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ESPECIALIDADE DE NANOTECNOLOGIAS

Multistage pancreas biomimetic construct based on the synergism of nanomedicines and stem cell-derived pancreatic cells

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**Multistage pancreas biomimetic construct based on
the synergism of nanomedicines and
stem cell-derived pancreatic cells**

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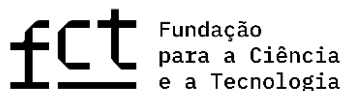


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*Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.*

- Marie Curie

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Resumo

Atualmente, a Diabetes *mellitus* é um dos maiores problemas de saúde a nível mundial, dado que cerca de 537 milhões de adultos em todo o mundo sofrem desta doença. A diabetes tipo 1 (T1D), que representa 10% de todos os casos de diabetes, é uma doença metabólica crónica caracterizada por uma secreção insuficiente de insulina devido à perda de células β , o que leva a níveis elevados de glicose no sangue. Apesar de a administração de insulina exógena ser o principal tratamento desta doença, muitos doentes continuam a sofrer de instabilidade glicémica grave, o que acarreta problemas a curto e longo prazo, tal como retinopatia, lesões microvasculares e nefropatia. Assim, uma estratégia de substituição das células β através do transplante de pâncreas ou dos ilhéus pancreáticos é uma abordagem mais fisiológica para estes doentes. No entanto, a escassez de doadores e a necessidade de terapia imunossupressora ao longo da vida colocam desafios significativos.

Como alternativa a estas estratégias, a diferenciação de células β e, mais recentemente, de células α a partir de células estaminais pluripotentes (PSCs) tem sido amplamente explorada nas últimas duas décadas. As PSCs oferecem uma fonte de células virtualmente ilimitada para transplante, ultrapassando a escassez de doadores associada ao transplante de ilhéus pancreáticos. Além disso, a encapsulação de células produtoras de insulina em matrizes de alginato tem sido explorada para as proteger do sistema imunitário, permitindo simultaneamente a troca de oxigénio, nutrientes e hormonas. No entanto, as abordagens de encapsulação na literatura apenas incluem células β diferenciadas a partir de PSCs, e a incorporação de outras células endócrinas pancreáticas derivadas de PSCs permitiria mimetizar de forma mais aproximada o ambiente nativo dos ilhéus pancreáticos.

Assim, o principal objetivo deste estudo foi desenvolver um novo pâncreas biomimético e explorar o seu potencial como tratamento para doentes com T1D. Este sistema é constituído por células β produtoras de insulina e células α produtoras de glucagon diferenciadas a partir de células estaminais pluripotentes induzidas humanas (hiPSCs), juntamente com glucose oxidase e nanopartículas (NPs) sensíveis ao pH que encapsulam um análogo do *glucagon-like peptide 1* (GLP-1) numa matriz de alginato. As células α diferenciadas foram incluídas neste sistema para melhor representar o ambiente nativo dos ilhéus pancreáticos e, conseqüentemente, regular melhor a função das células β diferenciadas. Ao incorporar também a glucose oxidase no sistema, o pH diminui transitoriamente em resposta a níveis elevados de glucose, levando à libertação do fármaco das NPs sensíveis ao pH de uma forma dependente da glucose. Além disso, a

inclusão de GLP-1 tem como objetivo melhorar a capacidade de resposta à glicose das células β diferenciadas, bem como diminuir a apoptose inicial sofrida pelas células após o transplante, reduzindo assim a percentagem de perda do material transplantado.

Neste trabalho, foram desenvolvidas e produzidas NPs sensíveis ao pH que encapsulam exenatide ou semaglutide através da técnica de dupla emulsão. As NPs apresentaram um tamanho médio de 145 nm, com uma eficiência de associação de 40% para o exenatide e de 55% para as NPs carregadas com semaglutide. Ambos os péptidos mantiveram a sua estrutura secundária após a libertação *in vitro* durante, pelo menos, 48 horas e mostraram um efeito semelhante na secreção de insulina das células INS-1E. As hiPSCs foram diferenciadas em células β e α e foram obtidas células positivas para a insulina (82 %), apesar da baixa capacidade de resposta à glucose, bem como células positivas para o glucagon (17,5 %). Ambos os tipos de células diferenciadas, assim como a glucose oxidase e as NPs responsivas à glucose, foram encapsulados em microesferas de alginato através da técnica de gelificação externa. As microesferas foram depois transplantadas para a cavidade peritoneal de ratinhos com T1D e as suas taxas de sobrevivência e níveis de glicemia foram avaliados durante um mês. O transplante do sistema desenvolvido mostrou um aumento na taxa de sobrevivência desses animais. Especificamente, o grupo tratado com o sistema desenvolvido teve uma taxa de sobrevivência de 50 % desde o dia 9 até ao final do estudo, enquanto o grupo de controlo (animais diabéticos sem tratamento) apresentou uma taxa de sobrevivência de 0 % ao dia 7. Além disso, os ratinhos diabéticos transplantados com células e exenatide apresentaram uma diminuição dos níveis de glucose.

No entanto, foi necessário efetuar mais otimizações no sistema e a adição de catalase contrabalançou com êxito o efeito citotóxico da glucose oxidase. Os ilhéus de dadores humanos ou ilhéus derivados de células estaminais foram incorporados em microesferas de alginato e a atividade metabólica (resazurina) bem como a sua função (secreção de insulina estimulada pela glucose) foram avaliadas em diferentes momentos. Os ilhéus de dadores encapsulados apresentaram uma maior atividade metabólica ao longo do tempo em comparação com os não encapsulados. Relativamente à secreção de insulina estimulada pela glucose, o impacto do sistema completo na secreção do péptido C dos ilhéus humanos não foi conclusivo. Por outro lado, os resultados sugerem que os ilhéus derivados de células estaminais apresentam uma maior (embora tardia) capacidade de resposta à glucose quando expostas a exenatide ou a NPs carregadas com exenatide no segundo dia após a encapsulação.

Em suma, neste trabalho foi desenvolvido um novo sistema pancreático biomimético como potencial tratamento para a diabetes tipo 1. Os resultados obtidos sugerem que as NPs sensíveis à glucose carregadas com análogos de GLP-1 podem tornar-se um aliado interessante para terapias baseadas em células estaminais destinadas à T1D. Além disso, o sistema pancreático bioartificial aqui descrito é bastante promissor para enfrentar alguns dos desafios apresentados pelas atuais terapias baseadas em células estaminais.

Palavras-chave: diabetes; terapia celular; análogos do GLP-1; nanopartículas sensíveis à glucose; pâncreas bioartificial.

Abstract

Diabetes *mellitus* is currently one of the world's greatest health problems, with around 537 million adults worldwide suffering from the disease. Type 1 diabetes (T1D), which accounts for 10% of all diabetes cases, is a chronic metabolic disease characterised by insufficient insulin secretion due to β -cell loss, leading to elevated blood sugar levels. Even though exogenous insulin administration is a lifesaving treatment, many patients still experience severe glycaemic lability, causing short- and long-term problems, such as retinopathy, microvascular damage and nephropathy. Thus, a β -cell replacement strategy through pancreas or pancreatic islet transplantation is the most physiological approach for these patients. However, donors' scarcity and the need for lifelong immunosuppressive therapy pose significant challenges.

As an alternative to these strategies, the differentiation of β -cells and more recently α -cells from pluripotent stem cells (PSCs) has been extensively explored over the past two decades. PSCs offer a virtually unlimited cell source for transplantation, addressing donor scarcity associated with islet transplantation. Additionally, encapsulating insulin-producing cells in alginate scaffolds has been explored to protect them from the immune system while allowing the exchange of oxygen, nutrients, and hormones. However, encapsulation approaches only include stem cell derived- β cells, and incorporating other stem cell-derived pancreatic endocrine cells would better mimic the native islet environment.

Therefore, the main goal of this study was to develop a novel biomimetic pancreas and to explore its potential as a treatment for T1D patients. This system comprises insulin-producing β - and glucagon-producing α -cells differentiated from human induced pluripotent stem cells (hiPSCs) together with glucose oxidase and pH-responsive nanoparticles (NPs) encapsulating a glucagon-like peptide 1 (GLP-1) analogue in an alginate matrix. Differentiated α -cells were included in this system to more closely resemble the native islet environment and consequently better regulate the function of differentiated β -cells. By incorporating glucose oxidase in the system, pH transiently decreases in response to high glucose levels, leading to drug release from pH-responsive nanoparticles in a glucose-sensitive manner. Moreover, the inclusion of GLP-1 aims to enhance the insulin secretion of differentiated β -cells, as well as to decrease the initial apoptosis experienced by cells after transplantation, thus reducing the percentage of graft loss.

Herein, pH-responsive NPs encapsulating exenatide or semaglutide were developed and produced by double emulsion technique. NPs showed an average size of 145 nm, with 40 % association efficiency for exenatide- and 55 % for semaglutide-loaded NPs. Both

peptides maintained their secondary structure after *in vitro* release for at least 48 h, and showed a similar effect on the insulin secretion of INS-1E cells. hiPSCs were differentiated into β - and α -cells and insulin-positive cells were obtained (82 %), despite low glucose responsiveness, as well as glucagon-positive cells (17.5 %). Both differentiated cell types, glucose oxidase and glucose-responsive NPs were encapsulated within alginate microspheres through the external gelation technique. Microspheres were then transplanted into the peritoneal cavity of T1D mice and their survival rates and glycemia levels were assessed for one month. The transplantation of the developed system showed an increase in the survival rate of those animals, specifically the group treated with the developed biomimetic construct had a 50 % survival rate from day 9 until the end of the study, while the control group (diabetic animals without treatment) had a 0 % survival rate at day 7. Moreover, diabetic mice transplanted with cells and exenatide showed a decrease in glucose levels.

Nonetheless, further improvements were performed in the system and the addition of catalase successfully counterbalanced glucose oxidase cytotoxic effect. Human donor islets or stem cell derived-islets were embedded in alginate microspheres and metabolic activity (resazurin) and function (glucose-stimulated insulin secretion) were assessed at different time points. Encapsulated donor islets showed higher metabolic activity over time compared to non-encapsulated ones. Regarding glucose-stimulated insulin secretion, the impact of the complete system on human islets C-peptide secretion was not conclusive. Conversely, stem cell-derived islets showed increased (although delayed) glucose-responsiveness when exposed to exenatide or exenatide-loaded NPs at day 2 post-encapsulation.

In conclusion, a novel biomimetic pancreatic construct was developed in this work as a potential treatment for type 1 diabetes. The results suggest that glucose-responsive GLP-1 analogue-loaded nanoparticles may be an interesting ally for stem cell-based therapies aimed at T1D. Besides, the multistage bioartificial pancreatic system herein described holds great promise to address some of the challenges presented by current stem cell-based therapies.

Keywords: Diabetes; stem cell therapy; GLP-1 analogues; glucose-responsive nanoparticle; bioartificial pancreas.

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List of Abbreviations and Symbols

ACDY	adenylate cyclase
<i>ACTB</i>	beta-actin gene
AE	association efficiency
Ala	alanine
ALK5i II	activin receptor-like kinase 5 inhibitor II
APC	antigen-presenting cell
ARX	Aristaless-related homeobox protein
AUC	area under the curve
AWERB	animal welfare and ethics review body
Axl	Axl receptor tyrosine kinase
BCR	B cell receptor
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BSA	bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
Cas9	CRISPR-associated protein 9
CAT	catalase
CD	circular dichroism
CMRL	Connaught Medical Research Laboratories
Con A	concanavalin A
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
<i>CTLA-4</i>	cytotoxic T-lymphocyte-associated protein 4 gene
CXCR4	C-X-C chemokine receptor type 4
D.I.T.	digital integration time
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DAPT	N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester
DC	dendritic cell
DE	definitive endoderm
DL	drug loading
DLS	dynamic light scattering
DM	Diabetes <i>mellitus</i>
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	deoxyribonucleic acid

dNTP	deoxynucleoside triphosphate
DPP-4	dipeptidylpeptidase-4
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EP	endocrine progenitors
ERK	extracellular-signal-regulated kinase
ESCs	embryonic stem cells
EXN	exenatide
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FOXA2	Forkhead Box A2
GBPs	glucose-binding proteins
GCG	glucagon
GCG	glucagon gene
GCGR	glucagon receptor
GDF8	growth differentiation factor 8
GHRH	growth hormone-releasing hormone
GHRHR	growth hormone-releasing hormone receptor
GIP	gastric inhibitory polypeptide
GIPR	gastric inhibitory polypeptide receptor
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLP-2R	glucagon-like peptide-2 receptor
GLUT2	glucose transporter 2
Gly	glycine
GOx	glucose oxidase
GPCR	G protein-coupled receptors
GSIS	glucose-stimulated insulin secretion
GSK	glycogen synthase kinase
HbA1c	glycated hemoglobin
HDAC	histone deacetylases

HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
hESCs	human embryonic stem cells
HFF	human foreskin fibroblast
HGF	hepatocyte growth factor
hiPSCs	human induced pluripotent stem cells
HLA	human leukocyte antigen
HNF	hepatocyte nuclear factor
HPLC	high-performance liquid chromatography
hPSCs	human pluripotent stem cells
HSA	human serum albumin
IBMIR	instant blood-mediated inflammatory reaction
ICM	inner cell mass
IDF	International Diabetes Federation
IGF	insulin-like growth factor
<i>IL2RA</i>	interleukin-2 receptor alpha chain
INS	insulin
<i>INS-VNTR</i>	insulin variable number of tandem repeats
iPSCs	induced pluripotent stem cells
i β -cell	immature β -cell
KGF	keratinocyte growth factor
LAR	long-acting release
LYP	lymphoid tyrosine phosphatase
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
M-MLV	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	messenger RNA
MRW	mean residue weight
m β -cell	mature β -cell
N-Cys	N-acetyl cysteine
ND	non-diabetic
NEP	neutral endopeptidase
NK	natural killer cells
NPs	nanoparticles
PARS	poly(ADP-ribose) synthetase
PBA	phenylboronic acid
PBS	phosphate-buffered saline

PBST	0.05 % Tween-20 in PBS
PDBu	Phorbol 12, 13-dibutyrate
PdI	polydispersity index
PD-L1	programmed death-ligand 1
PDX1	pancreatic and duodenal homeobox 1
PF	posterior foregut
PFA	paraformaldehyde
PGA	poly glycolic acid
PGT	primitive gut tube
pI	isoelectric point
PI3K	phosphoinositide 3-kinase
pKa	acid dissociation constant
PKC	protein kinase C
PLA	poly lactic acid
PLGA	poly(lactic-co-glycolic acid)
PP	pancreatic progenitors
PSCs	pluripotent stem cells
<i>PTPN22</i>	protein tyrosine phosphatase, non-receptor type 22 gene
RA	retinoic acid
RNA	ribonucleic acid
ROCK	rho-associated protein kinase
RT	room temperature
S.E.M.	scanning electron microscopy
SANT	Sonic hedgehog inhibitor
SCTR	secretin receptor
SC- β cells	stem cell-derived β cells
SEM	standard error of the mean
Shh	sonic hedgehog
SI	stimulation index
SMG	semaglutide
SOX17	SRY-Box transcription factor 17
SPB	saponin permeabilization buffer
SST	somatostatin
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes

T3	Triiodothyronine
TCR	T cell receptor
TF	transcription factor
TFA	trifluoroacetic acid
TGF- β	transforming growth factor- β
TPB	(2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl) phenyl)-2,4-pentadienoylamino) benzolactam
TPC	total polymer content
ULA	ultra-low attachment
USA	United States of America
VEGF	vascular endothelial growth factor
Vit C	vitamin C
Wnt	Wingless-related integration site
ZP	zeta potential

Relevant Background

The information provided in this chapter was partially based on the following publication:

- **Marques JM***, Nunes R*, Florindo H, Ferreira D, Sarmento B. Chapter 10 - A demanding path from iPSCs toward pancreatic β - and α -cells. In: Birbrair A, editor. Recent Advances in iPSC-Derived Cell Types. London: Academic Press, an imprint of Elsevier; 2021. p. 227-256. DOI: 10.1016/B978-0-12-822230-0.00002-8.

*Equal contribution.

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This research work contributes to the development of an innovative bioartificial pancreatic islet construct as a cell therapy for type 1 diabetes. This multidisciplinary thesis discusses different topics and research areas in order to provide a better understanding and scientific theoretical support of the work developed.

Therefore, this chapter begins by addressing the background problem through a comprehensive review of diabetes and its epidemiology, as well as the pathophysiology of type 1 diabetes and the healthy endocrine pancreatic environment (section 1.1), which is also important to understand the disease. In section 1.2, different available treatments for type 1 diabetes are addressed, followed by emerging therapeutical approaches (section 1.3) that have been recently developed to surpass some of the challenges posed by conventional treatments. In this section, nanotechnology-based drug delivery systems for type 1 diabetes are highlighted, as well as stem cell-derived pancreatic cells and their differentiation processes, followed by a selection of cell encapsulation strategies, which can be used to shield cells from the immune system.

1.1. Diabetes *mellitus*

1.1.1. Epidemiology and classification

Diabetes *mellitus* (DM) includes a group of chronic metabolic diseases characterised by high blood glucose levels due to deficiencies in insulin secretion, insulin action (or insulin resistance) or both.¹

Diabetes has no boundaries and is found in every population and all regions in the world (Figure 1). According to the International Diabetes Federation (IDF), DM affected 537 million adults (aged 20-79 years) worldwide in 2021, which represents 10.5 % of the global population in this age range. Besides, it is estimated that 6.7 million people of this age group died from diabetes-related causes in 2021.² As illustrated in Figure 2, the prevalence of diabetes has been growing in the last decades. If this tendency is maintained, it is estimated that the total number of adults with DM will be 643 million (11.3 %) by 2030 and 783 million (12.2 %) by 2045.²

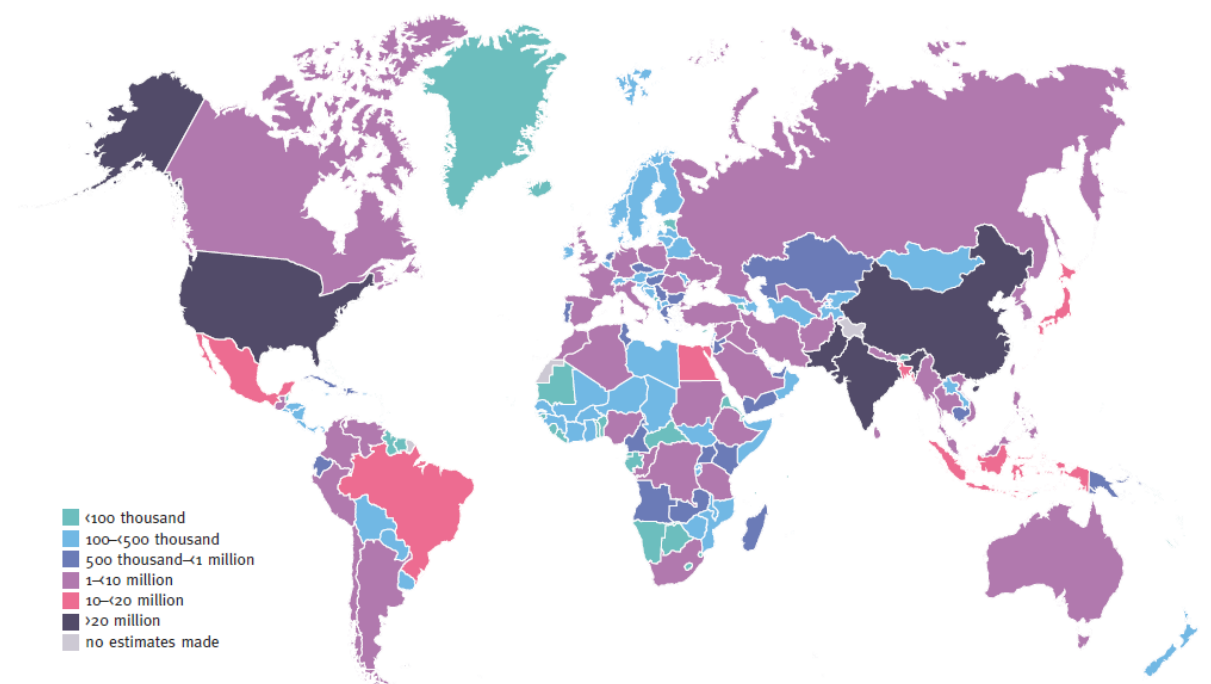


Figure 1 - Estimated total number of adults (20–79 years) with diabetes in 2021.²

Taking into account the global prevalence of DM and associated health problems, it is not surprising that diabetes-related health expenditure represents a significant burden in health systems. In fact, IDF estimates that 966 billion dollars had been spent only in 2021 for adults aged 20-79 years and this is expected to grow in the future, due to the increasing numbers of DM patients worldwide.² Therefore, there is an urgent need to develop new strategies to prevent, manage and treat this disease, as diabetes has and will continue to have a significant impact in societies and their health systems.

Besides hyperglycemia, diabetic patients may also show disturbances in carbohydrate, fat and protein metabolism. Typically, patients may show symptoms such as thirst, polyuria, blurred vision and weight loss. However, these symptoms are not always present or may be mild, which may hinder and delay diagnosis.³ When untreated, long-term hyperglycemia may lead to the development of several disabling and even life-threatening complications, which may be divided into microvascular and macrovascular complications, accounting for increased mortality and morbidity among diabetic patients.⁴ The first ones include retinopathy (which can lead to blindness), nephropathy (that can evolve to renal failure), and neuropathy (associated with foot ulcers and limb amputation). Macrovascular complications concern cardiovascular problems, such as coronary artery disease, peripheral arterial disease, and stroke.⁵

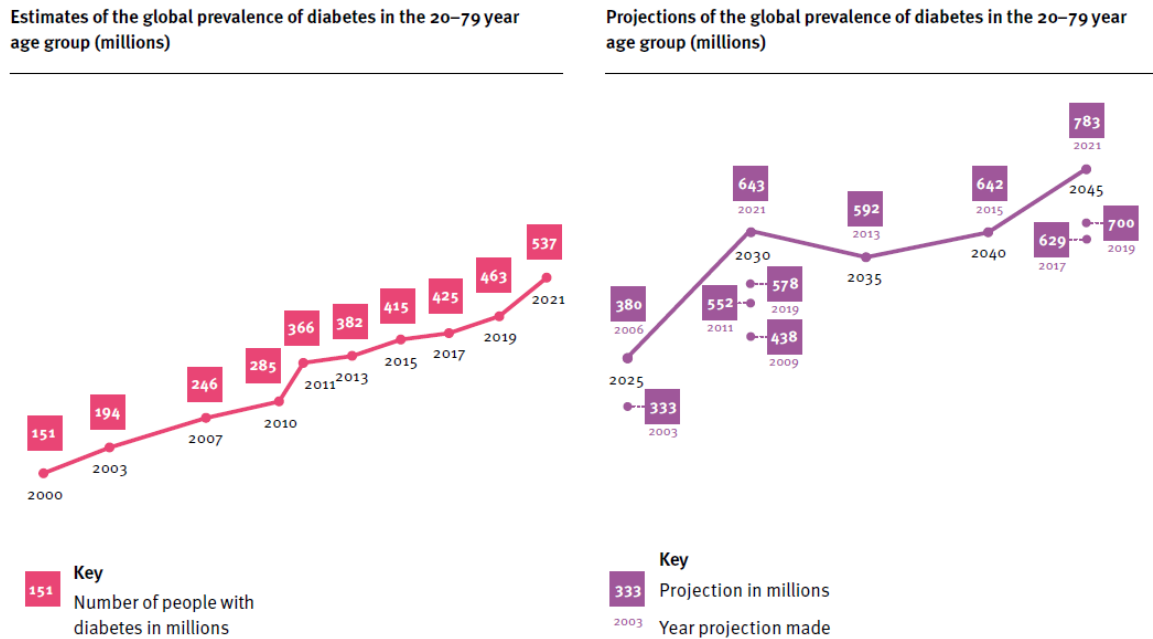


Figure 2 - Estimates and projections of the global prevalence of diabetes in the 20–79 year age group (millions).²

Depending on the underlying cause, DM can be classified into two major types: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D accounts for approximately 10 % of all DM cases, being characterised by an autoimmune destruction of insulin-producing cells (β -cells) in the islets of Langerhans within the pancreas, resulting in insulin depletion. T1D has an early-onset and is frequently diagnosed during childhood. Typically, T1D treatment consists of daily insulin injections in order to regulate blood glucose levels.⁶ On the other hand, T2D, which represents the majority of DM cases (around 90 %), is due to insulin resistance and/or abnormal insulin secretion. T2D therapy usually consists of diet and lifestyle changes and/or oral antidiabetic agents. However, in certain cases, when this is not enough to control patients' glycemia, they may also require insulin administration.^{1,3}

Besides these two major types, there are also other less common forms of diabetes (Table 1), such as hyperglycemia first detected during pregnancy, ketosis prone type 2 diabetes (which is a hybrid form of diabetes), monogenic diabetes, diseases of exocrine-pancreas, drug- or chemical-induced diabetes, among others.¹

Table 1 - Types of diabetes. Adapted from ¹.

Type 1 diabetes
Type 2 diabetes
Hybrid forms of diabetes
Slowly evolving immune-mediated diabetes of adults
Ketosis prone type 2 diabetes
Hyperglycemia first detected during pregnancy
Diabetes <i>mellitus</i> in pregnancy
Gestational diabetes <i>mellitus</i>
Other specific types
Monogenic diabetes
- Monogenic defects of β -cell function
- Monogenic defects in insulin action
Diabetes caused by diseases of the exocrine pancreas
Diabetes due to endocrine disorders
Drug- or chemical-induced diabetes
Infection-related diabetes
Uncommon specific forms of immune-mediated diabetes
Other genetic syndromes sometimes associated with diabetes (i.e. Prader-Willi syndrome, Down's syndrome, Friedreich's ataxia)

1.1.2. Type 1 Diabetes

Although T1D represents only 10 % of DM cases in adults, it is the most common form of diabetes in children and adolescents.² Despite decades of research concerning T1D aetiology, the answer is still not clear, but it is probably due to a combination of both genetic and environmental factors and their complex interactions. The fact that the triggering events frequently happen years before disease symptoms also hampers aetiology determination.⁷

A hallmark of T1D, which also distinguishes it from T2D, is the appearance of autoantibodies against β -cell antigens months or years prior to disease onset (presymptomatic T1D). The most common autoantibodies found in T1D patients are autoantibodies that target insulin,

glutamic acid decarboxylase, insulinoma-associated autoantigen 2, and zinc transporter 8.^{6,7} Besides their diagnostic relevance, these autoantibodies may also be useful to determine disease progression and whether there is an increased risk to develop the disease, for example in individuals with T1D relatives and also in general population.⁶ In fact, some studies point that individuals with two or more of these autoantibodies have a higher rate of progression to symptomatic T1D.^{8,9} In addition, the number of autoantibodies detected, age of autoantibody seroconversion (i.e. the age when a particular autoantibody is first detected in blood) and autoantibody titer, affinity and type are also important factors in determining the progression to the symptomatic phase of the disease.⁹

As previously mentioned, T1D is usually characterised by autoimmune destruction of β -cells. However, around 10 % of T1D patients are autoantibody-negative, which is an unusual form of T1D, known as idiopathic diabetes or type 1b. This type may include patients with unmeasurable levels of autoantibodies and rare forms of monogenic diabetes. The other 90 % of T1D patients develop autoimmunity against β -cells, which is also known as type 1a¹⁰ (in this work T1D refers to type 1a, unless otherwise stated).

In the past years, several genetic studies have identified so far more than 50 loci associated with T1D genetic susceptibility, whose genes are expressed in cells of the immune system and also in pancreatic β -cells.^{7,11} Those genes may be divided in human leukocyte antigen (HLA) and non-HLA genes, as the first ones represent about 50 % of genetic susceptibility to T1D.¹² In particular, two genotypes of HLA class II have been associated with an increased risk of developing autoantibodies and T1D, namely HLA DR and HLA DQ genotypes.⁷ HLA genes encode major histocompatibility complex (MHC) proteins, which have a crucial role in the immune system, as they present antigens on the surface of cells to be recognised by appropriate T cells.¹³ HLA class II genes are expressed in antigen-presenting cells (APCs), such as dendritic cells, macrophages or B cells. When APCs present β -cells antigens to T cells, a chronic immunological and pro-inflammatory response is elicited against them, leading to β -cells destruction. The triggering mechanism is not known. However, it is hypothesised that β -cell death could be elicited by a viral infection or physiological mechanisms, which would result in β -cell antigens release, their uptake by dendritic cells and consequent presentation of those antigens to T cells. Then, autoantigen-specific CD4⁺ and CD8⁺ T cells can start an immunological response against β -cells (Figure 3).^{3,7}

Non-HLA genes have also been associated with an increased T1D susceptibility, although their impact is less significant than HLA genes.¹⁰ Some of these genes include:

- insulin variable number of tandem repeats (*INS-VNTR*), that regulate the quantity of insulin mRNA in the thymus, which probably influences the development of insulin immune tolerance¹⁴;
- protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), which encodes lymphoid tyrosine phosphatase (LYP) (suppressor of T cell activation)¹⁵;
- cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*), which encodes a T cell receptor that acts as negative regulator of cytotoxic T cells¹⁶;
- interleukin-2 receptor alpha chain (*IL2RA* or *CD25*) that encodes one of the IL2 receptor chains, which is involved in regulation of regulatory T cells and immune tolerance.¹⁷

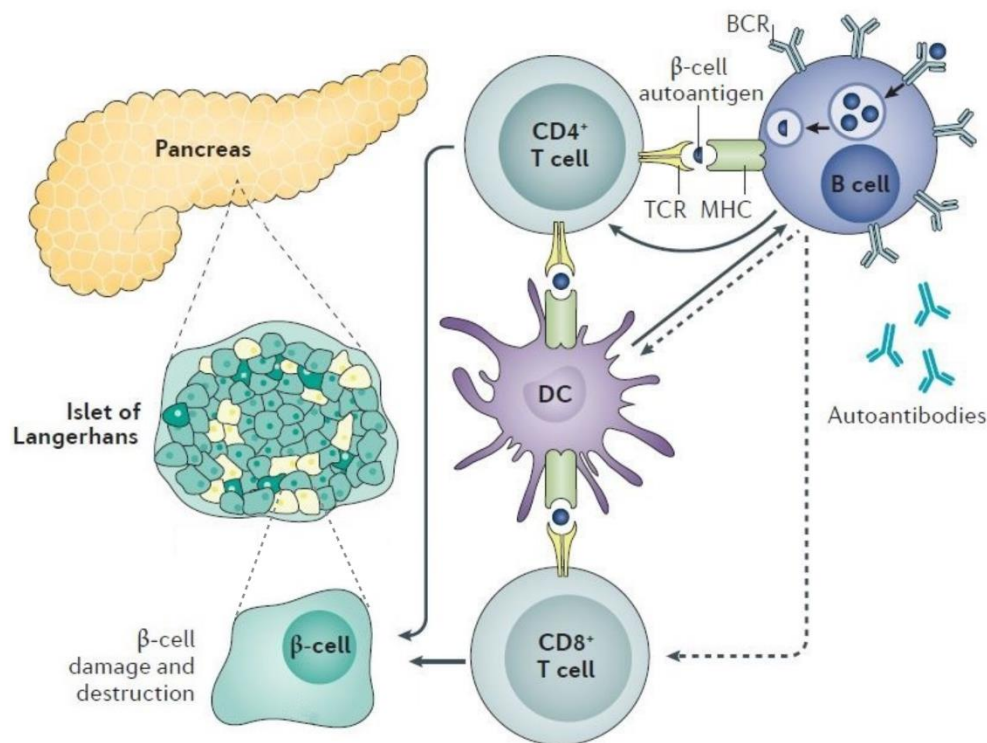


Figure 3 - Pathogenesis of T1DM. When antigen-presenting cells, like B cells and dendritic cells (DCs), interact with CD4+ and CD8+ T cells, presenting β -cells antigens through MHC proteins, a chronic immunological and pro-inflammatory response is elicited against β -cells, leading to their destruction. Additionally, activated B cells produce antibodies, in this case against β -cell autoantigens, which also serve as biomarkers of asymptomatic T1D. Dashed arrows indicate the potential interactions between B cells and CD8+ T cells and between B cells and DCs. BCR, B cell receptor; TCR, T cell receptor. Adapted from Katsarou *et al.*⁷

Despite the identification of numerous genetic factors associated with T1D, its aetiology is not fully explained by these genetic factors. Consequently, T1D is considered to be a polygenic disease influenced by environmental factors. In fact, monozygotic twins of T1D patients exhibit concordance rates (i.e. the probability that a pair of twins will have the same characteristic/trait) of T1D between 30 % and 50 %, depending on the follow-up duration.^{3,18} This demonstrates the complexity of the myriad of factors (both genetic and environmental) involved in T1D pathology and their multiple interactions.

Several environmental influences have been proposed to be involved in T1D development, namely viral infections, dietary factors (for example the timing of the first introduction of certain foods), high birth weight and growth rate, maternal factors (such as gestational infections) and toxic substances. Nevertheless, their influence on T1D is not completely understood nor the underlying mechanisms, being a combination of many genetic and non-genetic factors the probable trigger event for disease onset.^{3,7} A summary of the main putative genetic and environmental factors can be found in Table 2.

Table 2 - Summary of the main genetic and non-genetic risk factors associated with type 1 diabetes.

Genetic factors	Environmental factors
HLA genes (HLA class II)	Viral infections (cytomegalovirus, mumps virus)
Non-HLA genes	Dietary factors (cow milk, vitamin D)
(<i>INS-VTR</i> , <i>PTPN22</i> , <i>CTL-4</i> , <i>ILR2A</i>)	Maternal factors (gestational infections)
	High birth weight and growth rate
	Toxic substances

1.1.3. The pancreatic niche

To better understand the disease, it is also important to comprehend the pancreatic environment, particularly the endocrine pancreas.

The pancreas is composed of two morphologic and functional compartments: the exocrine pancreas (acinar cells and ductal cells) and the endocrine pancreas (islets of Langerhans) (Figure 4). The acinar cells of the exocrine pancreas produce and secrete digestive enzymes that are further released into pancreatic ducts and enter into the small intestine, participating in digestion processes. On the other hand, the endocrine pancreas,

representing less than 5 % of the total mass of the organ, is constituted by mixed populations of hormone-producing cells (the islets of Langerhans) embedded in the exocrine pancreas. Five major types of cells, which produce and secrete hormones involved in glucose homeostasis, constitute the islets populations, namely insulin-secreting β -cells, glucagon-secreting α -cells, somatostatin-secreting δ -cells, pancreatic polypeptide-secreting γ - or PP cells and ghrelin-secreting ϵ -cells. β -cells are the predominant cells of human islets (~60 %), followed by α -cells (~30 %) and the remaining 10 % are made up of γ -cells, PP cells and ϵ -cells, randomly distributed throughout the islets.^{19,20}

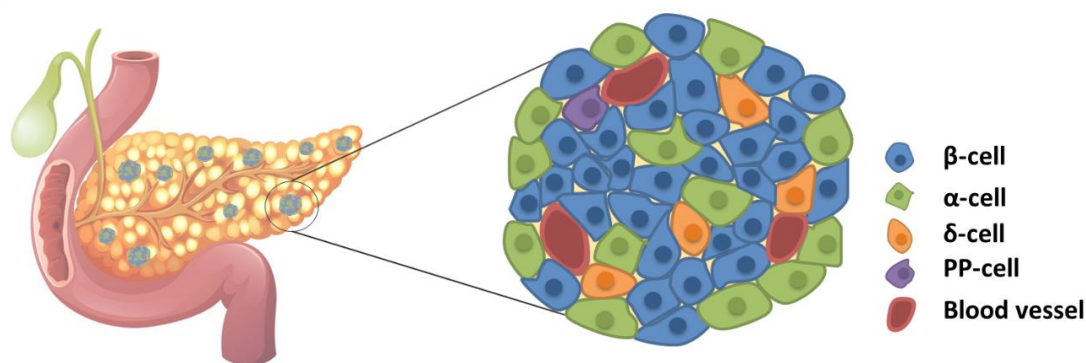


Figure 4 - Schematic representation of human pancreas and islet of Langerhans (magnified). The pancreas is constituted by exocrine and endocrine regions. The endocrine pancreas comprises the islets of Langerhans - mixed populations of hormone-releasing cells - scattered along the exocrine pancreas. Each islet is mainly composed of insulin-secreting β -cells, glucagon-secreting α -cells, somatostatin-secreting δ -cells, pancreatic polypeptide-secreting PP-cells and ghrelin-secreting ϵ -cells.

