation of FcRn-targeted NPs on Caco-2 cells was visualized over the non-targeted NPs.

To demonstrate the specific binding of FcBP₂ and Z_{FcRn} with the hFcRn receptor, the interaction between functionalized NPs in the presence of free FcBP₂ and Z_{FcRn}, which act as competitors regarding receptor attachment, was also evaluated. As shown in **Figure 15A**, the free ligands decreased the interaction between functionalized NPs and intestinal epithelial cells, considering that the MFI values were similar to the ones obtained for B-NPs, suggesting the functionalized NPs interacted with Caco-2 cells via hFcRn receptor. Hence, the results of flow cytometry and confocal microscopy analysis are in line with the results of SPR analysis, where the FcRn-targeted NPs bound to the hFcRn in a higher extension than B-NPs (the interaction was practically negligible). Moreover, these data also corroborated the XPS results, demonstrating a successful conjugation between FcBP₂ or Z_{FcRn} and PLGA-PEG-Mal.

Regarding the HeLa cells-NPs interaction, it was visualized (**Figure 15C**) that all the formulations interacted with the cells, and as expected, no significant differences were visualized between B-NPs and functionalized NPs. These results combined with those for Caco-2 cells suggest that the internalization of FcBP₂-NPs and Z_{FcRn}-NPs is mostly mediated via hFcRn receptor.

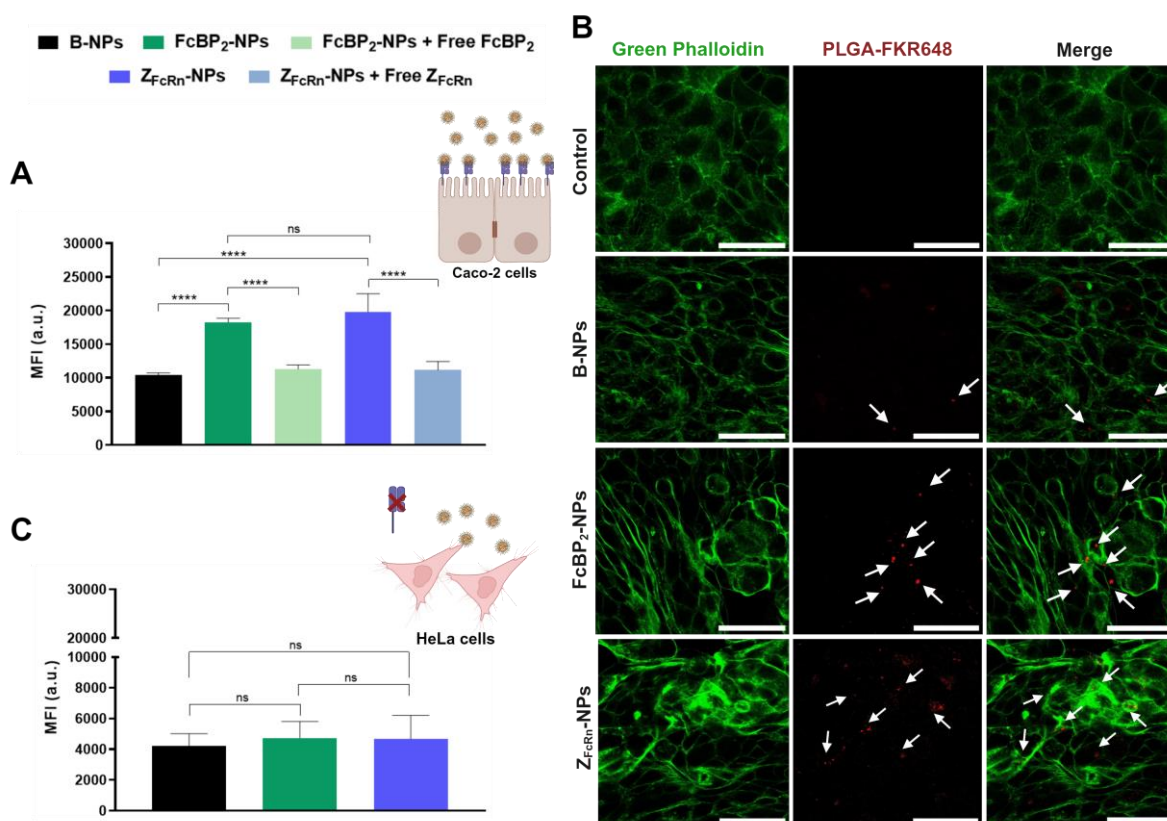


Figure 15. (A) Flow cytometry quantitative analysis of cellular internalization of B-NPs, FcBP₂-NPs, and ZFcRn-NPs in Caco-2, after 4 h of incubation. A competition assay was conducted, where the cells were incubated with the free FcRn-targeted ligands for 1 h, before incubation with the NPs. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the nanoformulations. (****) denotes significantly differences of $p < 0.0001$. All measurements were performed in triplicate and results are represented as mean $\pm$ SD ($n = 3$). (B) Confocal microscopy qualitative analysis of cellular internalization of B-NPs, FcBP₂-NPs, and ZFcRn-NPs in Caco-2 cells, after 4 of incubation. Flash PhalloidinTM Green 488 (green) was used for staining of actin filaments of the cells. 10% (w/v) of PLGA-FKR648 (red) was used during NPs production to confer fluorescence to the particles. Caco-2 cells without treatment were used as control. Scale bars represent 50 μ m. (C) Flow cytometry quantitative analysis of cellular internalization of B-NPs, FcBP₂-NPs, and ZFcRn-NPs in HeLa cells, after 4 h of incubation. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the non-functionalized and functionalized NPs and between both functionalized NPs. ns denotes non-significant differences. Measurements were performed in triplicate and results are represented as mean $\pm$ SD ($n = 3$)

4.8. Characterization of human intestinal organoids

The enterocytes and the mucus-producing goblet cells represent approximately 90% and 10% of the total epithelial cells of the intestine, respectively [288]. Enterocytes are the main intestinal cells responsible for the expression of hFcRn on the luminal surface, while the goblet cells are involved in the secretion of mucus, which acts as intestinal barrier, compromising the transport of drugs and particles throughout the gut environment [13, 17, 78, 176]. The presence of mucus-producing goblet cells and enterocytes in HIOs architecture was qualitatively analyzed by immunofluorescence assay. E-cadherin and

MUC2 were used as the specific markers for the epithelial cells and mucus-producing goblet cells, respectively. Moreover, to distinguish the lumen of the HIOs, villin marker was considered [291-293]. As shown in **Figure 16** and **Figure 17**, HIOs are fully polarized presenting the villin in the luminal surface and composed of goblet cells and enterocytes, since they are E-cadherin and MUC2 positive. HIOs also express the tight junction ZO-1 (**Figure 16** and **Figure 17**) that is responsible for epithelial proliferation, endothelial barrier formation, cell-cell tension and cell migration [294].

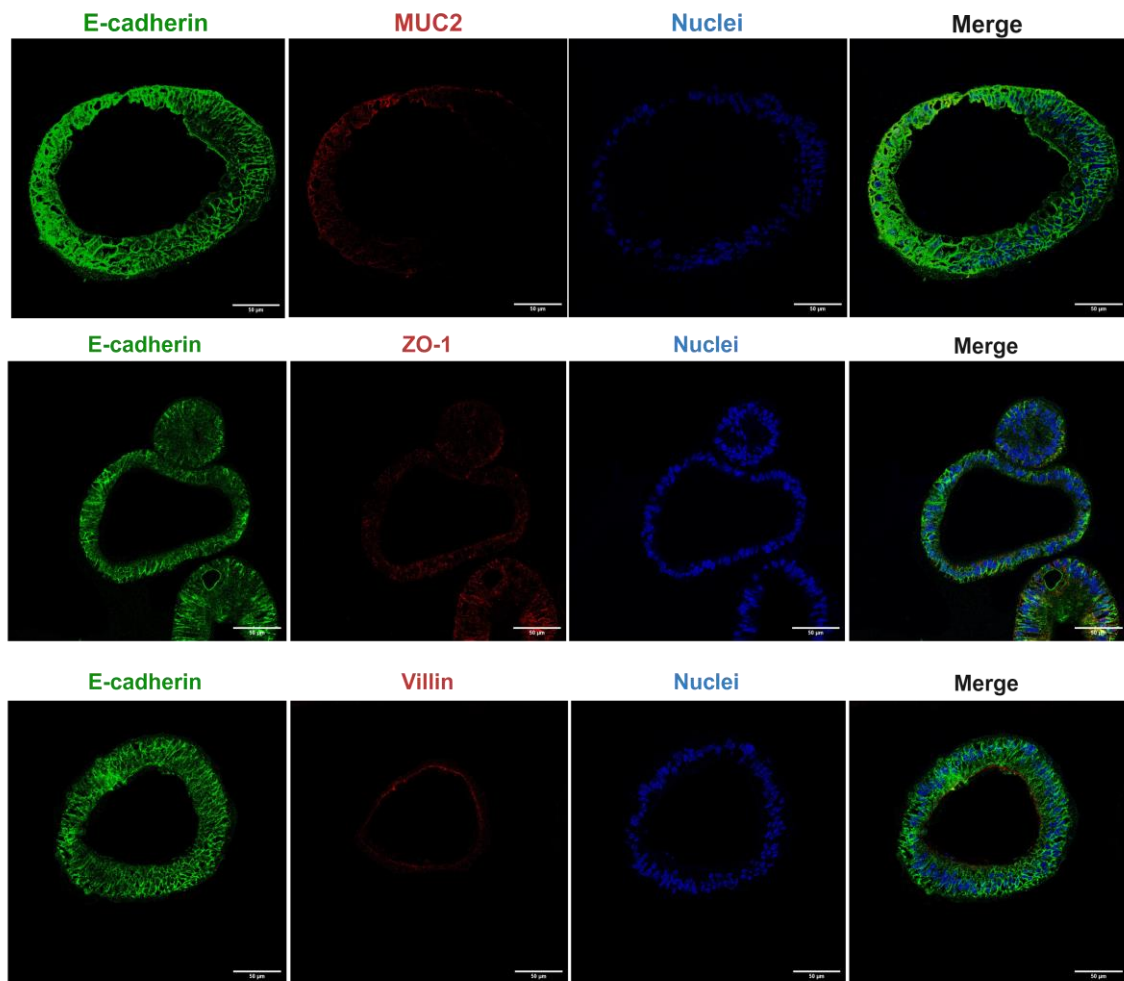


Figure 16. Representative images of the expression of E-cadherin (green) and MUC2, ZO-1, villin (red) in HUES9-derived HIOs sections. DAPI was used for staining the nuclei (blue) of the cells. Scale bars represent 50 μm.

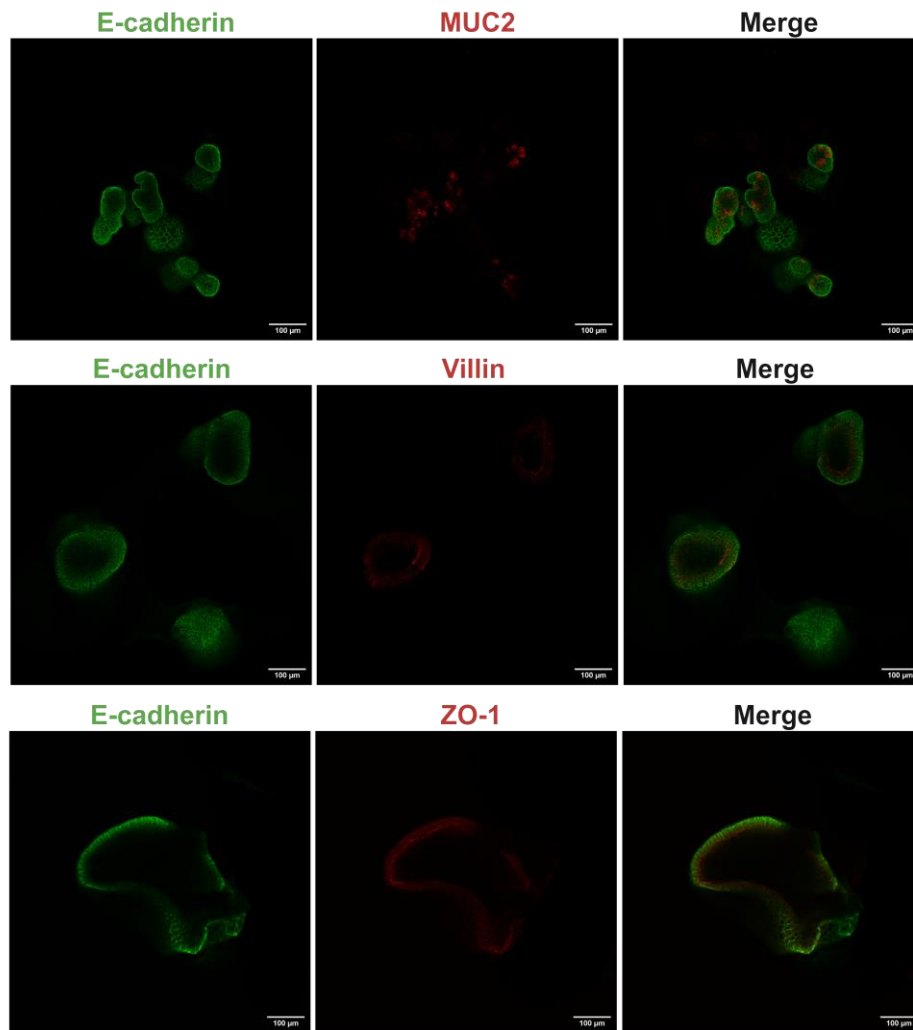


Figure 17. Representative images of the expression of E-cadherin (green), and MUC2, villin and ZO-1 (red) in HUES9-derived HIOs. Scale bars represent 100 µm.

4.9. Characterization of human FcRn expression in human intestinal organoids

To study the uptake of FcRn-targeted NPs, 3D HIOs were used as a model that can properly recapitulate the *in vivo* architecture, mimicking the biological complexity and functionality of human intestine. 3D HIOs presented proliferation and differentiation of intestinal stem cells to different cell type and provide extracellular matrix for intestinal epithelial cells [295-298]. To generate the 3D intestinal structures, HUES9 cells were differentiated into organoids, using a previously published protocol [295]. With embedding the mid/hind-gut spheroids in the Matrigel®, a highly organized structure with different cell types was obtained, with the lumen located in the center of the organoid and the lamina propria surrounding the organoids, which in this case corresponds to the Matrigel®. In humans, the hFcRn receptor is expressed in polarized epithelial cells of different sections of the intestine, including the colon and ileum, and less expressed in the jejunum and duodenum [17]. As previously

demonstrated in **Figure 16** and **Figure 17**, the enterocytes were abundantly detected in the HUES9-derived HIOs. According with this finding, it was hypothesized that the HIOs can also express the hFcRn receptor. Hence, the expression of hFcRn in HUES9-derived HIOs was assessed through immunofluorescence from organoids in sections (**Figure 18A**) and organoids in suspension (**Figure 18B**), and western blot (**Figure 18C** and **Figure 18D**) analysis. For western blot analysis, Caco-2 and HeLa cells were used as positive and negative controls, respectively. As demonstrated in **Figure 18A** and **Figure 18B**, HIOs express the hFcRn. In western blot analysis, a 42-kDa band was detected, corresponding to the molecular weight of the hFcRn heavy chain. A similar band was also observed in the Caco-2 cells, which was absent in HeLa cells, as expected. Therefore, the data supported that this model is suitable to evaluate the interaction of FcRn-targeted nanomedicines, due to the endogenous expression of hFcRn.

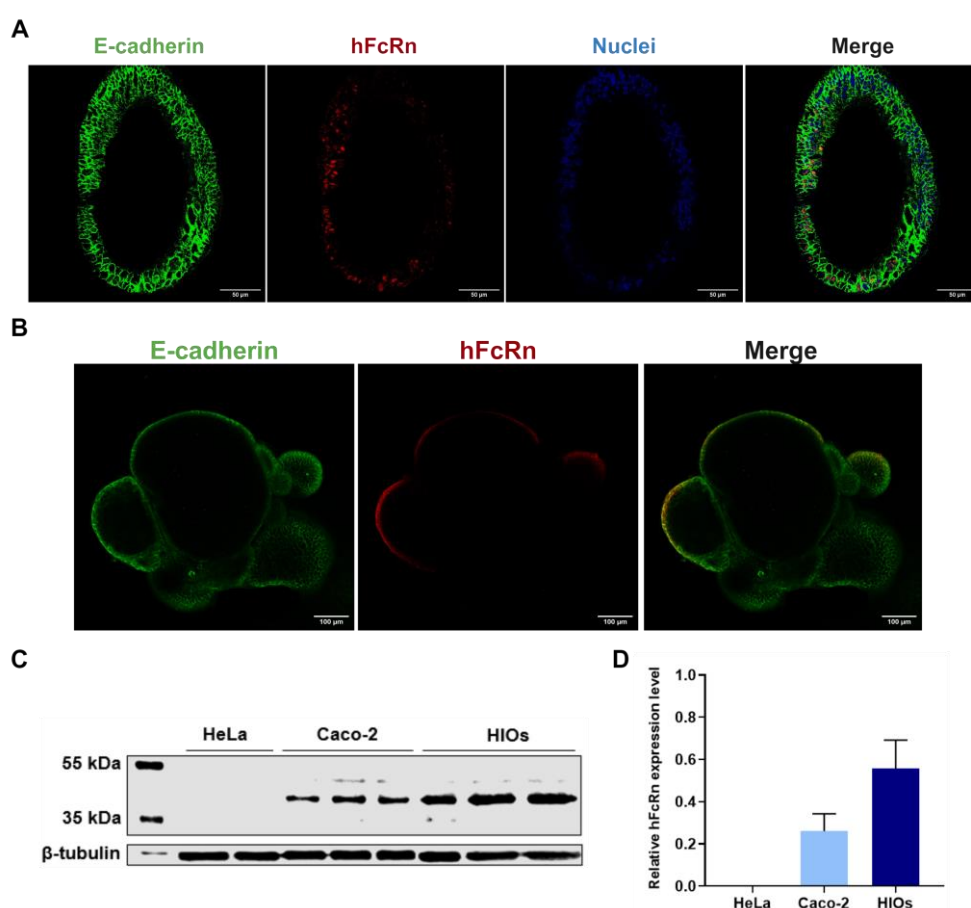


Figure 18. (A) Representative images of the expression of hFcRn (red) in HUES9-derived HIOs sections. Anti-h/mE E-cadherin goat IgG and DAPI were used for staining E-cadherin (green) and nuclei (blue), respectively. Scale bars represent 50 μ m. (B) Representative images of the expression of hFcRn (red) in HUES9-derived HIOs suspension. Anti-h/mE E-cadherin goat IgG was used for staining E-cadherin (green). Scale bars represent 100 μ m. (C and D) Western blot analysis of the hFcRn expression in HIOs. Caco-2 and HeLa cells were used as positive and negative controls, respectively. Monoclonal mouse anti- β -tubulin antibody was used to normalize the reaction.

4.10. Microinjection of polymeric nanocarriers into the lumen of human intestinal organoids

Currently, HIOs are being increasingly used for several purposes, such as cell biology, intestinal development, tissue regeneration, disease pathology, and host-pathogen interactions [299-310]. Nevertheless, the applicability of HIOs in the evaluation of cells-NPs interactions is still limited [311-313]. Here, it was investigated the potential of HUES9-derived HIOs being applied as a testing platform to assess the FcRn-targeted NPs interaction with the epithelial cells (**Figure 19A**).

The main drawback related with the study of epithelial cells-NPs interaction in HIOs is to reach the apical surface of the epithelium, due to the enclosed position of the lumen [314-317]. The hFcRn receptor is mainly expressed in the luminal surface of the enterocytes, in which the acidic pH of the lumen favors the binding between the FcRn-targeted NPs and the receptor [17]. Hereupon, for drugs, molecules, and other components reach the lumen of HIOs, three different approaches have been conducted. The first approach consists in forming 2D cell monolayers by dissociating the HIOs into single cells, enabling the access to the apical and basolateral sides. However, in this approach, the 3D structure of the organoids is lost, making the system less physiologically relevant [314-317]. The second approach focuses on establishing an organoid model with reversed polarity, in which the apical surface of the epithelium is facing the cell culture media, allowing direct access to the lumen. Notwithstanding, this approach can be time-consuming and, in some cases, unreachable [318-322]. The third approach comprises the use of the microinjection technique, injecting the compounds directly into the lumen of the organoids (**Figure 19A**). In this study, the microinjection technique was selected to assess the epithelial cells-NPs interaction since it is more informative as it better resembles the human physiology, comparatively with the other two approaches. To the best of our knowledge, this is the first time that the interaction between NPs and HIOs was evaluated by injecting the nanocarriers into the lumen of HIOs. Other studies were already conducted using this technique, but only to study the pathogen-host interaction [306, 310, 323-326]. The parameters applied to microinjection were previously optimized to reach a volume of NPs per injection of approximately 5 μL , and the total NPs volume injected was 20 μL , requiring 4 shots to achieve the required amount mentioned. The structure of the HIOs before and after injection into the intestinal lumen is depicted in **Appendix VI**. To ensure the nanosystems are being correctly injected in the lumen, a preliminary experiment was conducted, using B-NPs. As shown in **Figure 19B**, B-NPs were detected inside the HIOs lumen, immediately after injection, demonstrating the NPs were successfully injected into the lumen of HIOs.

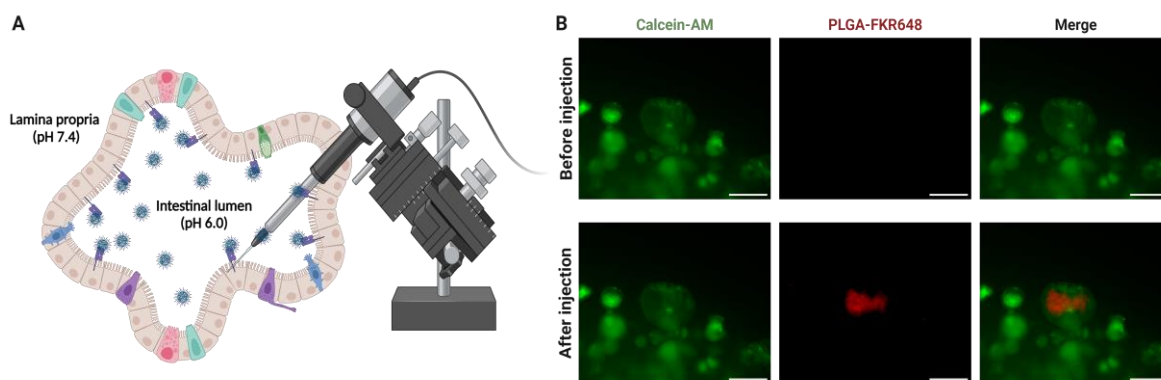


Figure 19. (A) Representative illustration of NPs injection into the lumen of the HUES9-derived HIOs. (B) Injection of fluorescent B-NPs (red) in the lumen of HUES9-derived HIOs. Calcein-AM was used to stain the organoids cells (green). Scale bars represent 500 μm .

4.11. Effect of nanoparticles in the metabolic activity of human intestinal organoids

The viability of HIOs was measured after 4 h of *post*-microinjection of a range of NPs concentrations between 3 mg.mL^{-1} and 15 mg.mL^{-1} , by the viability-based method of AlamarBlue[®]. The aims of this experiment were to evaluate the safety profile of the NPs (B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs) and to determine the concentration of particles that could be injected into the organoid's lumen for the *in vitro* interaction studies. As illustrated in **Figure 20A**, the HIOs 4 h *post*-microinjection had a viability higher than 70% in all the conditions, showing that all tested formulations were safe for intestinal organoids [289]. In **Chapter 4, section 4.6.**, the metabolic activity assay performed in Caco-2 cells already shown that this range of PLGA-NPs concentrations presented as safe formulation for the cells.

To further confirm the effect of NPs on the viability of the HIOs, a live-dead assay was performed using calcein-AM (green color) to label the live cells, and PI (red color) for the dead cells. Comparing the positive control group, in which the HIOs remained untreated, none of the treatments interfered in the viability of the organoids, as most of the cells of the HIOs were calcein-AM positive after 4 h of microinjection (**Figure 20B**). Notwithstanding, all the HIOs cells were PI positive after the injection of 1% (v/v) Triton[™] X-100, which was used as negative control in this assay. These results are in line with the results obtained in the AlamarBlue[®] assay.

Nevertheless, it was also tested if the microinjection procedure can influence the viability of HIOs by injecting the intestinal growth medium used to resuspend the NPs. The results (**Figure 20A** and **Figure 20B**) showed that the procedure used to inject the particles into the lumen of HIOs did not interfere with the organoids viability, demonstrating indeed, a similar viability response comparatively with the positive control.

Altogether, the tested concentrations of the non-functionalized and functionalized NPs were considered safe for HIOs.

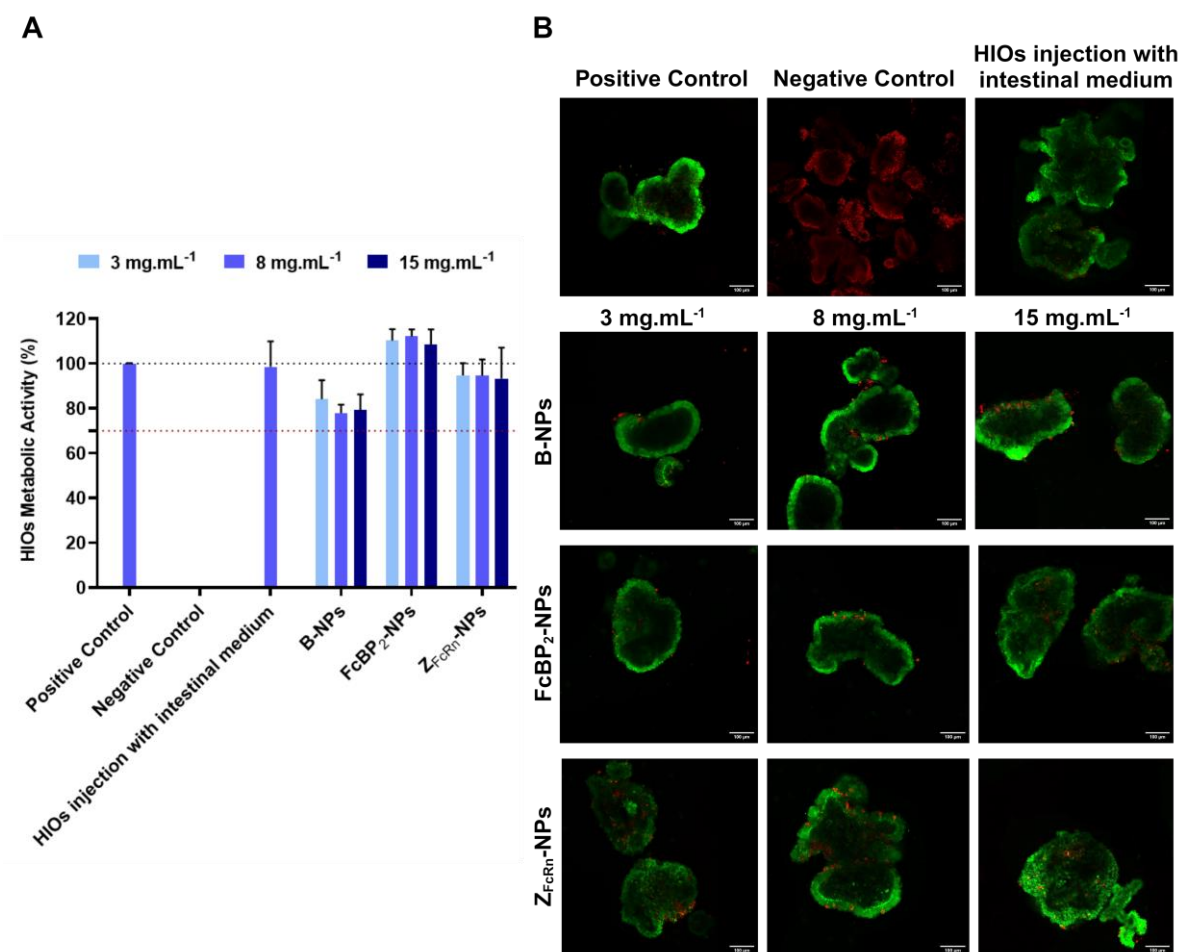


Figure 20. (A) Metabolic activity of HUES9-derived HIOs, after 4 h of incubation with non-functionalized (B-NPs) and functionalized NPs (FcBP₂-NPs and Z_{FcRn}-NPs). Values represent the mean $\pm$ SD ($n = 1$). Each condition was tested in 3 replicates. (B) Representative images of the live-dead staining assay. The images were taken by ZEISS CellDiscoverer 7 microscope, 4 h after the injection of the nanoformulations into the intestinal lumen of the organoids. Calcein-AM (green) was used for staining the live cells, while PI (red) was used for staining the dead cells. Scale bars represent 100 μ m.

4.12. FcRn-targeted nanosystems interact with human intestinal organoids

The main goal of these studies was to assess if the FcRn-targeted NPs are being internalized via hFcRn. The receptor-mediated internalization of the nanoformulations (B-NPs, FcBP₂-NPs, and Z_{FcRn}-NPs) was quantitatively and qualitatively studied through flow cytometry and confocal microscopy analysis, respectively. For this purpose, 15 mg.mL⁻¹ of the nanoformulations were used. The optimal concentration of NPs was established from HIOs viability assay (**Figure 20**), whereas a NPs concentration of 15 mg.mL⁻¹ did not cause a reduction in the viability of the organoids. The results of the interaction between NPs and HIOs-derived cells through flow cytometry analysis were expressed as percentage of

positive cells (**Figure 21A**) and MFI values (**Figure 21B**). All the formulations were able to interact with the HIOs-derived cells, however the HIOs incubated with FcBP₂-NPs and Z_{FcRn}-NPs had a significantly higher percentage of positive cells than B-NPs, being 54% and 44% of the HIOs-derived cells positive for FcBP₂-NPs and Z_{FcRn}-NPs, respectively, while only approximately 15% of the cells were positive for B-NPs. Additionally, the MFI values of FcBP₂-NPs and Z_{FcRn}-NPs were 2-fold and 2.5-fold higher than B-NPs. Therefore, the functionalization of the particles with the FcRn-targeted ligands increased the interaction of the nanoformulations with the intestinal organoids, as also previously visualized for Caco-2 cells (**Chapter 4, section 4.7.**). Analysis of the confocal microscope images also evidenced the B-NPs and FcRn-targeted NPs interacted with the 3D model, but an increased accumulation of FcRn-targeted NPs on the organoids over the non-targeted NPs was observed (**Figure 21C**).

To demonstrate the specific binding of FcBP₂ and Z_{FcRn} with the hFcRn receptor, the interaction between functionalized NPs in the presence of free FcBP₂ and Z_{FcRn} was also evaluated. As shown in **Figure 21A** and **Figure 21B**, the free ligands decreased the interaction between functionalized NPs and HIOs-derived cells to percentage of positive cells and MFI values similar to the ones obtained for B-NPs. The percentage of positive cells and MFI values for the cells incubated with free FcBP₂ or free Z_{FcRn}, followed by incubation with the FcBP₂-NPs or Z_{FcRn}-NPs, were respectively, 4-fold and 2-fold significantly lower than the cells only incubated with FcBP₂-NPs or Z_{FcRn}-NPs. These findings suggest that the functionalized NPs can be interacting with the cells of the organoids mainly via hFcRn receptor. Since the enterocytes represent approximately 90% of the total intestinal cells and are the main cell type responsible for the expression of the hFcRn receptor in the intestine, most of the positive cells containing FcRn-targeted NPs are probably enterocytes.