Around 3.2 million islets dwell the human pancreas and show wide islet-to-islet variability regarding their size, relative composition and spatial organisation of the different endocrine cells and arrangement of capillaries within islets.²⁰⁻²⁴ These structures can vary from small clusters of only a few cells to large aggregates of many thousands of cells, and present a compact or multi-lobular shape. Endocrine cells may distribute throughout islets in a mantle-like structure, where β -cells predominate in the inner core and are surrounded by scarce α - and δ -cells in the periphery or present a more complex architecture, with β -cells intermingled with α - and δ -cells in a more scattered organisation (Figure 5). Apparently, the architecture distribution of endocrine cells and vascularisation of human islets seem to be size-dependent.²⁵ Thus, while small islets (<100 μm in diameter) have a mantle-like structure composed mainly of β -cells and are poorly vascularised, larger islets (>100 μm in

diameter) present a more complex organisation, higher proportion of α -cells and more pronounced vascularisation.^{19,20,22,26} Indeed, highly vascularised islets are endowed by a capillary network that constitutes around 7-8 % of the islet volume, receiving around 85 % of the total pancreas blood flow.²⁷ In addition, single endocrine cells can be found scattered throughout the acinar and ductal tissue.²⁶

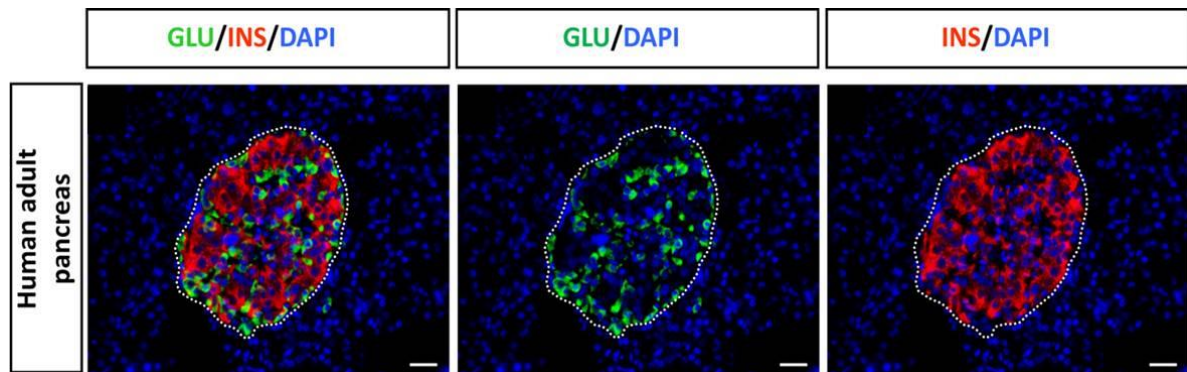


Figure 5 - Distribution of insulin-secreting β -cells and glucagon-secreting α -cells on a human islet of Langerhans. The images shown are from an adult human pancreas, stained for glucagon (GLU - green) and insulin (INS - red). Nuclear staining (blue) was performed with DAPI. Scale bars: 25 μ m. Adapted from Scharfmann *et al.*²⁸

β -cells produce, store and secrete insulin in response to blood glucose changes and this function is tightly regulated by glucose metabolism. The function of β -cells is the result of the sum of cell autonomous function in response to external stimuli and the interactions between cells within the islet.^{29,30} On the other hand, α -cells release glucagon in response to hypoglycemia. The two hormones have opposite actions on glucose regulation and consequently, the secretion of one hormone is controlled by the other one.³¹ Somatostatin inhibits the secretion of insulin, glucagon and pancreatic polypeptide under nutrients and Ca^{2+} stimulus.^{32,33} PP cells secrete pancreatic polypeptide that is mainly involved in satiety, although it seems to inhibit glucagon secretion under low glucose stimulation.³⁴ Overall, these hormones regulate each other and act together to maintain blood glucose homeostasis.

In conclusion, the islet niche plays a crucial role in the structure, interactions and regulatory functions of the different endocrine cells that operate within a complex regulatory network involving paracrine and neuronal control of energy homeostasis. For these reasons, T1D should not be regarded as a monohormonal disease, which is a crucial factor in the design and development of novel stem cell-based therapies.

1.2. Conventional therapeutic approaches for Type 1 Diabetes

1.2.1. Insulin

Currently, insulin replacement therapy is the first-line treatment for type 1 diabetes. Insulin was used successfully for the first time in humans in 1922 and at that time it was extracted from porcine and bovine pancreas.³⁵ However, it was not exempt from problems, which were related to immune-mediated side effects, insulin absorption or inefficient action, leading to ineffective glucose control. Later, humanized insulin forms have been developed and several types are currently available on the market (Table 3),³⁶ which better simulate the biologic action of endogenous insulin.

Table 3 - Different categories of insulin analogues. Adapted from ^{36,37}.

Type	Time action profile	Dose
Ultra-rapid-acting	The onset of action is typically at 3-4 min after administration, with peak of action at 1 h.	Three times a day, up to 20 min after the start of a meal
Rapid-acting	Usually begins 4-20 min after subcutaneous injection with peak at 1 h.	Three times a day, up to 15 min before food intake
Short-acting	Begins from 30 min after subcutaneous injection with peak action reaching 2-4 h.	Three times a day, 30 min before food intake
Intermediate-acting	Peak onset from 4-6 h, with duration of action until 14-16 h.	Once or twice daily subcutaneous
Long-acting	Beyond 24 h and up to 36 h.	Once daily subcutaneous, usually at the same time everyday with minimum 8 h interval between consecutive doses
Ultra-long-acting	The duration of action can last more than 40 h.	Once daily subcutaneous injection

Despite this improvement, T1D patients taking exogenous insulin injections not always achieve full control of their blood glucose levels, not only because it cannot completely compensate β -cell loss, but also due to the multifactorial nature of glucose control as it depends on diet, metabolism and physical activity.³ Therefore, diabetes management may be better achieved when a more physiological approach is adopted, in which pancreas secretes a basal low amount of insulin and a higher amount when glucose levels increase after a meal. Thus, a combination of different insulin analogues, such as intermediate- or long-acting preparations to provide basal insulin levels and ultra rapid-, rapid- or short-acting insulin analogues after a meal, offers a better and more precise glucose control than traditional insulin.^{7,37}

Moreover, in the past years some technological progresses have been achieved regarding diabetes management, namely insulin pumps and also glucose monitoring devices, which allow monitoring of blood glucose levels in real-time. The combination of these two technologies originated a device called sensor-augmented pump. Indeed, the usage of this device has increased, and some studies have already demonstrated some beneficial effects over the traditional injection therapy.³⁸ A randomised controlled trial reported that in adults and children with uncontrolled T1D, sensor-augmented pump therapy significantly reduced glycated hemoglobin (HbA1c, which reflects average plasma glucose over the past months and is an indicator used to monitor diabetes).³⁹ Also, a meta-analysis study of 33 randomised controlled trials showed improved glycemic control with continuous insulin infusion pumps when compared to multiple daily insulin injections.⁴⁰

As insulin pumps and continuous glucose monitors showed significant progress in diabetes management, this has encouraged further technological developments, by combining insulin pumps and continuous glucose monitors with a computer algorithm (also referred as an integrated closed-loop system, or artificial pancreas).³⁶ The algorithm takes into account the continuous glucose monitoring levels to determine the most appropriate quantity of insulin needed. Until now, these systems have shown promising results and a better overnight control of glycemia, by reducing the risk of nocturnal hypoglycemia.^{41,42} However, even with computer-assisted insulin delivery, a tight glycemic control is still challenging, mainly due to variable pharmacokinetics and absorption rates of insulin analogues. To overcome these issues, a dual-hormone artificial pancreas system has been developed, in which glucagon and insulin can be administered simultaneously, providing an improved glucose control, as well as better simulating the physiological mechanisms of the endocrine pancreas.⁴³ Given the beneficial metabolic outcomes of these systems, the Food and Drug

Administration (FDA) approved the first artificial pancreas system for use in T1D treatments in 2016.⁴⁴

Therefore, although insulin is not a cure, technological devices such as insulin pumps, continuous glucose monitors and artificial pancreas may greatly improve diabetes care and decrease associated comorbidities. Besides insulin therapy, a successful diabetes management should also combine a balanced diet and physical exercise, to decrease the risk of cardiovascular disease and obesity. Additionally, regular self-monitoring of glycemia and/or the use of continuous glucose monitors is crucial and has been shown to correlate with improved glycemic control.⁷

1.2.2. Whole-pancreas and islets transplantation

Despite the advances in insulin therapies and their impact on the improvement of the lives of diabetic patients, they still do not achieve the blood glucose control provided by functional β -cells.³⁶ Consequently, this unreliable control results in blood glucose level fluctuations outside the normal range. Prolonged periods of elevated blood glucose can cause microvascular damage, leading to long-term complications, such as diabetic nephropathy and retinopathy.⁵ Additionally, the constant need for individuals with T1D to monitor and regulate their insulin levels using costly medication is a chronic burden.

An alternative treatment strategy involves replacing the cell source (β -cells) instead of the missing hormone. In certain cases, specifically when T1D patients undergoing treatment with intensive insulin, pumps and/or continuous glucose monitoring show poor glucose control (such as recurrent hypoglycaemic episodes, hypoglycaemia unawareness, and glycaemic lability), transplantation of whole-pancreas or cadaveric islets offers an alternative to provide insulin independence.⁴⁵ Nevertheless, islet transplantation stands as a better option when comparing to whole-pancreas transplant, since it is a less invasive surgery with fewer post-transplant complications.^{36,45}

In 2000, the Edmonton protocol was established for islet transplantation, which improved efficiency and reliability in islet isolation procedure.⁴⁶ The initial results after transplant were promising, however, only 10 % of transplanted patients were non-dependent of exogenous insulin after 5 years.⁴⁷ In the past two decades further improvements have occurred in islet transplantation and currently 50 to 70 % of patients are insulin-independent at 5 years post-transplant, which is similar to the clinical outcomes of whole-pancreas transplantation alone.⁴⁸ The optimal site for transplantation has been islet injection into the hepatic portal vein, being a minimally invasive surgical procedure.⁴⁹

However, there are undoubtedly drawbacks associated with this procedure, namely donors' scarcity and the need for lifelong immunosuppression, which are also associated with whole-pancreas transplants and limit its broader application. Besides, there are also other problems, such as instant blood-mediated inflammatory reaction (IBMIR) immediately after transplant, loss of islet numbers and islet mass due to ischemia, apoptosis of islet cells, and harmful side effects of immunosuppressive therapy. Therefore, new strategies are also being developed to improve islet graft survival.⁵⁰ Nonetheless, the growing number of T1D cases every year and the shortage of pancreatic donors highlight the need for alternative approaches, such as stem-cell based replacement therapies (which will be addressed in more detail in section 1.3.2).

1.2.3. Peptide hormone-based therapies

New insulin analogues, incretins, and other hormones are being studied for their ability to improve T1D, since they are already widely used for T2D treatment. Besides, preclinical data strongly suggests beneficial effects of many peptide hormones, which also may show potential for the treatment of T1D.³⁶

Incretins are hormones produced by enteroendocrine cells, which are secreted into the bloodstream after a meal in response to nutrient ingestion, thus enhancing glucose-stimulated insulin secretion from β -cells. This is called the "incretin effect", which consists of a higher insulin secretion elicited by oral glucose than with intravenous administration of glucose, under similar plasma glucose levels.⁵¹ Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are the two most important incretins, accounting for approximately 50 to 70 % of insulin secretion from pancreatic β -cells after a meal.⁵²

1.2.3.1. Glucagon-like peptide 1 and its receptor

GLP-1 is a 30-amino acid peptide synthesised via the posttranslational modification of proglucagon in the neuroendocrine L cells found in the ileum and colon, despite recent studies suggesting that pancreatic α -cells also secrete GLP-1.⁵³ L cells secrete GLP-1 following nutrient intake, in response to elevated glucose levels, as well as other monosaccharides, lipids, and proteins.⁵⁴ The initial six amino acids of GLP-1 are cleaved from the N-terminus within the secretory vesicles, producing bioactive peptides that are subsequently secreted into the blood, specifically GLP-1 (7–36)-NH₂ and GLP-1 (7–37), with the first being more prevalent.⁵⁵

The half-life of native GLP-1 is relatively brief, ranging from approximately one to two minutes, depending on the species, due to two main factors: (a) the action of the enzymes dipeptidylpeptidase-4 (DPP-4) and neutral endopeptidase (NEP), and (b) renal elimination. DPP-4 cleaves GLP-1 (7–36)-NH₂ and GLP-1 (7–37) at the N-terminal dipeptide, forming GLP-1 (9–36)-NH₂ or GLP-1 (9–37), which are low affinity ligands for the GLP-1 receptor (GLP-1R), exhibiting a 100-fold lower binding affinity and more than 10,000-fold lower agonist activity when compared to the intact form.^{54,55} Both the intact forms of GLP-1 and its inactivated GLP-1 metabolites are rapidly cleared from the circulation via the kidneys. Although GLP-1 degradation is not influenced by kidney function, the clearance of both GLP-1 and, to a greater extent, its inactive metabolites, is delayed in patients with renal insufficiency.^{54,56}

GLP-1, GLP-2, glucagon, GIP, secretin, and growth hormone-releasing hormone (GHRH) belong to a group of structurally related peptides that exert their biological effects by binding to G protein-coupled receptors (GPCRs) of the class B family, which share structural similarities.⁵⁶ All members of this family are seven transmembrane G_αs-coupled receptors that elevate cAMP levels by activating adenylate cyclase. Each receptor of this family is named based on its specific endogenous ligand (GLP-1R, GLP-2R, GCGR, GIPR, SCTR, and GHRHR). Most studies indicate that there is no significant cross-reactivity between the peptide ligands and the receptors of this family under physiological conditions.^{54,57}

The human GLP-1R gene is expressed in multiple tissues, including pancreatic islets, brain, heart, intestine, kidney, liver, lung, and stomach.⁵⁸ Within the pancreas, GLP-1 enhances insulin biosynthesis and secretion from β -cells and suppresses glucagon secretion from α -cells in a glucose-dependent manner. Moreover, GLP-1 stimulates β -cell proliferation and inhibits β -cells apoptosis.^{59,60} Given the distribution of GLP-1R across different organs, GLP-1 exerts a plethora of other functions in the human body, summarised in Figure 6, which will not be detailed here since it is outside the scope of this work.

The clinical use of GLP-1 is hampered by its brief half-life, due to its fast metabolic degradation and renal clearance. Consequently, the amount of biologically active GLP-1 that reaches the blood represents only 10 to 20 % of the initial amount.^{61,62} Thus, to surpass the fast metabolism of human GLP-1 and prolong its half-life, GLP-1 analogues have been developed.

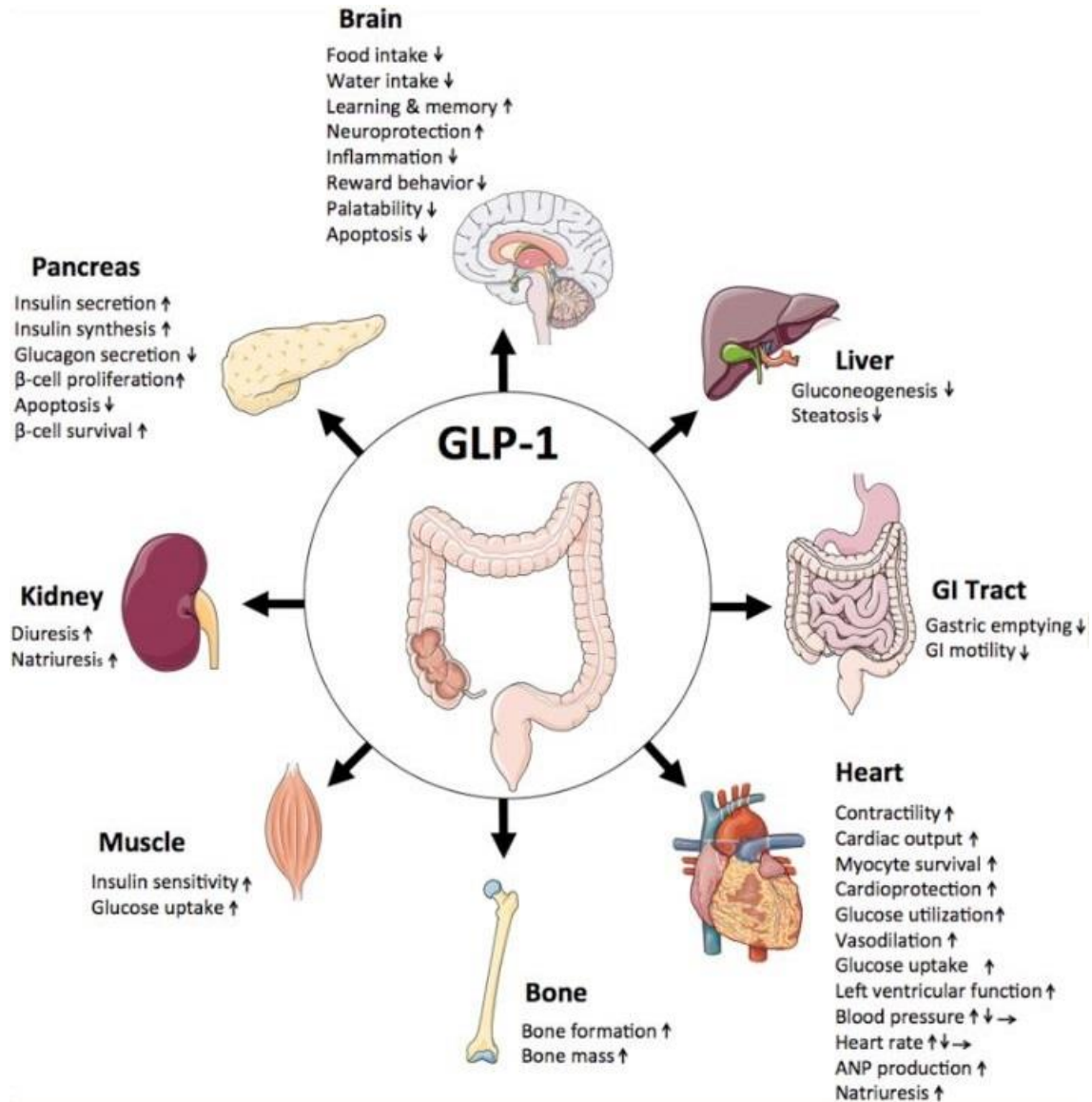


Figure 6 - Biological functions of GLP-1 in the human body. Adapted from Müller *et al.*⁵⁴

1.2.3.2. Glucagon-like peptide 1 analogues

GLP-1 analogues or GLP-1R agonists are synthetic molecules which replicate the physiological functions of GLP-1 but present modified amino acid sequences that confer resistance to enzymatic degradation by DPP-4.⁵³ These compounds represent a class of antidiabetic peptides and can be categorised into short- and long-acting, based on their pharmacokinetic and pharmacodynamic properties, as well as their glycemic control efficacy.⁴⁸ Currently, seven GLP-1R agonists have received FDA approval: albiglutide, dulaglutide, exenatide (short and long-acting release), liraglutide, lixisenatide, semaglutide and tirzepatide.⁴⁹

Exenatide (EXN), marketed as Byetta®, stands as the first FDA-approved GLP-1R agonist for the treatment of T2D.⁵⁰ This GLP-1 analogue is a synthetic variant of exendin4, which is a peptide derived from the saliva of the Gila monster lizard (*Heloderma suspectum*), exhibiting a 39-amino acid sequence with 53 % homology with human GLP-1.⁶³ EXN and GLP-1 display regions with distinct similarities, notably in the initial 11 N-terminal residues, where their identity is 82 %. However, in the subsequent 20 residues, the identity decreases to 35% (Figure 7). EXN demonstrates a superior binding affinity ($IC_{50} = 6 \text{ nM}$) to the isolated N-terminal domain of the GLP-1R compared to GLP-1 ($IC_{50} > 500 \text{ nM}$), possibly due to its greater helical propensity relative to GLP-1.⁴⁹ Moreover, EXN is not susceptible to degradation by DPP-4 due to the presence of glycine instead of alanine at position 8, leading to a prolonged duration of action compared to GLP-1. As an agonist of the GLP-1R, EXN induces glucoregulatory effects resembling those of GLP-1, encompassing glucose-dependent enhancement of insulin secretion, suppression of glucagon release, delayed gastric emptying, and promotion of β -cell proliferation.^{64,65} EXN is available in two distinct formulations, each with distinct pharmacokinetic characteristics. In the case of Byetta®, commercialised by AstraZeneca, EXN demonstrates a mean half-life of 2.4 h post-subcutaneous administration, accompanied by clinical effects lasting up to 8 h, primarily due to its rapid renal elimination. Byetta® is typically administered via subcutaneous injection twice-daily, with doses of 5 or 10 μg , and currently lacks a commercially available oral formulation. In contrast, EXN long-acting release, commercialized as Bydureon® (AstraZeneca), represents a slow-release subcutaneous formulation of EXN. Bydureon gradually releases EXN into the bloodstream, during approximately 10 weeks, through the poly(lactic-co-glycolic acid) (PLGA) microspheres present in the formulation, thereby prolonging its therapeutic action. This product was approved in 2012, both as an adjunct or monotherapy for patients with T2D, for once a week subcutaneous administration.^{53,54}

Regarding long-acting GLP-1R agonists, liraglutide (Victoza®) was the first approved for the treatment of T2D, showing improved resistance to DPP-4 enzymatic degradation and thus an extended half-life compared to native GLP-1 and short-acting GLP-1 analogues.⁵⁰ This prolonged half-life can be attributed to specific characteristics, namely acylation and binding to human serum albumin (HSA). Given that HSA has a half-life spanning several weeks, the improved binding affinity to albumin of GLP-1R agonists can significantly prolong their half-life by mitigating renal elimination.^{49,50} Semaglutide (SMG), commercialised as Ozempic® by Novo Nordisk, received approval for T2D therapy in 2017 and represents a next-generation of GLP-1 analogues with an extended half-life compared liraglutide (183 vs 11-15 h, respectively, following subcutaneous administration).^{50,51} The

chemical structure of SMG differs from that of the native human GLP-1 not only in two amino acids, namely in positions 8 (alanine to α -aminoisobutyric acid) and 34 (lysine to arginine), but also through the acylation of the lysine at position 26 with a linker composed of a glutamic acid moiety and a C-18 fatty diacid side chain. The substitution at position 8 renders SMG relatively resistant to DPP-4-induced degradation. The linker facilitates the interaction of SMG with HSA. The amino acid substitution of lysine at position 34 prevents the binding of C-18 fatty diacid to this position, thus restricting this binding to the only remaining lysine in the structure of SMG.⁵³

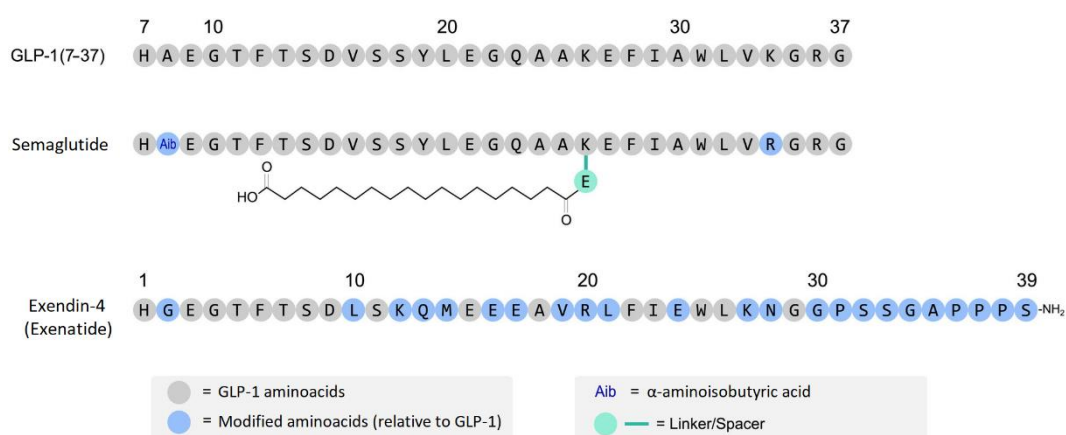


Figure 7 - Aminoacid sequences of GLP-1 (7-37), semaglutide and exenatide. Adapted from Mariam *et al.*⁶⁶

GLP-1 analogues are commonly used for the treatment of T2D. Nevertheless, some clinical studies have explored the therapeutic value of GLP-1 analogues as an addition to insulin therapy in T1D patients. These studies showed significant decrease of postprandial glucose and glucagon production, as well as delayed gastric emptying in patients taking insulin in combination with EXN or liraglutide, when compared with patients treated with insulin only.^{67,68} Moreover, another study revealed that diabetic mice with hyperglycemia treated with anti-IL-21 antibody and liraglutide returned to normoglycemia, showing an important role of immune modulators and incretin analogues in T1D treatment.⁶⁹ In fact, a clinical trial is currently ongoing to evaluate the effect of anti-IL-21 in combination with liraglutide in newly diagnosed T1D patients.⁷⁰

Therefore, the use of GLP-1 analogues may have a positive effect in conjugation with insulin therapy in patients with uncontrolled glycemia and mild obesity. Furthermore, their application associated with cell-based therapies, could also be a promising strategy for T1D

treatment. Given their short half-lives and degradation susceptibility (for example by proteases), nanotechnology could be an important way to shield these therapeutic agents, while also allowing a more controlled release (which will be addressed in the next section).

1.3. Emerging therapies for Type 1 Diabetes

1.3.1. Nano-based drug delivery systems

Although peptides may be promising therapeutic agents, their degradation susceptibility hinders their wide application. One possible strategy is to chemically modify the peptide, as mentioned in the previous section. However, a more precise and controlled release of the peptide in a specific target and/or in specific microenvironment conditions, besides protection from degradation, could be achieved with nanoparticulate or colloidal systems.⁷¹

A plethora of nanosystems have been developed and reported in the literature, from polymeric nanoparticles (NPs), to micelles, liposomes, solid lipid NPs, metallic NPs, carbon nanotubes, among others. However, these protein carriers may have some limitations depending on their composition and characteristics, including low encapsulation efficiency, physical instability and might pose some degree of toxicity to cells or tissues.⁷² NPs consist of particulate dispersions or solid particles ranging in size from 1 nm to 1000 nm, offering diverse structures suitable for customization. When developing NPs as delivery systems suitable for biomedical applications, a precise control of particle size, drug release profile and surface characteristics, is crucial to attain the desired pharmacokinetic and pharmacodynamic outcomes. Hence, an optimal NPs formulation exhibits high drug loading and encapsulation efficiency, with a sustained and controlled drug release, which does not affect the cargo structural integrity.^{72,73}

From the different nanosystems mentioned, polymeric NPs may represent one of the most effective for targeted drug delivery, due to their chemical versatility which enables the modification of physicochemical characteristics, drug release properties and biological behavior of NPs. Furthermore, their multifunctional structure can also be functionalized at the surface, allowing for enhanced bioavailability at target sites and/or response to environmental stimuli.^{73,74} Besides their wide application in the field of drug delivery, biodegradable polymers find extensive application in other areas, such as in regenerative medicine, tissue engineering or the development of implantable devices. Several types of polymers have been explored for the encapsulation of a wide range of bioactive molecules, such as peptides, drugs or DNA. For biomedical purposes, they should be biocompatible

and biodegradable materials, with the latter being influenced by the presence of labile chemical bonds in their structure, namely amides, phosphates, esters, orthoesters, anhydrides, urethanes and carbonates.⁷¹

The polymers used for protein delivery may be from natural or synthetic origin. Natural polymers are naturally abundant and cheap, however, synthetic polymers are advantageous for tissue engineering and drug delivery applications due to their consistent quality and low immunogenicity, such as polyethers, polyesters and polyamides. For instance, PLGA is an FDA-approved synthetic polyester widely used in protein delivery and consists of a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA) linked by ester bonds.⁷³ When this polymer is hydrolised, glycolic and lactic acid are generated, which can be easily metabolised in the body. For this reason PLGA is associated with reduced systemic toxicity, being frequently used for biomedical purposes. Due to the PLA and PGA different chemical properties, adjusting the ratio of the copolymers further enables the optimization of PLGA-based formulations. Nonetheless, PLGA is frequently copolymerised with other polymers due to its physical instability causing initial burst release of encapsulated proteins.^{73,75}

The success and broad application of PLGA nanosystems in drug delivery is supported by the numerous approved products on the market, such as Lupron Depot®, Bydureon® Somatuline® LA27, Sandostatin LAR® Depot and Decapeptyl®.^{76,77} Nevertheless, developing suitable delivery systems for EXN remains challenging, since its activity following injection lasts for a short period of time due to fast degradation/consumption. Besides, the need for regular EXN injections to allow a sustained pharmacological action is not desirable since it is painful for patients.⁷⁸

1.3.1.1. Glucose-sensitive drug delivery systems

The development of “*smart*” strategies in disease therapies offers many advantages, such as reducing the effective dose by increasing site-specific drug accumulation and decreasing off-target effects, spatiotemporal control, improved patient compliance, and control of therapy within the therapeutic window.⁷⁹

Glucose-responsive systems are particularly interesting for diabetes treatment applications since they allow to tune the release of the cargo according to glucose levels. These systems may be divided in three main types, regarding their mechanism (Figure 8): enzymatic (by using enzymes such as glucose oxidase (GOx)), through glucose-binding proteins (such as

Concavalin A (Con A)) and synthetic molecular recognition (such as phenylboronic acid (PBA)).^{79,80}

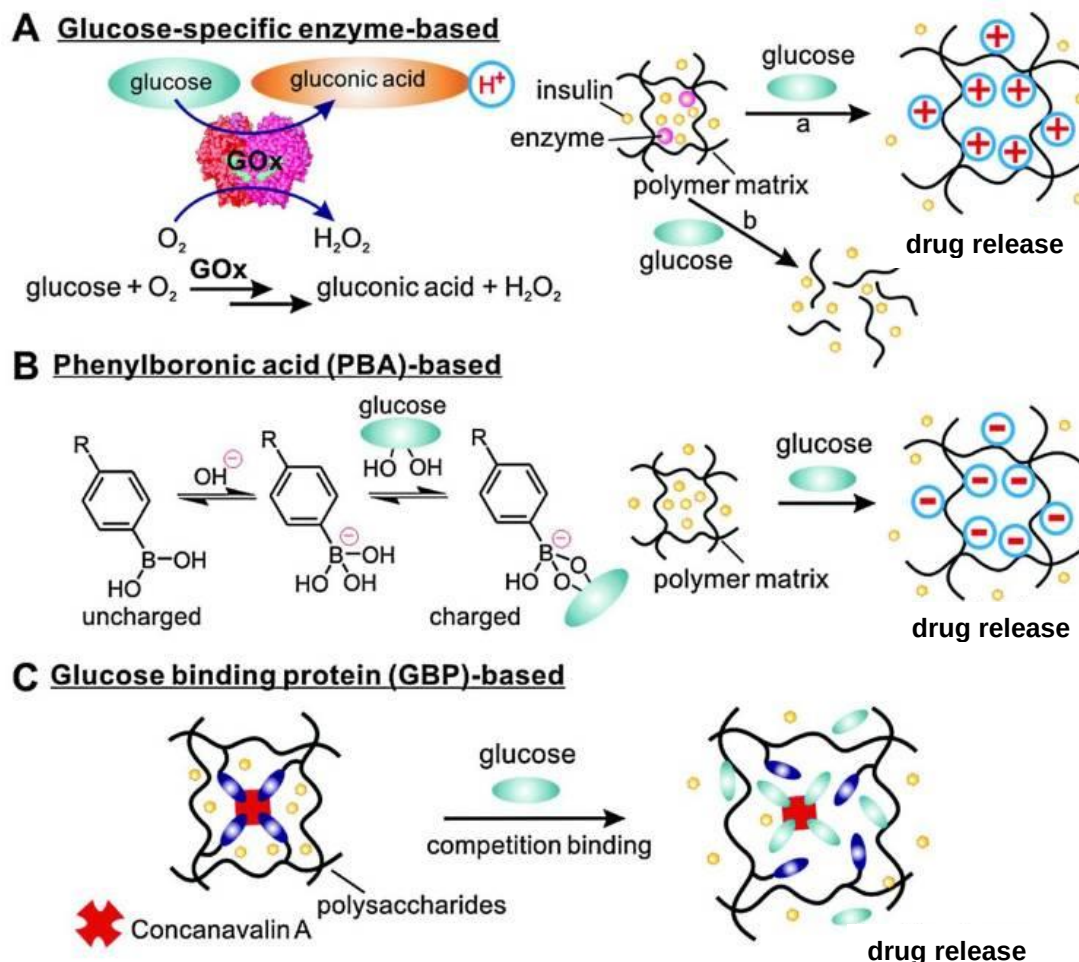


Figure 8 - Most common strategies for glucose-responsive drug release. Adapted from Mo et al.⁸⁰

GOx is an oxidoreductase (molecular weight: 160 kDa), commonly extracted from *Aspergillus niger*, which catalyzes with high specificity the oxidation of glucose into hydrogen peroxide and D-glucono- δ -lactone, which hydrolyzes to gluconic acid. This homodimeric enzyme contains, in each subunit, a flavin adenine dinucleotide (FAD) cofactor, which is used for glucose oxidation, as well as two intrasubunit disulfide bonds and a free sulfhydryl group. The rate of enzymatic production of gluconic acid is directly related to the local glucose concentration. It is also important to mention that GOx has high specificity for β -D-glucose, in contrast to other oxidases, that oxidise other sugars.^{81,82} Therefore, a typical closed-loop system using GOx is comprised of a pH-sensitive polymer matrix containing both GOx and

the peptide (therapeutic agent). The matrix undergoes a physical change such as hydrolysis or volume phase transition as the pH of the system decreases due to gluconic acid production, thereby releasing the entrapped peptide within the matrix in a glucose-dependent fashion. In some cases, catalase can be incorporated, since it is a peroxidase enzyme that quenches hydrogen peroxide (by-product produced by GOx) and converts it into an oxygen source for continued glucose oxidation by GOx.⁸³

Glucose-binding proteins (GBPs) can bind to glucose or glucosyl moieties in glycopolymers, being lectins the most common class of GBPs. Lectins are a family of proteins that can bind to carbohydrates, and have a significant role in cell signaling. Con A is a well-investigated plant lectin protein and shows a high affinity for glucose, polysaccharides and mannose. This protein may be incorporated in polymeric matrices that contain glycosylated moieties, creating a 3D structure that encapsulates a specific drug. When glucose levels increase, the interaction of Con A with the polymeric matrix decreases, since Con A preferentially binds to glucose, thereby releasing the loaded drug.⁸⁴ Alternatively, Con A can be used to control the release of glycosylated insulin (or other glycosylated drug) in the presence of hyperglycemic levels. Nonetheless, Con A also presents stability issues which limit its broader application in drug delivery systems.⁸⁵

Despite their advantages, protein-based glucose-sensing systems are sensible to environmental factors, such as pH and temperature and therefore, these must be tightly controlled to prevent protein denaturation. This limits glucose-sensing applications and sensor lifetimes of protein-based systems, as it poses challenges for the long term measurement of blood glucose by those sensors.⁸⁵ An alternative could be PBA and its derivatives as the glucose-sensing moiety. PBA compounds reversibly bind to polyols *cis*-1,2 or *cis*-1,3 diols, such as glucose, which stabilises a negative charge on the boronic acid. PBA presents two forms in equilibrium in solution, namely a charged, hydrophilic form and an uncharged form, which is relatively hydrophobic. When PBA is charged, it can interact with glucose through reversible covalent bonding, forming a stable complex and shifting the equilibrium towards the charged form of PBA.^{82,86} However, PBA presents a high pKa (8.6), which limits the application of PBA-based drug delivery systems in physiological conditions. Nevertheless, incorporating amine-containing groups in PBA contributes to the reduction of the effective pKa of neighboring boronic acids, thus enabling these systems to function at physiological pH.⁷⁹

Each approach has its own drawbacks, from possible immunogenicity of the protein components (in the case of GOx or Con A), to poor glucose selectivity and often limited function in physiologic conditions for PBA-based systems.⁸⁷ However, GOx is the most

commonly used option in glucose-sensitive drug delivery systems and can be physically entrapped or immobilised within pH-responsive polymeric matrices. In the presence of glucose, a transient pH decrease occurs in the microenvironment due to the generation of gluconic acid after glucose oxidation. This leads to the swelling or shrinkage of the pH-responsive carriers, ultimately leading to the release of the loaded drug.⁸⁸

These approaches are most frequently applied to hydrogels, which facilitate diffusion of glucose for more rapid sensing and equilibration, as well as tunable mesh size for selective release of the drug in response to glucose. However, the clinical applications of these platforms for diabetes therapy have some limitations, namely: bulk hydrogel systems exhibit slow response to changes in glucose concentration due to mass transport limitations, while GOx-containing membranes suffer from low mechanical strength, which can result in premature insulin leakage.⁸⁹ Therefore, the incorporation of these systems in close proximity with drug-loaded pH-sensitive nanoparticles could overcome some of these concerns and be used as an extra boost for differentiated β -cells after transplantation, in cell-based therapies for T1D.

1.3.2. Stem cell-based therapies

Pluripotent stem cells (PSCs) have the remarkable capacity to develop into cells of all three germ layers (ectoderm, mesoderm, and endoderm), but not extraembryonic structures (like placenta). PSCs comprise embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and are characterised by their capacity to divide and self-renew for long periods of time, while maintaining pluripotency.⁹⁰ ESCs can be obtained from the inner cell mass of blastocyst stage embryos (Figure 9a) however, their usage and manipulation still pose some problems, not only tissue rejection concerns, if they are used for cell transplantation, but also ethical issues regarding manipulation of human embryos. A possible solution to overcome these problems may be iPSCs, which consist of adult somatic cells that have been reprogrammed in order to express genes that confer them embryonic stem cell-like properties.⁹¹ The reprogramming methods used to generate iPSCs may vary from retroviral-mediated transduction to other methods that avoid genome integration, such as plasmid vectors, synthesised RNA delivery or use of recombinant proteins (Figure 9b).⁹²

Mouse iPSCs have been reported for the first time by Yamanaka and colleagues⁹³ in 2006 and one year later human iPSCs were reported by two different labs^{94,95}. More than 15 years later, many progresses have been achieved in stem cell biology and regenerative medicine.

Similar to embryonic stem cells, iPSCs have the same potential to expand and originate virtually any cell type of our body. Additionally, as terminally differentiated iPSCs may be derived from an autologous source of cells, they show significant benefits regarding immunological compatibility,⁹⁶ bringing the possibility of personalised medicine closer to reality. These features render iPSCs a great promise for cell-based therapies and tissue engineering (for diseases affecting organ systems, such as T1D), as well as disease modelling and drug screening.⁹⁷

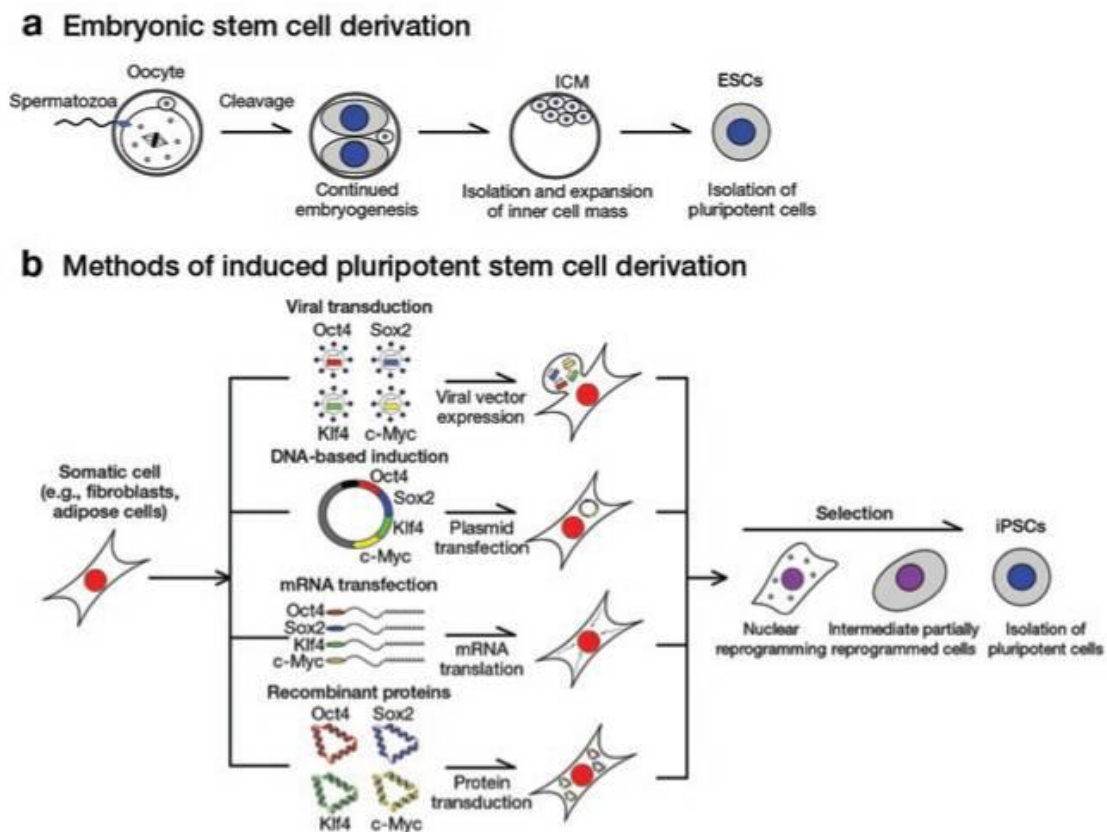


Figure 9 - Methods to obtain embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). (a) ESCs are obtained from the inner cell mass (ICM) of the blastocyst. (b) iPSCs consist of somatic cells that can be reprogrammed by several different techniques to achieve a pluripotent state. Adapted from Narsinh *et al.*⁹⁸

In the last two decades, β -cell generation research has been mainly focused on the differentiation of human ESCs (hESCs). Successive efforts have been made towards the successful differentiation of human PSCs (hPSCs) into pancreatic insulin-secreting β -cells, confirming the feasibility of hPSCs-based cell therapies for diabetes treatment. Indeed, developments in iPSCs technology triggered an increasing awareness for these cells. In

particular, the effective differentiation of pancreatic cell lineages from human iPSCs (hiPSCs) presents new opportunities for personalised autologous cell transplantation, without the need of immunosuppression, thus reducing immune rejection problems. However, there is still a long way to go and further developments are needed towards robust generation of hiPSCs and differentiation into pancreatic cell lineages.

This section provides a brief description of the potential of iPSCs for diabetes treatment, as well as an overview of the most recent advances on iPSCs differentiation towards pancreatic β - and α -cells.

1.3.2.1. The potential of iPSCs for diabetes treatment

One of the most common treatments for insulin-dependent diabetes (T1D and some T2D patients) is the exogenous insulin administration, which allows some regulation of the blood glucose levels. Nevertheless, even patients following this treatment cannot have a full glycemia control, as previously mentioned. Therefore, insulin therapy may not fully prevent some hypo- and hyperglycemic events through life on these patients.^{3,99} Despite technological advances in exogenous insulin delivery, like the insulin pumps⁹⁰ and artificial pancreas,¹⁰⁰ whole-pancreas or pancreatic islet transplantation constitute more physiologic alternatives to restore β -cell function.¹⁰¹ However, at the moment they are only recommended in specific cases, normally when patients show poor glucose control, due to some of their drawbacks, being the shortage of donors and the need for lifelong immunosuppressive therapy the main problems of these approaches.³ Taking this into account, the differentiation of iPSCs into endocrine cell types (particularly, but not only, β -cells) could be an extremely valuable solution to achieve an improved and personalised therapy for this disease and have a huge impact on global health.

Among all the pancreatic cells (endocrine and exocrine), β -cells are the most studied and explored in the field of iPSCs, due to their role in diabetes.¹⁰² However, the other endocrine cell types (mainly α -cells) are also important, given the fact that native human β -cells are in the heterogeneous environment of the islets of Langerhans, interacting with different endocrine cells (α , δ , PP and ϵ).^{103,104} As previously mentioned, these multiple interactions are essential for a correct regulation of insulin secretion, particularly α and δ cells, stressing the importance of cellular interactions in the islet environment and intra-islet paracrine signaling. Moreover, it has been reported that α -cells also affect the activity of β -cells through acetylcholine secretion in a glucose-dependent manner.¹⁰⁵ It is also noteworthy to mention that in the diabetes disease context, as β -cells are damaged or lost, the intra-islet

signaling events are deregulated and thus other endocrine cells may also be affected.¹⁰⁶ For these reasons, an islet-like structure composed of the different endocrine cell types would be a more effective way to regulate glucose metabolism rather than only focusing on β -cell therapies. Hence, while the main focus during the past years has been on β -cells differentiation, more studies are needed addressing other endocrine cells differentiation from iPSCs.

Overall, differentiation protocols for PSCs were developed based on knowledge obtained from pancreatic organogenesis, as a recapitulation of pancreas formation *in vitro*. However, most of this knowledge comes from animal studies and there are significant differences between humans and animals regarding pancreas formation.¹⁰⁷ Therefore, deeper knowledge of this process in humans will certainly give insights for better and more effective ways to differentiate endocrine cells from PSCs.

The generation of iPSCs-derived β - and α -cells provides a potentially useful step towards the generation of islets and whole pancreatic organs. In the last years, and with the development of increasingly effective differentiation protocols, iPSCs revealed the ability to differentiate *in vitro* into β -cells with similar efficiency and scalability of those obtained with ESCs.^{108,109} The *in vivo* proof of concept for the potential application of β -cells differentiated from iPSCs in the treatment of diabetes was established by Alipio *et al.*¹¹⁰ iPSCs derived from normal mouse skin fibroblasts were differentiated into insulin-secreting β -like cells and engrafted into the livers of a T2D mouse model via intraportal vein injection. The engraftment of insulin-secreting β -like cells restored insulin secretion and normoglycemia 2 days after implantation, persisting for more than 3 months. More importantly, engrafted iPSCs-derived β -like cells were able to survive within the tissue environment of the liver parenchyma. Similar results were obtained in a small cohort of streptozotocin (STZ)-treated T1D mouse model.¹¹⁰ More recently, β -cells differentiated from T1D patients derived-iPSCs (T1D SC- β cells) responded to blood glucose challenges over 16 weeks once transplanted underneath the kidney capsule of non-diabetic (ND) immunocompromised mice (Figure 10, a-c). After mouse β -cells destruction with alloxan, the engrafted β -cells continued functional over 5 months (Figure 10, d-g). No differences were observed between T1D-iPSCs derived β -cells and non-diabetic iPSCs-derived β -cells (ND SC- β cells).¹¹¹ Taking advantage of gene editing technology, Millman and collaborators took a step forward in the differentiation of β -cells from T1D-iPSCs.¹¹² They derived iPSCs from the skin of a patient with Wolfram syndrome and used CRISPR/Cas9 gene editing to correct the mutation on the gene that causes the disease (*WFS1*) before performing the differentiation into β -cells. *WFS1*-edited iPSCs-derived β -cells not only were able to efficiently differentiate into functional β -cells *in*

vitro, using a pre-established differentiation protocol by the same group,¹¹³ as well as reversed preexisting diabetes once transplanted into STZ-induced mice.¹¹²

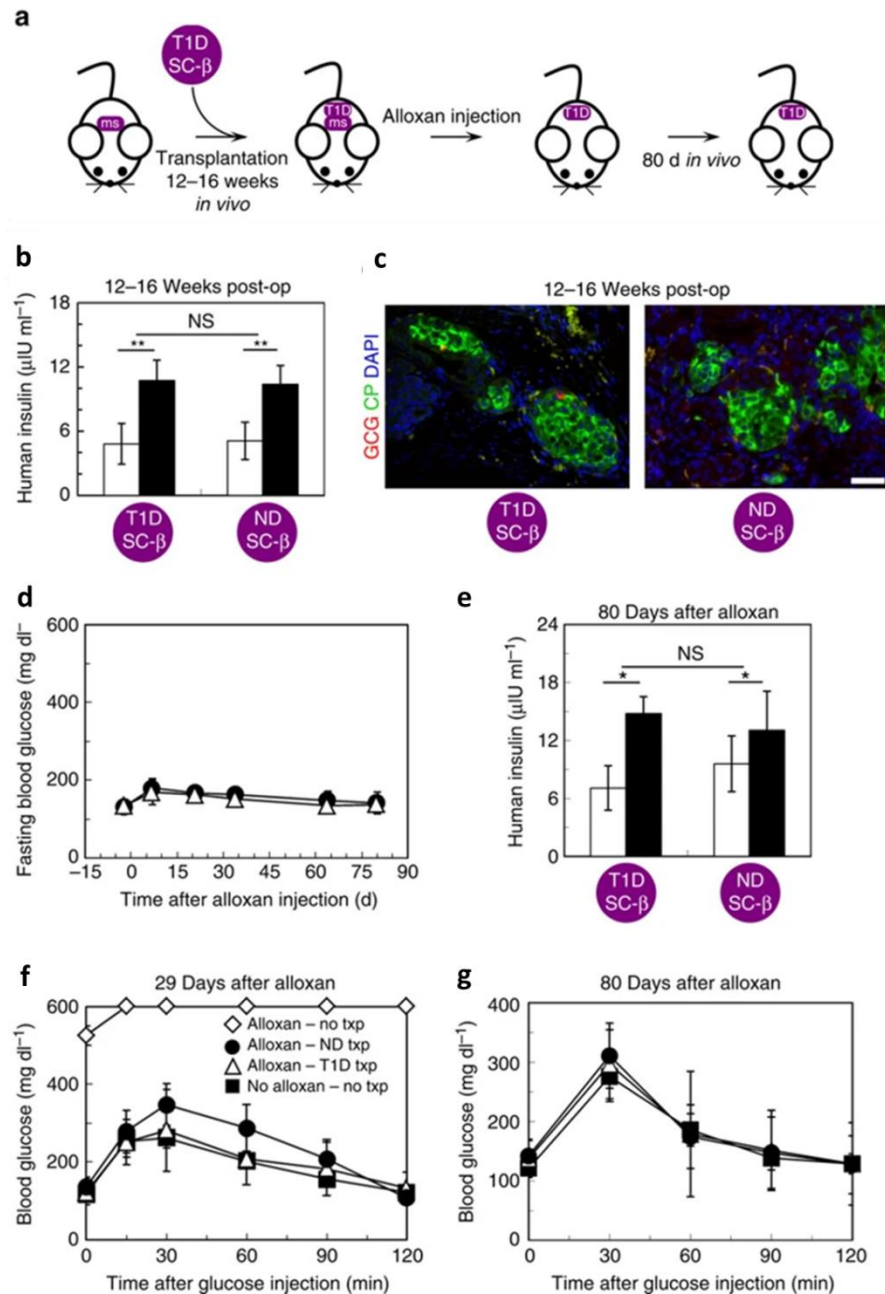


Figure 10 - T1D-iPSCs derived β -cells (T1D SC- β cells) function rapidly and persisted several months *in vivo* after transplantation. (a) Schematic summary of *in vivo* transplantation experiments. ms, endogenous mouse β -cells. T1D, T1D SC- β cells. (b) Levels of serum human insulin before (0 min) and after (30 min) a glucose injection for mice that were transplanted with T1D and ND SC- β cells 12–16 weeks before ($n=15$ and 18 for T1D and ND SC- β cells, respectively). (c) Representative immunostaining of grafts 12–16 weeks after transplantation. (d) Fasting blood glucose levels for a subset of mice that received alloxan injection to destroy endogenous mouse β -

cells. Mice that were transplanted with ND SC- β cells (closed circle; $n = 4$) and with T1D SC- β cells (open triangle; $n = 4$) are shown. (e) Levels of serum human insulin before (0 min) and after (30 min) a glucose injection for mice that were injected with alloxan 80 days prior ($n = 4$ and 4 for T1D and ND SC- β cells, respectively). (f) Blood glucose levels of mice after a glucose injection 29 days after alloxan treatment. Shown are mice that had not received a transplantation (open diamond; $n = 2$), were transplanted with ND SC- β cells (closed circle; $n = 4$), were transplanted with T1D SC- β cells (open triangle; $n = 4$), or did not receive alloxan treatment and did not receive a transplantation (closed square; $n = 5$) are shown. (g) Blood glucose levels after a glucose injection 80 days after alloxan treatment. Mice that were transplanted with ND SC- β cells (closed circle; $n = 4$), were transplanted with T1D SC- β cells (open triangle; $n = 4$), or did not receive alloxan treatment and did not receive a transplantation (closed square; $n = 4$) are shown. Mice were transplanted with cells 10–17 days in Stage 6. Scale bar, 100 μ m. * $P < 0.05$ and ** $P < 0.01$ (two-sided paired t-test). Data shown as mean $\pm$ standard error of the mean. Adapted from Millman *et al.*¹¹¹

To date, there are no reported protocols specifically developed for the iPSC-differentiation of δ , PP and ϵ cells nor exocrine cells. As a side note, the protocols developed for exocrine cells are for hESCs and as it is not a very explored area, the ones reported so far show very low differentiation efficiency when compared with endocrine cells differentiation.¹¹⁴ So, β -cells differentiation will be first addressed followed by α -cells.

1.3.2.2. From iPSCs towards β -cells

Most of the work pursuing *in vitro* generation of β -cells was made using ESCs either of mouse or human origin, since they have been extensively investigated as PSCs for the generation of many cell types.¹¹⁵⁻¹¹⁸ More recently, and with the emergence of iPSCs, most studies performed iPSCs and ESCs differentiations in parallel. Due to the similarities between iPSCs and ESCs, including *in vitro* differentiation patterns, the same differentiation protocols are frequently applied for the differentiation into β -cells.¹¹⁹ The most successful differentiation protocols towards functional β -cells are those simulating *in vitro* the native mouse or human embryonic pancreas and β -cell lineage development.^{117,120,121} Sequentially, hPSCs are exposed to different cocktails of key growth factors and small molecules, able to manipulate signaling pathways and transcription factors driving PSCs throughout different stages of pancreatogenesis, namely, definitive endoderm (DE),

primitive gut tube (PGT), posterior foregut (PF), pancreatic progenitors (PP), endocrine progenitors (EP) and finally, β -cells (Figure 11).

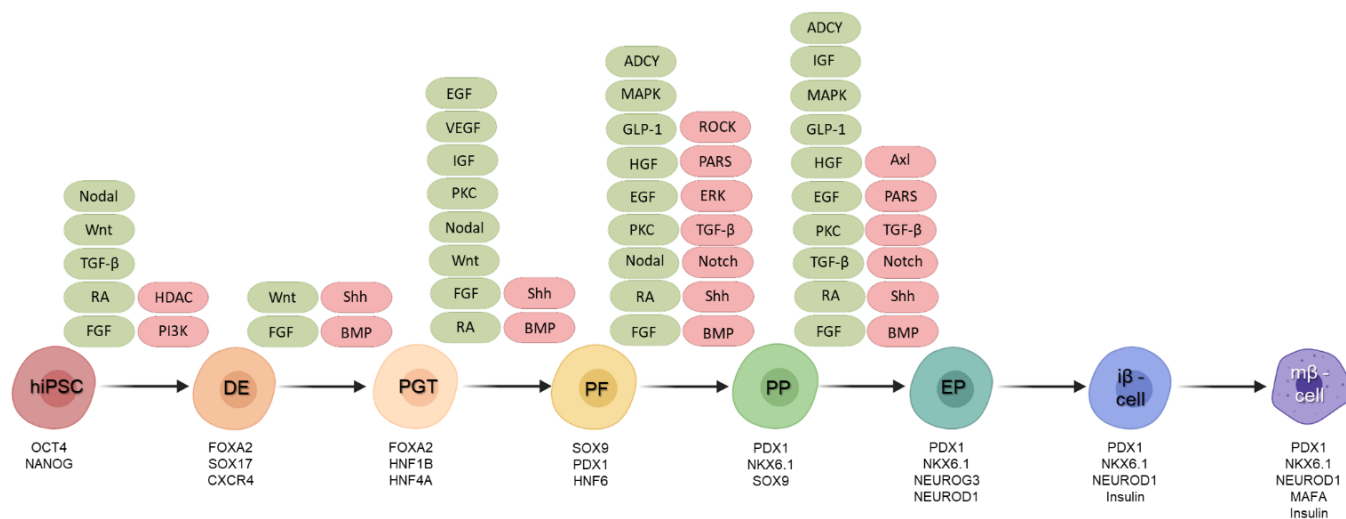


Figure 11 - Schematic representation of differentiation stages from hiPSCs towards mature β -cells. Activated signaling pathways at each stage of differentiation are represented in green boxes while inhibited signaling pathways are in red boxes. Markers for each stage of development are represented below. ADCY – adenylylate cyclase; Axl – Axl receptor tyrosine kinase; BMP – bone morphogenetic protein; DE – definitive endoderm; EGF – epidermal growth factor; ERK - extracellular-signal-regulated kinase; EP – endocrine progenitors; FGF – fibroblast growth factor; GLP-1 – glucagon-like peptide 1; HDAC – histone deacetylases; hiPSC – human induced pluripotent stem cell; HGF - hepatocyte growth factor; i β -cell – immature β -cell; IGF - insulin-like growth factor; MAPK – mitogen-activated protein kinase; m β -cell – mature β -cell; PARS – poly(ADP-ribose) synthetase; PF – posterior foregut; PGT – primitive gut tube; PI3K - phosphoinositide 3-kinase; PKC – protein kinase C; PP – pancreatic progenitors; RA – retinoic acid; ROCK – rho-associated protein kinase; Shh – sonic hedgehog; TGF- β – transforming growth factor- β ; VEGF - vascular endothelial growth factor; Wnt – Wingless-related integration site.

PSCs differentiation into DE is achieved through Nodal signaling pathway activation, by using high concentrations of Activin A.^{122,123} During this process, Nodal signaling pathway interacts positively or negatively with other key signaling pathways, including Wingless-related integration site (Wnt), transforming growth factor beta (TGF- β), fibroblast growth factor (FGF) and phosphoinositide 3-kinase (PI3K) signaling pathways. Thus, other growth factors and small molecules, such as bone morphogenetic protein 4 (BMP4),¹²⁴ basic fibroblast growth factor (bFGF),^{124,125} Wnt signaling activators (Wnt3A and the glycogen synthase kinase (GSK)-3 inhibitor CHIR99021),¹²⁶⁻¹²⁸ PI3K inhibitors (LY294002 and Wortmannin)^{121,127} and sodium butyrate¹²⁹ are used in combination with Activin A to enhance the efficiency of PSCs differentiation towards DE.^{117,130,131} The expression of high levels of

C-X-C chemokine receptor type 4 (CXCR4), SRY-Box Transcription Factor 17 (SOX17) and Forkhead Box A2 (FOXA2) marks the successful differentiation of PSCs into DE. DE generates both pancreatic and hepatic fates, being the latter inhibited in differentiation protocols towards pancreatic progenitors. The manipulation of retinoic acid (RA), BMP, FGF and Hedgehog signaling pathways directs DE into PGT stage and further into posterior foregut.¹¹⁷ FGF10 and FGF7 activate Notch signaling, promoting the proliferation of PDX1-expressing pancreatic progenitors.¹³²⁻¹³⁴ On the other hand, RA induces the expression of posterior foregut markers PDX1 and HNF6.¹¹⁷ Inhibitors of BMP signaling, namely Noggin,^{121,124,135-137} dorsomorphin^{127,138} and LDN-193180^{108,109} are used to suppress hepatic lineage differentiation.

The next stages involve the proliferation of pancreatic progenitors (PDX1, NKX6.1 and SOX9), multipotent cells able to generate the acinar, endocrine and ductal compartments of the pancreas, and their segregation into endocrine cell maturation. *In vitro* studies suggest the existence of two different populations of pancreatic progenitors that will define the differentiation fate.^{139,140} PP expressing only PDX1 will give rise to poly-hormonal, glucose-unresponsive endocrine cells, while the co-expression of PDX1 and NKX6.1 ensures differentiation into monohormonal glucose-responsive β -cells.¹²⁵ The final stages of pancreas development involve maturation of endocrine progenitors to mature hormone-producing cells.

iPSCs differentiation towards functional β -cells was described for the first time in 2008 by Tateishi *et al.*¹²⁹ In this study, iPSCs were derived by retroviral expression of human *Oct4*, *Sox2*, *Klf4*, and *c-Myc* in HFF-1 human foreskin fibroblast cells. Further, human dermal fibroblasts-derived iPSCs were successfully differentiated into functional islet-like clusters through definitive and pancreatic endoderm, using a four-stage differentiation protocol (Table 4).¹²⁹

Islet-like clusters expressed C-peptide and glucagon, releasing C-peptide after glucose stimulation, although in much lower levels than those of adult β -cells. In addition, hiPSCs underwent stage-specific morphological changes and expressed stage-specific genes and antigen markers for each developmental stage in a similar manner to those of hESCs. Overall, hiPSCs revealed similar efficiency of differentiation into insulin-secreting islet clusters as hESCs, although clonal variability between lines was noticed regarding their ability to differentiate.¹²⁹

Patient-specific iPSCs are a promising alternative for autologous cell transplantation. Maehr *et al.*¹²⁶ reprogrammed skin fibroblasts from T1D patients into iPSCs, followed by differentiation into insulin-producing cells (Table 4). The differentiation protocol directed

iPSCs towards definitive endoderm, gut tube endoderm, endocrine progenitors, pancreatic progenitors and β -like cells. T1D iPSCs-derived cells expressed key markers of the different stages. At the end of maturation, cells expressed the endocrine specific genes *INS*, *PDX1*, *NKX2.2*, *GCG* (glucagon) and *SST* (somatostatin), being positive for somatostatin, insulin, C-peptide and glucagon. Moreover, the differentiated cells were able to release C-peptide in a dose-dependent manner. T1D-iPSCs demonstrated similar differentiation efficacy to non-diabetic-derived iPSCs however, inpatient variations of differentiation propensity in patient-specific iPSCs clones was noted. iPSCs clones demonstrated similar differentiation patterns until definitive endoderm, but clonal variations became prominent towards gut tube and islet-like cells differentiation stages. This variability could be minimised by improving reprogramming strategies and iPSC differentiation protocols.¹⁴¹ Nevertheless, the use of T1D-derived iPSCs opens a new window of possibilities for iPSCs differentiated β -cells autologous transplantation. Despite these achievements, the low efficiency of most ESCs and iPSCs differentiation protocols is a major concern. In addition, generated β -cells are immature, expressing not only insulin but also other endocrine hormones, including glucagon and somatostatin.

In an attempt to improve differentiation efficiency and promote insulin-producing cell maturation *in vitro*, Zhang *et al.*¹²¹ differentiated human fibroblasts-derived iPSCs into PDX-1 pancreatic progenitors and used EGF to expand this population before proceeding towards maturation (Table 4). After maturation stage, differentiated islet cells expressed specific marker genes including *MAFA*, *GLUT2*, *PDX1* and *INS* in a similar manner to those expressed by cells differentiated from ESCs. PDX1⁺ and insulin-producing cells were obtained, but also amylase-producing cells, suggesting exocrine differentiation of hiPSCs. However, the responsiveness of hiPSC-derived islet-like cells to glucose was not assessed.¹²¹ Similarly, Nostro *et al.*¹²⁴ used a four-stage protocol (Table 4) to differentiate different ESCs and one hiPSC cell lines into insulin-producing cells, comparing the conventional monolayer culture with embryoid body format. The hiPSC cell line generated islet-like clusters expressing C-peptide but in lower amounts (~3%) than those obtained with ESCs cell lines (>6%). This study also stated the main advantage of using embryoid bodies, namely the higher scalability that allows obtaining a larger number of differentiated cells, although a faster differentiation was achieved using monolayers.¹²⁴ Nevertheless, most of these protocols give rise to polyhormonal cells more closely related to human fetal β -cells than with adult β -cells.¹⁴²

The search for large populations of relatively pure multipotent pancreatic progenitors able to generate monohormonal, insulin-secreting and glucose-responsive cells led to improved

second generation protocols, as those describe by Rezanian *et al.*¹⁰⁹ and Pagliuca *et al.*¹⁰⁸ Rezanian *et al.* induced hESCs and hiPSCs throughout 7 stages of differentiation and replaced the planar culture by an air liquid interface in the final stages of maturation (Table 4). The used iPSC line was able to differentiate towards NKX6.1⁺/insulin⁺ and insulin⁺/MAFA⁺ β -cells similarly to hESCs cell line, but with reduced efficiency when compared to the latter (~50%). Different results were obtained by Pagliuca *et al.*¹⁰⁸ that reported an *in vitro* scalable suspension-based differentiation protocol capable of efficiently generating high number of glucose-responsive β -cells from either hESCs (HUES8) or hiPSCs (hiPSC-1 and hiPSC-2) (Table 4). They performed a systematic testing of different combinations (>150) of numerous growth factors and small molecules (>70) to induce the differentiation of pancreatic progenitors towards functional β -cells. Obtained β -cells that closely resemble adult β -cells regarding gene expression patterns, glucose-responsive Ca²⁺ flux, package of insulin into secretory granules and amount of insulin secretion in response to multiple sequential glucose challenges *in vitro*.¹⁰⁸ However, *in vivo* studies were performed only with β -cells originated from the ESC line HUES8. Once transplanted into mice, β -cells were able to secrete insulin in response to blood glucose changes in healthy mice after 2 weeks and to improve hyperglycemia in diabetic mice after 18 weeks of implantation.¹⁰⁸ A similar time of 3-4 months is needed for *in vivo* maturation towards insulin-producing cells of transplanted pancreatic progenitors in diabetic mice.¹⁴³ Further, the same group obtained similar results using T1D patients-derived iPSCs.¹¹¹ More recently, other groups focused their attention in the manipulation of *in vitro* culture conditions during differentiation process in order to maximise the efficiency and scalability of the differentiation. Cell density, cell-cell contact and the effects of extracellular matrix (ECM) components seem to play a crucial role in the proliferation, differentiation and maturation of PSCs.^{128,144-146}

Table 4 - Summary of some protocols reported in the literature for differentiation of hiPSCs towards pancreatic β -cells.

iPSCs	DE	PGT	PF	PP	EP	Insulin-producing cells	Reference
Human dermal fibroblasts derived-iPSCs	Activin A, Na-Butyrate	--	EGF,bFGF, Noggin	EGF, Noggin	--	Nicotinamide, IGFII	Tateishi, 2008 ¹²⁹
T1DM-derived iPSCs	Wnt3a, Activin A	FGF10, Cyclopamine	--	FGF10, Cyclopamine, Retinoic Acid, Indolactam V	--	Exendin 4, DAPT, HGF, IGF	Maher, 2009 ¹²⁶
Human fibroblasts derived-iPSCs (C1, C2, C5 cell lines)	Activin A, Wortmannin	--	RA, Noggin, FGF7	--	--	Nicotinamide, bFGF, Exendin-4, BMP4	Zhang, 2009 ¹²¹
Human fibroblasts derived-iPSCs (38-2 from GM01390)	Activin A, BMP4, bFGF2, VEGF	--	FGF10, Wnt3a, DKK1, dosomorphin, Noggin	Vit C, Cyclopamine, Noggin, RA, FGF10	Vic, SB431542, Noggin	SB431542, Noggin, γ -secretase inhibitor L-685,458	Nostro, 2011 ¹²⁴
hiPSC derived from T1DM patients	Activin A, Wnt3a	FGF10, Cyclopamine	FGF10, Cyclopamine, Retinoic acid	FGF10, Cyclopamine, indolactam V	HGF, DAPT, IGF, GLP-1	--	Thatava, 2013 ¹⁴¹

(cont.)

hiPSC line 253G1	Activin A, CHIR99021, Wortmannin	--	--	FGF10, Noggin, dorsomorphin, RA, FR180204	--	Forskolin, Dexamethasone, Alk5 inh II, Nicotinamide	Takeuchi, 2014 ¹²⁷
hiPSC1 (ND-1), hiPSC2 (ND-2)	Activin A, CHIR99021	FGF7	--	PP1, FGF7, RA, SANT1, LDN193189, PdBu PP2: FGF, RA, SANT1	RA, SANT1, XXI, Alk5i II, T3, Heparin, Betacellulin.	T3, Alk5i	Pagliuca, 2014 ¹⁰⁸
hiPSC #A18945	GSK3 β inhibitor, GDF8	Vit C, FGF7	Vit C, FGF7, SANT1, RA, LDN193189, TPB	Vit C, FGF7, SANT1, RA, LDN193189, TPB, Y27632	SANT1, RA, LDN193189, T3, Alk5i II, zinc sulfate, heparin	i β cells: Alk5i II, T3, GS inh XX m β cells: Alk5i II, T3, N-Cys, AXL inh	Rezania, 2014 ¹⁰⁹
ND-1, ND-2 and fibroblasts derived iPSCs	Activin A + CHIR99021	FGF7	--	FGF7, RA, SANT1, Y27632, LDN193189, LDN, PdBu	RA, SANT1, XXI, ALK5i II, T3, Heparin, Betacellulin.	--	Millman, 2016 ¹¹¹
6IMR90-hiPSCs	Activin A, CHIR99021, Vit C, Y-27632, bFGF	CHIR99021, FGF10, Noggin, Vit C	RA, SANT1, FGF10, Noggin, Vit C; B27	Nicotinamide, EGF, Noggin, Vit C, B27	SANT1, RA, T3, Alk5i II, γ -secretase inh, betacellulin, heparin, Vit C, ITS-X100	--	Memon, 2018 ¹²⁸
IMR90-hiPSC	CHIR99021, Activin A, bFGF, Vit C	FGF10, Noggin, CHIR99021, Vit C	FGF10, Noggin, SANT1, Vit C	FGF10, Nicotinamide, EGF, Vit C	--	--	Aigha, 2018 ¹²⁵

Abbreviations: ALK5i II - Activin receptor-like kinase 5 inhibitor II; BMP4 - bone morphogenetic protein 4; DAPT - N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester; EGF – epidermal growth factor; FGF – fibroblast growth factor; GDF8 - growth differentiation factor 8; GSK3 β - glycogen synthase kinase 3 beta; HGF -

hepatocyte growth factor; hiPSC - human induced pluripotent stem cell; IGFII– insulin growth factor II; Na - sodium; N-Cys - N-acetyl cysteine; PDBu - Phorbol 12, 13-dibutyrate; RA – retinoic acid; SANT - Sonic hedgehog inhibitor; TPB - ((2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl) phenyl)-2,4-pentadienoylamino)benzolactam; T3 - Triiodothyronine; VEGF - Vascular endothelial growth factor; Vit C - Vitamin C; Y27632 - Rho-associated protein kinase inhibitor.

1.3.2.3. From PSCs towards α -cells

As previously mentioned, when differentiating iPSCs to β -cells it is commonly reported that an heterogeneous population is obtained, being some of those cells α -like or polyhormonal cells.^{147,148} Regarding α -cell differentiation, the protocols develop for ESCs were based on the protocol for β -cell differentiation of the respective laboratory^{138,149} and, more recently α -cells differentiated from iPSCs were also reported.¹⁵⁰

In one of those studies, authors established a six-stage protocol during four weeks,¹³⁹ which is summarised in Table 5. Throughout the protocol, cells went through different stages that mimicked human islets development, ending with α -cells that spontaneously formed clusters, similar to human islets size and shape. The obtained cells showed a strong expression of glucagon and also ARX, which is a transcription factor important for α -cell development.¹⁴⁹ After passing through a stage of co-expression of glucagon and insulin, cells went through a maturation stage *in vitro* or *in vivo* (in normoglycemic mice), losing insulin expression and other β -cell markers (such as MAFA and NKX6-1) and a simultaneous increase in α -cell markers. Differentiated cells were also challenged *in vivo* in order to confirm if their biological function was as expected, and they observed that biologically active glucagon was secreted in response to physiologic stimuli (like fasting or arginine challenge). However, glucose levels after 4 h fasting were almost the same between mice from control and test group (*i.e.*, with implanted α -cells) during the 18 weeks of glucose monitoring, with only two instances when it was slightly higher in the test group (as it would be expected). Moreover, three months after implantation of the cells, animals of the test group exhibited chronic hyperglucagonemia and a certain degree of glucagon resistance. Four months after transplant, implanted cells were harvested and characterised, and it was observed that cells showed an α -cell profile, pointing to a solid *in vitro* commitment to this lineage.¹³⁹

The Melton lab developed their protocol for α -cell differentiation with slight differences from the protocol mentioned above (Table 5).¹⁵⁰ Their protocol has only five stages but is significantly longer than the previously mentioned procedure (around 7 weeks vs 4 weeks). In this study, differentiated α -cells showed a transcription signature comparable to cadaveric human α -cells. Additionally, after transplantation in mice, the effect of differentiated α -cells on long-term blood glucose levels was assessed during 4 weeks and it was shown that basal glucose levels were higher in animals with transplanted α -cells. Furthermore, two of the main advantages of this protocol highlighted by the authors are the absence of exogenous growth factor matrices (like Matrigel) and also the possibility to achieve a greater production of differentiated cells, since they used a 3D culture system

using spinner flasks (versus the 2D culture of the previous protocol),¹⁵⁰ which are extremely important to allow its clinical application.