Hence, the results of flow cytometry and confocal microscopy analysis are in line with the results obtained for Caco-2 cells, whereas the FcRn-targeted NPs interacted with the cells in a higher extension than B-NPs. Therefore, a higher cellular association of FcRn-targeted NPs was observed with HUES9-derived HIOs than non-FcRn-targeted NPs, suggesting that the internalization of the functionalized particles might be occurring through hFcRn pathway, as also demonstrated for the intestinal epithelial cell line, Caco-2 cells.

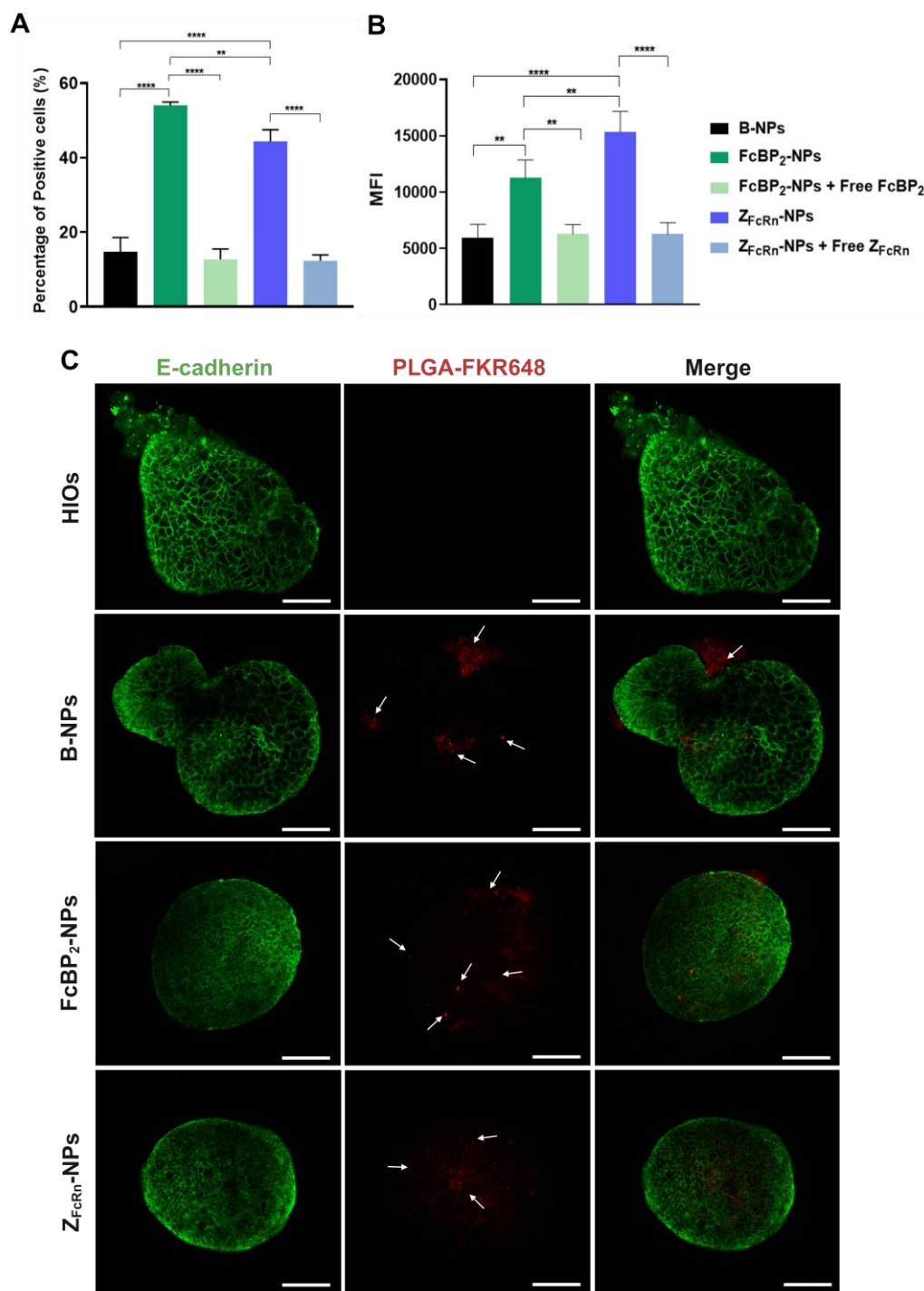


Figure 21. (A and B) Flow cytometry quantitative analysis of cellular internalization of B-NPs, FcBP₂-NPs, and ZFcRn-NPs in HUES9-derived HIOs, after 4 h of incubation at 37 °C. All measurements were performed in triplicate and results are represented as mean $\pm$ SD ($n = 1$). One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the non-functionalized and functionalized nanosystems and between both functionalized nanosystems. (**) and (****) denote significantly differences of $p < 0.01$ and $p < 0.0001$, respectively. (C) Confocal microscopy images of cellular internalization of nanoformulations in HUES9-derived HIOs, after 4 of incubation at 37 °C. Anti-h/mE E-cadherin goat IgG was used for staining of E-cadherin (green) and 10% (w/v) of PLGA-FKR648 (red) was used during NPs production to confer fluorescence to the particles. HUES9-derived HIOs microinjected with the intestinal cell growth medium was used as control. Scale bars represent 100 μ m.

4.13. Establishment of a type 2 diabetes mice model

The hFcRn is expressed in almost all cell types and tissues of the human body, and its expression is conserved throughout life, while in rodents it is downregulated immediately after weaning [246, 247]. Moreover, the interaction strength of the endogenous hFcRn-targeted ligands, IgG, and albumin, is different between mFcRn and hFcRn, due to the large cross-species binding differences. In fact, HSA binds poorly to mFcRn, while hIgG binds strongly to mFcRn, when compared to hFcRn, and therefore, the translation of the results obtained in mice to humans is unreliable. Even considering the binding-affinity of FcBPs and Z_{FcRn} to the receptor, this biomolecular parameter was only tested in hFcRn [29, 31]. To overcome the cross-species differences, several genetically engineered mouse strains have been developed, where mFcRn heavy chain has been replaced with the human counterpart, such as the homozygous hFcRn 32 transgenic mice model [251, 257-262]. Since this *in vivo* model expresses the hFcRn heavy chain instead of the mFcRn heavy chain, the binding-affinity between FcBP₂-NPs and Z_{FcRn} -NPs and the receptor can be conserved. Therefore, the hFcRn 32 transgenic mouse was the *in vivo* model selected in this work to evaluate the glucoregulatory effect and the biodistribution of FcRn-targeted NPs. The NA-STZ model was selected to induce T2DM in hFcRn transgenic mice. This model was designed by Masiello *et al.* in 1998 [227], in which STZ selectively destroys pancreatic β cells, while NA protects the cells from the cytotoxic effects of STZ, creating a state of partial insulin deficiency, similar to the condition of T2DM [217, 227, 327]. The NA-STZ model provides a stable and moderate hyperglycemia condition, reducing 40% the pancreatic β cells and 60% the insulin stores and it does not require the exogenous administration of insulin [207, 227]. The NA-STZ model was already used to evaluate the pharmacological effect of free GLP-1 analogs [328-333] and entrapped into NPs [118, 119]. Bearing this in mind, T2DM was induced in hFcRn transgenic mice through the NA-STZ model. Two different approaches to induce T2DM in mice were tested, following the protocol of Novelli *et al.* [231], with adaptations. In **Approach A**, animals received a single *i.p.* administration of NA followed by a single *i.p.* injection of STZ at day 0, while in **Approach B**, NA and STZ were intraperitoneally administered to the animals at the starting day of the experiment, as well as at day 2. The blood glucose levels of the mice were monitored for over 16 days, as depicted in **Figure 22A**. Regarding **Approach A**, it was observed that the body weight of the animals (**Figure 22B**), and the glycemia values (**Figure 22C** and **Figure 22D**) were practically maintained throughout the experiment, with blood glucose levels lower than 200 mg.dL⁻¹. In contrast, the administration of NA and STZ in days 0 and 2 (**Approach B**) decreased mice body weight and increased blood glucose for values higher than 200 mg.dL⁻¹, after 7 days of the first injection of NA and STZ (**Figure 22**), which is mainly attributed to the diabetic condition [334-336]. In **Approach B**, glycemia values were

maintained higher than 200 mg.dL⁻¹ from day 7 until day 16, meaning the animals were able to sustain the diabetic condition for over 9 days. Overall, **Approach B** was effective in inducing T2DM in the hFcRn transgenic mice model, being the methodology applied for further experiments.

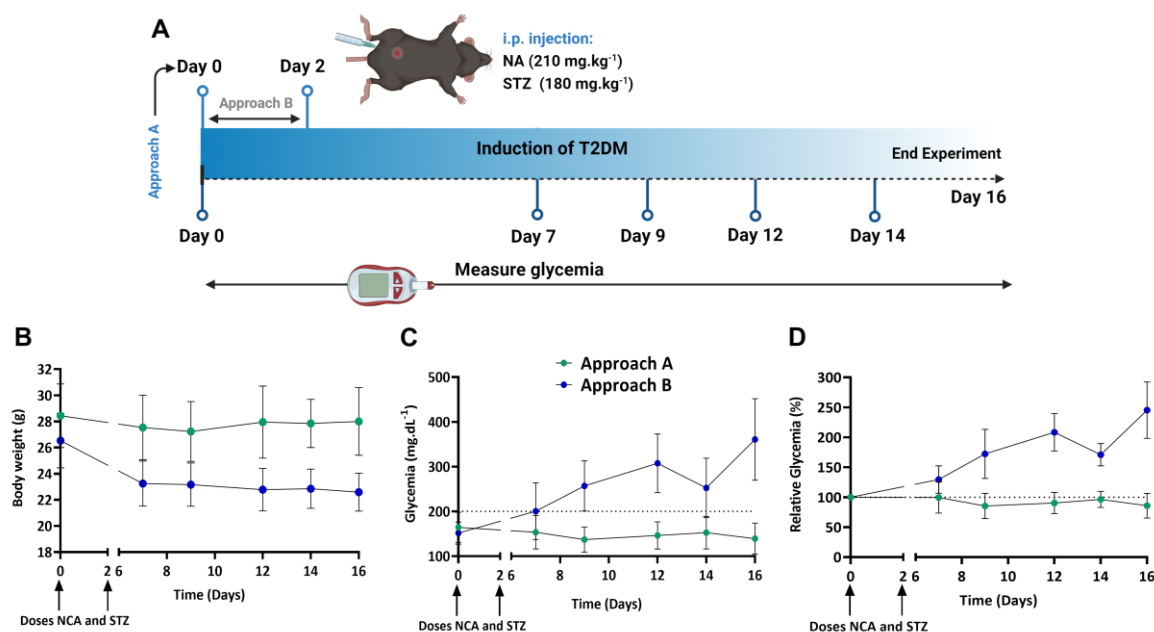


Figure 22. Establishment of a T2DM-induced mice model. (A) Representative illustration of T2DM induction following **Approach A** (*i.p.* injection of NA and STZ at day 0) and **Approach B** (*i.p.* injection of NA and STZ at days 0 and 2). (B) Body weight (g), (C) absolute glycemia (mg.dL⁻¹) and (D) relative glycemia (%) values. Bars represent mean $\pm$ SD ($n = 6$).

4.14. Biodistribution of FcRn-targeted nanoparticles through the gastrointestinal tract of type 2 diabetes-induced mice

The biodistribution of the orally administered Cy7.5-loaded NPs (B-NPs) and Cy7.5-loaded NPs modified with the FcRn-targeted ligands, FcBP₂-NPs, and Z_{FcRn}-NPs, was studied using an *in vivo* imaging system. **Figure 23** shows the whole-body imaging of the mice at 0.5, 2, 4 and 6 h *post-administration* of the nanosystems. The images demonstrated an intense fluorescence signal in the abdominal region of the mice for all the tested groups, but no further conclusions can be drawn. To properly estimate the distribution of the particles through the GIT, a humane end-point euthanasia was performed in T2DM-induced mice at specific time-intervals in order to collect the blood and organs. Further, stomach, small intestine, and large intestine were isolated to evaluate the fluorescence signal using NIR imaging. **Figure 24** represents the qualitative and semi-quantitative assessment of the distribution of the NPs in the GIT. The fluorescence signal of all NPs in the stomach decreased over time, which was translated into an increase in their accumulation in different

segments of GIT (**Figure 24A** and **Figure 24B**). At 0.5 h *post*-administration, the fluorescence signal of B-NPs and FcRn-targeted NPs was mainly concentrated in the proximal and middle sections of the small intestine (specifically in the duodenum and jejunum). However, 2 h *post*-administration, the fluorescence signal of the particles was stronger in the middle and distal sections of the small intestine (particularly in the jejunum and ileum), with Z_{FcRn} -NPs showing a tighter signal than B-NPs and FcBP₂-NPs (**Figure 24A** and **Figure 24C**). At 4 h after administration, an extensive decrease in the fluorescence signal of the NPs in the small intestine was visualized, which can be attributed to the distribution of the formulations to deep sections of the gut, namely the large intestine (cecum and colorectum) (**Figure 24A** and **Figure 24D**). The fluorescence signal of the nanosystems remained constant between 4 h to 6 h of the experiment in the large intestine. Notwithstanding, Z_{FcRn} -NPs showed a stronger fluorescence in the small intestine, followed by FcBP₂-NPs and B-NPs, 4 h *post*-administration. Moreover, 6 h of administration, both FcRn-targeted NPs were still found in the small intestine, particularly the Z_{FcRn} -NPs, while no signal of B-NPs was visualized. A stronger fluorescence signal was observed for the mice administered with Z_{FcRn} -NPs in the small and large intestine at 6 h compared with the other formulations. Therefore, the results strongly reinforce that FcBP₂-NPs and Z_{FcRn} -NPs remained in the gut for a longer period than B-NPs.

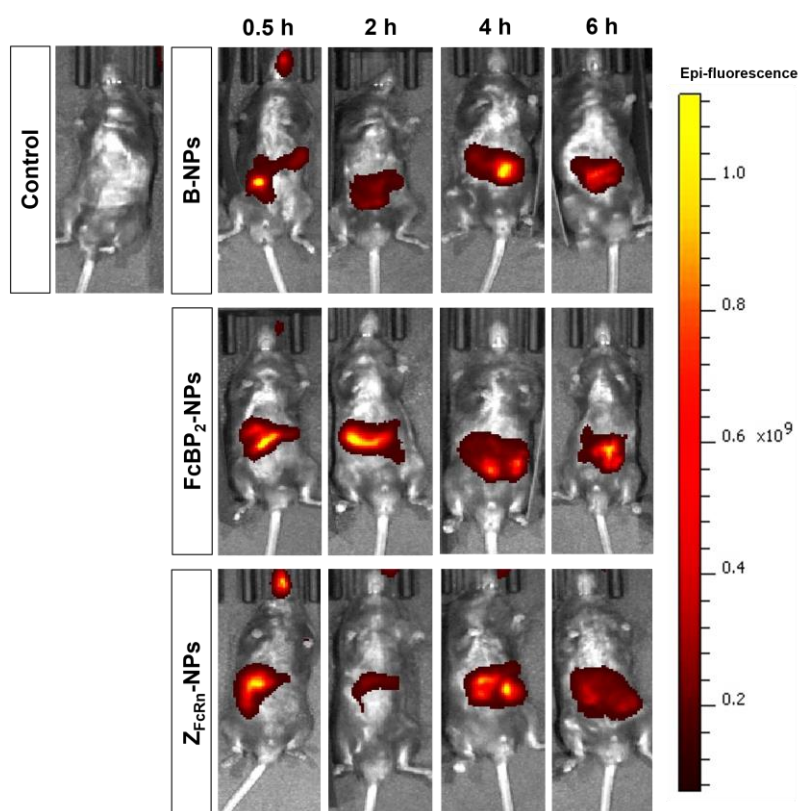


Figure 23. Qualitative assessment of the biodistribution of B-NPs, FcBP₂-NPs and Z_{FcRn} -NPs loaded with Cy7.5 in T2DM-induced mice using an *in vivo* imaging system.

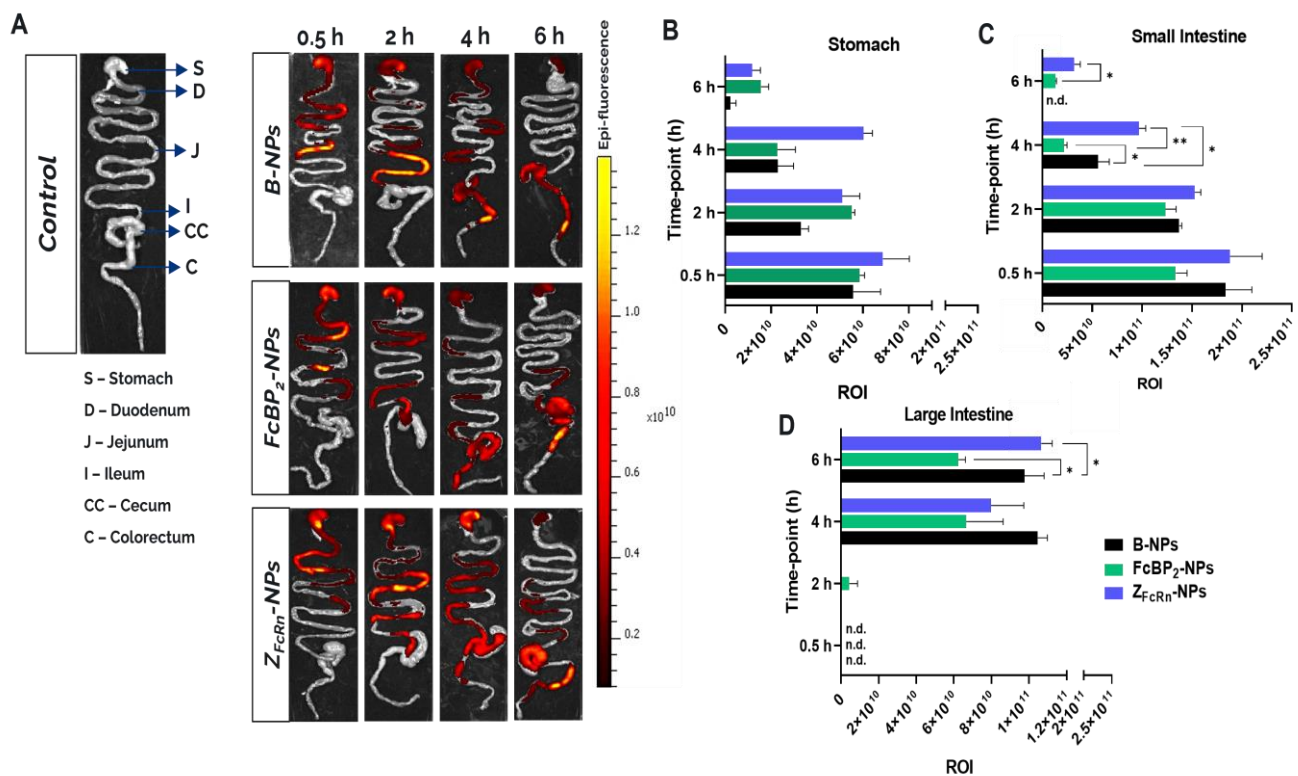


Figure 24. Biodistribution of the particles through the GIT of T2DM-induced mice. Qualitative (A) and semi-quantitative (B-D) assessment of the biodistribution of B-NPs, FcBP₂-NPs and ZFcRn-NPs loaded to Cy7.5 using an *in vivo* imaging system. The fluorescence signal of the Cy7.5 loaded NPs was semi-quantified from the total radiance signal from each segment of the GIT, including stomach (B), small intestine (C) and large intestine (D). One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the nanoformulations. (*) and (**) denote significant differences of $p < 0.05$ and 0.01 , respectively. Absence of * symbol indicates non-significant differences. n.d. means not detected. Results are represented as mean $\pm$ SEM ($n = 4$).

Furthermore, to understand the trafficking of B-NPs, FcBP₂-NPs, and ZFcRn-NPs along the full length of GIT, the amount of Cy7.5-loaded NPs, recovered from different sections of GIT, was quantified. As shown in **Figure 25**, at 0.5 h *post*-administration, the nanoformulations were mainly retained in the stomach and duodenum, and less in the jejunum (but only considering the B-NPs and FcBP₂-NPs). However, 2 h after oral administration, residual amounts of particles were present in the duodenum, being predominantly retained in the middle and distal sections of the small intestine (specifically jejunum and ileum). The levels of ZFcRn-NPs recovered from the jejunum was 2-fold higher than B-NPs, while FcBP₂-NPs were not detected. In contrast, the fluorescence levels of B-NPs recovered in ileum was 2-fold and 6-fold higher than FcBP₂-NPs and ZFcRn-NPs, respectively. These findings suggested that B-NPs exhibited a faster trafficking in the small intestine than both FcRn-targeted NPs. At a later time-interval, specifically 4 h, both FcRn-targeted NPs remained in ileum since considerable fluorescence intensities were detected,

whereas B-NPs were not found. Moreover, all nanoformulations reached segments of the large intestine, mainly the cecum, at 4 h *post*-administration. The amount of B-NPs recovered in the cecum was 4-times higher than Z_{FcRn}-NPs, while only scant levels of FcBP₂-NPs were detected. Also, B-NPs recovered from colorectum were 4-times greater than FcBP₂-NPs. Z_{FcRn}-NPs were present in residual amounts in the distal section of the large intestine. In addition, 6 h *post*-administration, Z_{FcRn}-NPs were the only nanoformulation that still remained in the distal part of the small intestine, the ileum. Additionally, the proportion of Z_{FcRn}-NPs detected in the cecum 6 h after treatment was similar to the B-NPs, whereas the FcBP₂-NPs were detected in scanty levels, as observed for the 4 h time-interval. In contrast, the amount of both FcRn-targeted NPs recovered in colorectum increased considerably in comparison with the same formulations at 4 h *post*-administration. Overall, the outcomes obtained in the quantitative analysis (**Figure 25**) are in accordance with the qualitative and semi-quantitative analysis (**Figure 24**), where NPs modified with FcRn-targeted ligands, particularly Z_{FcRn}, remained for a longer time in the small intestine, specifically the ileum, and in the large intestine, particularly the colorectum, compared to B-NPs. These findings can be attributed to the interaction of FcBP₂-NPs and Z_{FcRn}-NPs with hFcRn, which is mainly expressed in the ileum and colorectum, but somewhat less in the jejunum and duodenum [183, 246, 247].

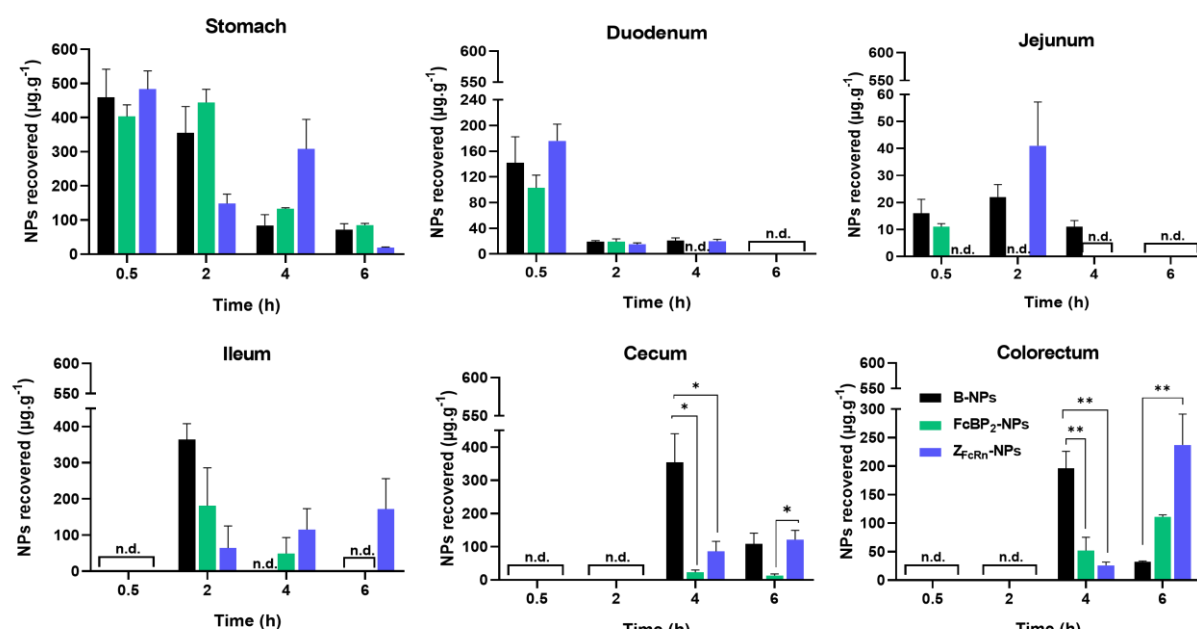


Figure 25. Quantitative distribution of the particles in different sections of GIT, including stomach, duodenum, jejunum, ileum, cecum and colorectum, after 0.5, 2, 4 and 6 h of oral administration. Results are expressed as the amount of recovered Cy7.5-loaded NPs and recovered Cy7.5-loaded NPs modified with FcBP₂ and Z_{FcRn}. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the nanoformulations. (*) and (**) denote significant differences of $p < 0.05$ and $p < 0.01$, respectively. Absence of * symbol indicates non-significant differences. n.d. means not detected. Columns represent mean $\pm$ SEM ($n = 3$).

Lastly, sections of GIT (stomach, duodenum, jejunum, ileum, cecum and colorectum) were processed for confocal fluorescence imaging, where the nuclei and the tissues were stained with DAPI and PhalloidinTM 546, respectively. This study was performed to evaluate if the particles were transported from the lumen towards the epithelium. At 0.5 h *post*-administration, nanoformulations were found into the duodenum and jejunum tissues (Z_{FcRn} -NPs was the only formulation not found in jejunum) (**Figure 26**), which is in accordance with the quantification analysis of the NPs recovered from these sections. After 2 h, B-NPs were found in the jejunum and ileum, while FcBP₂-NPs and Z_{FcRn} -NPs were located in the ileum and jejunum, respectively. Moreover, at later time-intervals, 4 h and 6 h *post*-administration, FcRn-targeted NPs, mainly Z_{FcRn} -NPs, were still found in the ileum. At these time-points, the formulations were also located in the cecum and colorectum, as shown in **Figure 26**. Interestingly, at all the time-points studied, the nanoformulations were located into the epithelial tissues, suggesting that the NPs were able to pass through the intestinal barrier and penetrate the intestinal epithelium of GIT sections.

Overall, these data indicate that the FcRn-targeted ligands, FcBP₂ and Z_{FcRn} , may enhance the distribution of NPs throughout the GIT and prolong their residence time, which can be occurring via the hFcRn pathway, as inferred from the previous report by Shi *et al.* [138].

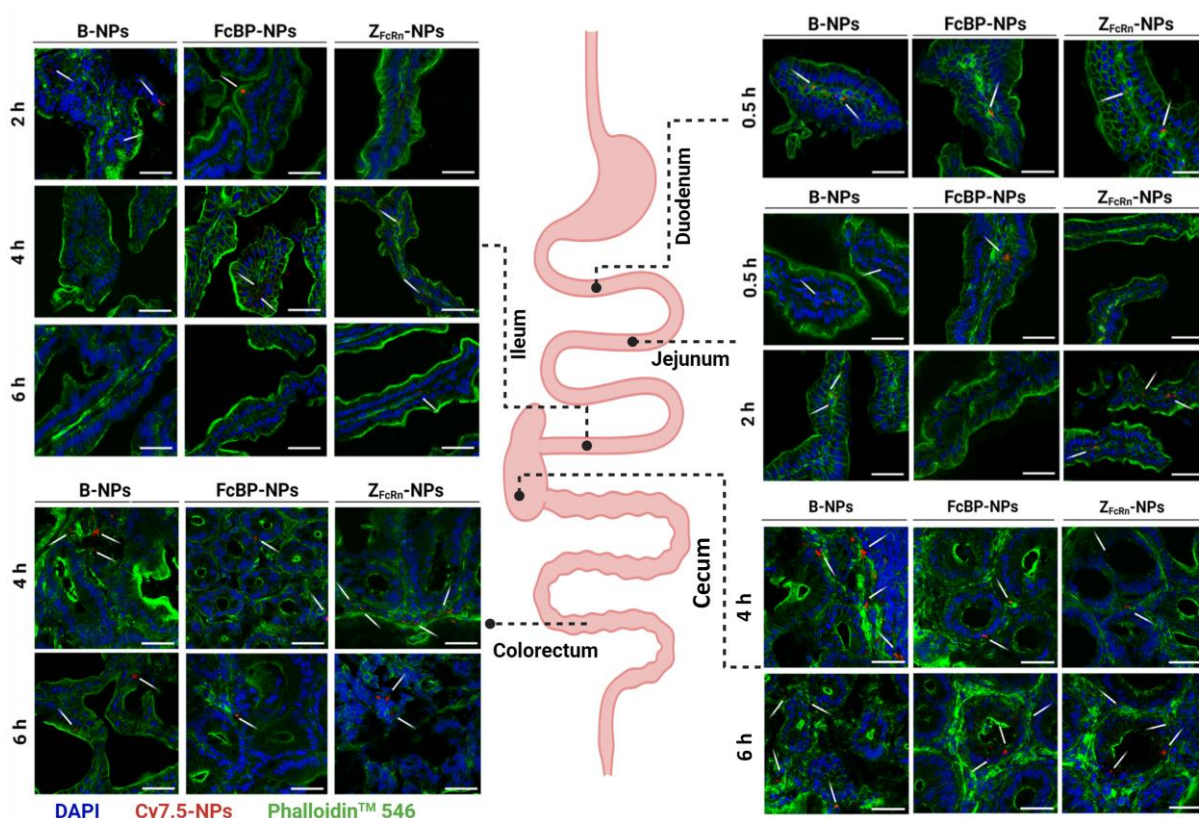


Figure 26. Distribution of NPs in different segments of GIT. Representative fluorescent microscopy images of transversal sections of duodenum, jejunum, ileum, cecum, and colorectum at 0.5, 2, 4 and 6 h *post*-administration of fluorescent Cy7.5-loaded NPs and FcRn-targeted Cy7.5-loaded NPs. Nuclei and cell tissue were stained with DAPI (blue), and Phalloidin™ 546 (green), respectively. The red color corresponds to Cy7.5-loaded NPs. White arrows highlight NPs penetrated in the intestinal epithelium of each section of GIT.