More recently, Yabe *et al.*¹⁵¹ demonstrated that NKX6.1 expression should be inhibited for a successful α -cell differentiation. In this study, the protocol shows greater similarities with previously described protocols for β -cell differentiation, also incorporating LDN193189 and EC23 (a retinoic acid agonist). However, differentiated α -cells obtained through this protocol still required further maturation *in vivo*.

Overall, although progress has been made in recent years regarding both β and α -cells there are still hurdles to overcome, mainly regarding their complete maturation *in vitro*.

Table 5 - Summary of the different protocols used for differentiation of hESCs towards α -cells.

Cell lines	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Reference
HUES8 and 1016 (hESCs lines)	Activin A, CHIR99021	KGF	RA, LDN 193189	LDN 193189 (1 day) no factors (4 days)	ALK5i II	Peterson, 2020 ¹⁵⁰
H1 (hESCs line)	Activin A, Wnt3a, bFGF	KGF, Cyclopamine -KAAD	KGF, Cyclopamine-KAAD, Noggin, RA	ALK5i II, Noggin, DAPT	ALK5i II	Rezania, 2011 ¹³⁹
TkDN4-M and 15M63 (hiPSCs line)	Activin A, Wnt3a, CHIR99021	FGF7, EC23, LDN193189	EC23, RA, Noggin, LDN 193189, Y-27632	FGF10, LDN 193189, DAPT	LDN 193189, EXN, ALK5i II	Yabe, 2021 ¹⁵¹

Abbreviations: ALK5i II - Activin receptor-like kinase 5 inhibitor II; bFGF - basic fibroblast growth factor; DAPT - N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester; hESC - human embryonic stem cell; KGF - keratinocyte growth factor; RA - retinoic acid.

1.3.3. Cell encapsulation strategies

Cell encapsulation is a technique used to involve cells in an artificial, partially permeable polymeric membrane, in order to confer them protection against the host's immune system after transplantation.¹⁵² Various encapsulation strategies are described in the literature, depending on the final form of the encapsulation.

1.3.3.1. The relevance of cell encapsulation for islet transplantation

Current clinical procedures for islet transplantation rely on the extraction of islets from deceased human donors. This strategy presents significant challenges, such as the need of a high quantity of islets to achieve significant functional effects, the short islet survival due to immune reactions and the toxicity of frequently used immunosuppressants.^{50,153} Prolonged immunosuppression requires continuous administration of drugs such as tacrolimus and sirolimus. Nonetheless, these drugs, while essential, pose significant risks: they are toxic not only to the kidneys and liver but also to β -cells.¹⁵⁴ In fact, tacrolimus and cyclosporine are diabetogenic, as they inhibit insulin secretion from β -cells, which likely contributes to the gradual decline in graft function.⁴⁸ Sirolimus, on the other hand, negatively impacts both β -cell function and proliferation.¹⁵⁴ Another critical drawback of these immunosuppressive therapies is the heightened susceptibility to infections due to a compromised immune response.¹⁵⁵ Taken together, these challenges make current transplantation strategies less cost-effective. As a result, research efforts are increasingly directed toward achieving immune tolerance for allogeneic islet grafts while minimizing the adverse effects of traditional immunosuppressive regimens. Thus, encapsulation of islets emerges as a promising approach to shield them from the immune system, thereby reducing the need for immunosuppression.

Encapsulation is a method used to envelope cells in a non-toxic biomaterial to protect them from immune reactions. One of the first reports of this technique was described in 1933 by Bisceglie, who encapsulated a mouse carcinoma and transplanted in a guinea pig to increase its survival.¹⁵⁶ Later, other authors used this technique, showing that encapsulated xenografts had increased survival, due to its separation from the host tissue while also exchanging oxygen and nutrients.^{157,158}

Currently, safeguarding encapsulated cells from immune responses remains a crucial factor of the materials employed for encapsulation purposes. These materials must allow the passage of small molecules, such as oxygen, nutrients and hormones, while preventing larger entities, such as antibodies and white blood cells from crossing the membrane.¹⁵⁹ Additionally, biomaterials used for encapsulation should also be biocompatible to avoid non-specific foreign body reactions, which may result in protein adsorption at the surface, resulting in fibrotic overgrowth that can ultimately lead to cell necrosis.^{147,148} Another disadvantage of islet encapsulation is the increased distance between blood vessels and the encapsulated cells, consequently extending the diffusion distance. Moreover, the amount of insulin reaching the blood decreases and cell necrosis due to hypoxia increases.¹⁵⁰ The size of capsules is typically in the range of 600 to 800 μm , resulting in a

significant diffusion distance. Considering that in the human body the average distance between cells and a capillary vessel is approximately 200 μm ,¹⁵¹ this poses a considerable challenge for diffusion. This is even more important for pancreatic islets, since they present greater capillary density than other tissues.¹⁵² However, small islets (< 150 μm) are less susceptible to these issues¹⁵⁰ and thus adjusting the size of microspheres can prove advantageous for the development of a bioartificial pancreas system.

1.3.3.2. Cell encapsulation techniques

Cell encapsulation techniques can be classified in two main groups, namely macroencapsulation and microencapsulation, which will be briefly discussed below.

1.3.3.2.1. Macroencapsulation

Macroencapsulation typically involves enclosing hundreds of cells or multiple islets within one encapsulation device. Macroencapsulation devices can be broadly categorised into two main groups: intravascular and extravascular.¹⁵³ The first ones are directly connected to the circulatory system, either through a shunt or a diffusion chamber surrounding a semi-permeable membrane surgically attached to the blood vessels of the recipient,¹⁵⁴ thereby allowing a closer contact with the bloodstream. However, there is a risk of thrombosis, blood leakage, or device clogging if the biomaterials selected lack sufficient biocompatibility, posing a serious hazard to the patient.¹⁵⁵ On the other hand, extravascular macroencapsulation devices can be readily implanted in highly vascularized sites (such as subcutaneously or in the peritoneal cavity) and typically consist of diffusion chambers housing a high quantity of islets, which are protected from the host immune system by a membrane.¹⁵⁴

A significant advantage of this approach is the ease of transplantation and the possibility of retrieving or reloading the device, if needed. Moreover, these devices show increased mechanical strength when compared to microencapsulation devices, but oxygen and nutrient diffusion may be compromised in the core of the device due to large diffusion distances.¹⁵⁶

1.3.3.2.2. Microencapsulation

Islet microencapsulation involves encapsulating individual islets, or a small number of islets, in spheres with size around hundreds of μm .¹⁵⁹ Due to the reduced size compared to macroencapsulation devices, this results in a higher surface-to-volume ratio, thereby enhancing diffusion of oxygen and nutrients and also allowing faster and more effective responses to changes in blood glucose levels.¹⁶⁰ Additionally, this approach typically does not require intricate or costly manufacturing processes, microcapsules can be implanted using a minimally invasive procedure, and their smooth surface, as well as decreased size, minimizes foreign body responses and inflammatory reactions.¹⁵⁹

The predominant method employed for the microencapsulation of islets, particularly within alginate hydrogels, involves an air-driven droplet generator, which is the approach used in this work and depicted in Figure 12. In this technique, alginate is extruded through a nozzle, forming a drop, that is separated from the nozzle tip by the surrounding airflow. Immediately after the droplet originates the gel by crosslinking when it contacts with the gelling bath underneath.¹⁶⁰

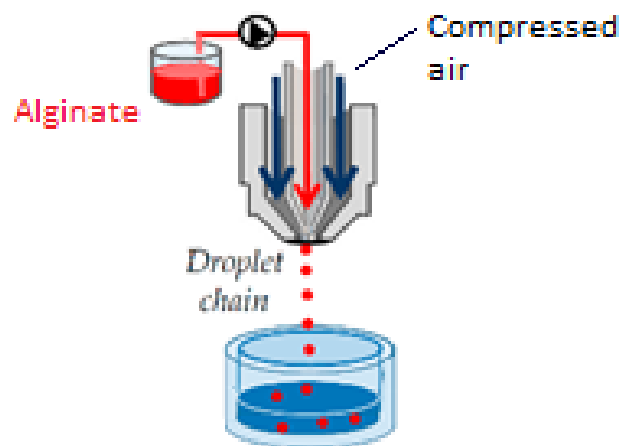


Figure 12 - Co-axial air flow technique - compressed air is used to separate alginate droplets at the tip of a nozzle. Adapted from Lengyel *et al.*¹⁶⁰

A different approach to cell encapsulation involves the formation of emulsions. Cells are suspended in a selected liquid, are mixed into an oil phase, generating an emulsion, and the cell suspension undergoes a gelation process. This is achieved by introducing a crosslinker, such as calcium ions for alginate-based hydrogels, or by reducing the temperature in the case of thermo-responsive hydrogels, like agarose.¹⁷⁰ This strategy enables the simultaneous generation of a great amount of microspheres within a short

timeframe, but it is associated with significant size variability and may result in the deposition of oily residues on the beads.¹⁷¹ Microfluidics can also be used for islet encapsulation, which offers the advantage of producing more uniformly sized beads than the previously mentioned approaches. However, to perform this technique, an additional material is needed to form the droplets, such as a mineral oil that can leave residues on the microspheres and negatively affect cell viability.¹⁷²

1.3.3.3. Biomaterials for cell encapsulation

Selecting the appropriate encapsulation material is a crucial factor: it should be biocompatible (avoiding fibrotic and immune reactions), shield cells from the host's immune response, and simultaneously provide a supportive environment for the transplanted cells.¹⁷⁰ Biomaterials for this purpose can be categorised into synthetic and naturally-occurring materials.

Natural biomaterials can be derived from plants, such as alginate from algae and agarose from seaweed, or from mammalian sources, for instance Matrigel, fibrin and collagen. They present higher biocompatibility and also some susceptibility to naturally-occurring chemical and enzymatic processes.¹⁷³ Depending on the application, this can either be advantageous, if it is intended as a temporary cell scaffold that can be resorbed in the body, or disadvantageous if cell protection is needed in the long term. Moreover, these materials often contain ligands that facilitate cell attachment and that can enhance cell signalling without the need for additional modifications.¹⁷⁴ However, these materials typically exhibit variability between batches, making it challenging to consistently control their physico-chemical properties.¹⁷⁵

Conversely, synthetic materials typically lack inherent biological activity. Yet, they can be customized for specific applications, for instance through the incorporation of certain ligands or motifs that are recognized by cells and enhance their adhesion and viability. Nonetheless, the production of these synthetic materials is often time-intensive and costly.¹⁷⁶

The most widely used polymer for cell encapsulation is alginate. It was the first material used for islet microencapsulation and continues to be the most popular choice due to its accessibility, low cost, and ease of production.¹⁷³ Alginate is a polyssacharide produced by brown algae, constituting 20-40% of the algae's dry weight.¹⁷⁷ It consists of linear chains formed by two monomers, β -D-mannuronic acid (M) and α -L-guluronic acid (G), joined by (1-4) glycosidic linkages, with free hydroxyl and carboxyl groups. Due to the low pKa values of G and M residues, at neutral pH, alginate is polyanionic.¹⁷³ Alginate molecular structure

may contain G and M monomers in different ratios and arrangements in homopolymeric (-GG- or -MM-) or heteropolymeric blocks (-GM-). The length and composition of each block are highly variable and depend on the algae species, regional and seasonal conditions and extraction process.¹⁷⁷

Alginate crosslinks when it contacts with a cation-rich solution, containing for instance as calcium ions. The G monomers interact with divalent cations through free carboxyl groups, originating a reticulated “egg-box” structure and forming a hydrogel. The mechanical properties of the gel formed are determined by factors such as the length and composition of G/M blocks, the alginate concentration, and the cation employed for crosslinking.¹⁷⁸ It is important to note that greater mechanical strength is linked to smaller pore sizes, potentially impacting the diffusion of oxygen and nutrients. Moreover, the GM ratio plays a role in the immunogenicity of the hydrogel, as high-M alginate has been associated with more pronounced immune reactions.^{179,180} The stability of alginate hydrogels is also dependant on the cations used for gelation, since alginate presents different affinities for distinct divalent cations. The following cations are presented in an increasing manner, according to the alginate affinity: Mg^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} , Ca^{2+} , Ni^{2+} , Sr^{2+} , Cu^{2+} , Cd^{2+} , Ba^{2+} .¹⁷⁸

Given its hydrophilic character, alginate does not promote protein adsorption, and cells also do not recognise the polymer. This is an advantageous property, since chemical modifications can be performed to promote specific biological interactions and also to adjust the hydrogel characteristics, such as degradation rate or release profile.¹⁸¹ A frequent modification involves integrating RGD (Arginine-Glycine-Aspartic acid) motifs. This three-amino-acid sequence is part of ECM proteins like laminin and fibronectin, which can thus promote cell adhesion to the hydrogel through the interaction with integrin receptors at the cell surface.¹⁸² Alginate can also undergo modifications with peptides that are recognized by matrix metalloproteinases (MMPs) derived from cells, thus allowing to remodel the surrounding hydrogel.¹⁸³

Alginate has demonstrated exceptional durability and biocompatibility, as evidenced by its ability to sustain xenotransplanted porcine islets in a human patient for 10 years. Nevertheless, to ensure optimal biocompatibility, the selected alginate should possess a medium to high molecular weight, high purity, with minimal presence of proteins, polyphenols, or endotoxins, and be crosslinked using an appropriate cation.¹⁸⁵

The encapsulation of allogeneic islets and SC- β -cells in alginate microspheres is particularly interesting to minimize immune reaction against the transplanted cells.¹⁸⁰ However, in the realm of cell-based therapies for T1D, cell encapsulation offers an additional advantage. During the islet isolation process, the surrounding islet ECM is

typically compromised, adversely affecting the function and survival of pancreatic cells.⁴⁹ Therefore, by encapsulating the islets or SC-pancreatic cells in alginate microspheres, this provides additional support for their survival and growth. Additionally, if alginate is chemically modified with RGD motifs, it allows to more closely mimic the native microenvironment of a human islet. This reasoning culminates in the development of a 'bioartificial pancreas', which tries to closely mimic the function and structure of the original organ.

Chapter 2

Motivation, Aims and Thesis Outline

2.1. Motivation and aims

T1D is a chronic metabolic disease characterised by insufficient insulin secretion due to β -cell loss, which leads to hyperglycemia.⁷ Exogenous insulin administration is the first-line treatment, but many patients still experience severe glycaemic lability, which can lead to microvascular damage and consequent long-term complications, such as diabetic nephropathy and retinopathy.¹⁶¹ For these patients, a β -cell replacement strategy through whole-pancreas or pancreatic islet transplantation is a better therapeutical approach, allowing the reestablishment of endogenous insulin secretion and improved glycemic control. These procedures show good clinical outcomes, with 50 to 70 % of patients being insulin-independent at 5 years post-transplant.⁴⁸ Additionally, islet transplantation has the advantage of being less invasive and resulting in fewer post-transplant complications compared to whole-pancreas transplantation.^{36,45} Nevertheless, these procedures are associated with significant challenges, including the scarcity of deceased pancreas donors (with multiple donors being frequently necessary for islet transplantation to fully treat one patient) and the need for lifelong immunosuppressive therapy to prevent transplant rejection.^{50,153}

As a potential alternative to these strategies, the differentiation of β -cells from PSCs has been extensively explored in the last two decades and more recently the differentiation of α -cells as well. The utilisation of PSCs to generate pancreatic cells is particularly interesting, since it provides a virtually unlimited cell source for transplantation,⁹⁰ overcoming the problem of donor scarcity associated with islet transplantation. However, even though the differentiation efficiency of β -cells has significantly improved over the years, stem cell-derived β -cells (SC- β cells) still more closely resemble their fetal than their adult counterparts, specially regarding insulin secretion, transcriptome and the expression of certain genes.¹⁶²

On the other hand, the entrapment of insulin-producing cells in alginate scaffolds has also been previously explored, protecting cells from the immune system while maintaining the exchange of oxygen, nutrients and hormones between the entrapped cells and the surrounding environment.^{163,164} However, the encapsulation approaches proposed so far only include SC- β cells, and the inclusion of other stem cell-derived pancreatic endocrine cells would allow to better mimic the native islet environment and consequently better regulate the function of transplanted SC- β cells. Moreover, the transplantation of SC-cells or islets has some underlying challenges, which should be minimised, such as early cell death after transplantation that results in significant graft loss.¹⁶⁵

Considering the physiological roles of GLP-1, previously addressed in section 1.2.3.1, GLP-1 analogues could be used to overcome or at least minimise some of the challenges mentioned above by: a) improving glucose-stimulated insulin secretion of SC- β cells, since they are not fully functional and mature after *in vitro* differentiation,^{162,166} and b) decreasing the initial apoptosis of SC- β cells after transplantation, thus reducing the percentage of graft loss, which is commonly reported.¹⁶⁵

Therefore, the main goal of this thesis was to develop a novel bioartificial pancreatic construct and to investigate its potential as a treatment for T1D patients, as depicted in Figure 13. This system combines pancreatic α - and β -cells differentiated from hiPSCs, GLP-1 analog-loaded glucose-responsive nanoparticles and GOx co-encapsulated in an alginate matrix. By using hiPSCs as the cell source, this allows the usage of patient-derived cells in future clinical applications to prevent allogeneic rejection of the graft. The inclusion of glucagon-producing cells will help to regulate insulin secretion by SC- β cells, better mimicking the endogenous feedback control of glycaemia. Additionally, differentiated cells will be entrapped in alginate microspheres in a way that mimics the physiological proportions found in pancreatic human islets. By encapsulating cells in alginate, once transplanted they will be shielded from an autoimmune attack without compromising graft function. Moreover, GOx was included in the system in order to accomplish a glucose-dependent peptide release from pH-sensitive NPs. To accomplish the main goal, the following specific objectives were proposed:

- (i) To develop and optimise glucose-responsive NPs loaded with GLP-1 analogues, by varying the proportion of the polymers and studying two GLP-1 analogues (exenatide and semaglutide);
- (ii) To characterise the developed NPs regarding their physico-chemical properties, as well as drug release kinetics and peptide stability in physiological and acid conditions;
- (iii) To assess and compare the glucoregulatory effect of exenatide and semaglutide;
- (iv) To differentiate hiPSCs into β - and α -cells *in vitro*;
- (v) To characterise differentiated β - and α -cells regarding their phenotype and biofunctionality *in vitro*;
- (vi) To co-encapsulate glucose-responsive NPs, GOx and differentiated β - and α -cells in alginate microspheres mimicking physiological proportions and assess the viability and biofunctionality of the system *in vitro*;

(vii) To evaluate the efficiency of the biofunctional system in reducing and controlling blood glucose levels in a T1D mouse model.

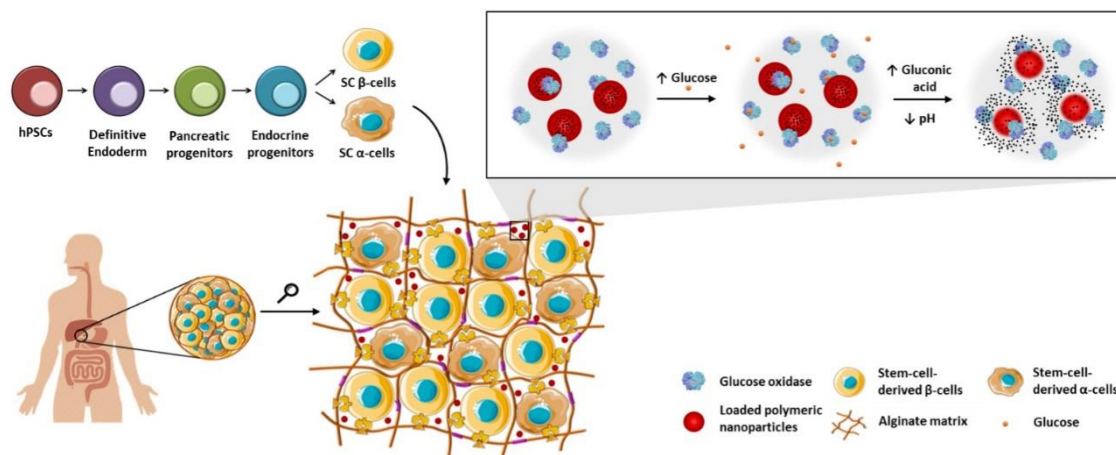


Figure 13 - Graphical abstract. The main aim of this thesis was to develop a novel bioartificial pancreatic construct and to evaluate its potential as a treatment for patients with T1D.

2.2. Thesis Outline

This thesis is organised in six chapters. Chapter 1 provided a literature review on the disease addressed in this work - type 1 diabetes - and the relevant topics supporting the development of the bioartificial pancreatic construct herein described. Besides type 1 diabetes, this chapter also addressed the currently available treatments for this disease and novel therapeutic strategies under development to surpass some of the challenges posed by existing treatments. In Chapter 2, the motivation for this thesis is presented, highlighting the main challenges of novel therapeutical approaches, which lead to the main goal and specific objectives of this project. Additionally, the structure of the thesis is presented as well as a short description of the collaborative work undertaken and the institutions involved in the development of this study. Chapter 3 describes the methodological details of the experiments performed. In Chapter 4, the results are outlined in different sections, according to their chronological development. Thus, this chapter begins with the formulation development (section 4.1), namely optimisation of the glucose-responsive GLP-1 analog-loaded NPs, as well as their physico-chemical characterisation and the assessment of drug release profile and peptide stability. In the following section, the glucoregulatory effect of both GLP-1 analogues (EXN and SMG) is assessed and compared (section 4.2). In section

4.3, the differentiation of β - and α -cells from hiPSCs is described along with their phenotypical characterisation and functional evaluation of SC- β cells *in vitro*. Then, the components of the bioartificial pancreatic system, which were detailed in the previous sections, and GOx were co-encapsulated in alginate microspheres and the efficiency of the system in controlling blood glucose levels was assessed in a T1D mouse model (section 4.4). Given the results obtained in the *in vivo* study, further improvements in the system had to be undertaken, which were explored *in vitro* on human islets and SC-islets through the assessment of their viability, insulin secretion and gene expression (section 4.5). Chapter 5 provides a final overview of the thesis, highlighting the main conclusions of the study as well as some future perspectives. Lastly, the final chapter is dedicated to a comprehensive list of all scientific contributions made by the author in the scope of this thesis (Chapter 6). The list of contributions includes a book chapter, publications in journals with peer-reviewed processes, poster and oral communications in conferences, as well as the attendance of scientific events and courses. At the end of the thesis, it is possible to find the Supplementary Data section, which contains additional data supporting the results described in Chapter 4.

The work presented in this thesis results from a collaborative effort between the Institute for Research and Innovation in Health (i3S) at the University of Porto (Portugal), the Leiden University Medical Centre (LUMC) (The Netherlands) and the Instituto Universitário de Ciências da Saúde (IUCS/CESPU) (Portugal). The research of the Nanomedicines & Translational Drug Delivery group at i3S is mainly focused on the development and characterisation of nanosystems for drug delivery applications and of 3D *in vitro* cell models to evaluate their efficacy and *in vitro/in vivo* correlation. Specifically, the group develops stimuli-responsive and/or functionalized nanoformulations carrying proteins or peptides of interest for diabetes treatment. The Islet group at the LUMC is specialised, as the name suggests, in the islets of Langerhans with both clinical and basic research projects. As the group is involved in the Dutch national human islet transplantation program and combines the expertise of physicians and biomedical scientists, its research establishes a close bridge from “bench to bedside”. Regarding basic research, this group is particularly interested in studying human islet cell identity, survival and function, and also strategies to generate stem cell-derived insulin-producing cells. On the other hand, clinical research is focused on the improvement of current human islet isolation and clinical β -cell replacement therapy, and on assessing islet function and damage in patients with severe β -cell failure (in particular T1D). Lastly, the mission of IUCS/CESPU is to conduct basic and translational research to better understand and address complex health problems, while developing and optimising drugs and analytical methods with impact on medicine, pharmaceutical industry, and environment.

The work developed on this thesis was achieved due to a strong collaboration between the institutions mentioned above and a solid teamwork spirit within each group. These partnerships enriched not only the scientific work here described, but also my personal and scientific experience. Most of the work was carried out at i3S, with the analytical analysis (high-performance liquid chromatography) being performed at IUCS/CESPU and later optimisations of the biofunctional system (section 4.5) were carried out at the LUMC.

Materials and Methods

3.1. Development of pH-responsive nanoparticles loaded with GLP-1 analogues

GLP-1 analog-loaded NPs were developed using a modified solvent emulsification-evaporation method based on a water-in-oil-in-water (w/o/w) double emulsion technique.¹⁶⁷ Several conditions were tested and optimised in order to obtain the desired formulation. The protocol described below was the one followed to produce the final formulation and the volumes are indicated per batch. The polymers used were PLGA (PURASORB® PDLG 7502A, 75/25 DL-lactide/glycolide copolymer, MW = 17 kDa), which was kindly offered by Corbion (The Netherlands) and Eudragit® E 100 (MW = 47 kDa) kindly provided by Evonik (Germany). The peptides used were exenatide acetate from Advanced ChemBlocks, Inc. (USA) and semaglutide, which was a kind offer from Novo Nordisk (Denmark).

Briefly, the organic phase consisted of a polymer solution at 40 mg/mL dissolved in ethyl acetate (Merck, USA) (1 mL) overnight at room temperature (RT). In the final formulation, the total polymer content (TPC) was composed of 50 % PLGA and 50 % Eudragit® E 100. The drug (either exenatide or semaglutide) was dissolved in Milli-Q® ultrapure water at a concentration of 44 mg/mL, of which 100 µL were added to 1 mL of the organic phase. Afterwards, this mixture was homogenized for 30 s in an ice bath at 70 % of amplitude, using a Vibra-Cell™ (VC 50) Ultrasonic Processor from Sonics & Materials, Inc. (USA), with a 2 mm sonication probe, forming the first emulsion - water-in-oil (w/o). Then, the first emulsion was transferred to a tube containing 4 mL of Kolliphor P 407 (BASF, Germany) aqueous solution (1 % W/V) and sonicated for 15 s under the same conditions, forming the second emulsion (w/o/w). The second emulsion was immediately transferred to a beaker containing 15 mL of Kolliphor P 407 aqueous solution (1 % W/V) and kept under magnetic stirring at 300 rpm for 3 h to promote ethyl acetate evaporation. NPs were concentrated by ultrafiltration, using Amicon® Ultra-15 centrifugal filter units (100 kDa cutoff; Merck, USA) and then washed twice with 10 mL of Milli-Q® ultrapure water, under the same conditions. The final NPs pellet was resuspended in 1 mL of Milli-Q® ultrapure water and stored at 4 °C for further experiments. Unloaded nanoparticles were produced following the same protocol without the addition of peptide in the aqueous phase.

When required, NPs were freeze-dried. After washing NPs, these were resuspended in 2 mL of Milli-Q® ultrapure water and poured into semi-stoppered glass vials with slotted rubber closures for lyophilization. Samples were stored at -80 °C overnight and then freeze-dried for 72 h at -80 ± 5 °C, using a FreeZone 2.5 Plus from Labconco (USA) at 0.09 mbar.

3.2. Characterisation of pH-responsive nanoparticles

3.2.1. Particle size, polydispersity index and zeta potential analysis

NPs were characterised regarding their average size and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta potential (ZP) through laser Doppler electrophoresis, using a Zetasizer Nano ZS (Malvern Panalytical Ltd., UK). To perform these measurements, samples were diluted (1:100) in a 10 mM sodium chloride aqueous solution. Values reported are the mean $\pm$ standard deviation (SD) of at least three different batches.

3.2.2. Morphological evaluation

The morphological features of NPs were analyzed by scanning electron microscopy (S.E.M.) using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: FEI Quanta 400 FEG ESEM (EDAX Genesis X4M). Samples were prepared by dropping 10 μ L of NPs suspension over a silicon wafer support and coating with a gold-palladium thin film by sputtering for 40 s with a 15 mA current, using the SPI Module Sputter Coater equipment. This procedure was performed at Centro de Materiais da Universidade do Porto (CEMUP).

3.2.3. Drug association efficiency and loading degree

The amount of loaded drug was directly quantified by high-performance liquid chromatography (HPLC) to determine the association efficiency (AE) and drug loading (DL) of the developed formulations by dissolving 3 mg of freeze-dried NPs in 5 mL of dimethylformamide (Honeywell, USA). AE and DL were determined following Equations (1) and (2), respectively.

$$AE (\%) = \frac{\text{Quantified loaded mass of cargo}}{\text{Initial mass of cargo}} \times 100 \quad (1)$$

$$DL (\%) = \frac{\text{Quantified loaded mass of cargo}}{\text{Total mass of freeze-dried NPs}} \times 100 \quad (2)$$

The HPLC method used to quantify exenatide and semaglutide is described in sub-section 3.2.5.

3.2.4. *In vitro* release of GLP-1 analogues from nanoparticles

For the *in vitro* release study, the sample and separate method¹⁶⁸ was used. Peptide release *in vitro* studies were performed after washing NPs to eliminate the non-encapsulated drug. Peptide-loaded NPs were dispersed in 5.0 mL of 25 mM phosphate buffer (pH 7.4) or 25 mM acetate buffer (pH 5), incubated at 37 °C under orbital agitation at 100 rpm. Each condition was analysed in triplicate. Aliquots of 200 µL were collected at specific time points (0.08, 0.5, 1, 2, 4, 8, 24 and 48 h) and the volume was replaced with the respective buffer, kept at the same temperature. Each aliquot was centrifuged at 10,000 ×*g* for 10 min and supernatant was stored at -20 °C until further analysis by HPLC, as described in the following sub-section (3.2.5).

3.2.5. Analytical HPLC method for the quantification of exenatide and semaglutide

Exenatide or semaglutide were quantified by reversed-phase HPLC with fluorescence detection using a Shimadzu UFLC Prominence System equipped with two pumps LC-20AD, an autosampler SIL-20AC, a column oven CTO-20AC, a degasser DGU-20A5, a fluorescence detector RF-10AXL, a system controller CBM-20A and the software LC solution, version 1.24 SP1.

For exenatide quantification, a LiChrospher® 100 RP-18 (5 µm) LiChroCART® 125-4 column was used as stationary phase with a LiChrospher® 100 RP-18 (5 µm) LiChroCART® 4-4 guard column (both from Merck, USA). Chromatographic analysis was performed using a gradient method at a flow rate of 0.9 mL/min. Mobile phases consisted of 0.1 % trifluoroacetic acid (TFA; Acros Organics, Belgium) in ultrapure water - eluent A - and acetonitrile (Fisher Scientific, USA) - eluent B. The gradient started at 67 % of eluent A decreasing to 55 % during the first 10 min. Then, eluent A increased again to 67% from 10 to 10.1 min and was kept constant until 24 min. The column was maintained at 35 °C during the analysis, and the injection volume was 20 µL. Detection was performed by fluorescence, with excitation at 295 nm and emission at 355 nm.

For semaglutide quantification, a Synergi Max-RP C12 column (4 µm; 4.6 x 250 mm) (Phenomenex, Inc., USA) was used as stationary phase with a LiChrospher® 100 RP-18 (5 µm) LiChroCART® 4-4 guard column (Merck, USA). Chromatographic analysis was performed using a gradient method at a flow rate of 1 mL/min. Mobile phases consisted of 0.1 % TFA in ultrapure water (eluent A) and 0.1 % TFA in acetonitrile (eluent B). The gradient started at 60% of eluent A decreasing to 20 % during the first 10 min. Then, eluent A increased again to 60 % from 10 to 11 min and was kept constant from 11 to 18 min. The injection volume was 20 µL and detection was performed by fluorescence, with excitation at 295 nm and emission at 350 nm.

3.3. Circular dichroism spectroscopy

In order to evaluate the secondary structure of exenatide and semaglutide released from NPs at different time points, circular dichroism (CD) spectroscopy was performed. Measurements were conducted using a Jasco J815 CD Spectrophotometer (Jasco Incorporated, USA) and the lamp housing was purged with nitrogen. Spectra were obtained from 190 to 260 nm using a 0.1 cm cell, a bandwidth of 1 nm, a data pitch of 1 nm, a digital integration time (D.I.T.) of 2 s, a scanning speed of 50 nm/min and an accumulation of 6 scans per sample. Since the voltage was higher than 600 V and absorbance was also high at wavelengths below 198 nm, data is represented between 198 and 260 nm. CD samples were obtained at specified time points from the *in vitro* release studies. Freshly prepared solutions of each peptide at 200 µg/mL were used as reference (native peptide). CD raw data were converted to mean residue ellipticity at wavelength λ ($[\theta]_{mrw,\lambda}$) given by:

$$[\theta]_{mrw,\lambda} = MRW \times \frac{\theta_{\lambda}}{10 \times d \times c} \quad (3)$$

where MRW is mean residue weight, θ_{λ} is the observed ellipticity (degrees) at wavelength λ , d is the pathlength (cm), and c is the concentration (g/ml). The MRW for the peptide bond is calculated by $MRW = M/(N - 1)$, where M is the molecular mass of the peptide (in Da), and N is the number of amino acids (the number of peptide bonds is $N-1$).¹⁶⁹

3.4. INS-1E culture conditions

INS-1E cells were routinely cultured in RPMI 1640 medium containing 10 mM HEPES (Merck, USA), supplemented with 10 % (V/V) of fetal bovine serum (FBS), 1x Penicillin-

Streptomycin Solution (100 IU/mL Penicillin and 100 µg/ml Streptomycin) (Corning®, USA) and 50 µM of β-mercaptoethanol (all purchased from Gibco, ThermoFisher). INS-1E cells were cultured in 75 cm² T-flasks at 37 °C, 5 % CO₂ and 95 % relative humidity with medium changes every 2 or 3 days.

3.5. Human islets culture conditions

Human pancreatic islets were isolated from pancreases of cadaveric donors, which were procured through the Eurotransplant multiorgan donation programme. Islets were used for research only if they could not be used for clinical purposes and if research consent was present, according to the local guidelines of the medical ethical committee of the Leiden University Medical Center and the Dutch Transplantation Foundation as the competent authority for organ donation in the Netherlands. Human islets with a purity >80 % were cultured in ultra-low attachment plates (ref. 3471; Corning, USA) with CMRL 1066 (Corning, 99-663-CV, 5 mmol/L glucose), supplemented with 10 % FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamycin, 20 µg/mL ciprofloxacin, 10 mmol/L HEPES and 1.2 mg/mL nicotinamide. Islet purity was assessed by dithizone (Sigma-Aldrich) staining, and islets were counted and scored using a standard algorithm for the calculation of 150 µm-diameter islet equivalent (IEQ) number (i.e., the number of “standard” islets with a diameter of 150 µm that would have the same total volume).^{170,171}

3.6. hiPSCs culture

3.6.1. hiPSCs source

WLS-4D1 hiPSC cell line was developed and kindly provided by the lab of William Stanford (Ottawa Hospital Research Institute).

3.6.2. Adherent culture of hiPSCs

Regular adherent culture and maintenance of WLS-4D1 cells was performed on tissue culture-treated 6-well plates (Falcon®, USA) coated with Matrigel® (Corning®, USA). After thawing, Matrigel® was diluted in cold DMEM/F-12 with 15 mM HEPES buffer (STEMCELL™ Technologies, Canada), according to the manufacturer’s instructions. Diluted Matrigel® solution was immediately used for coating tissue culture plates using 1

mL/well. Coated plates were incubated at room temperature for at least 1 hour and excess Matrigel® solution was removed from the wells immediately before use.

WLS-4D1 cells were maintained in mTeSR™ Plus (#100-0276, STEMCELL™ Technologies, Canada) with daily medium change. Cells were passaged as small aggregates (50 – 200 µm) every 5 – 7 days at dilutions ranging from 1:24 to 1:50, depending on aggregate density. Passaging was performed using ReLeSR™ (#05872, STEMCELL™ Technologies, Canada), an enzyme-free passaging reagent, according to the manufacturer's instructions. The well to be passaged was washed with D-PBS (Dulbecco's phosphate-buffered saline) without Ca⁺⁺ and Mg⁺⁺ (#37350, STEMCELL™ Technologies, Canada) and incubated with ReLeSR™ at room temperature for one minute. Afterwards, ReLeSR™ was removed, leaving only a thin layer of liquid covering the colonies. After incubation at 37°C for 4 – 5 min, mTeSR™ Plus was added. To detach the aggregates, the plate was held using one hand and firmly tapped on the side using the other hand for approximately 30 s. The aggregates were transferred to a tube containing mTeSR Plus™, diluted at the desired concentration and re-seeded in a Matrigel®-coated 6-well plate. Cells were incubated at 37°C, 5% CO₂ and undisturbed for 24 h.

Gentle Cell Dissociation Reagent (#100-0485, STEMCELL™ Technologies, Canada) was used for the preparation of single-cell suspensions. After washing with D-PBS without Ca⁺⁺ and Mg⁺⁺, cells were incubated with Gentle Cell Dissociation Reagent for 10 min at 37°C. Afterwards, to obtain a single-cell suspension, cells were pipetted up and down 2 – 3 times using a 1-mL micropipette and transferred to a tube containing an equal volume of DMEM/F-12. The well was then rinsed with DMEM/F-12 to ensure all cells were collected. After centrifugation at 300 x g, 20 °C for 5 min, cells were resuspended in an adequate volume of mTeSR™ Plus supplemented with 10 µM Y-27632 (#72304, STEMCELL™ Technologies, Canada).

3.6.3. Suspension culture of hiPSCs

To start a suspension culture from an adherent culture, WLS-4D1 were dissociated into single cells at the time of confluency. The obtained single-cell suspension was diluted in mTeSR™ Plus supplemented with 10 µM Y-27632 to an optimal seeding density of ~ 2 x 10⁵ cells/mL (static suspension culture) or ~ 3 x 10⁵ cells/mL (dynamic suspension culture). The cell suspension was transferred to a T-25 or T-75 flask (SPL Life Sciences, Pocheon-si, Korea) and incubated in the upright position at 37°C, 5% CO₂ for static suspension culture. Alternatively, for dynamic suspension culture, the cell suspension was transferred

to a 25 mL spinner-flask (#1967-00025, Bellco Glass, Vineland, United States of America) and incubated at 37°C, 5% CO₂ in a magnetic stirrer (Variomag® Biosystem 4, Thermo Fisher Scientific, United States of America) with a rotation speed of 5 rpm (revolutions per minute). After 48 h, the medium was replaced by mTeSR™ Plus without Y-27632. Afterwards, medium change was performed daily.

Suspension cultures were passaged every 4 – 5 days. For passaging, the suspension culture was collected into a tube and centrifuged at 300 x g, 20°C for 5 min. The aggregates were then dissociated into single cells using Gentle Cell Dissociation Reagent and seeded in mTeSR™ Plus supplemented with 10 µM Y-27632 at a concentration of $\sim 2 \times 10^5$ cells/mL (static suspension culture) or $\sim 3 \times 10^5$ cells/mL (dynamic suspension culture).

To estimate the number of aggregates in suspension, a sample of 500 µL was retrieved from the culture, centrifuged at 300 x g, 20 °C for 5 min and the pellet resuspended in 500 µL of DMEM/F-12. On the bottom of three wells of a 96-well plate, a '+' symbol was drawn to serve as a counting grid and 50 – 100 µL of the aggregate suspension were added to each well. Aggregates should be counted when they are bigger than 50 µm. The results from the three wells were averaged and the obtained average number was used to calculate the number of aggregates in the suspension culture.

For re-seeding in adherent culture, 200 – 300 aggregates were used per well of a 6-well plate. The required suspension volume was collected and centrifuged at 300 x g, 20 °C for 5 min. The aggregate pellet was resuspended in mTeSR™ Plus supplemented with 10 µM Y-27632 and 2 mL of the aggregate suspension were seeded per well. On the following day, the medium was replaced by mTeSR™ Plus without Y-27632 and the culture was maintained as regular adherent culture.

3.7. Immunocytochemistry of pluripotency markers

Cultured hiPSCs were regularly assessed for pluripotency by immunostaining. At around 80% confluency, cells were fixed with 4% paraformaldehyde (PFA, Thermo Fisher Scientific, USA) in 1x PBS for 10 min at RT. For intracellular staining, fixed cells were permeabilized with 1% V/V Triton™ X-100 (Fisher BioReagents™, USA) in 1x PBS for 5 min at RT. Fixed cells were incubated with blocking solution (BS) for 1 h at RT. BS composition was 1.5% W/V Bovine Serum Albumin (BSA) (Fischer BioReagents™, USA) and 5% V/V FBS (Biochrom AG, Germany) in 1x PBS. Afterwards, cells were incubated with the primary antibody diluted in BS (1:100) overnight at 4°C. The following primary

antibodies were used: Anti-Human OCT4 (#60093), Anti-Human SSEA-4 (#60062), Anti-Mouse SSEA-1 (#60060) and Anti-Human TRA-1-81 (#60065). All primary antibodies were acquired from STEMCELL™ Technologies (Canada). After three washing steps with 1x PBS (phosphate-buffered solution) for 5 min, cells were incubated with the secondary antibody diluted in BS (1:500) for 1 h at RT in the dark. The following secondary antibody was used: F(ab')₂-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594, (#A-11020, Thermo Fisher Scientific, USA). Cells were washed 3 times with 1x PBS for 5 min and stained with 4',6-diamidino-2-phenylindole dihydrochloride, DAPI, (#D9542, Sigma-Aldrich, USA) at 500 ng/mL for 5 min at RT. After one washing step with 1x PBS, 2 mL of 1x PBS were added to each well. Cell imaging was performed using ZOE™ Fluorescent Cell Imager (Bio-Rad, USA).

3.8. hiPSCs differentiation into pancreatic β-cells

Differentiation of WLS-4D1 into pancreatic progenitors (stage 4) was performed using STEMdiff™ Pancreatic Progenitor Kit (#05120, STEMCELL™ Technologies, Canada), according to the manufacturer's instructions.

Briefly, hiPSCs were seeded as single cells in Matrigel®-coated 6-well plates at a density of 2.5×10^6 cells/well in order to reach 90 – 100% confluency on the following day. The differentiation protocol requires daily medium changes, according to the following information (volumes are expressed per well of a 6-well plate):

- Stage 1 (definitive endoderm)

Day 1: 1.96 mL Endoderm Basal Medium + 20 µL Supplement MR + 20 µL Supplement CJ

Day 2: 1.98 mL Endoderm Basal Medium + 20 µL Supplement CJ

- Stage 2 (primitive gut tube)

Day 3: 1.96 mL Stage 2-4 Basal Medium + 20 µL Supplement 2A + 20 µL Supplement 2B

Days 4 and 5: 1.98 mL Stage 2-4 Basal Medium + 20 µL Supplement 2B

- Stage 3 (posterior foregut)

Days 6, 7 and 8: 1.98 mL Stage 2-4 Basal Medium + 20 µL Supplement 3

- Stage 4 (pancreatic progenitors)

Days 9 – 13: 1.98 mL Stage 2-4 Basal Medium + 20 μ L Supplement 4

On day 14 of the differentiation (end of stage 4), hiPSCs-derived pancreatic progenitors were transferred to an air-liquid interface to further differentiate into mature β -cells, following the protocol proposed by Rezania *et al.* ¹⁰⁹.

Cells were treated for 4 h with MCDB 131 medium (catalogue number 10372019, Gibco™, Amarillo, United States of America) supplemented with 10 μ M Y-27632. Afterwards, cells were dissociated into single cells using Gentle Cell Dissociation Reagent and centrifuged at 300 x *g* for 5 min at 20°C. The obtained cell pellet was resuspended in MCDB 131 medium supplemented with 10 μ M Y-27632 at a density of 0.5 x 10⁵ cells/ μ L. On the apical side of 6-well plate filter inserts (353493, Falcon®, United States of America), 5 – 10 μ L of the cell suspension were spotted (0.25 – 0.5 x 10⁶ cells/spot) with a total of around 10 spots per well. A volume of 1.5 mL of medium was used per well and added to the basolateral side of each insert. Medium changes were performed daily, according to the following:

- Stage 5 (endocrine progenitors)

Days 14 – 16: MCDB 131 medium supplemented with 20 mM glucose (Sigma-Aldrich, USA), 1.5 g/L sodium bicarbonate (Sigma-Aldrich, USA), 2% BSA (VWR, USA), 1x GlutaMAX™ (Gibco™, USA), 1:200 Insulin-Transferrin-Selenium-Ethanolamine, ITS-X, (Gibco™, USA), 0.25 μ M SANT-1 (Merck Millipore, USA), 0.05 μ M retinoic acid (Sigma-Aldrich, USA), 100 nM LDN193189 (Sigma-Aldrich, USA), 1 μ M 3,3',5-Triiodo-L-thyronine sodium salt, T3, (Sigma-Aldrich, USA), 10 μ M ALK5 inhibitor II (Enzo Life Sciences, USA), 10 μ M zinc sulfate (Sigma-Aldrich, USA) and 10 μ g/mL heparin (Sigma-Aldrich, USA)

- Stage 6 (immature β -cells)

Days 17 – 24: CMRL medium (Gibco™, USA) supplemented with 20 mM glucose, 1.5 g/L sodium bicarbonate, 2% BSA, 1x GlutaMAX™, 1:200 ITS-X, 100 nM LDN193189, 1 μ M T3, 10 μ M ALK5 inhibitor II, 100 nM γ -secretase inhibitor XX (Merck Millipore, USA), 10 μ M zinc sulfate and 10 μ g/mL heparin.

- Stage 7 (mature β -cells)

Days 25 – 31: CMRL medium supplemented with 20 mM glucose, 1.5 g/L sodium bicarbonate, 2% BSA, 1x GlutaMAX™, 1:200 ITS-X, 1 μ M T3, 10 μ M ALK5 inhibitor II, 1 mM N-acetyl cysteine (Sigma-Aldrich, USA), 10 μ M Trolox (Merck Millipore, USA), 2 μ M R428 (MedChemExpress, USA), 10 μ M zinc sulfate and 10 μ g/mL heparin.

3.9. hiPSCs differentiation into pancreatic α -cells

Differentiation of hiPSCs into pancreatic pre- α -cells was performed following the protocol proposed by Peterson *et al.*¹⁵⁰. WLS-4D1 hiPSCs were seeded as single cells in suspension culture at a concentration of 2×10^5 cells/mL in a T-75 flask in the upright position (static suspension). Cells were incubated at 37°C, 5% CO₂ and differentiation was initiated 2 days after seeding, when aggregates presented an average size of 100 – 200 μ m. Aggregates were allowed to settle for ~5 – 10 min and the old medium was removed by aspiration. Medium changes were performed according to the following schedule:

- Stage 1 (definitive endoderm)

Day 1: MCDB 131 medium supplemented with 8 mM glucose, 2.46 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:50,000 ITS-X, 0.25 ascorbic acid (Sigma-Aldrich, USA), 100 ng/mL Activin A (R&D Systems, USA) and 3 μ M CHIR99021 (Sigma-Aldrich, USA).

Day 2: MCDB 131 medium supplemented with 8 mM glucose, 2.46 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:50,000 ITS-X, 0.25 ascorbic acid and 100 ng/mL Activin A.

- Stage 2 (gut tube endoderm)

Day 4: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:50,000 ITS-X, 0.25 ascorbic acid and 50 ng/mL FGF7 (STEMCELL™ Technologies, Canada)

- Stage 3 (pancreatic progenitors)

Day 6: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:200 ITS-X, 0.25 ascorbic acid and 2 μ M retinoic acid.

Day 7: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:200 ITS-X, 0.25 ascorbic acid, 2 μ M retinoic acid and 200 nM LDN193189.

- Stage 4 (endocrine progenitors)

Day 8: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:200 ITS-X, 0.25 ascorbic acid and 200 nM LDN193189.

Days 9 and 11: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2% BSA, 2 mM GlutaMAX™, 1:200 ITS-X and 0.25 ascorbic acid.

- Stage 5 (pre- α -cells)

Days 13, 15, 17 and 19: MCDB 131 medium supplemented with 8 mM glucose, 1.23 g/L sodium bicarbonate, 2 % BSA, 2 mM GlutaMAX™, 1:200 ITS-X, 0.25 ascorbic acid and 10 μ M ALK5 inhibitor II.

3.10. Flow cytometry

Cells were analysed by flow cytometry at several time points of the differentiation protocols. Cells were dissociated into single cells using Gentle Cell Dissociation Reagent, resuspended in mTeSR™ Plus supplemented with 10 μ M γ -27632 and counted using a hemocytometer. For staining of intracellular antigens, 1×10^5 – 1×10^6 cells were aliquoted per sample into a 5 mL round bottom tube. Cells were centrifuged at 300 x g, 4°C for 5 min, resuspended in 100 μ L of diluted Zombie Green™ Fixable Viability Kit (BioLegend®, USA) solution (1:1000 in PBS) and incubated on ice in the dark for 15 min (optional step, performed only for differentiation into α -cells). After incubation, 2 mL of 2 % V/V FBS in PBS solution were added per tube and the samples were centrifuged at 300 x g, 4°C for 5 min. Cells were fixed using 2 % PFA for 15 min on ice. Afterwards, 1 mL of 2 % V/V FBS in PBS solution was added per tube and the samples were centrifuged at 300 x g, 4°C for 5 min. Cells were then resuspended in 500 μ L of saponin permeabilization buffer (SPB), which consists of 1 mg/mL saponin (Sigma-Aldrich, USA) and 1% W/V BSA in D-PBS without Ca^{++} and Mg^{++} , and incubated at room temperature for 15 min. After centrifugation at 300 x g, 4°C for 5 min, cells were resuspended in the antibody mix (or SPB for the unstained control) using 100 μ L per sample. The antibodies used, as well as respective dilutions and incubation conditions are listed in Table 6. After incubation, 1 mL of SPB was added to each tube and samples were centrifuged at 300 x g, 4°C for 5 min. Cells were resuspended in 2 % V/V FBS in PBS and filtered using a 100 μ m filtration nylon membrane. Samples were placed on ice and analysed using the BD Accuri™ C6 flow cytometer (BD Biosciences, USA). FlowJo v10 software was used to process flow cytometry data.

Table 6 - List of antibodies used for flow cytometry.

Antibody	Supplier	Reference	Dilution	Incubation conditions
APC-conjugated anti-human SOX17	R&D Systems (USA)	IC1924A	1:50	30 min, 4°C
PE Mouse anti-PDX-1 (Clone 658A5)	BD Biosciences (USA)	562161	1:20	1 h, 2 – 8°C
Alexa Fluor® 647 Mouse anti-NKX6.1 (Clone R11-560)	BD Biosciences (USA)	563338	1:20	1 h, 2 – 8°C
PE Mouse Anti-NKX6.1 (Clone R11-560)	BD Biosciences (USA)	563023	1:40	30 min, 4°C
Insulin (C27C9) Rabbit mAb (Alexa Fluor® 647 Conjugate)	Cell Signaling (USA)	#9008	1:80	1 h, 4°C
Alexa Fluor® 647 Mouse Anti-C-Peptide (Clone U8-424)	BD Biosciences (USA)	565831	1:50	1 h, 4°C
PE Mouse Anti-Glucagon (Clone U16-850)	BD Biosciences (USA)	565860	1:20	1 h, 4°C

3.11. Cells encapsulation

For alginate sterilisation, 1 % W/V low-viscosity alginate (PRONOVA™ UP LVG sodium alginate, NovaMatrix®, Norway) solution was prepared in sterile 0.9 % W/V NaCl (Honeywell, USA) and filtered using a Steriflip® filter unit (Merck Millipore, USA). The sterile solution was lyophilized for 72 h and stored at -20 °C until use.

For encapsulation, pre-sterilised alginate powder was dissolved at 2 % W/V in sterile 0.9 % W/V NaCl and stirred overnight at 4 °C.

Cells cultured in suspension were encapsulated as aggregates, while cells cultured in adherent conditions were encapsulated as single cells. Aggregate/cell suspension was centrifuged at 200 x g, 20 °C for 5 min and the pellet was resuspended in 2 % W/V sterile alginate solution. The aggregate/cell suspension in alginate was loaded into a 1-mL syringe (Henke Sass Wolf, Germany). Extrusion was performed under a coaxial air flow using a Var J1 encapsulation unit (Nisco Engineering AG, Switzerland) and a syringe pump, as previously described.¹⁷²

Sterile 100 mM CaCl₂ (VWR, USA) solution in 0.9 % W/V NaCl was used as the crosslinking solution. After extrusion, microspheres were allowed to crosslink for 10 min, washed with DMEM/F-12 and transferred to a 6-well plate. Microspheres were observed using ZOE™ Fluorescent Cell Imager and an inverted microscope. Encapsulated cells were maintained in their respective medium.

3.12. Metabolic activity assay

The metabolic activity of alginate-encapsulated cells was assessed by a resazurin assay adapted from a previously described protocol.¹⁷³ The resazurin (Sigma-Aldrich, USA) stock solution was prepared at 0.1 mg/mL and the respective working solution was prepared by diluting the stock solution at 20 % V/V in adequate cell culture media. To perform the assay, at each timepoint cell medium was removed and resazurin working solution (20 % V/V) was added. Cells were incubated in the dark for 2 h (INS-1E cells) or 3 h (human islets) at 37 °C and 5 % CO₂. Following incubation, 200 µL of the supernatant were transferred to a black-walled clear-bottom 96-well plate and fluorescence was measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). After the measurements, resazurin working solution was replaced by fresh cell culture media. To ensure the complete removal of resazurin from the alginate microspheres, culture media was replaced again after 30 min. Culture media with empty microspheres (no cells) was used as a blank, encapsulated cells were used as positive control and encapsulated cells treated with 1 % (V/V) Triton X-100 were used as negative control. All samples were analyzed in triplicate and normalised to the positive and negative controls, which were considered as 100 % and 0 % of metabolic activity, respectively. In accordance with the ISO 10993–5:2009 standard,¹⁷⁴ 70 % was considered as a threshold of cell viability.

3.13. Glucose-stimulated insulin secretion (GSIS)

3.13.1. Static GSIS

In order to assess the function of stem cell-derived β -cells (SC- β -cells) (stage 7 of differentiation) and the glucoregulatory effect of exenatide and semaglutide on INS-1E cells, a glucose-stimulated insulin secretion (GSIS) assay was performed, as described by Hogrebe *et al.*¹⁷⁵ INS-1E cells were seeded at 2×10^5 cells/mL in 24-well plates and cultured for five days, refreshing media on the third day. Cells were washed three times with Krebs buffer (Krb) and then pre-incubated in Krb for 30 min to remove residual insulin. Afterwards, cells were washed once, incubated in low (2 mM) glucose Krb for 30 min (at 37 °C and 5 % CO₂), and supernatants were collected. Then cells were incubated in high glucose (20 mM) Krb for 30 min, and the supernatant was collected. Krb buffer in low and high incubation steps had different concentrations of exenatide or semaglutide. Finally, cells were incubated in Krb containing 2 mM glucose and 30 mM KCl for 30 min, then the supernatant was collected. KCl-triggered depolarization increases cytosolic Ca²⁺ levels, leading to a peak in insulin secretion.

Supernatant samples containing secreted insulin were stored at -80 °C until further quantification. At the end of the experiment, cells were detached using Trypsin/EDTA (Sigma-Aldrich, USA) and cell number was counted manually using trypan blue and a hemocytometer.

Afterwards, cells were lysed to assess intracellular insulin content. Cells were centrifuged at 300 g for 5 min at 4 °C and washed twice with cold PBS. Cells were then incubated with ice-cold lysis buffer (cOmpleteTM protease inhibitor cocktail (Roche, Switzerland) + 1 % triton X-100 (Thermo Fisher Scientific, USA) in PBS 1x) for 30 min on ice, followed by centrifugation at 2000 g for 10 min at 4 °C. The supernatant was collected and acid-ethanol extraction was performed to extract protein content from lysates. The lysate was mixed with acid-ethanol (EtOH) (0.18 M HCl in 96 % (V/V) EtOH) in a 1:3 proportion of lysate and acid-ethanol solution and incubated at 4 °C for 12 h. Samples were then centrifuged to separate cellular debris. The supernatant was stored at -80 °C until further analysis by enzyme-linked immunosorbent assay (ELISA), described in sub-section 3.14.

The stimulation index (SI), which is extensively used as a quantitative descriptor for islet characterisation, was determined by calculating the ratio between insulin secretion at high and low glucose.¹⁷⁶

3.13.2. Dynamic GSIS

Functionality of human islets or SC-islets was assessed through dynamic GSIS. Approximately 20 islets per sample were collected, placed in filter-closed chambers (Suprafusion 1000; Brandel, Gaithersburg, MD) and perfused at 500 $\mu\text{L}/\text{min}$ at 37 °C. Firstly, a preconditioning of islets was performed by perfusing them with a low-glucose (1.7 mmol/L glucose) solution (20 mmol/L HEPES, 11.5 mmol/L NaCl, 0.5 mmol/L potassium chloride (KCl), 2 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 2.4 mmol/L NaHCO_3 , supplemented with 0.2 % human serum albumin in demineralised water) for 90 min. Afterwards, the assay was initiated and islets were perfused with the low-glucose solution for 20 min, followed by a high-glucose solution (20 mmol/L glucose) for 50 min, low-glucose solution again for 20 min, KCl low-glucose solution for 5 min (depolarization challenge) and a final incubation period of 10 min in low-glucose solution. Fractions were collected at 5 min intervals and quantified for human C-peptide by ELISA. For each timepoint, the C-peptide concentration was divided by the average of C-peptide concentration of the timepoints during the second low-glucose phase, to give a stimulation index at every time-point.

3.14. Enzyme-linked immunosorbent assay

Sandwich ELISA was used to quantify proinsulin and insulin content in GSIS samples. Briefly, high-binding 96-well plates (Corning Costar, USA) were coated with 50 μL of anti-insulin antibody (Abcam, ref. ab8304) diluted 1:1000 in PBS and incubated overnight at 4 °C under orbital agitation at 65 rpm. After incubation, wells were washed three times with 200 μL of 0.05 % Tween-20 in PBS (PBST) and blocked with 100 μL of 4 % skimmed milk (PanReac AppliChem, Spain) diluted in PBST for 1 h at RT. Wells were washed again three times with 200 μL of PBST. An insulin calibration curve was prepared in the range of 10 to 2000 pg/mL. Fifty microliters of standards and diluted samples were added in triplicates to wells and incubated for 2 h at RT. Wells were washed as described before, and 50 μL per well of HRP anti-insulin antibody (Abcam, ref. ab28063), diluted 1:2000 in PBS, were added for 1h at RT, followed by a washing step. Afterwards, 100 μL per well of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, USA) were added and incubated in the dark for 15 min. Then, 100 μL of 2 M of sulfuric acid was added to each well to stop the reaction. Absorbance was measured at 450 nm using SynergyMx™ MultiMode microplate reader.

At the LUMC, the GSIS samples were analysed using ELISA kits (Mercodia AB, Sweden) to quantify rat insulin (samples from INS-1E cells), human insulin and human C-peptide.

3.15. RT-qPCR

RNA (250–500 ng) was obtained from SC-islets after cell lysis with RLT buffer with β -mercaptoethanol, according to the manufacturer's instructions (micro RNeasy kit - Qiagen, Germany). Isolated RNA was reverse-transcribed to DNA with M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) (Invitrogen, Thermo Fisher Scientific, USA), oligo-dT primers (Qiagen, Germany), dNTP Mix (Promega, USA), dithiothreitol (DTT) (Invitrogen, Thermo Fisher Scientific, USA) and RNase-OUT™ Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific, USA). Quantitative PCR (qPCR) was performed with IQ SYBR green mix (170–8887, Bio-Rad, USA) and *ACTB* (beta-actin) was used as housekeeping gene. Amplification and detection were performed by using CFX systems (Bio-Rad, USA) and fold changes were obtained by using the $2^{-\Delta\Delta C_t}$ method.¹⁷⁷

3.16. *In vivo* study

Male C57BL/6 mice were acquired from Charles River Laboratories (Spain). Only male mice were used in this study since females are less sensitive to the toxin streptozotocin,¹⁷⁸ which was used for T1D induction. All animals involved in the experiments were handled in accordance with good animal practice, as defined by the European Union Directive 2010/63/EU and the national Decreto-Lei nº 113/2013. All animal experimental procedures described in this work were approved by the AWERB (animal welfare and ethics review body) at i3S (internal authorisation: BSm_2017_10) and conducted at the i3S animal facility, under authorisation by Direção-Geral da Alimentação e Veterinária (DGAV).

Five animals were housed per cage and were allowed to acclimatise in the facility for at least one week. Mice were housed at the i3S animal facility with ad libitum access to water and standard rodent food unless stated otherwise. Animals were kept under a 12-hour light/dark cycle, with 45-65 % humidity and temperatures between 20 and 24 °C. Mice were randomly assigned to each experimental group.

For the induction of T1D, 9-week-old mice were fasted during 4 h prior to STZ administration at 200 mg/kg by intraperitoneal injection. After STZ injection, 10 % sucrose water (to reduce morbidity and mortality) and standard rodent food were provided. Mice were closely

monitored every 2 h during 12 h for marked hypoactivity, unresponsiveness, or convulsions. Three days after STZ administration, 10 % sucrose water was replaced by regular water. Eleven days after STZ administration, treatments were initiated (experimental day 0) by transplanting the alginate microspheres in the peritoneal cavity.

Before transplantation, buprenorphine (0.1 mg/kg) in sterile saline solution was administered subcutaneously to ensure pain relief before mice regained consciousness. Mice were then anaesthetized with 5 % isoflurane at 2.5 mL/min, which was reduced to 2 % once the animal became unresponsive. Fur in the lower abdomen was shaved and skin was disinfected with chlorhexidine. An incision of 4–5 mm wide was made through the skin and muscle. Then the tip of the syringe loaded with the microspheres was slightly and gently introduced through the incision and alginate beads were transplanted in the mouse (1.76×10^6 cells/animal).

The incision was closed by suturing muscle and skin separately with absorbable suture wire (3–4 sutures). After surgery, animals received subcutaneous injections of buprenorphine (0.1 mg/kg) twice a day, for 3 days. A schematic representation of the *in vivo* experiment and transplantation procedure is shown in Figure 14.

During the entire duration of the study, animals were carefully monitored regarding body weight, blood glucose levels and their overall body condition. Blood glucose levels were measured with a FreeStyle Precision (Abbott®, USA) glucose meter, using a sample of capillary blood collected by pricking the lateral tail vein with a 27G needle. Animals were not fasted before blood glucose measurements to avoid inducing more stress. However, those measurements were performed at approximately the same time of the day in order to minimise the influence of their eating habits on the glucose levels measured. Animals were sacrificed on day 27 by CO₂ asphyxiation followed by cervical dislocation.

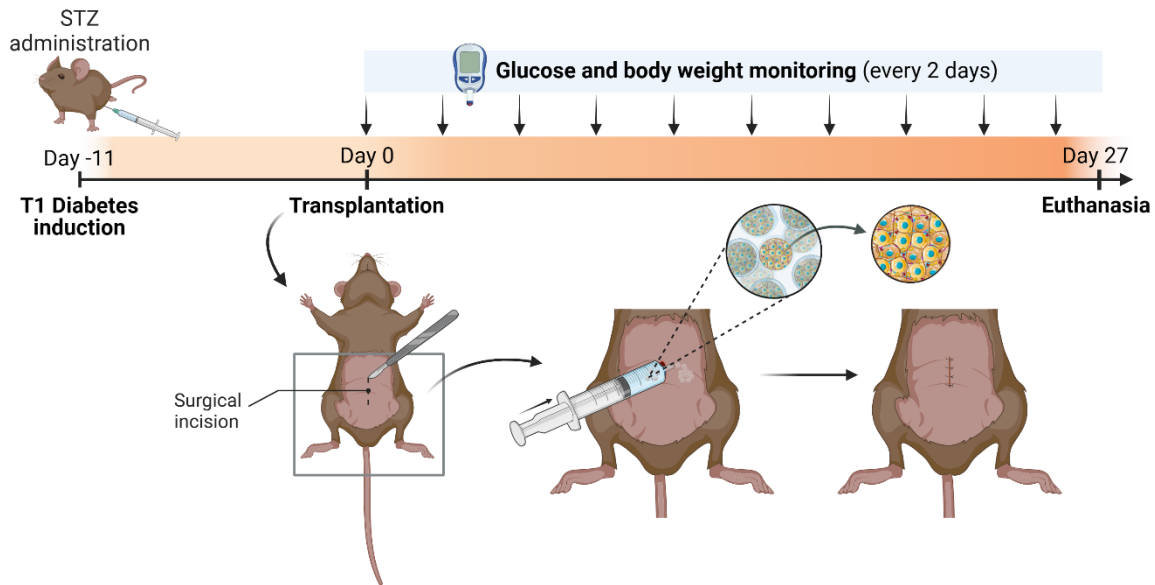


Figure 14 - Schematic representation of the *in vivo* experiment and transplantation procedure.

3.17. Statistical analysis

For statistical analysis, GraphPad Prism version 9.0.0 for Windows (GraphPad Software, USA) was used. Unless otherwise mentioned, data was analyzed using Student's t-test for comparison between two independent groups and ANOVA for comparison among three or more groups, with the level of significance set at probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results and Discussion

The information provided in this chapter was partially based on the following publication:

- **Marques JM**, Nunes R, Carvalho, AM, Florindo H, Ferreira D, Sarmento B. GLP-1 analogue-loaded glucose-responsive nanoparticles as allies of stem cell therapies for the treatment of Type I Diabetes. *ACS Pharmacol Transl Sci.* 2024; 7 (5): 1650-63. DOI: 10.1021/acsptsci.4c00173

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Protein or peptide drugs play a crucial role in treating or preventing various diseases by regulating physiological or pathological processes. However, their extensive use is limited by chemical and physical instabilities, enzymatic degradation, rapid clearance from circulation and potential risk for immunogenicity.^{179,180} Different strategies have been investigated in the past decades to overcome these limitations and increase their safety and efficacy, namely protein or peptide modification and encapsulation in drug delivery systems.¹⁷⁹ Nanoparticulate systems have the advantage of not only protecting the protein or peptide from enzymatic degradation, but also of controlling their release profile in a site- or stimuli-specific manner. Therefore, the formulation and characterisation of optimised drug delivery systems are crucial steps in modern pharmaceuticals.

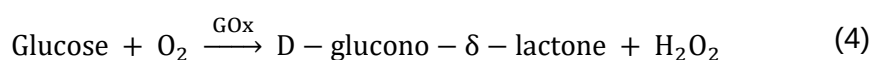
GLP-1 is an incretin hormone that acts in a glucose-dependent manner, stimulating insulin biosynthesis and secretion in islet β -cells while reducing glucagon secretion from α -cells. Besides, it also suppresses apoptotic cell death of β -cells and increases their proliferation. However, GLP-1 has a short plasma half-life (< 2 min) since it is rapidly degraded by the DPP-4 enzyme.¹⁸¹

In this work, the role of GLP-1 analogues in pancreatic cells is exploited and the incorporation of glucose-responsive GLP-1 analog-loaded NPs into the biomimetic pancreas system is proposed. This combination aims to: a) improve glucose-stimulated insulin secretion of SC- β cells, since they are not fully functional and mature after *in vitro* differentiation,^{162,166} and b) minimise immediate cell death after transplantation, thus reducing the percentage of graft loss, which is commonly reported.¹⁶⁵

Given the complexity of the system, it was developed by dividing it into different steps, starting with the development of the NPs, followed by the generation of SC- β cells and - α cells and then the encapsulation of all the components. In this chapter, the efforts undertaken towards the development of the bioartificial pancreatic construct are detailed. Section 4.1.1 begins with an explanation of the optimisation of the NPs formulation, followed by its physico-chemical characterisation, *in vitro* drug release and peptide stability studies (section 4.1.2). In section 4.3 the *in vitro* differentiation of α - and β -cells was described. Afterwards, these SC-pancreatic cells were co-encapsulated in alginate microspheres and assessed in an exploratory *in vivo* study (section 4.4). After further improvements in the system, its efficacy was tested in human cadaveric islets (section 4.5.1) and also SC-islets (section 4.5.2), which were differentiated in a different lab (LUMC), following a different *in vitro* differentiation protocol.

4.1. Development of GLP-1 analogue-loaded pH-responsive nanoparticles

To design glucose-responsive GLP-1 analog-loaded NPs, the enzyme GOx and a pH-sensitive polymer (Eudragit® E100) were included in the system. GOx catalyzes the oxidation of β -D-glucose to D-glucono- δ -lactone and hydrogen peroxide when molecular oxygen is present:



Subsequently, D-glucono- δ -lactone is hydrolyzed to form D-gluconic acid,¹⁸² leading to a transient decrease in the surrounding pH. Thus, by incorporating GOx and a pH-sensitive polymer in the system, peptide release can be adjusted based on glucose concentrations.

Among the great variety of GLP-1 analogues described in the literature,¹⁸³ EXN and SMG were selected for this study. Both peptides are FDA-approved drugs that mimic the physiological actions of the native GLP-1, but differ structurally from it, making them resistant to the action of DPP-4.¹⁸³ Given the hydrophilicity of EXN and the amphiphilic character of SMG, NPs were produced by a modified solvent emulsification-evaporation method based on the water-in-oil-in-water (w1/o/w2) double emulsion technique.¹⁶⁷

4.1.1. Formulation optimisation

Considering the aim of the study, Eudragit® E100 was selected due to its solubility below pH=5, which will be induced by increasing levels of gluconic acid resulting from glucose oxidation by GOx when glucose levels rise. Moreover, the inclusion of Eudragit® E100 can increase the association efficiency of negatively charged molecules (as is the case of EXN and SMG) due to the electrostatic interactions formed between the drug and the cationic polymer. The isoelectric point (pI) of EXN is 4.86 and of SMG is 5.4,^{64,184} thus at pH above the pI, the peptide will be negatively charged. Additionally, employing PLGA with free carboxylic end groups (such as PURASORB® PDLG 7502A) is expected to enhance encapsulation efficiency owing to the increased hydrophilicity of the polymer.¹⁸⁵

Previous work in the group, developed by a master's student working on the very initial steps of this project, namely on the NPs formulation, was used as a starting point. Therefore, the initial formulation here described, was produced using ethyl acetate as the organic solvent and Kolliphor P 407 aqueous solution (1 % W/V) as surfactant, since these

parameters had been previously optimised. Nevertheless, the formulation required further optimisation, specially regarding drug loading (which was below 0.8 %) and drug release profile. Hence, several parameters, such as internal phase composition and volume, sonication time, external phase composition and volume or polymer mass were tested until the final formulation was achieved (Table S 1). EXN was the peptide used in the optimisation tests.

Briefly, NPs were prepared by the double emulsion technique, using: 40 mg of polymer dissolved in 1 mL of ethyl acetate, 1% W/V Kolliphor P 407 as surfactant, 30 s in the 1st sonication step, 15 s in the 2nd sonication step and 700 μ L as internal phase volume.

A possible approach was to adjust the pH of the external aqueous phase to the nearest value of the drug pI and use a low percentage of surfactant in the internal aqueous phase. Regarding the first option, as exenatide pI is 4.86 and Eudragit E100 is soluble at pH under 5, it is not possible to use an external phase at pH 4.86. Therefore, 1 % Kolliphor P 407 in acetate buffer 0.1 M at pH 5.5 was tested. However, aggregates were also visible in all formulations. The second option was to use a low percentage of surfactant in the internal phase. In this case, 0.1 % of Kolliphor P407 was used, but these formulations also showed visible precipitates in all formulations (Figure S 1).

In order to avoid the formation of aggregates and to be able to tune other formulation parameters to increase drug loading, the time of sonication of the second step was also varied (30 s and 1 min), as well as the surfactant concentration of the external phase, which was increased from 1 % W/V to 2 % W/V. All the conditions tested showed visible aggregates. As the volume of the internal phase was 700 μ L, the volume was decreased to 100 μ L and the peptide concentration was increased to maintain the same amount of drug per batch. This time, the presence of macroscopic aggregates visibly decreased when compared to the previous conditions, although they were still present.

Considering this condition as a “new” starting point (Formulation A), since there were visible improvements, other parameters were tested. The concentration of surfactant of the external phase was increased to 2 % W/V in an attempt to further stabilise the formulation. Nevertheless, it had the opposite effect and all formulations tested showed significant aggregation. Another approach to stabilise the formulation was to try a different surfactant, namely polyvinyl alcohol (PVA). Two concentrations were tested (1 % and 2 % W/V), but precipitates were also observed after solvent evaporation.