4.15. Pharmacological effect of FcRn-targeted nanoparticles

4.15.1. FcRn-targeted nanoparticles reduce glycemia after a single administration

The glucoregulatory effect of FcRn-targeted semaglutide-NPs was evaluated for over 8 h after a single oral administration of the nanoformulations in T2DM-induced hFcRn transgenic mice (**Figure 27A**). **Appendix VII** and **Figure 27B** represent the absolute glycemia (mg.dL^{-1}) and the relative glycemia values (%) obtained for each group throughout the experiment, respectively. The animals subcutaneously treated with free semaglutide showed a notable decrease in blood glucose levels (**Figure 27B**), reaching the maximum blood glucose reduction ($\sim 60\%$) within 4 h (**Table 7**). This glucose-lowering effect is in accordance with the previous report of Liu *et al.* [273]. As expected, no changes in blood glucose levels were observed in the mice that received PBS by oral gavage (**Figure 27B**), with blood glucose concentrations always above 300 mg.dL^{-1} as at the start of the experiment (**Appendix VII**). A similar glycemic profile was observed for the animals treated with free semaglutide via oral gavage, suggesting that semaglutide might be enzymatically

degraded in the GIT [11, 80]. In contrast, non-targeted NPs (semaglutide-NPs) and both FcRn-targeted NPs (FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs) groups had a distinct glucose-lowering effect for the same dose of semaglutide after oral gavage. Semaglutide-NPs elicited a blood glucose level reduction in the mice of around 30% of the initial value, 6 h *post*-administration, remaining low until the end of the experiment (~ 25%). A similar decrease in blood glucose levels was observed for the mice treated with both FcRn-targeted semaglutide-NPs at the same time-point, but the maximum blood glucose decrease was reached at 8 h after administration, where animals treated with NPs achieved a glycemia decrease of ~ 35% (**Figure 27B**). In other reported studies, it was already observed that NPs incorporating GLP-1 analogs reached the lowest blood glucose levels 8 h *post*-administration [121, 122], which is in line with the current results. Hereupon, the glucose AUC values (0–8 h), shown in **Figure 27C**, demonstrated that the nanoformulations groups had a statistically significant effect ($p < 0.0001$) in reducing the overall blood glucose levels when compared with oral free semaglutide. Additionally, mice subcutaneously treated with free semaglutide exhibited the lowest AUC (0–8 h) value, indicating that this treatment effectively induced a pronounced glucose-lowering effect, consistent with the data presented in **Figure 27B** and **Appendix VII**. The BGD (%) parameter (calculated based on **Equation 7**) depicted in **Table 7** corroborated the obtained results, showing no reduction in blood glucose levels for PBS and oral free semaglutide groups, while semaglutide-NPs, FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide NPs had a BGD of 28 ± 11 %, 32 ± 24 % and 45 ± 12 %, respectively. An evident glucose-lowering effect was achieved by loading semaglutide into PEGylated PLGA-NPs, indicating that the nanosystems could efficiently improve the intestinal transcytosis and bioavailability of oral administered semaglutide. This was mainly attributed to the protective effect and sustained drug release of semaglutide-NPs in fluids that mimic the gastric and intestinal environment (**Chapter 4, section 4.4.**). However, no differences regarding the glucoregulatory effect between non-targeted NPs and FcRn-targeted NPs were found, highlighting the necessity for additional studies to expand the therapeutic window. Free semaglutide subcutaneously administered showed the highest BGD compared to the other groups (54 ± 4 %). Overall, the administration of a single dose of semaglutide entrapped into nanoformulations improved the glucose-lowering effect in diabetic mice compared to oral free semaglutide. It is noteworthy that the glucoregulatory effect observed in this study was similar or even higher than the results obtained in other reports, where GLP-1 analogs (e.g. GLP-1, exenatide and liraglutide) were incorporated into nanocarriers [118, 119, 121, 122, 135, 337].

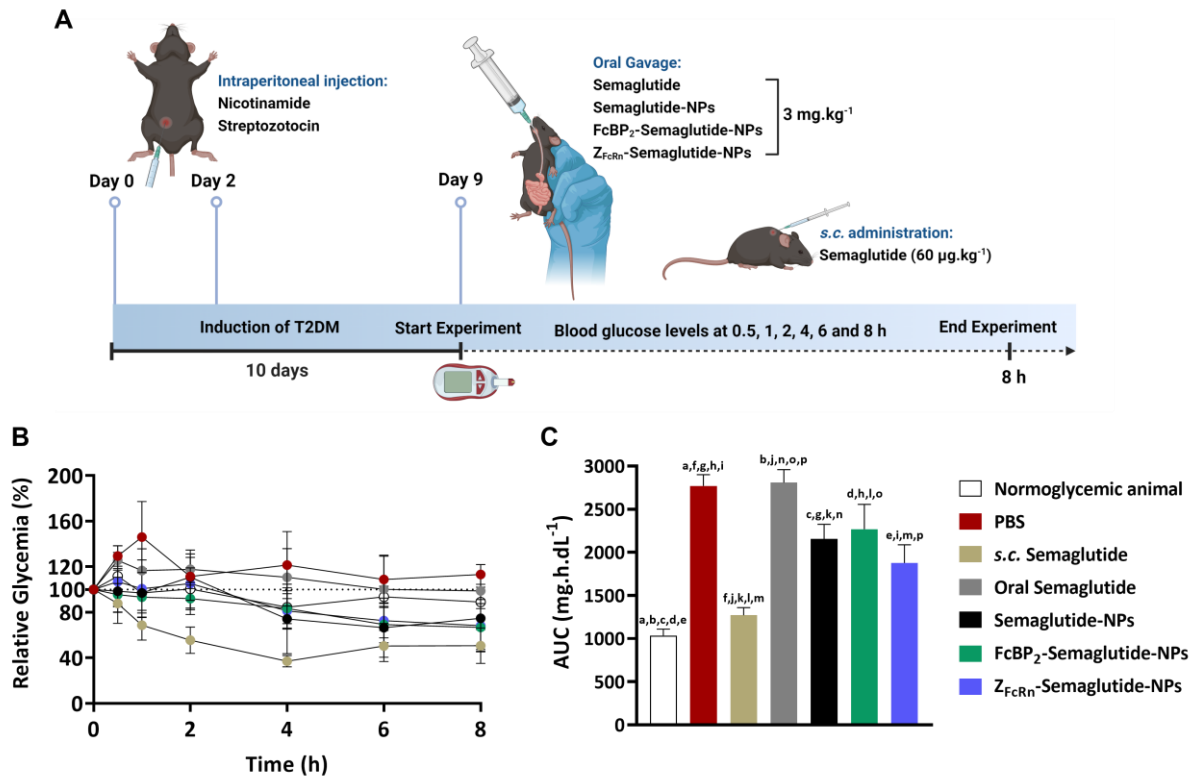


Figure 27. FcRn-targeted NPs reduce glycemia after a single administration. **(A)** Representative illustration of T2DM induction in hFcRn transgenic mice by *i.p.* injection of NA (210 mg.kg⁻¹) and STZ (180 mg.kg⁻¹) at days 0 and 2. Glucose levels were measured at day 9, which corresponds to 10 days after the first *i.p.* injection of NA and STZ. Animals with blood glucose levels ≥ 200 mg.dL⁻¹ were considered for the administration of the formulations. Glycemia was evaluated for 8 h at different time-points, upon a single administration of the samples. **(B)** Blood glucose levels of T2DM-induced mice were monitored for over 8 h *post-administration* of PBS, free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs through oral gavage, and free semaglutide via s.c. route. The relative glycemia (%) of each group was followed for over 8 h. Results are represented as mean $\pm$ SD ($n = 6$). **(D)** Blood glucose AUC (mg.h.dL⁻¹) from 0 h to 8 h, after oral and s.c. administrations of the formulations. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the groups. (a) denotes significant differences between normoglycemic animal and PBS, (b) corresponds the statistical differences between normoglycemic animal and oral free semaglutide, (c) represents statistical significance between normoglycemic and semaglutide-NPs, (d) denotes statistically significant differences between normoglycemic and FcBP₂-semaglutide-NPs, (e) corresponds the statistical differences between normoglycemic and Z_{FcRn}-semaglutide-NPs, (f) represents statistical significance between PBS and s.c. free semaglutide, (g) denotes statistically significant differences between PBS and semaglutide-NPs, (h) corresponds the statistical differences between PBS and FcBP₂-semaglutide-NPs, (i) represents statistical differences between PBS and Z_{FcRn}-semaglutide-NPs, (j) denotes statistically significant differences between s.c. free semaglutide and oral free semaglutide, (k) corresponds the statistical differences between s.c. free semaglutide and semaglutide-NPs, (l) represents statistical differences between s.c. free semaglutide and FcBP₂-semaglutide-NPs, (m) denotes statistically significant differences between s.c. free semaglutide and Z_{FcRn}-semaglutide-NPs, (n) corresponds the statistical differences between oral free semaglutide and semaglutide-NPs, (o) represents statistical difference between oral free semaglutide and FcBP₂-semaglutide-NPs, and (p) denotes significant differences between oral free semaglutide and Z_{FcRn}-semaglutide-NPs. The levels of significance were set at the probability of $p < 0.0001$. Scale bars represent mean $\pm$ SD ($n = 6$).

Table 7. Pharmacodynamic parameters of the formulations orally or subcutaneously administered to T2DM-induced hFcRn transgenic mice.

Group	Dose (mg.kg ⁻¹)	C _{min} (mg.dL ⁻¹)	t _{min} (h)	BGD (%)
PBS	-	315 ± 22	0	0 ± 13 ^{a,b,c,d}
s.c. semaglutide	0.060	110 ± 22	4	54 ± 4 ^{a,e,f}
oral semaglutide	3	320 ± 41	8	0 ± 6 ^{e,g,h,i}
semaglutide-NPs	3	222 ± 61	6	28 ± 11 ^{b,f,g}
FcBP ₂ -semaglutide-NPs	3	223 ± 120	8	32 ± 24 ^{c,h}
Z _{FcRn} -semaglutide-NPs	3	172 ± 70	8	45 ± 12 ^{d,i}

C_{min}: minimal concentration of glucose after administration of the formulations; t_{min}: time with minimal concentration of glucose after administration of the formulations; BGD: blood glucose decrease of each formulation for over 8 h of experiment; s.c.: subcutaneous; NPs: nanoparticles; FcBP₂: FcRn-targeted peptide 2; Z_{FcRn}: affibody.

The control selected for BGD calculation was the diabetic mice that received PBS by oral gavage. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between each group. (a) denotes statistically significant differences ($p < 0.0001$) between PBS and s.c. free semaglutide, (b) corresponds the statistically significant differences ($p < 0.01$) between PBS and semaglutide-NPs, (c) represents statistically significant differences ($p < 0.01$) between PBS and FcBP₂-semaglutide-NPs, (d) denotes statistically significant differences ($p < 0.0001$) between PBS and Z_{FcRn}-semaglutide-NPs, (e) corresponds the statistically significant differences ($p < 0.0001$) between s.c. free semaglutide and oral free semaglutide, (f) represents statistically significant differences ($p < 0.05$) between s.c. free semaglutide and semaglutide-NPs, (g) denotes statistically significant differences ($p < 0.01$) between oral free semaglutide and semaglutide-NPs, (h) corresponds the statistically significant differences ($p < 0.01$) between oral free semaglutide and FcBP₂-semaglutide-NPs, and (i) represents statistically significant differences ($p < 0.001$) between oral free semaglutide and Z_{FcRn}-semaglutide-NPs. C_{min} and BGD pharmacodynamic parameters are represented as mean ± SD ($n = 6$).

4.15.2. FcRn-targeted nanoparticles reduce glycemia after multiple administrations

Following the same format as the single-dose experiment, diabetic mice from each group were treated once a day for 7 days with PBS, free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs administered through oral gavage and free semaglutide administered via s.c. injection (**Figure 28A**). Glycemia was monitored in each group on the starting day of the experiment and on days 4 and 8, after daily oral administrations of the formulations, aiming to evaluate the glucose-lowering effect of the samples for a prolonged therapeutic window, better representing T2DM patients' clinical treatment. The mice subcutaneously treated with free semaglutide exhibited a 20% reduction in blood glucose levels on day 4 (**Figure 28B**), reaching the maximum decrease on day 8 (30% of reduction in glycemia). Regarding the mice orally treated with PBS or free semaglutide, no effect on blood glucose levels was observed throughout the experiment. The absence of glucoregulatory response in mice that received oral free semaglutide can be attributed to the degradation of the peptide by digestive enzymes from GIT [11, 80].

Furthermore, there was no observed effect in reducing blood glucose levels throughout the 8-day experiment with semaglutide-NPs, demonstrating a similar profile to PBS and oral free semaglutide groups. Conversely, both FcRn-targeted NPs groups demonstrated a marked reduction in blood glucose levels, which was similar (FcBP₂-semaglutide-NPs) or slightly higher (Z_{FcRn}-semaglutide-NPs) than the effect observed in the mice subcutaneously administered with free semaglutide. This reduction reached 40% on day 4, slightly increasing by day 8, yet remaining a noticeable glucoregulatory response. These findings reflect that the functionalization of the particles with FcRn-targeted ligands, FcBP₂ and Z_{FcRn}, induced a suitable pharmacological effect of semaglutide when compared to oral free semaglutide, and semaglutide-NPs, and similar or slightly higher in comparison with s.c. free semaglutide. The AUC values (0–8 days) of FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs, shown in **Figure 28C**, are consistent with the blood glucose levels described in **Figure 28B** and **Appendix VIII**, where FcRn-targeted NPs had a significantly higher pharmacological effect in reducing glycemia than oral free semaglutide ($p < 0.0001$) and non-targeted NPs ($p < 0.001$ for FcBP₂-semaglutide-NPs and $p < 0.0001$ for Z_{FcRn}-semaglutide-NPs). Notwithstanding, the AUC values of FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs were similar or even lower, respectively, when compared with the s.c. free semaglutide. Therefore, the FcRn-targeted NPs can be considered to replace the s.c. administration of semaglutide due to their similar glucose-lowering effect observed after 8 days of experiment. These findings suggest that the improved glucoregulatory effect of FcRn-targeted NPs over the other formulations upon multiple administrations can be related with the binding of the particles to hFcRn found on the luminal surface of the enterocytes, which enhanced the intestinal delivery of semaglutide [17]. The results are in agreement with the *in vitro* uptake studies (**Chapter 4, sections 4.7. and 4.12.**), where the FcRn-targeted NPs showed a higher interaction with intestinal models that endogenously express the hFcRn in comparison with non-targeted NPs, reinforcing the hypothesis of these nanosystems being transported through the intestinal epithelium *via* hFcRn pathway. Additionally, the reduction observed in blood glucose levels of diabetic mice orally treated with FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs was similar or higher than the reduction observed in other reports, in which GLP-1 analogs were entrapped into NPs [17, 121, 122, 337].

Overall, the FcRn-targeted NPs demonstrated to be suitable oral drug delivery nanoplatforms for improving the pharmacological effect of semaglutide, when compared with oral and s.c. free semaglutide groups and non-targeted semaglutide-NPs group. Although FcRn-targeted semaglutide-NPs showed ability to control glycemia of T2DM-induced hFcRn transgenic mice, their antidiabetic action is associated with the stimulation of insulin secretion and the decrease of glucagon release from pancreatic β and α cells,

respectively. Therefore, these nanoplateforms incorporating semaglutide are responsible to settle the hurdle of insufficient insulin secretion by β cells, which is commonly observed in T2DM patients. However, T2DM disorder is also characterized by resistance of the peripheral tissues (e.g., liver, muscle, and adipose tissue) to insulin. Exploring the glucose-lowering response of FcRn-targeted semaglutide-NPs, a combinatory therapy of these nanoplateforms with other antidiabetic drugs with ability to enhance the insulin sensitivity in peripheral tissues, such as biguanides (e.g., metformin) or thiazolidinediones (e.g., rosiglitazone or pioglitazone), can be considered. The combination of the NPs with these antidiabetic drugs can lead to a synergistic effect, resulting in sustained improvement in glycemic control, when compared with monotherapy [338-344]. Moreover, FcRn-targeted semaglutide-NPs can also be administered in combination with sulfonylureas (e.g., glimepiride or gliclazide) to potentiate their action. Sulfonylureas also stimulate insulin secretion from pancreatic β cells [344-347], which can complement the action of semaglutide-NPs. Additionally, other combination therapies can be considered, such as the co-administration of FcRn-targeted semaglutide-NPs with SGLT2 inhibitors (e.g., dapagliflozin, empagliflozin or ertugliflozin). Although the mechanism of action of SGLT2 inhibitors is not directly related with the two main factors responsible for the development of T2DM, insulin resistance and insufficient insulin secretion, this class is responsible for promoting urinary glucose excretion through the reduction of the renal glucose reabsorption, leading to a decrease in blood glucose levels [348-351].

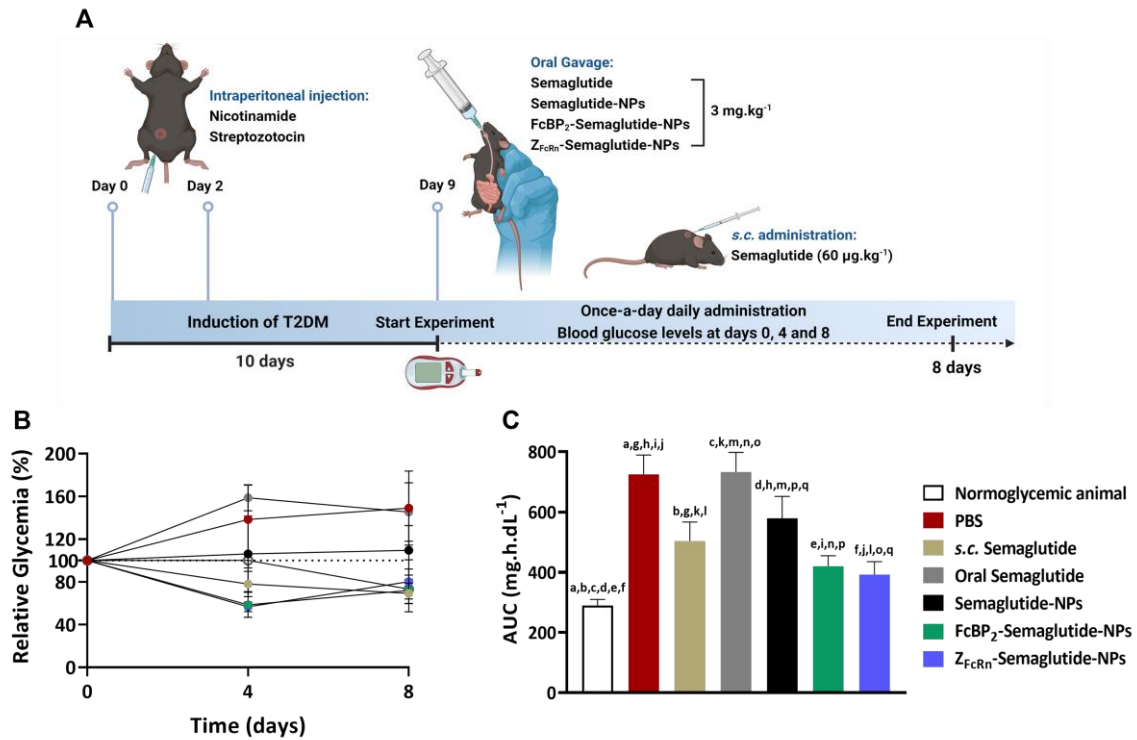


Figure 28. FcRn-targeted NPs reduce glycemia after multiple administrations. **(A)** Representative illustration of T2DM induction in hFcRn transgenic mice by *i.p.* injection of NA (210 mg.kg⁻¹) and STZ (180 mg.kg⁻¹) at days 0 and 2. Glucose levels were measured at day 9, which corresponds to 10 days after the first *i.p.* injection of NA and STZ. Animals with blood glucose levels ≥ 200 mg.dL⁻¹ were considered for the administration of the samples. Glycemia was evaluated at days 0, 4 and 8, upon daily administrations, once a day. **(B)** Blood glucose levels of T2DM-induced mice were monitored for over 8 days, after multiple administrations of PBS, free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs by oral gavage, and free semaglutide via s.c. route, once a day. Results are represented as mean $\pm$ SD ($n = 6$). **(D)** Blood glucose AUC from day 0 to day 8, after oral and s.c. administrations of the formulations. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the groups. (a) denotes statistically significant differences ($p < 0.0001$) between normoglycemic and PBS, (b) corresponds the statistically significant differences ($p < 0.0001$) between normoglycemic and s.c. free semaglutide, (c) represents statistically significant differences ($p < 0.0001$) between normoglycemic and oral free semaglutide, (d) denotes statistically significant differences ($p < 0.0001$) between normoglycemic and semaglutide-NPs, (e) corresponds the statistically significant differences ($p < 0.01$) between normoglycemic and FcBP₂-semaglutide-NPs, (f) represents statistically significant differences ($p < 0.05$) between normoglycemic and Z_{FcRn}-semaglutide-NPs, (g) corresponds the statistically significant differences ($p < 0.0001$) between PBS and s.c. free semaglutide, (h) denotes statistically significant differences ($p < 0.0001$) PBS and semaglutide-NPs, (i) represents the statistically significant differences ($p < 0.0001$) between PBS and FcBP₂-semaglutide-NPs, (j) corresponds the statistically significant differences ($p < 0.0001$) between PBS and Z_{FcRn}-semaglutide-NPs, (k) denotes statistically significant differences ($p < 0.0001$) between s.c. free semaglutide and oral free semaglutide, (l) represents statistically significant differences ($p < 0.05$) between s.c. free semaglutide and Z_{FcRn}-semaglutide-NPs, (m) corresponds the statistically significant differences ($p < 0.001$) between oral free semaglutide and semaglutide-NPs, (n) denotes statistically significant differences ($p < 0.0001$) between oral free semaglutide and FcBP₂-semaglutide-NPs, (o) represents statistically significant differences ($p < 0.0001$) between oral free semaglutide and Z_{FcRn}-semaglutide-NPs, (p) corresponds the statistically significant differences ($p < 0.001$) between semaglutide-NPs and FcBP₂-semaglutide-NPs, and (q) denotes statistically significant differences ($p < 0.0001$) between semaglutide-NPs and Z_{FcRn}-semaglutide-NPs. Scale bars represent mean $\pm$ SD ($n = 6$).

4.15.3. FcRn-targeted nanoparticles attenuate systemic inflammation

T2DM is a chronic inflammatory disease characterized by resistance to insulin, which is strongly related with an increase in the levels of proinflammatory cytokines, such as IL-1 β , IL-6, IL-18 and TNF- α [352-356]. GLP-1 analogs exhibit anti-inflammatory properties, being involved in the reduction of plasma levels of proinflammatory cytokines, and therefore, they are suitable to improve T2DM management [357-360]. Thus, it was hypothesized that semaglutide incorporated into polymeric NPs can progressively reduce the inflammatory scenario of T2DM-induced mice. The levels of IL-1 β , IL-6, IL-18 and TNF- α in plasma of diabetic mice were analyzed 8 h and 8 days after treatment with the formulations (**Figure 29**). Only IL-18 was detected in the plasma of the mice above the kit limit of detection. Increased circulating IL-18 levels have been previously described in both T2DM patients and hyperglycemic mice [354, 361, 362]. Pancreatic IL-18, produced by α cells, contributes to β cells proliferation and insulin production. β cells damage and number reduction, accompanied by IL-18 receptor loss at the pancreatic levels and elevated circulating IL-18, enhances macrophage recruitment and promotes hyperglycemia and insulin resistance [363].

Upon single administration, normoglycemic mice exhibited IL-18 levels statistically significantly lower than the PBS ($p < 0.01$) and the oral free semaglutide ($p < 0.05$) groups (**Figure 29A**). Moreover, the animals orally treated with FcRn-targeted NPs had a tendency for lower plasma IL-18 levels than the animals receiving oral free semaglutide or semaglutide-NPs. Furthermore, the animals receiving Z_{FcRn}-semaglutide-NPs had similar serum levels of IL-18 compared to the group receiving s.c. free semaglutide, while FcBP₂-semaglutide-NPs had lower serum levels of IL-18 than the group treated with s.c. free semaglutide. Similarly, the expression of IL-18 was also analyzed in the mice treated once a day for over one week with the nanoformulations (**Figure 29B**), which shows that the diabetic mice orally treated with PBS, free semaglutide or semaglutide-NPs had the highest plasma IL-18 levels, supporting the results previously discussed, in which those treatments did not exhibit a glucose-lowering effect. Animals receiving FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs presented lower plasma IL-18 levels than the groups receiving PBS, oral free semaglutide or semaglutide-NPs. Furthermore, animals receiving one of the FcRn-targeted nanosystems resulted in plasma IL-18 levels similar to the group receiving s.c. free semaglutide, suggesting that the oral administration of these NPs slightly controlled the inflammation in T2DM-induced mice. The absence of significant differences between mice groups receiving FcRn-targeted NPs and the other mice groups might be associated with the relatively short study period. A decrease in cytokines might be more pronounced in long-term studies [358].

Overall, the single administration and multiple administrations of FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs had a suitable *in vivo* pharmacological effect in controlling blood glucose levels while attenuating the systemic inflammation, which represented favorable therapeutic actions in the T2DM animal model employed.

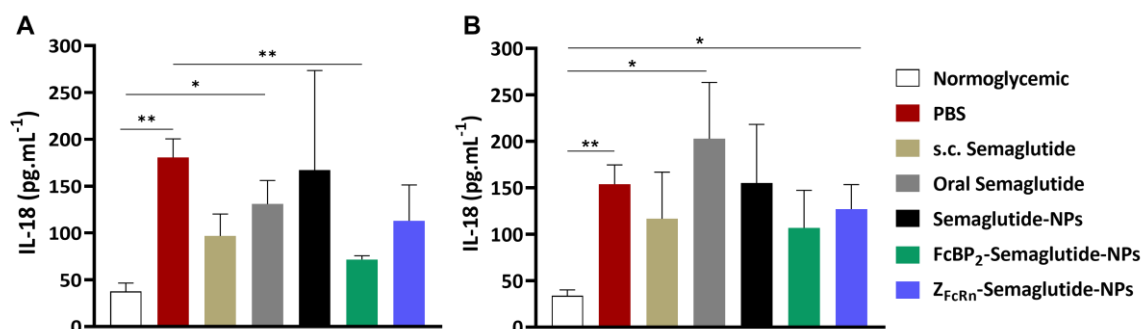


Figure 29. Amount of IL-18 detected in the plasma after single and multiple administrations of the samples in T2DM-induced mice. **(A)** Plasma IL-18 levels 8 h after administration of the samples to T2DM-induced hFcRn transgenic mice. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the groups. (**) denotes significant differences ($p < 0.01$) between normoglycemic animal and PBS, and FcBP₂-semaglutide-NPs and PBS. (*) represents significant differences ($p < 0.05$) between normoglycemic animal and oral free semaglutide. **(B)** Plasma IL-18 levels in T2DM-induced hFcRn transgenic mice 8 days *post*-administration. (**) denotes significant differences between normoglycemic animal and PBS ($p < 0.01$), (*) corresponds the statistical differences ($p < 0.05$) between normoglycemic animal and oral free semaglutide, and normoglycemic animal and Z_{FcRn}-semaglutide-NPs. Results are represented as mean $\pm$ SEM ($n = 4$).

4.15.4. FcRn-targeted nanoparticles increase recovery of insulin-producing β cells

Semaglutide, as a GLP-1 analog, triggers insulin secretion and suppresses glucagon release in a glucose-dependent manner thereby, reducing the risk of hyperglycemia [66, 364]. Thus, the administration of semaglutide at therapeutic doses can restore the insulin-secretory function of β cells in T2DM patients [365-367].

The plasma insulin levels (**Figure 30A** and **Figure 30B**) and the insulin pancreatic content (**Figure 30C** and **Figure 30D**) of the different experimental groups were evaluated at the end of 8 h or 8 days. Upon 8 h of single administration, the plasma insulin levels of semaglutide-NPs group ($p < 0.05$) and the Z_{FcRn}-semaglutide-NPs group ($p < 0.01$) were significantly higher than the oral free semaglutide and PBS groups (**Figure 30A**). Additionally, the FcBP₂-semaglutide-NPs group exhibited a tendency for higher levels of insulin in the plasma than the oral free semaglutide group. Notwithstanding, both FcRn-targeted NPs also resulted in a statistically higher amount of insulin in the pancreas compared to the PBS group ($p < 0.05$ for FcBP₂-semaglutide-NPs and $p < 0.001$ for Z_{FcRn}-semaglutide-NPs) and the oral free semaglutide group ($p < 0.05$ for FcBP₂-semaglutide-NPs and $p < 0.01$ for Z_{FcRn}-semaglutide-NPs); although no significant differences were

observed between semaglutide-NPs and both FcRn-targeted NPs (**Figure 30C**). These outcomes are in accordance with the glycemia profile, where the FcBP₂-semaglutide NPs and Z_{FcRn}-semaglutide NPs groups had a BGD of ~ 32% and ~ 45%, respectively, while the control groups, PBS and oral free semaglutide, did not showed a reduction in blood glucose levels throughout the experiment. Moreover, both FcRn-targeted NPs groups and the s.c. free semaglutide group presented a similar amount of insulin in the pancreas, suggesting that the entrapment of semaglutide into PLGA-NPs targeting the FcRn receptor induced a similar pharmacological effect to the s.c. administration of the antidiabetic peptide.

In contrast, upon daily administrations for 7 days, FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs induced a tendency for higher levels of plasma insulin compared to the control groups (PBS, oral free semaglutide and s.c. free semaglutide) (**Figure 30B**). Besides that, both FcRn-targeted NPs presented a statistically significantly higher insulin pancreatic content than PBS ($p < 0.01$ for FcBP₂-semaglutide-NPs and $p < 0.05$ for Z_{FcRn}-semaglutide-NPs) and oral free semaglutide ($p < 0.05$), as shown in **Figure 30D**. Importantly, the amount of insulin detected in the pancreas of the animals receiving FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs was notably higher than those receiving semaglutide-NPs ($p < 0.01$ for FcBP₂-semaglutide-NPs and $p < 0.05$ for Z_{FcRn}-semaglutide-NPs). Once again, these results are in line with the blood glucose level measurements, where both FcRn-targeted NPs demonstrated a suitable glucoregulatory response compared to PBS, oral free semaglutide and semaglutide-NPs. Notably, the s.c. free semaglutide group was the only group showing a glucose-lowering effect analogous to both FcRn-targeted NPs groups. Overall, the entrapment of semaglutide into FcBP₂-NPs and Z_{FcRn}-NPs caused a tendency increase in plasma insulin levels after oral daily administrations. As highlighted in previous studies, a significant rise in plasma insulin levels is not readily observed after daily oral administrations for over one week. However, such effects are primarily noticed in long-term studies lasting at least one month [118, 123, 124, 368-371], potentially accounting for the lack of significant differences between the particles and the control groups. Regarding the insulin pancreatic content, it was already reported in other studies that a single oral administration of GLP-1 analogs incorporated into nanoplateforms was able to improve the amount of insulin in the pancreas compared with the control (PBS group) or the oral free GLP-1 analogs [11, 119].