Other parameters were further tested: time of sonication of the second step (15 s, 45 s and 1 min), surfactant concentration of the external phase (which was increased again from 1%

W/V to 2 % W/V, but now with a different sonication time), polymer mass (which was decreased to 20 mg), surfactant type (Tween-80 was tested in two different concentrations - 1 % and 2 % W/V). Among all these tests, a few highlights: regarding the sonication time, 1 min in the second step was the condition showing the least amount of aggregation; concerning surfactant concentration the outcome was worse with 2 % W/V and more precipitates were visible when comparing to 1 % W/V; about polymer mass, the (macroscopic) outcome was similar for formulations with 20 mg and 40 mg, so the following tests were performed with 40 mg; regarding surfactant type, in which Tween-80 was tested, although macroscopically speaking there were no visible aggregates, the formulations were very light colored and after analyzing them on the DLS, it can be concluded that NPs were not formed or were formed in a very slight extent, since more than one population was observed ($PDI > 0.360$) and, in most of them, the major population had small sizes (< 50 nm), probably being micelles instead of NPs.

After these tests, the best condition obtained (Formulation B) was tested again, but this time loaded with exenatide (5 % drug loading). However, a high amount of aggregates was visible in all formulations. In an attempt to understand this striking difference observed between the loaded and unloaded formulations and to eliminate any incompatibility of the peptide (EXN) with the double emulsion technique, the same conditions were tested, but without any polymer in order to check if EXN would aggregate when subjected to these conditions alone. However, after subjecting the peptide to the entire process, no visible aggregate or precipitates were visible, showing that EXN alone is not forming the precipitates previously observed.

As the experimental conditions of Formulation B did not show positive outcomes when NPs were loaded with EXN, Formulation A conditions were again considered for further improvements. This time, instead of increasing surfactant concentration, a higher volume of surfactant was used in an attempt to further stabilise the formulations, since more molecules of surfactant are available for the same amount of NPs. The formulations obtained were significantly better as no macroscopic aggregates were visible and also showed promising physico-chemical characteristics (size around 145 nm and $PDI < 0.160$). Therefore, these experimental conditions were considered for further assays.

4.1.2. Characterisation of GLP-1 analog-loaded NPs

In this work, two peptides were explored, namely EXN and SMG. For each peptide, two formulations (PLGA:E100 (50:50) and E100) and three drug loadings (5, 10 and 15 %) were

tested and characterised regarding size, Pdl and ZP (Table 7). The results for SMG-loaded E100 NPs with 15 % of theoretical drug loading are not shown in Table 7 since this formulation showed precipitates. All formulations presented a mean size around 135-155 nm and a monodisperse population ($PdI < 0.200$), as well as a ZP between 17 and 42 mV, which decreased with increasing amounts of loaded peptide. This is due to the negative charge of both exenatide and semaglutide at pH 7, which interact with the positively charged E100 through electrostatic forces. S.E.M. images revealed the spherical shape of NPs and confirmed the size obtained by dynamic light scattering (DLS) (Figure 15, A and Figure 16, A).

Table 7 - Physicochemical properties of exenatide- and semaglutide-loaded NPs produced by double emulsion technique. Results are presented as mean $\pm$ standard deviation ($n = 3$).

Drug	Theoretical Drug Loading	Formulation	Size (nm)	Pdl	ZP (mV)	AE (%)	DL (%)
Exenatide (EXN)	5 %	PLGA:E100 (50:50)	149.4 $\pm$ 3.7	0.163 $\pm$ 0.016	31.1 $\pm$ 0.7	47.4 $\pm$ 6.3	2.4 $\pm$ 0.3
		E100	135.9 $\pm$ 5.4	0.155 $\pm$ 0.012	42.1 $\pm$ 1.1	49.0 $\pm$ 5.1	2.4 $\pm$ 0.3
	10 %	PLGA:E100 (50:50)	140.9 $\pm$ 2.5	0.164 $\pm$ 0.009	29.2 $\pm$ 1.2	41.4 $\pm$ 0.9	4.1 $\pm$ 0.1
		E100	134.8 $\pm$ 2.5	0.155 $\pm$ 0.016	25.8 $\pm$ 5.1	34.2 $\pm$ 1.1	3.4 $\pm$ 0.1
	15 %	PLGA:E100 (50:50)	146.0 $\pm$ 2.6	0.170 $\pm$ 0.017	21.7 $\pm$ 2.7	33.9 $\pm$ 0.8	5.1 $\pm$ 0.1
		E100	138.5 $\pm$ 4.0	0.167 $\pm$ 0.014	17.6 $\pm$ 4.2	36.9 $\pm$ 0.7	5.5 $\pm$ 0.1
Semaglutide (SMG)	5 %	PLGA:E100 (50:50)	149.7 $\pm$ 5.7	0.177 $\pm$ 0.016	29.3 $\pm$ 2.9	64.0 $\pm$ 2.8	3.2 $\pm$ 0.1
		E100	135.8 $\pm$ 3.5	0.154 $\pm$ 0.010	28.5 $\pm$ 4.0	60.1 $\pm$ 3.2	3.0 $\pm$ 0.2
	10 %	PLGA:E100 (50:50)	146.6 $\pm$ 5.1	0.165 $\pm$ 0.013	25.7 $\pm$ 2.9	50.1 $\pm$ 13.9	5.0 $\pm$ 1.4
		E100	153.9 $\pm$ 4.3	0.173 $\pm$ 0.016	25.1 $\pm$ 4.1	52.8 $\pm$ 2.4	5.3 $\pm$ 0.2
	15 %	PLGA:E100 (50:50)	153.1 $\pm$ 4.6	0.163 $\pm$ 0.011	16.9 $\pm$ 3.6	36.8 $\pm$ 2.3	8.5 $\pm$ 0.3

Abbreviations: polydispersity index (Pdl), zeta potential (ZP), association efficiency (AE) and drug loading (DL).

The association efficiency (AE) and drug loading (DL) of EXN were slightly lower than SMG in all cases when comparing the same theoretical drug loading and formulation (Table 7).

This could be explained by the presence of an 18-carbon fatty acid moiety in the structure of SMG,¹⁸⁶ which confers a higher degree of hydrophobicity, thus promoting the interaction between SMG and the polymers.

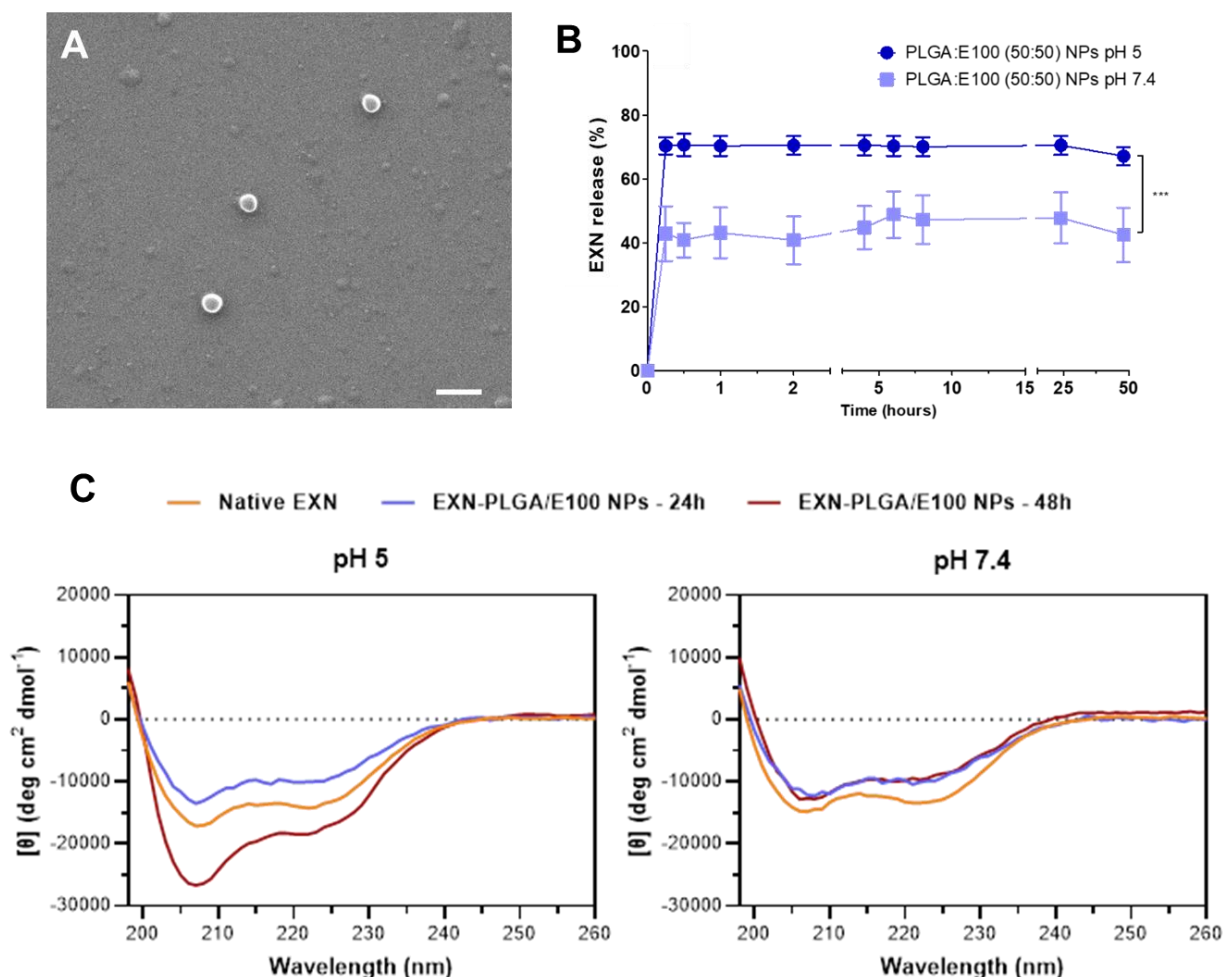


Figure 15 - Morphological features and drug release profile of EXN-loaded NPs. (A) S.E.M. image of EXN-loaded PLGA:E100 NPs (50:50) (Scale bar: 500 nm). (B) Release profile of EXN from NPs at pH 5 and pH 7.4. Points and vertical bars stand for mean and standard deviation (SD), respectively, and (***) indicates $p < 0.001$ ($n = 3$). (C) Far UV CD spectra for exenatide released from NPs during *in vitro* release assay at pH 5 (left) and pH 7.4 (right). Freshly prepared exenatide solution (native EXN) was used as reference. EXN-PLGA/E100 NPs denotes EXN-loaded PLGA:E100 (50:50) NPs.

The incorporation of a pH-responsive polymer (Eudragit® E100) in the NPs composition was used to modify the release profile of the loaded peptide, thereby increasing the release of their cargo when the optimum pH window is reached. Therefore, as the pH decreases, the formation of pores in the NPs matrix accelerates the release of the preloaded drug.¹⁸⁷

The release profiles of EXN and SMG were evaluated *in vitro*, during 48 h. The results demonstrated that PLGA:E100 NPs (50:50) exhibited a pH-dependent *in vitro* release profile, releasing approximately 70 % of the loaded EXN at pH 5 whilst this amount was only 40 % when NPs were exposed to pH 7.4 (Figure 15, B). Similarly, SMG-loaded NPs released 15 to 20 % more peptide in acidic conditions when compared to pH 7.4, depending on the formulation (Figure 16, B). This contrasts with the lack of pH-responsiveness behaviour of PLGA:E100 (90:10) NPs when tested in the same conditions, since these NPs have a significantly lower amount of E100 in their composition (Figure S 2). Although a burst release of EXN and SMG was observed within the first hour at both pH conditions, likely due to the leaching of the surface-adsorbed peptide, it was followed by a slow release up to at least 48 h.

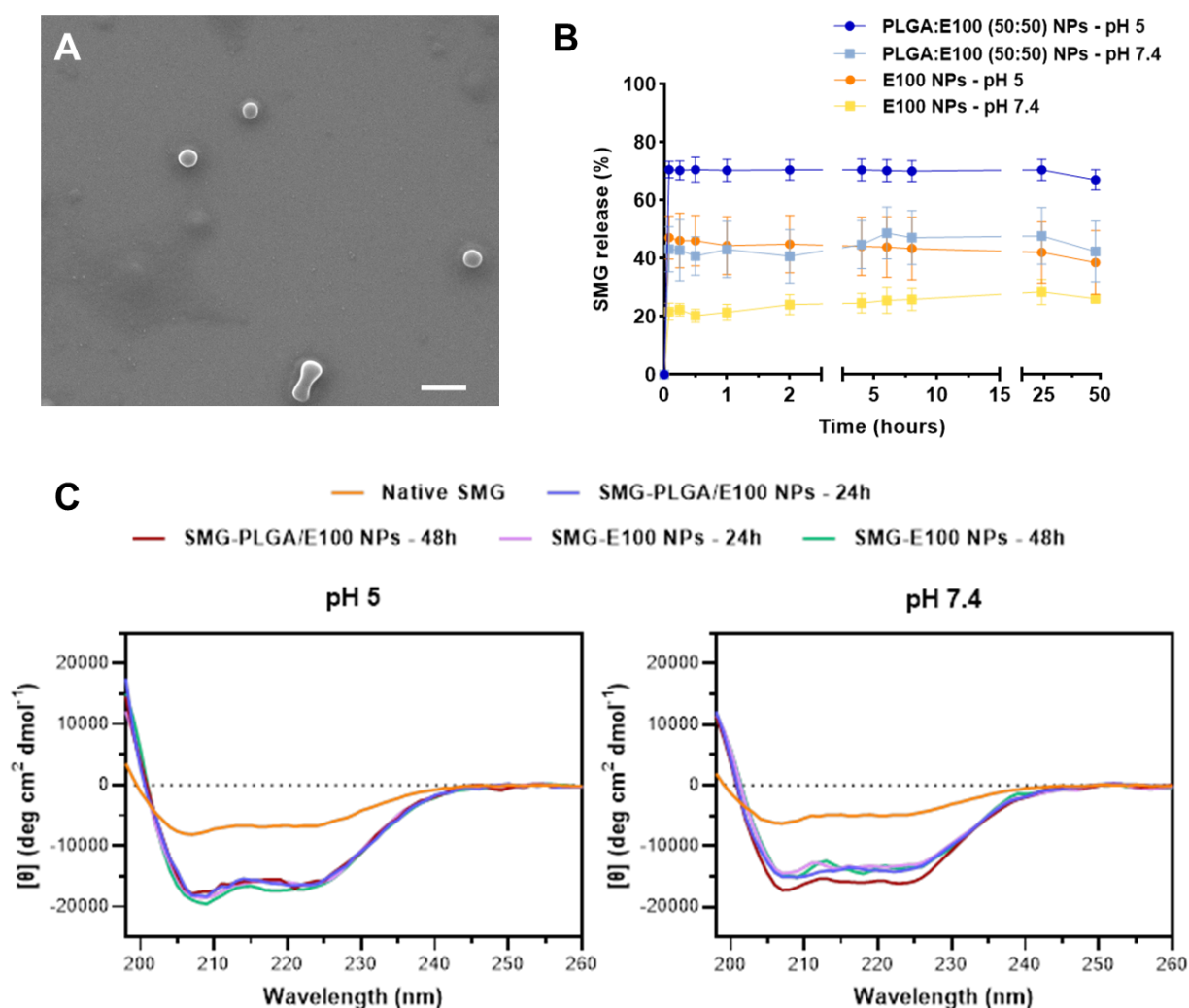


Figure 16 - Morphological features and drug release profile of SMG-loaded NPs. (A) S.E.M. image of SMG-loaded PLGA:E100 NPs (50:50) (Scale bar: 500 nm). (B) Release profile of SMG from NPs at pH 5 and pH 7.4. Points and vertical bars stand for mean and SD, respectively, and (***)

indicates $p < 0.001$ ($n=3$). (C) Far UV CD spectra for semaglutide released from NPs during *in vitro* release assay at pH 5 (left) and pH 7.4 (right). Freshly prepared semaglutide solution (native SMG) was used as reference. SMG-PLGA/E100 NPs denotes SMG-loaded PLGA:E100 (50:50) NPs and SMG-E100 NPs denotes SMG-loaded E100 NPs.

Circular dichroism (CD) spectroscopy analysis was performed to evaluate the secondary structure of each peptide after *in vitro* release from NPs. Samples were collected at 24 h and 48 h after the beginning of the assay at each pH condition (5 and 7.4). The results obtained reveal that there was no significant change in the secondary structure of both peptides after encapsulation and throughout the *in vitro* release study in all tested conditions since the CD spectra profile is maintained when compared to the native peptide (Figure 15, C and Figure 16, C). Results show that both EXN and SMG have a characteristic alpha-helix profile, with two negative peaks of similar magnitude at 222 nm and 208 nm,¹⁸⁸ as expected and similar to GLP-1 structure.^{184,189} Although not significant, there was a slight change in the magnitude of the peak at 208 nm for exenatide collected at 48 h after release from NPs at pH 5. It is reported that the relative magnitudes of the peaks at 222 nm and 208 nm may vary depending on H-bonding and the hydrophobicity of the environment.¹⁸⁸ Nevertheless, considering the physiologic conditions in which the complete system will be, the peptide will not be exposed to an acidic environment for 48 consecutive hours, but rather to intermittent variations of pH (between 7.4 and 5, depending on the glucose levels). Taking this into account and the physiologic conditions of the target application, both peptides can be considered stable for at least 48 h after encapsulation.

4.2. Glucoregulatory effect of exenatide and semaglutide on INS-1E cells

The inclusion of a GLP-1 analogue in the system intends to boost insulin secretion by SC- β -cells in response to glucose. The presence of glucose-sensitive NPs ensures the release and action of the GLP-1 analogue when high glucose levels are present. In order to determine which drug to include in the system and also the best drug concentration, glucose-stimulated insulin secretion assay was performed with the rat insulinoma cell line INS-1E (Figure 17). To the best of our knowledge, there are no previous *in vitro* studies comparing the insulinotropic effect of each drug on INS-1E cells in the same conditions.

Previous studies have been performed separately for EXN and SMG with different experimental conditions,^{190,191} which would make comparison between both peptides difficult.

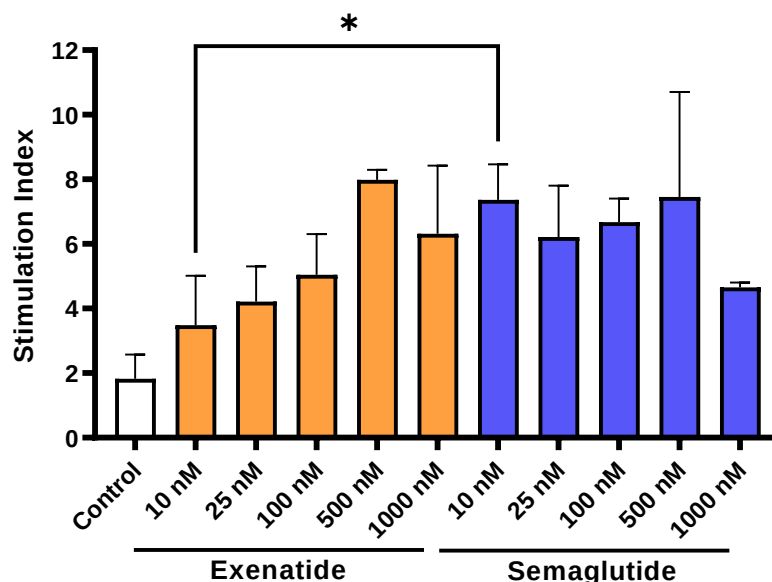


Figure 17 - Stimulation index (SI) after incubation of INS-1E cells with free GLP-1 analogues (EXN or SMG). Insulin secretion from INS-1E cells in response to low (2 mM) and high (20 mM) glucose concentrations in the presence of different concentrations of either EXN or SMG. Secreted insulin was quantified by ELISA. Bars represent the mean and error bars represent the standard deviation.

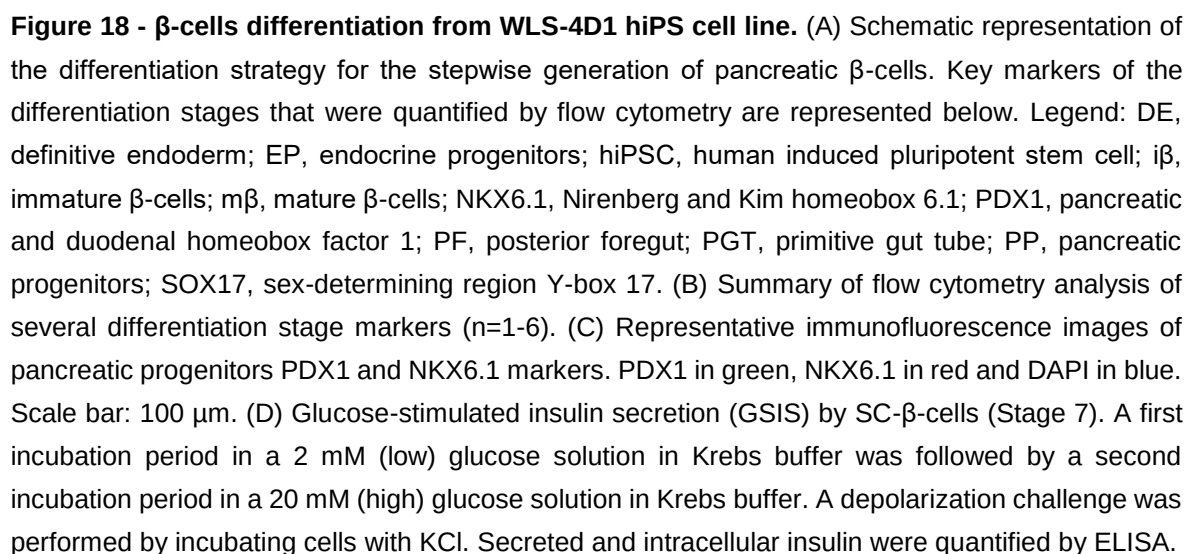
Cells were incubated under low (2 mM) or high (20 mM) glucose concentrations and the amount of insulin released was determined by ELISA (Figure 17). An increase in insulin secretion was observed for most of the tested concentrations of both peptides. However, there was no striking difference between the two peptides (except for 10 nM concentration), which could be attributed to their shared mechanism of action, despite having different potencies [EC_{50} : 0.11 nM (EXN) and 0.15 nM (SMG)].¹⁹² A steady increase in insulin secretion is observed with increasing concentrations of EXN, while SMG does not follow the same trend and shows similar stimulation index (SI) across the different concentrations tested. Taking these results into account and that EXN stimulates β -cells differentiation and proliferation,¹⁹³⁻¹⁹⁵ this peptide was chosen to proceed with the studies.

4.3. Generation of pancreatic cells from hiPSCs

Before diving into the results obtained for the differentiation of β - and α -cells from hiPSCs, it is important to highlight that our research group at i3S had no previous expertise in stem cell differentiation and, with the help of colleagues from another group at the same research institute (with expertise in the differentiation of stem cells into cardiac cells), considerable time and effort were invested in the implementation of these protocols. Therefore, several failed attempts were made before the results here presented. However, they are not here included since it was purely related with protocol implementation and it was not deemed relevant for the scientific discussion of this work.

Human induced pluripotent stem cell line WLS-4D1 was differentiated into β -cells following a stepwise protocol (Figure 18, A). The success of differentiation was confirmed by assessing the expression of specific markers at key differentiation stages by flow cytometry (Figure 18, B). At the end of the definitive endoderm (DE) stage, the vast majority (between 72 % and 96 %) of differentiating WLS-4D1 cells expressed sex-determining region Y-box 17 (SOX17). This result indicates a high commitment to the definitive endoderm lineage,¹⁹⁶ essential for β -cells differentiation. However, at the end of the pancreatic progenitors (PP) stage, the maximum percentage of Nirenberg and Kim homeobox 6.1 (NKX6.1) and pancreatic and duodenal homeobox factor 1 (PDX1) co-expression obtained in 5 independent experiments was only around 11 % (Figure 18, B and C). Both transcription factors (TFs) are reported to be markedly expressed in PP cells and in functional insulin-secreting β -cells. Evidence shows that PDX1 and NKX6.1 co-expression is crucial for the generation of glucose-responsive mono-hormonal β -cells^{197,198} and specifically, NKX6.1 has a key role in β -cell maturation and functionality.^{197,199} Therefore, the low co-expression of PDX1 and NKX6.1 observed could compromise further stages of differentiation. Nevertheless, variability in differentiation efficiency is reported for several protocols and cell lines and depends on the characteristics of the iPSC cell line and the robustness of the differentiation protocol.²⁰⁰

At the end of stage 4, differentiated PP cells were transferred to an air-liquid interface to proceed to the next differentiation stages, following the protocol described by Rezania *et al.*¹⁰⁹ At the end of Stage 6 and Stage 7 (days 25 and 32, respectively), insulin expression was above 50 % with low expression of NKX6.1 (Figure 18, B). This result (insulin-positive, NKX6.1-negative) indicates a polyhormonal population of immature β -cells and is reported in several *in vitro* protocols of β -cells differentiation.^{108,197,199}



The same cell line (WLS-4D1) was induced towards pre- α -cells following a different stepwise protocol (Figure 19, A and C) in static suspension culture,¹⁵⁰ instead of adherent conditions. For that, WLS-4D1 cells were successfully adapted to suspension conditions before proceeding to differentiation experiments. In order to assess their quality before undergoing differentiation, hiPSCs were stained for key pluripotency markers (OCT-4, SSEA-4 and TRA-1-81) and a differentiation marker (SSEA-1), as a negative control. Expression of OCT4, SSEA-4 and TRA-1-81 and the absence of SSEA-1 expression (Figure S 3) confirmed the pluripotency of cultured cells. These results hold significant importance for further differentiation experiments, since differentiation efficiency relies on the quality of the initial hiPSCs.

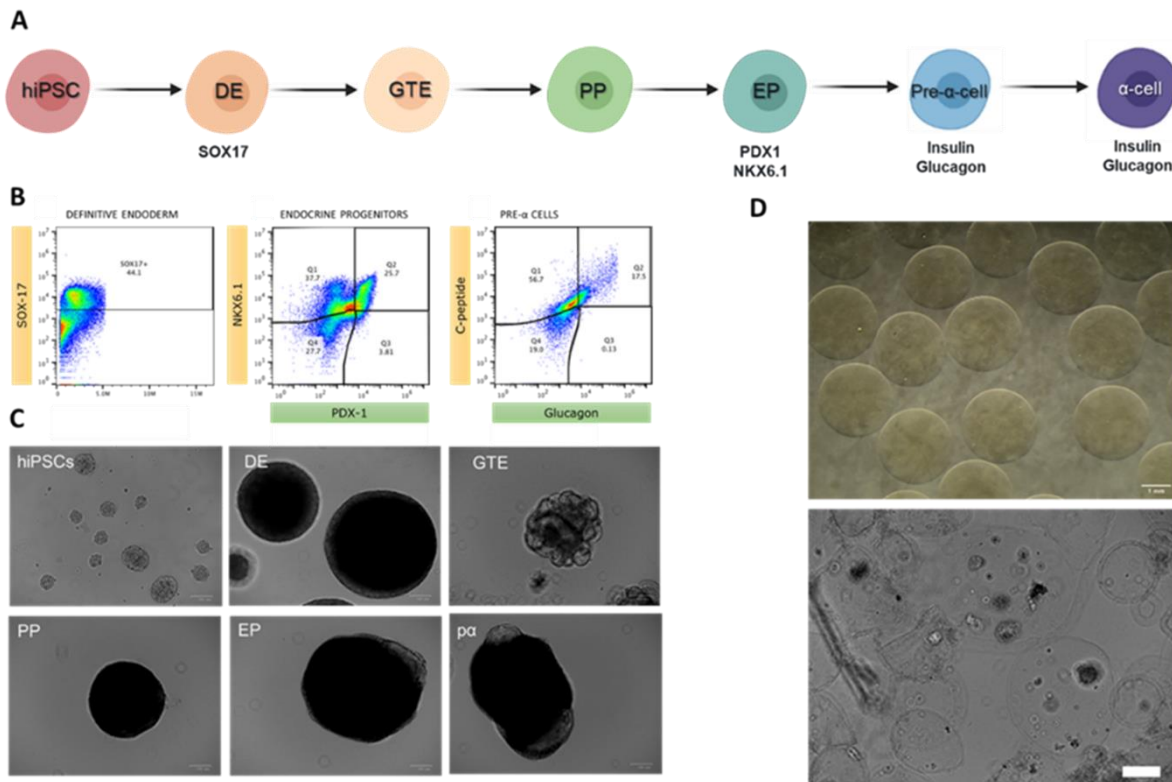


Figure 19 - α -cells differentiation from WLS-4D1 hiPS cell line. (A) Schematic representation of the differentiation strategy for the stepwise generation of pancreatic α -cells. Legend: hiPSC: induced pluripotent stem cell; DE: definitive endoderm; GTE: gut tube endoderm; PP: Pancreatic progenitors; EP: endocrine progenitors; α : pre- α -cells. (B) Flow cytometry dot plots of SOX17 expression in definitive endoderm cells, NKX6.1 and PDX-1 expression in endocrine progenitors cells and C-peptide and glucagon expression in pre- α -cells stage. (C) Representative brightfield images of the morphology of WLS-4D1 cells during differentiation until pre- α -cells. Scale bar: 100 μ m. (D) Representative stereoscopic images of EXN-loaded pH-sensitive NPs encapsulated within alginate microspheres (upper image); Representative brightfield image of differentiated cells encapsulated within the alginate system (lower image). Scale bar: 100 μ m.

The differentiation was followed by assessing the expression of specific markers by flow cytometry at day 4 (end of DE stage), day 13 (end of endocrine progenitors (EP) stage) and day 20 (end of pre- α -cells (p α) stage) (Figure 19, B). At the end of DE, SOX17 expression was approximately 44 %, which was slightly lower than previously reported by other authors.¹⁹⁶ The low expression of SOX17 is associated with a lower commitment to the endoderm lineage, which could compromise the efficiency of the differentiation protocol. On day 13 (end of EP stage), the expression of PDX1 and NKX6.1 was evaluated and around 4 % of the cell population was PDX1⁺/NKX6.1⁻, while 26 % was PDX1⁺/NKX6.1⁺. At this stage in α -cell differentiation the desired cell population is PDX1⁺/NKX6.1⁻,¹⁵¹ since PDX1 expression directs differentiation into endocrine progenitors and the absence of NKX6.1 leads cells towards the α -cell phenotype and expression of glucagon.^{150,197} At the end of p α stage (day 20), glucagon (GLU) and C-peptide (C-pep) expression were assessed and 17.5% of the differentiated cells were C-pep⁺/GLU⁺, which was similar to previously reported differentiation efficiencies at this stage.¹⁵⁰ Despite the early ending of the protocol, WLS-4D1 hiPSCs were successfully differentiated into glucagon-producing pre- α -cells, which, once transplanted, can convert to mature α -cells *in vivo*.¹⁵⁰

Both SC- β -cells and SC-pre- α -cells were encapsulated within alginate microspheres (2 % W/V) as well as the glucose-responsive NPs and GOx, as shown in Figure 19, D, to build the system to be tested in further studies.

4.4. *In vivo* study in diabetic mice

In an exploratory *in vivo* experiment, SC- β -cells and SC-pre- α -cells were encapsulated within alginate microspheres (2 % W/V), either alone (Cells), with the GLP-1 analogue EXN freely dispersed (100 nM) in the alginate matrix (Cells + Free drug) or with EXN-loaded glucose-responsive NPs (corresponding to 100 nM of drug) and the enzyme glucose-oxidase (10 μ g/mL) (Cells + NPs + GOx). SC- β - and pre- α -cells were combined in the alginate microspheres following a ratio of 60:40 (β -cells : pre- α -cells) to roughly recapitulate the proportion found in primary human islets. Alginate microspheres were then transplanted into the peritoneal cavity of single high-dose streptozotocin-induced diabetic C57BL/6 male mice (Figure 14). Animals were considered diabetic when at least 2 consecutive non-fasting blood glucose readings were ≥ 400 mg/dL. Animals were followed throughout 27 days regarding weight (Figure S 4) and blood glucose levels (Figure 20, A). The transplantation of differentiated cells embedded in an alginate matrix, regardless of the presence of EXN-loaded NPs or free drug, led to an improvement in the survival of transplanted animals,

since the controls (diabetic animals without treatment) had a higher mortality percentage, with 100 % at day 7 after transplantation (Figure 20, C). This was also a consequence of the severity of the induced disease that, in the absence of treatment, led to animals' death due to β -cells destruction. To confirm this, the pancreata of animals were collected and treated for hematoxylin and eosin (H&E) (Figure 20, D).

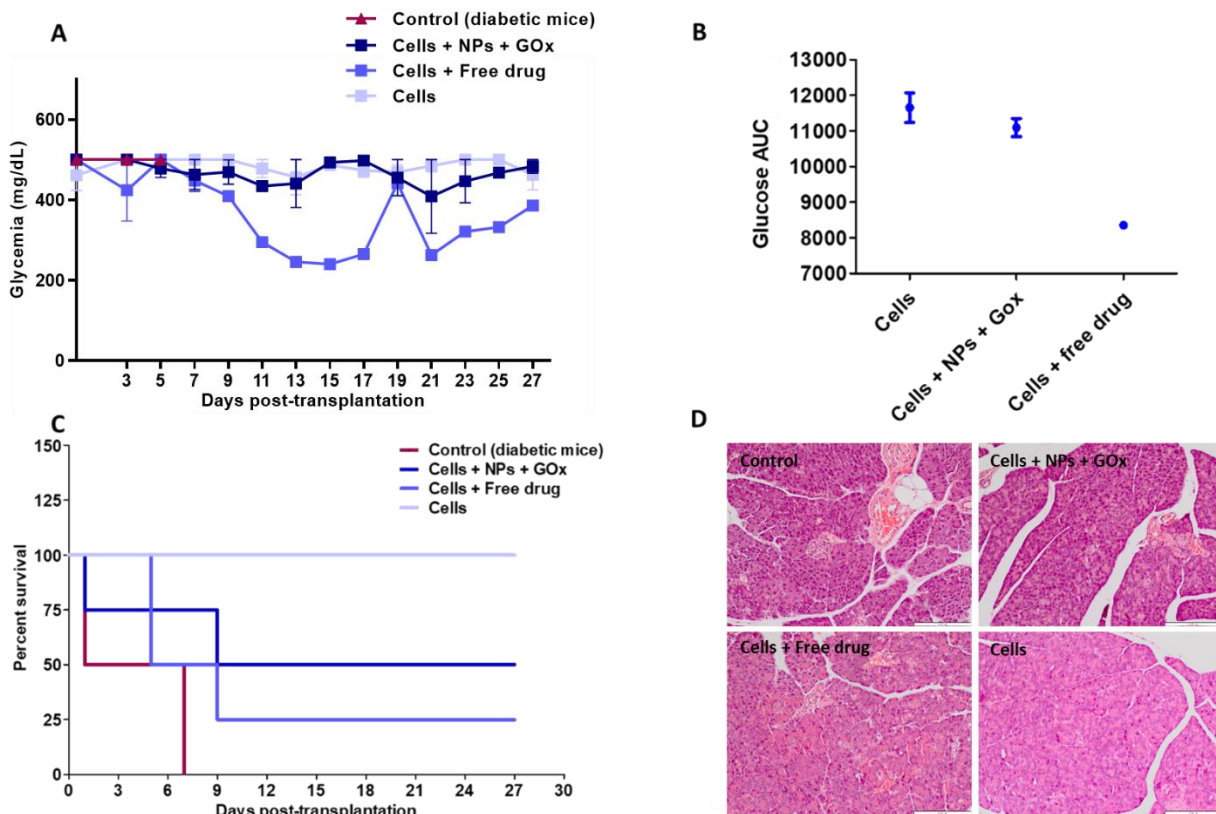


Figure 20 - *In vivo* characterisation of β - and pre- α -cells immobilised in an alginate matrix with glucose-responsive NPs. (A) Blood glucose concentration (mg/dL) and (B) AUC values for blood glucose levels of mice during 27 days after transplantation. Cells were encapsulated either alone (Cells), in combination with EXN-loaded glucose-responsive NPs and glucose oxidase (Cells + NPs + GOx) or in combination with EXN freely dispersed in the alginate matrix (Cells + Free drug). Blood glucose levels were monitored every two days for 27 days. Values are represented as mean $\pm$ standard error of the mean (SEM) (A) and mean $\pm$ SD (B). (C) Kaplan-Meier survival curve for mice transplanted with the different treatments. (D) Representative histology images of the pancreata of streptozocin-induced diabetic mice. Pancreatic sections of animals from different groups were stained with hematoxylin and eosin (H&E): Diabetic mice without treatment (Control); diabetic animal transplanted with Cells + NPs + GOx; diabetic animal transplanted with Cells + free exenatide and diabetic animal transplanted with Cells alone. Scale bar: 200 μ m.