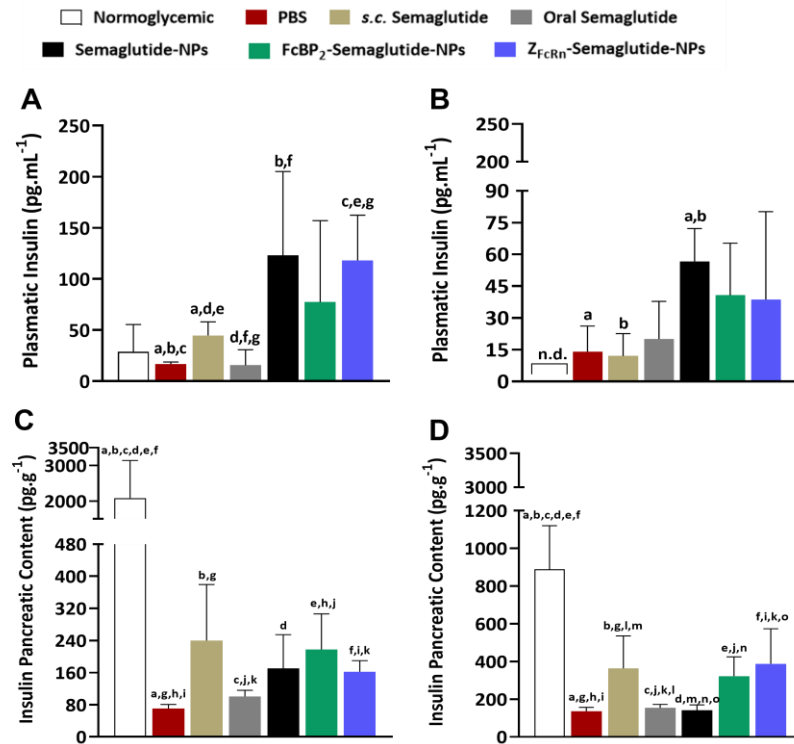


Figure 30. (A) Plasma insulin levels in T2DM-induced mice after 8 h of treatments. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the groups. (a) denotes significant differences of PBS and s.c. free semaglutide ($p < 0.05$), (b) corresponds statistical differences of PBS vs semaglutide-NPs ($p < 0.05$), (c) represents significant differences of PBS vs Z_{FcRn}-semaglutide-NPs ($p < 0.01$), (d) denotes significant differences of s.c. free semaglutide vs oral free semaglutide ($p < 0.05$), (e) corresponds statistical differences of s.c. free semaglutide vs Z_{FcRn}-semaglutide-NPs ($p < 0.05$), (f) represents significant differences of oral free semaglutide vs semaglutide-NPs ($p < 0.05$) and (g) denotes significant differences of oral free semaglutide vs Z_{FcRn}-semaglutide-NPs ($p < 0.01$). (B) Plasma insulin levels in T2DM-induced mice after 8 days of daily administration of the samples via oral gavage and s.c. route. (a) denotes significant differences of PBS vs semaglutide-NPs ($p < 0.01$), (b) corresponds statistical differences of s.c. free semaglutide and semaglutide-NPs ($p < 0.01$). n.d. means not detected. Insulin pancreatic content 8 h (C) and 8 days (D) post-administration of samples. (a) denotes statistically significant differences of normoglycemic animal vs PBS ($p < 0.001$), (b) corresponds statistical differences of normoglycemic animal vs s.c. free semaglutide ($p < 0.0001$ for (C) and $p < 0.001$ for (D)), (c) represents significant differences of normoglycemic animal vs oral free semaglutide ($p < 0.0001$), (d) denotes statistical differences of normoglycemic animal vs semaglutide-NPs ($p < 0.0001$), (e) corresponds significant differences of normoglycemic animal vs FcBP₂-semaglutide-NPs ($p < 0.0001$ for (C) and $p < 0.001$ for (D)), (f) represents statistical differences of normoglycemic animal vs Z_{FcRn}-semaglutide-NPs ($p < 0.0001$ for (C) and $p < 0.001$ for (D)), (g) denotes statistical differences of PBS vs s.c. free semaglutide ($p < 0.05$), (h) corresponds significant differences of PBS and FcBP₂-semaglutide-NPs ($p < 0.05$ for (C) and $p < 0.01$ for (D)), (i) represents statistical differences of PBS vs Z_{FcRn}-semaglutide-NPs ($p < 0.001$ for (C) and $p < 0.05$ for (D)), (j) denotes significant differences of oral free semaglutide vs FcBP₂-semaglutide-NPs ($p < 0.05$), (k) corresponds statistical differences of oral free semaglutide vs Z_{FcRn}-semaglutide-NPs ($p < 0.01$ for (C) and $p < 0.05$ for (D)), (l) represents significant differences of s.c. free semaglutide vs oral free semaglutide ($p < 0.05$), (m) denotes statistical differences of s.c. free semaglutide vs semaglutide-NPs ($p < 0.05$), (n) corresponds significant differences of semaglutide-NPs vs FcBP₂-semaglutide-NPs ($p < 0.01$) and (o) represents significant differences of semaglutide-NPs vs Z_{FcRn}-semaglutide-NPs ($p < 0.05$). Results are represented as mean $\pm$ SD ($n = 4$).

Additionally, the pancreas sections from each group were analyzed by immunohistochemistry to determine the effect of the treatments in the recovery of insulin-producing β cells mass. Representative micrographs of the islets from the mice treated with the nanoformulations are shown in **Figure 31A**, where insulin and glucagon are stained as **pink** and **brown** colors, respectively. The mice treated with FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs showed an increase in the amount of insulin staining compared to the other groups, and a closer resemblance with the normoglycemic group. A tendency for higher amount of β cells/mm² (**Figure 31B**) and β cells/ α cells ratio (**Figure 31D**) owing to the treatment with both FcRn-targeted NPs was obtained, suggesting that these nanoformulations were able to induce a recovery of β cell mass over the non-targeted NPs and the control groups (PBS, oral free semaglutide and s.c. free semaglutide). These results are in line with the literature since it is described that semaglutide can increase the islet cell proliferation and the recovery of β cells mass in diabetic patients [366]. Although FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs slightly increase the amount of α cells/mm² (**Figure 31C**), the β cells/ α cells ratio is still the highest values observed compared to the other diabetic mice groups. Therefore, both FcRn-targeted semaglutide-NPs demonstrated promising potential to improve the secretory insulin function of β cells.

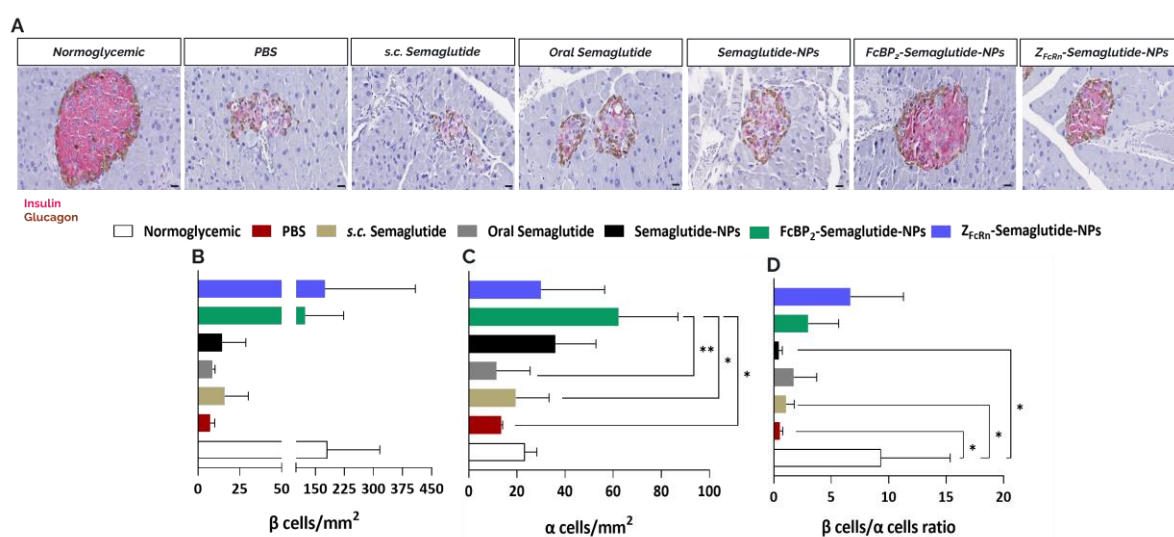


Figure 31. Insulin and glucagon detected in the pancreas tissues of the T2DM-induced mice treated for over 7 days with the nanoformulations. **(A)** Representative immunohistochemistry images of pancreas sections stained with primary antibodies mouse monoclonal anti-insulin antibody (**pink**) and rabbit polyclonal anti-glucagon antibody (**brown**) obtained in the scanning Photolmager software. Scale bars represent 20 μ m. Amount of **(B)** β cells/mm², **(C)** α cells/mm² of pancreas tissues, and **(D)** β cells/ α cells ratio. One-way ANOVA analysis of variance (Tukey's test) was used to analyze the differences between the groups. (*) and (**) denote significant differences of $p < 0.05$ and $p < 0.01$, respectively. Absence of * symbol indicates non-significant differences. Results are represented as mean $\pm$ SD of two sections per animals ($n = 2$).

4.15.5. FcRn-targeted semaglutide-loaded nanoparticles maintain morphology of type 2 diabetic mice organs

In the current study, H&E staining was used to evaluate the morphology and integrity of pancreas, heart, lung, liver, kidney, spleen, stomach, small intestine, and large intestine (**Figure 32**) of T2DM-induced mice, after daily administrations of free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs through oral gavage, and free semaglutide via s.c. route for over one week. Control groups, represented as PBS and normoglycemic mice, were included in the experiment.

In T2DM a reduction of 40% of pancreatic islets occurs, and as a consequence the secretion of insulin is compromised, leading to hyperglycemia [1]. As shown in **Figure 32**, the PBS group exhibited a noticeable reduction in the number and size of pancreatic islets compared to the normoglycemic group. This can be attributed to the induction of T2DM that causes the partial destruction of pancreatic β cells [228]. However, pancreatic islets of the mice treated with FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs were noticeably more alike in number and size to the normoglycemic group. These data can be related to the ability of semaglutide to improve the production of pancreatic islets [365-367].

In T2DM patients the onset of microvascular and macrovascular complications affecting the normal function of liver, kidney, heart, lung and other tissues is commonly observed when hyperglycemia is not properly managed [40]. However, in the current study, it was observed that the induction of T2DM in hFcRn transgenic mice did not affect the morphology and integrity of the tissues since no notable structural changes or histopathological signs were detected (**Figure 32**). Moreover, the architecture of the tissues was also devoid of any pathological features and toxicity signs after the treatments for one week, revealing that the formulations administered to the animals were safe.

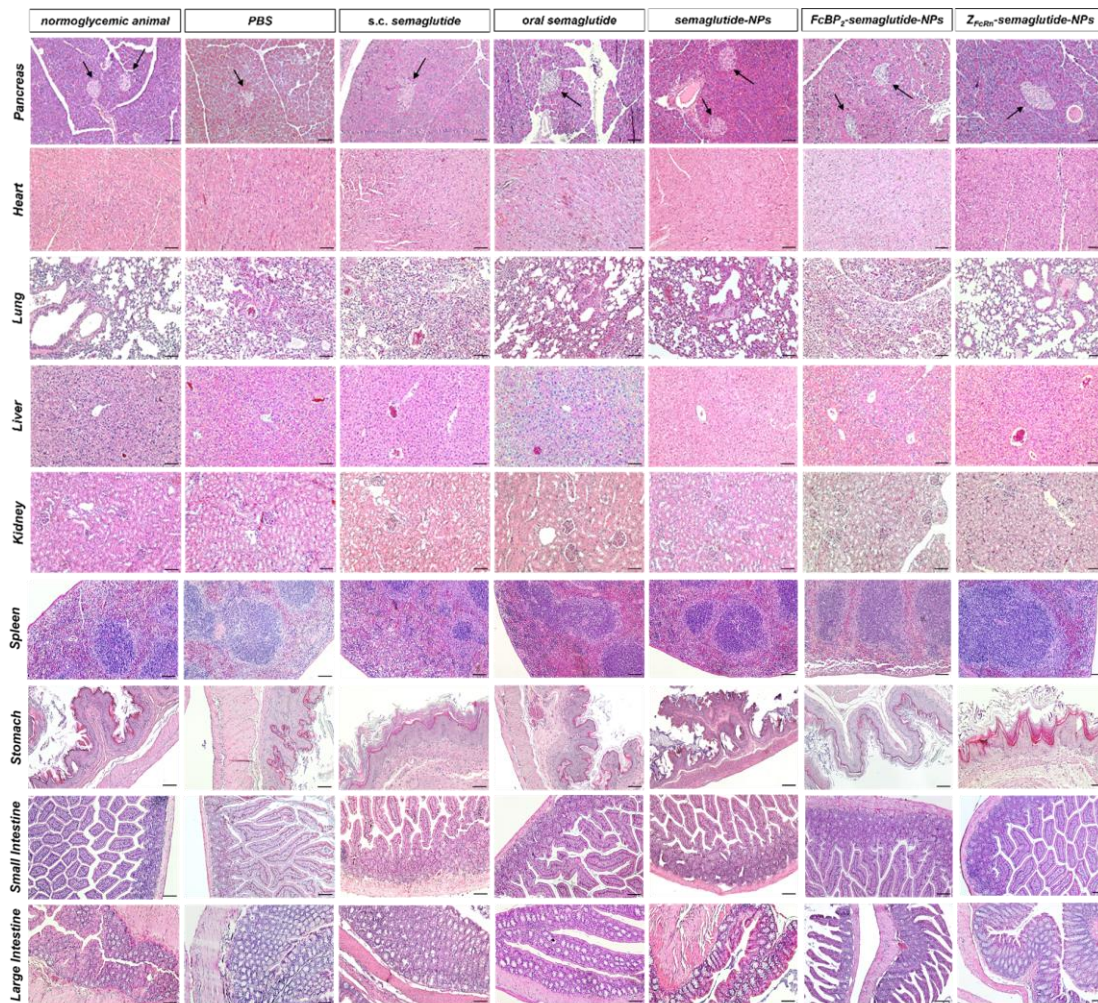


Figure 32. Representative micrograph of sections of pancreas, heart, lung, liver, kidney, spleen, stomach, and small and large intestine from each mice group with H&E staining. Scale bars represent 50 μ m.

Chapter | 5

Conclusions and Future Perspectives

5.1. Conclusions

In this thesis, semaglutide-loaded NPs were developed for intestinal-targeted delivery by means of surface-functionalization with ligands targeting the hFcRn receptor. The binding-affinity of FcRn-targeted ligands to the human receptor was tested by SPR analysis, showing that the FcBP₂ and the Z_{FcRn} had a stronger interaction with the receptor in an acidic pH-dependent manner. Further, both ligands were covalently linked with the PLGA-PEG-Mal co-polymer by Mal-thiol chemistry, resulting in CE values of approximately 30% and 80% for Z_{FcRn} and FcBP₂, respectively. The successful conjugation of PLGA-PEG-Mal:Z_{FcRn} and PLGA-PEG-Mal:FcBP₂ was additionally confirmed by XPS analysis, where the relative atomic percentages of oxygen and nitrogen were higher than the non-functionalized PLGA-PEG-Mal. It was also detected the presence of sulfur in both conjugated polymers, in which sulfur is found on the Cys residue located on the N-terminal and C-terminal of FcBP₂ and Z_{FcRn}, respectively. Semaglutide-loaded NPs were produced with the functionalized polymers through w/o/w double emulsion technique, resulting in monodisperse nanosystems with a Z-average of 170 nm, a neutral surface charge, and an AE and loading degree of semaglutide of 60% and 3%, respectively. Notably, the encapsulation of semaglutide into nanoplateforms has not been previously explored, highlighting the clinical relevance of this investigation. Regarding the production of NPs, it is important to determine whether the FcRn-targeted semaglutide-NPs could be produced on an industrial scale and how the physicochemical properties (Z-average, Pdl, ζ-potential, AE and loading degree of semaglutide, and CE of FcBP₂ and Z_{FcRn} with the PLGA-PEG-Mal co-polymer) of these nanosystems are maintained over time. For this, it is required to optimize the volume and amount of semaglutide, polymers, and surfactant agent, as well as the time of the production (*e.g.*, sonication time and evaporation time of the organic solvent), in order to obtain NPs with similar properties with the ones produced before the large-scale production. To reduce the variability between batches, large-scale systems that are managed with higher volumes and quantities of components will be used. After production, the NPs need to be purified to remove the excess of surfactant agent and the non-encapsulated semaglutide, and thus, techniques as ultracentrifugation, filtration, among others, need to be optimized for a large-scale purification. One of the main drawbacks related with the scaling-up of these nanosystems is the conjugation of PLGA-PEG-Mal co-polymer with the FcRn-targeted ligands. In this case, higher amounts of ligands will be required, which can be expensive. Hereupon, the ratio between polymer and ligands needs to be optimized to rationalize the amount of FcBP₂ and Z_{FcRn} spent in an industrial scale.

The interaction of FcBP₂-NPs and Z_{FcRn}-NPs with the hFcRn receptor was evaluated by SPR analysis, as performed for the free ligands. Both FcRn-targeted nanosystems (FcBP₂-

NPs and Z_{FcRn} -NPs) interacted with hFcRn in a pH-dependent manner, with a stronger interaction at acidic pH than at physiological pH, which was also observed for the free ligands. These data suggested that the Mal-thiol reaction performed to conjugate the ligands with the co-polymer PLGA-PEG-Mal and the manufacturing process applied to produce the nanosystems did not compromise the interaction of the ligands with the receptor. By *in vitro* uptake studies, it was demonstrated that both FcRn-targeted NPs had a 2-fold higher interaction with intestinal cells that endogenously express the hFcRn, Caco-2 cells, when compared to non-targeted NPs, due to the presence of the ligands on their surface. The FcRn-targeted capacity of NPs was also demonstrated in a 3D *in vitro* intestinal model, HIOs, due to its closer resemblance with the *in vivo* architecture, suitable mimicking the biological complexity and functionality of the human intestine. Firstly, it was proved that HIOs also endogenously express the human receptor and therefore, it could be interesting to evaluate the trafficking of the nanosystems through this model. However, the main drawback related to the study of epithelial cell–NP interactions in HIOs is to reach the apical surface of the epithelium, which corresponds to the center of the organoid. For this reason, the NPs were injected into the lumen to better mimic what happens *in vivo*. Only few studies were performed to evaluate the potential of HIOs on the screening of NPs, and none of them were conducted by injecting the NPs into the lumen of the organoids. By *in vitro* uptake studies, it was observed that the FcRn-targeted NPs demonstrated an enhanced interaction with HIOs than non-targeted NPs, which is in line with the results obtained for Caco-2 cells. Therefore, both FcRn-targeted NPs demonstrated to be a promising strategy for improving the intestinal delivery of semaglutide.

The glucoregulatory effect of FcBP₂-semaglutide-NPs and Z_{FcRn} -semaglutide-NPs in T2DM induced-transgenic hFcRn mouse model was evaluated after a single oral administration and after daily oral administrations for 7 days. T2DM was chemically induced to hFcRn transgenic mice through the NA-STZ model. Firstly, it was confirmed that the *i.p.* injection of NA and STZ at doses of 210 mg.kg⁻¹ and 180 mg.kg⁻¹, respectively, on days 0 and 2 of the experiment, were able to induce T2DM in hFcRn transgenic mice since the blood glucose levels achieved values equal or higher than 200 mg.dL⁻¹ and a decrease in the body weight of the animals was detected, probably due to the partial destruction of the pancreatic β cells. Regarding the glucose-lowering effect of the nanosystems, the oral administration of FcRn-targeted semaglutide-NPs to diabetic mice improved the glucoregulatory response over the oral free semaglutide in solution, after 8 h of a single oral administration (reduction of 30% of blood glucose levels) and after 4 days of daily oral administrations (reduction of 40% of blood glucose levels). Also, in the study conducted for 8 days, it was observed that both FcRn-targeted NPs had a similar glucoregulatory effect compared with s.c. free semaglutide. In fact, after 4 days of daily administrations, the

animals that received FcRn-targeted NPs had a more pronounced reduction in blood glucose levels (around 40%) than the animals subcutaneously administered with free semaglutide (around 30%). Therefore, FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs could be considered to replace the s.c. administration of free semaglutide. However, further studies are required to confirm this statement, including to evaluate the glucose-lowering response of these nanosystems in a long-term treatment (at least one month). Additionally, the non-targeted NPs were not able to reduce the blood glucose levels of diabetic mice, being the glycemic profile similar to the T2DM-induced mice treated with PBS. The loading of semaglutide into FcRn-targeted nanosystems increased the insulin pancreatic content and the plasma insulin levels, after multiple oral administrations of the particles. Interestingly, the insulin pancreatic content after 7 days of treatment was significantly higher for the mice treated with FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs, when compared to the mice treated with oral free semaglutide and non-targeted NPs. Notwithstanding, the animals that received FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs demonstrated a similar insulin pancreatic content with the animals treated with s.c. free semaglutide, reinforcing the hypothesis of using these nanosystems to replace the administration of the peptide via an invasive route. Despite the mice treated with FcRn-targeted NPs showed a favorable effect in controlling glycemia while attenuating the systemic inflammation (decrease of plasmatic IL-18 levels *post-treatment*), further studies are required to demonstrate the *in vivo* anti-inflammatory response of these nanosystems. Additionally, it must be considered the introduction of Rybelsus® as a control group of the *in vivo* study and compare its PD profile, insulin pancreatic content, and anti-inflammatory response with the groups treated with FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs, in order to consider these nanosystems as alternative to Rybelsus®. For that, the *in vivo* studies need to be performed using the same dose applied in the clinic for Rybelsus®. In this thesis, the nanosystems were administered to the mice considering a dose of semaglutide of 3 mg/kg, which corresponds to a human dose of semaglutide of 233 µg/kg, as described in **Chapter 3, section 3.9.3.1.**, which is the dose of Rybelsus® currently administered to T2DM patients. PK studies of the semaglutide incorporated into FcRn-targeted NPs need to be considered as well, and compared with the PK profile of Rybelsus®. It is expected that the FcRn-targeted NPs exhibited an improved oral bioavailability, and a higher glucose-lowering effect than the oral tablet form of semaglutide.

The biodistribution of the fluorescently labeled FcBP₂-NPs and Z_{FcRn}-NPs through the GIT of T2DM-induced mice was also evaluated. Both FcRn-targeted NPs were successfully transported throughout the GIT, exhibiting greater retention in the intestinal sections that highly express hFcRn, namely the ileum and the colorectum, 6 h *post-administration*, than the B-NPs. Hence, the results of the biodistribution assay corroborate the data obtained in

the *in vitro* uptake studies performed in 2D and 3D *in vitro* intestinal models, where FcBP₂-NPs and Z_{FcRn}-NPs had a higher internalization than B-NPs, reinforcing the statement that both FcRn-targeted NPs were interacting with intestinal epithelial cells via FcRn pathway. Although in this study it was considered the use of hFcRn transgenic mouse model that closer resemblance the interaction of the ligands FcBP₂ and Z_{FcRn} with the receptor in humans, the translation of the results obtained for both FcRn-targeted NPs from mice to humans needs to be considered with caution. Mice and humans differ in terms of physiology, immune response, metabolism, and PK/PD profile of drugs or nanosystems. Also, humans and mice have different expression of receptors, and other molecules that might compromise the overall response to the drug delivery nanosystem. Notwithstanding, in this thesis, T2DM was chemically induced to hFcRn transgenic mice following the NA-STZ model, which is well-known that does not fully resemble the T2DM disorder. NA-STZ model is mainly characterized by the partial destruction of the pancreatic β cells, leading to a substantially decrease in the secretion of insulin. However, T2DM is not only characterized by inadequate secretion of insulin, but also for the resistance of the peripheral tissues to insulin. Therefore, the results of *in vivo* studies might not fully predict what will happen in humans. Even though several models were already considered to induce T2DM (**Chapter 2, section 2.7.1.**), none of them fully resemble the pathophysiology of this disorder, and therefore, the translation of the results obtained from T2DM-induced mice to humans required careful consideration and validation. Nonetheless, to further protect the FcBP₂ and Z_{FcRn} located on the surface of the particles from GIT degradation, the NPs can be incorporated into gastro-resistant tablets or capsules. Therefore, the NPs need to be freeze-dried before their incorporation into tablets or capsules. To ensure the freeze-dried NPs will induce a similar or an enhanced response when compared with NPs in suspension, the physicochemical properties of the NPs, the secondary structure of semaglutide, and the binding of FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs to hFcRn must be evaluated after freeze-dried. If required, a cryoprotectant agent can be added to the nanoformulations before freeze-dried to enable the maintenance of their features.

According to the results obtained in this PhD thesis, the entrapment of semaglutide into nanocarriers targeting the intestinal hFcRn receptor was demonstrated to be a promising approach for improving the intestinal delivery of semaglutide and ultimately, for enhancing the treatment of T2DM.

5.2. Future perspectives

Although this PhD work demonstrated tremendous potential to improve the current treatment of T2DM, other relevant studies and new directions can be considered to highlight the discoveries found in this thesis.

Firstly, to protect the FcRn-targeted ligands from the GIT degradation, the nanosystems can be incorporated into gastro-resistant capsules, tablets, or a pH-responsive polymeric matrix composed of HPMC, Eudragit® L30D, or others. In the case of incorporating the FcRn-targeted NPs into gastro-resistant capsules or tablets, the nanosystems need to be freeze-dried and therefore, it is required to evaluate whether these systems are able to retain their physicochemical properties (Z-average, Pdl, ζ -potential, AE and loading degree of semaglutide), the secondary structure of semaglutide, and the preservation of the interaction between the ligands and the hFcRn receptor. Moreover, it would be important to analyze the protective effect of gastro-resistant capsules or tablets, or the pH-responsive polymeric matrix for NPs under conditions mimicking the gastric environment, by performing an *in vitro* release study, before moving forward with the *in vivo* assays.

In the *in vivo* studies, a higher number of animals per group could be included in the assays to suitably evaluate the significant differences observed between each animal group. Another experiment to be pursued is to evaluate the PK profile of FcRn-targeted semaglutide-NPs since only PD and biodistribution assays were performed. T2DM-induced mice treated with Rybelsus® could be included as a control group to compare its PK/PD profile and glucoregulatory response with both FcRn-targeted semaglutide-NPs. It is expected to observe a significant increase in the oral bioavailability of semaglutide incorporated into FcRn-targeted NPs and an increase in their glucose-lowering effect over Rybelsus®. This is intended to occur since NPs will be responsible for protecting the peptide from the harsh conditions of the GIT, and facilitate its transport through the mucosal intestinal barriers due to the covalent linkage of the particles with PEG (favoring the mucus transport), and the FcRn-targeted ligands FcBP₂ and Z_{FcRn} (promoting the intestinal transcytosis of NPs, avoiding the lysosomal pathway). Taking advantage of the fact that the animals orally treated once a day with FcBP₂-semaglutide-NPs and Z_{FcRn}-semaglutide-NPs for 7 days demonstrated a similar glucoregulatory response and insulin pancreatic content with the animals treated with s.c. free semaglutide, the FcRn-targeted NPs could be considered to replace the administration of semaglutide via an invasive route. However, it is required to evaluate the effect of these nanosystems in a long-term treatment of at least one month, to prove this hypothesis. A glucose tolerance test must be performed to evaluate the ability of the FcRn-targeted semaglutide-NPs in controlling blood glucose levels in diabetic mice. It was suggested that the FcRn-targeted semaglutide-NPs can have a potential anti-inflammatory effect in T2DM-induced hFcRn transgenic mice model, since in

a T2DM scenario the levels of these cytokines are commonly increased. Therefore, it would be interesting to analyze the levels of systemic proinflammatory cytokines (e.g., IL-1 β , IL-6, IL-18 and TNF- α) in the plasma, and peripheral tissues (liver, adipose tissues, and muscle) of diabetic animals, after a prolonged treatment.

Furthermore, other relevant parameter that needs to be pursued regarding the development of the nanoformulations and their clinical translation consist of testing the scaling-up of FcRn-targeted semaglutide-NPs. In this experiment, it is required to evaluate the ability of the nanosystems in retaining the same physicochemical properties when produced on a larger scale.

Notwithstanding, FcBP₂-semaglutide-NPs or Z_{FcRn}-semaglutide-NPs could be administered in combination with other antidiabetic peptides, such as biguanides, thiazolidinediones, sulfonylureas, or SGLT2 inhibitors. The combination of the FcRn-targeted semaglutide-NPs with biguanides or thiazolidinediones will induce a dual-effect in the management of T2DM, since the NPs incorporating the GLP-1 analog semaglutide are responsible to induce the insulin secretion from pancreatic β cells, while biguanides or thiazolidinediones are responsible to improve the sensitivity of peripheral tissues (e.g., liver, muscle, and adipose tissue) to insulin. Sulfonylureas combined with the FcRn-targeted semaglutide-NPs will amplify the response of the nanosystems, since they are also involved in insulin secretion. Other combination that could be considered is the co-administration of FcRn-targeted semaglutide-NPs with SGLT2 inhibitors, which promote the urinary excretion of glucose through the reduction of the renal glucose reabsorption, and consequently, induced an improved control in blood glucose levels.

The applicability of FcRn-targeted NPs incorporating semaglutide could also be considered for other metabolic disorders, such as obesity or overweight, and metabolic dysfunction-associated steatotic liver disease (MASLD). Additionally, the versatility of the FcRn-targeted PLGA NPs demonstrated that these nanocarriers could be employed for the oral delivery of other drugs (e.g., chemotherapeutic drugs), and consequently, to be explored for a variety of other therapeutics applications, such colorectal cancer, since it was already reported in the literature that the FcRn is expressed in cancer tissue and it is overexpressed in colorectal epithelial cells, showing the relevance of this strategy.

5.3. References

- [1] International Diabetes Federation, IDF Diabetes Atlas: 10th edition. 2021. Available on: <https://diabetesatlas.org/atlas/tenth-edition/> [accessed on 20th August 2023].
- [2] World Health Organization, Classification of diabetes *mellitus*: Global reports. 2019. Available on: <https://www.who.int/publications/i/item/classification-of-diabetes-mellitus> [accessed on 20th August 2023].
- [3] S.E. Alavi, P.J. Cabot, P.M. Moyle, Glucagon-like peptide-1 receptor agonists and strategies to improve their efficiency, *Mol. Pharm.* 16 (2019) 2278-2295.
- [4] Y. Zheng, S.H. Ley, F.B. Hu, Global aetiology and epidemiology of type 2 diabetes *mellitus* and its complications, *Nat. Rev. Endocrinol.* 14 (2018) 88-98.
- [5] S. Cornell, A review of GLP-1 receptor agonists in type 2 diabetes: a focus on the mechanism of action of once-weekly agents, *J. Clin. Pharm. Ther.* 45 (2020) 17-27.
- [6] S. Padhi, A.K. Nayak, A. Behera, Type II diabetes *mellitus*: a review on recent drug based therapeutics, *Biomed. Pharmacother.* 131 (2020) 110708.
- [7] American Diabetes Association Professional Practice Committee, Pharmacologic approaches to glycemic treatment: standards of medical care in diabetes - 2022, *Diabetes Care.* 45 (2021) 125-143.
- [8] D.J. Brayden, T.A. Hill, D.P. Fairlie, S. Maher, R.J. Mersny, Systemic delivery of peptides by the oral route: formulation and medicinal chemistry approaches, *Adv. Drug Deliv. Rev.* 157 (2020) 2-36.
- [9] S.T. Buckley, T.A. Bækdal, A. Vegge, S.J. Maabjerg, C. Pyke, J. Ahnfelt-Rønne, K.G. Madsen, S.G. Schéele, T. Alanentalo, R.K. Kirk, B.L. Pedersen, R.B. Skyggebjerg, A.J. Benie, H.M. Strauss, P.O. Wahlund, S. Bjerregaard, E. Farkas, C. Fekete, F.L. Søndergaard, J. Borregaard, M.L. Hartoft-Nielsen, L.B. Knudsen, Transcellular stomach absorption of a derivatized glucagon-like peptide-1 receptor agonist, *Sci. Transl. Med.* 10 (2018) eaar7047.
- [10] M.P. Kane, C.L. Triplitt, C.D. Solis-Herrera, Management of type 2 diabetes with oral semaglutide: practical guidance for pharmacists, *Am. J. Health. Syst. Pharm.* 78 (2021) 556-567.
- [11] F. Araújo, J. das Neves, J.P. Martins, P.L. Granja, H.A. Santos, B. Sarmiento, Functionalized materials for multistage platforms in the oral delivery of biopharmaceuticals, *Prog. Mater. Sci.* 89 (2017) 306-344.