Animals from the different groups showed an altered pancreatic structure, both in the exocrine and endocrine pancreas. The acinar cells presented some hypertrophy and vacuolization, while Langerhans islets were nearly absent or presented reduced dimensions, confirming the success of diabetes induction and maintenance.

The co-encapsulation of differentiated cells with EXN-loaded NPs appears to result in a slight reduction in hyperglycemia in diabetic mice when compared to encapsulated cells alone (Figure 20, A). This trend is supported by the lower area under the curve (AUC) values obtained for this group (Figure 20, B). Although this effect is more pronounced when the cells are encapsulated with the drug freely dispersed in the alginate matrix (Cells + Free drug), the higher mortality rate of this group jeopardises any conclusion. The duration of the *in vivo* experience (27 days) may have impaired the observation of a clear reduction in blood glucose levels of animals, since it typically takes approximately 2 weeks after cell transplantation to start to observe a reduction in blood glucose values and around 40 days to achieve normoglycemic levels.^{108,109} Moreover, the low differentiation efficiencies and the presence of polyhormonal β -cells in the final stage of differentiation may have also diminished the efficacy of the developed system, since the insulin release capacity of SC- β -cells was compromised *in vitro*. The number of cells transplanted per animal (~1.7 million) may have also limited the impact on blood glucose levels, since the majority of published studies use between 2-5 million cells per animal, taking into account the markers expression and depending on the implantation site.^{108,109,111,113,201}

Regarding the release of EXN from NPs, it is anticipated that during the initial 48 hours after transplantation, the peptide will experience an initial burst release, followed by a slower release, as evidenced by the *in vitro* results (Figure 20, B). Additionally, it is expected that an increase of up to 30 % in EXN release following meals will occur, given that the rise in glucose levels transiently decreases the pH in the microenvironment of the transplanted system, thereby accelerating the NPs solubilisation. Although it would be desirable to determine how long this formulation lasts until NPs are depleted, this presents significant challenges in both *in vitro* and *in vivo* settings. Specifically, the rate of drug release within the transplanted system, and consequently the depletion rate of the drug content from NPs, is significantly influenced by glycemic levels. These levels fluctuate throughout the day in response to the animal's activity, metabolism and health status and replicating these dynamic variations *in vitro* poses substantial difficulties, in addition to the inherent limitations of *in vitro* systems. Conversely, the determination of this specific parameter *in vivo* would require the use of a considerable number of animals, given the necessity of conducting several experimental endpoints. Given the scope of this study and the principles of the 3Rs,

it was considered more relevant to assess other parameters, such as glycemia and survival rates after transplantation.

Nevertheless, considering that transplanted cells experience several acute stressors immediately after transplant (including hypoxia, nutrient deprivation, and inflammation)¹⁶⁵ and that EXN promotes β -cells proliferation and differentiation,¹⁹³⁻¹⁹⁵ its release is especially important in the initial phase after transplantation to reduce the degree of β -cell loss. For these reasons, the initial EXN burst release observed *in vitro* (Figure 20, B) may be advantageous in improving the survival of the transplanted SC- β -cells immediately after transplantation, followed by a slow release in an intermediate phase, in a glucose-dependent manner, to promote insulin secretion. In later stages, after the depletion of the drug content from the NPs, the transplanted cells should be able to independently survive and function in their environment.

Previous studies have shown that hESC/iPSC-derived pancreatic progenitor cells when transplanted into ectopic sites in immunodeficient or T1D mice can undergo further differentiation and maturation *in vivo*.^{197,202,203} In these studies, PP cells differentiated into glucose-responsive insulin-secreting cells *in vivo*, which were able to reverse diabetes in the recipient mice. This shows that some growth factors and cues that are present *in vivo* are still missing in fully *in vitro* differentiation protocols. However, while it can be advantageous to transplant PP cells and allow the last differentiation phases to occur *in vivo*, there are some tumorigenicity and safety risks associated.²⁰⁴ Alternatively to SC- β -cells differentiated until stage 7, as in the present study, stage 6 SC- β -cells could be incorporated into the system here described and allowed to complete their maturation *in vivo*. This would be an interesting avenue for further investigation, as other authors have reported that the maturation of stage 6 SC- β -cells *in vivo* depends on the transplantation site and that these cells did not lead to overgrowths or tumour formation after transplantation.²⁰⁵ By combining these cells with EXN-loaded NPs, its initial release after transplant could play an important role in further stimulating stage 6 SC- β -cells differentiation and maturation *in vivo*.

4.5. Improving the survival of the biomimetic pancreas system

Taking into account the results obtained in this preliminary *in vivo* study, it was hypothesised that GOx concentration could also be playing a role in the viability of the system. The concentration of GOx used in this study (10 $\mu\text{g/mL}$) was based on a previous report in the literature,²⁰⁶ although with a different cell line. Despite far from ideal and with its inherent limitations (since different cell lines can be more or less susceptible to different external factors), in this case, the cytotoxicity test with the closest conditions would need to be performed with SC- β - and α -cells. However, due to the low amount of cells obtained at the end of each differentiation and also due to the high cost of each differentiation protocol, the GOx concentration according to a previous study was considered. Nevertheless, and considering the results obtained *in vivo*, this value was challenged and a metabolic activity assay was performed. INS-1E cells were co-encapsulated with different concentrations of GOx (1, 10 and 100 $\mu\text{g/mL}$) and metabolic activity was assessed at 24 h and 72 h after encapsulation (Figure 21).

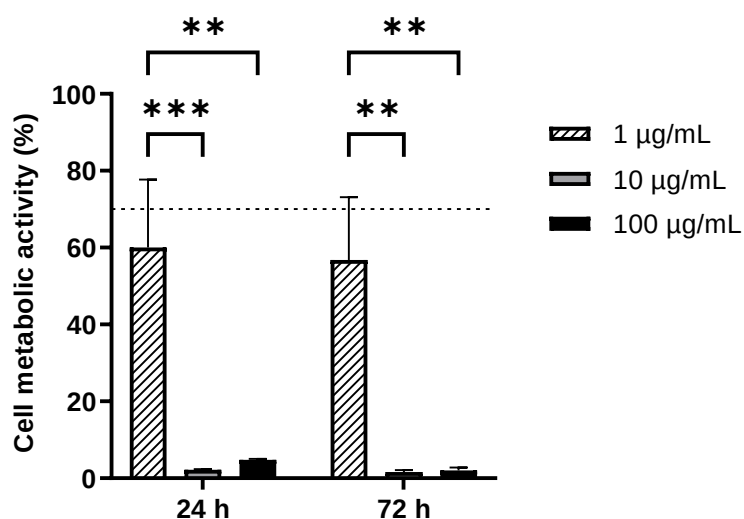


Figure 21 - Metabolic activity of INS-1E cells co-encapsulated with different concentrations of glucose oxidase at 24 h and 72 h after encapsulation. Results are represented as mean $\pm$ SEM.

In a previous study in which a prostate cancer cell line was used,²⁰⁶ GOx did not present a cytotoxic effect at 10 $\mu\text{g/mL}$. However, GOx significantly decreased the cell metabolic activity of INS-1E cells (an insulinoma cell line) when co-encapsulated at concentrations of 10 $\mu\text{g/mL}$ or above at 24 h after encapsulation (Figure 21). On the other hand, when GOx

was co-encapsulated at 1 $\mu\text{g/mL}$, metabolic activity was significantly higher in both timepoints, but still below the recommended value¹⁷⁴ to be regarded as safe (70 %).

This cytotoxic effect is due to the generation of hydrogen peroxide when glucose is oxidised.¹⁸² A possible strategy to overcome this deleterious effect of the presence of GOx in the system is the incorporation of the enzyme catalase (CAT), which catalyzes the conversion of two molecules of hydrogen peroxide into two molecules of water and one molecule of oxygen,²⁰⁷ thus obtaining completely unharmed substances for cellular activity.

Therefore, GOx and CAT concentrations and the ratio between them were optimised in order to counterbalance the cytotoxic effect produced by GOx. INS-1E cells were co-encapsulated with either GOx or CAT alone and also with different concentrations and ratios of both enzymes (1:3 and 1:30, GOx:CAT). After 24 h and 72 h post-encapsulation metabolic activity was evaluated (Figure 22).

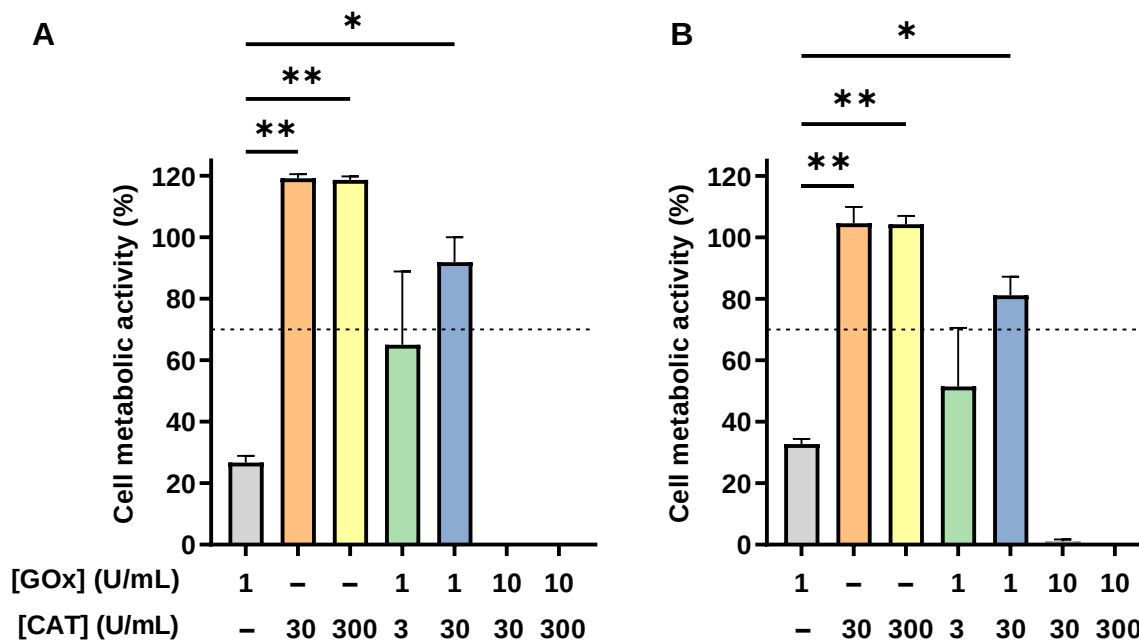


Figure 22 - Metabolic activity of INS-1E cells co-encapsulated with glucose oxidase (GOx) and catalase (CAT) at 24 h (A) and 72 h (B) after encapsulation. Results are represented as mean $\pm$ SEM.

Since different enzymes have different activities, in order to be able to better establish a ratio between GOx and CAT, the concentration GOx (in $\mu\text{g/mL}$) was converted to units per mL (U/mL), which is the unit used to express enzyme activity values. Regarding the GOx

used in this work, 1 U/mL $\approx$ 10 μ g/mL (according to the manufacturer). As can be seen in Figure 22, the optimum concentrations of GOx and CAT to be used in the system are 1 U/mL and 30 U/mL, respectively, since the metabolic activity of co-encapsulated INS-1E cells was above 70 % at day 1 and day 3 after encapsulation. Therefore, the optimum ratio to combine both enzymes in the system is 1:30 (GOx:CAT). However, this is only true when low concentrations of both enzymes are used (1 and 30 U/mL of GOx and CAT, respectively). When concentrations are increased by 10-fold, the metabolic activity of INS-1E cells drastically decreases to close to zero at day 1, despite maintaining the same ratio. As a negative control, INS-1E cells were co-encapsulated with CAT alone and no cytotoxic effect was observed, as would be expected.

4.5.1. System validation on human donor islets

Part of this work was performed in a different lab, namely in the Islet Group at the Leiden University Medical Centre (The Netherlands). At the LUMC there was access to human cadaveric islets, which were procured through the Eurotransplant multiorgan donation programme. These donor islets were used for research purposes only when they were not suitable for clinical islet transplantation and if there was research consent. Given the limited amount of sample available, most of the experiments performed with these islets had a low number of samples.

Firstly, the impact of the encapsulation procedure was assessed on human islets through the assessment of their metabolic activity. Human islets from healthy donors were encapsulated in 2 % alginate matrix by external gelation technique and kept *in vitro* in ultra-low attachment (ULA) well plates for 21 days, with medium changes every other day. Metabolic activity was assessed every 7 days and compared with non-encapsulated human islets. As can be seen in Figure 23, A, the encapsulation technique as well as the biomaterial chosen did not have a negative impact on the human islets metabolic activity. In fact, this had a protective effect on the islets from both donors, since the metabolic activity was always higher for encapsulated islets during the 21 days of the experiment when compared to non-encapsulated islets. Additionally, there were also morphological and structural differences between the two groups which were evident when islets were observed through the microscope (Figure 23, B). Specifically, non-encapsulated islets started to aggregate and lost their round shape, while encapsulated islets were able to maintain their structure and morphology across time.

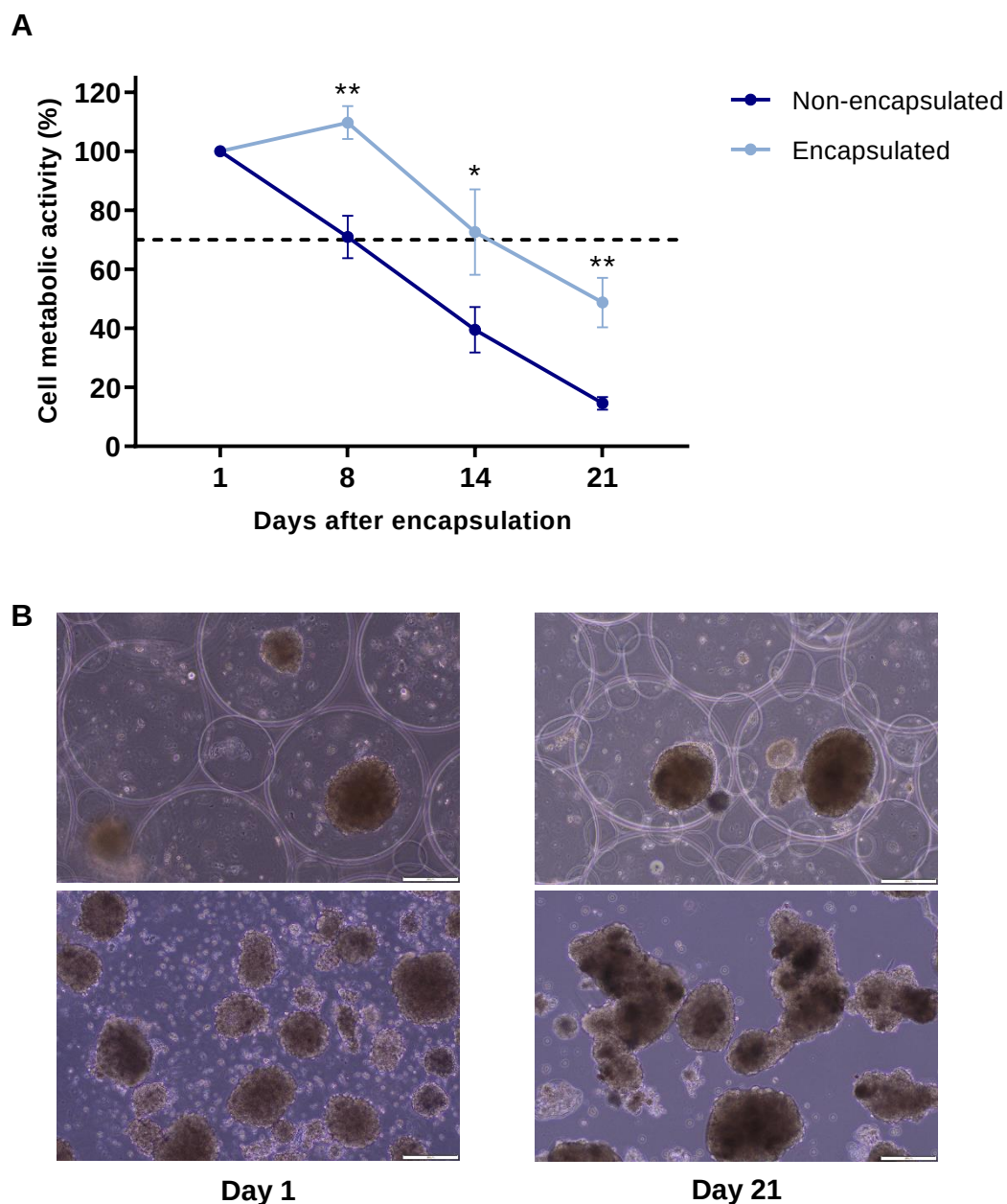


Figure 23 - Assessment of the impact of the encapsulation procedure on human donor islets.

(A) Metabolic activity of encapsulated and non-encapsulated human islets (2 donors, $n = 3$). Results are represented as mean $\pm$ SEM. (B) Representative bright-field images of encapsulated and non-encapsulated human islets at days 1 and 21 after encapsulation. Scale bar: 200 μ m.

Secondly, the impact of the complete system (i.e. with EXN-loaded NPs, GOx and CAT) on the metabolic activity of human islets was also assessed. As can be seen in Figure 24, A, the metabolic activity of non-encapsulated islets was below 40 % at day 8 after encapsulation and below 20 % after 21 days, while for all the other experimental groups it

was above 50 % during 21 days. This shows not only the importance of the alginate encapsulation in the preservation of islets survival and integrity, but also confirms that the presence of GOx is well balanced by the presence of CAT and does not have a cytotoxic effect on human islets, even after 21 days in culture. On the other hand, there was not a striking difference between the metabolic activity of encapsulated islets with and without EXN-loaded NPs, but the results suggest that when EXN is loaded in the NPs instead of being freely dispersed in the alginate matrix, the metabolic activity of encapsulated human islets increases. This is probably due to the slower release of EXN cargo from the NPs. However, the number of samples ($n = 1-2$) was too low to be able to draw robust conclusions.

Additionally, glucose-responsiveness was evaluated by dynamic GSIS at day 2 after encapsulation. Samples were later quantified by ELISA to assess the amount of C-peptide secreted and the results are presented in Figure 24, B. Islets from all conditions showed a similar profile. However, encapsulated islets with free exenatide (Encapsulated + EXN) showed a slightly higher glucose-responsiveness (at 25 min) and also a higher amount of C-peptide secreted after the depolarization challenge (at 90 min). This could be due to the fact that no media change was performed until two days after encapsulation. Since it is a closed system, the EXN that was initially freely dispersed in the alginate matrix, could migrate to the outside of the hydrogel but was still retained in the well. In contrast, if it were an open system, which would be closer to the human body, this difference would likely not be visible as cells would have a much shorter time in contact with EXN. Perhaps a different outcome would have been observed at later timepoints after encapsulation, which were initially planned and would be performed after medium change, but due to the limited amount of donor islets available it was not possible to perform. Despite the observed results after stimulation with high glucose (20 mM), it is important to mention that all experimental groups with islets co-encapsulated with EXN (either freely dispersed or in NPs) showed increased C-peptide secretion after the depolarization challenge (KCl), when compared to islets without EXN. This demonstrates that β - cells exposed to EXN synthesised higher amounts of insulin, which was stored in the intracellular secretory granules. However, the stimuli was not high enough to observe a difference between experimental groups, when they were subjected to high glucose levels (at 20 min). Possibly further tuning in the amount of loaded NPs would be needed to improve this outcome. Nevertheless, this is the behavior observed in mature human islets. SC-islets may have a different behavior, since they are not fully mature.

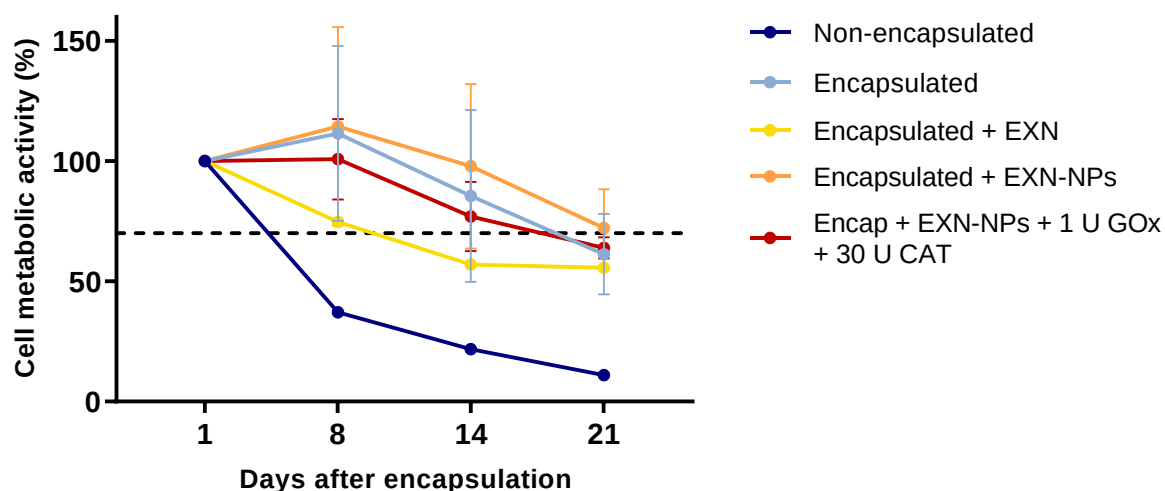
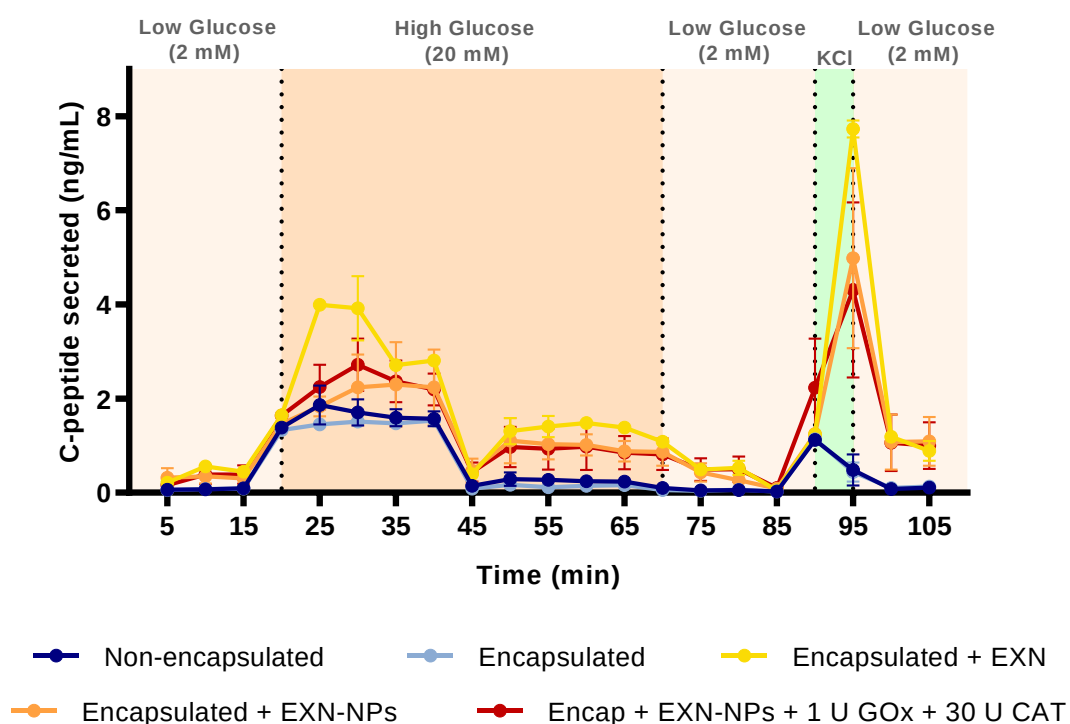
A**B**

Figure 24 - Evaluation of the effect of the complete system on the (A) metabolic activity ($n = 1 - 2$) and (B) glucose-stimulated insulin secretion of human islets ($n = 2$). Secreted C-peptide was quantified by ELISA. The condition “Encapsulated + EXN” denotes encapsulated human islets with 100 nM of exenatide freely dispersed in the alginate matrix, “Encapsulated + EXN-NPs” represents human islets co-encapsulated with exenatide-loaded NPs (100 nM of peptide) and “Encapsulated + EXN-NPs + 1 U GOx + 30 U CAT” is the complete system, i.e. human islets co-encapsulated with exenatide-loaded NPs (100 nM of peptide), 1 U of glucose oxidase and 30 U of catalase. Results are represented as mean $\pm$ SEM.

4.5.2. System validation on stem cell-derived islets

In this lab, a different approach to obtain SC- β - and α - cells was used and instead of generating each cell type independently, they were differentiated together under dynamic culture conditions. Therefore, at the end of the differentiation protocol (stage 7), SC-islets were obtained.

SC-islets were differentiated from HUES8 iPSC cell line until the end of stage 7. After day 27 of differentiation, SC-islets were cultured with the same medium used to culture islets from human donors (CMRL medium supplemented with FBS, nicotinamide, glutamine and HEPES) without the addition of further supplements. At day 33, SC-islets were encapsulated in 2 % alginate matrix by external gelation technique. SC-islets were kept in ULA well plates during the experiment with medium changes every two days (same *in vitro* conditions used for human islets).

Due to time constraints, it was only possible to perform two differentiation protocols and both differentiations had an unexpectedly low differentiation yield, which limited the assays that could be performed and the number of samples per experimental condition. In this case, it was only possible to perform dynamic GSIS assay and to collect RNA samples to evaluate gene expression (only one replicate per condition per differentiation). Metabolic activity could not be evaluated due to the lack of SC-islets.

Dynamic GSIS and RNA extraction were performed at days 2, 7 and 14 after encapsulation. SC-islets were compared with encapsulated SC-islets (encap SC-islets), encapsulated SC-islets with freely dispersed EXN (encap SC-islets + EXN) and the complete system (encap SC-islets + EXN-NPs + 1U GOx + 30U CAT). Of note, the protocol followed for dynamic GSIS of SC-islets was the same used for human islets. The results obtained for dynamic GSIS are shown in Figure 25. Importantly, in Figure 25, C there are only three experimental groups instead of four, since the amount of encapsulated SC-islets was not enough for a third timepoint and for that reason only the other experimental groups were assessed at day fourteen.

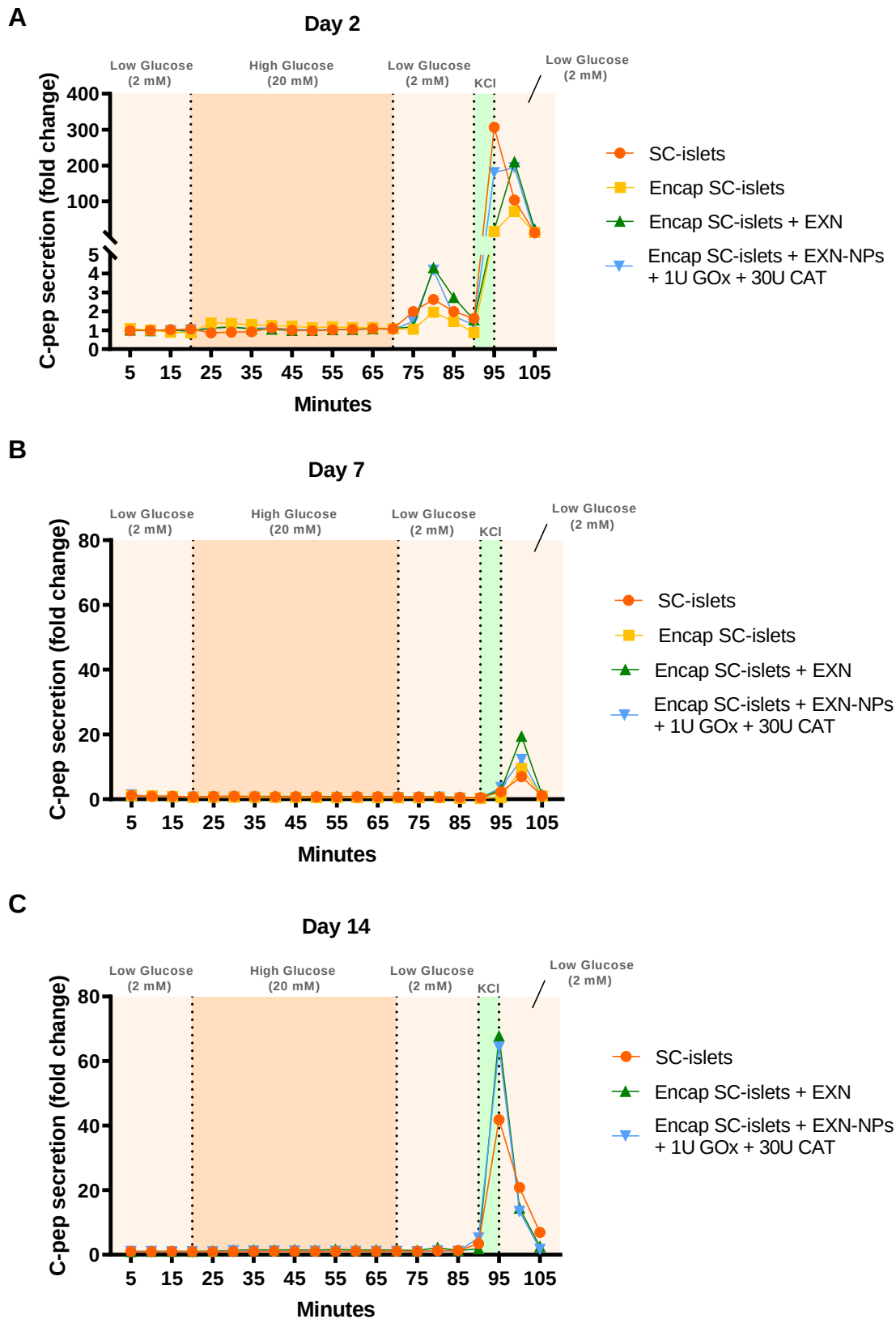


Figure 25 - Glucose-stimulated insulin secretion of non-encapsulated and encapsulated SC-islets on days 2 (A), 7 (B) and 14 (C) after encapsulation. Secreted C-peptide was quantified by ELISA ($n = 1$).

On day 2 after encapsulation (Figure 25, C), the stimulation index of SC-islets encapsulated with free EXN or in the complete system (i.e. EXN-NPs + GOx + CAT) was the double of the stimulation index of the control groups (non-encapsulated and encapsulated SC-islets). However, this was only around the 80 min timepoint, which corresponds to the second low glucose incubation period (after the high glucose incubation). This would not be expected if the SC-islets were fully mature (as the islets from human donors). However, this could be explained by either: a delayed response (since the peak is observed after the incubation with high glucose - 20 mM) or could be because immature SC-islets are sensitive to low glucose levels, instead of high glucose levels.¹⁴⁷ However, considering that during the first low glucose incubation period (0-15 min) the amount of C-peptide secreted is low, the first reason mentioned is the most probable scenario.

Regarding the dynamic GSIS profiles at days 7 and 14, their glucose-responsiveness is clearly even more decreased and impaired. This could be due to the fact that these SC-islets were still not fully mature at the end of stage 7 and by keeping them in culture in the absence of other supplements (which were being supplied in the medium until day 27) this could have a negative impact in their function. Thus, the culture conditions may not be fully optimised for this length of time after the differentiation protocol. Nevertheless, results at day 2 suggest indeed a positive outcome.

The results obtained for the gene expression of *INS*, *GLU*, *MAFA* and *PDX1* are shown in Figure 26. For all the genes analysed, the results suggest an increase in gene expression at day 14 when cells were either encapsulated with freely dispersed EXN in the alginate matrix or encapsulated in the complete system (EXN-NPs+GOx+cells). Although there was only one replicate per condition and the conclusions drawn are not robust, the results suggest a positive influence of EXN on the differentiation of the SC-islets, since the genes here analysed are typically expressed in mature β -cells.^{208,209} However, the way of delivering the peptide (either freely dispersed or loaded in NPs) does not seem to influence the expression of these genes during the time frame of this study, since the results were similar between the two groups. But it is also important to mention that SC-islets were not subjected to glucose variations during the study, which would not happen in a physiological environment. Thus, considering that glucose variations will happen when the system is transplanted, the NPs content will be released quicker and EXN may have a more notorious effect than the one here observed. Besides, since this experiment was performed *in vitro* in a closed system, the concentration of the peptide is constant (if not consumed by the cells). However, considering a transplantation scenario of this system, the freely dispersed peptide would very rapidly diffuse from the alginate matrix and only a small percentage of the initial

amount would be able to act on the transplanted cells. Thus, when the peptide is loaded in the NPs its release is expected to happen in a more controlled manner, enhancing its action on β -cells.

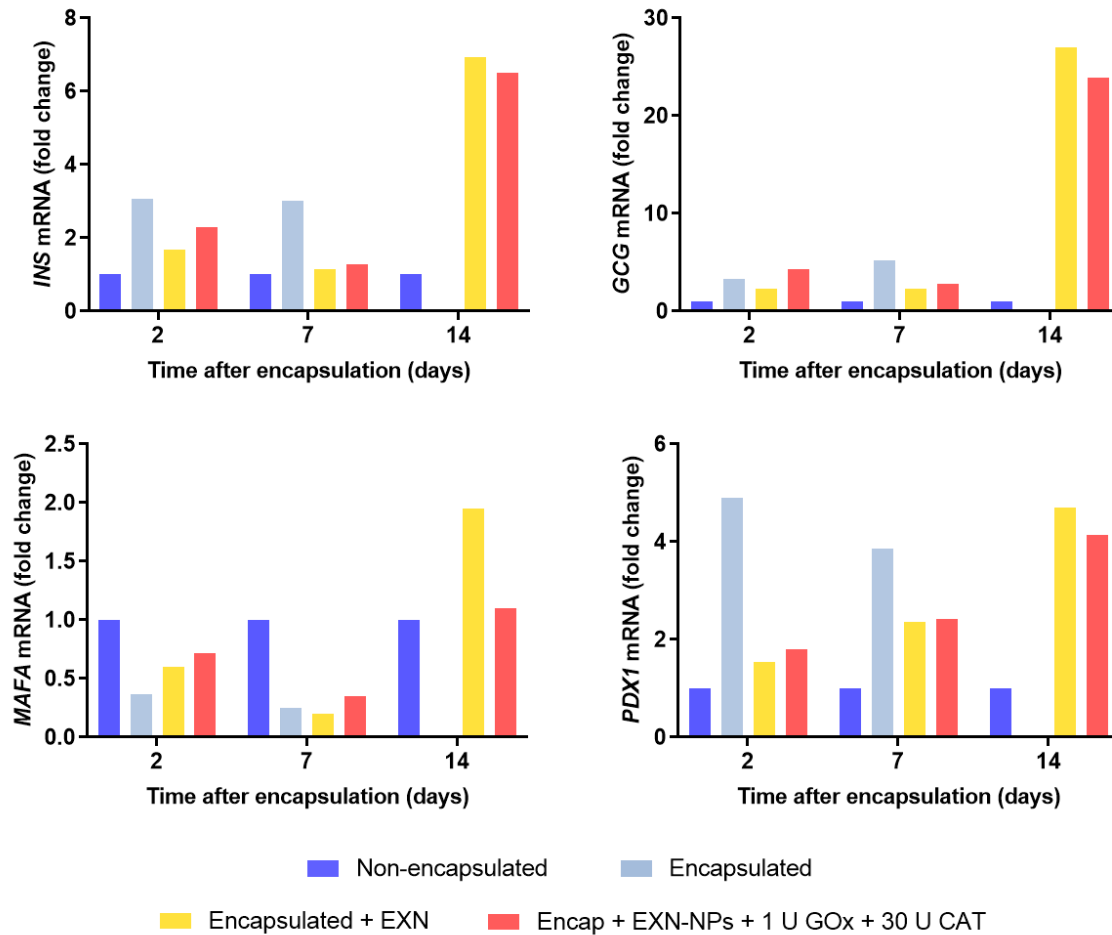


Figure 26 - Gene expression of *INS*, *GLU*, *MAFA* and *PDX1* in SC-islets at days 2, 7 and 14 post encapsulation. Gene expression was analysed by RT-qPCR and normalised to the housekeeping gene *ACTB*. Results are presented as fold change compared to the 'non-encapsulated' group.

Considering future clinical applications of this work, a(n almost) totally *in vitro* differentiation protocol which originates fully functional and mature β - and α -cells is desirable. Despite differentiation protocols have been improved and optimised in the past years, obtaining SC- β - and α -cells similar to adult pancreatic cells found in the human body is not the reality yet.²¹⁰ Therefore, the integration of advancements from different areas such as encapsulation techniques, differentiation protocols, and gene editing strategies aimed at immune system evasion, will facilitate the development of a cell replacement therapy for T1D.

In the particular case of T1D, it is essential to develop strategies to avoid an autoimmune reaction against the transplanted cells. One way to circumvent that is to use a suitable encapsulation material that allows cells to communicate with the outside of the graft and receive nutrients, but at the same time avoids the entrance of immune cells and antibodies, as explored in this thesis. These strategies are also being studied and explored by others, for example TheraCyte developed a durable and retrievable macroencapsulation device (with a size of 20 mm) consisting of a thin-membrane-based polymeric chamber with an outer vascularizing membrane that allows blood vessels infiltration and closer contact with cells and an inner impermeable membrane that avoids immune cells entrance.²¹¹ They already demonstrated the efficient protection of this device against the immune system, as well as the maturation of hPSCs into pancreatic endocrine cells *in vivo* inside the device, being able to reverse diabetes within 3 months upon implantation.^{203,212-214} Additionally, ViaCyte (recently acquired by Vertex Pharma) also developed similar devices, which have been evaluated in clinical trials. This was the first clinical trial for islet cell replacement therapy derived from stem cells for diabetes treatment. They developed three different strategies to encapsulate ESC-derived pancreatic progenitor cells, which were tested for safety and efficacy in T1D patients. One of these devices (PEC-Encap) is quite similar to the TheraCyte approach, also aiming to evade the immune system response against the implanted cells. However, it was observed that the insulin output was not sufficient for clinical benefit and therefore, further improvements in the system are being performed.²¹⁵ The other ViaCyte device (PEC-Direct) has a modified encapsulation membrane but does not prevent an immune reaction, thus it is aimed at T1D patients with a high risk of life-threatening acute complications. In addition, these devices also have the advantage of allowing the easy extraction of the cells if a problem occurs (such as tumorigenesis or dysfunction).²¹⁶ On the other hand, concerning T2D, there is not a need for cell encapsulation to avoid immune reaction if the cell source is autologous.

Despite the advantages of using an autologous source from an immunological standpoint, it is laborious, time-consuming and costly, which would hinder its widespread accessibility in the future. Thus, in the case of allogenic sources, it would be crucial to have HLA compatibility, but it is not easily feasible to have iPSCs stocks from HLA homozygous donors that are suitable for an entire population, due to HLA great variability.²¹⁷ Therefore, genome editing tools allied to stem cells in order to remove HLA genes may become a successful approach to achieve an “universal donor” or “off-the-shelf” cellular products, making these therapies cheaper and more accessible. Interestingly, some attempts have already been made, namely one study showed that by disrupting HLA-A and HLA-B while keeping HLA-C cells could escape CD8⁺ T and natural killer (NK) cells attack *in vitro* and *in*

vivo.²¹⁸ In another study, CRISPR technology was used to knockout HLA-B from heterozygous iPSCs and they demonstrated that the knockout cells had decreased immunogenicity when compared to the control group.²¹⁹ A different approach to escape immune system activity against grafts may be to overexpress CTLA4-Ig and PD-L1, which disrupt T cell costimulatory pathways and stimulate T cell inhibitory pathway, respectively. In this study, hESCs were genetically modified and the respective differentiated cells were immune-protected after implantation.²²⁰ Additionally, Han *et al.*²²¹ used genome editing tools to eliminate HLA class Ia and class II as well as introduction of the genes for PD-L1, HLA-G, and CD47, being the latter a “don’t-eat-me” signal that prevents macrophages engulfment. Authors observed that T cell response, NK cell killing and macrophage engulfment were reduced both *in vitro* and *in vivo*. Overall, these studies highlight the advantages of using immune modulation strategies to overcome some of the PSCs current problems related to graft rejection.²²¹

There are, of course, other concerns regarding pancreatic cells differentiated from hiPSCs, being some of them common to other iPSCs-differentiated cell types, namely:

- 1) risk of tumorigenicity: which may happen due to the possible incorporation of undifferentiated progenitor cells in the final product of differentiated cells or potential de-differentiation of mature endocrine cells after transplantation. Moreover, it could also happen because these cells are maintained in culture for long periods of time which increases the possibility for the incorporation of genomic alterations²⁰⁴;
- 2) safety issues, which are related to the type of approach used to derive iPSCs (integrating approaches, like retrovirus vs non-integrating approaches, like adenovirus or sendai virus vectors) and also the usage of the reprogramming factor c-Myc which is a well-known oncogene. However, human and mouse iPSCs have already been generated lacking c-Myc, despite decreased efficiency and cell quality of the differentiated cells²²²;
- 3) variation in differentiation efficiencies: depends of a variety of factors, such as cell origin, derivation procedure, differentiation protocol techniques and epigenetic factors;
- 4) formation of polyhormonal cells: as previously mentioned, the differentiation of pancreatic cells still needs improvement because it is common to observe that obtained cells exhibit polyhormonal profiles, which hampers their clinical application;
- 5) xenogeneic contaminations and unknown effects: due to their widespread availability it is quite common to use animal-derived products in cell culturing systems however, there is an urgent need to adapt techniques and protocols to use humanized or recombinant

components instead and avoid animal products, as it poses a higher risk of immunoreactivity and graft rejection;

6) lack of maturation: as previously addressed, the insulin-expressing cells or glucagon-expressing cells obtained through iPSCs differentiation *in vitro* lack some of the characteristics of their native counterparts, further maturing *in vivo* by not yet fully understood mechanisms which need to be further studied;

7) low survival rate: currently there is no well-established standard transplantation technique nor transplantation site for iPSCs-derived pancreatic cells. Besides this, the possibility of an immune rejection and also the low blood innervation inside the transplanted islets contribute to their low survival rate ²²³;

8) islet β -cell heterogeneity: although β -cell are often regarded as a homogeneous cell population, it has already been described β -cell heterogeneity in human pancreatic islets with distinct insulin-secretion characteristics.²²⁴ Four different subtypes have been identified and authors have shown that their frequencies may be altered in T2D patients.²²⁵ However, this heterogeneity is not taken into account in the differentiation approaches of β -cells differentiation from iPSCs or ESCs. Therefore, not only further studies are needed to better comprehend their different roles in healthy and disease context, but also to improve differentiation protocols to obtain a mature β -cell population with the different subpopulations.

Undoubtedly stem cell therapy, in particular hiPSCs, is a very promising strategy for diabetes treatment. Despite the many advances in the field, there is still a lot to be improved and overcome until their clinical application becomes a reality.

Final Considerations

This chapter provides a final overview, linking together the research findings covered in the previous chapter, as well as suggestions for future research directions.

5.1. Conclusions

T1D is a chronic metabolic disease marked by insufficient insulin secretion due to β -cell loss.¹ Although exogenous insulin is the primary treatment, many patients still suffer from glycemic instability, making β -cell replacement through whole-pancreas or pancreatic islet transplantation a better therapeutical approach for restoring endogenous insulin secretion.⁴⁵ Thus, T1D has long been the ideal disease candidate for a cell-based therapy since it is associated with the absence of one particular cell type: the pancreatic β -cells.

The main goal of this thesis was to develop a bioartificial pancreatic construct by combining β - and α -cells differentiated from hiPSCs with GLP-1 analog-loaded NPs and GOx in an alginate matrix. Both EXN-loaded NPs and SMG-loaded NPs were successfully developed, showing a pH-dependent release profile, with a 20-30 % increase in peptide release at pH 5. Moreover, both peptides proved to be stable after NPs production and release at both physiological and acidic conditions for at least 48 h. Additionally, differentiation of hiPSCs into β -like cells was attained. Insulin-positive cells were obtained at the end of stages 6 and 7, but the expression of NKX6.1 was negligible, which translated to a polyhormonal population of immature β -cells that lacked glucose sensitivity. Indeed, differentiated β -cells were able to secrete insulin, confirming the presence of intracellular insulin secretory granules. Nevertheless, this secretion was higher at low glucose levels, corroborating the polyhormonal profile of these cells, which has been previously reported in other *in vitro* protocols of β -cells differentiation.^{142,147,148} Regarding α -cells differentiation, this was carried out until $\text{p}\alpha$ -cells stage and around 17.5 % of cells expressed C-peptide and glucagon, which is the target population at this stage, as previously reported.¹⁵⁰ These differentiated cells were later co-encapsulated with the developed NPs and GOx in alginate microspheres, which were then transplanted in diabetic mice. Their glycemic levels were monitored for 27 days, as well as their survival rates. Despite the limitations of the *in vivo* study, there was a clear improvement on the survival of the animals after the transplantation of either the complete system (cells + NPs + GOx), cells + EXN or only cells when compared to the control group. Besides, diabetic mice transplanted with cells + EXN showed a decrease in glucose levels. Nevertheless, further improvements were performed in the system and the addition of catalase successfully counterbalanced GOx cytotoxic effect. Human donor islets or SC-islets were embedded in alginate microspheres and cell metabolic activity (resazurin)

and function (GSIS) were assessed at different time points. Encapsulated donor islets showed improved metabolic activity over time compared to non-encapsulated ones. However, regarding GSIS, the impact of the complete system on C-peptide secretion from human islets was not conclusive. On the other hand, results suggest an increased (but delayed) C-peptide secretion of SC-islets when exposed to EXN or EXN-loaded NPs at day 2. Additionally, results also indicate an increase in *INS*, *GCG* and *PDX1* expression on day 14 after encapsulation, when SC-islets are exposed to EXN (either freely dispersed or encapsulated).

Overall, PSCs have already shown their potential for future cell-based therapies applications, suggesting that this may be the right direction to pursue more effective T1D therapies. However, there are still various missing pieces, such as the lack of knowledge of the underlying mechanisms in differentiation and maturation of cells, which hampers their broad utilisation. Therefore, a deeper understanding of these mechanisms would provide a better control of this technology and consequently more effective therapeutic outcomes. In the particular case of T1D, hiPSCs alone will not be able to provide an effective treatment, since T1D is characterised by autoimmunity. Thus, the combination of stem cells with biomaterials and nanomedicine could provide a promising strategy for T1D treatment. Different glucose-responsive systems have already been developed, but their combination with stem cell-based therapies needs to be further explored to understand if these approaches could enhance hPSCs therapeutic application.

In summary, the work presented in this thesis provides some evidence that glucose-responsive GLP-1 analog-loaded nanoparticles may become an interesting ally for stem cell-based therapies aimed at T1D. Besides, the multistage bioartificial pancreatic system herein described holds great promise to address some of the challenges presented by current stem cell-based therapies. Nonetheless, several aspects still need further clarification and new questions have emerged during the development of this work that require additional research, which will be discussed in the following section.

5.2. Future Perspectives

In this section, a reflection on some limitations of the developed studies and opportunities for future research are provided.

In this work, GLP-1 analogue-loaded pH-sensitive NPs were developed to be included in the bioartificial pancreas in order to improve insulin secretion and β -cell survival. However,

NPs exhibited a burst release profile, a feature not favorable taking into account the final goal of the system. In order to attenuate the release profile observed, further improvements on peptide-loaded NPs formulation could be investigated. Specifically, through hydrophobic ion pairing of the peptides with counter ions that contain hydrophobic moieties, this would lead to a complex formation²²⁶ and consequently the encapsulation efficiency would be expected to improve. In addition, other polymers and/or the addition of pH-sensitive coatings to the NPs could also be investigated in order to improve even further their pH-responsiveness. Moreover, considering a future clinical application, the production of these nanoparticles could be tested on an industrial scale to determine whether they conserve the same properties and characteristics when batches are scaled up and whether the yield and time of production could be improved.

Regarding β -cell differentiation protocols, further optimisations would be needed from stage 3 onwards, given the low expression observed of the specific markers of each stage. This differentiation was carried out in adherent conditions however, β -cell differentiation protocols in dynamic suspension conditions have also been reported in the literature, which would be better to allow process scalability for clinical application. Concerning the differentiation of α -cells, although it was successful, it would also be desirable to evaluate their functionality *in vitro*. Glucagon secretion can be evaluated in an assay similar to GSIS, in which SC- α cells are subjected to consecutive glucose challenges. If the cells are functional, glucagon secretion will be promoted in the presence of low glucose concentrations and inhibited at higher glucose concentrations. Additionally, it would also be important to include the last stage of α -cells differentiation in the protocol, in order to obtain monohormonal α -cells instead of the $p\alpha$ cells that require further maturation *in vivo*. Alternatively, a different approach could be used for pancreatic cells differentiation and, instead of carrying out independent differentiation protocols for each cell type, a single differentiation protocol could be adopted to obtain SC-islets at the end of differentiation, as performed at the LUMC. This approach has been recently reported in the literature^{227,228} and was not available when this project started. Given the fact that differentiation protocols are long and very time-consuming, and that the number of experiments performed with SC-islets were limited, additional experiments would be needed to clarify the statistical significance of the results obtained, as well as to better test the efficacy and functionality of the system, since the populations of SC- β -cells present in the SC-islets were still immature.

Additional research would also be needed to further evaluate the impact of this cell therapy in the T1D diabetic mouse model, for instance by increasing the number of animals per group and also the duration of the study. It would also be interesting to include a group of

animals receiving insulin treatment instead of the transplant and compare the efficacy of the biofunctional system with gold standard treatment (insulin injections). Furthermore, glucose tolerance tests, which consist of measuring blood glucose levels over time after administration of a glucose bolus, should also be performed in order to further evaluate graft function *in vivo*. A deeper metabolic analysis could also be performed by collecting blood samples (after animal's euthanasia) and quantifying the levels of insulin (or C-peptide), glucagon and HbA1C.

Despite the challenges and limitations discussed, we expect this work provides a starting point for the development of novel cellular therapies for T1D that explore the synergy of nanomedicines and stem cell-based approaches.

Scientific Contributions

A comprehensive list of the scientific contributions stemming from the work developed in this thesis, as well as other collaborative works is provided in this chapter. Throughout the development of this thesis, various contributions have been made, primarily in the form of research articles, a book chapter and communications at scientific conferences. In addition to the scientific outputs, attendance of relevant scientific meetings and courses, as well as the organisation of a scientific meeting, participation in representative bodies within my main research institution (i3S), integration of the i3S COVID-19 task force and scientific outreach activities are also listed.

Articles in Peer-Reviewed Journals

- **Marques JM**, Nunes R, Carvalho AM, Florindo H, Ferreira D, Sarmento B. GLP-1 analogue-loaded glucose-responsive nanoparticles as allies of stem cell therapies for the treatment of Type I Diabetes. *ACS Pharmacol Transl Sci.* 2024; 7 (5): 1650-63. <https://doi.org/10.1021/acsptsci.4c00173>.
- Cristelo C, Nunes R, Pinto S, **Marques JM**, Gama FM, Sarmento B. Targeting β Cells with Cathelicidin Nanomedicines Improves Insulin Function and Pancreas Regeneration in Type 1 Diabetic Rats. *ACS Pharmacol Transl Sci.* 2023; 6 (10): 1544-60. <https://doi.org/10.1021/acsptsci.3c00218>.
- Hameedat F, Pinto S, **Marques JM**, Dias S, Sarmento B. Functionalized zein nanoparticles targeting neonatal Fc receptor to enhance lung absorption of peptides. *Drug Deliv and Transl Res.* 2023; 13 (6): 1699-1715. <https://doi.org/10.1007/s13346-022-01286-4>.
- Cristelo C*, Azevedo C*, **Marques JM***, Nunes R*, Sarmento B. SARS-CoV-2 and diabetes: New challenges for the disease. *Diabetes Res Clin Pract.* 2020; 164: 108228. <https://doi.org/10.1016/j.diabres.2020.108228>. (*equal contribution)

Book Chapter

- **Marques JM***, Nunes R*, Florindo H, Ferreira D, Sarmento B. Chapter 10 - A demanding path from iPSCs toward pancreatic β - and α -cells. In: Birbrair A, editor. *Recent Advances in iPSC-Derived Cell Types*. London: Academic Press, an imprint of Elsevier; 2021. p. 227-56. <https://doi.org/10.1016/B978-0-12-822230-0.00002-8>. (*equal contribution)

Oral Presentations in Scientific Events

- **Marques JM**, Carvalho AM, Nunes R, Neves J, Florindo H, Ferreira D, Sarmento B. Mimicking pancreatic islets with a twist: combining iPSC-derived pancreatic cells with glucose-responsive nanoparticles for T1D treatment. i3S Young Scientists Seminar; January 2024; Porto, Portugal.
- **Marques JM**, Carvalho AM, Nunes R, Neves J, Florindo H, Ferreira D, Sarmento B. Combining glucose-responsive nanoparticles and iPSCs differentiation: an innovative strategy to treat diabetes. XIVth Spanish-Portuguese Conference on Controlled Drug Delivery; January 2022; Online.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B. Multifunctional stimuli-responsive pancreas mimetic. New Frontiers in Biofabrication: From Biomolecules to Tissues and Organs; September 2019; Leuven, Belgium.

Poster Presentations in Scientific Events

- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B. Development of GLP-1 analogue-loaded nanoparticles in a bioartificial pancreas to treat type I Diabetes. IDF World Diabetes Congress 2022; December 2022; Online.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B. Development of glucose-responsive nanoparticles towards the design of a bioartificial pancreas for Diabetes therapy. 31st Conference of the European Society for Biomaterials; September 2021; Online.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B. NanoIntelligent bioartificial pancreas for Diabetes therapy. 47th Annual Meeting of the Controlled Release Society; June 2020; Online.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B.. Bioengineered glucose-responsive nanoparticles in artificial pancreas for Diabetes therapy. XIIIth Spanish-Portuguese Conference on Controlled Drug Delivery; January 2020; Santiago de Compostela, Spain.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmento B. Stimuli-responsive nanoparticles for pancreas mimetic construct. 8th i3S Annual Meeting; November 2019; Póvoa do Varzim, Portugal.

- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmiento B. Glucose-responsive nanoparticles embedded in a 3D-engineered pancreas. Training School on Advanced spectroscopic analytical methods and new technologies in drug delivery; October 2019; Helsinki, Finland.
- **Marques JM**, Nunes R, Florindo H, Ferreira D, Sarmiento B. Multifunctional stimuli-responsive pancreas mimetic. 2nd PhDay - PhD countdown: a time for Science; September 2019; Porto, Portugal.

Attendance of other Scientific Events

In addition to the aforementioned scientific events, where the dissemination of the PhD research work was undertaken through oral or poster presentations, other relevant scientific meetings were also attended whenever possible, namely:

- Interrogations at the Biointerface – 7th Advanced Summer School; June 2019; Porto, Portugal.
- VI Biotech Health Annual Symposium; July 2019; Porto, Portugal.
- 1st Discoveries Forum on Regenerative and Precision Medicine; September 2019; Porto, Portugal.
- VII BiotechHealth Annual Symposium; October 2020; Porto, Portugal.
- 3rd Spanish Portuguese Local Chapter of Controlled Release Society (SPLC-CRS) Young Scientists Virtual Meeting; January 2021; Online.
- International Society for Stem Cell Research (ISSCR) 2021 Annual Meeting; June 2021; Online.
- 31st Conference of the European Society for Biomaterials (ESB); September 2021; Online.

In this event, besides the poster presentation mentioned above, participation as session chair and evaluator of oral presentations was also undertaken.

- 4th PhDay - Break borders and build opportunities; November 2021; Porto, Portugal.
- International Society for Stem Cell Research (ISSCR) 2022 Annual Meeting; June 2022; Online.
- Pan3DP Conference - Engineering Multicellular Tissues; July 2022; Online.

- International Society for Stem Cell Research (ISSCR) 2023 Annual Meeting; June 2023; Online.
- 10th i3S Annual Meeting; November 2023; Póvoa do Varzim, Portugal.

Advanced Training

Throughout the development of this thesis, courses aimed at improving different research skills were also attended:

- Flow Cytometry Workshop - From basic knowledge to advanced applications; 7-8 October 2019; i3S, Porto, Portugal.
- Advanced Excel Training (21 h); 6-28 April 2020; Ciência e Letras, Online.
- Writing Global Health: A Virtual Workshop Series; 25 May - 25 June 2020; EUGLOH (host universities: Ludwig-Maximilians-Universität München & Université Paris-Saclay), Online.
- Training Course in Research Ethics / Integrity (monthly sessions); October 2020 - July 2021; i3S, Porto, Portugal.
- Experimental Design and Analysis of Data from Animal Experiments - 4th Edition; 16-18 February 2022; i3S, Porto, Portugal.
- Laboratory Animal Science Course - XXXIII Edition (FELASA accredited); 5-16 September 2022; i3S, Porto, Portugal.
- Workshop on Scientific Writing: How to streamline the process of writing a scientific paper; 4-5 December 2023; i3S, Porto, Portugal.

Organisation of a Scientific Event

During the PhD, there was also the opportunity to integrate the organising committee (from November 2019 to October 2020) of the following scientific event:

- 3rd PhDay - Back to the Future; 1 October 2020; Porto, Portugal (<https://www.i3s.up.pt/event?v=126>).

This meeting is held annually at i3S and is organised by PhD students for their peers, although all members of the i3S scientific community are invited to attend. This event provides a forum for the presentation of research conducted by i3S PhD students, while

fostering debate among the research community, not only from i3S, but also from international institutions.

Institutional Roles

In parallel with the work developed within the scope of this thesis, duties in two representative bodies at i3S were assumed for one year (November 2020 - December 2021), namely:

- PhD Students Representatives;
- Advisory Committee (representing PhD Students within this body).

This provided the chance to advocate for the needs and concerns of my colleagues and to contribute to institutional decision-making processes.

i3S COVID-19 Task Force

In the initial months of the COVID-19 pandemic in Portugal, the i3S scientific community was encouraged to integrate the i3S COVID-19 Task Force (https://covid19.i3s.up.pt/?page_id=1297). One of the areas of intervention of this task force was the molecular and serological testing for diagnostic purposes. Thus, participation in the diagnostics team was undertaken:

- Volunteer at COVID-19 i3S Task Force (diagnostic tests); May to July 2020; Porto, Portugal.

Scientific Outreach Activities

Given the importance of science dissemination to the general public, involvement in two editions of “Porto de Crianças” (<https://www.cm-porto.pt/programas-e-projetos/porto-de-criancas>), particularly in the scientific education project, was also undertaken. This initiative aims to promote the enrichment of the educational offer of kindergarten and primary public schools in Porto and to disseminate science to young children. Therefore, as a member of a team of i3S researchers, we did weekly visits (for one month in each edition) to 3rd and 4th grade classes of primary public schools to teach and perform scientific activities.

- Porto de Crianças; January 2020 and February 2022; Porto, Portugal.

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Supplementary Data

Table S 1 - Summary of parameters tested and optimised in the NPs formulation using double emulsion technique and physico-chemical characterisation of some batches (when aggregates were absent). All batches were produced in triplicate. Results are presented as mean $\pm$ standard deviation (n = 3). Text highlighted in bold and light grey refers to the parameter changed.

Changing parameter	Total Polymer mass (mg)	Polymer(s)	<u>Internal Phase:</u> Vol. (μ L) / composition (w/v)	<u>External Phase:</u> Vol. (mL) / composition (w/v)	Sonication time (1 st step, 2 nd step)	EXN Drug Loading (%)	Obs. ^{a)}	Size (nm)	PdI
External phase (adjustment of pH)	40	PLGA (7502A) : E100 (50:50)	700 μ L / Water	11.5 / 1 % Kolliphor P407 in 0.1 M acetate buffer (pH 5.5)	30 s, 15 s	5	***	-	-
	40	PLGA (7502A) : E100 (50:50)	700 μ L / Water	11.5 / 1 % Kolliphor P407 in 0.1 M acetate buffer (pH 5.5)	30 s, 15 s	-	***	-	-
	40	E100	700 μ L / Water	11.5 / 1 % Kolliphor P407 in 0.1 M acetate buffer (pH 5.5)	30 s, 15 s	-	***	-	-

(cont.)

Internal phase (addition of surfactant)	40	PLGA (7502A) : E100 (50:50)	700 µL / 0.1% Kolliphor P407	11.5 / 1 % Kolliphor P407	30 s, 15 s	5	***	-	-
	40	PLGA (7502A) : E100 (50:50)	700 µL / 0.1% Kolliphor P407	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	***	-	-
	40	E100	700 µL / 0.1% Kolliphor P407	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	***	-	-
Sonication time (2nd step)	40	PLGA (7502A) : E100 (50:50)	700 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 30 s	-	**	-	-
	40	PLGA (7502A) : E100 (50:50)	700 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 1 min	-	**	-	-
	40	E100	700 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 1 min	-	**	-	-
External phase conc.	40	PLGA (7502A) : E100 (50:50)	700 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 30 s	-	***	-	-
	40	E100	700 µL / Water	11.5 / 2% Kolliphor P407	30 s, 30 s	-	***	-	-
	40	PLGA (7502A)	700 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 30 s	-	***	-	-
	40	PLGA (7502A) : E100 (50:50)	700 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 1 min	-	***	-	-
	40	PLGA (7502A)	700 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 1 min	-	***	-	-

(cont.)

Internal phase volume	40	PLGA (7502A) : E100 (50:50)	100 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	*	<u>Formulation A</u>	
	40	E100	100 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	*		
	40	PLGA (7502A)	100 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	*		
External phase conc.	40	PLGA (7502A) : E100 (50:50)	100 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 15 s	-	***	-	-
	40	E100	100 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 15 s	-	***	-	-
	40	PLGA (7502A)	100 µL / Water	11.5 / 2 % Kolliphor P407	30 s, 15 s	-	***	-	-
External phase (surfactant)	40	PLGA (7502A)	100 µL / Water	11.5 / 1 % PVA	30 s, 15 s	-	***	-	-
	40	PLGA (7502A)	100 µL / Water	11.5 / 2 % PVA	30 s, 15 s	-	***	-	-
PLGA type (and surfactant)	40	PLGA (5004A)	100 µL / Water	11.5 / 1 % Kolliphor P407	30 s, 15 s	-	***	-	-
	40	PLGA (5004A)	100 µL / Water	11.5 / 1 % PVA	30 s, 15 s	-	***	-	-
	40	PLGA (5004A)	100 µL / Water	11.5 / 2 % PVA	30 s, 15 s	-	***	-	-

(cont.)

Sonication time (2nd step)	40	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 15 s	-	*	<u>Formulation A</u>	
	40	E100	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 15 s	-	*		
	40	PLGA 7502A	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 15 s	-	*		
	40	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 45 s	-	*	-	-
	40	E100	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 45 s	-	**	-	-
	40	PLGA 7502A	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 45 s	-	*	-	-
	40	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	NA	<u>Formulation B</u>	
	40	E100	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	*		
	40	PLGA 7502A	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	*		
External phase conc.	40	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 2% Kolliphor P407	30 s, 1 min	-	**	-	-
	40	E100	100 µL / Water	11.5 mL / 2% Kolliphor P407	30 s, 1 min	-	**	-	-
	40	PLGA 7502A	100 µL / Water	11.5 mL / 2% Kolliphor P407	30 s, 1 min	-	*	-	-

(cont.)

Polymer mass	20	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	*	-	-
	20	E100	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	*	-	-
	20	PLGA 7502A	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	-	*	-	-
External phase (surfactant)	20	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Tween-80	30 s, 1 min	-	*	48.06	0.474
	20	E100	100 µL / Water	11.5 mL / 1% Tween-80	30 s, 1 min	-	NA	72.79	0.433
	20	PLGA 7502A	100 µL / Water	11.5 mL / 1% Tween-80	30 s, 1 min	-	NA	74.15	0.362
External phase (surfactant conc.)	20	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 2% Tween-80	30 s, 1 min	-	NA	25.01	0.460
	20	E100	100 µL / Water	11.5 mL / 2% Tween-80	30 s, 1 min	-	NA	16.19	0.392
	20	PLGA 7502A	100 µL / Water	11.5 mL / 2% Tween-80	30 s, 1 min	-	NA	41.95	0.448
Drug loading (Formulation B conditions)	40	PLGA 7502A : E100 (50:50)	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	5	***	-	-
	40	E100	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	5	***	-	-
	40	PLGA 7502A	100 µL / Water	11.5 mL / 1% Kolliphor P407	30 s, 1 min	5	***	-	-

(cont.)

Surfactant volume	40	PLGA 7502A : E100 (50:50)	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	-	NA	140.90 ± 2.47	0.164 ± 0.009
	40	E100	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	-	NA	134.78 ± 2.46	0.155 ± 0.016
	40	PLGA 7502A	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	-	NA	143.51 ± 2.30	0.146 ± 0.012
Drug loading	40	PLGA 7502A : E100 (50:50)	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	5	NA	148.70	0.132
	40	E100	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	5	NA	141.67	0.158
	40	PLGA 7502A	100 µL / Water	19 mL / 1% Kolliphor P407	30 s, 15 s	5	NA	151.87	0.134

Notes:

PVA - polyvinyl alcohol; conc. - concentration.

a) Observations: *** - large quantity of macroscopic aggregates; ** - medium quantity of macroscopic aggregates; * - small quantity of macroscopic aggregates; NA - no macroscopic aggregates.

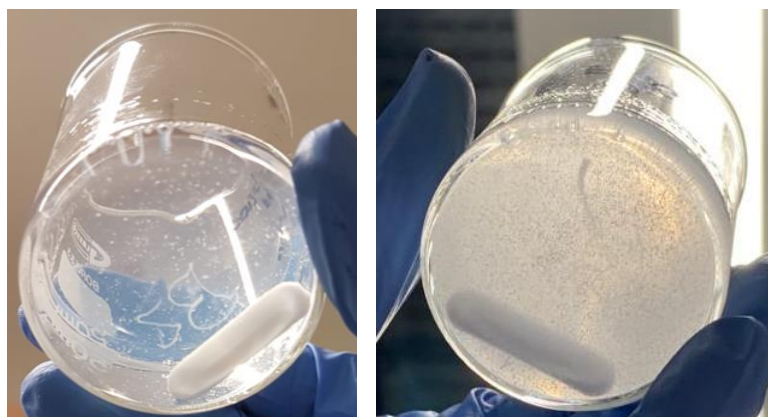


Figure S 1 - Examples of formulations that showed visible precipitates after solvent evaporation.

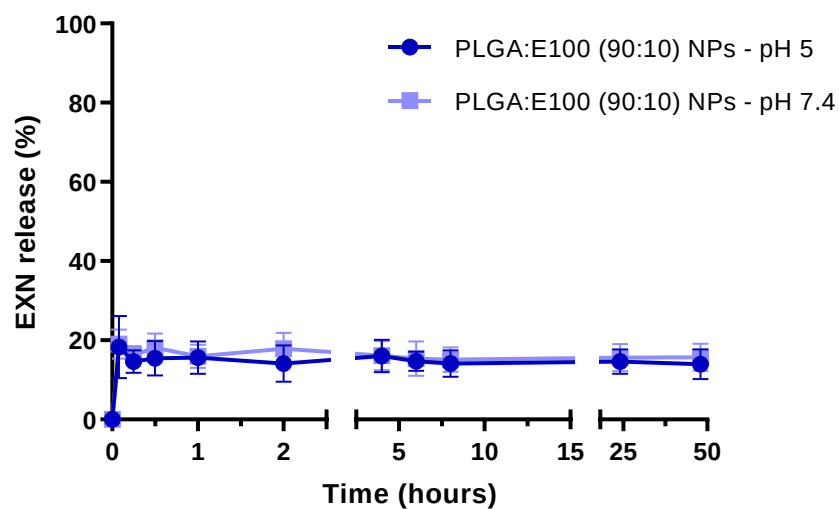


Figure S 2 - Release profile of EXN from PLGA:E100 (90:10) NPs at pH 5 and pH 7.4. Points and vertical bars stand for mean and standard deviation (SD), respectively.

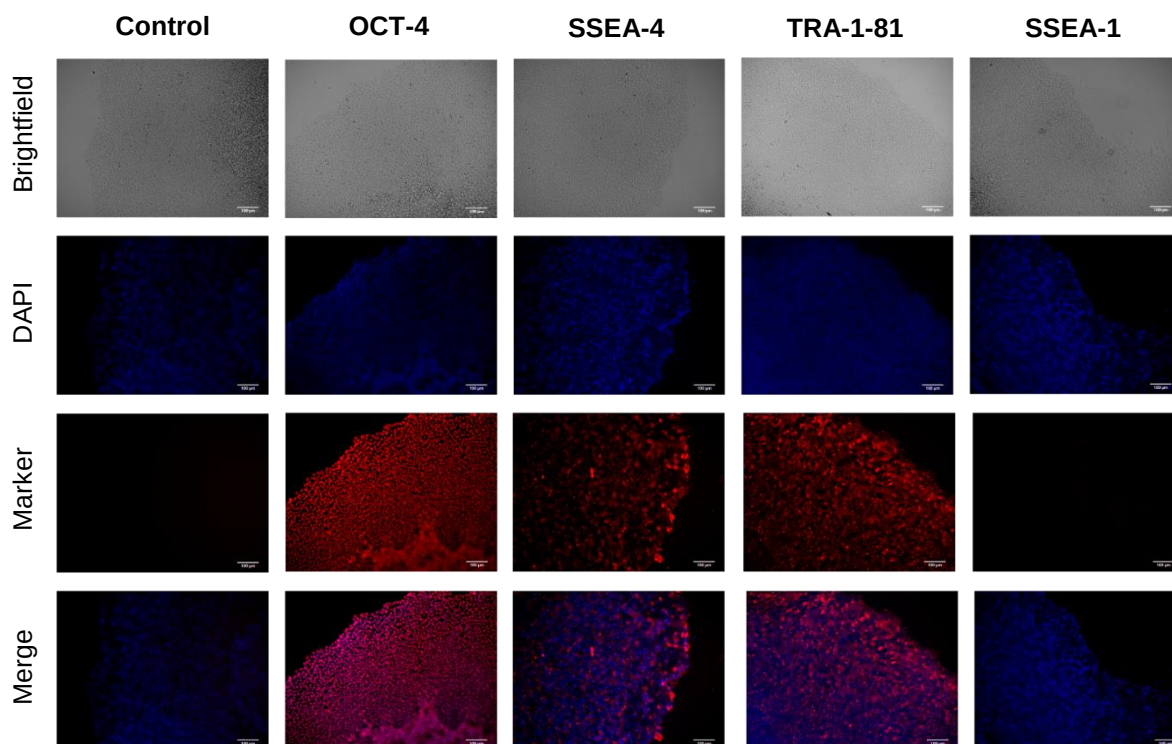


Figure S 3 - Quality control for pluripotency of hiPSCs previously cultured in static suspension conditions. WLS-4D1 cells were successfully maintained in static suspension culture for four passages and re-seeded in adherent culture two days after passage. Cells were stained for pluripotency markers (OCT-4, SSEA-4 and TRA-1-81) and differentiation marker (SSEA-1) in red and the nucleus (DAPI) in blue. Scale bar: 100 μ m.

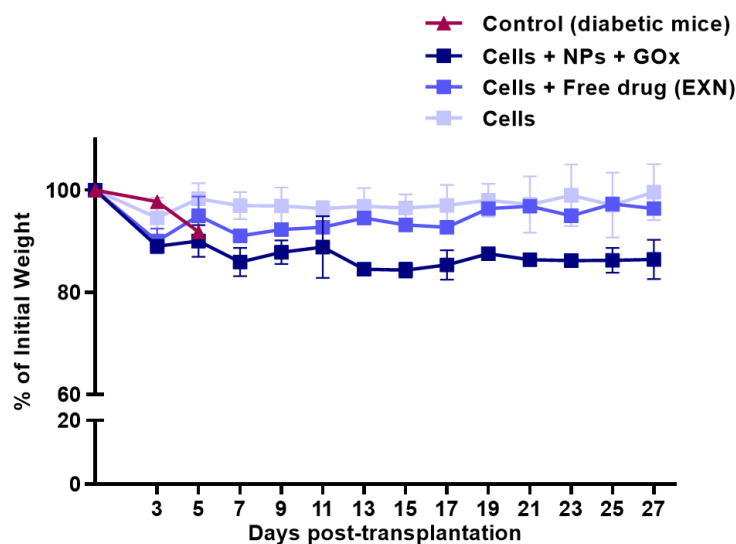


Figure S 4 - Body weight of streptozotocin-induced diabetic mice after transplantation. Cells were encapsulated either alone (Cells), in combination with EXN-loaded glucose-responsive NPs and glucose oxidase (Cells + NPs + GOx) or in combination with EXN freely dispersed in the alginate matrix (Cells + Free drug (EXN)). Body weight was monitored every two days for 27 days. Values are represented as mean $\pm$ SD.