- [12] A.A. Date, J. Hanes, L.M. Ensign, Nanoparticles for oral delivery: design, evaluation and state-of-the-art, *J. Control. Release.* 240 (2016) 504-526.
- [13] L.M. Ensign, R. Cone, J. Hanes, Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers, *Adv. Drug Deliv. Rev.* 64 (2012) 557-570.
- [14] J.T. Huckaby, S.K. Lai, PEGylation for enhancing nanoparticle diffusion in mucus, *Adv. Drug Deliv. Rev.* 124 (2018) 125-139.
- [15] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, *Adv. Drug Deliv. Rev.* 99 (2016) 28-51.
- [16] R. Nunes, F. Araújo, J. Tavares, B. Sarmiento, J. das Neves, Surface modification with polyethylene glycol enhances colorectal distribution and retention of nanoparticles, *Eur. J. Pharm. Biopharm.* 130 (2018) 200-206.
- [17] J.P. Martins, P.J. Kennedy, H.A. Santos, C. Barrias, B. Sarmiento, A comprehensive review of the neonatal Fc receptor and its application in drug delivery, *Pharmacol. Ther.* 161 (2016) 22-39.
- [18] B.L. Dickinson, K. Badizadegan, Z. Wu, J.C. Ahouse, X. Zhu, N.E. Simister, R.S. Blumberg, W.I. Lencer, Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line, *J. Clin. Invest.* 104 (1999) 903-911.
- [19] C. Chaudhury, C.L. Brooks, D.C. Carter, J.M. Robinson, C.L. Anderson, Albumin binding to FcRn: distinct from the FcRn-IgG interaction, *Biochemistry.* 45 (2006) 4983-4990.
- [20] A. Praetor, W. Hunziker, beta(2)-Microglobulin is important for cell surface expression and pH-dependent IgG binding of human FcRn, *J. Cell Sci.* 115 (2002) 2389-2397.
- [21] J.P. Martins, D. Liu, F. Fontana, M.P.A. Ferreira, A. Correia, S. Valentino, M. Kemell, K. Moslova, E. Mäkilä, J. Salonen, J. Hirvonen, B. Sarmiento, H.A. Santos, Microfluidic nanoassembly of bioengineered chitosan-modified FcRn-targeted porous silicon nanoparticles @ hypromellose acetate succinate for oral delivery of antidiabetic peptides, *ACS Appl. Mater. Interfaces.* 10 (2018) 44354-44367.
- [22] J.P. Martins, P. Figueiredo, S. Wang, E. Espo, E. Celi, B. Martins, M. Kemell, K. Moslova, E. Mäkilä, J. Salonen, M.A. Kostianen, C. Celia, V. Cerullo, T. Viitala, B. Sarmiento, J. Hirvonen, H.A. Santos, Neonatal Fc receptor-targeted lignin-encapsulated porous silicon nanoparticles for enhanced cellular interactions and insulin permeation across the intestinal epithelium, *Bioact. Mater.* 9 (2022) 299-315.

- [23] E.M. Pridgen, F. Alexis, T.T. Kuo, E. Levy-Nissenbaum, R. Karnik, R.S. Blumberg, R. Langer, O.C. Farokhzad, Transepithelial transport of Fc-targeted nanoparticles by the neonatal fc receptor for oral delivery, *Sci. Transl. Med.* 5 (2013) 213ra167.
- [24] C. Azevedo, J. Nilsen, A. Grevys, R. Nunes, J.T. Andersen, B. Sarmiento, Engineered albumin-functionalized nanoparticles for improved FcRn binding enhance oral delivery of insulin, *J. Control. Release.* 327 (2020) 161-173.
- [25] J.P. Martins, R. D'Auria, D. Liu, F. Fontana, M.P.A. Ferreira, A. Correia, M. Kemell, K. Moslova, E. Mäkilä, J. Salonen, L. Casettari, J. Hirvonen, B. Sarmiento, H.A. Santos, Engineered multifunctional albumin-decorated porous silicon nanoparticles for FcRn translocation of insulin, *Small.* 14 (2018) e1800462.
- [26] S. Ben Mkaddem, M. Benhamou, R.C. Monteiro, Understanding Fc receptor involvement in inflammatory diseases: from mechanisms to new therapeutic tools, *Front. Immunol.* 10 (2019) 811.
- [27] K.M.K. Sand, M. Bern, J. Nilsen, H.T. Noordzij, I. Sandlie, J.T. Andersen, Unraveling the interaction between FcRn and albumin: oportunities for design of albumin-based therapeutics, *Front. Immunol.* 5 (2015) 682.
- [28] Y. Ishima, T. Maruyama, M. Otagiri, V.T.G. Chuang, T. Ishida, The new delivery strategy of albumin carrier utilizing the interaction with albumin receptors, *Chem. Pharm. Bull.* 70 (2022) 330-333.
- [29] J.T. Sockolosky, M.R. Tiffany, F.C. Szoka, Engineering neonatal Fc receptor-mediated recycling and transcytosis in recombinant proteins by short terminal peptide extensions, *Proc. Natl. Acad. Sci. U.S.A.* 109 (2012) 16095-16100.
- [30] A. Datta-Mannan, J. Boyles, L. Huang, Z.Y. Jin, A. Peariso, A.T. Murphy, B. Ellis, N. Douglass, F. Norouziyan-Cooper, D.R. Witcher, Engineered FcRn binding fusion peptides significantly enhance the half-life of a Fab domain in cynomolgus monkeys, *Biotechnol. J.* 14 (2019) e1800007.
- [31] J. Seijsing, M. Lindborg, I. Höidén-Guthenberg, H. Bönisch, E. Guneriusson, F.Y. Frejd, L. Abrahmsén, C. Ekblad, J. Löfblom, M. Uhlén, T. Gräslund, An engineered affibody molecule with pH-dependent binding to FcRn mediates extended circulatory half-life of a fusion protein, *Proc. Natl. Acad. Sci. U.S.A.* 111 (2014) 17110-17115.
- [32] A.R. Mezo, K.A. McDonnell, C.A. Hehir, S.C. Low, V.J. Palombella, J.M. Stattel, G.D. Kamphaus, C. Fraley, Y. Zhang, J.A. Dumont, A.J. Bitonti, Reduction of IgG in nonhuman primates by a peptide antagonist of the neonatal Fc receptor FcRn, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 2337-2342.

- [33] C. Martins, F. Sousa, F. Araújo, B. Sarmento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv. Healthc. Mater.* 7 (2018).
- [34] M. Mir, N. Ahmed, A.u. Rehman, Recent applications of PLGA based nanostructures in drug delivery, *Colloids Surf. B Biointerfaces.* 159 (2017) 217-231.
- [35] F.Z. Syed, Type 1 diabetes *mellitus*, *Ann. Intern. Med.* 175 (2022) 33-48.
- [36] L.A. DiMeglio, C. Evans-Molina, R.A. Oram, Type 1 diabetes, *Lancet.* 391 (2018) 2449-2462.
- [37] S. Chatterjee, K. Khunti, M.J. Davies, Type 2 diabetes, *Lancet.* 389 (2017) 2239-2251.
- [38] Y. Wu, Y. Ding, Y. Tanaka, W. Zhang, Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention, *Int. J. Med. Sci.* 11 (2014) 1185-1200.
- [39] J.A. Kanaley, S.R. Colberg, M.H. Corcoran, S.K. Malin, N.R. Rodriguez, C.J. Crespo, J.P. Kirwan, J.R. Zierath, Exercise/physical activity in individuals with type 2 diabetes: a consensus statement from the American College of Sports Medicine, *Med. Sci. Sports Exerc.* 54 (2022) 353-368.
- [40] Y. Zheng, S.H. Ley, F.B. Hu, Global aetiology and epidemiology of type 2 diabetes *mellitus* and its complications, *Nat. Rev. Endocrinol.* 14 (2018) 88-98.
- [41] A. McGovern, Z. Tippu, W. Hinton, N. Munro, M. Whyte, S. de Lusignan, Comparison of medication adherence and persistence in type 2 diabetes: a systematic review and meta-analysis, *Diabetes Obes. Metab.* 20 (2018) 1040-1043.
- [42] N.F. Syafhan, R. Donnelly, R. Harper, J. Harding, C. Mulligan, A. Hogg, M. Scott, G. Fleming, C. Scullin, A.F. Hawwa, G. Chen, C. Parsons, J.C. McElroy, Adherence to metformin in adults with type 2 diabetes: a combined method approach, *J. Pharm. Policy Pract.* 15 (2022) 61.
- [43] K.M.P. Kumar, M. Chawla, A. Sanghvi, N.G. Sastry, C. Kotla, S. Phatak, S. Choudhari, Adherence, satisfaction, and experience with metformin 500 mg prolonged release formulation in indian patients with type 2 diabetes *mellitus*: a postmarketing observational study, *Int. J. Gen. Med.* 12 (2019) 147-159.
- [44] D. Hinnen, Glucagon-like peptide 1 receptor agonists for type 2 diabetes, *Diabetes Spectrum.* 30 (2017) 202-210.
- [45] D.J. Drucker, GLP-1 physiology informs the pharmacotherapy of obesity, *Mol. Metab.* 57 (2022) 101351.

- [46] M.P. Gilbert, R.E. Pratley, GLP-1 analogs and DPP-4 inhibitors in type 2 diabetes therapy: review of head-to-head clinical trials, *Front. Endocrinol.* 11 (2020) 178.
- [47] F. Araújo, P. Fonte, H.A. Santos, B. Sarmento, Oral delivery of glucagon-like peptide-1 and analogs: alternatives for diabetes control?, *J. Diabetes Sci. Technol.* 6 (2012) 1486-1497.
- [48] J.Y. Cheang, P.M. Moyle, Glucagon-like peptide-1 (GLP-1)-based therapeutics: current status and future opportunities beyond type 2 diabetes, *ChemMedChem.* 13 (2018) 662-671.
- [49] R. Ismail, I. Csóka, Novel strategies in the oral delivery of antidiabetic peptide drugs - insulin, GLP 1 and its analogs, *Eur. J. Pharm. Biopharm.* 115 (2017) 257-267.
- [50] M.H.A. Muskiet, L. Tonneijck, M.M. Smits, M.J.B. van Baar, M.H.H. Kramer, E.J. Hoorn, J.A. Joles, D.H. van Raalte, GLP-1 and the kidney: from physiology to pharmacology and outcomes in diabetes, *Nat. Rev. Nephrol.* 13 (2017) 605-628.
- [51] T.P. Vahl, B.W. Paty, B.D. Fuller, R.L. Prigeon, D.A. D'Alessio, Effects of GLP-1-(7-36)NH₂, GLP-1-(7-37), and GLP-1- (9-36)NH₂ on intravenous glucose tolerance and glucose-induced insulin secretion in healthy humans, *J. Clin. Endocrinol. Metab.* 88 (2003) 1772-1779.
- [52] M.A. Nauck, D.R. Quast, J. Wefers, A.F.H. Pfeiffer, The evolving story of incretins (GIP and GLP-1) in metabolic and cardiovascular disease: a pathophysiological update, *Diabetes Obes. Metab.* 23 (2021) 5-29.
- [53] J.J. Meier, GLP-1 receptor agonists for individualized treatment of type 2 diabetes *mellitus*, *Nat. Rev. Endocrinol.* 8 (2012) 728-742.
- [54] J.J. Holst, The physiology of glucagon-like peptide 1, *Physiol. Rev.* 87 (2007) 1409-1439.
- [55] M. Fineman, S. Flanagan, K. Taylor, M. Aisporna, L.Z. Shen, K.F. Mace, B. Walsh, M. Diamant, B. Cirincione, P. Kothare, W.I. Li, L. MacConell, Pharmacokinetics and pharmacodynamics of exenatide extended-release after single and multiple dosing, *Clin. Pharmacokinet.* 50 (2011) 65-74.
- [56] F.H. Moonschi, C.B. Hughes, G.M. Mussman, J.L. Fowlkes, C.I. Richards, I. Popescu, Advances in micro- and nanotechnologies for the GLP-1-based therapy and imaging of pancreatic beta-cells, *Acta Diabetol.* 55 (2018) 405-418.
- [57] L.V. Jacobsen, A. Flint, A.K. Olsen, S.H. Ingwersen, Liraglutide in type 2 diabetes *mellitus*: clinical pharmacokinetics and pharmacodynamics, *Clin. Pharmacokinet.* 55 (2016) 657-672.

- [58] Conjuchem, Albenatide, CJC-1134-PC. Available on: <https://synapse.patsnap.com/drug/3eef702f9ed747b98372452f4a10b8a0> [accessed on 25th March 2024].
- [59] E. Lilly, Prescribing information sheet of Trulicity® (dulaglutide). Available on: <https://pi.lilly.com/us/trulicity-uspi.pdf> [accessed on 22nd January 2024].
- [60] J. Rosenstock, B. Balas, B. Charbonnel, G.B. Bolli, M. Boldrin, R. Ratner, R. Balena, The fate of taspoglutide, a weekly GLP-1 receptor agonist, versus twice-daily exenatide for type 2 diabetes: the T-emerge 2 trial, *Diabetes Care*. 36 (2013) 498-504.
- [61] M. Giraudon, S. Sturm, N. Lambert, M. Niggli, J. Brumm, B. Mangold, C. Schmitt, Effect of varying degrees of renal impairment on the pharmacokinetics and tolerability of taspoglutide, *Diabetes Obes. Metab.* 19 (2017) 537-544.
- [62] D. Sharma, S. Verma, S. Vaidya, K. Kalia, V. Tiwari, Recent updates on GLP-1 agonists: current advancements & challenges, *Biomed. Pharmacother.* 108 (2018) 952-962.
- [63] J.P. Frias, J. Choi, J. Rosenstock, L. Popescu, E. Niemoeller, I. Muehlen-Bartmer, S. Baek, Efficacy and safety of once-weekly efpeglenatide monotherapy versus placebo in type 2 diabetes: the AMPLITUDE-M randomized controlled trial, *Diabetes Care*. 45 (2022) 1592-1600.
- [64] V.R. Aroda, J.P. Frias, L. Ji, E. Niemoeller, M.-L. Nguyễn-Pascal, K. Denkel, M. Espinasse, H. Guo, S. Baek, J. Choi, I. Lingvay, Efficacy and safety of once-weekly efpeglenatide in people with suboptimally controlled type 2 diabetes: the AMPLITUDE-D, AMPLITUDE-L and AMPLITUDE-S randomized controlled trials, *Diabetes Obes. Metab.* 25 (2023) 2084-2095.
- [65] P. Andreadis, T. Karagiannis, K. Malandris, I. Avgerinos, A. Liakos, A. Manolopoulos, E. Bekiari, D.R. Matthews, A. Tsapas, Semaglutide for type 2 diabetes *mellitus*: a systematic review and meta-analysis, *Diabetes Obes. Metab.* 20 (2018) 2255-2263.
- [66] A. Andersen, F.K. Knop, T. Vilsbøll, A pharmacological and clinical overview of oral semaglutide for the treatment of type 2 diabetes, *Drugs*. 81 (2021) 1003-1030.
- [67] L.B. Knudsen, J. Lau, The discovery and development of liraglutide and semaglutide, *Front. Endocrinol.* 10 (2019) 155.
- [68] C. De Block, C. Bailey, C. Wysham, A. Hemmingway, S.E. Allen, J. Peleshok, Tirzepatide for the treatment of adults with type 2 diabetes: an endocrine perspective, *Diabetes Obes. Metab.* 25 (2023) 3-17.
- [69] T. Min, S.C. Bain, The role of tirzepatide, dual GIP and GLP-1 receptor agonist, in the management of type 2 diabetes: the SURPASS clinical trials, *Diabetes Ther.*, 12 (2021) 143-157.

- [70] M. Christensen, F.K. Knop, J.J. Holst, T. Vilsboll, Lixisenatide, a novel GLP-1 receptor agonist for the treatment of type 2 diabetes *mellitus*, *I. Drugs*. 12 (2009) 503-513.
- [71] J. Rosenstock, C. Wysham, J.P. Frías, S. Kaneko, C.J. Lee, L.L. Fernández, H. Mao, X. Cui, C.A. Karanikas, V.T. Thieu, Efficacy and safety of a novel dual GIP and GLP-1 receptor agonist tirzepatide in patients with type 2 diabetes (SURPASS-1): a double-blind, randomised, phase 3 trial, *Lancet*. 398 (2021) 143-155.
- [72] S. Kalra, R. Sahay, A review on semaglutide: an oral glucagon-like peptide-1 receptor agonist in management of type 2 diabetes *mellitus*, *Diabetes Ther*. 11 (2020) 1965-1982.
- [73] M.E. Røder, Clinical potential of treatment with semaglutide in type 2 diabetes patients, *Drugs Context*. 8 (2019) 212585.
- [74] D.J. Drucker, Advances in oral peptide therapeutics, *Nat. Rev. Drug Discov*. 19 (2020) 277-289.
- [75] C. Twarog, S. Fattah, J. Heade, S. Maher, E. Fattal, D.J. Brayden, Intestinal permeation enhancers for oral delivery of macromolecules: a comparison between salcaprozate sodium (SNAC) and sodium caprate (C(10)), *Pharmaceutics*. 11 (2019).
- [76] NovoNordisk, Rybelsus® (semaglutide) tablets, for oral use. Available on: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/213051s000lbl.pdf [accessed on 28th May 2023].
- [77] B. Hodayun, X. Lin, H.J. Choi, Challenges and recent progress in oral drug delivery systems for biopharmaceuticals, *Pharmaceutics*. 11 (2019).
- [78] T.D. Brown, K.A. Whitehead, S. Mitragotri, Materials for oral delivery of proteins and peptides, *Nat. Rev. Mater*. 5 (2020) 127-148.
- [79] P. Kiptoo, A.M. Calcagno, T.J. Siahaan, Physiological, biochemical, and chemical barriers to oral drug delivery, *Drug Deliv*. (2016) 19-34.
- [80] S. Haddadzadegan, F. Dorkoosh, A. Bernkop-Schnürch, Oral delivery of therapeutic peptides and proteins: technology landscape of lipid-based nanocarriers, *Adv. Drug Deliv. Rev*. 182 (2022) 114097.
- [81] L.M. Ensign, R. Cone, J. Hanes, Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers, *Adv. Drug Deliv. Rev*. 64 (2012) 557-570.
- [82] M. Hu, X. Li, Barriers to oral bioavailability - an overview, *Oral Bioavailability* (2011) 1-5.
- [83] M. Goldberg, I. Gomez-Orellana, Challenges for the oral delivery of macromolecules, *Nat. Rev. Drug Discov*. 2 (2003) 289-295.

- [84] M.J. Mitchell, M.M. Billingsley, R.M. Haley, M.E. Wechsler, N.A. Peppas, R. Langer, Engineering precision nanoparticles for drug delivery, *Nat. Rev. Drug Discov.* 20 (2021) 101-124.
- [85] S. Ahadian, J.A. Finbloom, M. Mofidfar, S.E. Diltemiz, F. Nasrollahi, E. Davoodi, V. Hosseini, I. Mylonaki, S. Sangabathuni, H. Montazerian, K. Fetah, R. Nasiri, M.R. Dokmeci, M.M. Stevens, T.A. Desai, A. Khademhosseini, Micro and nanoscale technologies in oral drug delivery, *Adv. Drug Deliv. Rev.* 157 (2020) 37-62.
- [86] A. Ashkar, A. Sosnik, M. Davidovich-Pinhas, Structured edible lipid-based particle systems for oral drug-delivery, *Biotechnol. Adv.* 54 (2022) 107789.
- [87] M.S. Alqahtani, M. Kazi, M.A. Alsenaidy, M.Z. Ahmad, Advances in oral drug delivery, *Front. Pharmacol.* 12 (2021) 618411.
- [88] D. Guimarães, A. Cavaco-Paulo, E. Nogueira, Design of liposomes as drug delivery system for therapeutic applications, *Int. J. Pharm.* 601 (2021) 120571.
- [89] C. Tapeinos, M. Battaglini, G. Ciofani, Advances in the design of solid lipid nanoparticles and nanostructured lipid carriers for targeting brain diseases, *J. Control. Release.* 264 (2017) 306-332.
- [90] V.H. Nguyen, V.N. Thuy, T.V. Van, A.H. Dao, B.-J. Lee, Nanostructured lipid carriers and their potential applications for versatile drug delivery via oral administration, *Open Nano.* 8 (2022) 100064.
- [91] S. Asad, A.C. Jacobsen, A. Teleki, Inorganic nanoparticles for oral drug delivery: opportunities, barriers, and future perspectives, *Curr. Opin. Chem. Eng.* 38 (2022) 100869.
- [92] H. Bhardwaj, R.K. Jangde, Current updated review on preparation of polymeric nanoparticles for drug delivery and biomedical applications, *Next Nanotechnol.* 2 (2023) 100013.
- [93] J. Liu, P. Leng, Y. Liu, Oral drug delivery with nanoparticles into the gastrointestinal mucosa, *Fundam. Clin. Pharmacol.* 35 (2021) 86-96.
- [94] M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani, M.R. Mozafari, Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems, *Pharmaceutics.* 10 (2018) 57.
- [95] C. He, L. Yin, C. Tang, C. Yin, Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs, *Biomaterials.* 33 (2012) 8569-8578.
- [96] A. Banerjee, J. Qi, R. Gogoi, J. Wong, S. Mitragotri, Role of nanoparticle size, shape and surface chemistry in oral drug delivery, *J. Control. Release.* 238 (2016) 176-185.

- [97] S.P. Bandi, Y.S. Kumbhar, V.V.K. Venuganti, Effect of particle size and surface charge of nanoparticles in penetration through intestinal mucus barrier, *J. Nanopart. Res.* 22 (2020) 62.
- [98] S. Guo, Y. Liang, L. Liu, M. Yin, A. Wang, K. Sun, Y. Li, Y. Shi, Research on the fate of polymeric nanoparticles in the process of the intestinal absorption based on model nanoparticles with various characteristics: size, surface charge and pro-hydrophobics, *J. Nanobiotechnology.* 19 (2021) 32.
- [99] S.A. Kulkarni, S.S. Feng, Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery, *Pharm. Res.* 30 (2013) 2512-2522.
- [100] X.Y. Sun, Q.Z. Gan, J.M. Ouyang, Size-dependent cellular uptake mechanism and cytotoxicity toward calcium oxalate on Vero cells, *Sci. Rep.*, 7 (2017) 41949.
- [101] Y. Li, M. Kröger, W.K. Liu, Shape effect in cellular uptake of PEGylated nanoparticles: comparison between sphere, rod, cube and disk, *Nanoscale.* 7 (2015) 16631-16646.
- [102] Y. Li, T. Yue, K. Yang, X. Zhang, Molecular modeling of the relationship between nanoparticle shape anisotropy and endocytosis kinetics, *Biomaterials.* 33 (2012) 4965-4973.
- [103] A.B. Jindal, The effect of particle shape on cellular interaction and drug delivery applications of micro- and nanoparticles, *Int. J. Pharm.* 532 (2017) 450-465.
- [104] X.J. Du, J.L. Wang, S. Iqbal, H.J. Li, Z.T. Cao, Y.C. Wang, J.Z. Du, J. Wang, The effect of surface charge on oral absorption of polymeric nanoparticles, *Biomater. Sci.*, 6 (2018) 642-650.
- [105] P. Foroozandeh, A.A. Aziz, Insight into cellular uptake and intracellular trafficking of nanoparticles, *Nanoscale Res. Lett.* 13 (2018) 339.
- [106] Y. Wang, C. Pi, X. Feng, Y. Hou, L. Zhao, Y. Wei, The influence of nanoparticle properties on oral bioavailability of drugs, *Int. J. Nanomedicine.* 15 (2020) 6295-6310.
- [107] E.M. Pridgen, F. Alexis, O.C. Farokhzad, Polymeric nanoparticle drug delivery technologies for oral delivery applications, *Expert. Opin. Drug Deliv.* 12 (2015) 1459-1473.
- [108] Y. Wang, Y. Cui, Y. Zhao, Q. Zhao, B. He, Q. Zhang, S. Wang, Effects of surface modification and size on oral drug delivery of mesoporous silica formulation, *J. Colloid. Interface Sci.* 513 (2018) 736-747.
- [109] L. Martínez-Jothar, S. Doukeridou, R.M. Schiffelers, J. Sastre Torano, S. Oliveira, C.F. van Nostrum, W.E. Hennink, Insights into maleimide-thiol conjugation chemistry: conditions

for efficient surface functionalization of nanoparticles for receptor targeting, *J. Control. Release.* 282 (2018) 101-109.

[110] A. Baião, F. Sousa, A.V. Oliveira, C. Oliveira, B. Sarmento, Effective intracellular delivery of bevacizumab via PEGylated polymeric nanoparticles targeting the CD44v6 receptor in colon cancer cells, *Biomater. Sci.* 8 (2020) 3720-3729.

[111] P.J. Kennedy, F. Sousa, D. Ferreira, C. Pereira, M. Nestor, C. Oliveira, P.L. Granja, B. Sarmento, Fab-conjugated PLGA nanoparticles effectively target cancer cells expressing human CD44v6, *Acta Biomater.* 81 (2018) 208-218.

[112] T. Kantner, A.G. Watts, Characterization of reactions between water-soluble trialkylphosphines and thiol alkylating reagents: implications for protein-conjugation reactions, *Bioconjug. Chem.* 27 (2016) 2400-2406.

[113] A.R. Garizo, F. Castro, C. Martins, A. Almeida, T.P. Dias, F. Fernandes, C.C. Barrias, N. Bernardes, A.M. Fialho, B. Sarmento, p28-functionalized PLGA nanoparticles loaded with gefitinib reduce tumor burden and metastases formation on lung cancer, *J. Control. Release.* 337 (2021) 329-342.

[114] S. Gupta, A. Jain, M. Chakraborty, J.K. Sahni, J. Ali, S. Dang, Oral delivery of therapeutic proteins and peptides: a review on recent developments, *Drug Deliv.* 20 (2013) 237-246.

[115] M. Kaasalainen, J. Rytönen, E. Mäkilä, A. Näränen, J. Salonen, Electrostatic interaction on loading of therapeutic peptide GLP-1 into porous silicon nanoparticles, *Langmuir.* 31 (2015) 1722-1729.

[116] F. Araújo, N. Shrestha, M.A. Shahbazi, P. Fonte, E.M. Mäkilä, J.J. Salonen, J.T. Hirvonen, P.L. Granja, H.A. Santos, B. Sarmento, The impact of nanoparticles on the mucosal translocation and transport of GLP-1 across the intestinal epithelium, *Biomaterials.* 35 (2014) 9199-9207.

[117] F. Araújo, N. Shrestha, M. Shahbazi, D. Liu, B. Herranz-Blanco, E.M. Mäkilä, J.J. Salonen, J.T. Hirvonen, P.L. Granja, B. Sarmento, H.A. Santos, Microfluidic assembly of a multifunctional tailorable composite system designed for site specific combined oral delivery of peptide drugs, *ACS Nano.* 9 (2015) 8291-8302.

[118] F. Araújo, N. Shrestha, M.J. Gomes, B. Herranz-Blanco, D. Liu, J.J. Hirvonen, P.L. Granja, H.A. Santos, B. Sarmento, *In vivo* dual-delivery of glucagon like peptide-1 (GLP-1) and dipeptidyl peptidase-4 (DPP4) inhibitor through composites prepared by microfluidics for diabetes therapy, *Nanoscale.* 8 (2016) 10706-10713.

- [119] N. Shrestha, F. Araújo, M.A. Shahbazi, E. Mäkilä, M.J. Gomes, M. Airavaara, E.I. Kauppinen, J. Raula, J. Salonen, J. Hirvonen, B. Sarmento, H.A. Santos, Oral hypoglycaemic effect of GLP-1 and DPP4 inhibitor based nanocomposites in a diabetic animal model, *J. Control. Release.* 232 (2016) 113-119.
- [120] X. Li, C. Wang, R. Liang, F. Sun, Y. Shi, A. Wang, W. Liu, K. Sun, Y. Li, The glucose-lowering potential of exenatide delivered orally via goblet cell- targeting nanoparticles, *Pharm. Res.* 32 (2015) 1017-1027.
- [121] Y. Song, Y. Shi, L. Zhang, H. Hu, C. Zhang, M. Yin, L. Chu, X. Yan, M. Zhao, X. Zhang, H. Mu, K. Sun, Synthesis of CSK-DEX-PLGA nanoparticles for the oral delivery of exenatide to improve its mucus penetration and intestinal absorption, *Mol. Pharm.* 16 (2019) 518-532.
- [122] L. Zhang, Y. Shi, Y. Song, D. Duan, X. Zhang, K. Sun, Y. Li, Tf ligand-receptor-mediated exenatide-Zn(2+) complex oral-delivery system for penetration enhancement of exenatide, *J. Drug Target.* 26 (2018) 931-940.
- [123] Y. Xu, M. Van Hul, F. Suriano, V. Préat, P.D. Cani, A. Beloqui, Novel strategy for oral peptide delivery in incretin-based diabetes treatment, *Gut.* 69 (2020) 911-919.
- [124] Y. Xu, H. De Keersmaecker, K. Braeckmans, S. De Smedt, P.D. Cani, V. Préat, A. Beloqui, Targeted nanoparticles towards increased L cell stimulation as a strategy to improve oral peptide delivery in incretin-based diabetes treatment, *Biomaterials.* 255 (2020) 120209.
- [125] K.D. Ristroph, R.K. Prud'homme, Hydrophobic ion pairing: encapsulating small molecules, peptides, and proteins into nanocarriers, *Nanoscale Adv.* 1 (2019) 4207-4237.
- [126] L. Zhang, Y. Shi, Y. Song, X. Sun, X. Zhang, K. Sun, Y. Li, The use of low molecular weight protamine to enhance oral absorption of exenatide, *Int. J. Pharm.* 547 (2018) 265-273.
- [127] T.N.Q. Phan, R. Ismail, B. Le-Vinh, S. Zaichik, F. Laffleur, A. Bernkop-Schnürch, The effect of counterions in hydrophobic ion pairs on oral bioavailability of exenatide, *ACS Biomater. Sci. Eng.* 6 (2020) 5032-5039.
- [128] R. Ismail, T.N.Q. Phan, F. Laffleur, I. Csóka, A. Bernkop-Schnürch, Hydrophobic ion pairing of a GLP-1 analogue for incorporating into lipid nanocarriers designed for oral delivery, *Europ. J. Pharm. Biopharm.* 152 (2020) 10-17.
- [129] X. Bao, K. Qian, P. Yao, Oral delivery of exenatide-loaded hybrid zein nanoparticles for stable blood glucose control and β -cell repair of type 2 diabetes mice, *J. Nanobiotechnol.* 18 (2020) 67.

- [130] S.M. Lim, H.N. Eom, H.H. Jiang, M. Sohn, K.C. Lee, Evaluation of PEGylated exendin-4 released from poly (lactic-co-glycolic acid) microspheres for antidiabetic therapy, *J. Pharm. Sci.* 104 (2015) 72-80.
- [131] E.J. Park, S.M. Lim, K.C. Lee, D.H. Na, Exendins and exendin analogs for diabetic therapy: a patent review (2012-2015), *Expert Opin. Ther. Pat.* 26 (2016) 833-842.
- [132] J. Lau, P. Bloch, L. Schäffer, I. Pettersson, J. Spetzler, J. Kofoed, K. Madsen, L.B. Knudsen, J. McGuire, D.B. Steensgaard, H.M. Strauss, D.X. Gram, S.M. Knudsen, F.S. Nielsen, P. Thygesen, S. Reedtz-Runge, T. Kruse, Discovery of the once-weekly glucagon-like peptide-1 (GLP-1) analogue semaglutide, *J. Med. Chem.* 58 (2015) 7370-7380.
- [133] P. Uhl, C. Grundmann, M. Sauter, P. Storck, A. Tursch, S. Özbek, K. Leotta, R. Roth, D. Witzigmann, J.A. Kulkarni, V. Fidelj, C. Kleist, P.R. Cullis, G. Fricker, W. Mier, Coating of PLA-nanoparticles with cyclic, arginine-rich cell penetrating peptides enables oral delivery of liraglutide, *NBM.* 24 (2020) 102132.
- [134] N. Senduran, H.N. Yadav, V.K. Vishwakarma, P. Bhatnagar, P. Gupta, J. Bhatia, A.K. Dinda, Orally deliverable nanoformulation of liraglutide against type 2 diabetic rat model, *J. Drug Deliv. Sci. Technol.* 56 (2020) 101513.
- [135] Y. Liang, R. Ding, H. Wang, L. Liu, J. He, Y. Tao, Z. Zhao, J. Zhang, A. Wang, K. Sun, Y. Li, Y. Shi, Orally administered intelligent self-ablating nanoparticles: a new approach to improve drug cellular uptake and intestinal absorption, *Drug Deliv.* 29 (2022) 305-315.
- [136] Y. Shi, M. Yin, Y. Song, T. Wang, S. Guo, X. Zhang, K. Sun, Y. Li, Oral delivery of liraglutide-loaded poly-N-(2-hydroxypropyl) methacrylamide/chitosan nanoparticles: preparation, characterization, and pharmacokinetics, *J. Biomater. Appl.* 35 (2021) 754-761.
- [137] X. Bao, K. Qian, M. Xu, Y. Chen, H. Wang, T. Pan, Z. Wang, P. Yao, L. Lin, Intestinal epithelium penetration of liraglutide via cholic acid pre-complexation and zein/rhamnolipids nanocomposite delivery, *J. Nanobiotechnol.* 21 (2023) 16.
- [138] Y. Shi, X. Sun, L. Zhang, K. Sun, K. Li, Y. Li, Q. Zhang, Fc-modified exenatide-loaded nanoparticles for oral delivery to improve hypoglycemic effects in mice, *Sci. Rep.* 8 (2018) 726.
- [139] N.E. Simister, K.E. Mostov, An Fc receptor structurally related to MHC class I antigens, *Nature.* 337 (1989) 184-187.
- [140] N.E. Simister, A.R. Rees, Isolation and characterization of an Fc receptor from neonatal rat small intestine, *Eur. J. Immunol.* 15 (1985) 733-738.
- [141] W.P. Burmeister, A.H. Huber, P.J. Bjorkman, Crystal structure of the complex of rat neonatal Fc receptor with Fc, *Nature.* 372 (1994) 379-383.

- [142] T.T. Kuo, K. Baker, M. Yoshida, S.-W. Qiao, V.G. Aveson, W.I. Lencer, R.S. Blumberg, Neonatal Fc receptor: from immunity to therapeutics, *J. Clin. Immunol.* 30 (2010) 777-789.
- [143] A.P. West, Jr., P.J. Bjorkman, Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor, *Biochemistry.* 39 (2000) 9698-9708.
- [144] P.J. Bjorkman, M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, D.C. Wiley, The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens, *Nature.* 329 (1987) 512-518.
- [145] W.P. Burmeister, L.N. Gastinel, N.E. Simister, M.L. Blum, P.J. Bjorkman, Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor, *Nature.* 372 (1994) 336-343.
- [146] D. Plaksin, K. Polakova, M.G. Mage, D.H. Margulies, Rigidification of the $\alpha 2$ helix of an MHC class I molecule by a valine to proline mutation in position 165 does not prevent peptide-specific antigen presentation, *J. Immunol.* 159 (1997) 4408-4414.
- [147] M. Raghavan, V.R. Bonagura, S.L. Morrison, P.J. Bjorkman, Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants, *Biochemistry.* 34 (1995) 14649-14657.
- [148] C. Chaudhury, S. Mehnaz, J.M. Robinson, W.L. Hayton, D.K. Pearl, D.C. Roopenian, C.L. Anderson, The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan, *J. Exp. Med.* 197 (2003) 315-322.
- [149] J.T. Andersen, B. Dalhus, J. Cameron, M.B. Daba, A. Plumridge, L. Evans, S.O. Brennan, K.S. Gunnarsen, M. Bjørås, D. Sleep, I. Sandlie, Structure-based mutagenesis reveals the albumin-binding site of the neonatal Fc receptor, *Nat. Commun.* 3 (2012) 610.
- [150] C. Chaudhury, C.L. Brooks, D.C. Carter, J.M. Robinson, C.L. Anderson, Albumin binding to FcRn: distinct from the FcRn-IgG interaction, *Biochemistry.* 45 (2006) 4983-4990.
- [151] J.T. Andersen, J. Dee Qian, I. Sandlie, The conserved histidine 166 residue of the human neonatal Fc receptor heavy chain is critical for the pH-dependent binding to albumin, *Eur. J. Immunol.* 36 (2006) 3044-3051.
- [152] D.C. Roopenian, S. Akilesh, FcRn: the neonatal Fc receptor comes of age, *Nat. Rev. Immunol.* 7 (2007) 715-725.
- [153] S.W. de Taeye, T. Rispens, G. Vidarsson, The ligands for human IgG and their effector functions, *Antibodies.* 8 (2019).

- [154] E.O. Saphire, P.W. Parren, R. Pantophlet, M.B. Zwick, G.M. Morris, P.M. Rudd, R.A. Dwek, R.L. Stanfield, D.R. Burton, I.A. Wilson, Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design, *Science*. 293 (2001) 1155-1159.
- [155] J.K. Kim, M.F. Tsen, V. Ghetie, E.S. Ward, Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor, *Eur. J. Immunol.* 24 (1994) 2429-2434.
- [156] J.K. Kim, M.F. Tsen, V. Ghetie, E.S. Ward, Identifying amino acid residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis, *Eur. J. Immunol.* 24 (1994) 542-548.
- [157] M. Raghavan, M.Y. Chen, L.N. Gastinel, P.J. Bjorkman, Investigation of the interaction between the class I MHC-related Fc receptor and its immunoglobulin G ligand, *Immunity*. 1 (1994) 303-315.
- [158] J.K. Kim, M. Firan, C.G. Radu, C.H. Kim, V. Ghetie, E.S. Ward, Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn, *Eur. J. Immunol.* 29 (1999) 2819-2825.
- [159] W.L. Martin, A.P. West, Jr., L. Gan, P.J. Bjorkman, Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding, *Mol. Cell.* 7 (2001) 867-877.
- [160] C. Medesan, D. Matesoi, C. Radu, V. Ghetie, E.S. Ward, Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1, *J. Immunol.* 158 (1997) 2211-2217.
- [161] D.E. Vaughn, C.M. Milburn, D.M. Penny, W.L. Martin, J.L. Johnson, P.J. Bjorkman, Identification of critical IgG binding epitopes on the neonatal Fc receptor, *J. Mol. Biol.* 274 (1997) 597-607.
- [162] V. Oganessian, M.M. Damschroder, K.E. Cook, Q. Li, C. Gao, H. Wu, W.F. Dall'Acqua, Structural insights into neonatal Fc receptor-based recycling mechanisms, *J. Biol. Chem.* 289 (2014) 7812-7824.
- [163] T. Rath, T.T. Kuo, K. Baker, S.-W. Qiao, K. Kobayashi, M. Yoshida, D. Roopenian, E. Fiebiger, W.I. Lencer, R.S. Blumberg, The immunologic functions of the neonatal Fc receptor for IgG, *J. Clin. Immunol.* 33 (2013) 9-17.
- [164] J.T. Sockolosky, F.C. Szoka, The neonatal Fc receptor, FcRn, as a target for drug delivery and therapy, *Adv. Drug Deliv. Rev.* 91 (2015) 109-124.

- [165] H. M, E. Azzazy, R.H. Christenson, All about albumin: biochemistry, genetics, and medical applications. in: T. Peters (Ed.) *Clinical Chemistry*, Academic Press 43 (1997) 2014-2015.
- [166] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattacharya, M. Otagiri, S. Curry, Structural basis of the drug-binding specificity of human serum albumin, *J. Mol. Biol.* 353 (2005) 38-52.
- [167] S. Curry, H. Mandelkow, P. Brick, N. Franks, Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites, *Nat. Struct. Biol.* 5 (1998) 827-835.
- [168] M.M. Schmidt, S.A. Townson, A.J. Andreucci, B.M. King, E.B. Schirmer, A.J. Murillo, C. Dombrowski, A.W. Tisdale, P.A. Lowden, A.L. Masci, J.T. Kovalchin, D.V. Erbe, K.D. Wittrup, E.S. Furfine, T.M. Barnes, Crystal structure of an HSA/FcRn complex reveals recycling by competitive mimicry of HSA ligands at a pH-dependent hydrophobic interface, *Structure*. 21 (2013) 1966-1978.
- [169] K.M. Sand, B. Dalhus, G.J. Christianson, M. Bern, S. Foss, J. Cameron, D. Sleep, M. Bjørås, D.C. Roopenian, I. Sandlie, J.T. Andersen, Dissection of the neonatal Fc receptor (FcRn)-albumin interface using mutagenesis and anti-FcRn albumin-blocking antibodies, *J. Biol. Chem.* 289 (2014) 17228-17239.
- [170] K.M.K. Sand, M. Bern, J. Nilsen, B. Dalhus, K.S. Gunnarsen, J. Cameron, A. Grevys, K. Bunting, I. Sandlie, J.T. Andersen, Interaction with both domain I and III of albumin is required for optimal pH-dependent binding to the neonatal Fc receptor (FcRn), *J. Biol. Chem.* 289 (2014) 34583-34594.
- [171] C.L. Anderson, C. Chaudhury, J. Kim, C.L. Bronson, M.A. Wani, S. Mohanty, Perspective - FcRn transports albumin: relevance to immunology and medicine, *Trends Immunol.* 27 (2006) 343-348.
- [172] R.J. Ober, C. Martinez, C. Vaccaro, J. Zhou, E.S. Ward, Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn, *J. Immunol.* 172 (2004) 2021-2029.
- [173] M. Bern, J. Nilsen, M. Ferrarese, K.M.K. Sand, T.T. Gjølberg, H.E. Lode, R.J. Davidson, R.M. Camire, E.S. Bækkevold, S. Foss, A. Grevys, B. Dalhus, J. Wilson, L.S. Høydahl, G.J. Christianson, D.C. Roopenian, T. Schlothauer, T.E. Michaelsen, M.C. Moe, S. Lombardi, M. Pinotti, I. Sandlie, A. Branchini, J.T. Andersen, An engineered human albumin enhances half-life and transmucosal delivery when fused to protein-based biologics, *Sci. Transl. Med.* 12 (2020) eabb0580.

- [174] K.M. McCarthy, Y. Yoong, N.E. Simister, Bidirectional transcytosis of IgG by the rat neonatal Fc receptor expressed in a rat kidney cell line: a system to study protein transport across epithelia, *J. Cell Sci.* 113 (2000) 1277-1285.
- [175] S.M. Claypool, B.L. Dickinson, M. Yoshida, W.I. Lencer, R.S. Blumberg, Functional reconstitution of human FcRn in Madin-Darby canine kidney cells requires co-expressed human β 2-microglobulin, *J. Biol. Chem.* 277 (2002) 28038-28050.
- [176] P.J. Hornby, P.R. Cooper, C. Kliwinski, E. Ragwan, J.R. Mabus, B. Harman, S. Thompson, A.L. Kauffman, Z. Yan, S.H. Tam, H. Dorai, G.D. Powers, J. Giles-Komar, Human and non-human primate intestinal FcRn expression and immunoglobulin G transcytosis, *Pharm. Res.* 31 (2014) 908-922.
- [177] S. Tzaban, R.H. Massol, E. Yen, W. Hamman, S.R. Frank, L.A. Lapierre, S.H. Hansen, J.R. Goldenring, R.S. Blumberg, W.I. Lencer, The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity, *J. Cell Biol.* 185 (2009) 673-684.
- [178] A. Grevys, J. Nilsen, K.M.K. Sand, M.B. Daba, I. Øynebråten, M. Bern, M.B. McAdam, S. Foss, T. Schlothauer, T.E. Michaelsen, G.J. Christianson, D.C. Roopenian, R.S. Blumberg, I. Sandlie, J.T. Andersen, A human endothelial cell-based recycling assay for screening of FcRn targeted molecules, *Nat. Commun.* 9 (2018) 621.
- [179] M. Pyzik, T. Rath, T.T. Kuo, S. Win, K. Baker, J.J. Hubbard, R. Grenha, A. Gandhi, T.D. Krämer, A.R. Mezo, Z.S. Taylor, K. McDonnell, V. Nienaber, J.T. Andersen, A. Mizoguchi, L. Blumberg, S. Purohit, S.D. Jones, G. Christianson, W.I. Lencer, I. Sandlie, N. Kaplowitz, D.C. Roopenian, R.S. Blumberg, Hepatic FcRn regulates albumin homeostasis and susceptibility to liver injury, *Proc. Natl. Acad. Sci. U.S.A.* 114 (2017) e2862-e2871.
- [180] J. Chia, J. Louber, I. Glauser, S. Taylor, G.T. Bass, S.K. Dower, P.A. Gleeson, A.M. Verhagen, Half-life–extended recombinant coagulation factor IX–albumin fusion protein is recycled via the FcRn-mediated pathway, *J. Biol. Chem.* 293 (2018) 6363-6373.
- [181] V. Tenten, S. Menzel, U. Kunter, E.M. Sicking, C.R. van Roeyen, S.K. Sanden, M. Kaldenbach, P. Boor, A. Fuss, S. Uhlig, R. Lanzmich, B. Willemsen, H. Dijkman, M. Grepl, K. Wild, W. Kriz, B. Smeets, J. Floege, M.J. Moeller, Albumin is recycled from the primary urine by tubular transcytosis, *J. Am. Soc. Nephrol.* 24 (2013) 1966-1980.
- [182] U. Shah, B.L. Dickinson, R.S. Blumberg, N.E. Simister, W.I. Lencer, A.W. Walker, Distribution of the IgG Fc receptor, FcRn, in the human fetal intestine, *Pediatr. Res.* 53 (2003) 295-301.

- [183] P.R. Cooper, C.M. Kliwinski, R.A. Perkinson, E. Ragwan, J.R. Mabus, G.D. Powers, H. Dorai, J. Giles-Komar, P.J. Hornby, The contribution of cell surface FcRn in monoclonal antibody serum uptake from the intestine in suckling rat pups, *Front. Pharmacol.* 5 (2014) 225.
- [184] T.S. Ramalingam, S.A. Detmer, W.L. Martin, P.J. Bjorkman, IgG transcytosis and recycling by FcRn expressed in MDCK cells reveals ligand-induced redistribution, *EMBO J.* 21 (2002) 590-601.
- [185] G.V. Jerdeva, D.B. Tesar, K.E. Huey-Tubman, M.S. Ladinsky, S.E. Fraser, P.J. Bjorkman, Comparison of FcRn- and plgR-mediated transport in MDCK cells by fluorescence confocal microscopy, *Traffic.* 11 (2010) 1205-1220.
- [186] K.B. Degn, C.B. Juhl, J. Sturis, G. Jakobsen, B. Brock, V. Chandramouli, J. Rungby, B.R. Landau, O. Schmitz, One week's treatment with the long-acting glucagon-like peptide 1 derivative liraglutide (NN2211) markedly improves 24-h glycemia and alpha- and beta-cell function and reduces endogenous glucose release in patients with type 2 diabetes, *Diabetes.* 53 (2004) 1187-1194.
- [187] T. Karagiannis, I. Avgerinos, A. Liakos, S. Del Prato, D.R. Matthews, A. Tsapas, E. Bekiari, Management of type 2 diabetes with the dual GIP/GLP-1 receptor agonist tirzepatide: a systematic review and meta-analysis, *Diabetologia.* 65 (2022) 1251-1261.
- [188] M.K. Thomas, A. Nikooienejad, R. Bray, X. Cui, J. Wilson, K. Duffin, Z. Milicevic, A. Haupt, D.A. Robins, Dual GIP and GLP-1 receptor agonist tirzepatide improves beta-cell function and insulin sensitivity in type 2 diabetes, *J. Clin. Endocrinol. Metab.* 106 (2021) 388-396.
- [189] M.J.D. J.P. Frías, J. Rosenstock, F.C.P. Manghi, L.F. Landó, B.K. Bergman, B. Liu, X. Cui, K. Brown, Tirzepatide versus semaglutide once weekly in patients with type 2 diabetes, *N. Engl. J. Med.* 6 (2021) 503-515.
- [190] Y. Tang, L. Zhang, Y. Zeng, X. Wang, M. Zhang, Efficacy and safety of tirzepatide in patients with type 2 diabetes: a systematic review and meta-analysis, *Front. Pharmacol.* 13 (2022) 1016639.
- [191] L.L. Baggio, Q. Huang, X. Cao, D.J. Drucker, An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis, *Gastroenterology.* 134 (2008) 1137-1147.
- [192] J.-G. Kim, L.L. Baggio, D.P. Bridon, J.-P. Castaigne, M.F. Robitaille, L. Jetté, C. Benquet, D.J. Drucker, Development and characterization of a glucagon-like peptide 1-

albumin conjugate: the ability to activate the glucagon-like peptide-1 receptor *in vivo*, *Diabetes*. 52 (2003) 751-759.

[193] H. Tan, M. Sattler, P. Zou, Albumin-binding as a universal strategy for half-life extension, *Jpn. J. Res.* 3 (2022) 1-5.

[194] J. Rosenstock, J. Reusch, M. Bush, F. Yang, M. Stewart, Potential of albiglutide, a long-acting GLP-1 receptor agonist, in type 2 diabetes: a randomized controlled trial exploring weekly, biweekly, and monthly dosing, *Diabetes Care*. 32 (2009) 1880-1886.

[195] J. Nilsen, E. Trabjerg, A. Grevys, C. Azevedo, S.O. Brennan, M. Stensland, J. Wilson, K.M.K. Sand, M. Bern, B. Dalhus, D.C. Roopenian, I. Sandlie, K.D. Rand, J.T. Andersen, An intact C-terminal end of albumin is required for its long half-life in humans, *Commun. Biol.* 3 (2020) 181.

[196] C.B. Burness, L.J. Scott, Dulaglutide: a review in type 2 diabetes, *BioDrugs*. 29 (2015) 407-418.

[197] R.S. Weinstock, B. Guerci, G. Umpierrez, M.A. Nauck, Z. Skrivanek, Z. Milicevic, Safety and efficacy of once-weekly dulaglutide versus sitagliptin after 2 years in metformin-treated patients with type 2 diabetes (AWARD-5): a randomized, phase III study, *Diabetes Obes. Metab.* 17 (2015) 849-858.

[198] P.-Å. Nygren, Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold, *FEBS J.* 275 (2008) 2668-2676.

[199] J.T. Andersen, R. Pehrson, V. Tolmachev, M.B. Daba, L. Abrahmsén, C. Ekblad, Extending half-life by indirect targeting of the neonatal Fc receptor (FcRn) using a minimal albumin binding domain, *J. Biol. Chem.* 286 (2011) 5234-5241.

[200] J.Y. Lee, T. Park, E. Hong, R. Amatya, K.A. Park, Y.H. Park, K.A. Min, M. Jin, S. Lee, S. Hwang, G.S. Roh, M.C. Shin, Genetic engineering of novel super long-acting exendin-4 chimeric protein for effective treatment of metabolic and cognitive complications of obesity, *Biomaterials*. 257 (2020) 120250.

[201] S. Ben Mkaddem, M. Benhamou, R.C. Monteiro, Understanding Fc receptor involvement in inflammatory diseases: from mechanisms to new therapeutic tools, *Front. Immunol.* 10 (2019) 811.

[202] J.P. Oliveira, A.R. Prado, W.J. Keijok, P.W.P. Antunes, E.R. Yapuchura, M.C.C. Guimarães, Impact of conjugation strategies for targeting of antibodies in gold nanoparticles for ultrasensitive detection of 17 β -estradiol, *Sci. Rep.* 9 (2019) 13859.

- [203] K. Werengowska-Ciećwierz, M. Wiśniewski, A.P. Terzyk, S. Furmaniak, The chemistry of bioconjugation in nanoparticles-based drug delivery system, *Adv. Cond. Matter Phys.* 2015 (2015) 198175.
- [204] J.M.J.M. Ravasco, H. Faustino, A. Trindade, P.M.P. Gois, Bioconjugation with maleimides: a useful tool for chemical biology, *Chem. Eur. J.* 25 (2019) 43-59.
- [205] S. Keleştemur, M. Altunbek, M. Culha, Influence of EDC/NHS coupling chemistry on stability and cytotoxicity of ZnO nanoparticles modified with proteins, *Appl. Surf. Sci.* 403 (2017) 455-463.
- [206] D.N. Athmuri, P.A. Shiekh, Experimental diabetic animal models to study diabetes and diabetic complications, *MethodsX.* 11 (2023) 102474.
- [207] A. Ghasemi, S. Khalifi, S. Jedi, Streptozotocin-nicotinamide-induced rat model of type 2 diabetes, *Acta Physiol. Hung.* 101 (2014) 408-420.
- [208] S. Gheibi, K. Kashfi, A. Ghasemi, A practical guide for induction of type-2 diabetes in rat: incorporating a high-fat diet and streptozotocin, *Biomed. Pharmacother.* 95 (2017) 605-613.
- [209] R. Singh, M. Gholipourmalekabadi, S.H. Shafikhani, Animal models for type 1 and type 2 diabetes: advantages and limitations, *Front. Endocrinol.* 15 (2024) 1359685.
- [210] O.M. Akinlade, B.V. Owoyele, A.O. Soladoye, Streptozotocin-induced type 1 and 2 diabetes in rodents: a model for studying diabetic cardiac autonomic neuropathy, *Afr. Health Sci.* 21 (2021) 719-727.
- [211] S. Skovsø, Modeling type 2 diabetes in rats using high fat diet and streptozotocin, *J. Diabetes Investig.* 5 (2014) 349-358.
- [212] F. Marino, N. Salerno, M. Scalise, L. Salerno, A. Torella, C. Molinaro, A. Chiefalo, A. Filardo, C. Siracusa, G. Panuccio, C. Ferravante, G. Giurato, F. Rizzo, M. Torella, M. Donniacuo, A. De Angelis, G. Viglietto, K. Urbanek, A. Weisz, D. Torella, E. Cianflone, Streptozotocin-induced type 1 and 2 diabetes *mellitus* mouse models show different functional, cellular and molecular patterns of diabetic cardiomyopathy, *Int. J. Mol. Sci.* 24 (2023) 1132.
- [213] N. Rakieten, M.L. Rakieten, M.V. Nadkarni, Studies on the diabetogenic action of streptozotocin (NSC-37917), *Cancer Chemother. Rep.* 29 (1963) 91-98.
- [214] B.L. Furman, Streptozotocin-induced diabetic models in mice and rats, *Curr. Protoc. Pharmacol.* 70 (2015) 5.47.41-45.47.20.
- [215] A. Ghasemi, S. Jeddi, Streptozotocin as a tool for induction of rat models of diabetes: a practical guide, *Excli. J.* 22 (2023) 274-294.

- [216] L.J. Yan, The nicotinamide/streptozotocin rodent model of type 2 diabetes: renal pathophysiology and redox imbalance features, *Biomolecules*. 12 (2022) 1225.
- [217] C.O. Eleazu, K.C. Eleazu, S. Chukwuma, U.N. Essien, Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans, *J. Diabetes Metab. Disord*. 12 (2013) 60.
- [218] P. Brahmanaidu, V.V.S. Uddandrao, V. Sasikumar, R.R. Naik, S. Pothani, M.S. Begum, M.P. Rajeshkumar, C. Varatharaju, B. Meriga, P. Rameshreddy, A. Kalaivani, G. Saravanan, Reversal of endothelial dysfunction in aorta of streptozotocin-nicotinamide-induced type-2 diabetic rats by S-Allylcysteine, *Mol. Cell Biochem*. 432 (2017) 25-32.
- [219] A. Akbarzadeh, D. Norouzian, M.R. Mehrabi, S. Jamshidi, A. Farhangi, A.A. Verdi, S.M. Mofidian, B.L. Rad, Induction of diabetes by streptozotocin in rats, *Indian J. Clin. Biochem*. 22 (2007) 60-64.
- [220] D.K. Arulmozhi, A. Veeranjanyulu, S.L. Bodhankar, Neonatal streptozotocin-induced rat model of type 2 diabetes *mellitus*: a glance, *Indian J. Pharmacol*. 36 (2004) 217-221.
- [221] B. Ahrén, G. Pacini, Insufficient islet compensation to insulin resistance vs. reduced glucose effectiveness in glucose-intolerant mice, *Am. J. Physiol. Endocrinol. Metab*. 283 (2002) 738-744.
- [222] M. Rossmeisl, J.S. Rim, R.A. Koza, L.P. Kozak, Variation in type 2 diabetes - related traits in mouse strains susceptible to diet-induced obesity, *Diabetes*. 52 (2003) 1958-1966.
- [223] S.S. Ionova-Martin, J.M. Wade, S. Tang, M. Shahnazari, J.W. Ager, 3rd, N.E. Lane, W. Yao, T. Alliston, C. Vaisse, R.O. Ritchie, Changes in cortical bone response to high-fat diet from adolescence to adulthood in mice, *Osteoporos. Int*. 22 (2011) 2283-2293.
- [224] S. Andrikopoulos, A.R. Blair, N. Deluca, B.C. Fam, J. Proietto, Evaluating the glucose tolerance test in mice, *Am. J. Physiol. Endocrinol. Metab*. 295 (2008) 1323-1332.
- [225] M.J. Reed, K. Meszaros, L.J. Entes, M.D. Claypool, J.G. Pinkett, T.M. Gadbois, G.M. Reaven, A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat, *Metabolism*. 49 (2000) 1390-1394.
- [226] K. Srinivasan, B. Viswanad, L. Asrat, C.L. Kaul, P. Ramarao, Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening, *Pharmacol. Res*. 52 (2005) 313-320.
- [227] P. Masiello, C. Broca, R. Gross, M. Roye, M. Manteghetti, D. Hillaire-Buys, M. Novelli, G. Ribes, Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide, *Diabetes*. 47 (1998) 224-229.

- [228] T. Nakamura, T. Terajima, T. Ogata, K. Ueno, N. Hashimoto, K. Ono, S. Yano, Establishment and pathophysiological characterization of type 2 diabetic mouse model produced by streptozotocin and nicotinamide, *Biol. Pharm. Bull.* 29 (2006) 1167-1174.
- [229] A. Ahangarpour, A.A. Oroojan, L. Khorsandi, M. Kouchak, M. Badavi, Solid lipid nanoparticles of myricitrin have antioxidant and antidiabetic effects on streptozotocin-nicotinamide-induced diabetic model and myotube cell of male mouse, *Oxid. Med. Cell Longev.* 2018 (2018) 7496936.
- [230] P.L. Cruz, I.C. Moraes-Silva, A.A. Ribeiro, J.F. Machi, M.D.T. de Melo, F. Dos Santos, M.B. da Silva, C.M.C. Strunz, E.G. Caldini, M.C. Irigoyen, Nicotinamide attenuates streptozotocin-induced diabetes complications and increases survival rate in rats: role of autonomic nervous system, *BMC Endocr. Disord.* 21 (2021) 133.
- [231] M. Novelli, B. Bonamassa, M. Masini, N. Funel, D. Canistro, V. De Tata, M. Martano, A. Soleti, D. Campani, M. Paolini, P. Masiello, Persistent correction of hyperglycemia in streptozotocin-nicotinamide-induced diabetic mice by a non-conventional radical scavenger, *Naunyn Schmiedebergs Arch. Pharmacol.* 382 (2010) 127-137.
- [232] R. Shimizu, F. Sakazaki, T. Okuno, K. Nakamuro, H. Ueno, Difference in glucose intolerance between C57BL/6J and ICR strain mice with streptozotocin/nicotinamide-induced diabetes, *Biomed. Res.* 33 (2012) 63-66.
- [233] M. Novelli, D. Canistro, M. Martano, N. Funel, A. Sapone, S. Melega, M. Masini, V. De Tata, A. Pippa, C. Vecoli, D. Campani, R. De Siena, A. Soleti, M. Paolini, P. Masiello, Anti-diabetic properties of a non-conventional radical scavenger, as compared to pioglitazone and exendin-4, in streptozotocin-nicotinamide diabetic mice, *Eur. J. Pharmacol.* 729 (2014) 37-44.
- [234] C.C. Wu, C.N. Hung, Y.C. Shin, C.J. Wang, H.P. Huang, *Myrciaria cauliflora* extracts attenuate diabetic nephropathy involving the Ras signaling pathway in streptozotocin/nicotinamide mice on a high fat diet, *J. Food Drug Anal.* 24 (2016) 136-146.
- [235] W.C. Yu, R.Y. Huang, T.C. Chou, Oligo-fucoidan improves diabetes-induced renal fibrosis via activation of Sirt-1, GLP-1R, and Nrf2/HO-1: an *in vitro* and *in vivo* study, *Nutrients.* 12 (2020) 3068.
- [236] V. Rai, R. Moellmer, D.K. Agrawal, Clinically relevant experimental rodent models of diabetic foot ulcer, *Mol. Cell. Biochem.* 477 (2022) 1239-1247.
- [237] R.J. Fajardo, L. Karim, V.I. Calley, M.L. Boussein, A review of rodent models of type 2 diabetic skeletal fragility, *J. Bone Miner. Res.* 29 (2014) 1025-1040.

- [238] C.P.D. Kottaisamy, D.S. Raj, V. Prasanth Kumar, U. Sankaran, Experimental animal models for diabetes and its related complications-a review, *Lab. Anim. Res.* 37 (2021) 23.
- [239] S.J. Burke, H.M. Batdorf, D.H. Burk, R.C. Noland, A.E. Eder, M.S. Boulous, M.D. Karlstad, J.J. Collier, db/db mice exhibit features of human type 2 diabetes that are not present in weight-matched C57BL/6J mice fed a western diet, *J. Diabetes Res.* 2017 (2017) 8503754.
- [240] R. Roy, J. Zayas, S.K. Singh, K. Delgado, S.J. Wood, M.F. Mohamed, D.M. Frausto, Y.A. Albalawi, T.P. Price, R. Estupinian, E.F. Giurini, T.M. Kuzel, A. Zloza, J. Reiser, S.H. Shafikhani, Overriding impaired FPR chemotaxis signaling in diabetic neutrophil stimulates infection control in murine diabetic wound, *eLife*. 11 (2022) e72071.
- [241] R. Roy, J. Zayas, M.F. Mohamed, A. Aboonabi, K. Delgado, J. Wallace, M. Bayat, T.M. Kuzel, J. Reiser, S.H. Shafikhani, IL-10 dysregulation underlies chemokine insufficiency, delayed macrophage response, and impaired healing in diabetic wounds, *J. Invest. Dermatol.* 142 (2022) 692-704.
- [242] R. Roy, F. Mahmud, J. Zayas, T.M. Kuzel, J. Reiser, S.H. Shafikhani, Reduced bioactive microbial products (pathogen-associated molecular patterns) contribute to dysregulated immune responses and impaired healing in infected wounds in mice with diabetes, *J. Invest. Dermat.* 144 (2024) 387-397.
- [243] M.F. Mohamed, K. Gupta, J.W. Goldufsky, R. Roy, L.T. Callaghan, D.M. Wetzel, T.M. Kuzel, J. Reiser, S.H. Shafikhani, CrkII/Abl phosphorylation cascade is critical for NLRC4 inflammasome activity and is blocked by *Pseudomonas aeruginosa* ExoT, *Nat. Commun.* 13 (2022) 1295.
- [244] S. Latvala, B. Jacobsen, M.B. Otteneder, A. Herrmann, S. Kronenberg, Distribution of FcRn across species and tissues, *J. Histochem. Cytochem.* 65 (2017) 321-333.
- [245] S. Akilesh, G.J. Christianson, D.C. Roopenian, A.S. Shaw, Neonatal FcR expression in bone marrow-derived cells functions to protect serum IgG from catabolism, *J. Immunol.* 179 (2007) 4580-4588.
- [246] E.J. Israel, S. Taylor, Z. Wu, E. Mizoguchi, R.S. Blumberg, A. Bhan, N.E. Simister, Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells, *Immunology*. 92 (1997) 69-74.
- [247] M.G. Martín, S.V. Wu, J.H. Walsh, Ontogenetic development and distribution of antibody transport and Fc receptor mRNA expression in rat intestine, *Dig. Dis. Sci.* 42 (1997) 1062-1069.

- [248] M. Yoshida, S.M. Claypool, J.S. Wagner, E. Mizoguchi, A. Mizoguchi, D.C. Roopenian, W.I. Lencer, R.S. Blumberg, Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells, *Immunity*. 20 (2004) 769-783.
- [249] D.C. Roopenian, G.J. Christianson, T.J. Sproule, A.C. Brown, S. Akilesh, N. Jung, S. Petkova, L. Avanesian, E.Y. Choi, D.J. Shaffer, P.A. Eden, C.L. Anderson, The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs, *J. Immunol.* 170 (2003) 3528-3533.
- [250] M.C. Berin, M. Chehade, 40 - Mucosal immunology: an overview, in: D.Y.M. Leung, S.J. Szefer, F.A. Bonilla, C.A. Akdis, H.A. Sampson (Eds.) *Pediatric Allergy: Principles and Practice* (Third Edition), Elsevier 2016, pp. 365-370.
- [251] C. Kliwinski, P.R. Cooper, R. Perkinson, J.R. Mabus, S.H. Tam, T.M. Wilkinson, J. Giles-Komar, B. Scallon, G.D. Powers, P.J. Hornby, Contribution of FcRn binding to intestinal uptake of IgG in suckling rat pups and human FcRn-transgenic mice, *Am. J. Physiol. Gastroint. Liver Physiol.* 304 (2013) G262-G270.
- [252] C.M. Story, J.E. Mikulska, N.E. Simister, A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus, *J. Exp. Med.* 180 (1994) 2377-2381.
- [253] J.T. Andersen, M.B. Daba, G. Berntzen, T.E. Michaelsen, I. Sandlie, Cross-species binding analyses of mouse and human neonatal Fc receptor show dramatic differences in immunoglobulin G and albumin binding, *J. Biol. Chem.* 285 (2010) 4826-4836.
- [254] J. Nilsen, M. Bern, K.M.K. Sand, A. Grevys, B. Dalhus, I. Sandlie, J.T. Andersen, Human and mouse albumin bind their respective neonatal Fc receptors differently, *Sci. Rep.* 8 (2018).
- [255] J.T. Andersen, J. Cameron, A. Plumridge, L. Evans, D. Sleep, I. Sandlie, Single-chain variable fragment albumin fusions bind the neonatal Fc receptor (FcRn) in a species-dependent manner: implication for *in vivo* half-life evaluation of albumin fusion therapeutics, *J. Biol. Chem.* 288 (2013) 24277-24285.
- [256] J.T. Andersen, B. Dalhus, D. Viuff, B.T. Ravn, K.S. Gunnarsen, A. Plumridge, K. Bunting, F. Antunes, R. Williamson, S. Athwal, E. Allan, L. Evans, M. Bjørås, S. Kjærulff, D. Sleep, I. Sandlie, J. Cameron, Extending serum half-life of albumin by engineering neonatal Fc receptor (FcRn) binding, *J. Biol. Chem.* 289 (2014) 13492-13502.
- [257] G. Proetzel, D.C. Roopenian, Humanized FcRn mouse models for evaluating pharmacokinetics of human IgG antibodies, *Methods*. 65 (2014) 148-153.

- [258] L.B. Avery, M. Wang, M.S. Kavosi, A. Joyce, J.C. Kurz, Y.Y. Fan, M.E. Dowty, M. Zhang, Y. Zhang, A. Cheng, F. Hua, H.M. Jones, H. Neubert, R.J. Polzer, D.M. O'Hara, Utility of a human FcRn transgenic mouse model in drug discovery for early assessment and prediction of human pharmacokinetics of monoclonal antibodies, MABs. 8 (2016) 1064-1078.
- [259] T. Li, J.P. Balthasar, FcRn expression in wild type mice, transgenic mice, and in human tissues, Biomolecules. 8 (2018) 115.
- [260] C.M. Conner, D. van Fossan, K. Read, D.O. Cowley, O. Alvarez, S.X.-R. Xu, D.R. Webb, K. Jarnagin, A precisely humanized FcRn transgenic mouse for preclinical pharmacokinetics studies, Biochem. Pharmacol. 210 (2023) 115470.
- [261] Y.Y. Fan, L.B. Avery, M. Wang, D.M. O'Hara, S. Leung, H. Neubert, Tissue expression profile of human neonatal Fc receptor (FcRn) in Tg32 transgenic mice, MABs. 8 (2016) 848-853.
- [262] S.H. Tam, S.G. McCarthy, K. Brosnan, K.M. Goldberg, B.J. Scallon, Correlations between pharmacokinetics of IgG antibodies in primates vs. FcRn-transgenic mice reveal a rodent model with predictive capabilities, mAbs. 5 (2013) 397-405.
- [263] D. Valente, C. Mauriac, T. Schmidt, I. Focken, J. Beninga, B. Mackness, H. Qiu, P. Vicat, A. Kandira, K. Radošević, S. Rao, J. Darbyshire, M. Kabiri, Pharmacokinetics of novel Fc-engineered monoclonal and multispecific antibodies in cynomolgus monkeys and humanized FcRn transgenic mouse models, mAbs. 12 (2020) 1829337.
- [264] S.B. Petkova, S. Akilesh, T.J. Sproule, G.J. Christianson, H. Al Khabbaz, A.C. Brown, L.G. Presta, Y.G. Meng, D.C. Roopenian, Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease, Int. Immunol. 18 (2006) 1759-1769.
- [265] D.C. Roopenian, B.E. Low, G.J. Christianson, G. Proetzel, T.J. Sproule, M.V. Wiles, Albumin-deficient mouse models for studying metabolism of human albumin and pharmacokinetics of albumin-based drugs, mAbs. 7 (2015) 344-351.
- [266] D. Viuff, F. Antunes, L. Evans, J. Cameron, H. Dyrnesli, B. Thue Ravn, M. Stougaard, K. Thiam, B. Andersen, S. Kjærulff, K.A. Howard, Generation of a double transgenic humanized neonatal Fc receptor (FcRn)/albumin mouse to study the pharmacokinetics of albumin-linked drugs, J. Control. Release. 223 (2016) 22-30.
- [267] O.A. Mandrup, S.C. Ong, S. Lykkemark, A. Dinesen, I. Rudnik-Jansen, N.F. Dagnæs-Hansen, J.T. Andersen, L. Alvarez-Vallina, K.A. Howard, Programmable half-life and anti-

tumour effects of bispecific T-cell engager-albumin fusions with tuned FcRn affinity, *Commun. Biol.* 4 (2021) 310.

[268] J. Nilsen, I. Sandlie, D.C. Roopenian, J.T. Andersen, Animal models for evaluation of albumin-based therapeutics, *Curr. Opin. Chem. Eng.* 19 (2018) 68-76.

[269] M. Venanzi, M. Savioli, R. Cimino, E. Gatto, A. Palleschi, G. Ripani, D. Cicero, E. Placidi, F. Orvieto, E. Bianchi, A spectroscopic and molecular dynamics study on the aggregation process of a long-acting lipidated therapeutic peptide: the case of semaglutide, *Soft Matter*. 16 (2020) 10122-10131.

[270] F. Sousa, A. Cruz, P. Fonte, I.M. Pinto, M.T. Neves-Petersen, B. Sarmento, A new paradigm for antiangiogenic therapy through controlled release of bevacizumab from PLGA nanoparticles, *Sci. Rep.* 7 (2017) 3736.

[271] K.W. McCracken, J.C. Howell, J.M. Wells, J.R. Spence, Generating human intestinal tissue from pluripotent stem cells *in vitro*, *Nat. Protoc.* 6 (2011) 1920-1928.

[272] A. Ahangarpour, A.A. Oroojan, L. Khorsandi, G. Arzani, G. Afshari, Effects of betulinic acid on the male reproductive system of a streptozotocin-nicotinamide-induced diabetic mouse model, *World J. Mens Health.* 34 (2016) 209-216.

[273] D. Liu, J. Gu, W. Shao, J. Pang, X. Qian, T. Jin, Comparison of beneficial metabolic effects of liraglutide and semaglutide in male C57BL/6J mice, *Can. J. Diabetes.* 46 (2022) 216-224.

[274] G. Rakipovski, B. Rolin, J. Nøhr, I. Klewe, K.S. Frederiksen, R. Augustin, J. Hecksher-Sørensen, C. Ingvorsen, J. Pox-Wolf, L.B. Knudsen, The GLP-1 analogs liraglutide and semaglutide reduce atherosclerosis in ApoE(-/-) and LDLr(-/-) mice by a mechanism that includes inflammatory pathways, *JACC Basic Transl. Sci.* 3 (2018) 844-857.

[275] P. Janhavi, S. Divyashree, K.P. Sanjailal, S.P. Muthukumar, DoseCal: a virtual calculator for dosage conversion between human and different animal species, *Arch. Physiol. Biochem.* 128 (2022) 426-430.

[276] A.R. Mezo, V. Sridhar, J. Badger, P. Sakorafas, V. Nienaber, X-ray crystal structures of monomeric and dimeric peptide inhibitors in complex with the human neonatal Fc receptor, FcRn, *J. Biol. Chem.* 285 (2010) 27694-27701.

[277] Center for Drug Evaluation and Research, Product quality review(s): Rybelsus® (semaglutide) tablets - quality assessment. Available on: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/213051Orig1s000ChemR.pdf [accessed on: 25th February 2024].

- [278] C. He, L. Yin, C. Tang, C. Yin, Size-dependent absorption mechanism of polymeric nanoparticles for oral delivery of protein drugs, *Biomaterials*. 33 (2012) 8569-8578.
- [279] M. Caldorera-Moore, N. Guimard, L. Shi, K. Roy, Designer nanoparticles: incorporating size, shape and triggered release into nanoscale drug carriers, *Expert Opin. Drug Deliv.* 7 (2010) 479-495.
- [280] A.E. Pegg, Toxicity of polyamines and their metabolic products, *Chem. Res. Toxicol.* 26 (2013) 1782-1800.
- [281] B. Del Rio, B. Redruello, D.M. Linares, V. Ladero, P. Ruas-Madiedo, M. Fernandez, M.C. Martin, M.A. Alvarez, Spermine and spermidine are cytotoxic towards intestinal cell cultures, but are they a health hazard at concentrations found in foods?, *Food Chem.* 269 (2018) 321-326.
- [282] S.P. Patil, J.W. Yi, E.-K. Bang, E.M. Jeon, B.H. Kim, Synthesis and efficient siRNA delivery of polyamine-conjugated cationic nucleoside lipids, *MedChemComm.* 2 (2011) 505-508.
- [283] Y. Zhang, Z. Yuan, Y. Jin, W. Zhang, W.E. Yuan, Novel fluorinated spermine and small molecule PEI to deliver anti-PD-L1 and anti-VEGF siRNA for highly efficient tumor therapy, *Pharmaceutics*. 13 (2021) 2058.
- [284] R. Ribeiro, B. Sarmento, J. das Neves, Production and characterization of anti-CCR5 siRNA-loaded polycaprolactone nanoparticles for topical pre-exposure prophylaxis, *Methods Mol. Biol.* 2282 (2021) 403-416.
- [285] C. Pereira, J. Costa, B. Sarmento, F. Araújo, 3.3 - Cell-based *in vitro* models for intestinal permeability studies, in: B. Sarmento (Ed.) *Concepts and Models for Drug Permeability Studies*, Woodhead Publishing 2016, pp. 57-81.
- [286] Y. Lu, J. Qi, W. Wu, Chapter 20 - Lipid nanoparticles: *in vitro* and *in vivo* approaches in drug delivery and targeting, in: A.M. Grumezescu (Ed.) *Drug Targeting and Stimuli Sensitive Drug Delivery Systems*, William Andrew Publishing 2018, pp. 749-783.
- [287] T. Lea, Caco-2 Cell Line, in: K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, H. Wichers (Eds.) *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, Springer International Publishing 2015, pp. 103-111.
- [288] F. Araújo, B. Sarmento, Towards the characterization of an *in vitro* triple co-culture intestine cell model for permeability studies, *Int. J. Pharm.* 458 (2013) 128-134.

- [289] ISO. ISO 10993-5:2009: Biological evaluation of medical devices — part 5: tests for *in vitro* cytotoxicity. Available on: <https://www.iso.org/standard/36406.html> [accessed on 3rd September 2023].
- [290] K.v. Bilsen, P.M.v. Hagen, J. Bastiaans, J.v. Meurs, G.S. Baarsma, T. Missotten, R.W. Kuijpers, H. Hooijkaas, G.M. Dingjan, W.A. Dik, FcRn expression in the human retinal pigment epithelium, *Invest. Ophthalmol. Vis. Sci.* 51 (2010) 840-840.
- [291] G.M.H. Birchenough, M.E. Johansson, J.K. Gustafsson, J.H. Bergström, G.C. Hansson, New developments in goblet cell mucus secretion and function, *Mucosal Immunol.* 8 (2015) 712-719.
- [292] M.F. Izaguirre, D. Larrea, J.F. Adur, J.E. Diaz-Zamboni, N.B. Vicente, C.D. Galetto, V.H. Casco, Role of E-cadherin in epithelial architecture maintenance, *Cell Commun. Adhes.* 17 (2010) 1-12.
- [293] A.B. West, C.A. Isaac, J.M. Carboni, J.S. Morrow, M.S. Mooseker, K.W. Barwick, Localization of villin, a cytoskeletal protein specific to microvilli, in human ileum and colon and in colonic neoplasms, *Gastroenterology.* 94 (1988) 343-352.
- [294] W.-T. Kuo, M.A. Odenwald, J.R. Turner, L. Zuo, Tight junction proteins occludin and ZO-1 as regulators of epithelial proliferation and survival, *Ann. N.Y. Acad. Sci.* 1514 (2022) 21-33.
- [295] J.R. Spence, C.N. Mayhew, S.A. Rankin, M.F. Kuhar, J.E. Vallance, K. Tolle, E.E. Hoskins, V.V. Kalinichenko, S.I. Wells, A.M. Zorn, N.F. Shroyer, J.M. Wells, Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*, *Nature.* 470 (2011) 105-109.
- [296] C. He, F. Ruan, S. Jiang, J. Zeng, H. Yin, R. Liu, Y. Zhang, L. Huang, C. Wang, S. Ma, Z. Zuo, Black phosphorus quantum dots cause nephrotoxicity in organoids, mice, and human cells, *Small.* 16 (2020) 2001371.
- [297] T. Sato, H. Clevers, Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications, *Science.* 340 (2013) 1190-1194.
- [298] T. Sato, R.G. Vries, H.J. Snippert, M. van de Wetering, N. Barker, D.E. Stange, J.H. van Es, A. Abo, P. Kujala, P.J. Peters, H. Clevers, Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche, *Nature.* 459 (2009) 262-265.
- [299] M. Huch, J.A. Knoblich, M.P. Lutolf, A. Martinez-Arias, The hope and the hype of organoid research, *Development.* 144 (2017) 938-941.
- [300] A. Fatehullah, S.H. Tan, N. Barker, Organoids as an *in vitro* model of human development and disease, *Nat. Cell Biol.* 18 (2016) 246-254.

- [301] H. Clevers, Modeling development and disease with organoids, *Cell*. 165 (2016) 1586-1597.
- [302] R. Cruz-Acuña, A.J. García, Engineered materials to model human intestinal development and cancer using organoids, *Exp. Cell Res.* 377 (2019) 109-114.
- [303] S. Bartfeld, H. Clevers, Organoids as model for infectious diseases: culture of human and murine stomach organoids and microinjection of *Helicobacter pylori*, *J. Vis. Exp.* (2015) 53359.
- [304] V. Bozzetti, S. Senger, Organoid technologies for the study of intestinal microbiota-host interactions, *Trends Mol. Med.* 28 (2022) 290-303.
- [305] H.Q. Estrada, S. Patel, S. Rabizadeh, D. Casero, S.R. Targan, R.J. Barrett, Development of a personalized intestinal fibrosis model using human intestinal organoids derived from induced pluripotent stem cells, *Inflamm. Bowel Dis.* 28 (2022) 667-679.
- [306] J.L. Forbester, D. Goulding, L. Vallier, N. Hannan, C. Hale, D. Pickard, S. Mukhopadhyay, G. Dougan, Interaction of *Salmonella enterica* Seroovar *Typhimurium* with intestinal organoids derived from human induced pluripotent stem cells, *Infect. Immun.* 83 (2015) 2926-2934.
- [307] T. Grabinger, L. Luks, F. Kostadinova, C. Zimmerlin, J.P. Medema, M. Leist, T. Brunner, *Ex vivo* culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy, *Cell Death Dis.* 5 (2014) e1228.
- [308] K.B. Jung, H. Lee, Y.S. Son, M.O. Lee, Y.D. Kim, S.J. Oh, O. Kwon, S. Cho, H.S. Cho, D.S. Kim, J.H. Oh, M. Zilbauer, J.K. Min, C.R. Jung, J. Kim, M.Y. Son, Interleukin-2 induces the *in vitro* maturation of human pluripotent stem cell-derived intestinal organoids, *Nat. Commun.* 9 (2018) 3039.
- [309] M. Qu, L. Xiong, Y. Lyu, X. Zhang, J. Shen, J. Guan, P. Chai, Z. Lin, B. Nie, C. Li, J. Xu, H. Deng, Establishment of intestinal organoid cultures modeling injury-associated epithelial regeneration, *Cell Res.* 31 (2021) 259-271.
- [310] S.S. Wilson, A. Tocchi, M.K. Holly, W.C. Parks, J.G. Smith, A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions, *Mucosal Immunol.* 8 (2015) 352-361.
- [311] Z. Davoudi, N. Peroutka-Bigus, B. Bellaire, M. Wannemuehler, T.A. Barrett, B. Narasimhan, Q. Wang, Intestinal organoids containing poly(lactic-co-glycolic acid) nanoparticles for the treatment of inflammatory bowel diseases, *J. Biomed. Mater. Res. A.* 106 (2018) 876-886.

- [312] Z. Davoudi, N. Peroutka-Bigus, B. Bellaire, A. Jergens, M. Wannemuehler, Q. Wang, Gut organoid as a new platform to study alginate and chitosan mediated PLGA nanoparticles for drug delivery, *Mar. Drugs*. 19 (2021).
- [313] L. Bao, X. Cui, X. Wang, J. Wu, M. Guo, N. Yan, C. Chen, Carbon nanotubes promote the development of intestinal organoids through regulating extracellular matrix viscoelasticity and intracellular energy metabolism, *ACS Nano*. 15 (2021) 15858-15873.
- [314] K.L. VanDussen, J.M. Marinshaw, N. Shaikh, H. Miyoshi, C. Moon, P.I. Tarr, M.A. Ciorba, T.S. Stappenbeck, Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays, *Gut*. 64 (2015) 911-920.
- [315] K. Kozuka, Y. He, S. Koo-McCoy, P. Kumaraswamy, B. Nie, K. Shaw, P. Chan, M. Leadbetter, L. He, J.G. Lewis, Z. Zhong, D. Charmot, M. Balaa, A.J. King, J.S. Caldwell, M. Siegel, Development and characterization of a human and mouse intestinal epithelial cell monolayer platform, *Stem Cell Rep*. 9 (2017) 1976-1990.
- [316] Y. Wang, M. DiSalvo, D.B. Gunasekara, J. Dutton, A. Proctor, M.S. Lebhara, I.A. Williamson, J. Speer, R.L. Howard, N.M. Smiddy, S.J. Bultman, C.E. Sims, S.T. Magness, N.L. Allbritton, Self-renewing monolayer of primary colonic or rectal epithelial cells, *Cell Mol. Gastroenterol. Hepatol*. 4 (2017) 165-182.
- [317] G. Altay, E. Larrañaga, S. Tosi, F.M. Barriga, E. Batlle, V. Fernández-Majada, E. Martínez, Self-organized intestinal epithelial monolayers in crypt and villus-like domains show effective barrier function, *Sci. Rep*. 9 (2019) 10140.
- [318] P. Kakni, C. López-Iglesias, R. Truckenmüller, P. Habibović, S. Giselbrecht, Reversing epithelial polarity in pluripotent stem cell-derived intestinal organoids, *Front. Bioeng. Biotechnol*. 10 (2022) 879024.
- [319] J.Y. Co, M. Margalef-Català, X. Li, A.T. Mah, C.J. Kuo, D.M. Monack, M.R. Amieva, Controlling epithelial polarity: a human enteroid model for host-pathogen interactions, *Cell Rep*. 26 (2019) 2509-2520.
- [320] J.Y. Co, M. Margalef-Català, D.M. Monack, M.R. Amieva, Controlling the polarity of human gastrointestinal organoids to investigate epithelial biology and infectious diseases, *Nat. Protoc*. 16 (2021) 5171-5192.
- [321] T.J. Nash, K.M. Morris, N.A. Mabbott, L. Vervelde, Inside-out chicken enteroids with leukocyte component as a model to study host-pathogen interactions, *Commun. Biol*. 4 (2021) 377.

- [322] G. Stroulios, M. Stahl, F. Elstone, W. Chang, S. Louis, A. Eaves, S. Simmini, R.K. Conder, Culture methods to study apical-specific interactions using intestinal organoid models, *J. Vis. Exp.* (2021).
- [323] S. Bartfeld, T. Bayram, M. van de Wetering, M. Huch, H. Begthel, P. Kujala, R. Vries, P.J. Peters, H. Clevers, *In vitro* expansion of human gastric epithelial stem cells and their responses to bacterial infection, *Gastroenterology*. 148 (2015) 126-136.e126.
- [324] D.R. Hill, S. Huang, Y.H. Tsai, J.R. Spence, V.B. Young, Real-time measurement of epithelial barrier permeability in human intestinal organoids, *J. Vis. Exp.* (2017) 56960.
- [325] I.A. Williamson, J.W. Arnold, L.A. Samsa, L. Gaynor, M. DiSalvo, J.L. Cocchiaro, I. Carroll, M.A. Azcarate-Peril, J.F. Rawls, N.L. Allbritton, S.T. Magness, A high-throughput organoid microinjection platform to study gastrointestinal microbiota and luminal physiology, *Cell Mol. Gastroenterol. Hepatol.* 6 (2018) 301-319.
- [326] N. Bertaux-Skeirik, R. Feng, M.A. Schumacher, J. Li, M.M. Mahe, A.C. Engevik, J.E. Javier, R.M. Peek, Jr., K. Ottemann, V. Orian-Rousseau, G.P. Boivin, M.A. Helmrath, Y. Zavros, CD44 plays a functional role in *Helicobacter pylori*-induced epithelial cell proliferation, *PLoS Pathog.* 11 (2015) e1004663.
- [327] V. Polo, A. Saibene, A.E. Pontiroli, Nicotinamide improves insulin secretion and metabolic control in lean type 2 diabetic patients with secondary failure to sulphonylureas, *Acta Diabetol.* 35 (1998) 61-64.
- [328] S.P. Lee, F.Y. Kuo, J.T. Cheng, M.C. Wu, GLP-1 mediates the modulating effect of thymoquinone on feeding behaviors in diabetic rats, *Diabetes Metab. Syndr. Obes.* 12 (2019) 873-881.
- [329] E. Gumuslu, N. Cine, M. Ertan, O. Mutlu, I. Komsuoglu Celikyurt, G. Ulak, Exenatide upregulates gene expression of glucagon-like peptide-1 receptor and nerve growth factor in streptozotocin/nicotinamide-induced diabetic mice, *Fundam. Clin. Pharmacol.* 32 (2018) 174-180.
- [330] T. Chen, L. Kagan, D.E. Mager, Population pharmacodynamic modeling of exenatide after 2-week treatment in STZ/NA diabetic rats, *J. Pharm. Sci.* 102 (2013) 3844-3851.
- [331] E. Gumuslu, O. Mutlu, I.K. Celikyurt, G. Ulak, F. Akar, F. Erden, M. Ertan, Exenatide enhances cognitive performance and upregulates neurotrophic factor gene expression levels in diabetic mice, *Fundam. Clin. Pharmacol.* 30 (2016) 376-384.
- [332] S. Ozyazgan, N. Kutluata, S. Afşar, S.B. Özdaş, A.G. Akkan, Effect of glucagon-like peptide-1(7-36) and exendin-4 on the vascular reactivity in streptozotocin/nicotinamide-induced diabetic rats, *Pharmacology*. 74 (2005) 119-126.

- [333] P.E. Moustafa, N.F. Abdelkader, S.A. El Awdan, O.A. El-Shabrawy, H.F. Zaki, Liraglutide ameliorated peripheral neuropathy in diabetic rats: involvement of oxidative stress, inflammation and extracellular matrix remodeling, *J. Neurochem.* 146 (2018) 173-185.
- [334] J. Ventura-Sobrevilla, V.D. Boone-Villa, C.N. Aguilar, R. Román-Ramos, E. Vega-Avila, E. Campos-Sepúlveda, F. Alarcón-Aguilar, Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice, *Proc. West. Pharmacol. Soc.* 54 (2011) 5-9.
- [335] J. Szilvássy, I. Sziklai, P. Horvath, M. Szilasi, J. Németh, P. Kovács, Z. Szilvássy, Feeble bronchomotor responses in diabetic rats in association with decreased sensory neuropeptide release, *Am. J. Physiol. Lung Cell Mol. Physiol.* 282 (2002) L1023-1030.
- [336] M.L. Graham, J.L. Janecek, J.A. Kittredge, B.J. Hering, H.J. Schuurman, The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources, *Comp. Med.* 61 (2011) 356-360.
- [337] Y. Song, Y. Shi, L. Zhang, H. Hu, C. Zhang, M. Yin, X. Zhang, K. Sun, Oral delivery system for low molecular weight protamine-dextran-poly(lactic-co-glycolic acid) carrying exenatide to overcome the mucus barrier and improve intestinal targeting efficiency, *Nanomedicine.* 14 (2019) 989-1009.
- [338] J. Cuthbertson, S. Patterson, F.P. O'Harte, P.M. Bell, Addition of metformin to exogenous glucagon-like peptide-1 results in increased serum glucagon-like peptide-1 concentrations and greater glucose lowering in type 2 diabetes *mellitus*, *Metabolism.* 60 (2011) 52-56.
- [339] E. Cristiano, J.M. Miles, Side effect synergism between metformin and GLP-1 receptor agonists, *Am. Heart J.* 254 (2022) 240-241.
- [340] M. Zander, A. Christiansen, S. Madsbad, J. Juul Holst, Additive effects of glucagon-like peptide-1 and pioglitazone in patients with type 2 diabetes, *Diabetes Care.* 27 (2004) 1910-1914.
- [341] J. Ke, Y. Liu, J. Yang, R. Lu, Q. Tian, W. Hou, G. Wang, R. Wei, T. Hong, Synergistic effects of metformin with liraglutide against endothelial dysfunction through GLP-1 receptor and PKA signalling pathway, *Sci. Rep.* 7 (2017) 41085.
- [342] P.J. Larsen, E.M. Wulff, C.F. Gotfredsen, C.L. Brand, J. Sturis, N. Vrang, L.B. Knudsen, K. Lykkegaard, Combination of the insulin sensitizer, pioglitazone, and the long-acting GLP-1 human analog, liraglutide, exerts potent synergistic glucose-lowering efficacy in severely diabetic ZDF rats, *Diabetes Obes. Metab.* 10 (2008) 301-311.

- [343] M. Zander, A. Christiansen, S. Madsbad, J.J. Holst, Additive effects of glucagon-like peptide 1 and pioglitazone in patients with type 2 diabetes, *Diabetes Care*. 27 (2004) 1910-1914.
- [344] M.V. Udiawar, S.C. Bain, Liraglutide in combination with metformin or sulfonylurea for the treatment of type 2 diabetes in adult patients, *Clin. Med. Insights Ther.* 4 (2012) CMT.S7283.
- [345] Y. Seino, M.F. Rasmussen, T. Nishida, K. Kaku, Glucagon-like peptide-1 analog liraglutide in combination with sulfonylurea safely improves blood glucose measures vs sulfonylurea monotherapy in japanese patients with type 2 diabetes: results of a 52-week, randomized, multicenter trial, *J. Diabetes Investig.* 2 (2011) 280-286.
- [346] S. Kalra, S. Bahendeka, R. Sahay, S. Ghosh, F. Md, A. Orabi, K. Ramaiya, S. Al Shammari, D. Shrestha, K. Shaikh, S. Abhayaratna, P.K. Shrestha, A. Mahalingam, M. Askheta, A.R. AA, F. Eliana, H.K. Shrestha, S. Chaudhary, N. Ngugi, J.C. Mbanya, T.T. Aye, T.S. Latt, Z.A. Akanov, A.R. Syed, N. Tandon, A.G. Unnikrishnan, S.V. Madhu, A. Jawa, S. Chowdhury, S. Bajaj, A.K. Das, Consensus recommendations on sulfonylurea and sulfonylurea combinations in the management of type 2 diabetes *mellitus* - International Task Force, *Indian J. Endocrinol. Metab.* 22 (2018) 132-157.
- [347] A.J. Garber, Liraglutide in oral antidiabetic drug combination therapy, *Diabetes Obes. Metab.* 14 (2012) 13-19.
- [348] C. Li, J. Luo, M. Jiang, K. Wang, The efficacy and safety of the combination therapy with GLP-1 receptor agonists and SGLT-2 inhibitors in type 2 diabetes *mellitus*: a systematic review and meta-analysis, *Front. Pharmacol.* 13 (2022) 838277.
- [349] R. Marfella, F. Prattichizzo, C. Sardu, P.F. Rambaldi, C. Fumagalli, L.V. Marfella, R. La Grotta, C. Frigé, V. Pellegrini, D. D'Andrea, A. Cesaro, P. Calabrò, C. Pizzi, R. Antonicelli, A. Ceriello, C. Mauro, G. Paolisso, GLP-1 receptor agonists-SGLT-2 inhibitors combination therapy and cardiovascular events after acute myocardial infarction: an observational study in patients with type 2 diabetes, *Cardiovasc. Diabetol.* 23 (2024) 10.
- [350] P. Gourdy, P. Darmon, F. Dievart, J.-M. Halimi, B. Guerci, Combining glucagon-like peptide-1 receptor agonists (GLP-1RAs) and sodium-glucose cotransporter-2 inhibitors (SGLT2is) in patients with type 2 diabetes *mellitus* (T2DM), *Cardiovasc. Diabetol.* 22 (2023) 79.
- [351] J.E. Anderson, Combining glucagon-like peptide-1 receptor agonists and sodium-glucose cotransporter 2 inhibitors to target multiple organ defects in type 2 diabetes, *Diabetes Spectrum*. 33 (2020) 165-174.

- [352] C. Liu, X. Feng, Q. Li, Y. Wang, Q. Li, M. Hua, Adiponectin, TNF- α and inflammatory cytokines and risk of type 2 diabetes: a systematic review and meta-analysis, *Cytokine*. 86 (2016) 100-109.
- [353] J. Xiao, J. Li, L. Cai, S. Chakrabarti, X. Li, Cytokines and diabetes research, *J. Diabetes Res.* 2014 (2014) 920613.
- [354] E. Zaharieva, Z. Kamenov, T. Velikova, A. Tsakova, Y. El-Darawish, H. Okamura, Interleukin-18 serum level is elevated in type 2 diabetes and latent autoimmune diabetes, *Endocr. Connect.* 7 (2018) 179-185.
- [355] H. Zhuang, J. Han, L. Cheng, S.L. Liu, A positive causal influence of IL-18 levels on the risk of T2DM: a mendelian randomization study, *Front. Genet.* 10 (2019) 295.
- [356] J.F. Navarro-González, C. Mora-Fernández, The role of inflammatory cytokines in diabetic nephropathy, *J. Am. Soc. Nephrol.* 19 (2008) 433-442.
- [357] Y.S. Lee, H.S. Jun, Anti-inflammatory effects of GLP-1-based therapies beyond glucose control, *Mediators Inflamm.* 2016 (2016) 3094642.
- [358] J. Xia, Q. Li, Y. Liu, Q. Ren, J. Gao, Y. Tian, J. Li, B. Zhang, H. Sun, S. Liu, A GLP-1 analog liraglutide reduces intimal hyperplasia after coronary stent implantation via regulation of glycemic variability and NLRP3 inflammasome/IL-10 signaling in diabetic swine, *Front. Pharmacol.* 11 (2020).
- [359] V. Guarnotta, M.J. Bianco, E. Vigneri, F. Panto', B. Lo Sasso, M. Ciaccio, G. Pizzolanti, C. Giordano, Effects of GLP-1 receptor agonists on myokine levels and pro-inflammatory cytokines in patients with type 2 diabetes *mellitus*, *Nutr. Metab. Cardiovasc. Dis.* 31 (2021) 3193-3201.
- [360] G. Bendotti, L. Montefusco, M.E. Lunati, V. Usuelli, I. Pastore, E. Lazzaroni, E. Assi, A.J. Seelam, B. El Essawy, J. Jang, C. Loretelli, F. D'Addio, C. Berra, M. Ben Nasr, G. Zuccotti, P. Fiorina, The anti-inflammatory and immunological properties of GLP-1 receptor agonists, *Pharmacol. Res.* 182 (2022) 106320.
- [361] R.Z. Harms, D.N. Yarde, Z. Guinn, K.M. Lorenzo-Arteaga, K.P. Corley, M.S. Cabrera, N.E. Sarvetnick, Increased expression of IL-18 in the serum and islets of type 1 diabetics, *Mol. Immunol.* 64 (2015) 306-312.
- [362] Y. Moriwaki, T. Yamamoto, Y. Shibutani, E. Aoki, Z. Tsutsumi, S. Takahashi, H. Okamura, M. Koga, M. Fukuchi, T. Hada, Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes *mellitus*: relationship with diabetic nephropathy, *Metabolism.* 52 (2003) 605-608.

- [363] X. Zhang, S. Luo, M. Wang, Q. Huang, W. Fang, J. Li, T. Liu, Y. Zhang, Z. Deng, C.L. Liu, S. Guan, J.E. Ayala, R.A. Flavell, R.N. Kulkarni, P. Libby, J. Guo, Z. Liu, G.P. Shi, IL18 signaling causes islet β cell development and insulin secretion via different receptors on acinar and β cells, *Dev. Cell.* 57 (2022) 1496-1511.
- [364] J.J. Meier, Efficacy of semaglutide in a subcutaneous and an oral formulation, *Front. Endocrinol.* 12 (2021) 645617.
- [365] A. Ingersen, M. Schmücker, C. Alexandersen, B. Graungaard, T. Thorngreen, J. Borch, J.J. Holst, J.W. Helge, F. Dela, Effects of aerobic training and semaglutide treatment on pancreatic β -cell secretory function in patients with type 2 diabetes, *J. Clin. Endocrinol. Metab.* 108 (2023) 2798-2811.
- [366] T.S. Marinho, F.F. Martins, L.E.M. Cardoso, M.B. Aguila, C.A. Mandarim-de-Lacerda, Pancreatic islet cells disarray, apoptosis, and proliferation in obese mice. The role of semaglutide treatment, *Biochimie.* 193 (2022) 126-136.
- [367] C. Kapitza, K. Dahl, J.B. Jacobsen, M.B. Axelsen, A. Flint, Effects of semaglutide on beta cell function and glycaemic control in participants with type 2 diabetes: a randomised, double-blind, placebo-controlled trial, *Diabetologia.* 60 (2017) 1390-1399.
- [368] D.M. Hargrove, N.A. Nardone, L.M. Persson, J.C. Parker, R.W. Stevenson, Glucose-dependent action of glucagon-like peptide-1 (7-37) *in vivo* during short- or long-term administration, *Metabolism.* 44 (1995) 1231-1237.
- [369] S.J. Henderson, A. Konkar, D.C. Hornigold, J.L. Trevaskis, R. Jackson, M. Fritsch Fredin, R. Jansson-Löfmark, J. Naylor, A. Rossi, M.A. Bednarek, N. Bhagroo, H. Salari, S. Will, S. Oldham, G. Hansen, M. Feigh, T. Klein, J. Grimsby, S. Maguire, L. Jermutus, C.M. Rondinone, M.P. Coghlan, Robust anti-obesity and metabolic effects of a dual GLP-1/glucagon receptor peptide agonist in rodents and non-human primates, *Diabetes Obes. Metab.* 18 (2016) 1176-1190.
- [370] V.A. Gault, V.K. Bhat, N. Irwin, P.R. Flatt, A novel glucagon-like peptide-1 (GLP-1)/glucagon hybrid peptide with triple-acting agonist activity at glucose-dependent insulinotropic polypeptide, GLP-1, and glucagon receptors and therapeutic potential in high fat-fed mice, *J. Biol. Chem.* 288 (2013) 35581-35591.
- [371] Y. Li, T. Hansotia, B. Yusta, F. Ris, P.A. Halban, D.J. Drucker, Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis, *J. Biol. Chem.* 278 (2003) 471-478.

Chapter | 6

Appendix

Appendix I

Review paper: *New insights into nanomedicines for oral delivery of glucagon-like peptide-1 analogs*

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ADVANCED REVIEW



NANOMEDICINE AND NANOTECHNOLOGY

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New insights into nanomedicines for oral delivery of glucagon-like peptide-1 analogs

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Abstract

Type 2 diabetes *mellitus* (T2DM) is a metabolic disorder that arises when the body cannot respond fully to insulin, leading to impaired glucose tolerance. Currently, the treatment embraces non-pharmacological actions (e.g., diet and exercise) co-associated with the administration of antidiabetic drugs. Metformin is the first-line treatment for T2DM; nevertheless, alternative therapeutic strategies involving glucagon-like peptide-1 (GLP-1) analogs have been explored for managing the disease. GLP-1 analogs trigger insulin secretion and suppress glucagon release in a glucose-dependent manner thereby, reducing the risk of hyperglycemia. Additionally, GLP-1 analogs have an extended plasma half-life compared to the endogenous peptide due to their high resistance to degradation by dipeptidyl peptidase-4. However, GLP-1 analogs are mainly administered via subcutaneous route, which can be inconvenient for the patients. Even considering an oral delivery approach, GLP-1 analogs are exposed to the harsh conditions of the gastrointestinal tract (GIT) and the intestinal barriers (mucus and epithelium). Hereupon, there is an unmet need to develop non-invasive oral transmucosal drug delivery strategies, such as the incorporation of GLP-1 analogs into nanoplateforms, to overcome the GIT barriers. Nanotechnology has the potential to shield antidiabetic peptides against the acidic pH and enzymatic activity of the stomach. In addition, the nanoparticles can be coated and/or surface-conjugated with mucodiffusive polymers and target intestinal ligands to improve their transport through the intestinal mucus and epithelium. This review focuses on the main hurdles associated with the oral administration of GLP-1 and GLP-1 analogs, and the

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Appendix II

Research paper: *Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide*

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Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide

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ABSTRACT

Semaglutide is the first oral glucagon-like peptide-1 (GLP-1) analog commercially available for the treatment of type 2 diabetes. In this work, semaglutide was incorporated into poly(lactic-co-glycolic acid)-poly(ethylene glycol) (PLGA-PEG) nanoparticles (NPs) to improve its delivery across the intestinal barrier. The nanocarriers were surface-decorated with either a peptide or an antibody that target the human neonatal Fc receptor (hFcRn), located on the luminal cell surface of the enterocytes. Both ligands were successfully conjugated with the PLGA-PEG via maleimide-thiol chemistry and thereafter, the functionalized polymers were used to produce semaglutide-loaded NPs. Monodisperse NPs with an average size of 170 nm, neutral surface charge and 3% of semaglutide loading were obtained. Both FcRn-targeted NPs exhibited improved interaction and association with Caco-2 cells (cells that endogenously express the hFcRn), compared to non-targeted NPs. Additionally, the uptake of FcRn-targeted NPs was also observed to occur in human intestinal organoids (HIOs) expressing hFcRn through microinjection into the lumen of HIOs, resulting in potential increase of semaglutide permeability for both ligand-functionalized nanocarriers. Herein, our study demonstrates valuable data and insights that the FcRn-targeted NPs has the capacity to promote intestinal absorption of therapeutic peptides.

1. Introduction

Diabetes *mellitus* is a metabolic and chronic disorder and it is estimated that approximately 537 million people are living with diabetes, and this number will continue to rise up to 700 million by 2045 [1].

Specifically, type 2 diabetes (T2D) comprises around 90% of the total cases of diabetes worldwide, and it is mainly caused by inadequate secretion of insulin from pancreatic beta (β) cells and insulin resistance, leading to impaired glucose tolerance [1,2]. Currently, the treatment of T2D encompasses non-pharmacological actions (e.g., diet and exercise)

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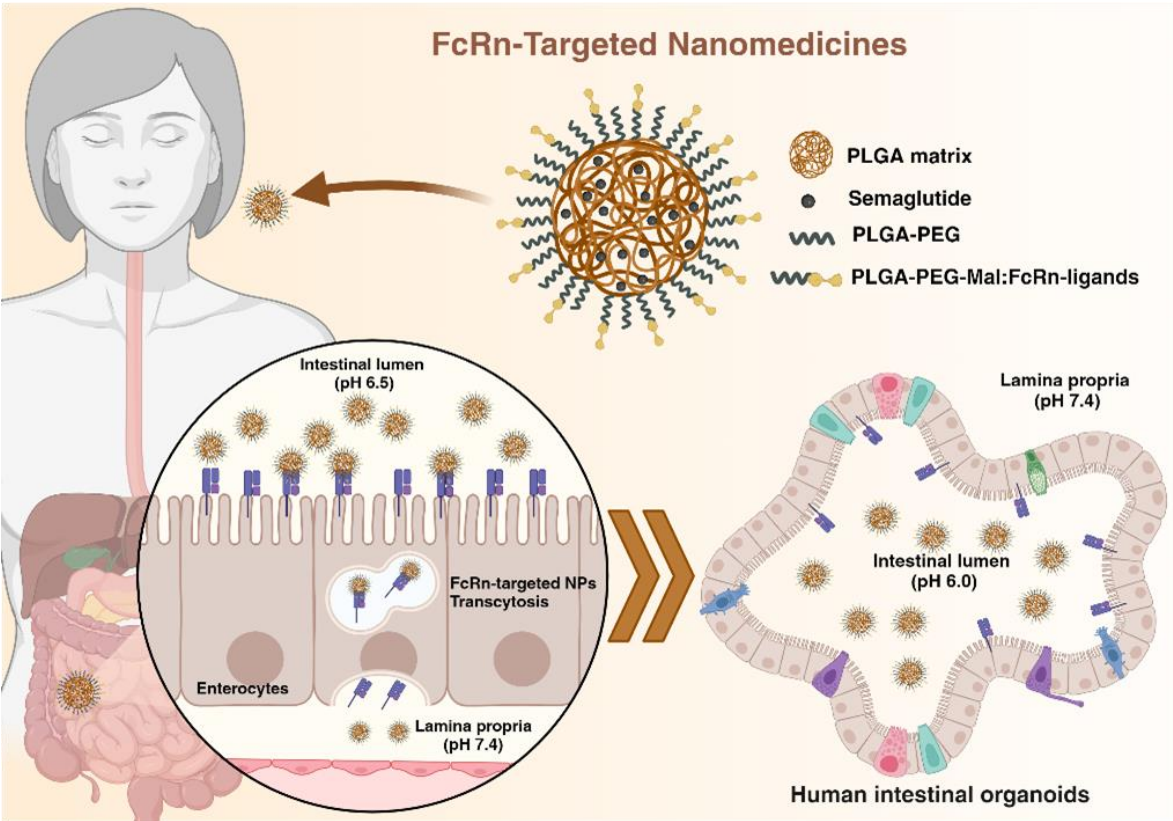
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Nanoparticles targeting the intestinal Fc receptor enhance intestinal cellular trafficking of semaglutide

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Appendix III

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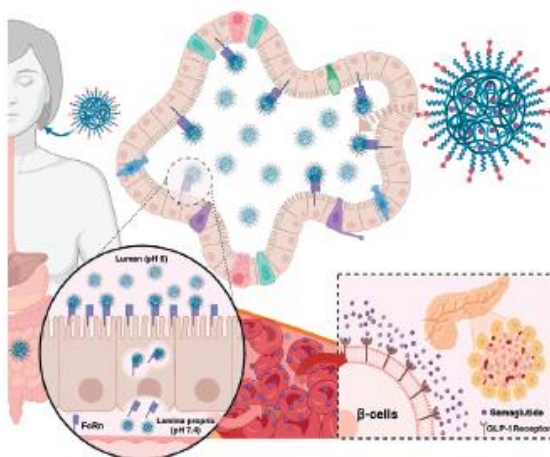


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Bioengineered neonatal Fc receptor-targeted nanomedicines improve the intestinal delivery of semaglutide in a 3D in vitro intestinal model

Appendix IV

Review paper: *Prevention of diabetes-associated fibrosis: Strategies in FcRn-targeted nanosystems for oral drug delivery*

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Prevention of diabetes-associated fibrosis: Strategies in FcRn-targeted nanosystems for oral drug delivery



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ABSTRACT

Diabetes mellitus is a chronic disease with an elevated risk of micro- and macrovascular complications, such as fibrosis. To prevent diabetes-associated fibrosis, the symptomatology of diabetes must be controlled, which is commonly done by subcutaneous injection of antidiabetic peptides. To minimize the pain and distress associated with such injections, there is an urgent need for non-invasive oral transmucosal drug delivery strategies. However, orally administered peptide-based drugs are exposed to harsh conditions in the gastrointestinal tract and poorly cross the selective intestinal epithelium. Thus, targeting of drugs to receptors expressed in epithelial cells, such as the neonatal Fc receptor (FcRn), may therefore enhance uptake and transport through mucosal barriers. This review compiles how in-depth studies of FcRn biology and engineering of receptor-binding molecules may pave the way for design of new classes of FcRn-targeted nanosystems. Tailored strategies may open new avenues for oral drug delivery and provide better treatment options for diabetes and, consequently, fibrosis prevention.

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Abbreviations: ABD, albumin-binding domain; AGEs, advanced glycation end-products; BSA, bovine serum albumin; β 2m, beta-2 microglobulin; DM, diabetes mellitus; DPP-4, dipeptidyl peptidase-4; ECM, extracellular matrix; EDC/NHS, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide; Fab, fragment antigen-binding; Fc, fragment crystallizable; Fc-NPs, Fc-decorated nanoparticles; FcRn, neonatal Fc receptor; FcRnBPs, neonatal Fc receptor-binding peptides; GIT, gastrointestinal tract; GLP-1, glucagon-like peptide-1; hFcRn, human neonatal Fc receptor; hIgG, human immunoglobulin G; HPMC, hypromellose acetate succinate; HSA, human serum albumin; HSA-NPs, HSA-decorated nanoparticles; IgG, immunoglobulin G; MAL, maleimide; mFcRn, mouse neonatal Fc receptor; MHC-I, major histocompatibility complex class I; mIgG, mouse immunoglobulin G; MSA, mouse serum albumin; NPs, nanoparticles; OI, Oral insulin; PEG, polyethylene glycol; PLA, poly(lactic acid); PLGA, poly(lactide-co-glycolic acid); RAAS, renin-angiotensin-aldosterone system; scFv, single-chain variable fragment; SiNPs, silica nanoparticles; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TGF- β , transforming growth factor beta; WT, wild-type.

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Appendix V

Selection of FcRn-targeted ligands with affinity for hFcRn

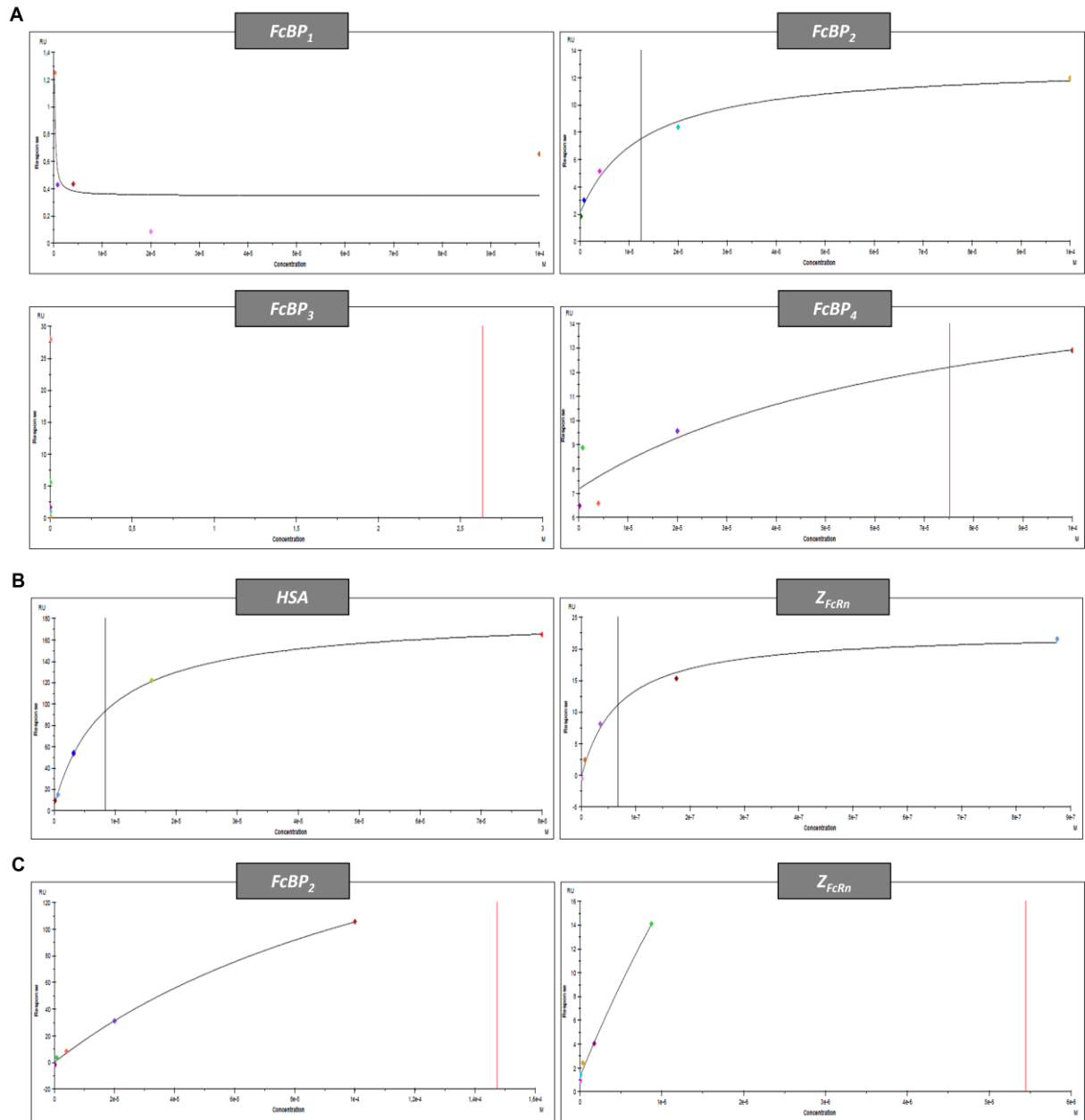
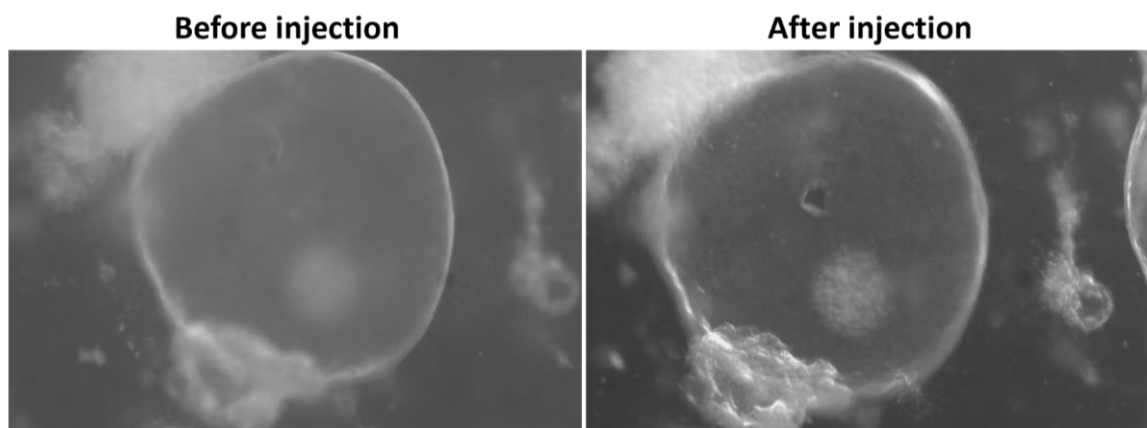


Figure A1. Saturation curves of the FcRn-targeted ligands interaction to hFcRn at pH 6.0 and pH 7.4. **(A)** Saturation curves of the FcBP₁, FcBP₂, FcBP₃, and FcBP₄ binding-affinity to hFcRn at pH 6.0. The peptide concentrations tested ranged from 0.16 to 100 μ M. It is noteworthy that only FcBP₂ peptide was able to interact with the receptor at acidic pH. **(B)** Saturation curves of the positive control (HSA) and the affibody Z_{FcRn} interaction to hFcRn at pH 6.0. The HSA concentrations tested ranged from 0.13 to 80 μ M, while the Z_{FcRn} concentrations tested ranged from 1.4 to 875 nM. Notably, both ligands were able to interact with the receptor at acidic pH. **(C)** Saturation curves of the FcBP₂ and Z_{FcRn} interaction to hFcRn at pH 7.4. The FcBP₂ concentrations tested ranged from 0.16 to 100 μ M, while the Z_{FcRn} concentrations tested ranged from 1.4 to 875 nM. The binding-affinity of FcBP₁, FcBP₃, and FcBP₄ to the receptor was not tested at physiological pH since these peptides did not bind to hFcRn at pH 6.0.

Appendix VI

Microinjection of polymeric nanocarriers into the lumen of human intestinal organoids



Objective 10x

Figure A2. Microinjection of nanosystems into the lumen of HIOs. The left image corresponds the HIOs structure, before NPs injection, and the right image corresponds the HIOs structure, after NPs injection into the lumen. It is noticeable the formation of a hole in the organoid, after injection of the particles. During the injection, the expansion of the organoids and the retraction, immediately after injection, were observed.

Appendix VII

FcRn-targeted nanoparticles reduce glycemia after a single administration

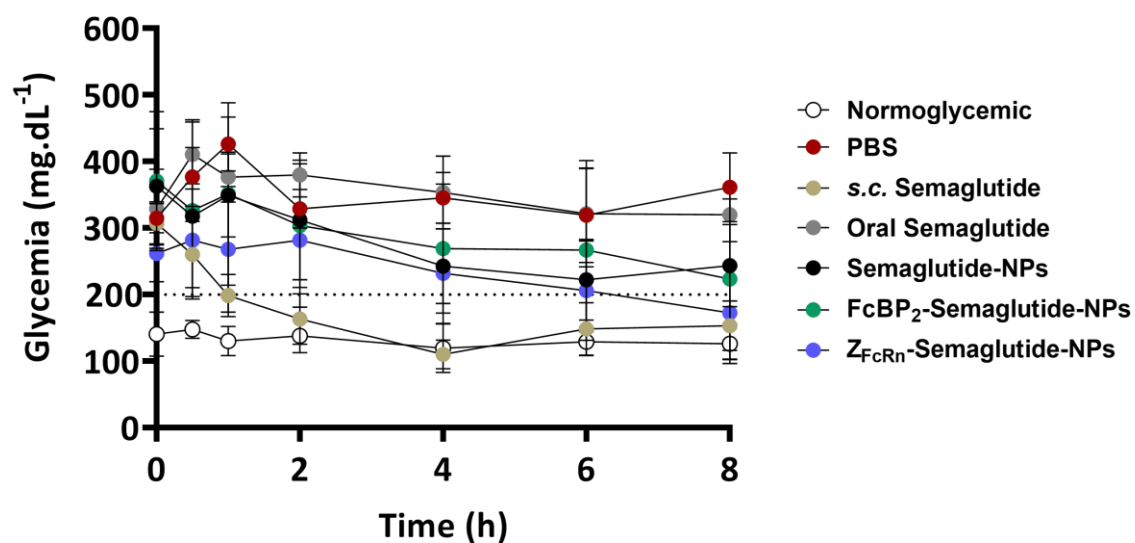


Figure A3. Blood glucose levels (mg.dL⁻¹) of T2D-induced mice monitored for over 8 h, after a single administration of PBS, free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs through oral gavage, and free semaglutide via s.c. injection. Results are represented as mean $\pm$ SD ($n = 6$).

Appendix VIII

FcRn-targeted nanoparticles reduce glycemia after multiple administrations

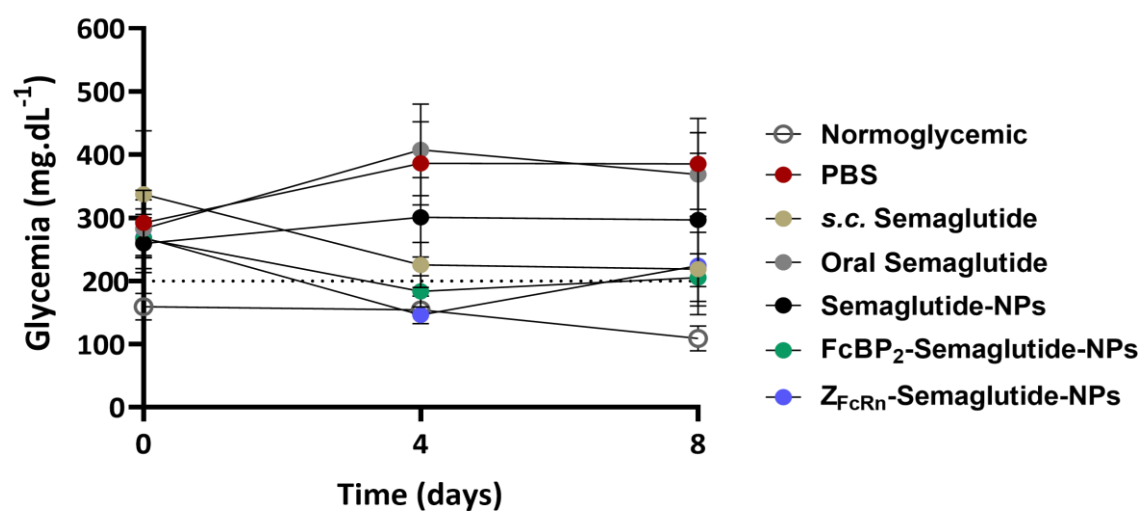


Figure A4. Blood glucose levels (mg.dL⁻¹) of T2D-induced mice monitored for over 8 days, after daily administrations of PBS, free semaglutide, semaglutide-NPs, FcBP₂-semaglutide-NPs, and Z_{FcRn}-semaglutide-NPs through oral gavage, and free semaglutide via s.c. injection. Results are represented as mean $\pm$ SD ($n = 6$).

Biographic note

Soraia Pinto was born on July 16, 1993, in Vila Nova de Gaia, Porto, Portugal. She obtained her master's degree in Pharmaceutical Sciences in 2018 from *Faculdade de Farmácia da Universidade do Porto*, Porto, Portugal. In 2019, she was congratulated with a master scholarship in a group specialized in the production and physicochemical characterization of nanosystems, NTDD group, at i3S, led by Professor Bruno Sarmento. Since 2020, Soraia is doing her PhD project in the PhD program Biomedical Sciences of ICBAS, *Universidade do Porto*, Porto, Portugal, at NTDD group, i3S, Porto, Portugal, under the supervision of Professor Bruno Sarmento. Her research interests focus on the nanotechnology field, particularly in the development of antidiabetic peptide-loaded nanosystems, functionalized with ligands that target an intestinal receptor, the FcRn. During her PhD journey, she obtained several skills regarding the development and characterization of NPs, as well as biomolecular techniques to evaluate the binding-affinity of ligands to the FcRn receptor. Additionally, she also acquired a broad experience in *in vitro* cell-based assays, including metabolic activity assays, uptake and internalization studies (flow cytometry and confocal microscopy analysis), and *in vivo* experiments, such as chemical induction of T2DM in rodents, *i.p.*, *s.c.* and oral administration of drugs, collection of blood samples from the tail vein, euthanasia through cardiac puncture followed by confirmation by cervical dislocation, collection of vital organs, and biodistribution assays. During the PhD pathway, Soraia had the opportunity to do a period abroad in the Department of Biomaterial & Biomedical Technology at UMCG, Groningen, The Netherlands, from October 2022 to April 2023, under the supervision of Professor Hélder Almeida Santos, where she had the outstanding chance to acquire expertise in the field of the 3D *in vitro* intestinal model, HIOs. She acquired skills related with the maintenance and growing of these 3D structures, and metabolic activity and cell-NPs interactions assays in organoids through the microinjection of the particles into their lumen. Soraia was also involved in the written of 4 review papers, 1 book chapter, and 7 research papers, since she was also involved in other collaborative projects. Additionally, she was also included in projects with pharmaceutical industries that ripen her knowledge regarding the nanotechnology field. She also did some courses regarding biomolecular interactions and laboratory animal science FELASA.

During her PhD period, she was also involved in other activities inside of i3S, Porto, Portugal, including to be part of the Organizing Committee of the PhDay conference and the PhD students seminars, as well as to be volunteer in *Porto de Crianças* initiative (which is based on give infants the opportunity to know a little bit more about the research field by visiting primary schools and discuss different subjects). Soraia with the help of her supervisors, also establish collaborations with Novo Nordisk company, Måløv, Denmark, and the Department of Protein Science, School of Engineering in Chemistry, Biotechnology

and Health, KTH Royal Institute of Technology, Stockholm, Sweden. She also participated in several national and international conferences that gave her the opportunity to present her PhD work and discuss science with researchers from different fields, and she was also abstract reviewer of one of the most important conferences in her field, the Controlled Release Society (CRS) conference 2023 and 2024. Recently, Soraia was selected as Trainee Representative of the Oral Delivery Focus Group of CRS, where she is having the chance to organize research events.